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MINIREVIEW

Microbial Products and Biofertilizers in Improving Growth and Productivity of Apple – a Review

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Abstract

The excessive use of mineral fertilizers causes many negative consequences for the environment as well as potentially dangerous effects of chemical residues in plant tissues on the health of human and animal consumers. Bio-fertilizers are formulations of beneficial microorganisms, which upon application can increase the availability of nutrients by their biological activity and help to improve soil health. Microbes involved in the formulation of bio-fertilizers not only mobilize N and P but mediate the process of producing crops and foods naturally. This method avoids the use of synthetic chemical fertilizers and genetically modified organisms to influence the growth of crops. In addition to their role in enhancing the growth of the plants, biofertilizers can act as biocontrol agents in the rhizosphere at the same time. Biofertilizers are very safe for human, animal and environment. The use of *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia*, *Bacillus*, *Paenibacillus* and some members of the *Enterobacteriaceae* is gaining worldwide importance and acceptance and appears to be the trend for the future.

Key words: apple productivity, biocontrol, biofertilization, bioproducts

Introduction

Apple (*Malus domestica* Borkh.) is the 3rd most important fruit crop worldwide, after citrus and banana (FAOSTAT, 2013). In 2013, the total apple production and harvest in the world was estimated at 80.8 million tons and 5.2 million hectares, respectively. Its cropping has expanded into subtropical and tropical zones (Karakurt and Aslantas, 2010) and is one of the most important cropped and consumed fruits in the world (Brown, 2012). Intensive farming practices, that warrant high yield and quality, require the extensive use of chemical fertilizers and pesticides, which are costly and create environmental problems. Hence, there has been a resurgence of interest in environmental friendly, sustainable and eco-friendly agricultural practices (Esitken *et al*., 2002). One potential way to decrease negative environmental impacts resulting from continued use of chemical fertilizers is inoculation with plant growth promoting rhizobacteria (PGPR). These bacteria improve nutrient (N, P, K, Fe, and Zn) bioavailability (Table I) and exert beneficial effects on plant growth and development, and therefore may be used as biofertilizers for agriculture. The natural role of the PGPR in maintaining soil fertility is more important than in conventional agriculture where higher use of agrochemicals minimizes their significance (Canbolat *et al*., 2006). Moreover, the applications of biofertilizers containing beneficial microorganisms instead of synthetic chemicals are known to improve fixation of nutrients in the rhizosphere, produce growth stimulants for plants, improve soil stability, provide biological control, biodegrade substances, recycle nutrients, promote mycorrhiza symbiosis, and develop bioremediation processes in soils contaminated with toxic, xenobiotic and recalcitrant substances (Rivera-Cruz *et al*., 2008). So the use of more sustainable technologies, such as biofertilization, is inevitable for the mitigation of environmental damage (Karakurt and Aslantas, 2010).

The influence of biofertilizers in improving apple growth and productivity

Applications of bio-fertilizers containing beneficial microorganisms instead of synthetic chemicals are known to improve plant growth through the supply

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Table I
Plant Growth Promoting Rhizobacterial (PGPR) strains improving nutrient (N, P, K, Fe and Zn) bioavailability

	Bacteria	References
N – Nitrogen, P – Phosphorus, K – Potassium, Fe – Iron	*Rhizobium leguminosarum*	Biswas *et al*., 2000
	Bradyrhizobium japonicum UCM B-6018	Tytova *et al*., 2013
N – Nitrogen, P – Phosphorus, Fe – Iron	*Pseudomonas aeruginosa* BS8	Goswami *et al*., 2015
N – Nitrogen, P – Phosphorus	*Bacillus megaterium, Bacillus mucilaginosus*	Han and Lee, 2005
N – Nitrogen, Fe – Iron	*Pseudomonas* strain GRP3	Sharma *et al*., 2003
	Pseudomonas fluorescens C7	Vansuyt *et al*., 2007
N – Nitrogen	*Azospirillum* spp.	Bashan and De-Bashan, 2010
	Pseudomonas alcaligens PsA15, *Mycobacterium phlei* MbP18	Egamberdiyeva and Höflich, 2004
	Azospirillum lipoferum, Azospirillum brasilense	Malik *et al*., 2002
	Klebsiella pneumonia, Pantoea agglomerans	Riggs *et al*., 2001
	Azotobacter spp.	Mrkovacki and Milic, 2001
	Azotobacter chroococcum	Wu *et al*., 2005
P – Phosphorus	*Streptomyces* spp.	Chang and Yang, 2009
	Micrococcus spp.	Dastager *et al*., 2010
	Achromobacter spp.	Ma *et al*., 2009
	Bacillus spp., *Burkholderia* spp.	Tao *et al*., 2008
	Bacillus megaterium	Wu *et al*., 2005
	Pseudomonas alcaligenes	Zhang *et al*., 2014
	Pseudomonas aeruginosa	Yadav *et al*., 2014
K – Potassium	*Bacillus edaphicus*	Sheng and He, 2006
Zn – Zinc	*Serratia* spp.	Abaid-Ullah *et al*., 2011
	Pseudomonas fluorescens	Di Simine *et al*., 1998
	Pseudomonas aeruginosa	Fasim *et al*., 2002
	Flavobacterium spp.	He *et al*., 2010
	Pseudomonas spp. PsM6, *P. jessenii* PjM15	Rajkumar and Freitas, 2008
	Acetobacter diazotrophicus	Saravanan *et al*., 2007
	Rhizobia spp.	Wani *et al*., 2008
	Pseudomonas sp. Z5	Yasmin, 2011

of plant nutrients and may help to sustain environmental health and soil productivity (O'Connell, 1992). A biofertilizer is a substance which contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003). Biofertilization is now a very important method for providing the plants with their nutritional requirements without having an undesirable impact on the environment (Abou El-Yazied and Sellim, 2007). Additionally, the use of biofertilizers can improve productivity per unit area in a relatively short time, consume smaller amounts of energy, mitigate contamination of soil and water, increase soil fertility, and promote antagonism and biological control of phytopathogenic organisms (Corpoica *et al*., 2007). Moreover, biofertilizers are known to improve fixation of nutrients in the rhizosphere, produce growth stimulants for plants, improve soil stability and provide biological control. They also biodegrade substances, recycle nutrients, promote mycorrhiza symbiosis and develop bioremediation processes in soils contaminated with toxic, xenobiotic and recalcitrant substances (Rivera-Cruz *et al*., 2008). Raghuwanshi (2012) stated that biofertilizers have a great potential as supplementary, renewable and environmental friendly sources of plant nutrients. Furthermore, they are an important component of integrated nutrient management and plant nutrition system. Application of biological potassium fertilizers (BPF), as preparation of silicate bacteria (liquid solution, containing two million bacteria per 1 ml, or packages of 500 g of peat-moss substrate, contains 2 million bacteria) and Azobacterin increased trunk cross-sectional area, mean shoot length, mean leaf area, total leaf area, number of fruits per tree, mean fruit weight and yield of "Charavnitsa" apple variety (Ryabtseva *et al*., 2005). Von-Bennewitz and Hlusek (2006) reported that

biofertilization is beneficial in stimulating the growth and fruiting of pome and stone fruits. Treatment of 'Topaz' and 'Ariva' apple trees with the biopreparations: Micosat F, Humus UP, Humus Active + Aktywit PM, BioFeed Amin, Vinassa, Florovit Eko and Florovit Pro Natura produced positive effects on the growth of apple roots and their mycorrhizal frequency, and the size of the populations of microorganisms in the rhizosphere soil (Derkowska *et al*., 2014). Besides, Rozpara *et al*. (2014) found also that Biofeed Amin preparation had a positive influence on the growth and development of 'Ariwa' apple trees growing. Tree trunk sectional area and yield of "Topaz" apple trees was improved with Florovit Natura and Yeast combined with *Pantoea* spp., *Pseudomonas fluorescens*, *Klebsiella oxytoca* and *Rhizobium* spp. bacteria species respectively as compared to NPK chemical fertilization (Mosa *et al*., 2016).

The effect of mycorrhiza on apple growth and yield

Abuscular mycorrhizal (AM) fungi are associated with the roots of over 90% of terrestrial plant species (Gadkar *et al*., 2001). They are a very important component within the rich biodiversity of microorganisms occurring in the rhizosphere (Turnau and Haselwandter, 2002). Xavier and Boyetchko (2002) have found that mycorrhizal fungi, in particular endomycorrhizal have a beneficial effect on plant growth and development, and that effect can be likened to the effects of biostimulators and biofertilizers on plants. Root inoculation with two biopreparations, Vambac® (VA-mycorrhiza genus *Glomus*, *Gigaspora* and the rhizospheric bacteria *Agrobacterium radiobacter*) and Amalgerol® (composed of vegetative and sea-algae oils and extracts) enhanced the uptake of phosphorus and vegetative growth of two-year-old apple trees cv. "Jonagold" grown on M.9 root stock (Von Bennewitz and Hlusek, 2006). Cavallazzi *et al*. (2007) stated that apple (*Malus prunifolia*) Colonization by *Glomus etunicatum* SCT110, *Scutellospora pellucida* SCT111, *Acaulospora scrobiculata* SCT112 and *Scutellospora heterogama* SCT113 fungal isolates significantly affected plant height, shoot and root dry weights, and root: shoot ratio. Moreover, mycorrhizal inoculation also significantly altered tissue concentrations of P, Zn, Cu, Ca, S, Na, N, K, Fe and Al.

Overall, *G. etunicatum* and *S. pellucida* were the most effective isolates to promote plant growth and nutrient uptake. Many investigations shows that AM symbiosis contributes to plant growth, nutrient uptake and improve fruit quality (Miransari, 2010). The positive and beneficial effects of AM fungi such as growth promotion, increased root length, leaf area and stem diameter (Sharma *et al*., 2011), transplant performance and tolerance to abiotic (water, nutrition) stresses (Göhre and Paszkowski, 2006), could be due to a positive interaction between AM fungi and other associated microorganisms such as *Azotobacter chroococcum* in a particular edaphic and agro-climatic conditions. Sharma *et al*. (2012) reported significant improvement in the vegetative growth parameters of 'Royal Delicious' apple saplings by using single and/or dual application of soil inoculation of *Glomus fasciculatum*, *Glomus mosseae*, and *A. chroococcum* strains namely, *A. chroococcum* strain-I (AZ_1) and *A. chroococcum* strain-II (AZ_2) at nursery stage under reduced inorganic fertilization. Grzyb *et al*. (2015) found that Florovit Eko + mycorrhizal fungi improved the tree trunk diameter of maiden trees of apple cv. "Topaz". Inoculation of three Arbuscular Mycorrhizal Fungi (AMF) species; *Glomus versiforme*, *Claroideoglomus etunicatum* and *Rhizophagus intraradices* could increase apple rootstocks (M.9, M.7 and MM.106) shoot height, stem diameter, leaf area, shoot fresh and dry weight and root fresh and dry weight and the concentration of N, P, Ca, Mg, Zn, and Fe compared to those of non – mycorhizal control plants (Hosseini and Gharaghani, 2015). Mosa *et al*. (2016) noticed that the combination of mycorrhizal fungi (*G. mosseae* and *Glomus intraradices*) and plant growth promoting bacteria (*Pantoea* sp., *P. fluorescens*, *K. oxytoca* and *Rhizobium* sp) improved the tree trunk, number and weight of fruits per tree of "Topaz" apple cultivar.

The influence of mycorrhiza in alleviating biotic and abiotic stresses in apple orchard

Runjin (1989) mentioned that sterilized soil inoculated with *G. versiforme* and *Glomus macrocarpum* improved water status and drought tolerance of the plants. Furthermore, arbscular mycorrhiza colonization in sterilized soils reduced the stomatal resistance and the permanent wilting as well enhanced the rate of recovery of the plant from the water stress. This was probably due to enhancing absorption and translocation of water by the external hyphae. Kaldorf and Ludwig-Müller (2000) observed that mycorrhiza-covered roots were better developed; especially the number of lateral and fine roots was significantly greater. The presence of mycorrhiza in the roots intensifies uptake of water and minerals from the soil by the root system. Al-Karaki (2004) showed that mycorrhizal fungi colonized more readily the roots of plants growing in an area with high water deficiency, and that the use of mycorrhizal inocula in dry areas had a favourable effect on the size and quality of the crop. Hamel (2004) reported that the network of extraradical mycorrhizal hyphae facilitate nutrient acquisition and transport

many ions to roots, particularly less mobile ions such as P, N, K, S, Ca and Zn. Arbuscular mycorrhizal fungi (*Glomus deserticola*) decreased soil EC and organic carbon and increased soil availability of N, P and K as well as leaf nutrient status of "Kinnow" mandarin (Usha *et al*., 2004). Inoculation of cherry rootstocks, 'Edabriz' and 'Gisela 5', plantelts with *Glomus clarum*, *Glomus caledonium*, *G. etunicatum*, *G. intraradices*, *G. mosseae* and mixture of these species increased Zn and P nutrient uptake than non-mycorrhizal plantlets (Aka-kaçar *et al*., 2010). It has been found that AM fungi can alleviate the unfavourable effects on plant growth of stresses such as heavy metals, soil compaction, salinity and drought (Miransari, 2010). Yang *et al*. (2014) studied the influence of *G. versiforme* on increasing one-year-old "Red Fuji" apple seedlings (*Malus hupehensis* Rehd. root stock) salt tolerance. They noticed that abuscular mycorrhizal fungi significantly increased the root length colonization of mycorrhizal apple plants under 2‰, and 4‰ salinity stress levels as compared to non-mycorrhizal plants. However, percent root colonization reduced as saline stress increased. Salinity levels were found to negatively correlate with leaf relative turgidity, osmotic potential irrespective of non-mycorrhizal and mycorrhizal apple plants, but the decreased mycorrhizal leaf turgidity maintained relative normal values under 2‰ and 4‰ salt concentrations. Under salt stress condition, Cl^- and Na^+ concentrations clearly increased and K^+ contents obviously decreased in non-mycorrhizal roots in comparison to mycorrhizal plants, this caused mycorrhizal plants to have a relatively higher K^+/Na^+ ratio in the root. Ascorbate peroxidase and catalase activities increased in mycorrhizal more than in non-mycorrhizal plants.

The role of some beneficial bacterial strains in improving nutrient uptake, soil fertility, apple growth and productivity

Use of biofertilizers containing beneficial microorganisms instead of synthetic chemical is known to improve plant growth through supply of plant nutrients and may help to sustain environmental health and soil productivity (O'Connell, 1992). In field trials, preplant inoculation with both *G. intraradices* and *G. mosseae* increased rootstock growth and leaf concentrations of P, Mg, Zn and Cu in fumigated plots but not in non-fumigated plots, indicating that colonization by native AM fungi in non-fumigated plots may have been sufficient for adequate nutrient acquisition (Forge *et al*., 2001). The plant promoting effect of the PGPB is mostly explained by the release of metabolites directly stimulating growth. The mechanisms by which PGPB promote plant growth are not fully understood, but are thought to include: (a) the ability to produce plant hormones, such as gibberellins (Gutierrez-Manero *et al*., 2001), cytokinins (De Salamone *et al*., 2001) and auxins (Egamberdiyeva, 2005) and inhibit ethylene production (Glick *et al*., 1995); (b) asymbiotic N2 fixation (Sahin *et al*., 2004); (c) solubilization of inorganic phosphate and mineralization of organic phosphate and/or other nutrients (Jeon *et al*., 2003). Esitken *et al*. (2003) found that *Bacillus strains*; OSU-142 and M-3 stimulated macro and micro-nutrient uptake such as N, P, K, Ca, Mg, Fe, Mn, Zn and Cu in apricot (*Prunus armeniaca* L. cv. Hacihaliloglu). Tenuta (2003) found that *Rhizobium*, *Bacillus* and *Pseudomonas* improve the uptake of nutrients like nitrogen, phosphorus, potassium, sulphur and iron. Recent studies confirmed that, a number of bacterial species mostly associated with the plant rhizosphere, are found to be beneficial for plant growth, yield and crop quality. They have been called 'Plant Growth Promoting Bacteria (PGPB)' including the strains in the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillium*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholdria*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium* and *Serratia* (Bashan and de-Bashan, 2005). Orhan *et al*. (2006) reported that *Bacillus* M3 (N2-fixing and phosphate solubilizing) alone or in combination with *Bacillus* OSU-142 (N2-fixing) increased the total N, available P, K, Ca, Mg, Fe, Mn, Zn contents in the soil and Fe and Mn contents in the leaves of raspberry cv. "Heritage". Aslantas *et al*. (2007) mentioned that floral and foliar applications of *Pseudomonas* BA-8 and *Bacillus* OSU-142 alone or in combination have the potential to increase yield, growth and nutrition of apple cultivars "Granny Smith and Stark Spur Golden". Karlidag *et al*. (2007) noticed that *Bacillus* M3, *Bacillus* OSU-142 and *Microbacterium* FS01 combinations stimulated plant growth and resulted in significant yield increases in apple cv. "Granny smith" by promoting abilities for auxin and cytokinin production, N2-fixation, phosphate solubilization and antimicrobial substance production. Karakurt and Aslantas (2010) evaluate the effects of four strains of plant growth promoting rhizobacteria (*Agrobacterium rubi* A-18, *Bacillus subtilis* OSU-142, *Burkholderia gladioli* OSU-7 and *Pseudomonas putida* BA-8) on growth and leaf nutrient content of 'Starking Delicious', 'Granny Smith', 'Starkrimson Delicious', 'Starkspur Golden Delicious' and 'Golden Delicious' apple cultivars grafted on semi-dwarf rootstock MM-106. They found that bacteria applications showed the desirable effects on plant growth and plant nutrient element contents. Mosa *et al*. (2016) showed the improvement in the growth, yield and fruit quality of "Topaz" apple trees following the addition of *Pantoea* sp., *P. fluorescens*, *K. oxytoca* and *Rhizobium* sp. bacteria species to Fertigo, Micosat, Humus UP, BioFeed Quality, BioFeed Amin, Yeast, Vinassa and Florovit Eko as compared to chemical NPK fertilization.

Some beneficial roles of bacterial strains in apple trees pest management

Biological control is considered a promising strategy for the management of fire blight and several biological control agents are now commercially available, including *P. fluorescens* A506 (Wilson and Lindow, 1993), *Pantoea agglomerans* E325 (Pusey, 1999), *B. subtilis* QST713 (Aldwinckle *et al*., 2002), *P. agglomerans* P10c (Vanneste *et al*., 2002) and *B. subtilis* BD170 (Broggini-Schärer *et al*., 2005) and *Pantoea vagans* C9-1 (Smits *et al*., 2010). *In vitro* – bacterized plantlets not only grew faster than nonbacterized controls but also were sturdier, with a better-developed root system and significantly greater capacities for withstanding biotic (Barka *et al*., 2000) and abiotic (Bensalim *et al*., 1998) stresses. Ramamoorthy *et al.* (2001) showed that some plant growth-promoting rhizobacteria (PGPR) induce systemic resistance by strengthening the physical and mechanical strength of the cell wall, as well as altering the biochemical and physiological reaction of the host plant that leads to the synthesis of chemical defense against the pathogen. Plant growth-promoting rhizobacteria can disrupt phytopathogen organization (Barka *et al*., 2002), stimulate developmental changes in host plants, induce systemic resistance to pathogens, affect phytohormone production, and improve nutrient and water management (Compant *et al*., 2005). *Pseudomonas* strains MRS23 and CRP55b inhibited the growth of pathogenic fungi, *i.e*. *Aspergillus* sp., *Fusarium oxysporum* f. sp. *ciceri* and *Rhizoctonia solani* under culture condition (Goel *et al*., 2002). Commercial formulations combining bacteria antagonistic to plant pathogenic microbes with ice nucleation-active bacteria have been utilized as an environmentally safe method to manage biotic and abiotic stress in plants (Lindow and Leveau, 2002). In addition, some of these bacteria, such as epiphytic or endophytic plant growth-promoting rhizobacteria, enhance plant growth while improving their resistance to stress (Dobbelaere *et al*., 2003). *Pseudomonas*, *Bacillus*, *Burkholderia*, *Agrobacterium* and *Streptomyces* suppress plant disease by production of antibiotics, siderophores, or by induction of systemic resistance or any other mechanism (Tenuta, 2003). The plant promoting effect of the PGPB are thought to do antagonism against phytopathogenic microorganisms by production of siderophores, the synthesis of antibiotics, enzymes and/or fungicidal compounds and competition with detrimental microorganisms (Lucy *et al*., 2004). Lactic acid – (LAB) active against *Erwinia amylovora* could be a novel approach for fire blight control, because they have been reported in the field of food technology as biopreservatives (Vermeirem *et al*., 2004), including fermented vegetables or fruit juices (Gomez *et al*., 2002). The capacities of certain species of LAB isolated from fresh plant products to control food-borne human pathogenic bacteria and postharvest fungi have been studied (Trias *et al*., 2008a; 2008b; 2008c). Also, strains of LAB have been reported as antagonistic to the plant pathogenic bacteria *Pectobacterium carotovorum*, *Xanthomonas campestris* and *Pseudomonas syringae* (Trias *et al*., 2008c). This antagonistic and bioprotective capacity is mainly due to a wide diversity of mechanisms of action including not only antibiosis (Cleveland *et al*., 2001), but also pre-emptive colonization of wounds and cuts (Trias *et al*., 2008a). In addition, LAB are not perceived as environmental and health hazards, because they have been considered with the status of "generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA, USA) and with the "qualified presumption of safety" (QPS) status by the European Food Safety Agency (EFSA). Ongena *et al.* (2005) showed the ability of *B. subtilis* strain M4, an important producer of a wide variety of fengycin-type lipopeptides, to protect wounded apple fruits against mold disease caused by *Botrytis cinerea*. The resistance of plants to root diseases as well as efficient nutrient assimilation is profoundly influenced by the presence and activity of beneficial microorganisms in the soil (Picardi *et al*., 2005). Orchard application of biological potassium fertilizers (BPF) increased the resistance of "Charavnitsa" apple trees to viral and bacterial diseases and to the sucking pests (Ryabtseva *et al*., 2005). Rhizobacteria are soil bacteria that colonize plant roots; they are able to multiply and occupy all the ecological niches found on the roots at all stages of plant growth (Antoun and Prévost, 2006). Such bacteria may negatively interact with plants, directly by competing for nutrients. Alternatively, the relationship between rhizobacteria and the host plant can be positive. For example, the bacteria may compete with pathogens for survival in the rhizosphere or they may promote mutualistic relationships with plants they were associated, allowing nutrient exchange and stimulating antibiotic production against phytopathogenic agents (Siddiki, 2006). Floral and foliar applications of *Bacillus* OSU-142 and BA-8 and OSU-142 decreased shot-hole disease in "Granny Smith" and "Star Spur Golden" young apple trees (Aslantas *et al*., 2007). Over 400 species of fungi and more than 90 species of bacteria which infect insects have been described including *Bacillus thuringiensis*, varieties of which are manufactured and sold throughout the world primarily for the control of caterpillar pests and more recently mosquitoes and black flies. So far, more than 40000 species of *B. thuringiensis* have been isolated and identified belonging to 39 serotypes. These organisms are active against either *Lepidoptera*, *Diptera* or *Coleoptera* pests (Moazami, 2007). *Burkholderia* species are able to synthesize a remarkable array of metabolites, including

siderophores, antibiotics, and phytohormones (Vial *et al*., 2007), and many strains belonging to this genus exhibit activities involved in bioremediation or biological control *in vitro* (Caballero-Mellado *et al*., 2007). Beneduzi *et al*. (2012) mentioned that bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR). Their effects can occur *via* local antagonism to soil-borne pathogens or by induction of systemic resistance against pathogens throughout the entire plant.

The effect of biocontrol agents in nematode control

Different fungal strains isolated from nematodes, soil and plants were shown to produce substances that inhibit nematode egg hatch or kill nematodes (Nitao *et al*., 1999). Khan *et al*. (2003) showed that the fungus *Paecilomyces lilacinus* penetrates nematode eggs and cuticles through the production of the lytic enzymes serine protease and chitinase. *Pseudomonas aeruginosa* (Siddiqui *et al*., 2000) and *Pseudomonas* spp. (Ali *et al*., 2002) have shown good results for the control of *Meloidogyne* spp. Besides, antagonistic bacteria have been repeatedly shown to be promising microorganisms for the biological control of plant-parasiticnematodes (Giannakou *et al*., 2004). Furthermore, many attempts have been made to use antagonistic bacteria and fungi to control root-knot nematodes (Khan *et al*., 2008). The damage caused by root-knot nematodes could be managed by application of microorganisms antagonistic to *Meloidogyne* spp., or compounds produced by these microbes (Ashraf and Khan, 2010). Mazzola *et al*. (2009) mentioned that the root-lesion nematode *Pratylenchus penetrans* is the most important nematode affecting apple production. This lesion may exhibit poor growth of "Gala" young apple trees grown on M26 stock apple, stunting and a gradual decline in yields. Severely infected root systems may lack feeder roots. Moreover, the author stated that this lesion can be controled by MeloCon WG (*P. lilacinus* strain 251) at 2 to 4 lb/A plus a soil wetting agent to established plants, although it might be better used when applied to plants just before planting.

Conclusions

- Biofertilizers are important components of integrated nutrients management and renewable source of plant nutrients to supplement chemical fertilizers in sustainable agricultural system.
- Biological fertilizers would play key role in productivity and sustainability of soil and also protect the environment as ecofriendly and cost effective inputs for the farmers.
- Beneficial microorganisms can be used as a tool in the apple orchard to improve greatly growth, yield and fruit quality.
- Biological pest mangement can be aim to reduce the usage of insecticides and maintain a clean environment and food safety, and then human health.

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MINIREVIEW

The Role of *Staphylococcus aureus* in Secondary Infections in Patients with Atopic Dermatitis (AD)

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Abstract

Staphylococcus aureus colonizes the mucous membrane of the nasal vestibule of a significant number of healthy people. These microorganisms are opportunistic pathogens, that in favorable conditions, may cause infections of various course, location or manifestation. Secondary infections emerge in cases when other risk factors contribute to such a change. One of the diseases during which *S. aureus* changes its saprophytic character to a pathogenic one is atopic dermatitis (AD), an allergic skin condition of a chronic and recurrent nature. Patients with AD are highly predisposed to secondary staphylococcal infections due to active *S. aureus* colonization of the *stratum corneum*, damage of the skin barrier or a defective immune response. Microorganisms present in skin lesions destroy the tissue by secreting enzymes and toxins, and additionally stimulate secondary allergic reactions. The toxins secreted by strains of *S. aureus* also act as superantigens and penetrate the skin barrier contributing to a chronic inflammation of the atopic skin lesions. The *S. aureus* species also releases proinflammatory proteins, including enzymes that cause tissue damage. When initiating treatment it is particularly important to properly assess that the onset of the secondary bacterial infection is caused by *S. aureus* and thus justifying the inclusion of antibiotic therapy. Depending on the severity and extent of the staphylococcal infection, topical antibiotics are used, usually mupirocin or fusidic acid, or general antibiotic treatment is introduced. Another therapeutic strategy without antibiotics has given a positive effect in patients.

Key words: *Staphylococcus aureus*, atopic dermatitis (AD), opportunistic infections, secondary staphylococcal infections, skin lesions

Introduction

Atopic dermatitis (atopic eczema, dermatitis eczema, eczematous dermatitis, neurodermititis), (latin: *dermatitis atopica*), also known as endogenous eczema is a chronic, relapsing inflammatory skin disease, which is characterized by skin lesions. The pathogenesis of AD is complex and still not fully understood. It is considered that all the interactions that occur between the genetically determined impairment of the structure and function of the epidermal barrier, the dysregulated immune and inflammatory response, environmental factors and infectious agents are engaged in the pathophysiology of AD (Ring *et al*., 2012). Lichenification of the skin and pruritis, a very burdensome symptom, are distinctive manifestations of AD. The Hanifin and Rajka diagnostic criteria for AD are used to diagnose this skin disease (Hanifin and Rajka, 1980).

Research conducted in recent years has brought two hypotheses to light that attempt to explain the pathogenesis of atopic dermatitis. The first hypothesis assumes that the initiating cause of the development of AD are immunological disorders. The initial stage of AD is characterized by a dominance of Th2 cells that secrete proinflammatory cytokines IL-4, IL-5, IL-13 and IgE. However, AD begins to shift into the chronic phase, Th1 cells begin to dominate. These immune aberrations cause inflammatory changes in the epidermis triggering epidermal barrier dysfunction. This hypothesis is called the "inside-to-outside hypothesis". Another concept presumes that there is a fundamental dysfunction of epithelial cells and so an impaired epidermal barrier. The presence of this defect may lead to the penetration of the epidermal barrier by allergens or other irritants inducing a secondary immune response in the skin. This hypothesis is named the "outside-to-inside hypothesis". Since the discovery of the mutation in the filaggrin (FLG) gene in a significant number of patients, researchers have begun to favour the second hypothesis as an explanation for the onset of AD (Palmer *et al*., 2006; Cork *et al*., 2009; Werfel, 2009; Bussmann *et al*., 2011).

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It is also known that dendritic cells (specifically Langerhans cells), keratinocytes, mast cells and eosinophils are key elements in the pathogenesis of AD. Dysfunction of these regulatory cells initiates a pathological process that leads to the development of symptomsin the course of atopic dermatitis (Werfel, 2009; Boguniewicz and Leung, 2011).

Epidemiology

Statistically, atopic dermatitis is one of the most common allergic conditions after hay fever and asthma. It affects nearly 30% of children and approximately 5% of adults who often develop secondary bacterial infections of the skin lesions (Nakamura *et al*., 2013). AD is an allergic condition that is showing a steady annual growth rate of 4% on a global scale. About 3% of adults suffer from AD in Poland. Residents of larger cities are more prone to develop AD than residents of rural areas (Sybilski *et al*., 2015). AD is often accompanied by other allergic conditions such as atopic asthma or allergic rhinitis (Lis *et al*., 2002).

The course of AD can be divided into the following phases: infantile, childhood, adolescent and adult. These phases are distinguished by the location of skin lesions and course of the disease (Nutten, 2015). Observations show a higher prevalence of AD development in women than in men (Sybilski *et al*., 2015).

Colonization of healthy skin by microorganisms

Changes in the microflora of patients with AD. The composition of a person's normal microflora depends on the colonized area of the human host. Generally however, the human microbial population consists of coagulase negative staphylococci, mainly *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*; *Propionibacterium acnes*, and yeasts of the genus *Malassezia*, most commonly the *Malassezia restricta* and *Malassezia globosa* species. All of these microorganisms are present on the skin surface and in skin folds as well as in hair follicles. Smaller populations of other species may also be identified: *Micrococcus* sp., *Aerobacter* sp. and *Proteus* sp. and in less abundance others (Wiburg *et al*., 1984; Grice and Serge, 2011).

The opportunistic pathogen *Staphylococcus aureus* may be a component of the normal microbial flora of the skin and according to various authors, it may be found in 5% to 100% of healthy individuals. However, a vast majority of people are either permanent or transient carriers of this species in the nasal vestibule (Kloos and Schleifer, 1986).

A characteristic feature of patients with AD is the abundant presence of bacteria of the genus *Staphylococcus* in comparison to healthy individuals (Table I). Results show an increased number of coagulase negative staphylococci that belong to the normal microflora as well as of *S. aureus* that triggers immune responses. Strains of the bacterial species *S. aureus* are present on the skin of patients and/or in the mucous membrane of the nasal vestibule of more than 80% of patients with disease exacerbation and in remission (Soares *et al*., 2013). The number of *S. aureus* cells in AD patients is 100-fold higher than in healthy individuals (Gloor *et al*., 1982; Hauser *et al*., 1985).

S. epidermidis strains found on human skin serve a protective function against *S. aureus* strains. The basis for this phenomenon is the antagonistic effect of *S. epidermidis* which produces antimicrobial peptides. Their task is to fight against other bacteria and to stimulate keratinocytes to produce antimicrobial peptides. The abundant number of *S. aureus* cells on the skin cause an increase in population of *S. epidermidis*, which is an additional burden for the skin (Cogen *et al*., 2010).

Table I
Microorganisms from the normal skin and from patients with atopic dermatitis (AD), in alphabetical order.

Microflora of the skin	normal	AD
Acinetobacter spp.	+	+
Aerobacter sp.	+	–
Brevibacterium spp.	+	+
Candida spp.	+	+
Corynebacterium spp.	+	+
Escherichia coli	+	+
coagulase-negative staphylococci	+	++
Klebsiella spp.	+/–	+
Malassezia spp. (*Pityrosporum* spp.)	+	++
Micrococccus spp.	+/–	+/–
Propionibacterium spp.	+	+
Proteus sp.	+/–	+/–
Salmonella spp.	+/–	+
Staphylococcus aureus	+	++
Streptococcus spp.	+/–	+

S. aureus, a pathogenic species – determinants of pathogenicity

S. aureus is capable of synthesizing virulence factors as well as of their extracellular release. These virulence factors demonstrate high biochemical activity. *S. aureus* exhibits a high resistance to a wide range of antibiotics and other antimicrobial agents.

S. aureus has the ability to adhere to components of the extracellular matrix. Adherence is mediated

by surface adhesins that belong to the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family of proteins. Examples include fibronectin-binding proteins A and B, (FnBPs), collagen-binding proteins, (CnBPs) and fibrinogen-binding proteins (Fb-BPs), (Gordon and Lowy, 2008; Schlievert *et al*., 2010). *S. aureus* produce numerous virulence factors that enable them to penetrate the host's protective barriers, spread into tissue and initiate infection, including the following: alpha-, beta-, delta-, gamma-hemolysins, lipase, serine proteinase (V8 protease) aureolysin (metalloproteinase), hyaluronidase, coagulase, fibrinolysin, staphylokinase, leukocidin, especially the Panton-Valentine leukocidin. Other important virulence factors include enterotoxins (*i.e*. SEA, SEB, SEC, SED, SEE and others), the Toxic shock syndrome toxin-1 (TSST-1) and epidermolytic toxins (Bukowski *et al*., 2010). The pathogenicity of *S. aureus* also depends on the presence of cell wall components such as: protein A, clumping factors (ClfA and ClfB) and teichoic acids and the bacterial capsule polysaccharide. Other notable virulence factors include peptidoglycan and lipoteichoic acid – major constituents of the cell wall (Gordon and Lowy, 2008; Kobayashi and DeLeo, 2009; Krishna and Miller, 2012; Otto, 2014).

S. aureus as an allergen

Colonization of the skin by *S. aureus* in AD causes the immune system to over-respond to their presence, has a toxic effect on keratinocytes and stimulates lymphocytes to secrete interferon (IFN) which consequently leads to the development of the chronic form of AD. The bacteria themselves as well as their excreted metabolites induce the activation of T lymphocytes, macrophages and antigen-presenting cells, leading, inter alia, to the increased production of immunoglobulins E (IgE) and G (IgG). An elevated level of IgE is one of the distinctive signs of an immune response to an allergen. Antistaphylococcal immunoglobin E (IgE) has been identified and measured in patients with AD and its level correlates with the severity of the disease (Adamek-Guzik *et al*., 2001; Ide *et al*., 2004).

Furthermore a high level of IgE in patients is associated with the coexistence of asthma, the length of the AD duration, as well as the severity of pruritus. Additionally, these responses trigger basophil activation (Reginald *et al*., 2011; Petry *et al*., 2012).

Secondary infections with *S. aureus* as a consequence of AD

Secondary infections, also known as superinfections are usually bacterial, fungal or viral infections that occur in the presence of an existing condition. Patients with AD are strongly predisposed to the development of secondary staphylococcal infections as a result of colonization of the *stratum corneum* by the *S. aureus* species, damage to the skin barrier or a defective immune response. Due to the fact that *S. aureus* is present in the skin of almost all patients with AD, the mere presence of these bacteria is not a sufficient criterion for the onset of a secondary infection in skin lesions (Lübbe, 2003; Gong *et al*., 2006).

Skin lesions in AD undergo impetiginization and become crusted, honey-coloured and weeping as a result of secondary infection. Pustules may sometimes appear on the skin of hands and feet (Lübbe, 2003).

The types of infections in atopic dermatitis

Patients with atopic dermatitis are exposed to infections caused by various groups of etiological factors as presented in Table I. The most common include: bacterial infections caused by various species of bacteria with infections predominantly caused by the *S. aureus* species; fungal infections, primarily caused by dermatophytes, *Malassezia* spp. and *Candida* spp., and viral infections, dominantly caused by the herpes simplex virus (HPV) and human parvovirus (HPV). However, epidemiological studies clearly indicate that greatest significance is given to infections caused by *S. aureus* or fungi (Ring *et al*., 1992; Lübbe, 2003; Sonesson *et al*., 2013).

Factors favoring *S. aureus* infections in patients with AD

The predisposition of patients with AD to the development of staphylococcal infections is associated with a defective epidermal barrier, raised adhesion activity of bacteria to skin cells, impaired elimination of these bacteria and impaired innate and acquired immunity. Abnormal lipid composition of the *stratum corneum*, sphingosine level reduction, altered skin pH values, low concentrations of IgA sweat gland secretions and a shortage of antimicrobial peptides, specifically cathelicidin LL-37, beta-defensins HBD-1, HBD-2, and HBD-3, as well as dermacidin, all of which have been reported in patients with AD, promote skin colonization by *S. aureus* and seriously hinder the elimination of the infectious agent. Additionally, scratching the affected area and use of various topical treatments makes patients with AD particularly vulnerable and prone to infections (Roll *et al*., 2004; Boguniewicz and Leung, 2011).

Virulence factors produced by *S. aureus* in atopic dermatitis. Microorganisms that are present in skin lesions cause tissue damage by secreting enzymes and toxins as well as by stimulating secondary allergic

responses. Bacterial strains, particularly *S. aureus* strains, isolated from the skin of patients with AD are capable of producing many toxins and enzymes such as aureolysin, serine proteinase (V8 protease) or phenol-soluble proteins known as Phenol-soluble modulins (PSMs), (Baran-Raunstrup *et al.* 1998; Międzobrodzki *et al.*, 2002; Rojo *et al.*, 2014). Staphylococcal enterotoxins, the toxic shock syndrome toxin-1 and other toxins such as alpha-toxin, play an important role in the development and sustenance of secondary infections in atopic skin lesions (Bogdali *et al.*, 2016). Enzymes and other substances that are secreted by bacteria into the tissue of the skin lesions during secondary infections are equally important. The structural components of bacterial cells such as the cell wall peptidoglycan or the staphylococcal pigment are also present significance (Lomholt *et al.* 2005; Soares *et al.*, 2013).

Staphylococcal superantigens – mechanism of action. *S. aureus* strains isolated from the skin of patients with AD release toxins, such as staphylococcal enterotoxins A, B, C (staphylococcal enterotoxin A, B, C) and the Toxic shock syndrome toxin-1 (TSST-1) which penetrate the epidermis and interact with the various cell types involved in the immune response, leading to an inflammatory response orchestrated by T cells. These virulence factors act as superantigens and are produced by almost 70% of *S. aureus* strains (Abeck and Mempel, 1998; Leung *et al.*, 2004; Soares *et al.*, 2013). Staphylococcal superantigens (SSAg,) trigger T cell activation by binding non-specifically to the T cell receptors (TCR) without the need for antigen presentation (Otto, 2014). Superantigens penetrate the skin barrier and contribute to the development of a chronic inflammation in the atopic skin lesions. Toxins stimulate lymphocytes to excessively produce cytokines such as IL-4. Additionaly, they promote the production of IgE against SSAg that activate mast cells and basophils to release inflammatory mediators. The direct stimulation of antigen presenting cells (APC) and keratinocytes causes the release of proinflammatory cytokines such as IL-1, TNF-α and IL-12 which increase the influx of T cells into the skin lesions. Superantigens may additionally make T cells unresponsive to topical glucocorticoids (GSs), making patients with AD insensitive to treatment with topical GSs. That is why combined treatment of atopic eczema with a weak glucocorticoid and antibiotic is more effective than treatment with only the potent topical corticosteroid (Schlievert *et al.*, 2008; Na *et al.*, 2012; Orfali *et al.*, 2015).

Other *S. aureus* virulence factors associated with AD. *S. aureus* strains are a source of proinflammatory proteins, *inter alia*, protein A, and have receptors with a high affinity to extracellular matrix proteins such as: collagen (collagen-binding protein, Cn-BP), fibrinogen (fibrinogen-binding protein, Fb-BP), lactoferrin (lactoferrin-binding protein, Lf-BP), and fibronectin (fibronectin-binding protein Fn-BP) (Międzobrodzki *et al.*, 1989; Naidu *et al.*, 1991). IL-4 and IL-13 which are present in the acute phase of eczema due to the increased expression of fibronectin and fibrinogen, enhance the adherence of staphylococci to inflamed skin.

The staphylococcal peptidoglycan induces the production of various cytokines including GM CSF (granulocyte macrophage colony-stimulating factor), a cytokine that is produced in excess in AD (Matsubara *et al.*, 2004).

Other enzymes produced by *S. aureus* also play an important role in the process of infection. Published show that *S. aureus* isolated from AD patients reports exhibits higher proteolytic activity than those isolated from healthy individuals without AD (Międzobrodzki *et al.*, 2002). These enzymes are known to not only cause damage to the skin barrier, facilitating the penetration of allergens and irritants, but can also modify endogenous protease inhibitors, initiate and enhance proinflammatory and allergic responses of the human immunology system and trigger the secretion of IgE by activating Th2 cells. Among the different significant enzymes that are involved in the development of secondary staphylococcal infections in patients with AD, phenol-soluble modulins (PSMs) (Cheung *et al.*, 2014) and the Panton-Valentine leukocidin (PVL) should be mentioned (Cavalcante *et al.*, 2015). These enzymes have the capacity to lyse hosts cells enable *S. aureus* to evade immune response. Moreover, *S. aureus* strains secrete proteins that inhibit chemotaxis (chemotaxis inhibitory protein of staphylococci – CHIPS), impairing the function of neutrophils. The onset and development of the infection also reduces monocyte chemotaxis (Ternowitz and Herlin, 1986; Międzobrodzki and Kaszycki, 2000). The golden carotenoid pigment and superoxide dismutase enzymes also play an important role in staphylococcal infections. These factors, secreted by *S. aureus*, inhibit the production of reactive oxygen species by the host's neutrophils (Międzobrodzki *et al.*, 2008; Krishna and Miller, 2012). Another important virulence factor that contributes to the onset of secondary staphylococcal infections in patients is aureolysin (metalloproteinase). This enzyme inhibits the activity of antimicrobial peptides such as cathelicidins. Furthermore, aureolysin is involved in the activation of other proteases secreted by *S. aureus* mainly serine proteases (Sabat *et al.*, 2008; Foelster Holst *et al.*, 2010). The alpha-toxin has also shown to be an important virulence factor which can quickly induce the release of TNF-alpha, arachidonic acid and platelet activating factor (PAF) from keratinocytes. The toxin forms transmembrane channels that act similarly to calcium channels (Jahreis *et al.*, 2000). Two other staphylococcal proteins

also play an essential role in the pathogenesis of AD, namely NP-taze and p70. These proteins induce the secretion of IL-2 and IFN-gamma from the mononuclear cells isolated from the peripheral blood of patients with AD (Jahreis *et al*., 2000).

Treatment of *S. aureus* infections in AD

In clinical practice, it is essential to properly assess that the onset of the secondary bacterial infection caused by *S. aureus* and to distinguish it from the appearing skin lesions, that have not been affected by AD, and thus justifying the inclusion of antibiotic therapy. Leyden and colleges proposed a quantitative approach to this question after having observed an increased effectiveness of antibiotic treatment of *S. aureus* infections when cell concentrations on the skin were above 10^6 CFU per 1 cm^2 (CFU – colony forming unit). It turns out that quantitative bacteriology is not always possible in a clinical setting, that is why it is recommended to firstly administer antibiotics, after having performed an antibiogram, for a period of 1 to 2 weeks after the appearance of the impetignized skin lesions, and then continue treatment with topical corticosteroids (Leyden *et al*., 1974).

Depending on the severity and extent of the staphylococcal infection, topical antibiotic therapy may be used or general antibiotic treatment is initiated. Affected areas of the skin may be treated by applying topical mupirocin or fusidic acid. Fusidic acid is available only in some European countries, North America and Oceania, excluding the United States. In recent years, more strains resistant to fusidic acid have appeared (fusidic acid-resistant *S. aureus*, FRSA), and so it is advisable to limit its use. Mupirocin is also often used to eradicate *S. aureus* carriage in patients with AD and their family members who are prone to frequent infections. When using this antibiotic it is essential to strictly follow the dosage regimen because there is a risk of placing selective pressure on the strain leading to its resistance to this antibiotic (Petry *et al*., 2012; Gelmetti, 2008).

The treatment regimen recommended for patients with a widespread or severe secondary staphylococcal infection include antibiotics such as erythromycin and next-generation macrolides, *i.e*. azithromycin or clarithromycin. In cases where *S. aureus* strains are resistant to macrolides, the application of penicillinase-resistant penicillins (dicloxacillin, oxacillin, cloxacillin) or next-generation cephalosporins is recommend (Gelmetti, 2008).

A series of studies has also been conducted that report the use of oral antihistamines and topical steroids in the treatment of AD without the use of antibiotics. Results showed that *S. aureus* had been eradicated from the skin in 70% of the patients. In the group of patients where *S. aureus* was not eliminated completely tests revealed an elevated level of IgE and a diminished proliferation of lymphocytes in response to SEB (Guzik *et al*., 2005).

Conclusions

Skin diseases, including atopic dermatitis, are not directly classified as life-threatening diseases. However, the symptoms and burden of the disease cause negative consequences in the lives of patients affected by them. The onset of a secondary infection caused by *S. aureus* in AD patients further increases the patient's burden, complicates and hinders treatment, as well as delays remission. This opportunistic bacterial species has a wide range of virulence factors that significantly exacerbate the disease. Toxins, enzymes and bacterial cell wall components trigger a strong immune response by the patient with a developing secondary infection. Data show that the number of patients with AD is steadily increasing. Finding effective medicines, as well as planning a comprehensive therapeutic strategy aimed both at treating disease symptoms and secondary infections that frequently accompany AD, is considered to be a big challenge both for science and practise. In order to obtain the desired results, it is essential to continue conducting research on both predisposed patients, as well as on the pathogenesis of infections caused by the opportunistic pathogen *S. aureus*.

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ORIGINAL PAPER

Molecular Characterization of Shiga Toxin-Producing *Escherichia coli* Strains Isolated in Poland

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) strains also called verotoxin-producing *E. coli* (VTEC) represent one of the most important groups of food-borne pathogens that can cause several human diseases such as hemorrhagic colitis (HC) and hemolytic – uremic syndrome (HUS) worldwide. The ability of STEC strains to cause disease is associated with the presence of wide range of identified and putative virulence factors including those encoding Shiga toxin. In this study, we examined the distribution of various virulence determinants among STEC strains isolated in Poland from different sources. A total of 71 Shiga toxin-producing *E. coli* strains isolated from human, cattle and food over the years 1996–2010 were characterized by microarray and PCR detection of virulence genes. As *stx1a* subtype was present in all of the tested Shiga toxin 1 producing *E. coli* strains, a greater diversity of subtypes was found in the gene *stx2*, which occurred in five subtypes: *stx2a, stx2b, stx2c, stx2d, stx2g*. Among STEC O157 strains we observed conserved core set of 14 virulence factors, stable in bacteria genome at long intervals of time. There was one cattle STEC isolate which possessed verotoxin gene as well as *sta1* gene encoded heat-stable enterotoxin STIa characteristic for enterotoxigenic *E. coli*. To the best of our knowledge, this is the first comprehensive analysis of virulence gene profiles identified in STEC strains isolated from human, cattle and food in Poland. The results obtained using microarrays technology confirmed high effectiveness of this method in determining STEC virulotypes which provides data suitable for molecular risk assessment of the potential virulence of this bacteria.

Key words: *Escherichia coli*, microarray detection, Shiga toxin-producing, virulence gene

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains also called verotoxin-producing *E. coli* (VTEC) represent one of the most important groups of food-borne pathogens that can cause several human diseases such as hemorrhagic colitis (HC) and hemolytic – uremic syndrome (HUS) (EFSA, 2010; Nataro and Kaper, 1998). Over 400 serotypes of STEC have been isolated from human. However, the majority of clinical STEC infections, particularly those associated with outbreaks and serious patient outcomes, are attributable to a subset of serogroups including *E. coli* O157:H7 (the prototype bacterium for enterohaemorrhagic *E. coli* – EHEC), O26: H11, O103:H2, O111:H8, O121:H19 and O145:H28 (Nataro and Kaper, 1998). In recent years, new serotypes of EHEC have also emerged (Bielaszewska *et al.*, 2011).

The ability of STEC strains to cause disease is associated with the presence of a wide range of identified and putative virulence factors including those encoding Shiga toxin (Nataro and Kaper, 1998). The Stx family consists of 2 major groups, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), which are well distinguished by their amino acid sequences. Several other virulence factor are known, including genes conferring the ability to cause attaching-effacing lesions located on LEE pathogenicity island. The LEE encodes intimin (Eae), translocated intymin receptor (Tir), a type III secretion system (EspA, EspB, EspD) and effector proteins translocated by the secretion system. Beyond Stx and LEE genes, "typical STEC" associated with human disease usually carry virulence factors encoded by 60-MDa plasmid as enterohemolysin (Ehx), serine protease (EspP), catalase peroxidase (KatP) and a type II secretion system (EtpD) (Nataro and Kaper, 1998).

As more atypical STEC strains have been reported, several proteins have been proposed as potential virulence determinants as autoagglutinating adhesion (Saa), subtilase cytotoxin (SubA), non-LEE encoded effectors (Nle) (Coombes *et al.*, 2008; Bugarel *et al.*, 2010; Paton and Paton, 2010). Moreover, recently there have been many scientific reports describing the presence of STEC isolated from humans, animals and the environment,

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carrying specific virulence determinants characteristic for different *E. coli* pathotypes as intermediate STEC/ETEC or EAEC/STEC virulence factor profiles (Bielaszewska *et al*., 2011; Prager *et al*., 2011). Therefore determining the virulence potential of STEC relies upon the determination of somatic and flagellar antigens, together with the identification of virulence genes what can be used to make a "molecular risk assessment" of the predict potential virulence of strains (Coombes *et al*., 2008).

Although the number of STEC isolates reported yearly is relatively low in Poland (few cases of human STEC infections), they are often responsible for serious illnesses or HUS complications (Jakubczak *et al.*, 2008; EFSA, 2010; Januszkiewicz *et al.*, 2010; 2012). What is interesting one outbreak of STEC infection related to international outbreak of STEC O104:H4 in 2011 was reported in Poland (Januszkiewicz *et al.*, 2012). There is no data containing molecular characterization of Shiga toxin-producing *E. coli* strains isolated in our country both from human infections as well as STEC strains isolated from animal and food. The aim of our study was to investigate the distribution of various virulence determinants among STEC strains isolated in Poland from different sources. To achieve our goal we used commercially available microtube array system designed to detect virulence genes in *E. coli* of various pathotypes.

Experimental

Material and Methods

Bacterial strains. A total of 71 Shiga toxin-producing *E. coli* (STEC) strains were analyzed (Table I). Those isolates were from collection of Department of Bacteriology (n = 38), Department of Food and Consumer Articles Research (n = 4) of National Institute of Public Health – National Institute of Hygiene (NIPH – NIH) and Department of Hygiene of Food of Animal Origin in National Veterinary Research Institute (NVRI) in Puławy (n = 29). STEC strains collected during 1996–2010 were isolated from human (n = 45), cattle (n = 16) and food (n = 10) and were classified into following groups: serotype O157 (n = 45), serotype O26 (n = 10), serotype O111 (n = 6) and NT (not typable using available Biomex latex assay) (n = 10). More than 90% of tested human STEC O157 isolates were from reported human cases in our country. All of them were from sporadic cases. Tested STEC strains presented different band patterns when typed with pulsed-field gel electrophoresis (PFGE) using XbaI enzyme (data not showed) which indicates that they had no epidemiological link.

Identification and virulence analysis of STEC strains. Classical tube test was used for *E. coli* re-identification. Serotyping was performing using latex agglutination test (Biomex). Stx production was confirmed by VTEC-RPLA assay (Oxoid). The cytotoxity assay with Vero cell monolayers (VCA) in 96 – well plates was performed as described previously (Januszkiewicz *et al*., 2010). The presence of *stx1*, *stx2* and *eae* genes were examined by PCR based methods according to protocols described previously (Januszkiewicz *et al*., 2010). Detection of Stx gene subtypes was performed by PCR based method according to WHO protocol (Scheutz *et al*., 2012). Presence of H7 antigen (*fli*C gene) in STEC O157 isolates was performed by PCR-RFLP (Januszkiewicz *et al*., 2010). A DNA microarray (Identibac Ec v. 03, Alere Technologies GmbH) was used to determine the presence of virulence genes among STEC strains. The microarrays were used according to the manufacturer's instructions. Array images were processed in IconoClust 3.0 (Clondiag, Germany) and signals were analyzed using the *gap*A-positive control gene for normalization and with cut-offs as recommended by manufacturer (> 0.4 = present, 0.4 to 0.3 = ambiguous, < 0.3 = absent, relative to the *gap*A signal).

Results

Phenotypic and genotypic assays of STEC identification. For a summary of results, see Table I and II. All of the STEC isolates produced a cytopathic effect on Vero cell monolayers, confirming their ability to express verotoxins. The Stx-cytotoxic activity was neutralized with antiserum. Production of appropriate verotoxins: Stx1, Stx2 or both were confirmed in tested strains by the RPLA assay. Genetic hallmarks of a appropriate toxins (*stx1* and *stx2*) and intimin (*eae*) were detected by PCR (Table I). All STEC O157 strains were positive for *fli*C (H7) gene. PCR subtyping of the *stx1* gene revealed subtype *stx1a* in all isolates which produced Stx1 toxin (Table II). STEC isolates produced Stx2 toxin possessed various subtypes of *stx2* gene: *stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2g* (Table II). Stx2c subtype was produced by STEC O157 only.

Microarray analysis. All tested STEC isolates were positive for control, species-specific genes *gad*, encoding glutamate decarboxylase and *ihf*A, encoding integration host factor subunit alpha. Genes known to be present in control strain STEC O157 EDL933 and which were represented on the array were successfully detected. There were genes encoding: Shiga toxins (*stx1, stx2*); adhesins: an outer membrane protein important for the attachment to host cells – intimin (*eae*A) and adherence-conferring protein (*iha*); toxins: heat stable enterotoxin (*ast*A), enterohemolysin (*ehx*), cytotoxin B (*tox*B); type III secretion system: non-LEE-encoded effector protein A (*nle*A), non-LEE-encoded effector protein B (*nle*B), non-LEE-encoded effector

Table I

Virulence genes in STEC isolated in Poland. Definition of virulence types (virulotypes) based on present genes/gene variants (●) as determined by Identibac Ec v. 3 microarray assay. Only genes positive for at least one STEC strain are showed. The genes present in at least 90% of STEC strains are highlighted: for STEC O157 in dark grey, for STEC O26 in grey, for STEC O111 in bright grey color. Black color marks the genetic determinants present only in single isolates. NT – isolates which were not typable using commercially available latex (Biomex Sp. z o.o.). The *stx* subtypes (bold black frame) were determined by PCR according to WHO protocol (WHO protocol).

Virulotype	Serotype	Source	No of strain	*stx1* subtype	*stx2* subtype	*stx1AB*	*stx2AB*	*eae*	*tir*	*nleA*	*espJ*	*iha*	*espF*	*ehx*	*tccP*	*etpD*	*nleC*	*espP*	*katP*	*toxB*	*nleB*	*cif*	*lpfA*	*espA*	*efa1*	*iss*	*espB*	*astA*	*cba*	*cdtB*	*celB*	*espI*	*mchA*	*mchB*	*mchF*	*mchC*	*saa*	*subA*	*st1a*	*epeA*	*ironN*
V12	O157	human	19/99	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	human	443/07	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	human	474/07	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	human	568/99	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	human	4/10	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	human	199/01	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
V2	O157	human	44/02	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●													
	O157	human	247/02	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●													
	O157	human	225/96	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●													
	O157	human	598/03	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●													
	O157	human	49/04	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●													
	O157	human	214/04	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●													
	O157	human	365/05	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●													
	O157	human	206/06	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●													
V3	O157	human	171/09		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	human	441		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	cattle	420		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	cattle	418		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	food	345/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	food	346/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
	O157	human	440/98		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●													
V4	O157	human	98/08	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																				
	O157	human	251/10	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																				
	O157	human	448	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																				

Table I
Continued

Virulotype	Serotype	Source	No of strain	*stx1* subtype	*stx2* subtype	*stx1AB*	*stx2AB*	*eae*	*tir*	*nleA*	*espJ*	*iha*	*espF*	*ehx*	*tccP*	*etpD*	*nleC*	*espP*	*katP*	*toxB*	*nleB*	*cif*	*lpfA*	*espA*	*efa1*	*iss*	*espB*	*astA*	*cba*	*cdtB*	*celB*	*espI*	*mchA*	*mchB*	*mchF*	*mchC*	*saa*	*subA*	*st1a*	*epeA*	*ironN*
V5	O157	food	349/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																				
	O157	food	445		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																				
V6	O157	food	1124	a		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●			●																
	O157	food	1128	a		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●			●																
VV7	O157	food	1125	a		●		●	●		●	●	●	●	●	●	●	●	●	●	●			●																
	O157	food	1126	a		●		●	●		●	●	●	●	●	●	●	●	●	●	●			●						●										
V8	O157	human	442		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																			
V9	O157	food	348/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●						●										
V10	O157	human	174/03		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●												
V11	O157	human	443	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●												
	O157	human	221/08	a	a	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●												
V12	O157	human	286/00		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●																
V13	O157	human	577/01	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		●	●																
V14	O157	human	175/06	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●												
V15	O157	cattle	447	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●								●											
V16	O157	Food	347/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●		●										
V17	O157	human	74/10		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●		●		●												
V18	O157	human	535/97		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																			
V19	O157	human	440	a		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●			●		●		●												
V20	O157	human	9/08		a		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●		●										
V21	O157	human	371/08		a		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●		●										
V22	O26	human	319/01	a		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●			●		●	●			●										
V23	O26	human	706/98	a		●		●	●	●	●	●	●	●	●	●		●	●	●			●	●	●	●	●	●												
V24	O26	human	50/97	a		●		●	●	●	●	●	●	●	●	●		●	●		●		●	●	●	●	●													

Table I
Continued

Virulotype	Serotype	Source	No of strain	*stx1* subtype	*stx2* subtype	*stx1AB*	*stx2AB*	*eae*	*tir*	*nleA*	*espJ*	*iha*	*espF*	*ehx*	*tccP*	*etpD*	*nleC*	*espP*	*katP*	*toxB*	*nleB*	*cif*	*lpfA*	*espA*	*efa1*	*iss*	*espB*	*astA*	*cba*	*cdtB*	*celB*	*espI*	*mchA*	*mchB*	*mchF*	*mchC*	*saa*	*subA*	*st1a*	*epeA*	*iroN*
V25	O26	human	169/97	a		●		●	●	●	●	●	●	●	●		●	●	●	●		●	●	●	●	●	●	●					●	●	●	●					
V26	O26	human	201/01		a		●	●	●	●	●	●	●	●	●		●	●	●		●	●	●	●	●	●	●			●											
V27	O26	human	368/01		a		●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●	●	●													
V28	O26	human	6/01	a	a		●	●	●	●	●		●	●			●		●			●	●	●	●	●	●														
V29	O26	human	317/01		B		●	●	●	●	●		●	●			●				●	●	●	●	●	●															
V30	O26	human	318/01		B		●	●	●	●	●	●	●		●		●				●	●	●	●	●	●															
V31	O26	human	237/05	a		●		●	●	●	●		●	●	●		●	●	●			●	●	●	●	●															
V32	O111	human	479	a		●		●	●	●	●	●	●		●						●	●	●	●	●				●												
	O111	cattle	477	a		●		●	●	●	●	●	●		●						●	●	●	●	●				●												
V33	O111	human	480	a		●		●	●	●	●	●	●	●	●						●	●	●	●	●				●												
V34	O111	human	481	a		●		●	●	●	●	●	●	●	●						●	●	●	●	●				●												
V35	O111	human	245/10		a		●	●	●	●	●	●	●	●	●						●	●	●	●	●				●												
V36	O111	cattle	483	a		●		●	●	●	●	●	●	●			●	●	●		●	●	●	●	●	●	●	●												
V37	NT	cattle	100/00	a		●		●	●	●	●		●	●	●		●		●		●	●	●	●	●	●		●												
V38	NT	cattle	102/00	a		●		●	●	●	●		●	●	●				●		●	●	●	●	●	●														
V39	NT	cattle	476	a		●		●					●	●	●									●	●		●					●								
V40	NT	cattle	474	a		●		●					●	●		●								●	●		●					●								
V41	NT	cattle	473	a		●		●					●	●										●	●		●					●								
V42	NT	cattle	475	a		●		●		●	●			●	●	●		●															●							
V43	NT	cattle	101/00	a		●		●														●	●				●												
V44	NT	cattle	489		g		●							●					●									●									●			
V45	NT	cattle	449	a		●					●							●								●			●	●		●		●					●
V46	NT	cattle	478		d		●					●		●				●					●							●	●						●			

Table II
Prevalence of *stx1* and *stx2* subtypes in the genome of 71 STEC strains

STEC serotype (number of strains)	Number of STEC strains with the *stx* gene subtype:								
	stx1	*stx2*						*stx1* + *stx2*	
	stx1a	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2g*	*stx2a* + *stx2c*	*stx1a* + *stx2a*	*stx1a* + *stx2c*
O157(n = 45)	5	2	0	11	0	0	5	1	21
Non-O157 (n = 26)	18	4	2	0	1	1	0	0	0
Total	22	7	2	11	1	1	5	1	21

protein C (*nle*C), EspA protein (*esp*A), EspF effector protein (*esp*F), non-LEE-encoded EspJ effector protein (*esp*J), Tir – cytoskeleton coupling protein (*tcc*P); type II secretion pathway related protein (*etp*D); serin protease autotransporters – SPATE (*esp*P); peroxidase-katalase (*kat*P) and translocated intimin receptor (*tir*).

All STEC O157: H7/H-isolates (n = 45) tested positive for markers encoding adhesins (*eae*A, *iha*), toxins (*ehx*, *tox*B), secretion systems proteins (*nle*A, *nle*B, *nle*C, *esp*F, *esp*J, *etp*D, *tcc*P), serin protease autotransporters – SPATE (*esp*P), peroxidase-katalase (*kat*P) and translocated intimin receptor (*tir*), while genes encoding heat stable enterotoxin AstA, colicin B activity protein Cba and cytolethal distending toxin subunit B CdtB, secretion III system protein EspA, Efa adhesin and assiociated with increased serum survival marker Iss were variable absent or present (Table I).

All STEC O26 (n = 11) and STEC O111 (n = 6) were positive for 13 and 11 markers presented on the array respectively (Table I). A positive spot signal in STEC O26 was detected for 13 genes encoding adhesins (*eae*, *iha*, type III secretion system EspB protein – *esp*B and lymphocyte inhibitory factor A-*efa1*), toxin (*ehx*), secretion systems proteins (*nle*A, *esp*A, *esp*F, *esp*J and cell cycle inhibiting factor – *cif*), major fimbrial subunit (*lpf*A), assiociated with increased serum survival marker *iss* and translocated intimin receptor (*tir*), while few strains were additionally present for genes encoding AstA, ToxB, CelB, Cba toxins, microcin M truncated protein MchcA, microcin H47 activity protein MchcB, putative microcin L transport protein MchcF, member of the microcin operon MchcC, secretion systems proteins (NleB, NleC, TccP) and EspP and KatP.

All STEC O111 (n = 6) were positive for 11 markers presented on the array encoding: adhesins (*eae*A, *iha*, *efa1*), secretion systems proteins (*nle*A, *nle*B, *esp*A, *esp*F, *esp*J, *cif*), major fimbrial subunit *lpf*A and translocated intimin receptor *tir* while adhesin *esp*B, toxins (*ehx*, *ast*A, *cel*B, *cba*), secretion systems proteins (*nle*C, *tcc*P) *esp*P, *kat*P and *iss* were variable present or absent.

Virulence gene profile. Based on presence or absence of virulence genes the tested STEC isolates were divided into 46 virulotypes (see Table I for details). Among 45 STEC O157 tested strains 21 virulotypes were distinguished (from V1 to V21) (Table I). STEC non-O157 strains isolated from cattle (n = 10), not typable using available latex assay, had different panel of virulence markers than tested isolates belonging to O157, O111 and O26 serotypes. Among STEC non-O157 strains isolated from cattle there were two strains which possessed virulence markers not present in any tested isolates. One of those strains (no. 478) possessed in *sub*A, *saa* and *epe*A genes (Table I) which are considered to encode subtilase toxin, autoagglutinating adhesion and serine protease autotransporters of *Enterobacteriaceae* (SPATE) respectively. The second strain (no. 489) had *st1a* gene encoded heat-stable enterotoxin ST1a and the third strain (no. 449) possessed *iro*N marker, encoded outer membrane protein receptor for siderophore.

Discussion

Besides Stx toxins, it is known that the STEC strains carry other virulence markers *E. coli* engagement in pathogenesis of STEC infection (Nataro and Kaper, 1998). Those virulence markers could be typical for STEC pathotype or acquire from other pathogenic *E. coli*. Therefore, in this study we conducted a comprehensive analysis of virulence gene profile in STEC strains from different origin isolated in Poland. To have the opportunity to detect a large number of target sequences present in genome of different *E. coli* pathotypes, the commercially available DNA microtube array was used. To the best of our knowledge, such a wide range of determinants of pathogenesis of STEC isolated in Poland have never been defined.

Using microarray data analysis the STEC strains isolated in Poland have been distinguished into 46 virulotypes. Interestingly, among STEC O157 strains, which constituted the most representative serological group in investigated strains collection, we observed conserved core set of virulence factors. Apart from the Stx toxin, all isolates possessed the LEE-associated genes for intimin (*eae*A), the translocated intimin receptor (*tir*) and the effector protein (*esp*F). Large virulence plasmid pO157 genes for enterohemolysin (*ehx*), toxin B (*tox*B),

type II secretion pathway related protein (*etp*D) were detected in all STEC O157 isolates. Moreover all isolates tested positive for the gene for adhesin Iha, *tcc*P and *esp*J markers and finally genetic markers for effector proteins NleA, NleB, NleC were present in all STEC O157 isolates assayed. Moreover, in the group of STEC O157 strains there were human, animal and food isolates which were isolated at long intervals of time. This data suggest that those panel of virulence factors might be essential for the survival of STEC O157 in a host organism and causing the infection.

These data are in agreement with the results of other researchers (Wu *et al.*, 2008; Söderlund *et al.*, 2010; Bugarel *et al.*, 2011). They confirmed that the panel of genetic determinants of virulence in the STEC O157 strains genomes are highly typical for this serotype. It is worth noting that only Söderlund *et al.* (2010) used commercially available EC 03 array to define virulence genetic determinants in STEC O157 strains so far. Results of the Swedish researchers clearly showed the presence of stable core of virulence markers in STEC O157 genome isolated from cattle (Söderlund *et al.*, 2010). The range of virulence determinants identified in STEC O157 strains using Array Tube Ec03 was the same as examined in the presented study.

Interestingly, we observed the stability of virulence determinants composition in STEC O157 genome for long intervals of time. It was in fact the presence of the same virulotypes in strains from different sources isolated in Poland in different years (virulotypes: V1, V2, V3, V4) (Table I). This may indicate the correlation between this virulotype and serotype and suggest that the set of genes, observed in the majority of STEC O157 isolates in the world, might be essential for the survival of these organisms in the host organism and induce them to infection.

Next to Shiga toxins, intimin, encoded by LEE pathogenicity island, is the main virulence marker, whose presence is routinely determined in diagnostics of clinical STEC isolates. It is important factor in pathogenesis of EPEC and STEC isolates because it enables direct contact between the bacterial cell surface and epithelial cells of the intestine. Our results revealed that almost all STEC isolates possessed *eae* gene. Only four STEC non-O157 strains isolated from cattle were included in the LEE – negative group (not carry *eae* gene). LEE-negative STEC non-O157 strains have been isolated from human and animals from several years (Galli *et al.*, 2010; Irino *et al.*, 2010). Those isolates may carry other adhesion factors as LpfA, Iha and Saa which play role in pathogenesis of human infection of this bacteria (Galli *et al.*, 2010; Irino *et al.*, 2010). The results presented in the hearing, as well as the observations made by other authors, lead to the conclusion that the presence of LEE-negative STEC non-O157 strains is common and therefore there is a need to pay special attention in the diagnosis of infections caused by them.

Interestingly, there was one strain (no. 478) among LEE-negative STEC isolates which carried genetic determinant responsible for production of the other cytolysin – subtilase (SubAB). According to scientific reports, STEC strains producing subtilase were isolated from people with HC and HUS (Galli *et al.*, 2010; Irino *et al.*, 2010; Paton and Paton, 2010). Characteristic of genetic markers in subtilase – producing STEC isolates were described by other authors (Galli *et al.*, 2010; Irino *et al.*, 2010). The isolates no. 478 possessed *saa*, *epe*A, *iha*, *ehx*, *esp*P, *lpf*A, *cdt*B and *cel*B, but did not carry *eae* and *tir* markers. Study conducted in Argentina by Galli *et al.* (2010) have shown that among the VTEC strains isolated from humans with HC and HUS, there were isolates which had *sub*AB, *saa*, *lpf*A, *ehx*, *iha*, *cdt*B markers. Similar observations made Irino *et al.* (2010), who showed that the subtilase – producing *E. coli* strains which all produced enterohemolysin, they also had the *saa*, *lpf*A, *iha* genes responsible for the expression of adhesins. Understanding the profile of genetic determinants in the genome of LEE – negative STEC strains may determine their pathogenicity potential, resulting from the co-occurrence of rare genetic markers present.

Recently there have been many scientific reports describing the presence of *E. coli* isolated from humans, animals and the environment, carrying specific virulence determinants characteristic for different *E. coli* pathotypes (Bielaszewska *et al.*, 2011; Prager *et al.*, 2011). Using microarray technology it was possible to confirm the presence of a wide range of genetic markers present in different *E. coli* pathotypes in a single experiment. The results of our analysis showed that among 71 STEC strains there was one isolate (no. 489) which possessed Shiga toxin gene as well as *sta1* gene encoded heat-stable enterotoxin STIa characteristic for enterotoxigenic *E. coli*. Moreover this isolate as the only in the STEC collection in this study produced Stx2g subtypes. Our results indicate that in our country there may exist *E. coli* strains with specific genetic determinants for both Shiga toxin producing (STEC) and for enterotoxigenic (ETEC) *E. coli*. The occurrence of strains with similar properties were also found in Germany (Prager *et al.*, 2011). Molecular characterization of 24 Shiga-toxin producing isolates producing Stx2g, obtained from clinical material, animals and the environment, have found that all tested isolates, in addition to *stx2g* gene carry the *st1a* gene and did express STIa, which typically is associated with enterotoxin-producing *E. coli* (Prager *et al.*, 2011). The emergence of infections in humans strains with combination of virulence genes of different pathotypes of intestinal pathogenic *E. coli* may indicate the spread in the environment of a new, intermediate and emerging pathotype

(Bielaszewska *et al*., 2011; Mellmann *et al*., 2011; Prager *et al*., 2011). This phenomenon was observed in the current year, during one of the biggest so far HUS outbreaks in Europe, which caused an epidemic strain of *E. coli* O104: H4. This strain exhibited features of two *E. coli* pathotypes: enteroaggregative (EAEC) and Shiga toxin producing (STEC) (Bielaszewska *et al*., 2011; Mellmann *et al*., 2011). Given a rising number of intermediate pathotypes becoming described among *E. coli,* a wider range of virulence markers should be included in the regular pathotype diagnostics.

In the routine diagnosis of infections caused by STEC tests are also used based on the amplification of gene fragments encoding Shiga toxins. The Shiga toxin genes occur in many different nucleotide sequences-subtypes (Scheutz *et al*., 2012). Therefore in the routine identification of the subtypes of Shiga toxins genes in the genomes of STEC strains is extremely important because it provides valuable data that are used in monitoring of the STEC infections in many countries (Leotta *et al.*, 2008). In addition, this method is applied in the routine diagnostics of STEC infections, especially in the situation of carrying out epidemiological investigations during outbreaks caused by these microorganisms (Bielaszewska *et al*., 2011; Scheutz *et al*., 2011).

It is worth noting that among the STEC producing Stx2, isolated in Poland (n = 49), the subtype *sxt2c* (n = 37) was dominated. Moreover, these subtype was present only in STEC from O157 serotype and performed alone (n = 11) or with *stx1a* (n = 21) or *stx2a* (n = 5). These results are accordance with the results obtained by other authors (Leotta *et al*., 2008; Aspán and Eriksson, 2010; Käppeli *et al*., 2011). In Switzerland all STEC O157 strains (n = 44) isolated from people from 2000 to 2009 years possessed *stx2a* or *stx2c* subtypes (Käppeli *et al*., 2011). According to Eriksson and Aspan studies, STEC O157 strains isolated from cattle in Sweden carried *stx2a* or *stx2c* subtypes (Aspán and Eriksson, 2010). These subtypes of *stx2a* gene also predominated in STEC O157 strains isolated in Argentina, Australia and New Zealand (Leotta *et al.*, 2008).

It must be underline that the determination of the subtypes of Shiga toxin genes is extremely important because it provides information about the pathogenic potential of the STEC strain. Several studies have revealed that the individual *stx* subtypes differ in biological activity which were observed using various animal models and *in vivo* cytotoxicity assay (Fuller *et al*., 2011). Moreover STEC strains possessed *stx2a* as their sole gene, or in combination with *stx2c* have been described as more closely associated with HUS, than STEC strains with other Stx gene combinations (Friedrich *et al*., 2002). Further, purified Stx2, in contrast to purified Stx1 can elicit signs of HUS in baboons (Stearns-Kurosawa *et al*., 2010). In addition, a recent study in mice model of disease (Fuller *et al*., 2011) demonstrated that Stx2b and Stx2c had potencies similar to that of Stx1, while Stx2a, Stx2d, and elastase-cleaved Stx2d were 40 to 400 times more potent than Stx1. These findings may partly explain the high virulence of STEC O104:H4 strains, which is responsible for causing the outbreak of hemolytic uremic syndrome in May 2011 in Germany (Bielaszewska *et al*., 2011; Prager *et al*., 2011; Scheutz *et al*., 2011). Epidemic *E. coli* O104: H4 strain possessed stx2a subtype, which could explain the high incidence of HUS in infected patients (one case of HUS in four cases of bloody diarrhea) (Bielaszewska *et al*., 2011; Prager *et al*., 2011; Scheutz *et al*., 2011). Our results showed that STEC strains isolated in the country are diverse in terms of occurrence of *stx* subtypes, which according to scientific publications, are more potent (*stx2a*, *stx2d*) than other subtypes of the *stx* genes.

In conclusion, this is the first comprehensive analysis of virulence gene profiles identified in STEC strains isolated from human, cattle and food in Poland. Simultaneous detection of virulence markers provides data suitable for molecular risk assessment of the potential virulence of STEC isolates. The results obtained using microarrays technology confirmed high effectiveness of this method in determining STEC virulotypes, which certainly supports the implementation of this method for the diagnosis of STEC isolates in our country in selected provincial sanitary – epidemiological laboratories.

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ORIGINAL PAPER

Genetic Variability and Proteome Profiling of a Radiation Induced Cellulase Mutant Mushroom *Pleurotus florida*

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Abstract

We report the genetic similarity changes between a mutant mushroom (*Pleurotus florida*, designated as PfCM4) having increased cellulolytic activity developed through radiation mutagenesis and its wild type by amplified fragment length polymorphism (AFLP). On average, 23 AFLP fragments were amplified per primer combination, and a total of 286 polymorphic fragments (78.57% polymorphism) with maximal fragment length of 1365 base pairs (bp) were obtained. The genetic similarity between wild type and PfCM4 was found to be 22.30%. In addition, mycelial and secreted protein profiling by 2D-PAGE showed at least three and five different protein spots in the range of 25 kD to 100 kD, respectively, in PfCM4. It seems that the variation in genetic similarity and different expression of both mycelial and secreted proteins in PfCM4 in comparison to the wild type could likely be correlated with its increased cellulolytic activity effected by the irradiation.

Key words: *Pleurotus florida*, 2D-PAGE, AFLP, genetic variability, mushroom

Introduction

Oyster mushrooms (*Pleurotus* spp.) are economically important in the world mushroom market and hold second place in the world's production of edible mushrooms, after the popular white button mushroom, *Agaricus bisporus* (Chang, 1999; Berne *et al*., 2008). In addition, the genus *Pleurotus* comprises about 40 species (Jose and Janardhanan, 2000). These mushrooms are known not only for their culinary and medicinal properties, but also for their potential applications in multifarious fields, including disposing of agro-lignocellulosic wastes, important industrial production, and environmental conservation (Sathesh-Prabu and Lee, 2012). Mutation breeding is a process by which mutant variants with desirable traits to be bred are developed, without altering the remaining genotype, by physical, chemical and biological mutagenic agents (Sathesh-Prabu and Lee, 2011). In a previous study, we developed a potent cellulase mutant of *Pleurotus florida,* designated as PfCM4, induced by gamma radiation at a dose of LD_{99} (0.51 kGy) (Sathesh-Prabu and Lee, 2012) that showed 17.24% more cellulolytic activity than wild type ($p < 0.05$). Boominathan *et al*., (1990) observed that gamma-ray radiation can change the genetic diversity of filamentous fungi and induce positive mutants. The genetic similarity of mycelia and basidiospores was altered according to the dose of gamma radiation (Lee and Chang, 1999). Amplified fragment length polymorphism (AFLP) is a highly accurate fingerprinting method to detect polymorphisms among individuals, populations, and independently evolving lineages (Mueller and Wolfenbarger, 1999). Reproducibility, reliability, and specificity are the main advantages of the AFLP technique that has already been applied to establish genetic differences among *Pleurotus* sp. (Mueller and Wolfenbarger, 1999; Urbanelli *et al*., 2007; Pawik *et al*., 2012). Exposure to gamma radiation results in more extensive transcriptional changes (Fry *et al*., 2006) and might change the expression of proteins. Proteomic technologies are powerful tools for examining alterations in protein profiles (Dubey and Grover, 2001). The 2D-PAGE (Two-dimensional polyacrylamide gel electrophoresis) approach to protein profiling is an accessible, economical, and robust technique that possesses high resolving power and enables the detection of hundreds of proteins on a single gel plate (Issaq and Veenstra, 2008). In this vein, the present study was carried out to evaluate the effect of gamma radiation on the genetic similarity of the cellulase mutant *P. florida* (PfCM4) by using AFLP, and differentially expressed mycelial and secreted proteins were investigated by 2D-PAGE.

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Experimental

Materials and Methods

DNA extraction. DNA extractions were performed by the standard CTAB method with slight modifications. Fresh mycelia of wild type and PfCM4 (0.1 g) were collected from 7-day old cultures grown in potato dextrose broth by centrifugation at 15000 × g for 10 min and frozen with liquid nitrogen. Frozen mycelia were ground with a sterile mortar and pestle and kept in a 1.5 ml micro-tube. The preheated (65°C for 30 min) 2X CTAB extraction buffer (700 µl) was added to each of the micro-tubes and the mixture incubated at 65°C for 30 min, 700 µl of Chloroform:Isoamylcohol (24:1) was then added followed by centrifugation at 14000 rpm for 30 min at 4°C. Afterwards, the top aqueous upper phase was transferred to a 1.5 ml micro-tube and two volumes of 95% alcohol were added and incubated on ice for 10 min followed by centrifugation at 15000 × g for 10 minutes at 4°C. Subsequently, the collected pellet was washed with 70% alcohol, then air dried, and the DNA pellet was resuspended in 200 µl of TE buffer. The purity and concentration of the extracted DNA were estimated by nanodrop spectroscopy (Nanodrop-ND1000), and the quality of the DNA was obtained by means of electrophoresis in 1% agarose gels, followed by staining with ethidium bromide. A DNA stock solution was kept at –20°C for further analyses.

Amplified fragment length polymorphism. The AFLP analysis was carried out as previously described by Vos *et al*., (1995) with some modifications and the instruction manual for the AFLP analysis system for microorganisms (Invitrogen, CA). The adapter, primer sequences and reaction buffers employed for AFLP were procured from Invitrogen and are listed in Table I.

DNA restriction and ligation of adapters. Aliquots of the extracted DNA (250 ng) were digested with 2.5 U each of EcoRI and MseI in 5 µl of a 5X restriction buffer (50 mM Tris-HCl pH7.5, 50 mM Mg-acetate, 250 mM K-acetate) in a final volume of 25 µl for 2 h at 37°C. After incubation, the mixture was incubated at 70°C for 15 min to inactivate the restriction endonucleases. Twenty-four micro litres of an adapter ligation solution (EcoRI/MseI adapters, 0.4 mM ATP, 10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate) and 1 U of T4 DNA ligase (in 10 mM Tris-HCl pH 7.5, 1 mM DTT, 50 mM KCl, 50% glycerol) was added to 25 µl of restricted DNA and incubated for 2 h at 20°C. After ligation, the reaction mixture was diluted tenfold in a 1X TE buffer (10 mM Tris-HCl pH 8.0, 0.1 MM EDTA) and used as a template for amplification reactions.

Non-selective PCR amplification. Non-selective PCR was performed to generate a template DNA with AFLP primers, each having zero selective nucleotides.

Table I
Adapter, primer sequences and primer combinations used for AFLP analysis.

EcoRI – adapter 1 EcoRI – adapter 2	5'-CTC GTA GAC TGC GTA CC-3' 5'-AAT TGG TAC GCA GTC TAC-3'
MseI – adapter 1 MseI – adapter 2	5'-GAC GAT GAG TCC TGA G-3' 5'-TAC TCA GGA CTC AT-3'
EcoRI primer (E)	5'-GAC TGC GTA CCA ATT C-3'
MseI primer (M)	5'-GAT GAG TCC TGA GTA A-3'
Selective primers EcoRI+2 (E+2) EcoRI+AC EcoRI+AA MseI+1 (M+1) MseI+A MseI+C MseI+G MseI+T	 5'-GAC TGC GTA CCA ATT CAC-3' 5'-GAC TGC GTA CCA ATT CAA-3' 5'-GAT GAG TCC TGA GTA AA-3' 5'-GAT GAG TCC TGA GTA AC-3' 5'-GAT GAG TCC TGA GTA AG-3' 5'-GAT GAG TCC TGA GTA AT-3'
Primer combinations Set I (E+2/M+1) E+AC/M+A E+AC/M+C E+AC/M+G E+AC/M+T E+AA/M+A E+AA/M+C E+AA/M+G E+AA/M+T	 Set II (E+1/M+1) E+A/M+A E+C/M+A E+G/M+A E+T/M+A E+C/M+C E+T/M+C E+G/M+T E+T/M+T

Amplification was performed on 5 µl of ligated DNA in a total volume of 51 µl containing EcoRI+0 and MseI+0 primers, 5 µl of a 10X PCR reaction buffer (100 mM Tris-HCl pH 8.3, 15 mM MgCl2, 500 mM KCl), and 5 U of Taq DNA polymerase (PKT, Korea). Amplifications were carried out on a thermal cycler (MJ Research Inc., Korea) with the following conditions: 20X (94°C for 30 s, 56°C for 1 min and 72°C for 1 min). After amplification, the samples were diluted to 1:50 with a TE buffer and used for the selective PCR amplification.

Selective PCR amplification. The non-selective amplified products were further amplified using EcoRI (E) and MseI (M) primers with two or one selective nucleotides. Two sets containing 16 primer combinations (Set I: E+2/M+1; Set II: E+1/M+1) were prepared (Table I). Selective amplification was performed on a thermal cycler (MJ Research Inc., Korea) with the following conditions as follows: one cycle of 94°C for 2 min, 65°C for 30 s, and 72°C for 2 min, followed by 13 cycles of amplification with an annealing temperature decrease by a 0.7°C/cycle starting with 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, and ended with 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min.

Analysis of AFLP polymorphism. The selective amplified PCR products were run in an automated electrophoretic separator (Labchip GX II, Caliper life sciences, MA). Bands were scored manually as binary

data for the presence (1) or absence (0) of the fragments. Bands that could not be scored unambiguously and bands with low intensity value (< 0.01 ng) were excluded. The binary information was used to calculate Jaccard's pairwise similarity coefficients using an SPSS-20 package.

Proteome profiling by 2D-PAGE

Cell lysis and precipitation of mycelial proteins. Extraction of mycelial proteins of the wild type and mutant was carried out according to Nandakumar *et al*., (2003) with modifications. The harvested mycelia biomass (500 mg wet weight) and an equal amount of acid washed glass beads (0.5 mm, BioSpec, USA) were added to a 2 ml screwcap tube containing 1 ml of a cell lysis buffer (20 mM Tris-HCl pH 7.6, 10 mM NaCl, 0.5 mM deoxycholate and 0.5 mM PMSF). The mixture was agitated in a Mini-BeadBeater (BioSpec, USA) at maximum speed for 8 min (repeated cycles of 30 s on followed by 30 s cooling on ice). The homogenate was centrifuged (15000 × g for 10 min at 4°C), and the collected supernatant was treated with DNase/RNase (7 µl/ml of DNase/RNase/Mg mix). To precipitate the proteins, 2 volumes of 10% tricholoroacetic acid (TCA) in acetone and 0.07% 2-mercaptoethanol (2-ME) were added and precipitated overnight at –20°C. Subsequently, the precipitated proteins were separated by centrifugation (15000 × g for 20 min at 4°C). The pellet was washed three times with cold-acetone containing 0.07% 2-ME to remove the TCA and dried under a speed vacuum. Thirty microlitres of 0.2 M NaOH was added to the TCA precipitated dry powder for 2 min after which a 1 ml solubilization buffer (9 M urea, 4% w/v CHAPS, 1% w/v DTT and ampholytes 2% v/v 3–10 nonlinear) was added (vortexed occasionally for 1–2 min) followed by sonication on ice for 20 min. Insoluble material was removed by centrifugation (15000 × g for 20 min at 4°C), and the supernatant was collected and stored at –20°C for further use. The protein concentration was determined according to the method of Bradford (1976) with reference to bovine serum albumin using Bio-Rad protein assay reagents (Bio-Rad Laboratories).

Precipitation of secreted proteins. Culture supernatants of the wild type and mutant were collected from 7-day old cultures grown in a 1% CMC medium by centrifugation (15000 × g for 20 min at 4°C). The supernatant was concentrated by freeze-drying according to Fragner *et al*., (2009). Subsequent precipitation and quantification of secreted proteins were carried out by the methods described above.

Protein separation by 2D-PAGE. Immobiline Dry Strips (IPG strip, Bio-Rad Laboratories, 17 cm, pH 4–7 NL) were rehydrated using an immobiline dry strip re-swelling tray. The IPG strips were allowed to rehydrate with protein samples (50 µg) in the rehydration buffer (8 M urea, 2% w/v CHAPS, 2% v/v ampholytes 3–10 nonlinear, 0.002% bromophenol blue and 7 mg DTT/2.5 ml) for 16 h. The strip was overlaid with mineral oil to avoid evaporation. Subsequently, the first dimensional isoelectric focusing (IEF) of the rehydrated strips was performed in an IPGphor system (GE Healthcare) at 20°C in gradient mode. The IEF was performed under the following conditions: 500 V for 1 h, 1000 V for 1 h, 8000 V for 3 h, and finally 8000 V until it reaches 21.2 kVh. The maximal current per strip was set to 50 µA. Prior to the second dimensional electrophoresis, the focused strips were equilibrated with equilibration buffer I (6 M urea, 75 mM Tris-HCl pH 8.8, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 1% DTT) and equilibration buffer II (equilibration buffer I excluding DTT) for 10 min each in a shaker. Second-dimensional vertical SDS-PAGE was performed in a Protean II xi system (Bio-Rad Laboratories) with a Laemmli tris-glycine SDS electrophoresis buffer. Strips were overlaid with an agarose sealing solution containing bromophenol tracking dye. Electrophoresis was performed at 16 mA/gel for 30 min followed by 24 mA/gel for 5 h or until the tracking dye reached within 1 cm of the gel bottom.

Silver staining and imaging of the gel. After electrophoresis, the gels were stained with a silver stain as described previously (Shevchenko *et al*., 1996) with slight modifications. Briefly, gels were fixed in 50% methanol and 10% acetic acid for 1 h and followed by washing twice with 50% ethanol for 20 min each. The gels were sensitized with a solution containing 0.2 g/l $Na2S_2O_3 \cdot 5H_2O$ for 90 s followed by three rinses with water for 20 s each. The gels were then agitated with impregnation solution containing 2 g/l $AgNO_3$ and 750 µl of 37% HCOH/l for 40 min followed by two rinses with water for 20 s each. The gels were then developed with a developer containing 60 g/l Na_2CO_3, 0.5 ml of 37% HCOH/l, and a 20 ml/l sensitizing solution with intensive shaking until the desired intensity of staining was achieved. The development was terminated by discarding the reagent, followed by washing the gel with 5% acetic acid. The gels were dried using a GelAir drying system (Bio-Rad Laboratories) according to the manufacturer's instructions and subsequently scanned for further analysis.

Results and Discussion

To obtain better quality and productivity in edible mushrooms such as *Pleurotus* sp., mutations could be applied, resulting in the desired characteristics that could be economically beneficial (Flegg *et al*., 1985; Djajanegara and Harosoyo, 2009). In the present study,

a potent cellulase mutant of *P. florida* (PfCM4) was employed to study the genetic variability effected by gamma irradiation using AFLP and to investigate the differentially expressed mycelial and secreted proteins by 2D-PAGE. In our previous study, a mutant (PfCM4) was induced by gamma radiation at a dose of LD_{99} (0.51 kGy) at the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Korea (Sathesh-Prabu and Lee, 2012). To summarize, among 16 mutants, Pf CM4 showed 17.24% more cellulolytic activity than the wild type ($p < 0.05$). This characteristic (increased cellulolysis) was found to be stable at up to four generations of sub-culturing. It was observed that Pf CM4 can utilize all kinds of carbon sources tested for their mycelia growth. Starch, xylan, and glucose favourably supported the radial mycelia extension, and the opposite was recorded in sorbose and chitin as a carbon source. Yeast extract and NH_4NO_3 have been recorded as the best organic and inorganic nitrogen sources, respectively. Pf CM4 was found to grow significantly faster, even at high temperature (30°C), than wild type ($p < 0.05$), and the optimal pH was 5.5–6.5.

The AFLP, a PCR-based technique, provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin and complexity (Vos *et al.*, 1995). AFLP showed enough sensitivity to detect polymorphisms among the strains (Urbanelli *et al.*, 2007) and it is suitable for the identification and differentiation of intraspecies level as well as for determining their genomic relationships (Majer *et al.*, 1996; Pawik *et al.*, 2012). In the present study, the restriction enzymes, ECoRI and MseI, along with two sets of primer combinations (Set I: E+2/M+1; Set II: E+1/M+1), 8 combinations each, were used to generate AFLP fingerprinting of the wild type and mutant, PfCM4. The consolidated results are presented in Table II. All primer combinations gave successful AFLP amplification bands in both the wild type and mutant (Fig. 1). A total of 286 polymorphic fragments with a maximal fragment length of 1365 base pairs (bp) were scored. The primer combination of E+AA/M+A amplified the highest number of fragments (72), while the lowest number of fragments (5) were observed with the combination of E+AC/M+A. A significant genomic similarity change was observed

Table II
Polymorphism of wild type and mutant PfCM4 by AFLP.

Primer combinations	Total no. of fragments	No. of polymorphic fragments	Maximal fragment length	Polymorphism (%)
E+AC/M+A	5	4	590	80.00
E+AC/M+C	35	31	1289	88.57
E+AC/M+G	31	29	1261	93.55
E+AC/M+T	11	10	1275	90.91
E+AA/M+A	72	50	1322	69.44
E+AA/M+C	43	37	1365	86.05
E+AA/M+G	16	13	1361	81.25
E+AA/M+T	8	7	665	87.50
E+A/M+A	20	17	974	85.00
E+C/M+A	29	20	943	68.97
E+G/M+A	17	10	877	58.82
E+T/M+A	21	14	962	66.67
E+C/M+C	15	11	1072	73.33
E+T/M+C	18	11	1166	61.11
E+G/M+T	11	10	970	90.91
E+T/M+T	16	12	1110	75.00
Sum	368	286	–	–
Average	23	17.88	–	78.57

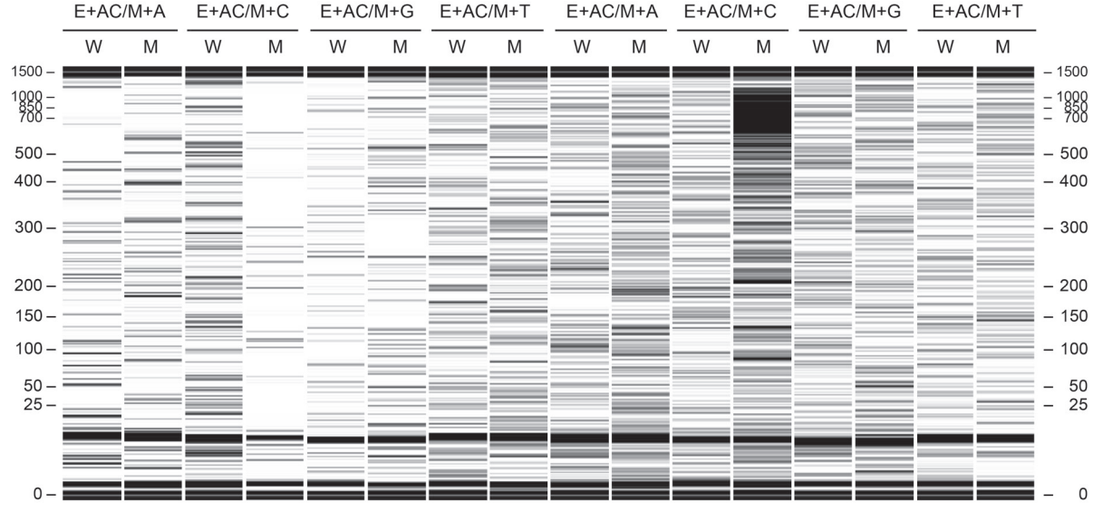

Fig. 1. AFLP fingerprinting of wild type (W) and mutant PfCM4 (M) was generated using the Set I (E+2/M+1) primer combination. Bands that could not be scored unambiguously and bands with low intensity value (< 0.01 ng) were excluded.

by an analysis of the AFLP profile. The genetic similarity between the wild and PfCM4 was found to be 22.30%. In this study, as the genetic similarity was found to be low, the sequence of the internal transcribed spacer (ITS1-5.8S-ITS2) regions of the wild type and PfCM4 was analysed and found that both the wild type and PfCM4 belonged to *P. florida* (data not shown). The variable ITS regions have an advantage of the phylogenetic analysis and identification of the closely related fungal species (Kim *et al*., 1999; Park *et al*., 2001). Lee and Chang (1999) reported the genetic similarity percentage among the gamma irradiated mycelia in the range of 23–36% and 22–25% for the gamma irradiated basidiospores of *Pleurotus ostreatus*. Kwon *et al*. (2007) obtained 6–58% of genetic dissimilarity between wild and irradiated mycelia of *P. ostreatus* and found that genetic similarity alteration is proportional to the radiation dose.

In this study, on average, 23 AFLP fragments were amplified per primer combination and a total of 286 polymorphic fragments (78.57% polymorphism) were obtained. This efficiency is a little higher than the value obtained by an AFLP analysis in the sporeless mutants of *Pleurotus eryngii* (17.8 per primer combination) and in the congeneric species *Pleurotus pulmonarius* (18.0 per primer combination) (Okuda *et al*., 2009; 2012). Using only one restriction endonuclease (PstI) and four selective primers, the AFLP fingerprinting of 21 *Pleurotus* isolates was obtained with the genetic similarity between *Pleurotus* isolates ranging from 0–75% (Pawlik *et al*., 2012). Urbanelli *et al*. (2007) generated a total of 94 AFLP polymorphic fragments of 90 specimens belonging to three taxa of *P. eryngii* complex with restriction enzymes, ECoRI and *Taq*I together with eight primer combinations. In the present study, AFLP primers with selective nucleotides determined the number of amplicons and corresponding percentage of polymorphism. Among all combinations, E+AC/M+G gave the highest percentage of polymorphism (93.55%). Set I primer combinations gave 81.90% of the average polymorphism, whereas Set II gave 71.43%. It was observed that the Set I primer combinations showed 50.34% more generation of total fragments than Set II. Our results are partially consistent with Meng *et al*. (2003). Among the different combinations of AFLP primers used for AFLP profiling of 14 *P. ostreatus* strains, E+3/M+3 gave more amplified fragments than others such as E+2/M+1 or E+2/M+3 and found that the genetic distance ranged from 19–75% between strains (Meng *et al*., 2003). In contrast, Pawlik *et al*. (2012) showed that the primers with three selective nucleotides amplified fewer restriction fragments compared to those with one and two selective nucleotides. Vos *et al*. (1995) demonstrated that the number of amplified fragments is affected by the selective nucleotides at the ends of the AFLP primers. The change in genetic similarity evaluated by the AFLP suggested that the genetic similarity of *P. florida* could be altered by the gamma irradiation.

Mutation can alter the coding region or non coding region of a gene. Mutation altering the non coding region will cause different protein production or have no effect on mRNA maturation (Elliot and Langton, 1981). The estimated mycelial and secreted protein concentration in the wild type and mutant PfCM4 by the Bradford method was 289.07 ± 29.60; 398.88 ± 48.98 and 307.69 ± 21.53; 430.77 ± 39.42 μg/ml, respectively. It was found that mutant PfCM4 gave 37.90 and 40.00% increased mycelial and secreted protein concentrations, respectively, when compared to that of the wild type. In several studies, mutations that produce higher metabolites are mostly obtained by radiation mutagenesis (Slater, 2000). In this study, we used glass beads for a mechanical lysis of the cell to liberate cytoplasmic proteins because this approach has been more efficient than either chemical or enzymatic extraction methods (Nandakumar, 2002). To examine the expression of secreted and mycelial proteins in the wild type and PfCM4, we successfully employed the commonly utilized proteomics technique, 2D-PAGE. As mentioned by Kim *et al*. (2007), proteomic analysis is a powerful tool that can provide a systematic understanding of events at the molecular level.

In the present study, the proteins samples were first isoelectrofocused on a broad pH range IPGstrip, pH3-10, and it was observed that 95% of the protein spots were concentrated in acidic to a neutral pH range (data not shown). Hence, a narrow pH range IPGstrip, 4–7, was employed for the protein profiling. In the present study, four 2D PAGE gels were obtained corresponding to the mycelial and secreted proteins of the wild type and mutant PfCM4. Fig. 2 shows the quantitative comparison of the mycelial and secreted proteome profile prepared from wild type and mutant PfCM4 indicating the presence of protein spots with a wider range of molecular weights (10 kD to 150 kD) and acidic to neutral p*I* (4 to7). Protein spots that were determined to be reproducibly present in all three replicates performed for the mycelial and secreted proteins of wild type and mutant PfCM4 were taken for the study. About 145 and 182 protein spots were visualized from the mycelial and secreted proteins. However, the number of 2D-PAGE protein spots observed for both wild type and mutant PfCM4 seemed to be low when compared with those for other filamentous fungi (Nandakumar *et al*., 2003; Lakshman *et al*., 2008). It could be explained that a loss of proteins during solubilization of TCA precipitate is apparent in the preparation of samples for 2D-PAGE, and there is no single sample preparation protocol available to achieve a better sample preparation for electrophoresis thus avoiding protein modification

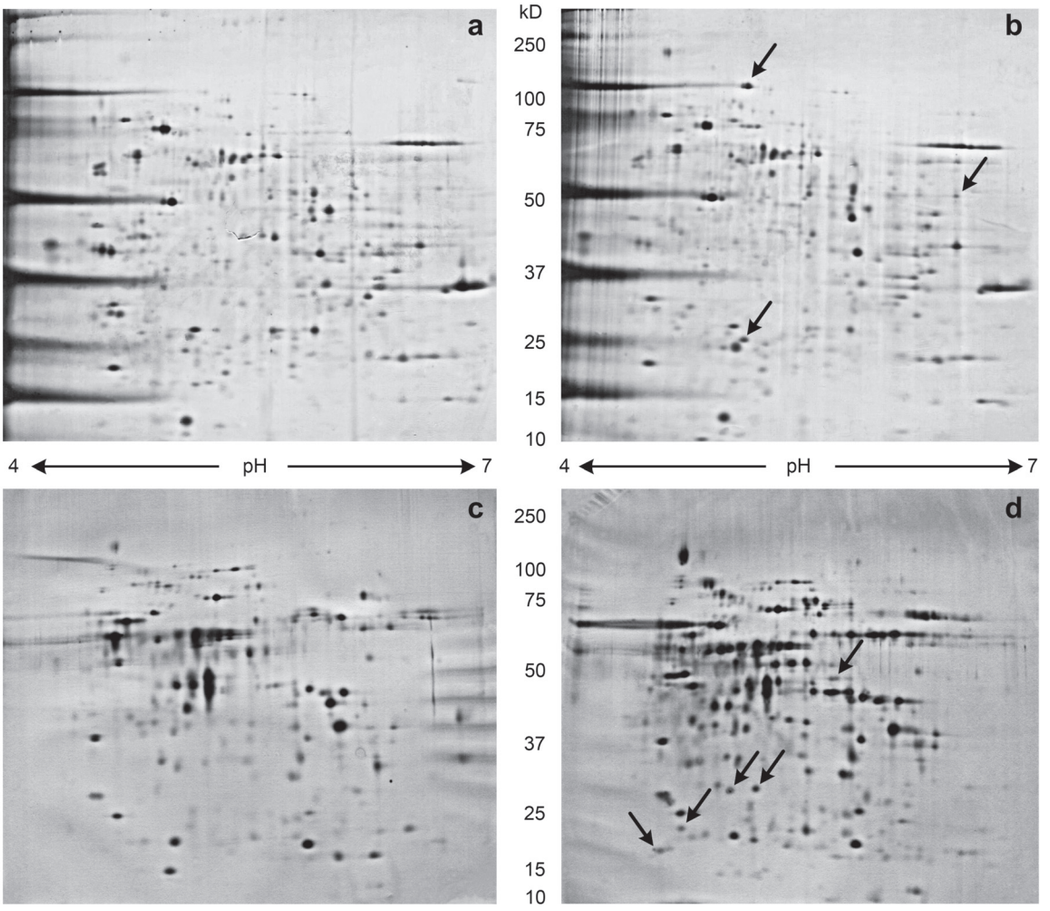

Fig. 2. 2D-PAGE profiles of mycelial proteins of wild type (a) and mutant PfCM4 (b); and secreted proteins of wild type (c) and mutant PfCM4 (d). Protein spots that were determined to be reproducibly present in all triplicates were taken into account. Arrows represent protein spots that were differently expressed than those of the wild type.

or degradation and/or a quantitative loss of proteins (Harder *et al*., 1999; Nandakumar *et al*., 2003). It was observed that there were three different protein spots (100 kD; p*I* 4.5–5.0, 45–50 kD; p*I* 6.0 and 25 kD; p*I* 4.5–5.0) in the culture of PfCM4 (mycelial proteins) as compared to the wild type. Hernandez-Macedo *et al*. (2002) studied the intracellular filamentous fungal proteomics of *Phanerochaete chrysosporium* and *Lentinula edodes* using 2D-PAGE, and visualized 21 proteins related to iron uptake in these ligninolytic fungi. In the case of secreted proteins, a higher number of protein spots were visualized compared to mycelial proteins and it was found that five protein spots (50 kD; p*I* 5.5–6.0), 2 spots (25–37 kD; p*I* 5.0–5.5, 20 kD; p*I* 4.5–5.0 and 15 kD; p*I* 4.5–5.0) were differently expressed in the culture of PfCM4. Patel *et al*. (2013) obtained an extra band of protein in irradiated culture of *Pleurotus sajor caju* with two other more intense bands in comparison to the wild culture extract. In addition, in the present study, no streaking or tailing was observed, and no precipitation of proteins during gel running or staining was evident. Identi ication of differently expressed protein spots by MALDI-TOF MS and ESI-MS/MS is now underway. It seems that the variation in genetic similarity and different expression of both mycelial and secreted proteins in PfCM4 in comparison to the wild type could likely be correlated with its increased cellulolytic activity effected by the irradiation.

Conclusion

The genetic similarity of the irradiated mycelia of *P. florida* could be changed by the gamma radiation and subsequently results in a different expression of mycelial and secreted proteins. The genetic similarity between the wild type and PfCM4 was found to be 22.30% as analysed by AFLP. Protein profiling of the mycelial and secreted proteins by 2D-PAGE showed at least three and five different protein spots in the range of 25 kD to 100 kD and p*I* 4 to 7, respectively, in PfCM4. Further studies will focus on the identification and

characterization of differently expressed protein spots for a better understanding of the gamma-ray radiation effect on *P. florida* at the molecular level with the special reference to the increased cellulolytic activity.

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ORIGINAL PAPER

Partial Characterization of Bacteriocin Produced by Halotolerant *Pediococcus acidilactici* Strain QC38 Isolated from Traditional Cotija Cheese

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Abstract

During a screening of lactic acid bacteria producing bacteriocin from Cotija cheese, the strain QC38 was isolated. Based on the 16S rRNA gene nucleotide sequencing (516 pb accession no KJ210322) and phylogenetic analysis, the isolate was identified as *Pediococcus acidilactici*. Neutralized cell-free supernatant was tested for antimicrobial activity against 17 Gram-negative and Gram-positive pathogens. Growth inhibition was achieved against *Listeria monocytogenes* (supplier or indication or source), *Staphylococcus aureus*, *Vibrio vulnificus*, *Vibrio cholerae* O1 Ogawa, *Vibrio cholerae* NO 01 and *Salmonella enterica* subsp. *Enterica* serovar Typhimurium. Bacteriocin-like substance, after heating at 121°C for 15 min it remained stable and its antimicrobial activity was observed at pH ranging from 1.0 to 10.0 but inactivated by α-chymotrypsin and proteinase K. Strain QC38 was able to grow in 1–9% NaCl concentration. The plate overlay assay showed an approximate size of bacteriocin-like substance between 3.4 and 6.5 kDa. *P. acidilactici* QC38 harboured a plasmid that contains a gene for a pediocin (PA-1).

Key words: *Pediococcus acidilactici*, bacteriocin, cheese, cotija, lactic acid bacteria

Introduction

According to the U.S. Food and Drug Administration, some of the most common foodborne pathogenic bacteria are *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringes*, pathogenic *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enteritidis*, *Samonella* Typhimurium, *Shigella*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Yersinia enterocolitica*. Foodborne pathogens in the United States, cause losses in several billions of dollars (Hoffmann *et al*., 2012). Since the therapeutic antibiotics are restricted in foods, the utilization of antimicrobial peptides from lactic acid bacteria (LAB) that target food pathogens without toxic or other adverse effects has received important attention.

Bacteriocins produced by LAB are a diverse group of ribosome-synthesized antimicrobial peptides that may be divided into two main groups: class I peptides, which contain post-translational modifications, and class II, or unmodified peptides (Rea *et al*., 2011). Their production is a desirable trait among LAB from the perspective of controlling microbial populations in fermented foods in order to extend product shelf-life and also for safety purposes (Zacharof and Lovitt, 2012).

Cotija cheese is an artisanal raw-milk product named after a village of Michoacán State in Mexico. The texture of this cheese is hard and crumbly with a strong salty flavor. It is made of unpasteurized cow's milk in a very warm climate with 4% salt as a preserve without addition of lactic acid starters, usually 25 kg cylinders with a cream color crust and aged for about 3 months up to a year (Cervantes *et al*., 2008). The contribution of native microbiota may play an important role in its flavor, sensory properties and the quality of the Cotija cheese being produced (Wouters *et al*., 2002). Halotolerant (*Lactobacillus pentosus* and *Weisella thailandensis*) as well as the halophilic LAB (*Lactobacillus acidipiscis*

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and *Tetragenococcus halophilus*) have previously been detected in Cotija cheese (Morales *et al*., 2011) without identification of those producing bacteriocins.

The present work, aimed to identify the halotolerant lactic acid bacteria isolated from Cotija cheese and its antimicrobial activity. The bacteriocin produced by the isolate were also characterized.

Experimental

Materials and Methods

Screening for bacteriocin-producing lactic acid bacteria. Cotija cheese samples were collected from the mountain range of Michoacán, México as 20 g of cheese were added to 180 ml of peptone water. Then, the mixture was serially diluted with peptone water and plated on MRS and M-17 agar (Oxoid, Basingstoke, Hampshire, England) and supplemented with cycloheximide at 1 µg/ml to prevent growth of yeast and molds. The plates were incubated at 28°C for two to three days. The isolates were evaluated by cell morphology and Gram's stain reaction. The isolates were screened for bacteriocin production using the spot on the lawn assay using MRS plates (Moraes *et al*., 2010). Five µl of each isolated strain was spotted on MRS plates and incubated at 28°C for 24 h. After incubation, the plates were overlaid with 10 ml of BHI soft agar and inoculated with 10^5 CFU/ml of every tested pathogenic indicator strain. Then, the inhibition zone around the spots revealed potency of antibacterial activity.

Biochemical identification of isolated bacteriocin-producers. Phenotypic identification was performed using Gram´s staining and an H_2O_2 production test. Fermentation of carbohydrates was done by API 50 CHL system (BioMérieux, Inc,.Hazelwood Missouri, USA).

Identification of the isolate by 16S rDNA sequencing and phylogenetic analysis. Bacteriocin-like substance producers were identified by 16S rDNA sequencing to confirm the results obtained from the biochemical identification. DNA was extracted using the modified method described by Wilson (2001). Briefly, the pellet was suspended in 400 µl of TE buffer (1 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8). Ten mg/ml of lysozyme, and 70 U/ml mutanolysin were added to the tube. The mixture was incubated for 30 min at 37°C, then 30 µl of 10% sodium dodecyl sulphate and 5 µl of proteinase K (20 mg/ml) were added. The tube was mixed and incubated at 37°C for 30 min. The cell debris were removed by precipitation with 100 µl of 5 M NaCl and 80 µl hexadecyltrimethylammonium bromide-NaCl (CTAB-NaCl) solution at 65°C for 10 min. Thereafter, 500 µl of chloroform-isoamyl alcohol (24:1) was added, mixed and centrifuged at 12000 × *g* for 10 min and the aqueous layer was transferred to a new tube containing an equal volume of isopropanol, mixed gently and stored at –20°C overnight. DNA was recovered by centrifugation and washed with 70% ethanol, dried under vacuum, suspended in 30 µl of molecular grade water and stored at –20°C until use. DNA was used to amplify the variable region (V1-V3) of the 16S rRNA gene (about 510 bp) using primer 4F and pD as previously described (Kommedal *et al*., 2011).

After agarose gel electrophoresis, the PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

To test the relationship of the 16S rRNA sequence obtained, against a set of most similar *Pediococcus* 16S sequences deposited in GeneBank, a Maximum Likelihood analysis (ML) was performed. The 16S *Pediococcus* sequence (QC38) was uploaded to the GenBank under accession number KJ210322. GenBank accession numbers included in the study were: AB219053, AB680266, AB682548, AB682554, DQ294959, DQ294960, EF059986, EF059987, EU147314, EU147315, FJ538506, FJ844959, FJ844984, FJ915729, GU904688, GQ421474, GQ421479, GQ222392, GQ222393, GQ222394, HQ288527, HQ315858, HQ315859, JN039348, JN836485, JQ806707, JQ806718, JX431046, and KF193970. Before analysis, all sequences were aligned with Clustal X ver. 2.1 (Thompson *et al*., 1997). Regarding the ML method, the algorithm implemented in the program aLRT-PHYML (Guindon and Gascuel, 2003) was used, along with an optimized base frequency and an estimated ratio of transition/transversion. Optimization of tree topology (rather than branch length) was selected. The reliability of each node was estimated *via* the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006) with the Shimodaira-Hasegawa-like option. The *Lactobacillus plantarum* (strain B-2-7) was used as the outgroup (accession no. GQ183911).

Indicator pathogenic strains. *S. aureus* ATCC 6538, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) ATCC14028 (supplier, sources are missing everywhere there, *Brucella abortus* 2308, *Brucella melitensis* 16M, *Brucella suis* 1330, *E. coli*, *Pseudomonas aeruginosa*, *V. vulnificus*, *V. cholerae* O1 Ogawa, *V. cholerae* non-01, *Vibrio alginoliticus*, *V. parahemolyticus*, *L. monocytogenes* ATCC19115, *Listeria innocua*, *Listeria ivanovii* and *Y. enterocolitica* were used as indicator strains for inhibitory effect. All strains, except *V. vulnificus*, *V. cholerae* O1 Ogawa, *V. cholerae* non-01, *V. alginoliticus*, *V. parahemolyticus*, *B. abortus* 2308, *B. melitensis* 16M and *B. suis* 1330 were cultured in Brain heart infusion broth agar (BHI) (BD, Maryland, USA) but *V. vulnificus*, *V. cholerae* O1 Ogawa, *V. cholerae* non-01, *V. alginoliticus* and *V. parahemolyti-*

cus were cultured onto Tryptone soy agar supplemented with 1% NaCl. Finally, *B. abortus* 2308, *B. melitensis* 16M and *B. suis* 1330 were cultured in Tryptone soy agar (BD, Maryland, USA).

Antimicrobial activity assay. Strain QC38 was grown in MRS broth at 37°C for 24 h. The culture was centrifuged at 8000 × *g* for 10 minutes. The cell free supernatant (CFS) was then adjusted to pH 6.5, treated with catalase (1 mg/ml) and filtered through a 0.22 μm filter (Millipore, Carrigtwohill, Co. Cork, Ireland). Then, agar well diffusion assay (AWDA) was used for detection of antimicrobial activity against the investigated indicator pathogenic strain (Ponce *et al*., 2008). Soft BHI agar (1% w/v) containing approximately 10^5 CFU/ml of indicator strains was overlaid onto MRS plates. Wells of 5 mm in diameter were created in the agar indicator pathogen overlaid plates and then filled with 50 μl of the CFS and overnight incubated at 37°C. Then inhibition zones were measured.

Production of bacteriocin. Strain QC38 was grown in MRS broth (pH 6.5) during 18 h. Then, the cells (2% v/v) were transferred to a bottle with MRS broth and incubated at 37°C. Incubated broth samples were aseptically taken from the culture at periodical time intervals in order to measure optical cell density at 600 nm, pH and antimicrobial substance production. The antimicrobial activity of CFS was measured by the AWDA method as described above. The antimicrobial substance concentration Arbitrary Unit per milliliter (AU/ml) was calculated as the inverse of the most concentrated dilution, which induces the inhibition of *L. monocytogenes* (Todorov, 2008).

Effect of pH and enzymes on bacteriocin activity. The effect of pH on bacteriocin activity in the CFS was evaluated by adjusting the pH from 1 to 10 with 1 M HCl or 1 M NaOH. After 2 h of incubation at 30°C, the samples were adjusted to pH 6.5 and the antimicrobial activity was determined (Ponce *et al*., 2008). The CFS was treated with proteinase K, α-chymotrypsine, and pronase E (final concentration 1 mg/ml) for 1 h, then the antimicrobial activity was measured by the AWDA method using *L. monocytogenes* as an indicator.

Effect of temperature and extender storage. The CFS was heated at 37, 50, 70, 80 and 90°C for 1 h, then the samples were cooled at 4°C and the effect of extender storage at 4 and –20°C for 5, 10, 20 days and 1 year was also evaluated. Untreated CFS was used as a positive control in each experiment. Residual activity was determined by AWDA, as described above.

Effect of NaCl concentration on the growth of *P. acidilactici* QC38 and bacteriocin production. *P. acidilactici* QC38 was inoculated (1% v/v) into MRS broth containing different concentrations of NaCl (from 0.5–10% w/v) and incubated at 37°C during 24 h. The optical density at 600 nm wavelength was measured to evaluate bacterial growth in the broth culture. Production of bacteriocin in each concentration of NaCl was evaluated by the AWDA assay using *L. monocytogenes* as the indicator of inhibition (Verluyten *et al*., 2004).

PCR of bacteriocin gene. Plasmid DNA isolation was performed by the modified alkaline lysis method (Anderson and McKay, 1983) and DNA was used as a template for the amplification of bacteriocin gene. PCR was performed using 20 μl reaction mixture consisting of 1X PCR buffer, 1 U of *Taq* DNA polymerase, 3 mM of $MgCl_2$, 0.4 mM of dNTPs and 0.2 μM of each primer (PedF and PedR) (Suwanjinda *et al*., 2007). The PCR conditions were an initial denaturation step at 94°C during 5 min, followed by 30 cycles of denaturation at 94°C during 1 min, annealing (51°C, 40 s) and extension (72°C, 3 min). The products were analyzed on 1.5% agarose gel and staining with ethidium bromide.

Molecular size of bacteriocin. The molecular size of bacteriocin present in CFS was determined by tricine-SDS-PAGE (Schagger and von Jagow, 1987) and a low-molecular weight maker (polypeptide SDS-PAGE, Bio-rad) was used. After electrophoresis, one half gel was fixed and stained with Coomasie blue. The other half was used to determine the position of bacteriocin in the gel (Powell *et al*., 2007). Briefly, the gel was overlayed with *L. monocytogenes* (10^6 CFU/ml), suspended in BHI soft agar and incubated at 37°C for 24 h.

Results

Isolation of the bacteriocin-producing strains. A total of 178 LAB strains were isolated from Cotija cheese. After preliminary identification the isolate, called QC38 was Gram-positive, catalase and oxidase tests negative, and identified by carbohydrate fermentation profile (API 50CH L system) as *P. acidilactici*.

Phylogenetic analysis of isolate QC38. The resulting ML phylogeny revealed that its 16S sequence has a phylogenetic close relationship with other *Pediococcus* 16S sequences deposited in the GenBank. The phylogeny was resolved showing two main clades with strong nodal support (aLRT > 90, not shown; Fig. 1). One of these clades has approximately three times more sequences than the other one. The smaller clade contains our 16S *Pediococcus* sequence (QC38), was closely related to *P. acidilactici* strains.

Antimicrobial activity and production of bacteriocin. Inhibitory activity was observed against *E. coli*, *L. monocytogenes*, *L. innocua*, *S. aureus*, *S.* Typhimurium, *V. cholerae* NO 01, *V. cholerae* O1 Ogawa and *V. vulnificus* (Table I). As bacteriocin production of *P. acidilactici* QC38 was evaluated, the antimicrobial activity was observed since the exponential growth phase but the activity reached its highest bacteriocin

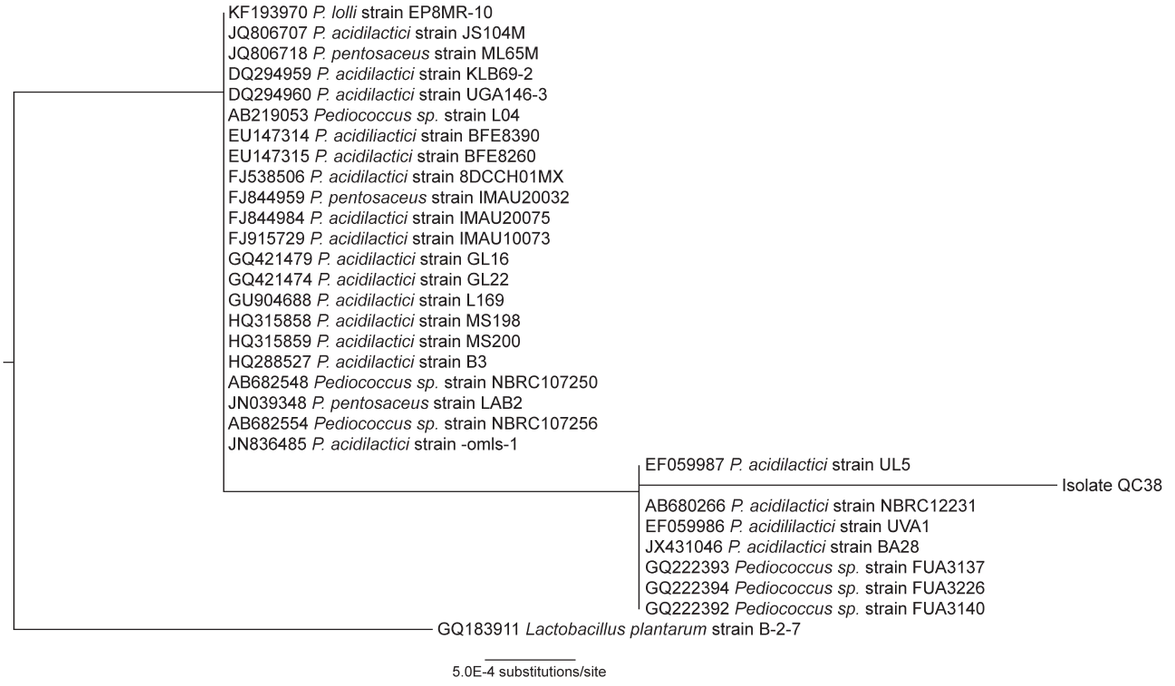

Fig. 1. ML tree reconstruction based on 516 bp from 16S *Pediococcus* species.

Branch support values in the two main clades are > 90 (aLRT). Letters at the beginning of tip names correspond to GenBank accession numbers, followed by *Pediococcus* species and strain. *L. plantarum* strain B-2-7 was used as the outgroup.

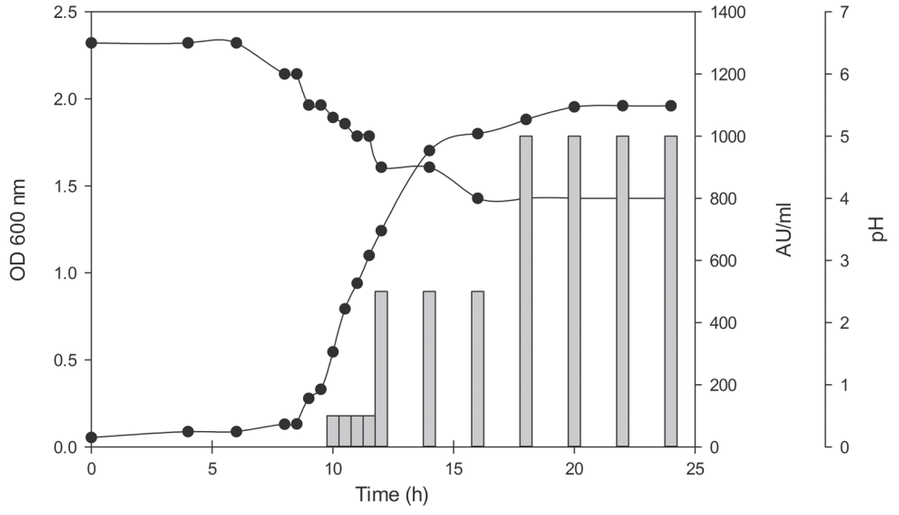

Fig. 2. Bacteriocin production during the growth of *P. acidilactici* QC38 at 37°C.

Changes in optical density measured at 600 nm (●). Antimicrobial activity is presented as AU/ml (bars) against *L. monocytogenes*.

production level in the late exponential phase. The highest antimicrobial activity was maintained during the stationary phase (Fig. 2).

Effect of enzymes, pH and temperature. When CFS of *P. acidilactici* QC38 was treated with different proteolytic enzymes total inactivation of the inhibitory activity was observed with proteinase K and α-chymotrypsin but partially with pronase E. Concerning the pH stability of bacteriocin produced by *P. acidilactici* QC38, the inhibitory activity remained stable after incubation for 2 h at pH values between 1.0 and 10.0 and the CFS was stable at all temperatures tested, even after treated at 121°C, 15 psi pressure for 15 min (Table II).

Growth of *P. acidilactici* QC38 in NaCl and the effect on the bacteriocin production. *P. acidilactici* QC38 was able to grow in 1–9% NaCl. Bacteriocin

Table I
Antimicrobial activity spectrum of *P. acidilactici* QC38.

Indicator microorganism	Activity
Bacillus cereus	–
Brucella abortus 2308	–
Brucella melitensis 16M	–
Brucella suis 1330	–
Escherichia coli	+
Listeria innocua	+++
Listeria ivanovii	–
Listeria monocytogenes	+++
Pseudomonas aeruginosa	–
Salmonella Typhimurium	+
Staphylococcus aureus	++
Vibrio parahemolyticus	–
Vibrio alginoliticus	–
Vibrio cholerae non-01	+
Vibrio cholerae O1 Ogawa	+
Vibrio vulnificus	+
Yersinia enterocolitica	–

(–) No inhibitory activity; (+) 1–9 mm (low inhibitory effect on growth); (++) 9.1–19 mm (medium effect); and (+++) > 19.1 mm (high effect).
*Antimicrobial activity was measured by AWDA.

production was unaffected by 1–3% NaCl, however, higher concentrations of NaCl (4–9%) reduced bacteriocin production.

PCR bacteriocin gene and molecular size. After purification of a plasmid from *P. acidilactici* QC38 it was used as a template to amplify a fragment of the gene encoding for pediocin PA-1 300 bp that indicates the presence of this encoding gene in *P. acidilactici* QC38

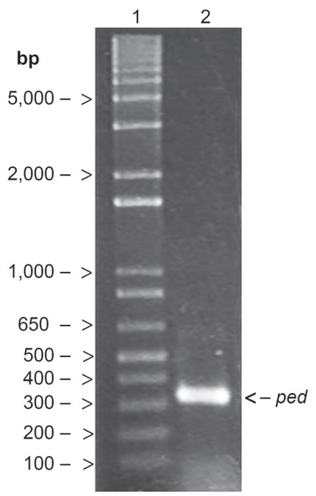

Fig. 3. DNA fragments obtained after PCR with plasmid DNA from *P. acidilactici* QC38 using Pediocin PA1-specific primers.

(Lane 1) 1 kb Plus ladder, and Lane 2) 300 bp amplicon obtained using plasmid DNA.

Table II
Effects of proteolytic enzymes, temperature, and pH on the activity of the bacteriocin produced by *P. acidilactici* QC38.

Treatment	Residual bacteriocin activity (%)
Proteinase K[b]	0
Pronase E[b]	69
α-chymotrypsin[b]	0
50°C[c]	98
70°C[c]	98
80°C[c]	84
90°C[c]	89
121°C, 15 psi for 15 min[d]	84
4°C, 5 days[e]	100
4°C, 10 days[e]	100
4°C, 20 days[e]	98
4°C, 1 year[e]	51
–20°C, 5 days[e]	100
–20°C, 10 days[e]	100
–20°C, 20 days[e]	98
–20°C, 1 year[e]	89
pH[f]	
1	96
2	98
3	100
4	100
5	100
6	100
7	100
8	96
9	84
10	82
Control[g]	100

[b] Bacteriocin treated with proteolytic enzymes at 37°C for 1 h.
[c] Bacteriocin incubated at different temperatures for 1 h each.
[d] Bacteriocin autoclaved at 121°C, 15 psi for 15 min.
[e] Bacteriocin storage at various temperatures and time intervals.
[f] Bacteriocin treated at different pH values at 37°C for 2 h each.
[g] Untreated bacteriocin. Antimicrobial activity was measured by AWDA.

(Fig. 3). The molecular size of bacteriocin produced by *P. acidilactici* QC38 was between 3.4–6.5 kDa, as determined by tricine-SDS-PAGE (Fig. 4).

Discussion

The food processing industry has recently focused on applying new strategies for food conservation in order to guarantee their quality and safety. Application of bacteriocin can be an useful alternative, since these peptides do not alter the food flavor, aroma and texture.

Bacteriocins are commonly produced by LAB and have been isolated from fermented foods such as dairy

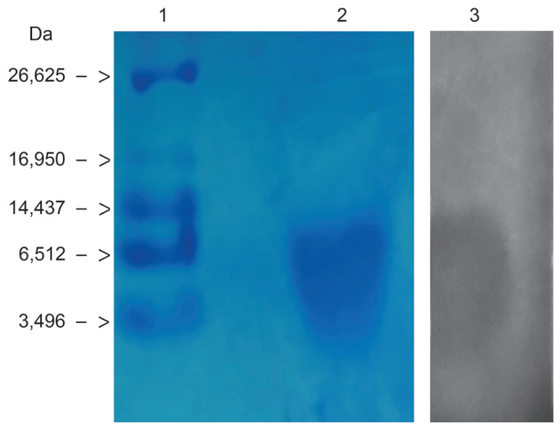

Fig. 4. Tricine-SDS-PAGE of bacteriocin produced by *P. acidilactici* QC38.
Lane 1) Molecular weight marker; Lane 2) Bacteriocin produced by *P. acidilactici* QC38 stained with Coomassie blue; Lane 3) Zone of growth inhibition.

products. It has been reported that LAB isolated from dairy products are good candidates for evaluating antimicrobial substance production, as they are not pathogenic bacteria and most of them have been used as probiotic strains (De Vuyst and Leroy, 2007).

In Mexico, different kinds of cheeses are manufactured without starter cultures such Cotija cheese. In this work different LAB were isolated from Cotija cheese, but one of them was able to inhibit pathogenic Gram-positive and Gram-negative bacteria. It was identified as *P. acidilactici* belonging to LAB which is involved in different fermentation processes. The ability of LAB to inhibit the growth of pathogenic and undesirable bacteria in food is well known due to the production of organic acids, hydrogen peroxide or bacteriocins (De Vuyst and Leroy, 2007). The proteinaceous nature of bacteriocin produced by *P. acidilactici* QC38 was confirmed by using proteolytic enzymes. Anti-Listerial activity has been reported for other *P. acidilactici* strains isolated from dairy and fermented products, these bacteriocins have a molecular weight of about 4.6–15 kDa (Gurira and Buys, 2005; Abbasiliasi *et al.*, 2012; Zhang *et al.*, 2012). Bacteriocins produced by strains of *P. acidilactici* varied in their antimicrobial activity whereas those isolated from a sausage inhibited *L. monocytogenes* but could not inhibit *S. aureus* or *Salmonella* Typhimurium (Albano *et al.*, 2007). Nevertheless, *P. acidilactici* QC38, was not only able to inhibit the growth of *L. monocytogenes* but also *E. coli*, *L. innocua*, *S. aureus*, *S.* Typhimurium, *V. cholerae* NO 01, *V. cholerae* O1 Ogawa, and *V. vulnificus*. The inhibitory activity against Gram-positive and Gram-negative is less prevalent in the bacteriocin reports. Since Cotija cheese is made of raw unpasteurized milk, *Brucella* spp. was evaluated as an indicator, however the three tested strains were not inhibited in the study. The antimicrobial activity of bacteriocin of *P. acidilactici* QC38 showed similar results of those produced by different LABs previously reported (Cladera-Olivera *et al.*, 2004; Todorov and Dicks, 2006). PCR plasmid investigation of *P. acidilactici* QC38 detected the presence of the gene encoding the pediocin PA-1 in the purified plasmid which is a bactericidal peptide produced *via* plasmid-linked operon (Halami and Chandrashekar, 2005; Manjulata and Halami, 2011). The bacteriocin produced by *P. acidilactici* QC38 showed a size between 3.4–6.5 kDa similar to that one reported for pediocin, however bacteriocin produces by QC38 showed antimicrobial activity against broad range of foodborne pathogenic bacteria not reported previously.

Conclusions

Due to strict regulations in the food industry, there is an increasing demand in controlling food-borne pathogenic bacteria such as *L. monocytogenes*, *S. aureus*, *S.* Typhimurim *etc*. To our knowledge, this is the first study on a bacteriocinogenic strain of *P. acidilactici* isolated from Cotija cheese. The bacteriocin reported in this work was able to inhibit some virulent Gram-positive and Gram-negative food-borne pathogens and it was resistant to a wide range of pH storage and NaCl rather than being a heat stable bacteriocin. All these properties nominates this peptide as a potential preservative for food products.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Simultaneous Biodegradation of Phenol and n-Hexadecane by Cryogel Immobilized Biosurfactant Producing Strain *Rhodococcus wratislawiensis* BN38

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Abstract

The capability of the biosurfactant-producing strain *Rhodococcus wratislawiensis* BN38 to mineralize both aromatic and aliphatic xenobiotics was proved. During semicontinuous cultivation 11 g/l phenol was completely degraded within 22 cycles by *Rhodococcus* free cells. Immobilization in a cryogel matrix was performed for the first time to enhance the biodegradation at multiple use. A stable simultaneous hydrocarbon biodegradation was achieved until the total depletion of 20 g/l phenol and 20 g/l n-hexadecane (40 cycles). The alkanotrophic strain *R. wratislawiensis* BN38 preferably degraded hexadecane rather than phenol. SEM revealed well preserved cells entrapped in the heterogeneous super-macroporous structure of the cryogel which allowed unhindered mass transfer of xenobiotics. The immobilized strain can be used in real conditions for the treatment of contaminated industrial waste water.

Key words: *Rhodococcus* sp., biodegradation, immobilization, n-hexadecane, phenol

Introduction

Pollution of the environment is one of the major challenges of today's civilization (Kumar and Sumangala, 2012). Phenolic compounds are among the most frequently found xenobiotics in rivers, industrial effluents, and landfill runoff waters. Phenols come from several types of industries – from coal refineries, phenol manufacturing pharmaceuticals, paper mills, paints, dyes, petrochemicals, and textiles (Kumar *et al.*, 2005). Phenol and its derivatives are harmful both for the living bodies and non living environment (Nair *et al.*, 2008). These are toxic either by ingestion or by contact or inhalation even at low concentrations. Industrial effluents containing phenol require proper treatment prior to its discharge into the environment. Pollution, due to petroleum oil, is also a prevalent ecological hazard and hence, microbial degradation of hydrocarbons is a top issue (Cameotra and Singh, 2009). The capacities of aerobic microorganisms for biodegradation of aromatic compounds as well as aliphatic alkanes, often found together in different wastes, are of particular relevance (Dawson *et al.*, 2007). Biodegradation is generally preferred due to economic issues and the lack of production of hazardous byproducts to the use of conventional processes. The biochemical potential of actinobacteria of the genus *Rhodococcus* has been increasingly studied because of their high catabolic diversity and unique enzymatic capabilities, as well as stable cell physiology (van der Geize and Dijkhuizen, 2004).

To enhance the biodegradation capabilities of microorganisms different approaches were applied (Shetty *et al.*, 2007; Li *et al.*, 2013). For example, immobilization technique could lead to the accomplishment of quite effective technological processes for bioremediation of contaminated sites (Yordanova *et al.*, 2009). The advantages of the process based on immobilized biomass include enhancing microbial cell stability, allowing continuous process of operation and avoiding the biomass-liquid separation requirement. Physical entrapment of organisms inside a polymeric matrix is one of the most widely used techniques for whole-cell immobilization (Meggyes and Simon, 2000). Super-macroporous polymer cryogels are an interesting class

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of materials due to their unique heterogeneous open porous structure, which significantly increases the equilibrium sorption properties and allows unhindered diffusion of solutes, nano-particles and micro-particles. Usually, cryogels possess spongy-like structure of huge pores (50–200 μm) containing free water surrounded by thin walls and, therefore, they are often used for immobilization of enzymes and cells by entrapment inside the channels of interconnected pores (Lozinsky *et al.*, 2003).

Many bacterial strains have been isolated with the abilities to degrade n-hexadecane and phenol separately, which are often used to represent the aliphatic and aromatic pollutant (Yordanova *et al.*, 2009; Abdel-Megeed *et al.*, 2010; Tambekar *et al.*, 2012). However, few strains have been reported to have the dual abilities (Sun *et al.*, 2012).

This paper aims to report the first study on simultaneous biodegradation of n-hexadecane and phenol by *Rhodococcus wratislawiensis* BN38 immobilized in hydroxypropylcellulose/poly (N-isopropylacrylamide) cryogel matrix.

Experimental

Materials and Methods

Microorganism, media and cultivation. The *R. wratislawiensis* BN38, employed in this study was isolated from soil polluted with hydrocarbons by a standard enrichment technique (Tuleva *et al.*, 2008). Minimal salt medium (MSM) with the following composition (g/l): $K_2HPO_4 \cdot 3H_2O$, 4.8; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; supplemented with trace element solution (mg/l): $CaCl_2 \cdot 2H_2O$, 2.0; $MnCl_2 \cdot 4H_2O$, 0.4; $NiCl_2 \cdot 6H_2O$, 0.4; $ZnSO_4 \cdot 7H_2O$, 0.4; $FeCl_3 \cdot 6H_2O$, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.2 and phenol and n-hexadecane at 500 mg/l, unless otherwise mentioned, was used. The pH of the medium was adjusted to 7.0. The MSM was solidified as MSM phenol agar by addition of 1.8% agar when necessary. Cultures grown on MSM agar supplemented with 500 mg/l phenol were used to inoculate 500 ml Erlenmeyer flasks, containing 100 ml liquid MSM. At each cycle MSM was supplemented to 100 ml with fresh sterile medium without cell transfer and only phenol was added. Cultures were incubated during the long-term semicontinuous biodegradation processes while shaking (120 rpm) at 29°C. Inocula with cell density (OD_{610} nm) of 0.4 were employed in the processes of biodegradation. The pH values were 6.7–6.8 all the time, due to the buffering activity of the nutrient medium used.

Bacterial growth was assessed by determination of the optical density (OD_{610} nm) of the culture. Acclimatization procedures were carried out by multiple passages on MSM agar with phenol as a sole carbon and energy source. Ten passages were carried out with increasing concentrations of the xenobiotic to achieve an adapted culture.

Analytical methods. Phenol concentrations were determined colorimetrically with 4-aminoantipyrine according to American Public Health Association (1999).

Trehaloselipids were quantified after alkaline hydrolysis with the anthrone method (Pan *et al.*, 1996). From the dichloromethane extracts of *n*-hexadecane grown BN38 cultures 4 ml samples were taken, freed from the solvent and hydrolyzed for 15 min with 1 ml 1 M NaOH in a boiling water bath under frequent shaking. After cooling, equimolar amount of 1 M HCl was added and the mixture was centrifuged 15 min at 15000 × g to separate residual *n*-alkane. Then one hundred and thirty micro liters of the aqueous phase was mixed with 3-fold volume of freshly prepared anthrone reagent (0.2 g anthrone in 96% sulphuric acid) and the mixture was heated 15 min in a boiling water bath. After cooling, the OD_{620} was measured and compared with a calibration curve prepared with trehalose in a concentration range from 0 to 0.3 mM.

Biodegradation of hexadecane was measured as substrate depletion. At certain time points, whole cultures were extracted with equal volumes of *n*-hexane and residual *n*-hexadecane was quantified by gas chromatography using a Hewlett-Packard model 5859 instrument equipped with a flame ionization detector.

Cell surface hydrophobicity. Cell surface hydrophobicity was determined by the bacterial adhesion to hydrocarbons (BATH) and measured spectrophotometrically as described by Rosenberg *et al.* (1980). The difference between the OD of the aqueous phase before and after the mixing time was used to calculate the adhesion as a percentage: $100 \times [1\text{-}(OD_{600}$ nm after mixing/OD_{600} nm before mixing)].

Detection of biosurfactant production. Two simple preliminary methods were used for detection of biosurfactant production: (1) The surface tension (ST) of the supernatant fluid was measured, after centrifugation at 8000 × g for 20 min, by the du Noüy ring method using a tensiometer (Krüss, Hamburg, Germany). Before each measurement, the instrument was calibrated against triple distilled water. (2) The emulsifying activity of the culture supernatant was estimated by adding 0.5 ml of sample fluid and 0.5 ml of kerosene to 4.0 ml of distilled water. The tube was vortexed for 10 sec, held stationary for 1 min, and then visually examined for turbidity of the stable emulsion.

Immobilization. Separately, 0.2 g hydroxypropylcellulose (MW 1.15×10^6 g/mol, Aqualon Division, USA) was dissolved in 7 ml de-ionized water under stirring at 20°C and kept overnight to ensure complete dissolution of the polymer. Then, 0.2 g N-isopropyl-

acrylamide (Aldrich, Germany) and 0.04 g crosslinking agent (*N*,*N*'-methylene bisacrylamide; Merck, Darmstadt, Germany) both dissolved in 3 ml distilled water, and 0.067 ml photoinitiator (H_2O_2, 30% aqueous solution; Merck, Germany) were added and the obtained mixture was poured into Teflon dishes (portions of 1 ml in 10 dishes with a diameter of 20 mm) forming a 4 mm thick layer. The samples were frozen at –20°C for 2 h and irradiated with the full spectrum of UV-Vis light from a 400-W metal halide flood lamp (Dymax 5000-EC; Dymax Corporation, Torrington, CT, USA) for 5 min (irradiation dose rate: 5.7 J/cm^2 min; input power: 93 mW m^{-2}). The cryogels were extracted in distilled water for seven days, frozen and freeze dried in an "Alpha 1–2 Freeze Drier" (Martin Christ) at –55°C and 0.02 mbar for 24 h. Finally, the freeze dried cryogels were immersed in 7 ml cell suspension. Cells were harvested by centrifugation at 8000 × g and resuspended in phosphate buffer (0.06 M, pH 7.0 at 20°C) to obtain a cell density of 40×10^9 g^{-1} carrier material. The cells were immobilized into HPC/PNIPAAm cryogel pores by soaking freeze dried disks in cell suspension till 90% of the suspension was swollen by the matrix.

Scanning Electron Microscopy (SEM). Cryogel disks with and without cells were frozen in a freezer at –20°C, fractured and freeze dried in an "Alpha 1–2 Freeze Drier"(Martin Christ) at –55°C and 0.02 mbar for 24 h. Then, gel specimens were fixed on a glass substrate and coated with a thin layer of gold for 60 sec. The interior and surface morphology of the gels and immobilized bacteria were studied by using a JEOL JSM-5300 SEM operating at 10 kV at magnification from 150 to 1000.

Statistical analysis. All data are presented as means± standard deviation. The significance of differences between the treatments was evaluated by one-way analysis of variance (ANOVA) and a Bonferroni post hoc test, using InStat (GraphPad Software Inc., La Jolla, CA, USA). Values of $P < 0.05$ were considered significant.

Results

Catabolic activity of free cells after acclimatization. The acclimatization of the selected isolate was performed in a phenol containing MSM to increase its biodegradation potential. Initially, the strain was grown in MSM with increasing concentrations of phenol (250, 500, 750 and 1000 mg/l). During the acclimatization process, the bacterial cells became adapted to the increasing concentrations of phenol with no signs of cell lysis and 500 mg/l appeared to be the most appropriate concentration for conveying the process. This adaptability of *R. wratislawiensis* BN38 was clearly revealed by the numerous numbers of cycles with active processes of biodegradation. Each cycle started with introduction of the substrate in the medium and finished with its depletion. The whole added phenol quantity (11 g/l) was mineralized with a high rate and stability during 22 cycles of operation with xenobiotic concentration of 500 mg/l at each cycle (Fig. 1).

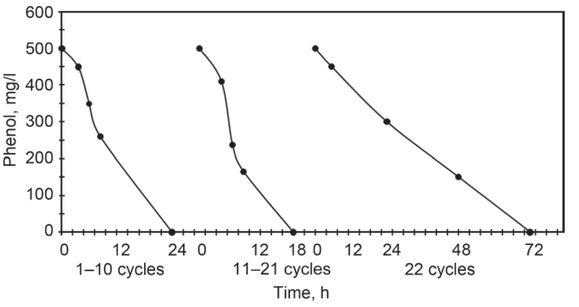

Fig. 1. Dynamics of phenol biodegradation (—●—) by free acclimated cells of *R. wratislawiensis* BN38. The results are presented as average values ($x_{av.} \pm \sigma$) for the first 20 cycles and separately for the last 22nd cycle.

Simultaneous biodegradation of aromatic and aliphatic xenobiotics by free cells. The simultaneous biodegradation of an aromatic and aliphatic xenobiotic was also accomplished by *R. wratislawiensis* BN38 free cells. After the addition of equal quantities of phenol and hexadecane, full depletion of phenol was achieved for 18–24 h in the range of 16 active cycles, while hexadecane was totally degraded for only 12 h (Fig. 2). During these 16 active cycles of biodegradation 8 g/l phenol and 8 g/l hexadecane were degraded.

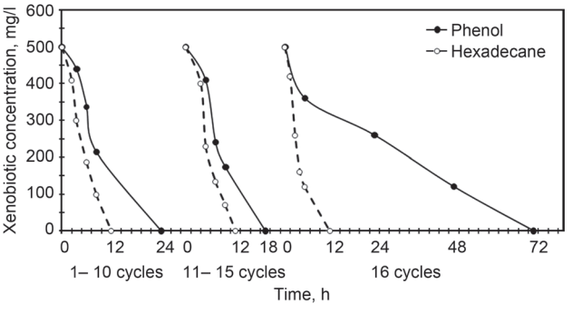

Fig. 2. Simultaneous biodegradation of aromatic (—●—) and aliphatic (––○––) xenobiotics by free cells of *R. wratislawiensis* BN38.

Phenol biodegradation by cryogel immobilized cells. The possibilities for intensification of the biodegradation processes by the use of *R. wratislawiensis* BN38 cells immobilized in a polymer cryogel were investigated. Fourty consecutive active cycles of phenol biodegradation were successfully performed. The overall amount of degraded xenobiotic was 20 g/l (Fig. 3). Cryogel matrix based on hydroxypropylcellulose/poly

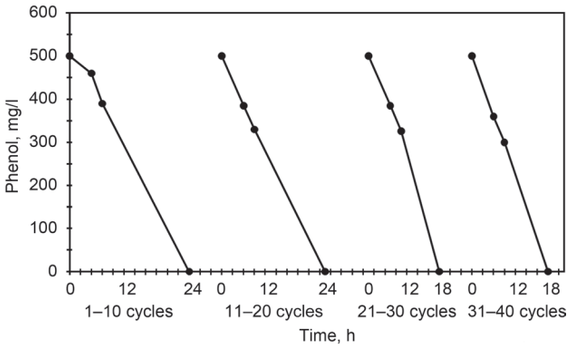

Fig. 3. Semicontinuous processes of phenol biodegradation by the immobilized *R. wratislawiensis* BN38 cells (—●—), presented as average values ($x_{av.} \pm \sigma$) for residual phenol for every 10 cycles.

(N-isopropylacrylamide) co-network is used for the first time for the immobilization of the investigated strain. It was established that during 40 cycles the catabolic potential of the immobilized cells remained at a very high level. These results confirmed that the method of immobilization appeared to be very effective for a long period of time-40 days (Fig. 3). The optical density was 0.25–0.3 OD, which value is negligible compared to the cell density of entrapped quantity of cells (1.6 OD).

Scanning Electron Microscopy (SEM). As seen from SEM micrographs of the studied samples, the obtained cryogel had a heterogeneous super-macroporous structure (Fig. 4A). Bacterial cells were homogeneously distributed and adhered to all the surface spaces of the carrier, forming a biofilm. The uniform distribution is an important criterion for the proper adsorption and degradation of phenol on the whole surface area of the bacterial colonies immobilized. The colonies that were formed by the cells of the strain are well visualized by the SEM (Fig. 4B, C). Moreover, scanning electron microscopy studies confirmed that the cells preserved their shape and their regular distribution after many cycles of use.

Simultaneous biodegradation of phenol and hexadecane by cryogel immobilized cells. These experiments were also performed at a semicontinuous mode of operation by the addition of both xenobiotics at equal concentrations. The end of each cycle was marked when the whole phenol quantity was depleted. After multiple addition of phenol and n-hexadecane to the medium (40 active cycles), it was found that both xenobiotics were totally degraded, but at different rates. Apparently, the alkanotrophic strain *R. wratislawiensis* BN38 preferably degraded hexadecane rather than phenol. The total amount of each degraded compound was 20 g/l (Fig. 5).

Biodegradation at gradual increase of phenol concentration. Estimation of the maximal biodegradation capacity of the immobilized cells was also performed by

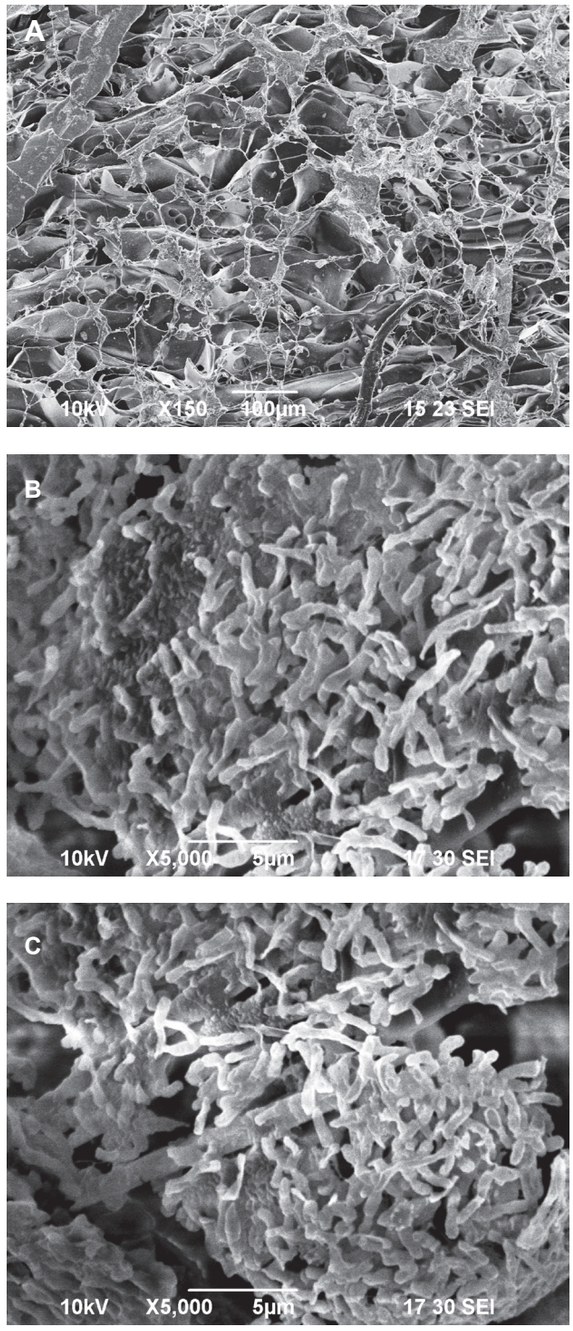

Fig. 4. Scanning Electron micrographs (A) cryogel matrix; (B) cells immobilized in the cryogel matrix at phenol biodegradation, and (C) immobilized cells in the cryogel matrix at phenol and hexadecan biodegradation.

the addition of phenol with increased concentration at every cycle that followed. The total depletion of the phenol at nine different initial concentrations, from 0.6 to 2.2 g/l, was registered. The experiments demonstrated that the immobilized *R. wratislawiensis* BN38 cells have high catabolic ability up to a phenol concentration of 2.4 g/l (Fig. 6). Decrease in the biodegradation rate was

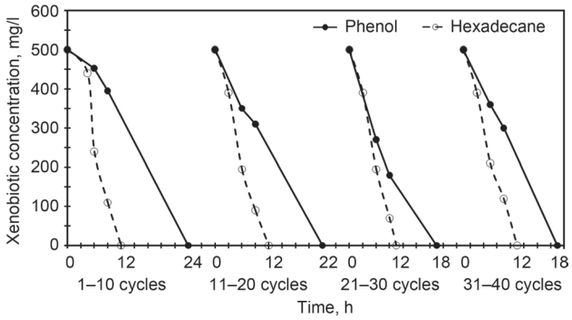

Fig. 5. Semicontinuous biodegradation processes of two xenobiotics by the immobilized *R. wratislawiensis* BN38 cells.
The results are presented as average values ($x_{av.} \pm \sigma$) for the residual phenol (—●—) and hexadecane (––○––) for every 10 cycles.

registered at a concentration of 2.4 g/l phenol, probably due to the high concentration of the xenobiotic.

Simultaneous biodegradation at gradual increase of phenol and hexadecane concentrations. Simultaneous biodegradation of phenol and hexadecane, at equal concentrations of both xenobiotics from 0.6 up to 2.4 g/l, showed a similar trend as compared to the biodegradation of phenol without hexadecane (Fig. 7). Gas chromatography analyses proved that hexadecane was totally degraded and did not inhibit the process of phenol biodegradation. However, at a concentration of 2.4 g/l, 96% of the added hexadecane was degraded for 96 h, while the degradation of phenol was retarded and 55% of it remained in the medium. Parallel carbon uptake of phenol and hexadecane in fact resulted in twofold higher consumption of the toxic carbon substrates.

Role of biosurfactants in the biodegradation processes. When only phenol was present in the aqueous media, trace amounts of surfactant production from free cells were registered (50 mg/l). Cell surface hydrophobicity was high (80%) and remained constant till the end of the process. The surface tension was slightly decreased to 50 mN/m. During the biodegradation of both hydrophilic and hydrophobic xenobiotics, enhanced biosurfactant production (500 mg/l) was quantified. Biosurfactant levels increased exponentially and reached maximum levels in stationary growth as described previously (Tuleva *et al.*, 2008). A significant decrease of the surface tension to 32 mN/m was also registered, accompanied by the formation of stable emulsions with kerosene. Cell surface hydrophobicity declined from 80% to 32% in the stationary growth.

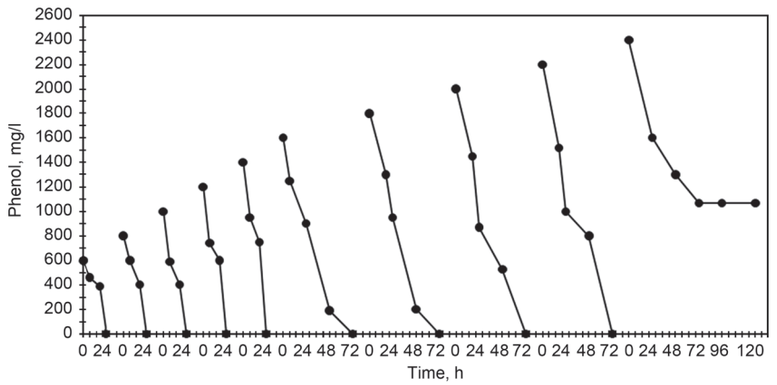

Fig. 6. Biodegradation of phenol by immobilized *R. wratislawiensis BN38* cells (—●—) at gradual increase of xenobiotic concentration.

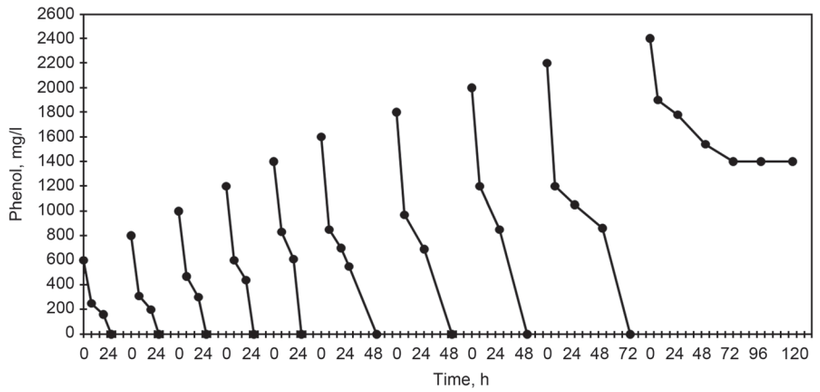

Fig. 7. Biodegradation of phenol in the presence of hexadecane by immobilized *R. wratislawiensis* BN38 cells (—●—) at gradual increase of xenobiotics concentration.

Discussion

The main objective of this study was to assess the potential of free and immobilized *R. wratislawiensis* BN38 cells to degrade both aromatic and aliphatic waste substrates.

The multiple passages on solid media with phenol as a sole carbon and energy source showed that biodegradation of industrial wastes could be improved if the microorganisms are previously adapted to the toxic compounds. Changes in the membrane fluidity and induction of certain enzymes during acclimatization of *R. wratislaviensis*, could be the major responses of the bacterium to the presence of this xenobiotic (Kumar *et al.*, 2005). In our experiments during the first 10 cycles of biodegradation, phenol was completely degraded for 24 h and in the ensuing cycles, the time for phenol degradation even slightly decreased (18 h). Similar results were reported by Soudi and Kolahchi (2011), where phenol with the same concentration (500 mg/l) was degraded for 32 h by *Rhodococcus erythropolis* SKO-1 but in a single cycle. During the last cycle, prolonged time of degradation was observed probably due to decrease of the biodegradation capabilities of the strain.

Representatives of the genus *Rhodococcus* have been immobilized in different types of matrices (Prieto *et al.*, 2002). Other studies describe the performance in 10 active cycles of phenol biodegradation by immobilized in Ca alginate *Rhodococcus* sp. cells (Pai *et al.*, 1995). Quek *et al.* (2006), reported on the immobilization and performance of a hydrocarbon-degrading *Rhodococcus* sp. F92 on polyurethane foam with a successful result in the bioremediation of petroleum hydrocarbons for both free and immobilized cells. In our study, with the application of the immobilization technique, a model system was developed for a stable in time and very effective biodegradation process. The formation of a polymer co-network from HPC and PNIPAAm (1:1 mass ratio) provided a biocompatible cryogel matrix with good mechanical properties. Moreover, it was established that the immobilization procedure employed preserves the cells viability and biodegradation capability. Similar results were described by Velickova *et al.* (2010). The increased stability of the cells during the long time of use with preserved vitality and catabolic activity could be explained by the protecting effect of the matrix in which they were immobilized. There is a diffusion barrier formed that prevents the sharp intrusion of different xenobiotics into the cell (Basha *et al.*, 2010).

Most notable part of our results was the capability of *R. wratislawiensis* BN38 immobilized in the cryogel for a simultaneous biodegradation of a hydrophilic and a hydrophobic xenobiotic under semicontinuous processes. In comparison to the catabolic ability of the free cells of the same strain, the biodegradation capability of the immobilized cells was much higher, taking into account the number of active cycles of operation (40). During these cycles the multiple addition of phenol and hexadecane resulted in their total mineralization. In the case of free cells their biodegradation capability decreased in the last cycles, while immobilized cells preserved their ability to degrade both xenobiotics with increasing concentrations up to 2.4 g/l.

To date there are very few studies carried out on the simultaneous degradation of a hydrophilic and a hydrophobic xenobiotic. Thus, Sun *et al.* (2012) reported that three *Acinetobacter* strains were able to degrade phenol and hexadecane (400 mg/l) in a single active cycle. The active simultaneous biodegradation of phenol and hexadecane is due to the presence of two membrane connected enzymes: phenolhydroxylase and alcanemonooxigenase. In addition, the channels for the transfer of hydrophilic and hydrophobic xenobiotics are situated on different sites of the cells (van Beilen and Funhoff, 2007; Ullrich and Hofrichter, 2007).

A well known feature of Gram(+) bacteria, especially the actinobacteria is their ability to degrade alkanes (Finnerty, 1992). Further more, their growth on aliphatic hydrocarbons is usually accompanied by the synthesis of biosurfactants. Suggested mechanisms for the uptake of hydrophobic contaminants by degrading bacteria include direct contact of the substrates with microorganisms having a high cell surface hydrophobicity and biosurfactant-mediated uptake by microorganisms capable of producing biosurfactants (Zhao *et al.*, 2011). Thus, when grown on phenol and hexadecane strain BN38 produced a bisurfactant possessing the same fragmentation pattern from mass spectra analysis of trehalose tetraester as already described (Tuleva *et al.*, 2008). Initially the cells of *R. wratislawiensis* BN38 showed 80% adhesion to hexadecane indicating high cell surface hydrophobicity. Later, they became more hydrophilic most probably due to the cell-bound biosurfactants that expose their hydrophilic moieties towards the water phase. These observations are consistent with previous reports (Kundu *et al.*, 2013).

For the hydrophilic xenobiotic phenol, the processes were different. In our studies no surfactant activity and no changes in cell surface hydrophobicity were registered. Most probably phenol acts in a different manner by changing the function of the cell membrane by transformation of its fluidity and the protein/lipid ratio (Sikkema *et al.*, 1995). Thus, these changes together with the induction of a number of catabolic enzymes are the main cell response to the presence of the xenobiotic phenol in the medium (de Carvalho and da Fonseca, 2005).

Conclusions

(1) Successful simultaneous biodegradation of the aromatic xenobiotic phenol and the aliphatic one

n-hexadecane by *R. wratislawiensis* BN38 cells immobilized in a hydroxypropylcellulose/poly (N-isopropylacrylamide) cryogel was achieved for the first time.

(2) The system possesses capability to degrade the two xenobiotics up to concentrations of 2.4 g/l and can be exploited for multiple use (40 active operating cycles).

(3) Such model system is very near to the parameters found in different waste waters, where the toxic compounds do not appear separately but in mixtures.

(4) The ability of the investigated strain to degrade different xenobiotics combined with the immobilization technique makes possible the preparation of active and stable operating system that can be involved in a biotechnological scheme for the bioremediation of waste waters, containing these pollutants.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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ORIGINAL PAPER

Complex Biochemical Analysis of Fruiting Bodies from Newly Isolated Polish *Flammulina velutipes* Strains

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Abstract

The present study examined Polish strains of *Flamulina velutipes* as a potential source of nutraceuticals and found that their nutritional value is dependent on the fruiting bodies gathering time. To prove the above hypothesis protein, carbohydrate and phenolic substances concentration were determined. Moreover, catalase, superoxide dismutase, cellobiose dehydrogenase activities were assayed. In order to prove the healing properties of Enoki fruiting bodies the obtained extracts were tested for antioxidant and bacteriostatic abilities. We have proved that Polish *F. velutipes* fruiting bodies may be a rich source of antioxidants and that they are capable of inhibiting *Staphylococcus aureus* growth.

Key words: *Flammulina velutipes*, antibacterial activities, antioxidative activities, fruiting body, phenolic compounds

Introduction

It is presumed that there are more than 14000 species of mushrooms including at least 2000 with various degrees of edibility, of which about 200 edible mushrooms are wild species (Chang, 1987; 2008; Kalac, 2013; Zhang *et al.*, 2013a). During thousands of years, mushrooms have been valued throughout the world as both food and medicine due to their taste and nutritional value as well as healing properties (Wright, 2004; Adebayo *et al.*, 2014). Over time, the importance of mushrooms in human diet has led to the cultivation of certain species. It seems that *Auricularia auricularis* (the wood ear mushroom) was the first fungal species cultivated in China around AD 600 (Kues and Liu, 2000). Over the centuries, the number of cultivated species has been growing together with various applications not only in cuisine but also in medicine. Nowadays, mushrooms are important ingredients of baked dishes (*ex. Pleurotus* sp., *Ganoderma* sp., *Lentinula* sp.), soups (*Lentinula* sp., *Agaricus bisporus*), or drinks (*Ganoderma lucidum*). Moreover, they are used instead of *Saccharomyces* to produce alcohol (*e.g. Agaricus blazei*, *Flammulina velutipes*, *Pleurotus ostreatus*) (Moon and Lo, 2013). To date, the reported healing properties of edible fungi encompass anticancer (*Ganoderma* sp. (Wu *et al.*, 2013a), *Lentinula edodes* (Cao *et al.*, 2013)), antiviral (*Ganoderma pfeifferi* (Niedermeyer *et al.*, 2005), *Pleurotus ostreatus* (Santoyo *et al.*, 2012)), immunomodulatory (*Grifola frondosa* (Wu *et al.*, 2013b)), anti-diabetic (*A. blazei* (Di Naso *et al.*, 2010)), and cardiovascular (*Volvariella volvacea* (Chiu *et al.*, 1995)) activity. Moreover, a number of edible fungal species possess antioxidant properties (*G. lucidum*, *Hericium erinaceus*) (Deepalakshmi *et al.*, 2013; Han *et al.*, 2013). Recently, an explosion of investigations describing bioactive compounds from fungi has been observed, and with every study, the number of isolated and characterized compounds is growing. Each paper brings to light new healing properties of fungal glucans (Wiater *et al.*, 2012), proteins (Jaszek *et al.*, 2013), enzymes (Xu *et al.*, 2011) and other (Mahmood *et al.*, 2010; Ma *et al.*, 2011). Enoki (*F. velutipes*) is one of the most valuable species in Asian countries with little importance in Europe. Despite its culinary values, cultivation and merchandise thereof in certain European countries is even illegal. At the same time, recent papers have proved that *F. velutipes* possess anticancer, immunomodulatory, anti-inflammatory, and antioxidant activities (Chang *et al.*, 2013; Zhang *et al.*, 2013b; Gunawardena *et al.*,

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2014). Beside its obvious medicinal properties, Enoki has been confirmed as a source of laccase, proteases, sterols, endo-β-1,3-galactanase, or asparaginase (Eisele *et al.*, 2011; Kotake *et al.*, 2011; Iketani *et al.*, 2013; Kim *et al.*, 2013; Tong *et al.*, 2014). Despite laboratory experiments with Enoki mycelium, little is known about the nutritional composition and medicinal properties of wild *Flammulina* fruiting bodies (Ergonul *et al.*, 2013; Lin *et al.*, 2013) and even less about cultivated ones (Cai *et al.*, 2013). Given the dynamics of production of bioactive compounds by fungal mycelium, it is obvious that the same effect may be observed in its fruiting bodies. To retain the maximum medicinal value of consumed mushrooms, they should be harvested at the time of their maximum activity. The aim of the paper was to prove not only the medicinal potential of new *F. velutipes* strains isolated in Polish ecological regions, but also to show that fruiting bodies grown in mushroom farms should be harvested at an appropriate time to preserve their maximum healing values.

Experimental

Materials and Methods

Strains, medium and growth processing. *F. velutipes* strains Fv4, Fv10, and Fv11 were obtained from the culture collection of the Department of Vegetable Crops, Poznań University of Life Sciences. The strains were identified by ITS sequencing previously (Janusz *et al.*, 2014). Pure cultures were isolated by cutting out a piece of trama from the inner part of carpophores and placing it onto 20 g/kg malt agar medium in a Petri dish. Stock cultures of the strains were grown for 7 days at 30°C on potato dextrose agar. They were stored at 4°C and subcultured every month. The cultures were used for producing the grain spawn (wheat grain) by a convenient method. The prepared spawn was stored at 4°C until it was used for inoculation.

Fruiting conditions. Cultivation of *F. velutipes* fruiting bodies was carried out at the Department of Vegetable Crops of Poznan University of Life Sciences. The applied substrate was a mixture of oak and beech sawdust (1:1, v/v). The above-mentioned sawdust mixture was enriched by addition of wheat bran in the amount of 200 g/kg DM (Dry Matter) of the sawdust as well as saccharose and gypsum in the amount of 10 g/kg. The experimental substrate was wetted with distilled water to reach a moisture content of 650 g/kg and used to fill polypropylene sacks equipped with a micro-filter. Each sack contained 2.5 kg wet substrate. The substrate was subjected to sterilization at the temperature of 121°C for 1.5 h and, after cooling it down to the temperature of 25°C, it was inoculated with the mycelium of the *F. velutipes* strains. Incubation was conducted in darkness at the temperature of 25°C and relative air humidity of 80–85% until the entire substrate was overgrown by mycelium. Next, the sacks were transferred to the cultivation facility and the foil was cut off in their upper part directly over the surface of the substrate. Throughout the trial, the temperature in the facility was maintained at 14–15°C and relative air humidity at 85–90%. The facility was additionally lit with fluorescent light (day-light) of 4500 cd intensity and ventilated to keep the CO_2 concentration below 1 lm^3. The fruiting bodies were harvested depending on the strain growth to achieve the same stage of growth (Fig. 1). Therefore, for strain Fv4 time zero means 43 days, Fv10 – 36 days and for Fv11 – 51 days. Next, the fruiting bodies were harvested at 0, 1, 2, 3, and 4 days.

Preparation of extracts from the fruiting body biomass. Fungal fruiting bodies were harvested periodically during 96 hours after the beginning of the fruiting (at 0, 1, 2, 3, and 4 days). The fruiting body biomass was homogenized in distilled water in the proportion 1:1 with a glass Potter homogenizer at 4°C. After centrifugation (15 min, 10000 × g), portions of the crude supernatant were frozen and used for the conducted experiments.

Analytical methods

Determination of carbohydrates, proteins, and phenolic compounds. The carbohydrate concentration of the extracts isolated from the fruiting bodies was measured by the phenol-sulfuric acid assay according to DuBois *et al.* (1956) with D-glucose as a standard. The reducing sugar was determined by the Somogyi-Nelson methods based on the procedure described by Hope and Burns (1987) with some modifications. The protein content was analyzed according to the Bradford (1976) method using bovine serum albumin (BSA) as a standard. In addition, the phenolic compounds in the extracts from the fruiting body were quantified by the DASA test (Malarczyk, 1989). The changes in absorbance were measured at 500 nm and compared with the standard curve of vanillic acids.

Laccase activity assay. Laccase activity was measured using syringaldazine (4-hydroxy, 3,5-dimetoxybenzaldehyde) as a reaction substrate (Leonowicz and Grzywnowicz, 1981). The catalytic activity of enzyme was expressed in nanokatals per milligram of protein.

Cellobiose dehydrogenase activity assay. Cellobiose dehydrogenase (CDH) activity was measured by monitoring the change in absorbance of the two-electron acceptor 2,6-dichloroindophenol (DCIP) (Sigma Chemical Co., St. Louis, MO, USA) at 520 nm ($\varepsilon 520 = 6.8\ mM^{-1}cm^{-1}$), pH 4.5, and 30°C using a Shimadzu UV160A (Shimadzu, Tokyo, Japan) spectrophotometer. The reaction mixture consisted of DCIP (50 µl,

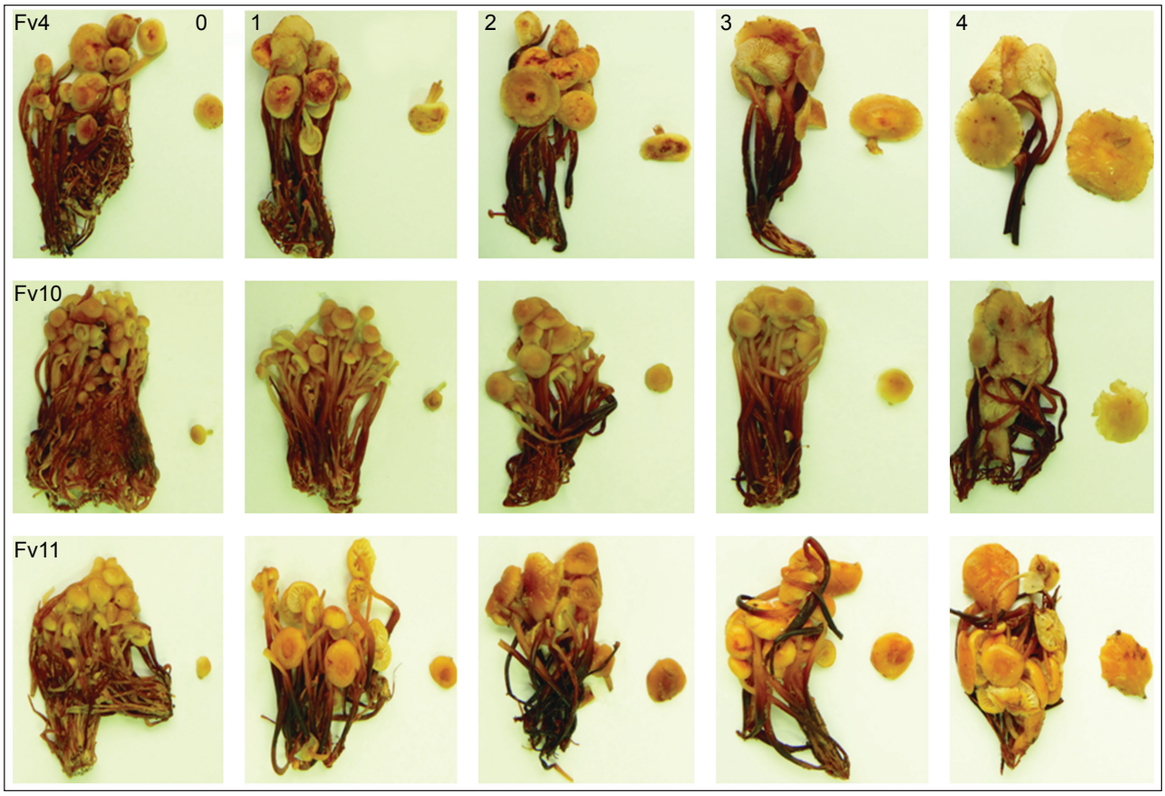

Fig. 1. Visualization of the different stages of development of the fruiting bodies of the *F. velutipes* Fv4, Fv10, and Fv11 strains 0, 1, 2, 3, and 4 days (0, 24, 48, 72, and 96 hours) after the beginning of fructification.

3 mM in water containing 10% v/v ethanol), sodium fluoride (50 µl, 80 mM in water as an inhibitor for potentially present laccases), lactose (100 µl, 300 mM in 100 mM sodium acetate buffer, pH 4.5), 700 µl of the same buffer, and 100 µl of the enzyme solution appropriately diluted in a 1 ml glass microcuvette. The reaction was started by addition of the enzyme and the decrease in absorbance was monitored during the first 60 s. The final enzyme activity was expressed as nkat per liter (Baminger *et al.*, 2001; Karapetyan *et al.*, 2006).

Assay of the relative level of superoxide anion radicals (SOR). The SOR level was estimated according to the method for rapid detection of superoxide anion presence in fungal material (Pazdzioch-Czochra *et al.*, 2003). The spectrophotometric measurements were based on the detection of superoxide-induced formation of formazan from nitrotetrazolium blue (NBT) under alkaline conditions, as described previously (Jaszek *et al.*, 2006). The alkaline conditions were introduced to prevent precipitation of formazan for about 40 min.

Antioxidant activity assays

ABTS Radical-Scavenging Test. The ABTS radical-scavenging ability of the extracts isolated from the fruiting bodies were recorded according to the procedure of Re *et al.* (1999) with some modification. For detection of the antioxidant capacity, 10 µl of the investigated compounds at concentrations ranging from 15 to 500 mg/ml were mixed with 990 µl of the ABTS radical solution. The determination of absorbance stability in the range of 1 to 15 minutes was checked for both mushroom preparations. The stability of absorbance of the samples was observed after 6 minutes of incubation. Accordingly, this time (60 s) was then used in the assays. The percentage of reduction of ABTS oxidation was calculated by the presented formula:

$$\text{ABTS}^{+} \text{ scavenging effect } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 means the absorbance of the control samples and A_1 stands for the absorbance at 734 nm of the investigated compounds/standards.

DPPH Free Radical-Scavenging Test. The DPPH free radical-scavenging ability of the extracts isolated from the fruiting bodieswas determined according to the method described by Paduch *et al.* (2008). The analysed compounds (0.1 ml) at concentrations ranging from 6.25 to 600 mg/ml were added to 0.1 ml of a DPPH· solution (0.2 mg/ml in ethanol). Trolox and ascorbic acid (standards with strong antioxidant activities) were used as positive markers. Absorbance at 515 nm was determined at room temperature after 2,

5, 10, 15, 20, and 30 min of incubation. The optimal time of incubation in the presented measurement was 10 min. The percentage of reduction of the DPPH oxidation rate was calculated according to the presented formula

$$\text{DPPH scavenging effect (\%)} = [A_0 - (As - Ac)/A_0] \times 100$$

where A_0 means the absorbance of the control sample (with DPPH), and As means the absorbance of the standards or investigated compounds (with DPPH), Ac means the absorbance of the investigated compounds (without DPPH). The Trolox calibration curves for both tests were prepared for a concentration range from 15 to 500 mg/ml and EC_{50} values were indicated as described previously (Jaszek *et al.*, 2013).

Analysis of the antibacterial activity of extracts from the fruiting body. The antibacterial effect of the extract isolated from the fruiting bodies was tested using *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 25923 bacterial strains. Before testing, inocula of each bacterium were grown in the Luria-Bertani (LB) medium. The tested substances (100 µl) were added to 48-well sterile polystyrene plates containing 1 ml of the LB medium. The inoculum was determined by measuring the optical density and expressed as the number of cells per ml. The inocula were used in infecting doses as follows: *E. coli*: 1×10^4, 1×10^6 cells/ml and *S. aureus:* 1×10^4, 2×10^6cells/ml. During a 24-hour incubation period (at 37°C), the LB medium samples from each experiment were tested for bacterial growth by measuring the OD (optical density) on a plate reader at 660 nm.

Electrophoretic visualization of the activities of superoxide dismutase (SOD) and catalase (CAT) – enzymatic antioxidants and protein profiles. The samples of the prepared homogenate were separated by ultrafiltration using the Microcon Centrifugal Filter Units, 3000 NMWL designed by Millipore. Afterwards, 15 µg of proteins from the samples were loaded into each well of 10% native polyacrylamide gel. The gels were run at 4°C and 145 V. After separation, SOD activities were visualized based on the method of Beyer and Fridovich (1987). The CAT activity bands were visualized using ferricyanide negative staining according to the Wayne and Diaz (1986) methodology. For the detection of protein bands, Coomassie brilliant blue (R-250) staining was used.

Statistical analysis. Statistical analysis of data from independent experiments repeated three times was performed on three replicates from each treatment with Excel program Microsoft Office 2010 package and the results presented in the paper were a mean ± SD from three experiments and three repetitions (n = 9). Multiple comparisons of means were performed by the Tukey Honest Significance Post Hoc Test. P values ≤ 0.05 were considered significant for all the tests. Analysis of variance ANOVA was used to determine significance of differences between values.

Results

Chemical composition of extracts from *F. velutipes* fruiting bodies. The extracts from the fruiting bodies of three *F. velutipes* strains were evaluated in terms of the concentration of protein, total sugar, reducing sugar, and phenolic compounds. The results obtained show that the maximum concentration of the substances varied during the development of the analyzed fungal fruiting bodies. The maximum concentration of proteins (over 10 mg/g dry weight) was found in fruiting bodies harvested on the first or second day. Total sugars achieved their maximum concentration later (up to even 4 days) and reached up to 15.4 mg/g of dry weight, whereas the concentration of reducing sugars seemed to be changing during these days and the pattern was hard to be noticed. In all the analyzed strains, the maximum concentration of phenolic substances (up to 9.57 mM/g dry weight) was observed in the fruiting bodies harvested on the second or third day of fructification. It appears that strains Fv4 and Fv10 are richer in proteins than Fv11 (Table I). Moreover, the electrophoretic profiles of Fv4 and Fv10 differ from that of Fv11 (Fig. 2). It was observed that the amount of protein contained in the fruiting bodies was decreased with the

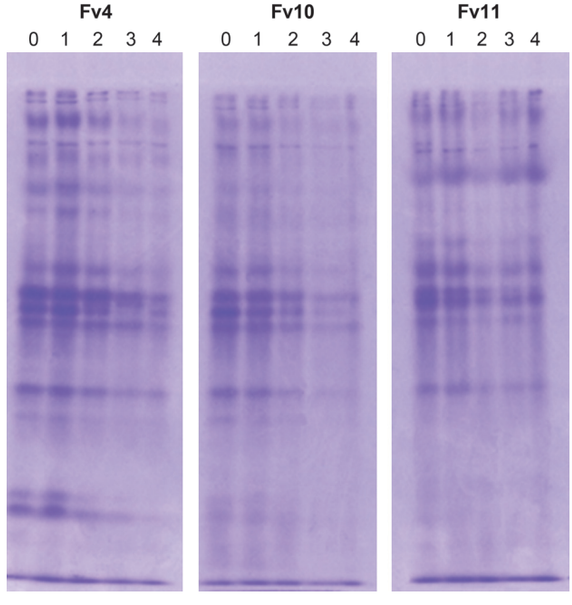

Fig. 2. Native electrophoretic analysis of the protein profile of extracts prepared from *F. velutipes* Fv4, Fv10, and Fv11 strains fruiting bodies harvested at 0, 1, 2, 3, and 4 days (0, 24, 48, 72, and 96 hours) after the beginning of fructification.

Table I
Chemical composition of extracts isolated from fruiting body of *F. velutipes* yield of proteins, total sugars, reducing sugars and concentration of phenolic compounds. Data are mean ±SD for three experiments and three replicates (n = 9). The mean values in columns marked by the same lowercase letter are not significantly different at significance of $p \leq 0.05$ determined according Tukey's honest significance test.

Samples	Protein (mg/g*)	Total sugars (mg/g*)	Reducing sugars (mg/g*)	Total phenolic (mM/g*)
Fv4/0	6.7 ± 0.10^{c}	6.7 ± 0.40^{g}	2.9 ± 0.02^{b}	7.5 ± 0.20^{b}
Fv4/1	10.9 ± 0.10^{a}	6.1 ± 0.07^{g}	2.6 ± 0.06^{c}	9.3 ± 1.20^{a}
Fv4/2	6.8 ± 0.05^{c}	7.9 ± 0.40^{f}	3.4 ± 0.10^{a}	9.6 ± 0.20^{a}
Fv4/3	5.2 ± 0.20^{e}	11.4 ± 0.30^{c}	3.2 ± 0.02^{a}	6.2 ± 0.10^{c}
Fv4/4	5.3 ± 0.10^{e}	7.6 ± 0.30^{f}	2.3 ± 0.08^{d}	6.4 ± 0.10^{c}
Fv10/0	10.4 ± 0.03^{a}	9.8 ± 0.10^{e}	2.0 ± 0.07^{d}	7.6 ± 0.05^{b}
Fv10/1	6.8 ± 0.08^{c}	9.8 ± 0.02^{e}	1.8 ± 0.05^{e}	5.9 ± 0.20^{d}
Fv10/2	4.7 ± 0.10^{f}	13.1 ± 0.20^{b}	2.1 ± 0.06^{d}	6.3 ± 0.08^{c}
Fv10/3	3.3 ± 0.01	8.8 ± 0.50^{f}	1.3 ± 0.10^{f}	4.2 ± 0.20^{e}
Fv10/4	4.5 ± 0.09^{g}	6.5 ± 0.70^{g}	2.2 ± 0.10^{d}	4.0 ± 0.10^{e}
Fv11/0	5.3 ± 0.10^{e}	9.9 ± 0.10^{e}	2.1 ± 0.25^{d}	9.6 ± 0.70^{a}
Fv11/1	6.1 ± 0.10^{d}	15.4 ± 0.80^{a}	2.5 ± 0.03^{c}	8.1 ± 0.50^{b}
Fv11/2	4.1 ± 0.10^{h}	10.1 ± 0.10^{d}	1.4 ± 0.20^{f}	5.9 ± 0.30^{d}
Fv11/3	4.8 ± 0.08^{f}	10.6 ± 0.05^{d}	2.0 ± 0.10^{e}	6.6 ± 0.04^{c}
Fv11/4	7.3 ± 0.09^{b}	13.7 ± 1.40^{b}	1.6 ± 0.10^{f}	5.8 ± 0.06^{d}

Mean values in columns with same lowercase letters are not significantly different at $p \leq 0.05$.
* – g dry weight of fruiting body

time of its development. In addition, the electrophoretic analysis confirmed the fact the highest amount of protein was observed in the fruiting bodies of strain F4. However, this strain seems to possess more total sugars than Fv4 and Fv10 (Table I).

Enzyme activities: CDH, CAT, SOD. The highest activities of cellobiose dehydrogenase (CDH), catalase (CAT), and superoxide dismutase (SOD) were observed in extracts isolated from Fv4. CDH and SOD seemed to be most active in the fruiting bodies of the strain when they were harvested on the second day. The activities of CDH were at least twice as high as in other two strains (Fig. 3). Moreover, the maximum activity of CDH in the Fv10 fruiting bodies was observed on the same day (24 hours), whereas the highest activity in Fv11 appeared 24 hours earlier and was continuously declining. The highest activity of (CAT) was noted in the fruiting bodies of Fv4 and remained almost unchanged over the time, in contrast to Fv10 and Fv11, in which they were obviously decreasing day after day (Fig. 4A). The electrophoretic analysis of SOD activities proved existence of two clearly visible isoforms in all the analyzed strains. The highest SOD activities were observed in the Fv4 strain, contrary to those of Fv11. In all the investigated fruiting bodies, the level of SOD decreased during the fructification period (Fig. 4B). No laccase or manganese peroxidase activities were detected in all the analyzed fruiting body extracts.

Antioxidant and antibacterial activities. To allow comparison of the antioxidant properties of the tested strains, we checked the level of superoxide anion radicals. The results obtained allow a conclusion that the highest concentration was observed in the fruiting bodies of the Fv4 strain harvested after 1 day. A similar pattern was found in the case of strain Fv11; however, the level of superoxide anion radicals was approx. 20% lower. The same peak was observed on the second day for the fruiting bodies of Fv10; however, both Fv10 and Fv11 had another maximum within 4 days, and the second peak was slightly higher for Fv10 (Fig. 5). Since

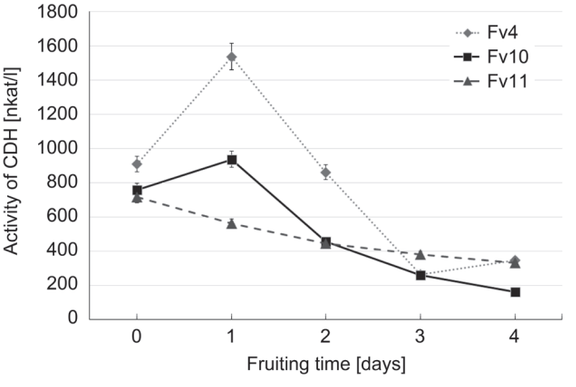

Fig. 3. The effect of the developmental stages of *F. velutipes* Fv4, Fv10, and Fv11 strains fruiting bodies harvested at 0, 1, 2, 3, and 4 days (0, 24, 48, 72, and 96 hours) after the beginning of fructification on CDH activities. Data are mean ±SD for three experiments and three replicates (n = 9). The mean values marked by the same letter are not significantly different at significance of $p \leq 0.05$ determined according Tukey's Honest Significance test.

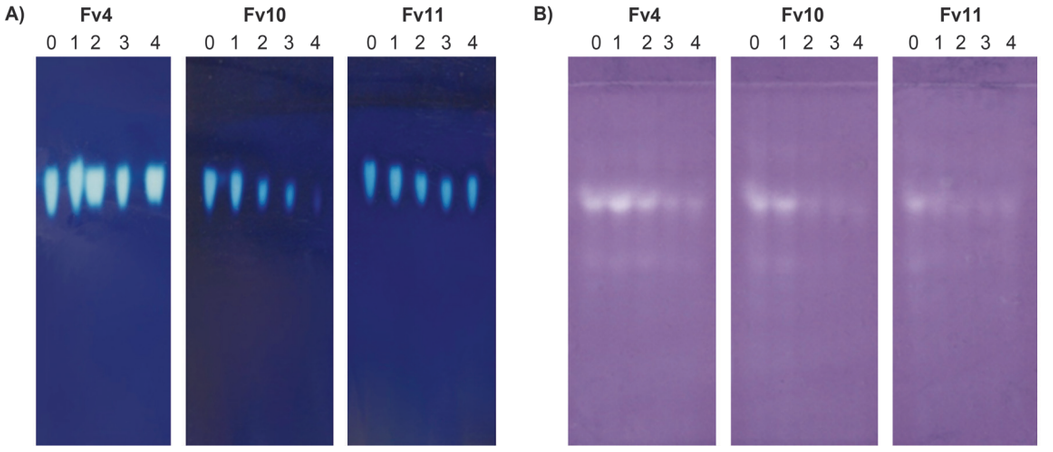

Fig. 4. Native PAGE electrophoresis of CAT (A) with and SOD (B) activities in the *F. velutipes* Fv4, Fv10, and Fv11 strains fruiting bodies harvested at 0, 1, 2, 3, and 4 days (0, 24, 48, 72, and 96 hours) after the beginning of fructification (10% polyacrylamide gels stained according to Wayne and Diaz (A) and Beyer and Fridrovich (B))

no laccase activity was detected in the analyzed extracts, we used the ABTS and DPPH method to estimate the antioxidant potential of the tested *F. velutipes* fruiting bodies. The results obtained showed clearly that all the strains exhibited the highest antioxidant activity during the first 24 hours. The scavenging abilities of *F. velutipes* fruiting body extracts assayed with the ABTS method at the concentration range of 15–500 mg/ml were between 13.6 and 90.5% for Fv4, 0.5 and 79.9% for Fv10, and 3.6 and 77.1% for Fv11 (Fig. 6). In the case of the DPPH method, the maximum of the scavenging effect was 57.2% for Fv4, 58.3% for Fv10 and 58.1% for Fv11 (Fig. 7). The calculation of normalized EC_{50} values specified the concentrations of extracts isolated from the fruiting bodies of *F. velutipes* that are able to scavenge 50% of free radicals present in the tested mixture (Table II). The antioxidant activity of the analyzed fungal extracts seemed to be increasing together with their

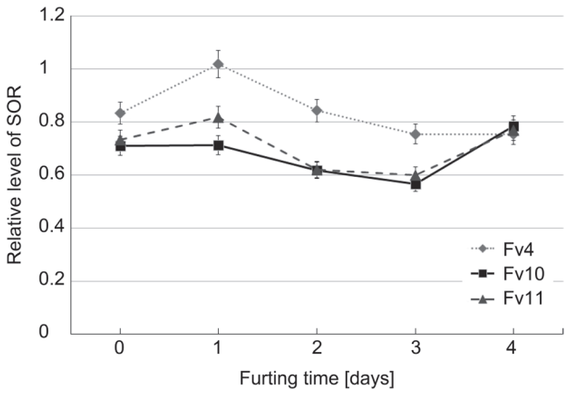

Fig. 5. Relative levels of superoxide anion radicals (SOR) in extracts prepared from the fruiting bodies of the *F. velutipes* Fv4, Fv10, and Fv11 strains harvested at 0, 1, 2, 3, and 4 days (0, 24, 48, 72, and 96 hours) after the beginning of fructification. Data are mean ±SD for three experiments and three replicates (n = 9). The mean values marked by the same letter are not significantly different at significance of $p \leq 0.05$ determined according Tukey's Honest Significance test.

Table II

EC_{50} values (half of the maximum scavenging effect) of extracts isolated from *F. velutipes* fruiting body submerged cultures with trolox as control. Data are mean ±SD for three experiments and three replicates (n = 9). $EC_{50} > 500$ mg/ml cannot be calculated from the graphs. The mean values in columns marked by the same small lowercase and in rows marked by the same uppercase letter are not significantly different at significance of $p \leq 0.05$ determined according Tukey's honest significance test (HSD).

Samples	EC_{50}(mg/ml)	
	DPPH method	ABTS method
Fv4/0	236.2 ± 1.1^{e}	57.9 ± 0.6^{k}
Fv4/1	246.1 ± 1.0^{g}	56.9 ± 0.5^{k}
Fv4/2	206.9 ± 0.9^{h}	70.2 ± 0.6^{j}
Fv4/3	267.6 ± 1.2^{f}	107.1 ± 0.9^{i}
Fv4/4	>500	112.2 ± 0.0^{h}
Fv10/0	$>500^{a}$	125.0 ± 0.6^{g}
Fv10/1	$>500^{a}$	130.2 ± 0.5^{h}
Fv10/2	237.1 ± 0.9^{e}	191.7 ± 0.6^{d}
Fv10/3	$>500^{a}$	221.6 ± 0.7^{c}
Fv10/4	$>500^{a}$	315.6 ± 0.9^{a}
Fv11/0	452.1 ± 2.1^{a}	121.3 ± 0.5^{l}
Fv11/1	430.2 ± 2.0^{b}	155.3 ± 0.4^{f}
Fv11/2	437.0 ± 1.9^{c}	178.8 ± 0.5^{d}
Fv11/3	398.0 ± 2.0^{d}	125.0 ± 0.6^{g}
Fv11/4	$>500^{a}$	261.1 ± 0.8^{b}
Trolox	8.1 ± 1.0^{h}	8.04 ± 1.1^{m}

Mean values in columns with same lowercase letters are not significantly different at $p \leq 0.05$.
ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid),
DPPH – di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium.

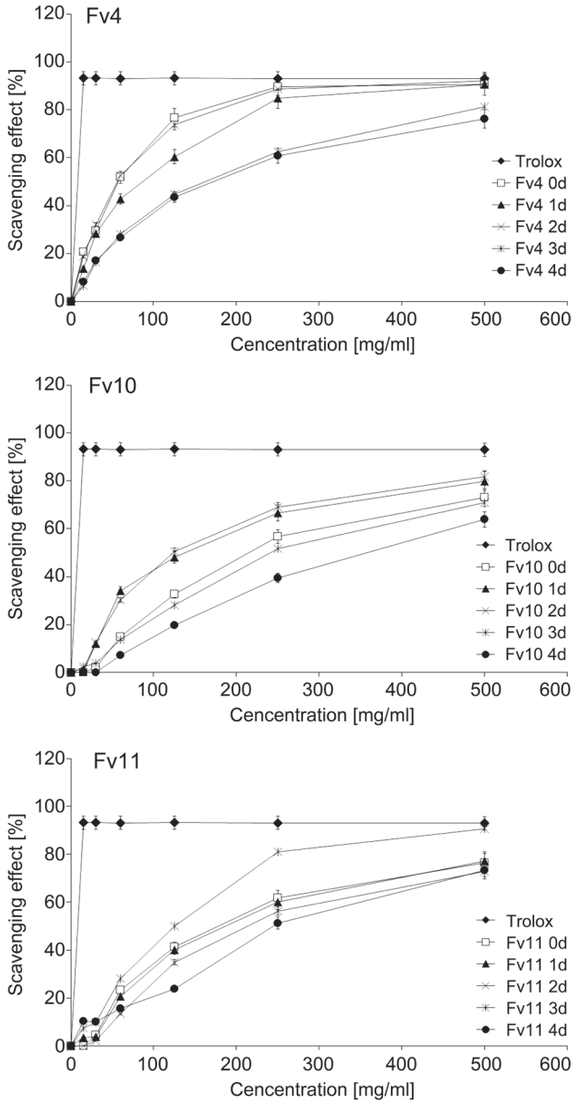

Fig. 6. The ABTS radical-scavenging test of extracts prepared from *F. velutipes* Fv4, Fv10, and Fv11 strains fruiting bodies harvested at 0, 1, 2,3, and 4 days (0, 24, 48, 72, and 96 hours) after the beginning of fructification. Data are mean ±SD for three experiments and three replicates (n = 9).

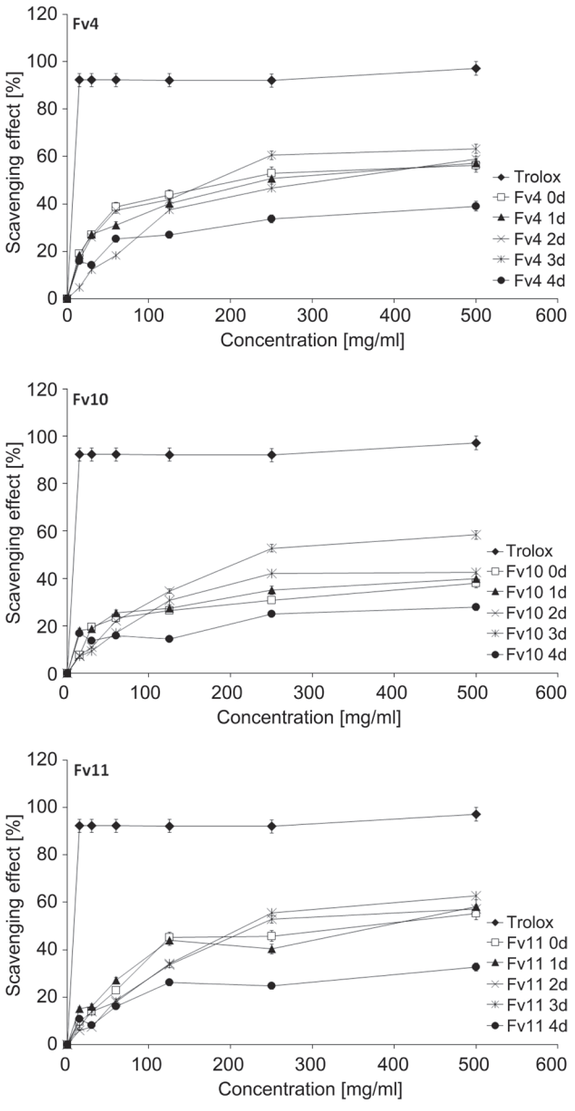

Fig. 7. The DPPH radical-scavenging test of extracts prepared from *F. velutipes* Fv4, Fv10, and Fv11 strains fruiting bodies harvested at 0, 1, 2,3, and 4 days (0, 24, 48, 72, and 96 hours) after the beginning of fructification. Data are mean ±SD for three experiments and three replicates (n = 9).

concentration, achieving a plateau at 250 g/l. Bearing in mind that antioxidant properties of fungal extracts may be related to antibacterial potential, we tested their ability to inhibit growth of *S. aureus*. All the *F. velutipes* fruiting bodies harvested during the first two days appeared to inhibit growth of the mentioned bacterial strain, however, to a different extent. The highest antibacterial activities were detected in the extracts from Fv11, which were even capable of complete inhibition ($OD_{660nm} = 0$) of bacterial growth introduced as inoculum of 1×10^6 cells/ml (Table III). Strain Fv10 appeared to be slightly less active, as it inhibited *S. aureus* growth introduced as lower inoculum (1×10^4 cells/ml).

Discussion

In recent years, an explosion in interest in healthy nutrition has been observed. Many products originating from plants or animals are labeled as ecological or good for human health. Nowadays, food products are carefully examined with respect to composition, nutritional values and finally possible medicinal properties/activities. Recent advances in biotechnology allowed introducing a number of products, including microorganisms or the result of cultivation thereof, to markets all over the world. Among them, fungi form a large group that not only is crucial for brewery products

Table III
Optical density (OD 660 nm) measurement of *E. coli* and *S. aureus* introduced as 1×10^4 cells/ml and incubated 1 day in LB medium at 37°C in the presence of extracts isolated from fruiting body of *F. velutipes*. Data are mean ±SD for three experiments and three replicates (n = 9). The mean values in columns marked by the same lowercase letter are not significantly different at significance of $p \leq 0.05$ determined according Tukey's Honest Significance test.

Samples	OD at 660 nm			
	E. coli		*S. aureus*	
	1×10^4/ml	1×10^6/ml	1×10^4/ml	1×10^6/ml
Fv4/0	0.4 ± 0.02^{dA}	0.4 ± 0.02^{dA}	0.1 ± 0.02^{fB}	0.1 ± 0.03^{fB}
Fv4/1	0.6 ± 0.02^{bA}	0.5 ± 0.02^{cB}	1.1 ± 0.03^{aC}	1.1 ± 0.02^{aC}
Fv4/2	0.3 ± 0.09^{eC}	0.4 ± 0.03^{dB}	0.7 ± 0.02^{bA}	0.1 ± 0.01^{fD}
Fv4/3	0.3 ± 0.01^{eB}	0.4 ± 0.04^{cA}	0.2 ± 0.01^{eC}	0.2 ± 0.02^{eC}
Fv4/4	0.5 ± 0.03^{cB}	0.5 ± 0.04^{cB}	0.4 ± 0.02^{cC}	0.7 ± 0.03^{bA}
Fv10/0	0.4 ± 0.02^{dB}	0.5 ± 0.01^{dA}	0.0^{gD}	0.1 ± 0.02^{fC}
Fv10/1	0.4 ± 0.05^{dB}	0.5 ± 0.03^{dA}	0.0^{gD}	0.1 ± 0.03^{fC}
Fv10/2	0.7 ± 0.01^{aA}	0.7 ± 0.02^{aA}	0.0^{gC}	0.2 ± 0.01^{eB}
Fv10/3	0.5 ± 0.02^{cB}	0.6 ± 0.05^{bA}	0.0^{gD}	0.2 ± 0.02^{eC}
Fv10/4	0.6 ± 0.03^{A}	0.5 ± 0.02^{cB}	0.4 ± 0.02^{cC}	0.5 ± 0.04^{cB}
Fv11/0	0.4 ± 0.01^{dB}	0.6 ± 0.05^{bA}	0.0^{gC}	0.0^{gC}
Fv11/1	0.5 ± 0.02^{cA}	0.5 ± 0.02^{cA}	0.0^{gB}	0.0^{gB}
Fv11/2	0.4 ± 0.03^{dB}	0.5 ± 0.04^{cA}	0.0^{gD}	0.2 ± 0.01^{eC}
Fv11/3	0.4 ± 0.03^{dB}	0.5 ± 0.02^{cA}	0.1 ± 0.01^{fD}	0.2 ± 0.01^{eC}
Fv11/4	0.5 ± 0.02^{cB}	0.6 ± 0.09^{aB}	0.3 ± 0.01^{dC}	0.3 ± 0.02^{dC}

Mean values in columns with same lowercase letters are not significantly different at $p \leq 0.05$.
Mean values in rows with same uppercase letters are not significantly different at $p \leq 0.05$.
OD – optical density

but also their importance in medicine is increasing, as indicated by many papers describing new fungal compounds or their application. In the beginning, mushrooms were valued for their flavor in preparation of dishes; nowadays, they are rather regarded as a source of nutraceuticals. Recent analyses have proved that mushrooms are important in our diet due to the high protein and low fat content (Barros *et al.*, 2008); moreover, a number of papers have evidenced their value as antimicrobial, antioxidant, and anticancer compounds (Janes *et al.*, 2006; Moradali *et al.*, 2007; Karaman *et al.*, 2010; Lemieszek and Rzeski, 2012). The results of our experiments support the findings that *F. velutipes* fruiting bodies may be a source of nutraceuticals in human diet. The paper presents not only three newly isolated Enoki strains but also proves that the harvesting time of fruiting bodies may be important for the level of bioactive compounds. Focusing on the nutraceutical value of Enoki, it should be underlined that the highest protein and carbohydrate concentration is observed in fruiting bodies harvested during the first days. However, there are species that contain more proteins or carbohydrates (Barros *et al.*, 2008); nevertheless, high nutrient value tends to be observed in young fruiting bodies (Barros *et al.*, 2007a; 2007b). Cheung *et al.* (2003) proved that the total phenolic compounds were responsible for the antioxidant properties of extracts from wild growing mushrooms. In our experiments, the highest total phenolic compound content in early harvested fruiting bodies support this hypothesis; however, the differences among the strains suggest that more compounds may be engaged in the *F. velutipes* antioxidant properties. Emerging information related to antioxidant properties of fungal cellobiose dehydrogenase (Nyanhongo *et al.*, 2013; Sulej *et al.*, 2013) we showed CDH activities in *F. velutipes* fruiting bodies in contrary to manganese peroxidase and laccase, which were not detected. To our knowledge, CDH activity has not been previously demonstrated in Enoki fruiting bodies; however, the role of this enzyme was proved to be important in pigmentation of *Pycnoporus cinnabarinus* (Temp and Eggert, 1999). Since we have found a correlation between cellobiose dehydrogenase (Fig. 3) and the scavenging effect (Table II) of fungal extracts, the results obtained may support CDH involvement in the antioxidant properties *F. velutipes.* Besides this cellulose-degrading enzyme,

two other key antioxidant enzymes with high activities were found in the early harvested Enoki fruiting bodies. Both catalase and superoxide dismutase are produced by many organisms to prevent free radical damage (Garcia *et al.*, 2003; Mau *et al.*, 2004; Rahman, 2007). Ma *et al.* (2014) have proved that activities of catalase and SOD are dependent on hydratation of fruiting bodies in *Auricularia auricula-judae*; moreover, SOD is the most efficient enzyme scavenging superoxide anion radicals (SOR). It is possible that reactions catalyzed by SOD and catalase are responsible for decreasing the superoxide anion radical concentration. Barros *et al.* (2007a; 2007b) suggested that the decrease in the antioxidant activity with maturation of fruiting bodies (*Lactarius* sp.) may be caused by their involvement in defense against aging processes. In nature, antibacterial fungal compounds are supposed to protect fruiting bodies from microbial infections and they were found useful in biotechnology (Barros *et al.*, 2007a; 2007b). The results of our experiments have proved that *F. velutipes* fruiting bodies may have antibacterial and bacteriostatic activities against *S. aureus*. At an early stage of maturation, strain Fv11 was able to inhibit growth of *S. aureus* completely at both tested concentrations (even in 2×10^6). The results obtained are similar to those of Barros *et al.* (2007a; 2007b), who indicated that loss of antibacterial activity during maturation of fruiting bodies may be related to loss of antioxidant properties. However, some fungal species are unable to stop the growth of *S. aureus* (Ramesh and Pattar, 2010) completely. Camelini *et al.* (2005) has proved that the concentration of glucan in *Agaricus brasiliensis* is related to cap formation and the glucan concentration is high only in mature fruiting bodies (cap open). Considering the *F. velutipes* images and carbohydrate concentration (Table I), there may be a similar correlation on the second and third day, which may be useful information for harvesting fruiting bodies without the need for complicated tests. In conclusion, the results obtained prove that Polish strains of Enoki have nutritional and nutraceutical values as edible mushrooms. Moreover, the results of our experiments indicate that the age of fruiting bodies is important for their healing properties.

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ORIGINAL PAPER

Prevalence of Biofilm Formation and Wide Distribution of Virulence Associated Genes among *Vibrio* spp. Strains Isolated from the Monastir Lagoon, Tunisia

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Abstract

In the current study, 65 *Vibrio* spp. were isolated from the Monastir lagoon water, were characterized phenotypically and genotypically. In addition, we looked for the presence of three *Vibrio parahaemolyticus* virulence genes (*tlh*, *trh* and *tdh*) and ten *Vibrio cholerae* virulence genes (*ctx*A, *vpi*, *zot*, *ace*, *tox*R, *tox*T, *tos*S, *tox*RS, *tcp*A and *cp*P). We also investigated the antibiotic susceptibilities and the adherence ability of the identified strains to abiotic material and to biotic surfaces. The cytotoxicity activity against HeLa and Vero cell lines were also carried out for all tested strains. All *Vibrio* isolates were identified to the species level and produced several hydrolytic exoenzymes. The results also revealed that all strains were expressing high rates of resistance to tested antibiotics. The minimum inhibitory concentration (MIC) values showed that tetracycline and chloramphenicol were the most effective antibiotics against the tested bacteria. *Vibrio alginolyticus* and *V. cholerae* species were the most adhesive strains to both biotic and abiotic surfaces. Besides, *V. alginolyticus* isolates has the high levels of recombination of genes encoding *V. cholerae* and *V. parahaemolyticus* virulence factors. *In vitro* cytotoxic activities of several *Vibrio* extracellular product were also observed among HeLa and Vero cells.

Key words: *Vibrio* spp., antibiotic susceptibility, biofilm, Monastir lagoon, virulence genes

Introduction

Vibrio species are widely distributed in marine environments, estuarine waters, sediments and hatcheries microbiota (Costa *et al.*, 2010; Mechri *et al.*, 2012). They have been associated with some human infections (Barton and Acton, 2009; Reilly *et al.*, 2011) and can cause several epizootics in many aquatic animals, especially in fish, shellfish and crustaceans (Ben Kahla-Nakbi *et al.*, 2006; Rebouças *et al.*, 2011).

The basis of pathogenicity of *Vibrio parahaemolyticus* depends on three major virulence factors having several biological activities, the thermostable direct haemolysin (*tdh*); the TDH-related haemolysin (*trh*); and the thermolabile haemolysin (*tlh*) (Matsumoto *et al.*, 2000; Nair and Hormazabal, 2005). *Vibrio cholerae* carries a wealth of pathogenic determinants encoded by two separate genetic elements; the cholera toxin genes encoded by the filamentous phage, *CTX*φ and the putative prophage *VPI*φ, which encodes several genes clusters required for toxin co-regulated pilus (*TCP*) production, accessory colonization factors (ACF) and the *tox*T, *tcp*P, *tcp*H and *tcp*I regulatory proteins (Peterson, 2002). Other factors have been associated with enteropathogenicity including two membrane regulatory proteins (*tox*R and *tox*S) (Miller *et al.*, 1987; Miller *et al.*, 1989), a zonula occludens toxin (*zot*) (Fasano *et al.*, 1991) and an accessory cholera enterotoxin (*ace*) (Trucksis *et al.*, 1993).

In most ecosystems, bacterial communities often adopt a sessile biofilm lifestyle in the target to increase their surviving chances by protecting themselves from adverse environmental stressful conditions (Hall-Stoodley *et al.*, 2004; Hoffman *et al.*, 2005). Biofilms exhibits complex spatial organization composed by capillary water channels allowing the flow of nutrients and oxygen into the interior of the biofilm-associated bacteria and allow toxic metabolites to diffuse out of the biofilm (Costerton *et al.*, 1995).

The present study was aimed for isolation and identification of three *Vibrio* species (*Vibrio alginolyticus*, *V. cholerae* and *V. paraheamolyticus*) from the Monastir lagoon water, for detection of biofilm formation and

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for investigation of the presence of three *V. parahaemolyticus* virulence genes (*tlh*, *trh* and *tdh*) and ten *V. cholerae* virulence genes (*ctx*A, *vpi*, *zot*, *ace*, *tox*R, *tox*T, *tos*S, *tox*RS, *tcp*A and *tcp*P). The isolates were also tested for their cytotoxic activity towards two epithelial cells. Their pattern of resistance to antibiotics was also carried out.

Experimental

Materials and Methods

Study area and sample collection. The lagoon of Monastir is situated on the eastern littoral of Tunisia, between the experimental fish and shellfish hatcheries of the National Institute of Marine Sciences and Technologies and a private hatchery of *Sparus aurata* and *Dicentrarchus labrax*. This part of the lagoon is used for supplying the fish and clam hatcheries with rearing water and also used for clam (*Ruditapes decussatus*) farming. The water samples were collected every ten days for a period of 12 months (January to December 2009). All the samples were collected in sterile glass containers (500 ml) and transported in isothermal condition to the laboratory for analysis within 2 h.

Isolation and bacterial characterization. *Vibrio* species were isolated using the membrane filtration technique. The water samples were filtrated through a sterile 0.45 µm pore size cellulose nitrate membrane filter (Millipore, Germany). These filters were transferred in alkaline peptone water (pH 8.6, 1% NaCl) and incubated at 37°C for 24 h. The enrichments were streaked onto Thiosulfate Citrate Bile Salts Sucrose agar (TCBS agar) supplemented with 2% NaCl to increase the detection of *Vibrio* species and incubated at 37°C for 24 h.

Preliminary identification of the strains had been performed on the bases of colony morphology on TCBS (Scharlau Microbiology, Spain) supplemented with 2% NaCl, Gram nonstaining (KOH) method, cytochrome oxidase activity, motility (Mannitol-Motility agar; Pronadisa, Madrid, Spain), resistance to vibriostatic O129 (10 and 150 µg), salt requirement (growth on 0%, 2%, 4%, 8% and 10% NaCl medium) and growth at 23 and 37°C. All of the isolates were processed using API 20E strips (bioMerieux), following the manufacturer's instructions. Ability of *Vibrio* isolates to produce extracellular enzymes such as lipase, amylase, lecithinase, caseinase and Dnase was performed as described previously (Liu *et al*., 1996). *Vibrio* strains were assessed for hemolytic activity on blood base agar supplemented with 5% (v/v) human blood. The strains were conserved as frozen stocks at –80°C in tryptic soy broth (TSB; Bio-Rad, France) with 2% NaCl plus 15% (v/v) glycerol.

Antibacterial susceptibility. Antibiotic susceptibility tests were performed using the disk diffusion method on Mueller-Hinton agar (bioMérieux, France) plates supplemented with 1% NaCl as described by Ottaviani *et al.* (2001). The commercial disks (Bio-Rad, France) containing the following antibiotics were used: ampicillin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), gentamicin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracyclin (30 µg), erythromycin (15 µg), kanamycin (30 µg) and carbenicillin (100 µg). After incubation at 37°C for 18–24 h, the diameters of the inhibition zone were interpreted according to the "Comité de la Société Française de l'Antibiogramme" (Cavallo *et al*., 2006) and followed by the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2002), the strains were categorized as susceptible or resistant to the drug. *Escherichia coli* ATCC 25922 was used as a quality control strain.

Determination of minimum inhibitory concentration (MIC). Minimum inhibitory concentration of six antibiotics (Sigma-Aldrich, USA): ampicillin sodium salt, erythromycin, tetracycline hydrochloride, streptomycin sulfate, gentamycin sulfate and chloramphenicol against *Vibrio* isolates were carried out using the broth microdilution method in Muller Hinton broth (bioMérieux, France) supplemented with 2% NaCl (M7-A7; CLSI, 2006). All *Vibrio* strains were cultured on Trypticase Soy Agar plates (TSA) supplemented with 2% NaCl and incubated at 30°C for 24–48 h. The tested isolates were suspended in 0.85% saline to a turbidity equivalent to a 0.5 McFarland standard (1×10^8 CFU/ml) and serially diluted to obtain a concentration of 10^5 CFU/ml in sterile U shaped bottom 96-well microtiter plates containing the test concentrations of antibiotics (0.125–256 mg/l). The plates were incubated at 35°C for 18–20 h after which they were examined for the presence or absence of growth. *E. coli* ATCC 25922 was used as a control microorganism.

Chromosomal DNA preparation. *Vibrio* isolates were grown aerobically on TSA plates containing 1% NaCl at 37°C overnight. Genomic DNA was extracted using Wizard genomic DNA purification kit (Promega, France) according to the manufacturer's instructions.

Molecular characterization. *Vibrio* strains identified by microbiological methods were subjected to polymerase chain reaction assays to assess the presence of genes encoding the heat shocking protein 40 (Hsp-40) specific to *V. alginolyticus*, the outer membrane protein (Omp W) specific to *V. cholerae* and the regulatory toxin protein (ToxR) specific to *V. parahaemolyticus* (Table I). Amplification reactions contained 5 × PCR buffer (Promega, France), 200 µmol/l of each desoxyribonucleotide triphosphate, 1.5 mmol/l of $MgCl_2$, 1 U Taq polymerase (Promega, France), 1 µmol/l of each primer, and 2 µl of the template in a final reaction volume of 25 µl. PCR amplifications were carried out in a thermal cycler (Eppendorf, Mastercycler per-

Table I
PCR primers used in this study

Target genes	PCR primer sequences (5'–3')	Product size (bp)	Reference
hsp-40	VM-F, 5'-CAGGTTTGYTGCACGGCGAAGA-3' V.al2-MmR, 5'-GATCGAAGTRCCRACACTMGGA-3'	144	Nhung *et al*., 2007
toxR-Vp	toxR-Vp1, 5'-GTCTTCTGACGCAATCGTTG-3' toxR-Vp1, 5'-ATACGAGTGGTTGCTGTCATG-3'	678	Lin *et al*., 1993
omp-W	ompW1, 5'-CACCAAGAAGGTGACTTTATTGTG-3' ompW2, 5'-GAACTTATAACCACCCGCG-3'	588	Nandi *et al*., 2000
*tox*RS	toxR0, ATGAGTCATATTGGTACTTAAATT toxS2, AACAGTACCGTAGAACCGTGA	1397	Sechi *et al*., 2000
*tox*T	toxT1, TTGCTTGGTTAGTTATGAGAT toxT2, TTGCAAACCCAGACTGATAT	581	Sechi *et al*., 2000
*tox*R	toxR1, CCT TCG ATC CCC TAA GCA ATA C toxR2, AGG GTT AGC AAC GAT GCG TAA G	779	Rivera *et al*., 2001
*tox*S	toxS1, CCACTGGCGGACAAAATAACC toxS2, AACAGTACCGTAGAACCGTGA	640	Sechi *et al*., 2000
zot	zot1, ACGTCTCAGACATCAGTATCGAGTT zot2, ATTTGGTCGCAGAGGATAGGCCT	198	Colombo *et al*., 1994
ace	ace1, GCTTATGATGGACACCCTTTA ace2, TTTGCCCTGCGAGCGTTAAAC	284	Colombo *et al*., 1994
*tcp*P	tcpP1, CGAATGCAGTAATCAAGTCT tcpP2, CAGTCAGCTTCATCAACAAT	320	Sechi *et al*., 2000
*tcp*A	tcpA1, CACGATAAGAAAACCGGTCAAGAG tcpA2, ACCAAATGCAACGCCGAATGGAGC	617	Keasler and Hall, 1993
vpi	VPI1, GCAATTTAGGGGCGCGACGT VPI2, CCGCTCTTTCTTGATCTGGTAG	680	Sechi *et al*., 2000
*ctx*A	ctx2, CGGGCAGATTCTAGACCTCCTG ctx3, CGATGATCTTGGAGCATTCCCAC	563	Fields *et al*., 1992
tlh	tlhf1, AGC GGA TTA TGC AGA AGC AC tlhr2, ATC TCA AGC ACT TTC GCA CG	150	Xie *et al*., 2005
trh	trhf1, TTG GCT TCG ATA TTT TCA GTA TCT trhr1, CAT AAC AAA CAT ATG CCC ATT TCC G	500	Bej *et al*., 1999
tdh	tdhf1, CCA TTC TGG CAA AGT TAT T tdhr1, TTC ATA TGC TTC TAC ATT AAC	534	Xie *et al*., 2005

sonal). The reaction mixture was subjected to an amplification of 35 cycles. Apart from the primer annealing temperature, each cycle consisted of denaturation at 94°C for 30 sec, annealing for 30 sec, and primer extension at 72°C for 1 min, then the mixtures were kept at 72°C for 10 min. The annealing temperature was 60°C for *hsp-40* and 64°C for *omp*W and *tox*R. PCR products were electrophoresed through 1.5% agarose gel to resolve the amplified products which were visualized under UV light after ethidium bromide staining.

Virulence gene. Oligonucleotide primers used in this study were listed in Table I. Amplification was carried out in a thermal cycler (eppendorf, Mastercycler personal) with a standard PCR reaction mixture that contained 10 µl of 5 × PCR reaction buffer (Promega, France), 200 µmol/l of each of the four dNTPs, 1.5 µmol/l $MgCl_2$ (Promega, France), 1 µmol/l of each primer, 1 µl extracted DNA (50 ng), 1.25 U Taq polymerase (Promega, France) and sterile ultrapure water to make the volume to 50 µl. The mixtures were incubated for 5 min at 94°C, followed by 35 cycles of amplification. Except for the primer annealing temperature, each cycle consisted of denaturation at 94°C for 40 sec, annealing for 40 sec, and primer extension at 72°C for 1 min and the mixtures were kept at 72°C for 10 min. The annealing temperature was 48°C for *tdh*, 54°C was used for *tox*RS, *tox*R, *tox*T and *tlh*, 58°C was used for *tcp*P, *tcp*A, *tox*S, *trh* and *ace* whereas the temperature was 60°C for *vpi*, *zot* and *ctx*A. The amplified products were electrophoresed in a 1.6% agarose gel at 90 V for 30 min, stained with ethidium bromide then visualized and photographed using Gel Doc XR apparatus (Bio-Rad, Milan, Italy).

Adherence to PE and PVC surfaces. The quantitative estimate of biofilm formation of *V. alginolyticus* strains on PE and PVC surfaces was determinate using the protocol described by Cerca *et al.* (2006). *Vibrio* strains from fresh agar plates were harvested with sterile

PBS and diluted to a standard concentration equal to an OD of 1.0 at 540 nm (1×10^9 CFU/ml). The 1 cm PE and PVC squares were inserted in the bottom of 24-well microtitre plates (Greiner Bio-One Cellstar, Germany) and 2 ml of each cell suspension was added to each well. Adhesion to each material was allowed to occur for 2 h at room temperature, with gentle shaking.

Negative control wells without bacterial cells were filled with PBS. At the end of the experiment, each well was washed twice with PBS to remove non-adherent or loosely adherent bacteria. After the last wash the pieces were removed from each well and immersed in a new microtiter plate with 1 ml of 98% (w/v) methanol in each well (Henriques *et al*., 2005). The methanol was discarded after 15 min of contact and the pieces were allowed to dry at room temperature. Aliquots of crystal violet were added to each well and incubated for 5 min. After the pieces were washed in water, they were left to dry, then immersed in 1 ml of 33% acetic acid to release and dissolve the stain. The OD of the obtained solution was measured at 570 nm using a spectrophotometer (Jenway 6405 uv/vis). All strains were tested in triplicate, and the bacteria were classified according to Stepanovic *et al.* (2000) as follows (0): $OD \leq ODc$; weakly adherent (+): $ODc < OD \leq 2 \times ODc$; moderately adherent (++): $2 \times ODc < OD \leq 4 \times ODc$; and strongly adherent (+++): $4 \times ODc \leq OD$. This classification was based on the cut-off OD (ODc) value defined as three standard deviation values above the OD of the negative control.

Cell culture conditions. Two cell monolayers were used to examine the adhesive properties of *Vibrio* strains: Hep-2 (human larynx carcinoma) and Vero (kidney epithelial cells of African Green Monkey). For the cytotoxicity assay, we used Vero cells and HeLa (human cervical epitheloid carcinoma) cells.

The cells were grown in MEM (Minimum Essential Medium, Sigma) supplemented with 10% of foetal calf serum (Sigma), 1% of antibiotic solution (streptomycin-penicillin 5000 U, Sigma), and 1% of non-essential aminoacids (Sigma). Cells were seeded on 24-well tissue culture plates (2×10^4 cell/ml), and incubated at 37°C in 5% CO_2 for 24 h (Baffone *et al*., 2005).

Adherence assay. Bacterial adherence was performed as described previously by Snoussi *et al.* (2008). Briefly, 100 µl of 10^7 cells /ml was added to Vero and Hep-2 cells and the 24-well plates were incubated at 37°C for 3 h in 5% CO_2. The cells were washed three times in sterile PBS to remove non-adherent bacteria, fixed in methanol and stained with Giemsa for microscopic examination under oil immersion. Uninoculated cell lines served as negative controls. All organisms were tested twice. The adhesion index was assayed as NA = no adhesive (0–10 bacteria/cells); W = weak adhesion (10–20 bacteria/cells); M = medium adhesion (20–50 bacteria/cells); S = strong adhesion (50–100 bacteria/cells).

Cytotoxicty assay. *In vitro* cytotoxicity was examined on HeLa and Vero cell lines as performed by Baffone *et al.* (2005). *Vibrio* isolates were inoculated in TSB (Bio-Rad, France) supplemented with 1% of NaCl, and incubated at 37°C for 18–24 h. At the end of incubation, each flask contents were transferred to sterile tubes (50 ml) and centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a 0.22 µm pore size filter membrane (Millipore, Germany). The bacterial cell-free filtrates were serially diluted (dilutions of 1:10, 1:50 and 1:100), were added to HeLa and Vero cells, previously washed in PBS, and incubated at 37°C in 5% CO2 for 24 h. At the end of incubation, cells were observed under light inverted microscopy and checked for cytotoxic effect (rounding and shrinking to ≥ 50% of cells). All tests were performed in duplicate. The filtrates showing cytotoxic activity at a 1:10 dilution were considered to be weak (W) producers of toxin, those at a 1:50 dilution were moderate (M) producers, and those at a 1:100 dilution were strong (S) producers (Barbieri *et al*., 1999).

Statistical test. All data were analyzed with SPSS for Windows, version 16.0. The correlation between presence and absence of the virulence genes was studied by the Crosstabs method. For all test P-values < 0.05 were considered statistically significant.

Results

A total of 65 *Vibrio* isolates were obtained on the selective TCBS agar plates and then they were characterized through the API 20E miniaturized system. Three environmental *Vibrio* species were identified on the basis of their biochemical profile as *V. alginolyticus* (n = 48), *V. cholerae* (n = 12) and *V. parahaemolyticus* (n = 5). The majority of *Vibrio* isolates were positive for lysine decarboxylase, indole production, glucose fermentation and mannitol fermentation. *V. cholerae* and *V. parahaemolyticus* strains gave positive results with ornithine decarboxylase and gelatinase. The five *V. parahaemolyticus* isolates were able to utilize citrate and to assimilate rhamnose (Table II). All *Vibrio* strains tolerated low concentrations of NaCl (2 and 4%). While only 5 (41.66%) *V. cholerae* strains and 4 (80%) *V. parahaemolyticus* strains grow in a nutrient broth prepared with 6% NaCl. Of the 48 *V. alginolyticus* isolates, 12 (25%) were capable of growing at 10% NaCl added to a nutrient broth. *Vibrio* isolates produced several hydrolytic exoenzymes such as amylase, lecithinase, lipase, caseinase, gelatinase and Dnase. Thirty seven of the forty-eight (77.08%) *V. alginolyticus* and 2/12 (16.66%) *V. cholerae* isolates were β-hemolytic. The PCR-based identification

Table II
Biochemical and enzymatic characterization of *Vibrio* isolates

Characteristic		*V. alginolyticus* no. (%)[a]	*V. cholerae* no. (%)[a]	*V. parahaemolyticus* no. (%)[a]
No. of tested strains		48	12	5
Gram		–	–	–
Motility		+	+	+
Oxydase		+	+	+
β-Galactosidase		0	12 (100)	3 (60)
Adenine dehydrolase		0	0	0
Lysine decarboxylase		47 (97.91)	12 (100)	5 (100)
Ornithine decarboxylase		24 (50)	12 (100)	5 (100)
Citrate utilization		8 (16.66)	10 (83.33)	5 (100)
H_2S production		4 (8.33)	2 (16.66)	0
Urea hydrolysis		0	0	1 (20)
tryptophan deaminase		6 (12.5)	0	0
Indole production		48 (100)	12 (100)	5 (100)
Voges Proskauer		10 (20.83)	3 (25)	0
Gelatinase		32 (66.66)	12 (100)	5 (100)
Fermentation of:	Glucose	48 (100)	12 (100)	5 (100)
	Mannitol	47 (97.91)	12 (100)	5 (100)
	Inositol	0	0	0
	Sorbitol	4 (8.33)	0	0
	Rhamnose	0	0	5 (100)
	Sucrose	48 (100)	12 (100)	0
	Melibiose	0	0	0
	Amygdalin	21 (43.75)	4 (33.33)	4 (80)
	Arabinose	0	0	3 (60)
O/129:	10 μg	R	R	R
	150 μg	S	S	S
Growth at:	0% NaCl	0	0	0
	2% NaCl	48 (100)	12 (100)	5 (100)
	4% NaCl	48 (100)	12 (100)	5 (100)
	6% NaCl	48 (100)	5 (41.66)	4 (80)
	8% NaCl	48 (100)	0	0
	10% NaCl	12 (25)	0	0
Growth at:	23°C	48 (100)	12 (100)	5 (100)
	37°C	48 (100)	12 (100)	5 (100)
Exoenzymes:	Amylase	37 (77.08)	9 (75)	3 (60)
	Lecithinase	41 (85.41)	12 (100)	4 (80)
	Lipase	48 (100)	12 (100)	5 (100)
	Caseinase	48 (100)	10 (83.33)	4 (80)
	Gelatinase	44 (91.66)	10 (83.33)	5 (100)
	Dnase	48 (100)	12 (100)	5 (100)
	β-hemolytic	37 (77.08)	2 (16.66)	0

[a] – Number and percentage of positive tests; S – sensitive; R – resistant.

of studied *Vibrio* strains yielded amplicon size of 144, 588 and 678 bp for *V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae,* respectively (Fig. 1).

Antibiogram patterns obtained for the *Vibrio* spp. are presented in Table III. Tests for antimicrobial susceptibility revealed that bacterial strains belonging to different species of *Vibrio* genera exhibited some common pattern of antibiotic resistance or susceptibility. In fact, all strains displayed a total resistance to ampicillin and more than 70% of them showed a significant

Table III
Antibiotic resistance pattern expressed in (%) and minimum inhibitory concentration of *Vibrio* strains expressed in mg/L (%)

Antibiotics	*V. alginolyticus* (n = 48)	*V. cholerae* (n = 12)	*V. parahaemolyticus* (n = 5)
Ampicillin (10 µg)	100	100	100
Chloramphenicol (30 µg)	62.5	33	0
Cotrimoxazole (25 µg)	58.33	0	0
Gentamicin (10 µg)	75	25	0
Nalidixic acid (30 µg)	70.8	75	80
Streptomycin (10 µg)	83.3	75	100
Tetracyclin (30 µg)	83.3	25	0
Erythromycin (15 µg)	100	75	80
Kanamycin (30 µg)	95.8	16.6	60
Carbenicillin (100 µg)	100	25	40
Ampicillin	16 (12.5)	16 (33.3)	16 (80)
	32 (35.4)	32 (41.6)	32 (20)
	64 (22.9)	64 (16.6)	–
	128 (20.8)	128 (8.3)	–
	256 (8.3)	–	–
Erythromycin	16 (20.8)	4 (33.3)	4 (40)
	32 (39.6)	8 (25)	8 (20)
	64 (22.9)	16 (25)	16 (20)
	128 (16.6)	32 (16.6)	–
	–	–	–
Tetracyclin	2 (10.4)	0.5 (16.6)	0.5 (20)
	4 (16.6)	1 (33.3)	1 (80)
	8 (41.6)	2 (16.6)	–
	16 (25)	4 (8.3)	–
	32 (6.2)	8 (8.3)	–
Streptomycin	4 (6.2)	2 (33.3)	4 (80)
	8 (10.4)	4 (41.6)	16 (20)
	16 (45.8)	8 (16.6)	–
	32 (27)	16 (8.3)	–
	64 (10.4)	–	–
Gentamycin	4 (12.5)	1 (25)	1 (20)
	8 (35.4)	2 (33.3)	2 (80)
	16 (43.7)	4 (16.6)	–
	32 (8.3)	8 (25)	–
Chloramphenicol	1 (18.7)	0.5 (16.6)	0.25 (20)
	2 (18.7)	1 (25)	0.5 (20)
	4 (31.2)	2 (50)	1 (60)
	8 (31.2)	4 (8.3)	–

resistance to streptomycin, nalidixic acid and erythromycin. *V. alginolyticus* strains had the highest multi-drug resistance showing a strong resistance to ampicillin, erythromycin, carbenicillin, streptomycin, kanamycin and tetracycline. The resistance to chloramphenicol was observed in 62.5% of the analyzed *V. alginolyticus* strains and in 33% of the *V. cholerae* isolates.

The MIC results for *Vibrio* isolates were summarized in the Table III. MIC values of antimicrobials observed throughout the study showed that all investigated isolates were highly susceptible to chloramphenicol (0.25–8 mg/l) and were moderately sensitive to both tetracyclin (0.5–32 mg/l) and gentamycin (0.5–32 mg/l). The MIC values of different tested anti-

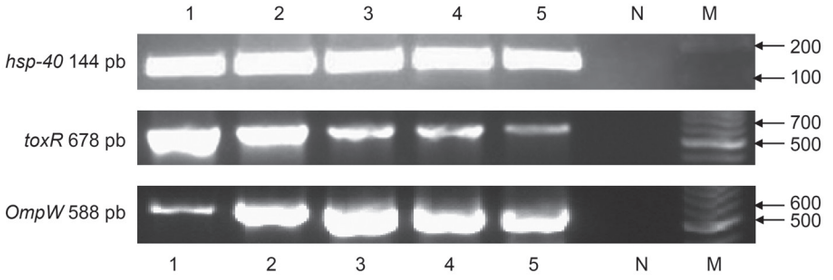

Fig. 1. Agarose gel electrophoresis of 1.5% agarose of the amplification products of isolates obtained with PCR for the *Hsp-40* (*V. alginolyticus*: 1, AMa_1, 2, BN_3, 3, CJ4, 5, CJ_3, 6, DS_3); PCR for the *toxR* (*V. parahaemolyticus*: 1, AA_2, 2, DM_4, 3, DJ_1, 4, CAt_4, 5, BJ_3) and PCR for the *OmpW* (*V. cholerae*: 1, BJ_1, 2, AM_1, 3, BN_2, 4, CF_3, 5, BJ_2). N, negative control, M, molecular weight marker 100 bp ladder (Promega, France).

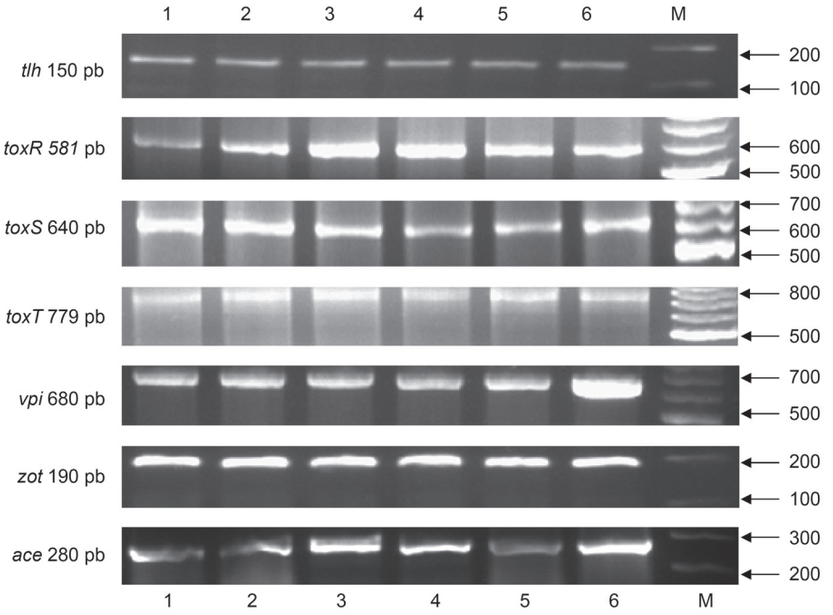

Fig. 2. Virulence genes espression of *Vibrio* strains isolated from Monastir lagoon. Agarose gel electrophoresis (1.6% agarose) of the *ace* (*V. alginolyticus*: 1, AMa_1, 2, BN_3, 3, CJ_4, 4, DMa_3, 5, BJt_1, 6, CO_1), the *zot* (*V. alginolyticus*: 1, AJ_3, 2, BAt_3, 3, CAt_3, 4, DS_3, 5, AAt_2, 6, DAt_3), the *vpi* (*V. alginolyticus*: 1, AAt_1, 2, AAt_2, 3, BJ_2, 4, CJ_3, 5, DJt_4, 6, DS_3). the *toxT* (*V. cholera*: 1, BJ_1, 2, AJt_3, 3, BN_2 and *V. aliginolyticus*: 4, AMa_1, 5, CJ_3, 6, DS_3), the *toxS* (*V. cholera*: 1, CF_3, 2, AM_1, 3, BJ_2, *V. parahaemolyticus*: 4, CAt_4 and *V. alginolyticus*: 5, CAt_3, 6, DMa_1), the *toxR* (*V. cholera*: 1, CF_3, 2, AM_1, 3, BJ_2, *V. parahaemolyticus*: 4, CAt_4 and *V. alginolyticus*: 5, BJt_3; 6, CMa_4) and the *tlh V. parahaemolyticus*:1, AA_2, 2, DM_4, 3, DJ_1, 4, CAt_4, *V. alginolyticus*: 5, BM_2, 6, AJ_3). M, molecular weight marker 100 bp ladder (Promega, France).

biotics for *V. parahaemolyticus* strains were lower than those found among other *Vibrio* species. In other hand, the vast majority of *V. alginolyticus* isolates showed a strong resistance to ampicillin (87.4% ≥ 32 mg/l); erythromycin (79% ≥ 32 mg/l); tetracyclin (31.5% ≥ ≥ 16 mg/l); streptomycin (37.4% ≥ 32 mg/l) and gentamycin (52% ≥ 16 mg/l).

The distribution of *V. cholera*e and *V. parahaemolyticus* virulence-associated genes among the tested *Vibrio* strains was presented in the Table IV. The presence of the *tox*R and the *tox*S genes was detected in the majority of *V. cholerae* (100% and 83%, respectively) strains and *V. alginolyticus* (73% and 58%, respectively) strains, while only one *V. parahaemolyticus* isolates was positive to these genes (Fig. 2). The *tox*T fragment was amplified from the chromosome of 10/12 (83%) *V. cholerae* strains whereas 13/48 (27%) *V. alginolyticus* isolates gave a positive result to this gene. Only the *V. alginolyticus* strains exhibited the presence of three *V. cholerae* virulence genes: *vpi* (25%), *ace* (19%) and *zot* (29%).

Table IV
Biofilm formation on biotic and abiotic materials, virulence genes distribution and cytotoxic activity of *Vibrio* isolates

Strain	Strain number	Virulence genes (%)						Adherence										Cytotoxic effect					
								Materials OD_{570}						Hep-2 (%)			Vero (%)	HeLa (%)			Vero (%)		
								PVC (%)			PE (%)												
		*tox*R	*tox*S	*tox*T	*vpi*	*ace*	*zot*	*tlh*	**W**	**M**	**S**	**W**	**M**	**S**	**W**	**M**	**S**	**W**	**M**	**S**	**W**	**M**	**S**
VA	48	73	58	27	25	19	29	37	20	37	41	41	39	19	37	14	8	31	24	10	42	21	6	31	25	10
VC	12	100	83	83	–	–	–	–	17	33	50	33	25	42	42	33	8	50	25	17	33	17	8	42	33	–
VP	5	20	20	–	–	–	–	100	60	20	–	20	20	–	60	–	–	60	20	–	20	–	–	60	20	–

VA – *V. alginolyticus*; **VC** – *V. cholerae*; **VP** – *V. Parahaemolyticus*; **PVC** – polyvinyl-chloride; **PE** – polyethylene; **W** – weak; **M** – moderate; **S** – strong.

All *V. parahaemolyticus* isolates were positive to the *tlh* virulence gene, while 18/48 *V. alginolyticus* strains possessed this gene. The crosstabs method revealed a significant relationship ($P < 0.05$) between the presence of the *tox*R gene and the *tox*S gene. On other hand, a positive correlation was observed between the presence of the *vpi* gene and the *tox*R gene ($P = 0.039$), *tox*S gene ($P = 0.007$) and the *tox*T gene ($P = 0.005$). However, no significant relationship was observed between the presence of *V. cholera*e and *V. parahaemolyticus* virulence genes. All isolates gave negative results for the amplification of *tox*RS*I tcp*P, *tcp*A, *tdh* and *trh.*

The results of the biofilm formation by *Vibrio* species on PVC and PE surfaces showed that *V. cholerae* and *V. alginolyticus* strains were strongly adhesive to both abiotic materials than other isolates. In fact, 50% (6/12) of *V. cholerae* isolates and 41% (20/48) of *V. alginolyticus* exhibited high adherence ability to PVC pieces. *V. cholerae* isolates presented better adherence ability on PE surface than *V. alginolyticus* strains (42 and 19%, respectively).

Adherence ability was observed in 11 of 12 (92%) of the analyzed *V. cholerae* strains in Vero cells, while 10 (83%) isolates were found adhesive when Hep-2 cell line was used. The other tested *Vibrio* species revealed that were lower adhesive to both cell lines than *V. cholerae* isolates. We also noted that only *V. alginolyticus* strains showed a strong adherence to Hep-2 cells (Table IV). About 2 of 48 (4%) *V. alginolyticus* strains and one of 12 (8%) *V. cholerae* strains were able to adhere strongly to both epithelial cell lines (Fig. 3).

The cytotoxic activity of extracellular products (ECPs) of the three studied *Vibrio* species against HeLa and Vero cell lines showed that more than 60% of *V. alginolyticus* strains have cytotoxic effect with different degrees to both epithelial cell lines. About 5 of 48 (10%) *V. alginolyticus* isolates showed a strong cytotoxicity against Vero monolayer while only 3 strains gave the same results when Hep-2 cells were used. However, most strains of *V. cholera*e and *V. parahaemolyticus* exhibited essentially weak and moderate cytotoxic activities (Table IV).

Discussion

The past two decades have witnessed remarkable increasing frequency of *Vibrio* species isolated from diseased aquatic animals and from human infections. *V. alginolyticus* is recognized as one of the major causative agent of vibriosis in cultured fish and shellfish in Mediterranean coastal environment (Gomez-Leon *et al.*, 2005; Sonia and Lipton, 2012). Other studies reported that this specie is considered as an important human opportunistic pathogen usually associated with otitis

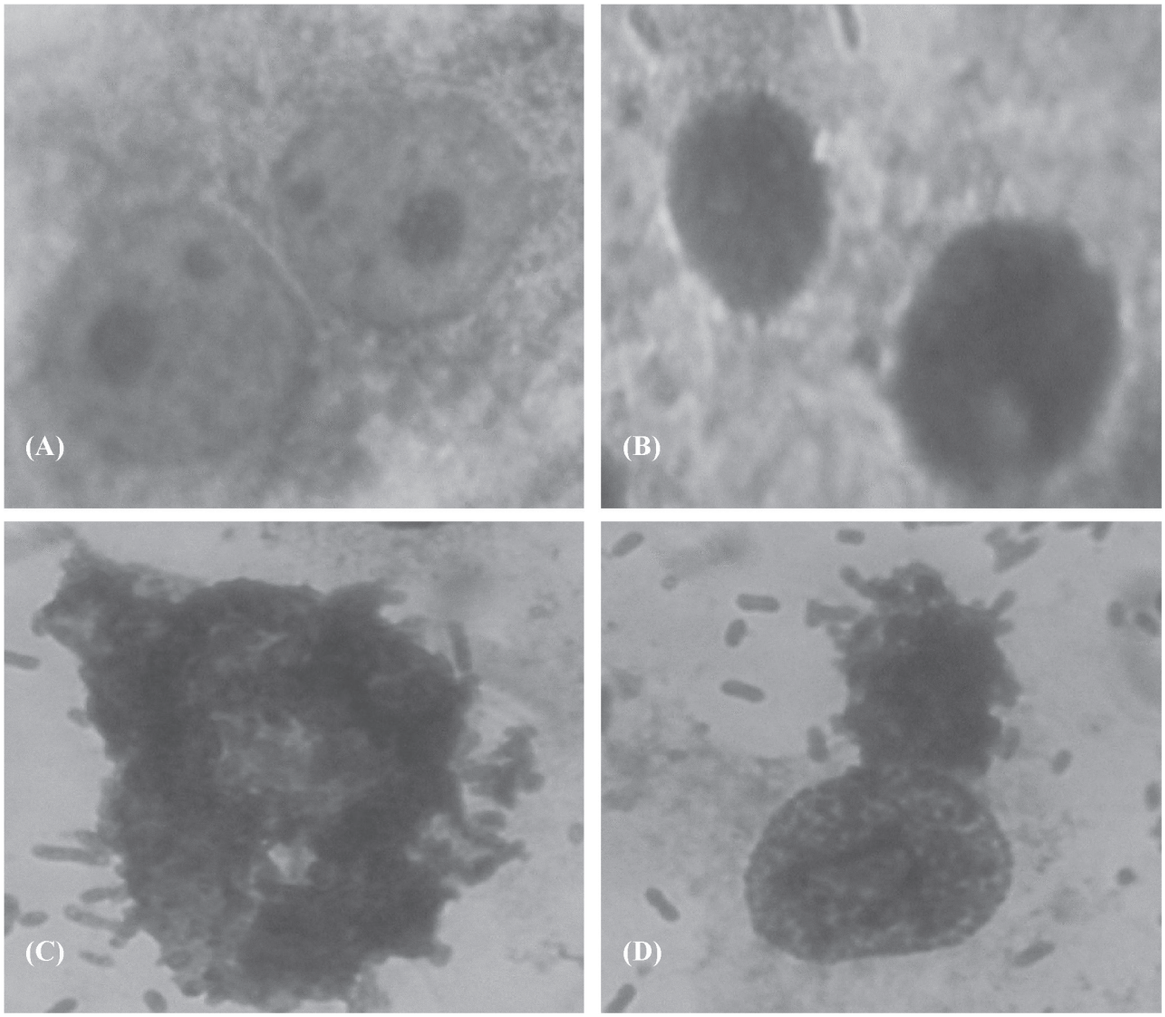

Fig. 3. Optic microscopy showing the high adherence ability of *Vibrio alginolyticus* (strain Bat4) to both Vero and Hep-2 cell lines. Giemsa stain: magnification (×1000). (a) and (B): Negative control for Vero and Hep-2 cells. (C) and (D): Vibrio alginolyticus strain Bat4 strongly adhesive to Vero and Hep-2 cells respecctively.

externa, endophthalmitis and wound infections (Li *et al*., 2009; Reilly *et al*., 2011). It's also well documented that *V. cholerae* and *V. parahaemolyticus* are most often incriminated in food-borne and waterborne gastroenteritis outbreaks (Nair *et al*., 2007; Yoder *et al*., 2008).

Sixty-five *Vibrio* spp. strains were isolated from water samples collected from the Monastir lagoon and biochemically characterized using the commercial miniaturized Api 20E kit. The phenotypic characteristics of *Vibrio* isolates were in accordance with those described previously by Snoussi *et al.* (2006). However, these findings are in discordance with Ben Kahla-Nakbi *et al.* (2007) who showed that a majority of *V. alginolyticus* isolates recovered from dead and moribund fish samples were negative to indole test. The environmental strains of *V. alginolyticus*, *V. cholerae* and *V. parahaemolyticus* were genetically identified to the species levels using the *hsp-40*, *omp*W and the *tox*R genes, respectively (Lin *et al*., 1993; Nandi *et al*., 2000).

In the present study, *Vibrio* isolates exhibited multidrug resistance to at least four antibiotics. Vaseerahan *et al.* (2005) carried a study of 80 *Vibrio* strains isolated from Indian shrimp culture ponds and hatcheries for determination of their susceptibility to the most used antibiotics in the shrimp farming, all tested isolates were resistant to ampicillin, which corroborate with our findings. Other studies, reported that *V. alginolyticus* strains showed resistance to erythromycin, streptomycin, gentamycin, tetracyclin and chloramphenicol (Gomathi *et al*., 2013; Mechri *et al*., 2013b). These data are in keeping with our results.

The MIC's obtained from the study showed that all *Vibrio* strains were sensitive to chloramphenicol (MIC's ≤ 8 mg/l), while most of them expressed high

rates of resistance to ampicillin (MIC's ≥ 32 mg/l) and erythromycin (MIC's ≥ 8 mg/l). In a previous study, Manjusha *et al.* (2005) reported strong resistance against amoxicillin, ampicillin, carbenicillin, cefuroxime, rifampicin and streptomycin in *Vibrio* spp. isolated from Indian coastal and brackish areas. Another study, showed that *Vibrio* isolates recovered from aquaculture structure expressed moderate resistance to chloramphenicol, gentamycin, tetracyclin and erythromycin (Akinbowale *et al*., 2006).

Previous work showed that *Vibrio* species represents an important recipient of some *V. cholerae* and *V. parahaemolyticus* virulence genes transfers (Xie *et al.*, 2005). Snoussi *et al.* (2008), reported the diffusion of six *V. cholerae* virulence genes among 28 *V. alginolyticus* strains isolated from the Mediterranean seawater. Our results corroborate with these findings and represents the first report describing higher frequencies of *V. cholerae* and *V. parahaemolyticus* virulence genes distribution, among environmental *V. alginolyticus* isolates, than observed previously (Ren *et al*., 2013; Khoudja *et al*., 2014). These data supports the evidence of genetic extensive exchange of virulence determinants between *V. alginolyticus* and other *Vibrio* species in marine and estuary environments.

Biofilm formation constitutes an efficient adaptive strategy utilized among numerous *Vibrio* species, which remarkably promotes bacterial persistence in the environment and/or colonization of eukaryotic hosts (Morris and Visick, 2010). In this study, *V. cholerae* and *V. alginolyticus* strains exhibited high capacity of adherence to both PVC and PE surfaces, while *V. parahaemolyticus* isolates showed low to moderate adhesion to the same materials. These data corroborate previous studies showing that environmental *Vibrio* species were able to form biofilm on abiotic surfaces of different degrees (Mechri *et al*., 2013a).

The attachment of bacterial pathogens to eukaryotic cells represents an essential first step in the colonization and the production of disease. This propriety seems to be diffused among *Vibrio* species (Scoglio *et al*., 2001; Mohammadi-Barzelighi *et al.*, 2011). Our data showed that *V. cholerae* and *V. alginolyticus* isolates exhibited an important adherence ability to both tested cell lines. These findings may explain a possible interaction between these strains and the epithelial cell lines used in this study.

Several studies reported cytotoxic effects of extracellular products of some *Vibrio* spp. against a variety of cell lines (Hiyoshi *et al*., 2010; Mechri *et al*., 2013b). Our investigation showed that *V. alginolyticus* isolates exhibited the most important cytotoxic activity against Vero and HeLa cell lines. Balebona *et al.* (1998), suggested that cytotoxicity in cell lines can be directly related to the virulence of *V. alginolyticus* strains.

Conclusions

This study highlights the incidence of multiple antibiotic resistance in three environmental *Vibrio* species and the wide distribution of some *V. cholerae* and *V. parahaemolyticus* virulence genes among the studied strains. Besides, it is clearly shown that tested bacteria present a high ability to adhere to biotic and abiotic surfaces though at varying levels. These isolates exhibited also a significant cytotoxicity against HeLa and Vero cell lines.

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ORIGINAL PAPER

Distribution and Identification of Endophytic *Streptomyces* Species from *Schima wallichii* as Potential Biocontrol Agents against Fungal Plant Pathogens

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Abstract

The prospective of endophytic microorganisms allied with medicinal plants is disproportionally large compared to those in other biomes. The use of antagonistic microorganisms to control devastating fungal pathogens is an attractive and eco-friendly substitute for chemical pesticides. Many species of actinomycetes, especially the genus *Streptomyces,* are well known as biocontrol agents. We investigated the culturable community composition and biological control ability of endophytic *Streptomyces* sp. associated with an ethanobotanical plant *Schima wallichi.* A total of 22 actinobacterial strains were isolated from different organs of selected medicinal plants and screened for their biocontrol ability against seven fungal phytopathogens. Seven isolates showed significant inhibition activity against most of the selected pathogens. Their identification based on 16S rRNA gene sequence analysis, strongly indicated that all strains belonged to the genus *Streptomyces*. An endophytic strain BPSAC70 isolated from root tissues showed highest percentage of inhibition (98.3 %) against *Fusarium culmorum* with significant activity against other tested fungal pathogens. Phylogenetic analysis based on 16S rRNA gene sequences revealed that all seven strains shared 100 % similarity with the genus *Streptomyces*. In addition, the isolates were subjected to the amplification of antimicrobial genes encoding polyketide synthase type I (PKS-I) and nonribosomal peptide synthetase (NRPS) and found to be present in most of the potent strains. Our results identified some potential endophytic *Streptomyces* species having antagonistic activity against multiple fungal phytopathogens that could be used as an effective biocontrol agent against pathogenic fungi.

Key words: *Schima wallichii*, biocontrol agent, endophytic *Streptomyces*, nonribosomal peptide synthetase (NRPS), polyketide synthase (PKS-I)

Introduction

Loss in crop production caused by fungal diseases is a major concern resulting in loss of 25% yield in western countries and almost 50% in developing countries (Gohel *et al*., 2006). Several economically important crops in tropical and sub-tropical regions are affected due to diverse fungal diseases, so it is important to control fungal diseases for constant food supply to an ever increasing world population (Oskey, 2009). Synthetic pesticides can keep fungal infections at an acceptable level. However, their extensive use can lead to several drawbacks such as environmental pollution, lack of specificity, development of resistant fungal strains or accumulation of compounds potentially hazardous to other life forms as well, including humans (Dahiya *et al*., 2006; Evangelista-Martinez, 2014). Therefore, control of fungal pathogens requires a more environmental friendly approach. Henceforth, microbe-based biocontrol methods could be an alternative to control devastating fungal diseases (Zhao *et al*., 2012; Patil *et al*., 2014). Microbial antagonists are commonly used for the biocontrol of fungal diseases by the use of various groups of microorganism like bacteria, algae, fungi and actinomycetes (Brimner and Boland, 2003).

Actinomycetes are characteristic as biological control agents against fungal plant pathogens by having the ability to produce secondary metabolites and biologically active compounds such as enzymes and antibiotics (Adegboye and Babalola, 2012; Mingma *et al*., 2014), of

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which genus *Streptomyces* is of meticulous importance (Evangelista-Martinez, 2014). Among the 10000 antimicrobial compounds produced by microorganisms, more than 50% were isolated from actinomycetes and about 60% of the bioactive compounds developed for agricultural use originated from the genus *Streptomyces* (Anderson and Wellington, 2001). It is well known that *Streptomyces* sp. can produce a wide variety of secondary metabolites including antibiotics, enzymes and alkaloids, which may be the causative agent for antagonistic activity (Hayakawa *et al*., 1996).

Bioactive compounds such as benzoquinones (Rothrock and Gottlieb, 1984), aminoglycosides (Godfrey, 1995; Qin *et al*., 1994), polyenes (Smith *et al*., 1990; Raatikainen *et al*., 1994), nucleoside antibiotics (Hwang *et al*., 1994; Trejo-Estrada *et al*., 1998) are some agriculturally useful metabolites produced by genus *Streptomyces*. Several members of genus *Streptomyces* have been reported to significantly inhibit growth of plant fungal pathogens (Taechowisan *et al*., 2005; Maldonado *et al*., 2010; Evangelista-Martinez, 2014). To name some, *Streptomyces rochei* in combination with *Trichoderma harzianum* was used to control root rot in pepper (Ezziyyani *et al*., 2007), *Streptomyces aureofaciens* improved protection against anthracnose disease in mango caused by *Colletotrichum goeosporioides* (Haggag and Abdall, 2011) and *Streptomyces griseus* was used to control *Fusarium* wilt in tomato (Anitha and Rabeeth, 2009). Among the known commercial products produced by *Streptomyces,* Actinovate® and Mycostop® are the two most useful commercial products against foliar, root rot and wilt diseases marketed to date (Evangelista-Martinez, 2014).

In recent years, endophytic actinobacteria have attracted the attention of researchers as biocontrol agent against plant pathogens due to their better plant colonizing ability and antifungal activities. Their antagonistic capability has been proved against different phytopathogens, including *Rhizoctonia solani*, *Verticillium dahlia*, *Fusarium oxysporum*, *Colletotrichum orbiculare* (Coombs *et al*., 2004; Hasegawa *et al*., 2006; El-Tarabily *et al*., 2009; Shimizu *et al*., 2009).

Attempts were made to isolate actinomycetes as endophytes from various plants, where they live in symbiotic manner without causing any apparent damage to the host plant (Stone *et al*., 2000). Biological control ability of endophytic actinomycetes has been reported both *in vitro* and *in vivo* (Cao *et al*., 2005; Taechowisan *et al*., 2003). Evidences indicate that new endophytic actinomycetes were isolated from various organs of medicinal plants, and produced various bioactive compounds with a novel chemical structure, which would further increase the potential effectiveness as a biological control agent (Godfrey, 1995; Nimnoi *et al*., 2010). Therefore, there is a need for the isolation and characterization of actinomycetes from different geographical locations is important in order to identify new and commercially valuable genetic resources (Evangelista-Martinez, 2014). However, there is no study on the isolation of endophytic actinomycetes from medicinal plant *Schima wallichii* as biocontrol agents. Further, we used degenerate primers to amplify the antimicrobial genes like Polyketide synthase (PKS) type I and nonribosomal peptide synthetase (NRPS) because most of the biosynthetic pathways for the production of secondary metabolites are associates with these genes.

In our study, we have attempted to isolate endophytic actinomycetes from surface sterilized organs of medically important plant *S. wallichii.* The isolates were screened for their biocontrol ability against common fungal phytopathogens such as *F. oxysporum*, *Fusarium proliferatum*, *Fusarium oxysporum* f. sp. *ciceri*, *Fusarium culmorum*, *Fusarium graminearum*, *Alternaria* sp. and *Colletotrichum* sp. The potential strains selected as efficient biocontrol agents were identified by using 16S rRNA gene sequence. Further, biosynthetic potential of the potent strains was examined by amplification of PKS-I and NRPS genes.

Experimental

Materials and Methods

Sample collection. Healthy and disease-free tissues of the plant (*S. wallichii*) were collected from Dampa tiger reserve forest (23°44'N; 92°39'E) during the period of October 2013, based on ethanobotanical history, commonly used by the local tribes to heal the wounds caused by insects like spider and scorpion, as antiseptic and as external application in snake bite. Roots were dug out carefully so that sufficient amount of root material was collected. The tissues were placed in sterile bags and brought to the lab immediately and processed within 6 h of collection.

Surface sterilization and isolation of endophytic actinomycetes. Surface sterilization is the first and obligatory step for the recovery of true endophytes in order to kill the surface microbial population. The plant tissues were normally treated with oxidant or general sterilant for a period, followed by sterile rinse. The different plant tissues (leaf, fruit, root and bark) were used for isolation of endophytic actinomycetes and washed in running tap water for 5–10 minutes to remove adhered debris. The tissues were cut into small pieces (1.0×0.5 cm) and surface sterilized by using three step procedures as described by Taechowisan and Lumyong (2003). Tissues were sterilized by sequential immersion in 0.1% Tween 20 for few seconds and transferred to clean conical flask, followed by 70% ethanol for 2 min,

and a solution of 0.1% NaOCl for 1 min. Samples were washed in sterile water minimum three times to remove all surface disinfectants. An aliquot (0.1 ml) of the last wash was spreaded on starch casein agar (SCA) plates and incubated at 28 ±2°C for three weeks to proof the authenticity of the surface sterilization protocol (Kuster and Williams, 1964). The sterilized tissues were kept on autoclaved blotting paper to remove the any trace of water and inoculated on five different agar media viz. Starch Casein Nitrate Agar Media (SCNA), Actinomycetes Isolation Agar Media (AIA) and Tap Water Yeast Extract Agar Media (TWYE) at the rate of 10–15 tissue bits per plate. Nalidixic acid and Cycloheximide (80 µg/ml) were added to the media to inhibit the fungal and eubacterial growth. The plates were incubated at 28 ± 2°C for 3–4 weeks, actinomycetes colonies were transferred and maintained on ISP2 media by repetitive streaking.

Identification of isolated endophytic actinomycetes. The isolates were identified based on cultural and morphological characteristics, including, colonies on the plate, aerial and substrate color, spore mass color, production of melanoid pigments and color of diffusible pigments (Goodfellow and Haynes, 1984; Shimon *et al*., 1999). Based on Bergey's manual of systematic bacteriology, we classified the isolates by looking onto the aerial and substrate mycelia color in the following series: gray, white, red, yellow, green, blue, and violet (Buchnan and Gibbons, 2000). Microscopic characteristics using light microscopy and gram-stain properties were also performed and observed that the spore chains under light microscope, showed various spore characters like straight or flexuous chains, spira, extended, long and open coils. The spore chain morphology and surface of spore were examined by field emission gun-scanning electron microscopy (FEG-SEM) of 10-day old cultures grown on ISP4 according to the method described previously Kumar *et al.* (2011). Different biochemical tests like starch hydrolysis, Citrate utilization test, Indole test, methyl red test, Vogus-Proskauer test, catalase test were performed to characterize actinomycetes till genus level.

***In vitro* antifungal bioassay.** The endophytic actinomycetes isolates were evaluated for their antagonistic activity against seven major plant pathogenic fungi: *F. oxysporum*: CABI-293942; *F. oxysporum* f. sp. *ciceri* MTCC-2791, *F. proliferatum*: MTCC-286, *F. culmorum* MTCC-2090, *F. graminearum* MTCC-1893, *Alternaria* sp. MTCC-9601 and *Colletotrichum* sp. MTCC-3405 by dual-culture *in vitro* assay according to Bredholdt *et al.* (2007). All the pathogens were maintained on potato dextrose agar and maintained in a molecular microbiology and systematic laboratory, Department of Biotechnology, Mizoram University. An agar block of fungal pathogen was prepared by using sterile cork borer with diameter of 8 mm, and placed at the centre of PDA plate. Two endophytic actinomycetes discs (8 cm) 7 days old, grown on starch casein agar incubated at 28°C, were placed on the opposite sides of the plates, 3 cm away from the fungal block. Plates without endophytic actinomycetes discs were served as control. All plates were inoculated at 28°C for 14 days and percentage of inhibition was calculated by using the formula C-T/C × 100, where, C is the colony growth of fungal pathogen in control, and T is the colony growth in dual culture. All experiments were carried out in triplicates. Antagonistic activity was considered positive (+) if the zone of inhibition of the growth zone was more than 3 mm.

Molecular identification by using 16S rRNA gene amplification. The identity of the selected strains was determined based on the amplification and sequencing of 16S rRNA gene. Total genomic DNA was extracted by the Puregene Yeast/Bact Kit B (QIAGEN). The integrity of the genomic DNA was visualized by gel electrophoresis in 0.8% (w/v) by using the gel documentation system XR$^+$ system (BioRed, Singapore).

Fragment of 16S rRNA gene were amplified by PCR using universal primers- PA: 5'-AGA GTT TGA TCC TGG CTC AG-3') and PH: 5'-AAG GAG GTG ATC CAG CCG CA-3' (Qin *et al.,* 2009). The PCR reaction mixture was carried out in 25 µl total volume, containing 50 ng of genomic DNA, 2.5 µl (10 x) Taq Buffer, 1.5 µl (15 mM) MgCl2, 2.0 (2.5 mM) dNTPs, 0.5 µl (10 pmol) each primer and 1 µl (1 U) *Taq* DNA Polymerase. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 30 amplification cycles of denaturation at 94°C for 1 min, annealing at 57°C for 40 sec, extension at 72°C for 1 min and a final extension of 10 min at 72°C. The amplified PCR product was separated on 1.2 % agarose gel using TAE buffer and examined under gel documentation system XR+ (BioRed). The amplified amplicon was purified by using the Purlink PCR Purification Kit (In-vitrogen) and was sequenced commercially at SciGenom Labs Pvt. Ltd, India. Sequences were assembled and trimmed by using Finch TV version 1.4 (Geospiza inc.).

Phylogenetic analysis. Sequences of 16S rRNA gene were analyzed for homology using BlastN search program and very closely related species showing high level of identity (97–100%) was considered as closest match. These sequences, along with other actinomycetes strains retrieved from NCBI GenBank (www.ncbi.nlm.nih.gov) were used for the construction of phylogenetic tree after pairwise aligned using ClustalW (Thompson *et al*., 1997). All the assembled sequences were submitted to NCBI Genebank and accession numbers were obtained. A Maximum-Likelihood Tree was constructed using Kimura 2-Parameter (Kimura, 1980) and reliability of phylogenetic tree was evaluated by bootstrap analysis

with 1000 resamplings (Felsenstein, 1985) with Mega 5.05 software, taking *Escherichia coli* as outgroup.

PCR amplifications of antimicrobial genes (PKS-I and NRPS). Two antimicrobial genes Polyketide synthase (PKS) type I and nonribosomal peptide synthetase (NRPS) were amplified by using degenerate primers: K1F 5'-TSAAGTCSAACATCCGBCA-3' and M6R 5'-CGCAGGTTSCSGTACCAGTA-3' and A3F 5'-GCSTACSYSATSTACACSTCSGG-3' and A7R 5'-SASGTCVCCSGTSGCGTAS-3' respectively (Ayuso-Sacido and Genilloud, 2005). The PCR was performed in Veriti thermal cycler (Applied Biosystems, Singapore) in a final volume of 25 µl containing 25 ng of genomic DNA, 1.0 U of Taq DNA polymerase, 1 mM $MgCl_2$, 0.5 mM of dNTPs, 1.0 µM of each primer and 10% DMSO. PCR conditions were one denaturation step at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 60 s, annealing at 59°C for 60 s, and extension at 72°C for 2 min. Final extension step was done at 72°C for 10 min. A negative control reaction mixture without DNA template was also included with each set of PCR reactions. The PCR product was visualized on 1.5% agarose gel as stated above.

Statistical analysis. The data were calculated statically by using Microsoft Excel XP 2007 and significance difference ($P \leq 0.05$) was estimated by one way analysis of variance (ANOVA) between antimicrobial activities of different isolates by using SPSS software version 16.0.

Results

The distribution and identification of endophytic actinomycetes associated with different organs of medicinal plant *S. wallichii*, collected from Dampa Tiger Reserve, the largest wildlife sanctuary in Mizoram, Northeast, India, along with their *in vitro* antifungal activities to test the ability of the isolates to suppress the growth of fungal pathogens was studied. Twenty two isolates from 68 tissues were preliminarily characterized morphologically and biochemically according to international *Streptomyces* project (ISP method) and by following Bergey's manual of determinative bacteriology. The Scanning electron microscope (SEM) result showed that the aerial mycelia produce spiral spore chains (Fig. 1). Isolated cultures were designated as BPSAC (2, 8, 16, 20, 32, 40, 42, 48, 54, 57, 60, 65–72, 75 and 81) (Table I and II). The distribution of endophytic actinomycetes was found to be highest in roots (n = 9, 40.9%) followed by bark (n = 6, 27.2%), leaves (n = 4, 18.1%)

Table I
Morphological and microscopic characteristics of endophytic actinomycetes isolates with their different media

Isolate No. and NCBI Genbank accession No.	Isolate identified	Growth and colony nature	Aerial Mycelia	Substrate Mycelia	Pigmen tation	Media name
BPSAC2	*Streptomyces* sp.	slow and rough	white	white	no	SCNA
BPSAC8	*Streptomyces* sp.	slow and powdery	brownish white	light brown	no	ISP2
BPSAC16	*Streptomyces* sp.	slow and smooth	yellow	light yellow	no	ISP5
BPSAC20	*Streptomyces* sp.	slow and rough	orange	light orange	no	AIA
BPSAC22	*Streptomyces* sp.	slow and powdery	brownish white	brown	yellowish brown	SCNA
BPSAC32	*Streptomyces* sp.	slow and sticky	orange	light orange	no	TWYE
BPSAC40	*Streptomyces* sp.	slow and rough	brownish white	brownish white	no	AIA
BPSAC42	*Streptomyces* sp.	slow and firm	gray	light brown	no	SCNA
BPSAC48	*Streptomyces* sp.	slow and sticky	orange	orange	no	SCNA
BPSAC54	*Streptomyces* sp.	slow and rough	brownish white	brownish white	no	AIA
BPSAC57	*Streptomyces* sp.	slow and powdery	white	white	no	SCNA
BPSAC60	*Streptomyces* sp.	slow and smooth	yellow	light yellow	no	SCNA
BPSAC65 (KJ914903)	*S. sampsonii*	slow and rough	brownish white	brownish white	yellowish brown	TWYE
BPSAC66 (KJ914904)	*S. olivaceus*	slow and firm	brownish white	light brown	no	AIA
BPSAC67	*Streptomyces* sp.	slow and powdery	light brown	brown	no	TWYE
BPSAC68 (KJ914906)	*Streptomyces* sp.	slow and sticky	yellow	yellow	no	TWYE
BPSAC69 (KJ914907)	*Streptomyces* sp.	slow and rough	cream white	cream white	light brown	SCNA
BPSAC70 (KJ914908)	*Streptomyces* sp.	slow and rough	brownish white	brown	light brown	AIA
BPSAC71 (KJ914909)	*S. tempisquensis*	slow and powdery	gray	light brown	light brown	AIA
BPSAC72 (KJ914910)	*S. anulatus*	slow and firm	light brown	light brown	no	TWYE
BPSAC75	*Streptomyces* sp.	slow and sticky	yellow	yellow	no	SCNA
BPSAC81	*Streptomyces* sp.	slow and rough	white	white	no	ISP5

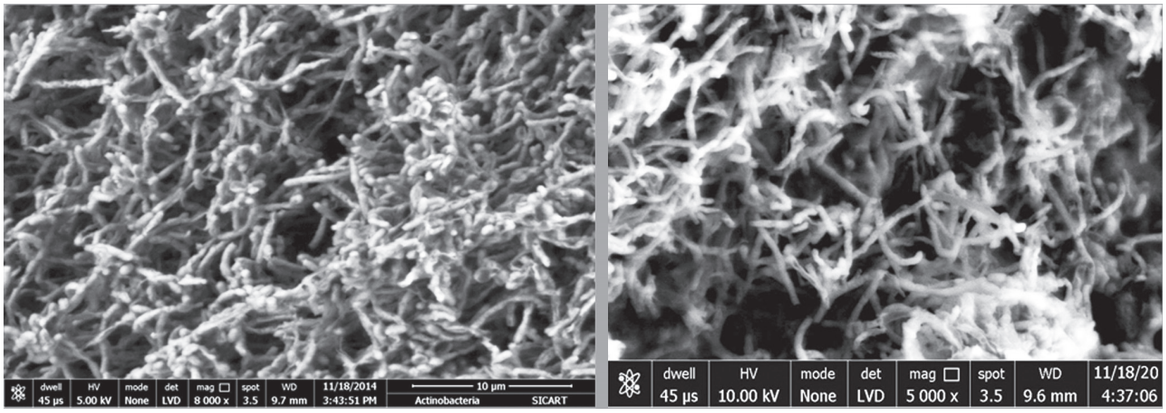

Fig. 1. Scanning electron microscope showing spore chain morphology of BPSAC70 strain and grown on AIA (actinomycetes isolation agar) media after 2 weeks at 28°C.

and fruit (n = 3, 13.6%). Five different nutritional media were used to understand the best suitable media for the recovery of endophytic actinomycetes and found that starch casein nitrate agar media (SCNA) (n = 8, 36.3%) was most effective followed by actinomycetes isolation agar media (AIA) (n = 6, 27.0%), tap water yeast extract agar media (TWYE) (n = 5, 22.0%), glycerol asparagine agar media (ISP5) (n = 2, 14.0%) and yeast and malt extract agar media (ISP2) (n = 1, 9.0%) (Fig. 2).

Fifteen of the 22 isolates showed significant growth inhibitory activity against at least two tested pathogens, when assayed against seven fungal phytopathogens (*F. culmorum* MTCC-2090, *F. proliferatum* MTCC-286, *F. oxysporum* CABI-293942, *F. graminearum* MTCC-1893, *F. oxysporum* f. sp. *ciceri* MTCC-2791, *Alternaria* sp. MTCC-9601 and *Colletotrichum* sp. MTCC- 3405). The results showed that most of the strains suppressed the test fungi with percentage of inhibition ranging from

Table II
Biochemical characterization of endophytic *Actinomycetes* isolates

Isolate No	Indole	Catalase	Urease	Oxidase	Nitrate	Starch
BPSAC2	+	+	+	+	+	+
BPSAC8	–	+	+	–	+	–
BPSAC16	–	–	+	+	–	–
BPSAC20	–	–	+	–	+	+
BPSAC22	–	+	–	+	–	+
BPSAC32	–	+	–	+	–	–
BPSAC40	–	+	+	+	+	–
BPSAC42	–	+	+	–	–	–
BPSAC48	–	–	+	+	+	+
BPSAC54	–	–	–	–	+	–
BPSAC57	–	–	+	–	+	–
BPSAC60	–	–	–	+	–	–
BPSAC65	+	+	+	–	+	+
BPSAC66	–	+	+	+	+	+
BPSAC67	–	+	–	–	+	–
BPSAC68	–	+	+	+	–	–
BPSAC69	–	+	–	+	–	–
BPSAC70	+	+	+	+	+	+
BPSAC71	+	+	+	+	+	+
BPSAC72	+	+	+	+	–	+
BPSAC75	–	+	+	–	+	+
BPSAC81	+	+	+	+	–	–

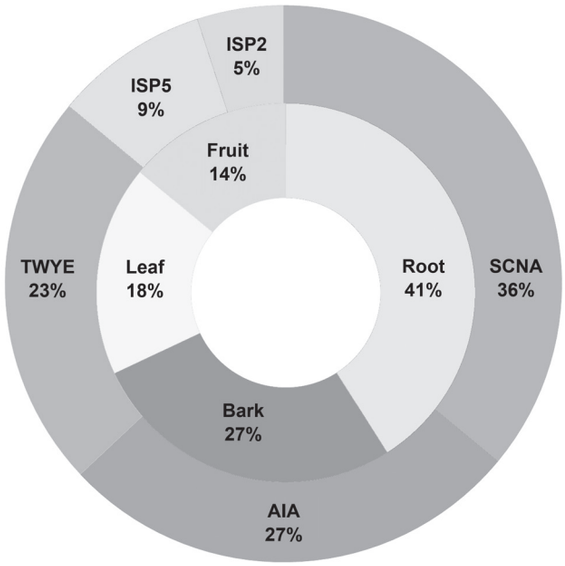

Fig. 2. Pie chart of isolated endophytic actinomycetes based on different isolation media and plant tissues.

26% to 98%. All isolates showed antagonistic activity against *F. culmorum* and *F. proliferatum*, whereas four isolates BPSAC (65, 68, 70 and 71) exhibited positive antagonistic effect against all selected pathogens (Fig. 3). Antagonistic activity of isolate number BPSAC 70 showed most prominent antagonism against *F. culmorum* (98.3%) and *F. graminearum* (92.3%), whereas isolate numbers BPSAC 66, 68 and 71 showed the highest antagonistic activities against *Alternaria* sp. (90.6%), *F. oxysporum* f. sp. *ciceri* (92.3%) and *F. proliferatum* (90.6%), respectively. The use of several indicator organisms will help us in selecting the broad spectrum antifungal strains. Among all selected pathogens *Alternaria* sp. was the most susceptible fungal pathogen against most of the endophytic actinomycetes isolates with the exception to BPSAC (2, 22, 32, 42, 54, 60 and 69) (Table III).

The selected potential isolates BPSAC (65, 66, 68, 69, 70, 71 and 72) which showed highest antagonistic activity against most of the pathogens were identified by amplification of 16S rRNA gene. The sequences were aligned by BLAST analysis along with the type strains downloaded from NCBI GenBank databases. Analysis of partial 16S rRNA gene sequences (565–830 bp) of potential seven strains exhibited high level of sequence similarity (97–99%) with sequences of *Streptomyces* species deposited in NCBI GeneBank. This indicates that all strains were closely related with the members of genus *Streptomyces*. The phylogenetic tree was constructed based on maximum likelihood method (Fig. 4) with Kimura 2-parameter model (R = 1.26) according to lowest BIC values using Mega 5.05. Gaps were treated by pairwise deletion and the estimated Transition/Transversion bias (R) was 1.25. The phylogenetic tree also confirmed the above results and the potent isolates were shorted out into four groups along with their closest relatives retrieved from NCBI GenBank.

Table III
Antifungal activity of endophytic actinomycetes against plant fungal pathogens

Isolate No	Percentage of inhibition zone (PI) ± SD against						
	F. oxysporum CABI-293942	*F. graminearum* MTCC-1893	*F. culmorum* MTCC-2090	*F. proliferatum* MTCC-286	*F. oxy. ciceri* MTCC-2791	*Alternaria* sp. MTCC-9601	*Colletotrichum* sp. MTCC-3405
BPSAC2	0.0 ± 0.0^{a}	46.6 ± 2.8^{a}	44.0 ± 3.4^{a}	40.6 ± 5.1^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	37.6 ± 2.5^{a}
BPSAC16	0.0 ± 0.0^{a}	41.3 ± 3.2^{bc}	37.6 ± 2.5^{bc}	44.0 ± 3.4^{bc}	0.0 ± 0.0^{a}	35.3 ± 3.2^{bc}	0.0 ± 0.0^{bc}
BPSAC22	40.6 ± 5.1^{bc}	0.0 ± 0.0^{bde}	35.3 ± 3.2^{bde}	$26.6 \pm 2.8b^{de}$	31.3 ± 3.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{bc}
BPSAC32	0.0 ± 0.0^{a}	0.0 ± 0.0^{bde}	40.6 ± 5.1^{bdfg}	37.6 ± 2.5^{bdfg}	40.6 ± 5.1^{bde}	0.0 ± 0.0^{a}	37.1 ± 2.8^{a}
BPSAC40	0.0 ± 0.0^{a}	0.0 ± 0.0^{bde}	39.6 ± 4.0^{bdfhi}	30.2 ± 2.8^{bdfhi}	0.0 ± 0.0^{a}	35.3 ± 3.2^{bc}	0.0 ± 0.0^{bc}
BPSAC42	0.0 ± 0.0^{a}	0.0 ± 0.0^{bde}	36.3 ± 3.2^{bdfhjk}	40.6 ± 5.1^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{bc}
BPSAC54	0.0 ± 0.0^{a}	0.0 ± 0.0^{bde}	85.3 ± 5.1^{bdfhjlm}	29.7 ± 2.8^{bdfhi}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{bc}
BPSAC60	44.0 ± 3.4^{bde}	72.3 ± 2.5^{bdfg}	44.0 ± 3.4^{a}	30.7 ± 2.8^{bdfhi}	31.3 ± 3.2^{bc}	0.0 ± 0.0^{a}	40.6 ± 5.1^{bde}
BPSAC65	90.0 ± 2.0^{bdfg}	84.5 ± 0.5^{bdfhi}	74.6 ± 5.0^{bdfhjlno}	85.0 ± 0.5^{bdfhjk}	75.3 ± 2.5^{bdfg}	62.6 ± 0.57^{bde}	73.3 ± 0.57^{bdfg}
BPSAC66	72.3 ± 2.5^{bdfhi}	$0.0 \pm 0.0b^{de}$	64.6 ± 0.5^{bdfhjlnpq}	79.0 ± 1.0^{bdfhjlm}	72.3 ± 2.5^{bdfhi}	90.6 ± 0.5^{bdfg}	0.0 ± 0.0^{bc}
BPSAC68	85.3 ± 5.1^{bdfhjk}	76.67 ± 2.8^{bdfhjk}	$72.3 \pm 2.5^{bdfhjlnprs}$	72.3 ± 2.5^{bdfhjlno}	92.3 ± 2.5^{bdfhjk}	76.67 ± 2.8^{bdfhi}	64.6 ± 0.5^{bdfhi}
BPSAC69	0.0 ± 0.0^{a}	72.3 ± 2.5^{bdfg}	$72.3 \pm 2.5^{bdfhjlnprs}$	$76.67 \pm 2.8^{bdfhjlnpq}$	79.0 ± 1.0^{bdfhjlm}	0.0 ± 0.0^{a}	52.5 ± 0.5^{bdfhjk}
BPSAC70	90.6 ± 0.5^{bdfg}	92.3 ± 5.1^{bdfhjlm}	$98.3 \pm 2.8^{bdfhjlnprt}$	$86.0 \pm 1.7^{bdfhjlnprs}$	76.67 ± 2.8^{bdfhjln}	64.6 ± 0.5^{bdfhjk}	74.6 ± 5.0^{bdfhjlm}
BPSAC71	74.6 ± 5.0^{bdfhjlm}	85.3 ± 2.5^{bdfhjlno}	$74.6 \pm 5.0^{bdfhjlnotuv}$	$90.6 \pm 0.5^{bdfhjlnprtu}$	44.0 ± 3.4^{bdfhjlno}	56.6 ± 2.8^{bdfhjlm}	72.3 ± 2.5^{bdfhjlno}
BPSAC72	72.3 ± 2.5^{bdfhi}	85.3 ± 5.1^{bdfhjlm}	$76.67 \pm 2.8^{bdfhjlnprtuwx}$	$74.6 \pm 5.0^{bdfhjlnprtvw}$	0.0 ± 0.0^{a}	87.0 ± 1.7^{bdfhjlno}	85.3 ± 5.1^{bdfhjlnpq}
Control	35.4 ± 2.5^{bdfhjln}	38.2 ± 2.2^{bdfhjlnp}	$30 \pm 0.0^{bdfhjlnprtuwy}$	$25.1 \pm 2.7^{bdfhjlnprtvx}$	35.4 ± 2.5^{bdfhjlnp}	28.0 ± 2.3^{bdfhjlnp}	26.6 ± 2.8^{bdfhjlnpr}

Mean (±SD) followed by the same letter(s) in each column are not significantly different at $P < 0.05$ using Duncan's new multiple range test.

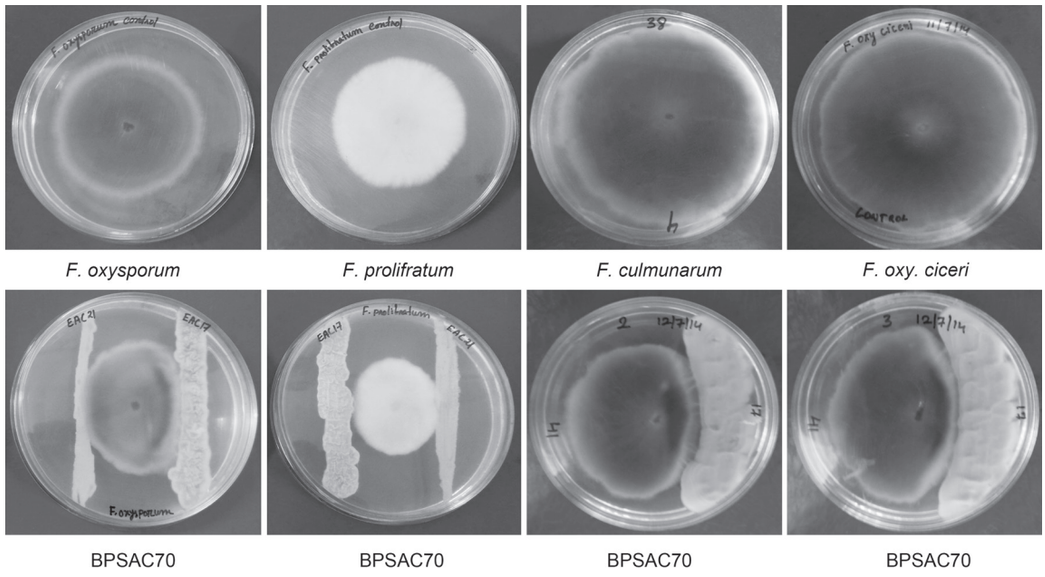

Fig. 3. Antagonistic activity of endophytic actinomycetes against some plant fungal pathogens.

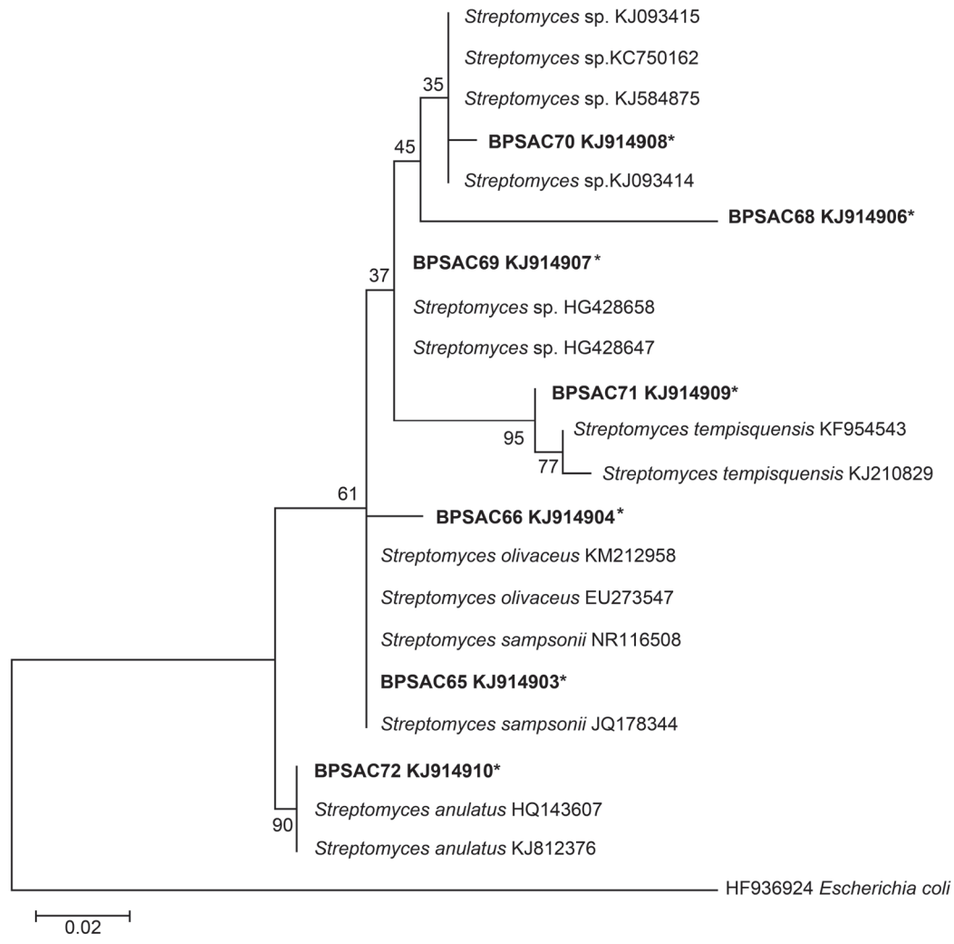

Fig. 4. Maximum-Likelihood phylogenetic tree generated by Kimura 2 parameter model based on 16S rRNA genes of endophytic actinomycetes.

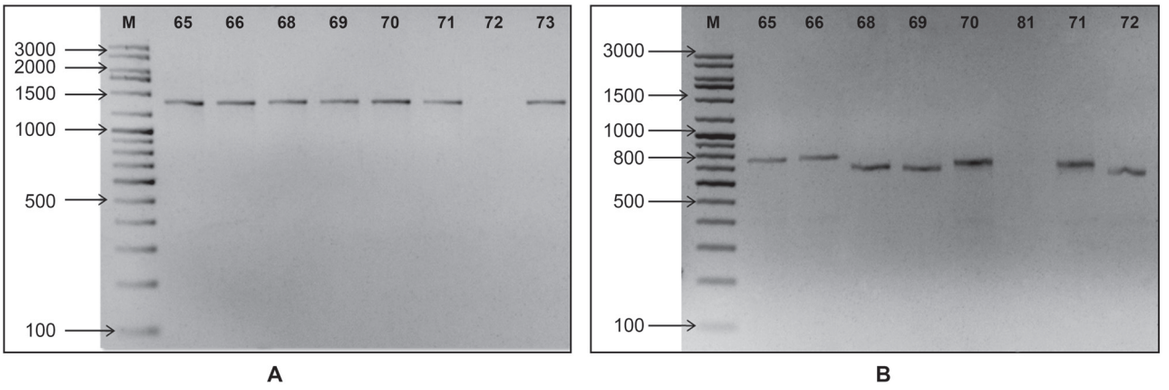

Fig. 5. Amplification of 1200–1400 bp of PKSI gene using K1F/M6R primer (A) and 700–800 bp of NRPS gene using A3F/A7R primers (B) from endophytic actinomycetes strains.

Isolates BPSAC (68, 69 and 70) had 96–99% similarity with type strains *Streptomyces* sp. and interestingly falls in one group. Other isolates conform to be a branch with *Streptomyces sampsonii*, *Streptomyces olivaceus*, *Streptomyces tempisquensis* and *Streptomyces anulatus*.

All twenty two isolates were subjected to the detection of PKS and NRPS genes and it was found that nine strains BPSAC (2, 32, 65, 66, 68, 69, 70, 71 and 72) were positive for the PKSI and 11 strains BPSAC (16, 32, 54, 60, 65, 66, 68, 69, 70, 71 and 72) were positive for NRPS gene fragments whereas other strains could not showed any amplification. Interestingly, all seven isolates which showed antagonistic activity, also showed the presence of PKS type I and NRPS gene fragments, which further proves them to be potential antagonistic strains and needs further attention (Fig. 5).

Discussion

Chemical-mediated suppression of plant pathogens is generally the primary method to repress the plant diseases. Indeed, the range of secondary metabolites produced by *Streptomyces* and other microorganism offers a great potential to fight many soil borne pathogens. As compared to *Streptomyces* in the rhizosphere, endophytic *Streptomyces* are expelled from the competition with other soil microorganisms and can efficiently colonize plant tissues. Thus, endophytic *Streptomyces* plays an important role in the development of plant by enhancing nutrient assimilation or by producing secondary metabolites (Kizuka *et al*., 2002). Endophytic *Streptomyces* have been investigated in many studies as biocontrol agent and revealed as a promising resource for agricultural industry (Cao *et al*., 2005; Shimizu *et al*., 2006). Previous studies have proved the use of endophytic *Streptomyces* as biocontrol agent against *F. oxysporum* (Sardi *et al*., 1992), bacteria, yeast and filamentous bacteria (Shimizu *et al*., 2001).

Plants growing in biodiversity rich areas with ethano-botanical history are likely to house endophytes with greater potential. Endophytes acquired a specific ability that allows them to sustain under the living tissues without any detectable infectious symptoms to the host. Thus, they are of immense importance to the host due to their capability to produce a wide array of natural bioactive compounds (Sardi *et al*., 1992; Strobel and Daisy, 2003; Cao *et al*., 2005; Kim *et al*., 2012).

However, this study was conducted for the first time to understand the endophytic actinomycetes population distribution and their potential as a biocontrol agent associated with traditional medicinal plant *S. wallichii*. In total twenty two isolates were obtained, among them maximum were isolated from root tissues (n = 9, 40.9%) followed by bark (n = 6, 27.2%), leaves (n = 4, 18.1%) and fruit (n = 3, 13.6%). Our results are in consensus with several studies, indicating the population of endophytic actinomycetes as highest in root tissues (De-Araujo *et al*., 2000; Taechowisan and Lumyong, 2003; Cao *et al*., 2005; Passari *et al*., 2015). To name a few, Verma *et al.* (2009) reported the highest percentage of endophytic actinomycetes was obtained from roots (55%) followed by other tissues of 20 different *Azadirachta indica* trees.

The reason may be due to the fact that actinomycetes present in rhizosphere can easily penetrate to root tissues and since plants collect nutrient and water through their roots, this may be the major source for the recovery of actinomycetes (Nimnoi *et al*., 2010). Though, Kayini and Pandey (2010) have reported the recovery of endophytic fungi from *S. wallichii* but, to the best of our knowledge, this is the first attempt made to understand the distribution of endophytic actinomycetes and their potential as biocontrol agent from *S. wallichii*. Five different nutrient media were used for the recovery of endophytic actinomycetes and found that maximum isolates (36.3%) used SCNA as a source of nutrition, which was in contradictory with the find-

ings of Coombs and Fransco (2003) and Khamna *et al.* (2009a; 2009b), who suggested that TWYE and HV agar medium were the best media for the isolation of endophytic actinomycetes.

All isolates were screened for their antagonistic ability against selected fungal phytopathogens and interestingly, seven isolates identified as *Streptomyces* sp. found to inhibit growth of most of the selected pathogens. Genus *Streptomyces* has an excellent track record for the discovery of bioactive metabolite and for the production of natural antibiotics (Baltz, 2006; Mingma *et al*., 2014).

Microbial metabolites may have an active role in resistance development by functioning as signals mediating cross-talk between the endophytes and their host (Graner *et al*., 2003). Since the endophytic actinomycetes were isolated from medicinal plant and that strains expected to produce a wide variety of antifungal and plant growth regulatory bioactive metabolites (Bredi, 1989; Franco and Cautinho, 1991), they can be exploited as novel sources of natural products and novel biocontrol agents as well. Endophytic actinomycetes are reported by many researchers for their role in plant protection against fungal pathogens. Two endophytic *Streptomyces* sp. were found to be active against all tested fungal pathogens including strains of *Fusarium*, *Colletotrichum* and *Alternaria* which was in agreement with earlier works of Khamna *et al*. (2009a; 2009b) and Intra *et al.* (2011). *Streptomyces* sp. were reported to suppress or inhibit plant pathogen *F. oxysporum*, the causative agent of cucumber *Fusarium* wilt (Zhao *et al*., 2012). Similarly, Verma *et al.* (2009) demonstrated the antifungal activity of endophytic actinomycetes against numbers of fungal pathogens.

Seven isolates were characterized by 16S rRNA gene sequence analysis and diversity of the potential isolates was found. The sequence of most isolates showed identity of 97–100% with BlastN sequences. All the isolates were classified as *Streptomycetaceae* family and identified as *Streptomyces* sp., *S. sampsonii*, *S. tempisquensis*, *S. olivaceus* and *S. anulatus.* Isolate BPSAC66 (*S. olivaceus*) was morphologically similar with BPSAC65 (*S. sampsonii*) and they were found to cluster together.

To understand the biosynthetic potential of the isolates, detection of genes encoding polyketide synthase and nonribosomal peptide synthetase mainly responsible for the synthesis of most biologically active polyketide and peptide compounds have been broadly used (Khamna *et al*., 2009a; 2009b). However, our results and findings from other researchers suggest that the antimicrobial potential of the culturable endophytic actinomycetes may only be assessed by screening of antimicrobial activity against desired pathogens. In our study, most of the isolates showed the presence of PKSI and NRPS genes, also showed antifungal activity against most of the tested pathogens which is contrary to the findings of Qin *et al.* (2009), who stated that antimicrobial activity results and the detection of functional genes showed no direct relationship.

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ORIGINAL PAPER

Characterization of Rhizobial Bacteria Nodulating *Astragalus corrugatus* and *Hippocrepis areolata* in Tunisian Arid Soils

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Abstract

Fifty seven bacterial isolates from root nodules of two spontaneous legumes (*Astragalus corrugatus* and *Hippocrepis areolata*) growing in the arid areas of Tunisia were characterized by phenotypic features, 16S rDNA PCR-RFLP and 16S rRNA gene sequencing. Phenotypically, our results indicate that *A. corrugatus* and *H. areolata* isolates showed heterogenic responses to the different phenotypic features. All isolates were acid producers, fast growers and all of them used different compounds as sole carbon and nitrogen source. The majority of isolate grew at pHs between 6 and 9, at temperatures up to 40°C and tolerated 3% NaCl concentrations. Phylogenetically, the new isolates were affiliated to four genera *Sinorhizobium*, *Rhizobium, Mesorhizobium* and *Agrobacterium*. About 73% of the isolates were species within the genera *Sinorhizobium* and *Rhizobium*. The isolates which failed to nodulate their host plants of origin were associated to *Agrobacterium* genus (three isolates).

Key words: 16S rDNA sequencing, arid areas, PCR-RFLP, phenotypic properties, rhizobial bacteria

Introduction

Rhizobia or "legume nodulating bacteria (LNB)" or "root nodule bacteria' (RNB)" are defined as nitrogen-fixing bacteria that form nodules on legume plants. In the last few years, a large diversity of LNB has been revealed, which has caused deep changes in the taxonomy of these bacteria. Rhizobia currently consist of 98 species belonging to 13 different genera. The predominant symbionts for most legume species in habitats throughout the world are found in the *a*-class of *Proteobacteria*: *Rhizobium*, *Azorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Mesorhizobium*, *Bradyrhizobium*, *Methylobacterium* (Jaftha *et al*., 2002; Jourand *et al*., 2004), *Devosia* (Rivas *et al*., 2003), *Shinella* (Lin *et al*., 2008), *Ochrobactrum* (Trujillo *et al*., 2005; Zurdo-Pineiro *et al*., 2007), *Phyllobacterium* (Valverde *et al*., 2005; Mantelin *et al*., 2006) and *Microvirga* (Ardley *et al*., 2012). Moreover, about eight species within two genera of *β*-class of *Proteobacteria – Burkholderia* and *Cupriavidus* have been reported (Moulin *et al*., 2001; Chen *et al*., 2001; 2006; 2008; Klonowska *et al*., 2012). In addition, bacteria from *γ*-class of *Proteobacteria* have also been reported (Benhizia *et al*., 2004; Muresu *et al*., 2008; Mahdhi *et al*., 2012). On the other hand, many *Agrobacterium*-like strains have been isolated from root nodules of different legumes species (Gurtler *et al*., 1991; Liu *et al*., 2005; Mahdhi *et al*., 2008), but all of them failed to nodulate their original plant hosts and until now no definitive explanation of the presence of these bacteria inside nodules could be demonstrated.

Legumes belonging to the genera *Astragalus* and *Hippocrepis* are distributed in northern Africa, southern Europe and East Asia. Several *Astragalus* species are used as herbal medicine and *Hippocrepis* species have a wide range of uses as minor crops including consumption, fodder, forage and land stabilization. Despite the high number of these legume species (3000 species for *Astragalus* and 20 for *Hippocrepis*), only few of them have been considered for their nitrogen symbiotic fixation. Previous studies reported that microsymbionts associated to root nodules of some *Astragalus* species belonged to *Sinorhizobium*, *Rhizobium*, *Agrobacterium, Bradyrhizobium* and *Mesorhizobium* (Zhang *et al*., 2000; Gao *et al*., 2001; 2004; Wei *et al*., 2003; Zhao *et al*., 2012;

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Guerrouj *et al*., 2013; Gnat *et al*., 2014). Surprisingly, Muresu *et al.* (2008) reported that strains isolated from *Hippocrepis* unisiliquosa are identified as members of the genus *Bacillus* or as uncultured bacteria.

At Tunisia, *Astragalus* and *Hippocrepis* nitrogen-fixing symbiotic associations are poorly documented (Zakhia *et al*., 2004; Mantelin *et al*., 2006). Previous research's reported that rhizobia associated to *Astragalus glombiformis*, *Astragalus armatus*, *Astragalus corrugatus* and *Astragalus algerianus* were assigned to the genus of *Rhizobium* and *Phyllobacterium*, and only one isolate from nodules *Hippocrepis areolata* was affiliated to the genus *Sinorhizobium* (Zakhia *et al*., 2004).

Considering the potential value of the *Astragalus* and *Hippocrepis* species in the arid regions of Tunisia and the little information available about the diversity of their root nodulating bacteria, the present paper aim to determine the taxonomic diversity of 57 bacterial collection isolated from root nodules of *A. corrugatus* and *H. areolata* by using polyphasic approach including phenotypic and PCR-RFLP analysis and 16S rRNA gene sequencing.

Experimental

Materials and Methods

Bacterial isolation and growth conditions. Fifty seven isolates and six reference strains (Table I) representing different rhizobial species belonging to *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* were used in this study. Rhizobial bacteria were isolated from naturally occurring root nodules collected in four arid soils of Tunisia (Table I). For rhizobia isolation, healthy nodules dissected from roots were surface sterilized with ethanol (70%) and sodium hypochlorite (2%). Then nodules were separately crushed and the nodule juice was streaked on plates of yeast-mannitol agar (YMA) (Vincent, 1970) and incubated at 28°C for the isolation of the rhizobia. The obtained bacterial colonies were purified by being repeatedly streaked on the same medium. Pure isolates were stored with 25% (wt/vol) glycerol at −80°C.

Nodulation test. To assess nodulation, seeds were surface-sterilized in 98% sulphuric acid for 30 min and germinated on H_2O-agar plates (0.8%) at 25°C. Seedlings were transferred into vermiculite, inoculated with individual isolates and grown in a growth chamber at 25°C with12–16 h photoperiod. Nitrogen-free nutrient solution was used for plant watering (Vincent, 1970). Controls, not inoculated, were included. Four replicates were maintained for each treatment. Four weeks post inoculation, the plants were uprooted and the occurrence of nodulation in each plant was checked.

Phenotypic characterization. All isolates were initially tested for their phenotypic features. For bacterial growth, bacteria were cultivated in 50 ml of YM broth into 250 ml Erlenmeyer flasks and incubated in a gyratory shaker at 180 g and 28°C. Growth was followed by measuring the optical density at 600 nm every 2 h and generation time of each isolate was deduced from the exponential phase of the growth curves.

Growth of the isolates at different temperatures (28, 37, 40, 42, 45°C), the ability to grow in the presence of different NaCl concentrations (1, 2, 3, 4, 5%) and at different pH levels (4, 5, 7, 9, 11) were determined by growth on supplemented YMA as described by Mohamed *et al.* (2000).

The modified-YMA medium (Somasegaran and Hoben, 1994) was used to investigate the ability of isolates to use carbohydrates (1% glucose, galactose, fructose and sucrose) and amino-acids (0.1% L-proline, L-arginine, L-tyrosine and L-leucine) as a sole carbon and nitrogen sources respectively. Production of acid or alkali was determined on YMA supplemented with 0 ± 0025% (w/v) bromothymol blue as pH indicator. All phenotypic tests were performed in triplicate.

PCR amplification and RFLP analysis of 16S rRNA gene. Total genomic DNA was extracted as described by Mhamdi *et al.* (2002). Primers fD1 and rD1 (Weisburg *et al*., 1991), were used for PCR amplification of 16S rRNA gene. PCR was carried out in Gen Amp PCR system 9700 (Applied Biosystems) in a 25 µl containing template DNA extract as described previously by Mahdhi *et al.* (2012). PCR amplification was analyzed by horizontal 1% (w/v) agarose gel electrophoresis stained with ethidium bromide. The amplified DNA fragments of 16S rRNA gene were digested with *Rsa*I, *Hinf*I, *Hae*III, *Cfo*I, *Nde*II and *Msp*I restriction enzymes (Promega products). The restriction patterns were checked by horizontal 4% (w/v) agarose gel electrophoresis stained with ethidium bromide.

Different 16S rDNA types were designed based upon the combined RFLP patterns obtained from the six enzymes, *e.g.* isolates is defined as a unique 16S rDNA type if it has one band different from other isolates in the six digestions

Sequencing of 16S rRNA gene. Bacterial genomic DNA extracted according to Mhamdi *et al.* (2002) was used as templates. For five isolates 16S rDNA gene chosen as representative of different 16S rDNA types, were amplified using universal primers fD1 and rD1 as described above. The PCR products were purified and sequenced using the ABI PRISM BigDye Terminator cycle sequencing kit according to the manufacturer's protocol and analysed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequences were assembled using ChromasPro and were aligned with Clustal X. The acquired sequences were deposited

Table I
New isolates and reference strains used in this study.

Isolates	*Site of origin*	*Host plants*	*Nodulation test*	*16SrDNA type*
ACM1	Menzel Habib	*Astragalus corrugatus*	–	1*
ACM2	Menzel Habib	*Astragalus corrugatus*	+(12)	2†
ACM3	Menzel Habib	*Astragalus corrugatus*	+(11)	2†
ACM4	Menzel Habib	*Astragalus corrugatus*	+(10)	2†
ACN1	Nefta	*Astragalus corrugatus*	+(15)	3ϕ
ACN2	Nefta	*Astragalus corrugatus*	+(14)	3ϕ
ACN3	Nefta	*Astragalus corrugatus*	+(11)	3ϕ
ACN4	Nefta	*Astragalus corrugatus*	+(11)	3ϕ
ACN5	Nefta	*Astragalus corrugatus*	+(10)	2†
ACN6	Nefta	*Astragalus corrugatus*	+(10)	2†
ACN7	Nefta	*Astragalus corrugatus*	+(10)	2†
ACN8	Nefta	*Astragalus corrugatus*	+(13)	2†
ACN9	Nefta	*Astragalus corrugatus*	+(15)	2†
ACN10	Nefta	*Astragalus corrugatus*	–	1*
ACN11	Nefta	*Astragalus corrugatus*	+(14)	2†
ACN12	Nefta	*Astragalus corrugatus*	+(14)	3ϕ
ACN13	Nefta	*Astragalus corrugatus*	+(13)	3ϕ
ACN14	Nefta	*Astragalus corrugatus*	+(11)	2†
ACN15	Nefta	*Astragalus corrugatus*	+(11)	2†
ACN16	Nefta	*Astragalus corrugatus*	+(14)	3ϕ
ACZ1	Zarzis	*Astragalus corrugatus*	+(13)	4‡
ACZ2	Zarzis	*Astragalus corrugatus*	+(15)	4‡
ACZa1	Zárate	*Astragalus corrugatus*	+(15)	4‡
ACZa2	Zárate	*Astragalus corrugatus*	+(15)	4‡
ACZa3	Zárate	*Astragalus corrugatus*	+(12)	4‡
HBM1	Menzel Habib	*Hippocrepis areolata*	+(12)	3ϕ
HBM2	Menzel Habib	*Hippocrepis areolata*	+(13)	3ϕ
HBM3	Menzel Habib	*Hippocrepis areolata*	+(14)	3ϕ
HBM4	Menzel Habib	*Hippocrepis areolata*	+(11)	3ϕ
HBM5	Menzel Habib	*Hippocrepis areolata*	+(10)	3ϕ
HBM6	Menzel Habib	*Hippocrepis areolata*	+(10)	3ϕ
HBM7	Menzel Habib	*Hippocrepis areolata*	+(11)	3ϕ
HBM8	Menzel Habib	*Hippocrepis areolata*	+(12)	3ϕ
HBM9	Menzel Habib	*Hippocrepis areolata*	+(13)	3ϕ
HBN1	Nefta	*Hippocrepis areolata*	+(14)	3ϕ
HBN2	Nefta	*Hippocrepis areolata*	+(12)	3ϕ
HBN3	Nefta	*Hippocrepis areolata*	+(11)	3ϕ
HBN4	Nefta	*Hippocrepis areolata*	+(10)	5†
HBN5	Nefta	*Hippocrepis areolata*	+(11)	3ϕ
HBN6	Nefta	*Hippocrepis areolata*	+(12)	5†
HBN7	Nefta	*Hippocrepis areolata*	+(12)	3ϕ
HBN8	Nefta	*Hippocrepis areolata*	+(12)	5†
HBN9	Nefta	*Hippocrepis areolata*	+(13)	3ϕ
HBN10	Nefta	*Hippocrepis areolata*	+(10)	5†
HBZ1	Zarzis	*Hippocrepis areolata*	+(10)	2†
HBZ2	Zarzis	*Hippocrepis areolata*	+(15)	2†
HBZ3	Zarzis	*Hippocrepis areolata*	+(14)	3ϕ

Table I
New isolates and reference strains used in this study.

Isolates	*Site of origin*	*Host plants*	*Nodulation test*	*16SrDNA type*
HBZ4	Zarzis	*Hippocrepis areolata*	+(13)	2†
HBZ5	Zarzis	*Hippocrepis areolata*	+(12)	2†
HBZ6	Zarzis	*Hippocrepis areolata*	+(14)	2†
HBZ7	Zarzis	*Hippocrepis areolata*	+(14)	2†
HBZ8	Zarzis	*Hippocrepis areolata*	+(13)	5†
HBZ9	Zarzis	*Hippocrepis areolata*	+(12)	5†
HBZ10	Zarzis	*Hippocrepis areolata*	+(11)	5†
HBZ11	Zarzis	*Hippocrepis areolata*	–	1*
HBZ12	Zarzis	*Hippocrepis areolata*	+(10)	2†
HBZ13	Zarzis	*Hippocrepis areolata*	+(15)	2†
R. mongolense STM246^T = LMG1941^T	Mongolia, China	*Medicagoruthenica*	Nt	6
R. galegae HMBI540^T = LMG6214^T	Finland	*Galegae orientalis*	Nt	7
M. loti ORS664 = LMG6125^T	New Zealand	*Lotus tenuis*	Nt	8
M. mediterraneum ORS2739^T = LMG17148^T	Spain	*Cicer arietinum*	Nt	9
S. meliloti ORS665^T = LMG6133^T	Virginia, USA	*Medicagosativa*	Nt	3
B. japonicum NZP5549^T = LMG6138^T	Japon	*Glycine max*	Nt	10

Note: STM: collection du laboratoire des Symbioses tropicales et méditerranéennes; HAMBI: Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; LMG: Collection of Bacteria of the Laboratorium voor Microbiologie, Universiteit Ghent, Belgium; NZP: Culture Collection of the Department for Scientific and Industrial Research, Biochemistry Division, Palmerston North, New Zealand; ORS: Collection, Laboratoire commun de Microbiologie, BP 1386, Dakar, Senegal; ^{T} type strain. *: isolates grouped in genus *Agrobacterium*; †: isolates grouped in genus *Rhizobium*; ϕ: isolates grouped in genus *Sinorhizobium*, ‡: isolates grouped in genus *Mesorhizobium*. + Positive test, – no nodulation. Numbers in parentheses indicate the number of nodules per plant, Nt: not tested

in the GenBank database and were analysed for homologies to related sequences obtained from GenBank. The phylogenetic analyses were performed using mega 3.1 software (Kumar *et al*., 2001). A neighbour-joining tree was constructed using Kimura two-parameter model (Kimura, 1980) of and support of internal branches was assessed using 1000 bootstrap replications. The GenBank accession numbers for the 16S rRNA gene sequences reported in this paper are KR108303 (ACN5), KR108304 (HBN4), KR108300 (ACM1), KR108301 (ACN1) and KR108302 (ACZ1).

Results

Nodulation test and phenotypic characterisation. A nodulation test was performed for all isolates. Result showed that only two *A. corrugatus* isolates (ACM1 and ACN10) and one *H. areolata* isolate (HBZ11) affiliated to *Agrobacterium* by 16S rRNA gene sequencing analysis (see below) failed to nodulate their host plant of origin. The other isolates formed ten to fifteen nodules per plantlet after four weeks post-inoculation (Table I).

Phenotypically, (Table II) all isolates were acid producers, fast growers (Generation times < 6 h). Five *A. corrugatus* isolates (described by the analysis of 16S rRNA sequences as *Mesorhizobium* have a generation time between 4 and 6 h. All tested isolates used all tested compounds as sole carbon and nitrogen sources and were able to grow at pHs between 6 and 9, but none of them could grow at pHs 4 and 11.

The majority of the isolates grew at 28, 37 and 40°C. Only two isolates (ACN1 and ACN4) continued to grow at 42°C, but not at 45°C. Most of the tested isolates tolerated NaCl concentrations from 1 to 3%. Three *A. corrugatus* isolates (ACN1, ACN4, and ACN13) and one *H. areolata* isolate (HBZ1) continued to grow in 4% NaCl and none of them tolerated 5% NaCl.

PCR-RFLP analysis of 16S rRNA gene. The new isolates of 16S ribosomal DNA and reference strains was PCR-amplified and a single band of the expected size of approximately 1500 bp was produced. PCR products were digested with six restriction enzymes *Rsa*I, *Hinf*I, *Hae*III, *Cfo*I, *Nde*II and *Msp*I. Ten 16S rDNA types were distinguished among the 57 isolates and the six reference strains. Each 16S rDNA type comprised 1 to 23 isolates (Table I). Five rDNA types were identified among the new isolates. Types 1, 2, 3 of 16S rDNA included new isolates originating from both *A. corrugatus* and *H. areolata* microsymbionts. The type 4 and 5 of 16S rDNA contained only *A. corrugatus* and *H. areolata* isolates respectively. Type 3 of 16S rDNA consisted of

Table II
Phenotypic characteristics of the isolates

Characteristics		*Sinorhizobium* isolates	*Rhizobium* isolates	*Mesorhizobium* isolates	*Agrobacterium* isolates
Number of isolates		23	26	5	3
Generation time	**G < 4 h**	+	+	–	+
	4 ≤ G < 6	–	–	+	–
Growth at pH	4	–	–	–	–
	9	+	+	+	+
	11	–	–	–	–
Acid production		+	+	+	+
Alkali production		–	–	–	–
NaCl tolerance	2%	+	+	+	+
	3%	+	+ (20)	+	–
	4%	+ (3)	+ (1)	–	–
	5%	–	–	–	–
Utilisation of sugars	glucose	+	+	+	+
	galactose	+	+	+	+
	fructose	+	+	+	+
	sucrose	+	+	+	+
Utilisation of amino acids	L-arginine	+	+	+	+
	L-proline	+	+	+	+
	L-leucine	+	+	+	+
	L-tyrosine	+	+	+	+
Growth at temperature	40°C	+	+ (24)	+ (4)	–
	42°C	+ (2)	–	–	–
	45°C	–	–	–	–

Note: (+) positive growth/ present; (–) no growth/absent;
Number in parentheses indicate the number of positive isolates of the total number of isolates tested

both new isolates and a reference strain *(Sinorhizobium meliloti* LMG6133^T).

Sequencing of 16S rDNA. A total of five *A. corrugatus* and *H. areolata* isolates representing the five different 16S rDNA types were selected to undergo 16S rDNA gene sequencing. New *A. corrugatus* and *H. areolata* strains exhibited 99–100% 16S rDNA sequence similarity with reference species already described in GenBank. In the reconstructed phylogenetic tree (Fig. 1), strain ACZ1 (Representative of 16S rDNA type 4) was phylogenetically related to *Mesorhizobium temperatum* CCNWSX0012-2 and to *Mesorhizobium* sp. LAC831. The two isolates ACN5 and HBN4, representative of 16S rDNA types 2 and 5 respectively, were grouped in *Rhizobium* branch; with ACN5 strain was associated to *Rhizobium* sp. STM 394, while HBN4 strain was affiliated related to *Rhizobium* sp. STM 4037. The sequences of 16S rDNA of the strain ACN1 was closely related to the 16S rDNA sequences of *S. meliloti* LMG6133^T and *Sinorhizobium* sp. STM4038, while the strain ACM1 was phylogenetically related to *Agrobacterium tumefaciens* 2002000903 and *Agrobacterium* sp. STM4035.

Discussion

Currently, in the rhizobia taxonomy the polyphasic approach, based on phenotypic and genomic criteria is used (Graham *et al.*, 1991; Vandamme *et al.*, 1996; Małek and Sajnaga, 1999). Among the phenotypic features characteristic related to the microorganism ecological niches are considered as the most interesting. In the present studies a collection of 57 isolates was obtained from *A. corrugatus* and *H. areolata* root nodules covering four regions of Tunisia and characterized by a polyphasic approach including phenotypic features, PCR-RFLP of 16S rDNA and 16S rDNA sequence analysis. All isolates, except ACM1, ACN10 and HBZ11, re-induce nodules in their host plant. The three non-re-nodulating strains can be considered as opportunistic endophytes as already proposed (Zakhia *et al.*, 2006; Mahdhi *et al.*, 2007; 2012).

Phenotypically (Table II) our results indicate that *A. corrugatus* and *H. areolata* isolates showed heterogenic responses to the different phenotypic features. This heterogeneity may contribute the nodulation of

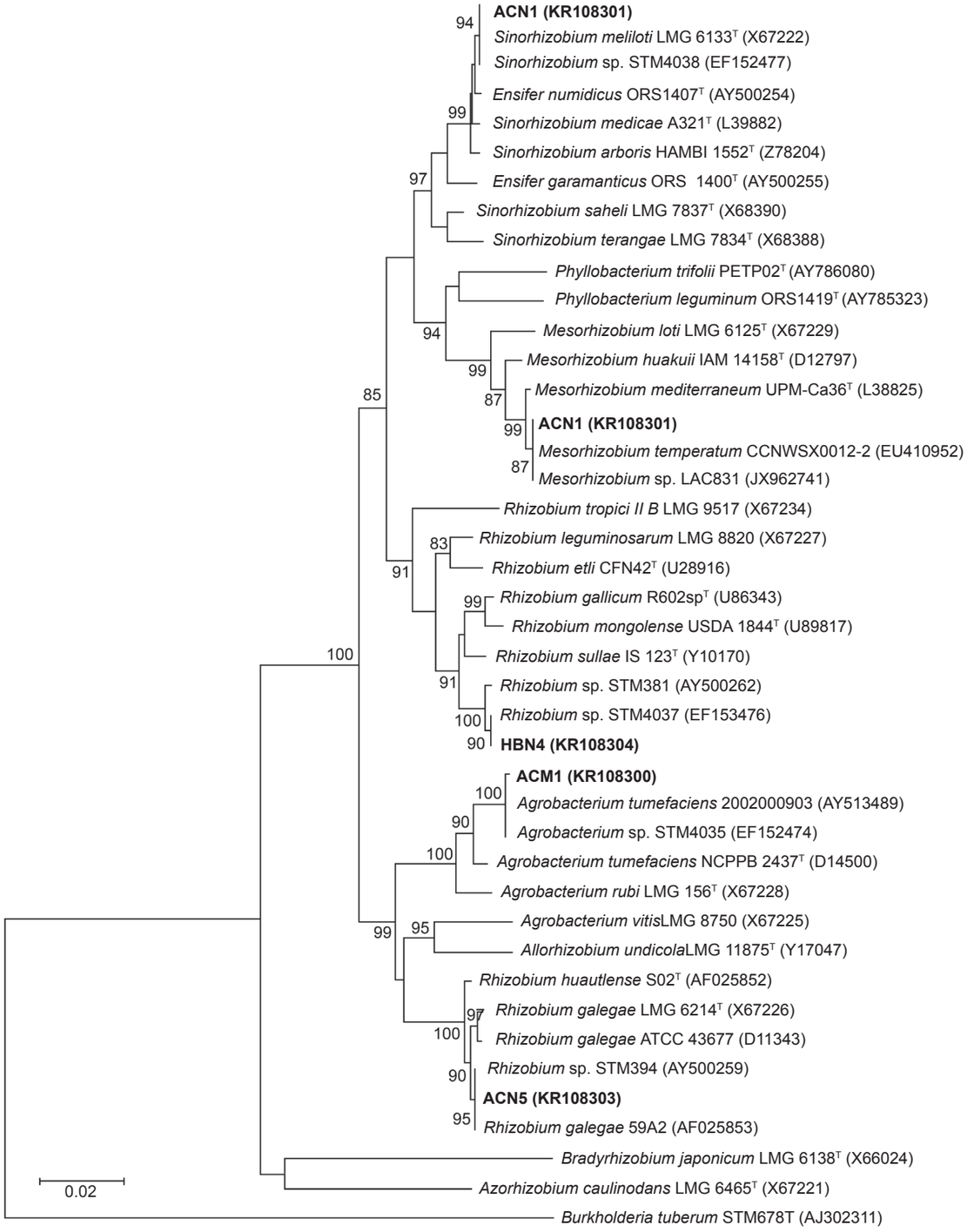

Fig. 1. 16S rRNA gene sequence-based dendrogram obtained by neighbor-joining method showing the phylogenetic positions of *A. corrugatus* and *H. areolata* isolates. Only significant bootstraps (> 80%) are shown (1000 replications). Sequence accession numbers are listed in parentheses.

legumes in different conditions (Wei *et al.*, 2008). All new isolates are acid producers, fast growers (Generation times < 6 h) like *Rhizobium*, *Sinorhizobium* and *Mesorhizobiun* species (Małek and Sajnaga, 1999). All tested isolates are able to use all tested compounds as sole carbon and nitrogen sources. Similar results were reported by Guerrouj *et al.* (2013) for rhizobia nodulating *A. glombiformis* in Eastern Morocco. This ability to use a wide range of carbon sources could be beneficial for the bacterial life cycle in the soil and may be related

to their high competitiveness in a natural environment. Elkan (1992) reported that carbohydrate sources could be used to differentiate fast-growing rhizobia from the slow-growing bradyrhizobia.

As for salinity, temperature and pH tolerance, our results showed that most of the isolates are able to grow at 3% NaCl, at pHs between 6 and 9 and at high temperature (40°C), except of two of them which were continued to grow at 42°C. These results corroborate our earlier reports on the root nodule bacteria isolated from wild legumes in Tunisia (Mahdhi *et al*., 2007; 2008; Rejili *et al*., 2009; Fterich *et al*., 2011). Similarly, Guerrouj *et al.* (2013) reported that rhizobial strains associated to *A. glombiformis* are tolerant to 342 mM NaCl and 40°C in Eastern Morocco. In China, Wei *et al.* (2003) reported that some rhizobial strains nodulating *Astragalus* species tolerate only 2% NaCl. The capacity of new isolates to tolerate high temperatures and high NaCl concentrations could be considered a specific adaptation to high soil temperatures and salinity in arid regions as described by Karanja and Wood (1988).

By using the comparative 16S rRNA gene sequence analysis, the new isolates were grouped on the phylogram in the *Sinorhizobium*, *Rhizobium*, *Mesorhizobium* and *Agrobacterium* genera; with 73% of the new isolates are species within the genera *Sinorhizobium* and *Rhizobium* as are many other indigenous legume symbionts from Tunisia (Zakhia *et al*., 2004; Ben Romdhane *et al*., 2005; Mahdhi *et al*., 2008). By 16S rRNA gene sequencing of isolates ACN5 (16S rDNA type 2), HBN4 (16S rDNA type 5) and ACN1 (16S rDNA type 3) are closely related to Tunisian legume nodulating bacteria belonging to *Rhizobium* sp. STM 394, *Rhizobium* sp. STM 4037 and *Sinorhizobium* sp. STM4038 which were isolated from *Astragalus cruciatus* and *Argyrolobium uniflorum* growing on the same Tunisian soils (Zakhia *et al*., 2004; Mahdhi *et al*., 2008). So, it would now be interesting to test the cross-nodulation capacity of our new isolates on *A. cruciatus* and *A. uniflorum* legumes.

It has been previously reported that *A. corrugatus* is nodulated by strains belonging to the genera *Rhizobium, Sinorhizobium, Brdyrhizobium, Mesorhizobium, Agrobacterium* and *Phyllobacterium* (Wei *et al*., 2003; Gao *et al*., 2004; Zakhia *et al*., 2004; Mantelin *et al*., 2006; Guerrouj *et al*., 2013; Zheng *et al*., 2013; Gnat *et al*., 2014). Our results thus confirm the previous studies showing the large diversity of *A. corrugatus* rhizobia that belong to *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Agrobacterium*. However, no *Phyllobacterium* and *Bradyrhizobium* were recovered in our results. Others studies (Guerrouj *et al*., 2013; Gnat *et al*., 2014) showed that *Astragalus glycyphyllos* in Poland and *A. glombiformis* in Morocco were nodulated by rhizobial bacteria belonging only to the genus *Mesorhizobium*. Among our collection only five *A. corrugatus* isolates (16S rDNA type 5) were phylogenetically grouped in *Mesorhizobium* branch, closely related to *M. temperatum* CCNWSX0012-2 and to *Mesorhizobium* sp. LAC831 nodulating *Lotus creticus* from arid regions of Tunisia (Rejili *et al*., 2009; 2012; 2013). This diversity of rhizobia nodulating *Astragalus* species may be in relation with climatic and edaphic conditions. Our study confirms that the *Astragalus* species nodulated by several rhizobial genomspecies can be qualified as promiscuous and that their rhizobia have very diverse genomic and symbiotic gene backgrounds (Zhao *et al*., 2008; Gnat *et al*., 2014)

Until now, nodulation of *Hippocrepis* species has been poorly documented (Zakhia *et al*., 2004; Muresu *et al*., 2008). In addition, only two strains were included in these studies. In our collection, *H. areolata* isolates are identified as *Rhizobium*, *Sinorhizobium* and *Agrobacterium* genera.

Several studies have reported the presence of *Agrobacterium* strains in nodules of some legumes (de Lajudie *et al*., 1999; Gao *et al*., 2001; Liu *et al*., 2005; Mhamdi *et al*., 2005; Mrabet *et al*., 2006), but all them failed to nodulate their original host plants. Here we isolated three *Agrobacterim* isolates (16S rDNA type 1) that also failed to nodulate their host plants *in vitro*. The sequences of 16S rDNA of one isolated *Agrobacterium* isolate (ACM1) is closely related to the 16S rRNA gene sequence of *Agrobacterium* sp. STM4035, which was isolated by Mahdhi *et al.* (2008) from the root nodules of the pastoral legume *A. uniflorum*.

In conclusion, our study is the first report on the characterisation of *A. corrugatus* and *H. areolata* in Tunisia. Our investigation showed that LNB originating from nodules of these legumes was genetically diverse and affiliated with *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Agrobacterium*. Most of *A. corrugatus* and *H. areolata* isolates were related to previously described LNB in Tunisian soils. However, rhizobia from other locations that were not covered in this study should be investigated to provide further information about the diversity of bacteria nodulating these legumes in Tunisia

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ORIGINAL PAPER

Characterization of a Highly Enriched Microbial Consortium Reductively Dechlorinating 2,3-Dichlorophenol and 2,4,6-Trichlorophenol and the Corresponding *cpr*A Genes from River Sediment

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Abstract

Anaerobic reductive dechlorination of 2,3-dichlorophenol (2,3DCP) and 2,4,6-trichlorophenol (2,4,6TCP) was investigated in microcosms from River Nile sediment. A stable sediment-free anaerobic microbial consortium reductively dechlorinating 2,3DCP and 2,4,6TCP was established. Defined sediment-free cultures showing stable dechlorination were restricted to *ortho* chlorine when enriched with hydrogen as the electron donor, acetate as the carbon source, and either 2,3-DCP or 2,4,6-TCP as electron acceptors. When acetate, formate, or pyruvate were used as electron donors, dechlorination activity was lost. Only lactate can replace dihydrogen as an electron donor. However, the dechlorination potential was decreased after successive transfers. To reveal chlororespiring species, the microbial community structure of chlorophenol-reductive dechlorinating enrichment cultures was analyzed by PCR-denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments. Eight dominant bacteria were detected in the dechlorinating microcosms including members of the genera *Citrobacter*, *Geobacter*, *Pseudomonas*, *Desulfitobacterium*, *Desulfovibrio* and *Clostridium.* Highly enriched dechlorinating cultures were dominated by four bacterial species belonging to the genera *Pseudomonas*, *Desulfitobacterium*, and *Clostridium*. *Desulfitobacterium* represented the major fraction in DGGE profiles indicating its importance in dechlorination activity, which was further confirmed by its absence resulting in complete loss of dechlorination. Reductive dechlorination was confirmed by the stoichiometric dechlorination of 2,3DCP and 2,4,6TCP to metabolites with less chloride groups and by the detection of chlorophenol RD *cpr*A gene fragments in dechlorinating cultures. PCR amplified *cpr*A gene fragments were cloned and sequenced and found to cluster with the *cpr*A/*pce*A type genes of *Dehalobacter restrictus*.

Key words: 16S rDNA, chlorophenol, *cpr*A gene, DGGE, reductive dechlorination

Introduction

Chlorinated phenols (CPs) are common chemical pollutants harmful to human health that are found in ground water due to unmanaged industrial discharges (WHO, 1998). Chlorinated phenols have been extensively used in the manufacture of pesticides, herbicides and dyes and are therefore commonly found in sediments, soil and water resources near industrial wastewater effluents (WHO, 1989; Takeuchi *et al*., 2000). Concern about these compounds relates directly to their high toxicity and persistence in a variety of habitats to levels exceeding natural limits. Chlorinated phenols tend to accumulate in anaerobic habitats, evading aerobic microorganisms that are able to degrade them (Villemur, 2013). The biodegradation of CPs in anaerobic environments has been extensively studied (Häggblom, 1992; McAllister *et al*., 1996; Masunaga *et al*., 1996). Recently, much attention has been focused on anaerobic bacteria that can dechlorinate chlorinated hydrocarbons in a unique process called reductive dechlorination (El Fantroussi *et al*., 1998; Holliger *et al*., 2003). Reductive dechlorination is driven by the activity of a class of enzymes called reductive dehalogenases (RDs). In this process, organohalide-respiring bacteria couple dechlorination to energy generation and growth using chlorinated hydrocarbons as electron acceptors (Holliger, *et al*., 1998; Smidt *et al*., 2000; Löffler *et al*., 2003; Smidt and de Vos, 2004). Dehalorespiring bacteria are found in soil, sediments, and wastewater sludge, and are able to dechlorinate different types of chloroorganics *via* a reductive dechlorination process (Häggblom and Bossert, 2003). However, many of these bacteria remain uncultured. Dehalorespiration was first reported by Deweed *et al*. (1990) in *Desulfomonile tiedjei*, an organism that couples reductive dechlorination

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of 3-chlorobenzoate to formate oxidation. A number of dehalorespiring bacteria have since been described, however, those reducing chlorinated phenols are restricted to a few strains belonging to a few genera. A facultative anaerobic *Myxobacterium*, *Anaeromyxobacter dehalogenans* 2CP-1, has been described as a halorespiring bacteria reducing 2CP and 2,6-dichlorophenol (2,6DCP) (Cole *et al*., 1994; Sanford *et al*., 2002). In this case, dechlorination was found mostly to be site specific. *Desulfovibrio dechloracetivorans* SF3 showed *ortho* dechlorination of 2CP and 2,6DCP (Sun *et al*., 2000). The genus *Desulfitobacteria* was found to contain a relatively large number of strains capable of dechlorination of both aliphatic and aromatic hydrocarbons (Villemur *et al*., 2006). The first *Desulfitobacterium* strain, DCB-2, was isolated in 1992 (Madsen and Licht, 1992). *Desulfitobacterium dehalogenans* strain JW/IU-DC1, first documented by Utkin *et al.* (1994), was reported for its dechlorination of 2,3DCP, 2,6DCP, and 2,4,6TCP (Wiegel *et al*., 1999). Subsequently, other strains belonging to the genus *Desulfitobacterium* with dechlorination activity toward CPs were isolated and characterized, these included *Desulfitobacterium chlororespirans* strain Co23 (Sanford *et al*., 1996; Cupples *et al*., 2005), *Desulfitobacterium hafniense* strain DCB-2 (Christiansen and Ahring, 1996), strain TCP-A (Breitenstein *et al*., 2001), and strain PCP-1 (Bouchard *et al*., 1996; Villemur, 2013). The obligate anaerobic bacterium *Dehalococcoides ethenogenes* strain 195 known for its ability to dechlorinate tetrachloroethene (Maymo-Gatell *et al*., 1997) was recently reported to possess diverse dehalogenation ability. Genomic studies revealed the presence of 17 putative dehalogenase gene homologues in strain 195 that reflect its ability to dechlorinate different chlorinated aromatic hydrocarbons including different chlorophenol congeners (Fennell *et al*., 2004). Dehalogenation in the environment, particularly dechlorination activities in sedimentary environments, is an area of research interest due to its important ecological prospective (Häggblom and Bossert, 2003; Leys *et al*., 2013). Dechlorination of mono- and di-chlorophenol isomers in estuarine anaerobic sedimentary environments has been studied and chlorophenol-dechlorinating microbial communities in such environments have been characterized (Itoh *et al*., 2010). The sludge of related wastewater industrial effluents is a good candidate for studying reductive dechlorination of aryl halides. The dechlorination of pentachlorophenols was detected in the sludge from a pulp and paper mill (Karn *et al*., 2011). Reductive dechlorination of CPs, biphenyls, and benzenes has been reported in a number of sedimentary environments (Wu *et al*., 1998; Fagervold *et al*., 2005; Zanaroli *et al*., 2010; Kjellerup *et al*., 2014; Kuokkaa *et al*., 2014; Vandermeeren *et al*., 2014).

This study aims to assess whether the potential for microbial reductive dechlorination of aryl halides, such as CPs, exists in River Nile sediments and to characterize the bacterial community structure in reductive dechlorinating enrichment cultures developed for the establishment of a highly enriched bacterial consortium for reductive dechlorination of CPs.

Experimental

Materials and Methods

Sampling and anaerobic microcosms. Sediment samples were collected in 100-ml leak-proof stopper-supported screw-capped Duran bottles from the River Nile at active industrialized areas located in Helwan, Egypt (29°51′N, 31°20′E). Sediment samples were spiked with 2,3DCP and 2,4,6-trichlorophenol (2,4,6TCP), flushed with hydrogen, and stored anaerobically in the dark for almost one year prior to use. Screening for dechlorinating bacteria was performed after the establishment of active microcosms and enrichment cultures. Microcosms were initiated by the addition of 10 g of sediment slurry into 90 ml of basal mineral medium in 160 ml serum bottles capped with Teflon-coated butyl rubber stoppers and sealed with aluminum caps. The head space was flushed with hydrogen gas. 2,3DCP and 2,4,6TCP were separately added to triplicate microcosms to a final concentration of 100 μM. Original unamended sediment slurry was used as a control. Microcosms were kept at room temperature for monitoring purposes at different time intervals.

Anaerobic enrichment cultures. Microcosms and anaerobic enrichment cultures were established using defined mineral salts medium containing (per liter): NH_4Cl, 0.5 g; K_2HPO_4, 0.4 g; $MgCl_2 \cdot 6H_2O$, 0.49 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; KCl, 0.052 g; metal solution, 1 ml; vitamin solution, 20 ml. The metal solution had the following composition (per liter): $ZnSO_4 \cdot 7H_2O$, 0.001 g; $CuSO_4 \cdot 5H_2O$, 0.002 g; $MnSO_4 \cdot 5H_2O$, 0.001 g; $NaMoO_4.2H_2O$, 0.0005 g; $CoCl_2 \cdot 6H_2O$, 0.0005 g; $NiCl_2 \cdot \cdot 6H_2O$, 0.001 g; $Na_2SeO_4 \cdot 10H_2O$, 0.001 g; $FeSO_4 \cdot 7H_2O$, 0.005 g. The vitamin solution had the following composition (per liter): biotin, 0.002 g; folic acid, 0.002 g; pyridoxine-HCl, 0.01 g; thiamine-HCl, 0.005 g; riboflavin, 0.005 g; nicotinic acid, 0.005 g; p-aminobenzoic acid, 0.005 g; vitamin B12, 0.001 g. Acetate (5 mM) was used as the carbon source. The pH of the medium was adjusted to 7.0 using HCl. From the stock medium solution, 9 ml were dispensed into 22 ml anaerobic vials and 2,3DCP or 2,4,6TCP were added as the main terminal electron acceptor after autoclaving to a final concentration of 50 μM. Vials were capped with Teflon-coated butyl rubber stoppers and sealed with aluminum

caps. The head space was flushed with hydrogen gas. To remove dissolved oxygen and ensure anaerobic conditions, the medium was reduced with Ti(III)-citrate, with resazurin added as an indicator. Active cultures were maintained by transferring 1 ml into 9 ml of freshly prepared medium. Enrichment sediment-free cultures were obtained after at least five successive transfers of active cultures (10%) into new medium containing CPs as electron acceptors. All cultures were incubated inverted at 30°C in the dark without shaking and were monitored for dechlorination activity by gas chromatographic analysis. Cultures showing dechlorination were diluted in the same medium and reincubated under the same conditions until sediment-free cultures were obtained.

Tested electron donors and acceptors. Different electron donors including, hydrogen, formate, lactate, pyruvate, and acetate at 5-mM concentrations were tested. Chlorinated phenols, formate, lactate, pyruvate, acetate, and thiosulfate were tested as electron acceptors. When being used as a main electron acceptor, CPs were added to cultures at a final concentration of 50 μM.

Chemicals. Chlorinated phenol standards were obtained from Sigma-Aldrich (Deisenhofen, Germany) and were used as authentic samples to characterize unknown compounds.

Chemical analyses. Chlorinated phenols were analyzed after extraction from culture medium with methylene chloride using a gas chromatograph (GC-396B, GL Science, Japan) equipped with an Aquatic column (60 m × 0.32 mm I.D., 1.0 μm df; GL Science) with an online Tekmar Dohrman headspace autosampler. A GC-4000 gas chromatograph (GL Science) equipped with a J&W Scientific column (30 m × 0.53 mm I.D., 1.0 μm df) was also used for direct sample injection and analysis. The following program was used to achieve maximum separation between peaks: column oven temperature, 40°C for 3 min, 40°C to 80°C at a rate of 8°C/min, 80°C to 190°C at 44°C/min, 190°C for 5 min. Helium was used as the carrier gas. Chlorophenols concentrations were represented as means of three measurements from three independent experiments (±SD).

DNA extraction and PCR amplification. Total community DNA was extracted using an Ultra Clean Soil DNA purification kit (Mo Bio Laboratories, Solana Beach, CA, USA). About 0.5 g from established microcosms or harvested cells from enrichment cultures were transferred to bead beating tubes and vortexed horizontally for 2 min at room temperature. DNA was precipitated and purified according to the instruction manual. Amplification of the 16S rRNA genes for DGGE analysis was performed using GC-clamp primers (EUB341F-GC: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAGCAG-3′ and EUB517R: 5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al*., 1993). Amplification was performed in a 25 μl reaction containing: 2.5 μl of 10 × *Taq* buffer (100 mM Tris-HCl, pH 8), 1.25 mM $MgCl_2$, 100 μM dNTPs (Invitrogen, Carlsbad, CA, USA), 1.2 μM forward primer and reverse primer (Invitrogen), 0.5 U *Taq* DNA polymerase (Invitrogen), and about 5 ng of template DNA. PCR was performed in a Thermal Cycler (2720; Applied Biosystems, Foster City, CA, USA). A touchdown PCR program was implemented as follows: initial denaturation step at 95°C for 5 min; 5 cycles of 94°C for 40 s, annealing at 65°C for 40 s, and extension at 72°C for 40 s; 5 cycles of 94°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 50°C for 40 s, and extension at 72°C for 40 s were performed, followed by a final hold at 72°C for 7 min. Amplicons were analyzed by electrophoresis on 1% agarose gels with size markers (1 kb DNA ladder, Invitrogen) and visualized using ethidium bromide. Amplification of the *cpr*A genes was performed with a combination of forward (dehaloF4: 5′-(C/T)(C/A)T(G/T)GGTTA(C/T)(A/T)ATGC-3′) and reverse (dehaloR2: 5′-T(C/T)TGTACCATAGCC-3′) primers, which were designed from the conserved region in the *cpr*A gene of *D. dehalogenans* (von Wintzingerode *et al*., 2001). The amplification protocol included an initial denaturation step at 95°C for 5 min, 30 cycles of 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 2 min, followed by a final elongation step at 72°C for 7 min. The amplified fragment was cloned into the pGEM-T Easy vector for sequencing.

DGGE. DGGE was performed using the Dcode Mutation Detection System (Bio-Rad Laboratories Ltd., Hertfordshire, UK). PCR products were electrophoresed on 8% polyacrylamide gels containing a 25% to 50% denaturing gradient of formamide and urea. Electrophoresis was performed in 0.5 × TAE buffer (1 × TAE buffer contains 0.04 M Tris base, 0.02 M sodium acetate, and 10 mM EDTA, pH 7.4) at 60°C for 5 h at 200 V. Gels were stained with SYBR Green I nucleic acid gel stain (Cambrex Bio Science, Rockland, MA, USA) and analyzed for DGGE band profiles with a UV Gel Doc XR documentation system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Selected DGGE bands were excised and eluted by incubation in 100 μl of TE buffer at 100°C for 5 min. The supernatant was used as a template for reamplification of the 16S rRNA genes using bacterial primers EUB314F, without the GC clamp, and EUB517R, prior to sequencing.

Cloning and sequencing. PCR products were ligated to the pGEM-T Easy vector (Promega, Madison, WI, USA) and used to transform *Escherichia coli* JM109 (Takara, Tokyo, Japan) according to the manufacturer's recommendations. Recombinant *E. coli* clones were

screened for correct inserts by colony PCR. Recombinant plasmid DNA of positive clones was extracted using the Wizard Plus SV Miniprep DNA purification system (Promega) and used for sequencing. BigDye terminator cycle sequencing (Sanger *et al*., 1977) was performed with an ABI 310 genetic analyzer (Applied Biosystems) using dye terminator-based PCR products amplified with the vector-specific forward primer M13F according to the instruction manual.

Sequence and phylogenetic analysis. Obtained sequences were processed for analysis using the *Genetyx-Win* MFC application software version 4.0. Sequences were identified and compared with their nearest phylogenetic neighbors using the BLAST search program, National Center for Biotechnology Information (NCBI), National Library of Medicine, USA (www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997). Sequence alignments were performed using *Clustal W*1.83 XP software (Thompson *et al*., 1997) and phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) and *MEGA6* software (Kumar *et al*., 2004).

Nucleotide sequence accession numbers. Sequences of the 16S rDNA gene identified in this study have been deposited in the GenBank database under the accession numbers (LC010626-LC010637). The *cpr*A gene sequences have been deposited under the accession numbers (LC010638-LC010645).

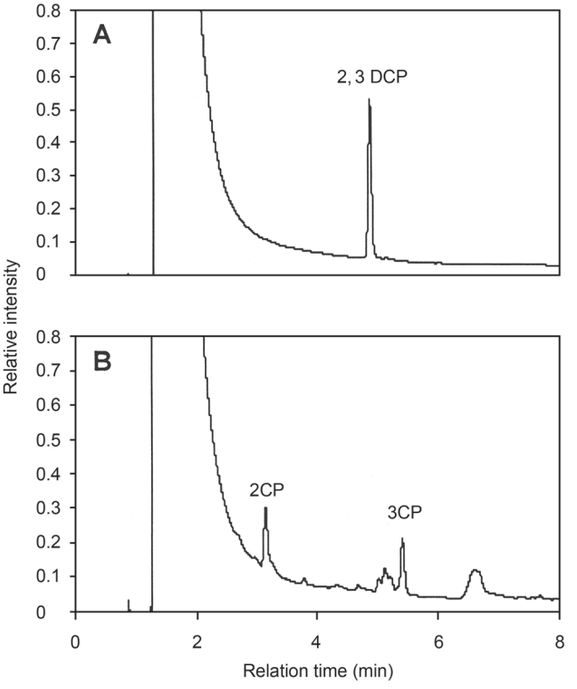

Fig. 1. Detection of dechlorination activity in established microcosms.

(A) GC chromatogram showing 2,3DCP as a starting compound spiked to microcosms. (B) GC chromatogram showing transformation of 2,3DCP to 2CP and 3CP after prolonged time of incubation as a result of dechlorination activity.

Results

Dechlorination in established anaerobic cultures. Microcosms and enrichment cultures maintained under optimum conditions of temperature and pH showed a dechlorination activity against CPs. Figure 1 shows the detection of 2CP and 3CP as dechlorination products from microcosms amended with 2,3DCP. In enrichment cultures, complete dechlorination of 50 μM 2,3DCP to 3CP and 2,4,6TCP to 2,4DCP, 4CP within 3 weeks of incubation was observed (Fig. 2). Dechlorination activity was favored with *ortho* chlorine, however, detection of trace amounts of 2CP in 2,3DCP-dechlorinating cultures revealed the weak dechlorination activity of *meta* chlorine. Dechlorination was found to be mainly site specific. 2,4,6TCP exhibited dechlorination of *ortho*-chlorine producing mainly 2,4DCP. Dechlorination beyond 2,4DCP yield 4CP mainly with trace amounts of 2CP. Figure 3 shows the proposed reductive dechlorination routes for both 2,3DCP and 2,4,6TCP in established cultures. Dechlorination and growth were found to be dependent on the presence of acetate as a carbon source in the culture medium. Hydrogen was found to be necessary as an electron donor for efficient dechlorination of 2,3DCP and 2,4,6TCP. Among different organic acids tested, only lactate can replace hydrogen as an electron donor. However, the rate of dechlorination was decreased and cultures lost dechlorination activity after successive transfers.

Bacterial community structure in dechlorinating cultures. The DGGE fingerprints obtained in this study with metagenomic DNA from microcosms amended with 2,3DCP and 2,4,6TCP showed changes in the bacterial community structure from the original sediment. Community fingerprints of microcosms supplemented with either 2,3DCP or 2,4,6TCP were stable and showed no significant changes in the DGGE profiles even after a prolonged incubation time. The DGGE profiles for 2,3DCP or 2,4,6TCP dechlorinating microcosms showed the predominance of eight major Operational Taxonomic Units (OTUs) (Fig. 4A). Sequencing of the dominant DGGE bands of 16S rRNA gene assigned these OTUs to members of the genera *Citrobacter*, *Geobacter*, *Pseudomonas*, *Desulfitobacterium*, *Desulfovibrio*, and *Clostridium* (Table I). Different DGGE profiles were observed when enrichment cultures were compared with dechlorinating microcosms. Enrichment resulted in loss of at least four of the microcosm bacterial members, represented by bands A1 (*Citrobacter* sp.), A2 (*Geobacter* sp.), A6 (*Desulfovibrio* sp.), and A7 (*Clostridium* sp.). Highly enriched dechlorinating

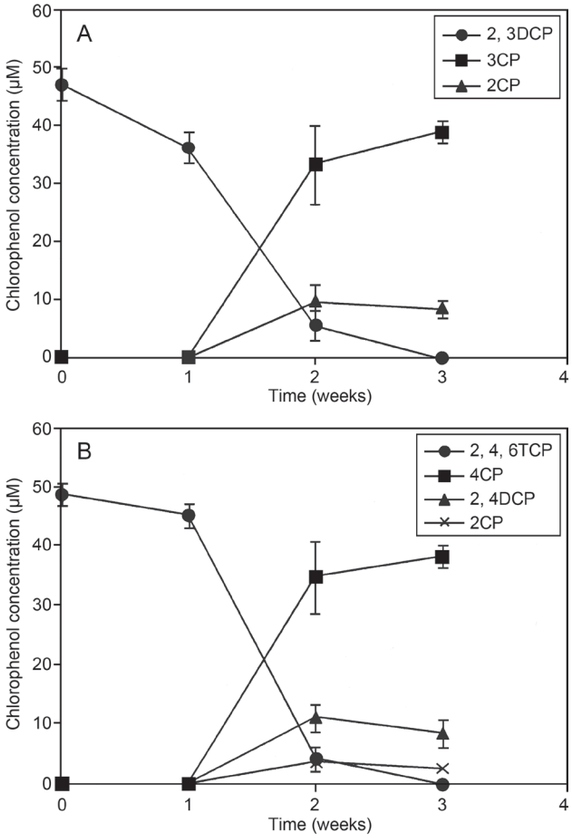

Fig. 2. Changes in chlorophenol concentration in enrichment cultures over time.

(A) Dechlorination of 2,3DCP to 3CP and 2CP. (B) Dechlorination of 2,4,6TCP to 2,4DCP with further dechlorination to 4CP and 2CP. Each bar represents the mean and standard deviation of three replicates samples.

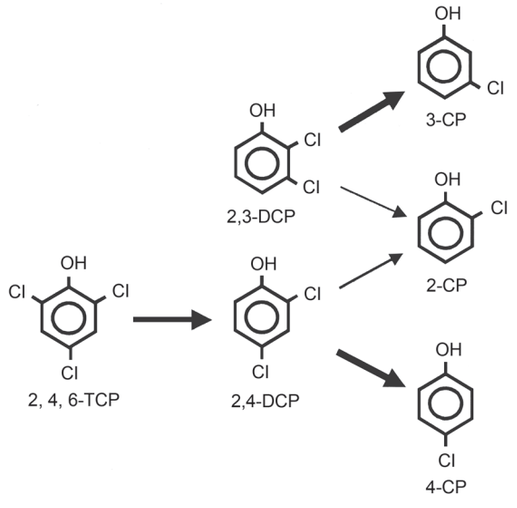

Fig. 3. Proposed pathway for 2,3DCP and 2,4,6TCP dechlorination in highly enriched cultures.

Dechlorination was favored at *ortho* position. Low dechlorination activity was observed at *meta* position of 2,3DCP, or *para* position of 2,4,6TCP. TCP was dechlorinated at *ortho* position successively giving 2,4-DCP and finally 4-CP. Small amount of 2-CP was detected.

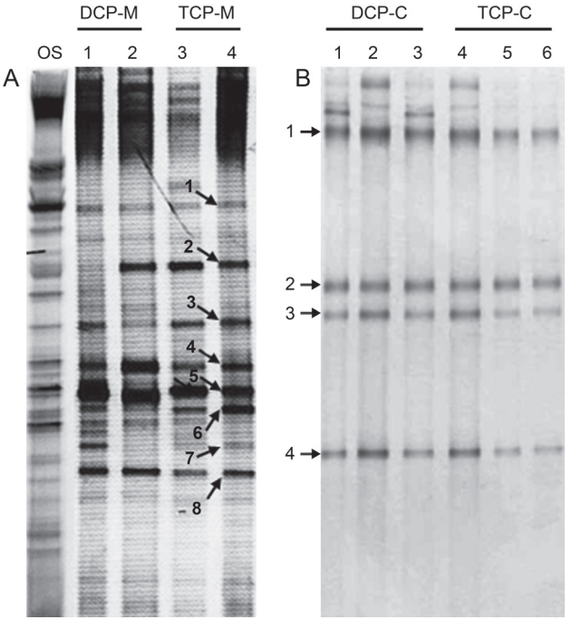

Fig. 4. DGGE fingerprints of 16S rRNA gene fragments from established dechlorinating microcosms (A) and enrichment cultures (B).

Lane OS represents bacterial fingerprint of original sediment. Lane 1,2 represent fingerprints obtained from genomic DNA of DCP dechlorinating microcosms while lane 3,4 represent those from TCP dechlorinating microcosms. Lane 1,2,3 corresponds to bacterial populations of highly enriched DCP dechlorination cultures, while lane 4,5,6 correspond to those of TCP dechlorination cultures.

cultures comprised four major OTUs (Fig. 4B), which were identified as members of the genera *Pseudomonas*, *Desulfitobacterium* and uncultured *Clostridium*.

Identification of dechlorinating bacteria. This study also investigated the microorganisms responsible for dechlorination in sediment microcosms or enrichment cultures. The DGGE microcosm OTU A4 is similar to OTU B2, which was detected in enriched dechlorinating cultures. Both OTUs have been assigned to the genus *Desulfitobacterium*, which is known for its ability to couple dechlorination to growth *via* halorespiration. The DGGE pattern of cultures lacking dechlorination activity revealed the absence of OTU B2, indicating its direct involvement in dechlorination. Direct microscopic examination of highly enriched dechlorinating cultures showed the predominance of curved rod-shaped bacteria, a characteristic morphology of *Desulfitobacterium*. Taken together, these results provide compelling evidence that both OTUs A4 and B2 identified as *Desulfitobacterium* sp. were responsible for dechlorination of 2,3DCP and 2,4,6TCP, lowing the concentration of CPs.

Detection of the chlorophenol RD gene (*cprA*). To detect the chlorophenol RD encoding gene (*cprA* gene) from microcosms and dechlorinating cultures, PCR amplification using the primer set dehaloF4/

Table I
OTUs identified in established microcosms and enrichment cultures with their phylogenetic affiliations.

DGGE bands	Accession No.	Closest match			Phylogenetic affiliation
		Identity	Accession No.	Similarity (%)	
A1	LC010626	*Citrobacter freundii* MW-D 2220	KC835086	98	Gammaproteobacteria
		Citrobacter sp. F3-7	EF491835	98	Gammaproteobacteria
		Enterobacter sp. 18A	HQ289882	98	Gammaproteobacteria
		Klebsiella sp. SUS9K	KF991505	98	Gammaproteobacteria
		Uncultured Enterobacteriaceae bacterium C146500177	JX531197	98	Gammaproteobacteria/Environmental sample
A2	LC010627	*Geobacter lovleyi* Geo7.1A	JN982204	99	Deltaproteobacteria
		Geobacter thiogenes K1	NR028775	99	Deltaproteobacteria
		Uncultured *Geobacter* sp. clone MFC-1-L16	JX944529	99	Deltaproteobacteria/Environmental sample
		Uncultured delta proteobacterium clone CF_05	EF562566	99	Deltaproteobacteria/Environmental sample
		Geobacter sp. enrichment culture clone MC18B6-5	JQ256498	99	Deltaproteobacteria/Environmental sample
A3	LC010628	*Pseudomonas fulva* Z47	KF835747	100	Gammaproteobacteria
		Pseudomonas putida P1	KJ960183	99	Gammaproteobacteria
		Pseudomonas aeruginosa P17	KJ960182	99	Gammaproteobacteria
		Pseudomonas stutzeri 54DMH1	KM025368	99	Gammaproteobacteria
		Pseudomonas sp. IMARCUG-3	KJ918745	99	Gammaproteobacteria
A4	LC010629	*Desulfitobacterium* sp. CR1	AB299028	100	Clostridia
		Desulfitobacterium sp. RPf35Ei	AY548779	97	Clostridia
		Desulfitobacterium dichloroeliminans LMG P-21439	NR102483	96	Clostridia
		Desulfitobacterium hafniense DCB-2	NR121797	95	Clostridia
		Desulfitobacterium dehalogenans JW/IU-DC1	NR044641	95	Clostridia
A5	LC010630	*Pseudomonas putida* strain P1	KJ960183	99	Gammaproteobacteria
		Pseudomonas aeruginosa P17	KJ960182	99	Gammaproteobacteria
		Pseudomonas stutzeri 54DMH1	KM025368	99	Gammaproteobacteria
		Pseudomonas sp. IMARCUG-3	KJ918745	99	Gammaproteobacteria
		Pseudomonas sp. JS-C55	KJ921736	99	Gammaproteobacteria
A6	LC010631	*Desulfovibrio vulgaris* Miyazaki F	CP001197	100	Deltaproteobacteria
		Desulfovibrio sp. wpp1	KF601938	100	Deltaproteobacteria
		Uncultured *Desulfovibrio* sp. clone immo-6	AB936633	100	Deltaproteobacteria/ Environmental sample
		Desulfovibrio sp. enrichment culture clone 2	JF714419	100	Deltaproteobacteria/ Environmental sample
		Uncultured delta proteobacterium clone SRB4	DQ069228	100	Deltaproteobacteria/ Environmental sample
A7	LC010632	Uncultured *Clostridium* sp. clone BacBotHigh001	JF731763	100	Clostridia/Environmental sample
		Clostridium sp. M-43	AB504378	99	Clostridia
		Uncultured bacterium clone PSM25	AB639316	99	Environmental sample
		Clostridium sp. S11-3-10	AB838978	98	Clostridia
		Uncultured *Clostridium* sp. clone XT41	KF511913	97	Clostridia/Environmental sample
A8	LC010633	Uncultured *Clostridium* sp. DGGE-GZ-C7	KC529382	100	Clostridia/Environmental sample
		Uncultured *Clostridium* sp. clone A05	JX545163	100	Clostridia/Environmental sample
		Clostridium sp. enrichment culture DGGE-gz-5d-15	JX470312	100	Clostridia/Environmental sample

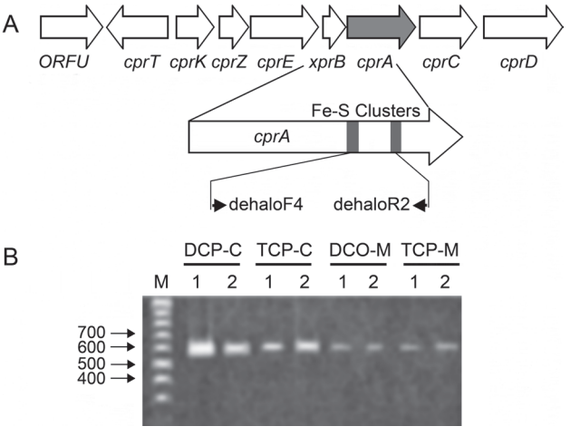

Fig. 5. Detection of chlorophenol reductive dehalogenase encoding gene, *cpr*A in Nile river sediment microcosms and established enrichment cultures.

(A) Schematic representation of the *cpr* gene locus in *D. dehalogenans* (Smidt *et al.*, 2000) showing target *cpr*A gene and the sequenced region in this study covering up the Fe-S cluster. (B) PCR amplification of Fe-S cluster region of *cpr*A clones from DCP and TCP cultures as well as dechlorinating microcosms. Lane M represents the size marker (1 Kb DNA ladder, Invitrogen, USA).

dehaloR2 was performed with an expected product size of approximately 600 bp (Fig. 5B). Eight clones were recovered from DCP and TCP dechlorinating cultures. Sequence analysis of the obtained clones from DCP and TCP dechlorinating cultures showed sequence similarity of 99% to the *cpr*A gene from uncultured bacterium DCB and 96–98% to the *cpr*A gene from *Dehalobacter restrictus*.

Phylogenetic analysis of the *cpr*A genes. When aligned to available sequences in the public databases,

Table I continued

DGGE bands	Accession No.	Closest match			Phylogenetic affiliation
		Identity	Accession No.	Similarity (%)	
A8	LC010633	*Clostridium* sp. enrichment culture clone N6	JQ420069	100	Clostridia/Environmental sample
		Uncultured bacterium clone LBAC143	KJ601180	100	Environmental sample
B1	LC010634	*Pseudomonas stutzeri* YC-YH1	KJ786450	99	Gammaproteobacteria
		Pseudomonas pseudoalcaligenes	LK391695	99	Gammaproteobacteria
		Pseudomonas sp. NEAU-ST5-5	JQ762269	99	Gammaproteobacteria
		Uncultured *Pseudomonas* sp. clone 1-22-20	KJ650703	99	Gammaproteobacteria/Environmental sample
		Uncultured Pseudomonadaceae bacterium c:S2-8094	KF786991	99	Gammaproteobacteria/Environmental sample
B2	LC010635	*Desulfitobacterium* sp. CR1	AB299028	100	Clostridia
		Desulfitobacterium sp. RPf35Ei	AY548779	97	Clostridia
		Desulfitobacterium dichloroeliminans LMG P-21439	NR102483	96	Clostridia
		Desulfitobacterium hafniense DCB-2	NR121797	95	Clostridia
		Desulfitobacterium dehalogenans JW/IU-DC1	NR044641	95	Clostridia
B3	LC010636	*Pseudomonas putida* strain P1	KJ960183	99	Gammaproteobacteria
		Pseudomonas aeruginosa P17	KJ960182	99	Gammaproteobacteria
		Pseudomonas stutzeri 54DMH1	KM025368	99	Gammaproteobacteria
		Pseudomonas sp. IMARCUG-3	KJ918745	99	Gammaproteobacteria
		Pseudomonas sp. JS-C55	KJ921736	99	Gammaproteobacteria
B4	LC010637	Uncultured *Clostridium* sp. DGGE-GZ-C7	KC529382	100	Clostridia/Environmental sample
		Uncultured *Clostridium* sp. clone A05	JX545163	100	Clostridia/Environmental sample
		Clostridium sp. enrichment culture DGGE-gz-5d-15	JX470312	100	Clostridia/Environmental sample
		Clostridium sp. enrichment culture clone N6	JQ420069	100	Clostridia/Environmental sample
		Uncultured bacterium clone LBAC143	KJ601180	100	Environmental sample

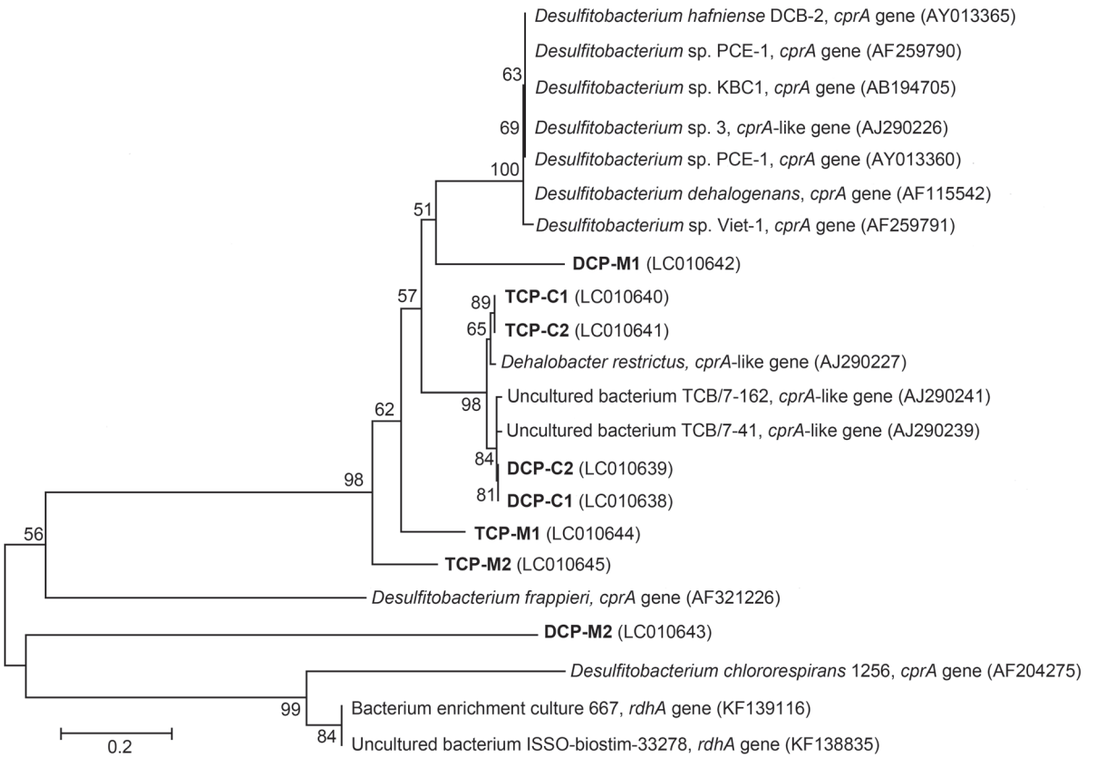

Fig. 6. Neighbour-joining tree showing the phylogenetic distribution of *cpr*A gene fragments from microcosms and enrichment cultures. The dendrogram was constructed from a *ClustalW* alignment and *Mega 3* analysis. Nodes supported by bootstrap values greater than 50 are indicated. Accession numbers of clones (boldface) and reference sequences from GenBank are indicated in parentheses. The scale bar represents substitutions per nucleotide.

the *cpr*A genes cloned from microcosms was closely related to the *cpr*A gene from uncultured bacterium TCB (97%), *D. restrictus* (96%), *Desulfitobacterium frappieri* (82%), *D. hafniense* (82%), and *D. chlororespirans* (84%). The *cpr*A genes cloned from enriched cultures were closely related to those from uncultured bacterium DCP (99%), *D. restrictus* (98%), *D. chlororespirans* (85%), *D. hafniense* (82%), and *D. frappieri* (86%). Figure 6 shows the phylogenetic relationships between the detected *cpr*A clones and those from reported dechlorinating bacteria.

Discussion

Organohalide respiration is a unique metabolic process that is implicated in the global halogen cycle and is of environmental significance for remediation purposes. As part of anaerobic dehalogenation processes, reductive dechlorination has triggered considerable interest for the potential detoxification of anthropogenic groundwater contaminants (Hug and Edwards, 2013). Anaerobic reductive dechlorination has been studied in a number of sedimentary environments (Lake *et al.*, 1992; Alder *et al.*, 1993; Wu *et al.*, 1998; Fagervold *et al.*, 2005; Fung *et al.*, 2009; Itoh *et al.*, 2010). These investigations were made possible using laboratory microcosms. Anaerobic reductive dechlorination of chlorinated benzenes for example was investigated in microcosms containing chloroaromatic-contaminated river sediment (Vandermeeren *et al.*, 2014). Dechlorination activity in enrichment cultures showed at least three different dechlorination reactions. A distinct *ortho*-dechlorinating path for 2,3DCP, 2,4DCP, and 2,4,6TCP was predominant. However, *meta*- and *para*-dechlorination paths also existed with slow rates and restriction to 2,3DCP and 2,4DCP, respectively. Although some strains were reported to exhibit chlorophenols dechlorination of all three substituent (*ortho*, *meta*, and *para*) positions (Thibodeau *et al.*, 2004; Gauthier *et al.*, 2006), this pattern of dechlorination was restricted to the *ortho* position, consistent with dechlorination reported for the anaerobic bacterium, strain PCE1 (Gerritse *et al.*, 1996). In enrichment cultures, dechlorination beyond monochlorophenols was not detected. This pattern of dechlorination is most similar to that reported for *D. chlororespirans*, which was able to dechlorinate *ortho* chlorines of polysubstituted phenols to monohalophenol end products (Sanford *et al.*, 1996). However, the chlorophenol concentration may

represent a limiting factor for dechlorination activity as a higher concentration of CPs may result in reduction of dechlorination activity due to the cytotoxicity of these compounds. It has been reported that bacteria may develop some traits to overcome the cytotoxicity of higher CP concentrations. Surfactant production was found to have a role in overcoming the toxicity of chlorophenols (Chrzanowski *et al*., 2009). Rhamnolipids, in particular, were found to reduce the toxicity of CPs due to their accumulation in biosurfactant micelles and hydrophobic interactions with rhamnolipid-based dissolved organic carbon (Chrzanowski *et al*., 2011).

Dechlorination activity could only be serially transferred in enrichments containing hydrogen as an added electron donor. Dechlorinating cultures have a strict requirement for hydrogen. A variety of alternative electron donors have been reported to support reductive dechlorination (Gerritse *et al*., 1999), however, in this study, electron donors other than hydrogen failed to support dechlorination. Lactate has been found to be used by a number of dehalorespiring bacteria as an electron donor (Li *et al*., 2013). *D. chlororespirans* strain Co23 was able to grow by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate and 2,3-DCP (Sanford *et al*., 1996). However, it was found in this study that cultures growing with lactate were not stable and lost dechlorination activity after successive transfers. Different organic acids are used as the electron donors by some bacterial species; however, hydrogen is thought to be the most important electron donor for dehalorespiration, because of the high affinity of dehalorespiring bacteria for hydrogen (Luijten *et al*., 2004). Competition for hydrogen is known to exist among hydrogen-utilizing microorganisms in anoxic environments. In such habitats, where the hydrogen concentration is limited, the threshold ecosystem hydrogen concentration is controlled by microbial populations that couple hydrogen oxidation to thermodynamically favorable electron accepting reactions, including reductive dehalogenation (Mazur and Jones, 2001; Mazur *et al*., 2003). The establishment of sediment-free highly enriched reductive dechlorinating cultures would require hydrogen amendment to replace that provided by co-cultured bacteria. The presence of acetate was found to be important for sustaining stable dechlorinating cultures. It is suggested that acetate may play an important role as a carbon source to support the growth of dechlorinating bacteria and other associated bacteria. This differs from the reported role of acetate as an electron donor for growth coupled to reductive dechlorination (Sun *et al*., 2000).

The diversity of organohalide-respiring bacteria has not yet been fully described. Ongoing research in this field may lead to the discovery of novel strains, species and even new genera that could be implemented in bioremediation processes (Hug *et al*., 2013). The metagenomes of microbial communities are currently under investigation to reveal the metabolic interactions within organohalide-respiring consortia. Some consortia have been developed and characterized for organohalide-respiring populations and associated bacteria (Duhamel and Edwards, 2007; Rowe *et al*., 2008; Brisson *et al*., 2012; Hug, 2012; Maphosa *et al*., 2012). PCR-DGGE analysis using 16S rRNA genes usually yields patterns that reflect the composition of dominant microorganisms, including the nonculturable members (Gelsomino *et al*., 1999). The DGGE patterns obtained in this study with total community DNA showed changes in the dominant bacterial populations of native river sediment compared with CP amended microcosms. However, the bacterial community structure of 2,3DCP and 2,4,6TCP dechlorinating microcosms were similar and have remained largely intact over time. The DGGE profile for 2,3DCP or 2,4,6TCP dechlorinating microcosms revealed the predominance of eight major OTUs identified as members of the genera *Citrobacter*, *Geobacter*, *Pseudomonas*, *Desulfitobacterium*, *Desulfovibrio*, and *Clostridium*. Based on these detected genera, dechlorination has been assigned to *Desulfitobacteria* due to its reported ability to dechlorinate a variety of aryl halides and couple dechlorination to growth for its respiration (Smidt *et al*., 2000; Löffler *et al*., 2003; Holliger *et al*., 1998). The presence of *Desulfovibrio* in dechlorinating microcosms suggests its possible indirect role in dechlorination *via* syntrophic association with existing dehalorespiring bacteria (Drzyzga *et al*., 2001). *Geobacter*, as well as being detected in established dechlorinating microcosms, has also been suggested to play a role in organohalide respiration (Wagner *et al*., 2012). Cultures devoid of the distinct DGGE band assigned to Desulfitobacterium, lack any dechlorination activity, providing clear evidence that *Desulfitobacterium* sp. play a key role in the dechlorination of 2,3DCP and 2,4,6TCP. *Desulfitobacterium* spp. have been cited in many studies as dechlorinating bacteria (Bouchard *et al*., 1996; Breitenstein *et al*., 2001; Cupples *et al*., 2005; Villemur *et al*., 2006). Highly enriched cultures showed the prevalence of curved rods, a characteristic feature of *Desulfitobacterium* spp. (Villemur *et al*., 2006). Although some physiological characteristics of *Desulfitobacterium* spp. were reported, they did not match the dechlorination requirements in established dechlorinating cultures, suggesting a novel type of *Desulfitobacterium* with alternative physiological requirements. The requirements for dechlorination in this study were found to be similar to those reported for Dehalobacter sp. strain TCP1 when dechlorinating 2,4,6-TCP to 4-CP with hydrogen as the sole electron donor and acetate as the carbon source (Wang *et al*., 2014). Further attempts to isolate dechlorinating

bacteria were unsuccessful indicating the possible existence of syntrophic interactions, as co-culture consortia, within dechlorinating communities.

Reductive dehalogenase (RD) encoding genes (*rdh*A) have been identified in a wide variety of strictly anaerobic aryl halide respiring bacteria (Löffler *et al*., 1996; Christiansen *et al*., 1998; van de Pas *et al*., 1999; Thomas *et al*., 2008). Hug and Edwards (2013) reported that the RD complement within an organism, enrichment culture, or contaminated site, is of critical importance for determining the potential dechlorination activity. In this study, *cpr*A RD genes were detected in dechlorinating microcosms and enrichment cultures (Fig. 5A, B), showing similarity to the *cpr*TKZEBACDA operon of *D. dehalogenans* (Smidt *et al*., 2000). The *cpr*A gene was not detected in cultures lacking dechlorination activities suggesting its key role in catalyzing orthochlorine removal from either 2,3 DCP or 2,4,6-TCP. Analysis of *cpr*A gene clones retrieved from both DCP and TCP dechlorinating cultures revealed eight distinct sequences, some with high sequence similarity to *D. restrictus* and some with relatively low sequence similarly to *Desulfitobacterium* spp. DGGE profiling revealed only one OTU affiliated to *Desulfitobacterium* sp., suggesting the presence of different copies of the *cpr*A gene. *D. hafniense* strain PCP-1 was reported to possess two RDs encoded by *crd*A and *cpr*A5 (Villemur *et al*., 2002). These two RD genes have also been found in several other *Desulfitobacterium* strains (Gauthier *et al*., 2006). The fact that several RD encoding genes exist in *Desulfitobacterium* spp. suggests the presence of distinct but related enzymes to achieve the dehalogenation of several chlorinated compounds.

Conclusion

The current study demonstrated the potential for reductive dechlorination of CPs in the sediment from a contaminated site of the River Nile by naturally occurring bacteria. Highly enriched, stable dechlorinating cultures were established using acetate as the carbon source, hydrogen as the electron donor, and CPs as electron acceptors. Metagenomic analysis of a highly enriched chlorophenol-reductive dechlorinating consortium revealed the predominance of *Pseudomonas*, *Desulfitobacterium*, and two uncultured *Clostridia*. Dechlorination was mainly assigned to *Desulfitobacterium*, as its absence resulted in complete loss of dechlorination. Chlorophenol RD *cpr*A gene fragments were retrieved from dechlorinating cultures. The *cpr*A gene was only detected by amplification in active dechlorinating cultures confirming dehalorespiration of chlorophenols. Several copies of the *cpr*A genes were detected with high similarity to the RD of *D. restrictus*. Phylogenetic analysis based on *cpr*A sequences indicated that dechlorinating cultures had a wide distribution of *cpr*A types. Collectively, these results provide valuable insights into potential bioremediation applications especially in anaerobic environments.

Acknowledgments

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SHORT COMMUNICATION

The Effect of Local Platelet Rich Plasma Therapy on the Composition of Bacterial Flora in Chronic Venous Leg Ulcer

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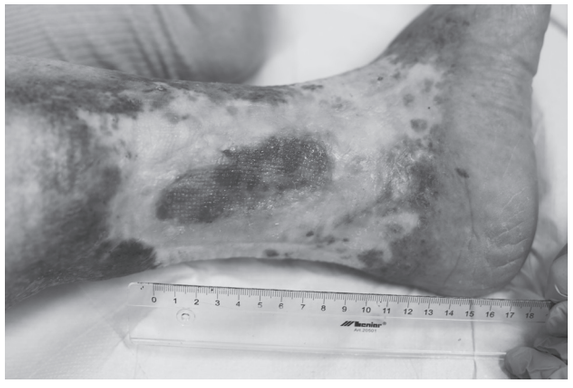
Abstract

Microbial colonisation of chronic venous ulcers and synergism between bacterial species slow down the healing process. The study aimed at performing qualitative analysis of microbial flora in venous leg ulcers treated with platelet rich plasma (PRP). Twenty two women and twelve men aged 47–90 years were treated with PRP at our department between 2012 and 2015. Ulcer cultures collected before and after PRP therapy yielded 83 and 110 microbial isolates, respectively, of Gram positive, Gram negative bacteria and candida. *Pseudomonas aueruginosa* and *Staphylococcus aureus* were the most common pre- and post-treatment isolates. PRP therapy and increased the variety of microbial flora.

Key words: antibacterial activity, microflora of venous leg ulcer, platelet-rich plasma (PRP), venous leg ulcer

Chronic venous leg ulcers (Fig. 1) are a significant diagnostic and therapeutic challenge. In most patients, venous ulcer develops secondarily to chronic venous insufficiency. The recurrent infection affects up to 72% of cases, and after skin graft the annual relapse rate can be as high as 48% (Morimoto *et al*., 2014). Chronic nature of venous ulcer, tissue damage, wound exudate and the decreased partial oxygen pressure promote colonisation and uncontrolled microbial growth. Whereas the colonisation itself does not significantly alter healing process, except for the cases of host immune response, the increase in bacterial count and synergistic interaction between the individual species may hinder ulcer healing, leading to further tissue damage (Bowler, 2002).



Antimicrobial treatment is difficult and often insufficient as antibiotics have poor penetration to the infection site (Alinovi *et al*., 1986). Therefore the treatment includes improvement of blood flow to the affected site and other types of experimental therapies (Howard *et al*., 2008; Rosales *et al*., 2010). One of the therapeutic alternatives in refractory venous leg ulcers is platelet rich plasma (PRP) successfully used in orthopaedics, rheumatology, maxillofacial surgery or aesthetic medicine. PRP is a rich source of autologous growth factors, which participate in coagulation, immune response, angiogenesis and wound healing (Lubkowska *et al*., 2012). There are reports of improved ulcer healing after PRP therapy in comparison with the controls, as well as converse observations (Saad Setta *et al*., 2011; Martinez-Zapata *et al*., 2012). During our initial clinical attempts to treat venous leg ulcers with PRP, the more severe sings of microbial growth (increased inflammatory exudate, more intense smell of decomposition) were observed, hence the decision to address this issue.

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Therefore, we conducted a qualitative analysis of bacterial flora in venous leg ulcers during local treatment with platelet rich plasma.

Between 2012 and 2015, 34 patients with venous leg ulcers were treated at the First Department of General and Vascular Surgery, Second Faculty of Medicine with the English Division and the Physiotherapy Division, Medical University of Warsaw. The inclusion criteria for the treatment involved completed causal treatment, *i.e.* the absence of superficial and perforator vein reflux as well as the restored iliac vein patency. The exclusion criteria included leg ulcers secondary to atherosclerosis-related ischaemia, diabetes, rheumatoid arthritis, lymphatic obstruction or phlebitis. The essential eligibility criterion for local treatment with platelet rich plasma was the lack of progress of spontaneous ulcer healing despite completed causal treatment. The allowed time window was 6 months, *i.e.* if the ulcer has not resolved or has not presented signs of progressing reepithelialisation within this period, it was eligible for PRP therapy. The culture specimens from the ulcer surface were collected 7–14 days prior to treatment. The specimens were collected using collection and transport system swabs and tubes with Amies BO91 medium (ZTS Hagmed). The collected biological material was cultured on the growth media for aerobic bacteria, anaerobic bacteria and fungi using the streak plate method on Columbia Agar with 5% Sheep Blood, Haemophilus agar, MacConkey agar, Chapman agar, Scheadler agar, Sabouraud Dextrose agar, as well as MRSA and Strep B chromogenic media. Antibiotic susceptibility of each bacterial and fungal isolate was determined using either automated method (Vitek 2 Compact, BioMerieux) or Kirby Bauer disk diffusion method. The MIC gradient strips (E-test, The bacterial colony count was not determined, but only roughly estimated based on the number of colonies grown after plating and noted for diagnostic purposes as: 1 – scanty, 2 – few, 3 – moderate, 4 – numerous.

Platelet rich plasma was prepared from 18–27 ml of blood drawn with syringe from the superficial veins on the forearm. The blood was then transferred to sterile 9 ml test tubes containing sodium citrate (Vacuette 9NC, Greiner Bio-One GmbH, Austria). After mixing, the tubes were left on the tube rack until the RBCs separated from the plasma. The process typically lasted for 3–4 hours. Afterwards, using the new syringe and venflon cannula (Vasofix Certo 16G 2" B. Braun Melsungen, Germany) the supernatant (plasma) was collected. The collected plasma was transferred to sterile 9 ml test tubes (Vacuette Z Greiner Bio-One GmbH, Austria). The test tubes containing plasma were centrifuged for 15 minutes at 3000 rpm (Low Speed Centrifuge 80-2C, Pioway Medical Lab Equipment Co., Ltd., China). After centrifuging, using the new syringe and venflon cannula (Vasofix Certo 16G 2" B. Braun Melsungen, Germany) the upper half content of the test tube was collected (4.5 ml). The remaining part of plasma was mixed (Vortex-Genie 2 Scientific Industries INC, USA) for 30 seconds in order to obtain homogeneous platelet solution. 0.5 ml of the obtained solution was collected for platelet count (Cell Dyn 1700 Abbott Diagnostics, USA). The assay was compared to the automated full blood count results. Platelet concentration ratio ranged from 2.2 to 4.1. Next, 0.1 ml of calcium chloride (10% Calcium chloratum, WZF) was added to each test tube of prepared platelet rich plasma solution. The platelet preparation was administered to the ulcer margins as an intradermal injection, using the 1 ml syringes with 30 G needle (0.3 × –4 mm). Additionally, mesotherapy was performed on ulcer surface and after that, the ulcer was covered with platelet rich plasma. After plasma agglutination on ulcer surface, the entire area was covered with a proper size sterile hydrogel wound dressing Aqua-Gel (Kikgel). Wound dressing was changed every 3 days so as to avoid removing the plasma from ulcer surface. Control cultures were collected 3 weeks after the PRP treatment. During that period no antibiotics – local or systemic – were administered. Treatment efficacy was assessed visually based on the ulcer size decrease and the re-epithelialisation.

The study group (34 subjects) consisted of 22 women (64.7%) and 12 men (35.3%) whose age ranged from 47 to 90 years. The mean age was 70.5 years (SD 9.8). The surface area of the ulcer ranged from 3 to 92 cm^2 with the mean surface area of 31.94 cm^2. Single ulcer was present in 25 (73.5%) patients. Double ulcer was shown in 4 (11.8%) patients, whereas 5 subjects (14.7%) presented with multiple ulcers (3 or more). Symptom duration ranged between 11 and 192 months, with the mean duration of 55.8 months.

After PRP therapy, a significant healing improvement was shown in 21 subjects (61.8%), as assessed by decrease in wound size, whereas in 13 remaining cases (38.2%) there was no improvement.

Microbiological analysis of bacterial flora a in the wounds prior the treatment yielded 83 microbial isolates. The most commonly isolated bacterial species included (in the order of frequency): *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacteroides fragilis*. Table I shows quantitative distribution of isolated bacterial and fungal species among patients. The majority of cultures from single swab from the patient (73.5%) showed the presence of multiple species.

Gram-positive bacteria were isolated from over 30% of patients, Gram-negative bacteria were isolated from 59% of patients, with anaerobic bacteria and fungi making up 9.6% and 1.2%, respectively. The most commonly isolated Gram-positive bacteria were *S. aureus*, *E. faeca-*

Table I
Quantitative distribution of microbial species before and after PRP therapy.

Type	Species	No. of isolates	
		before PRP therapy	after PRP therapy
Gram-positive cocci	*Methicillin-susceptible Staphylococcus aureus (MSSA)*	10	16
	Enterococcus faecalis	6	7
	Methicillin-resistant Staphylococcus aureus (MRSA)	3	3
	Streptococcus group C	2	2
	Streptococcus agalactiae (group B)	2	3
	Streptococcus pyogenes (group A)	1	0
	Streptococcus group G	1	1
	Staphylococcus hominis	0	1
	Staphylococcus lugdunensis	0	1
Gram-negative bacilli	*Serratia marcescens*	4	1
	Morganella morganii	4	7
	Escherichia coli	3	10
	Proteus mirabilis	3	6
	Klebsiella oxytoca	3	4
	Enterobacter cloacae	3	5
	Pantoea agglomerans	2	1
	Proteus vulgaris	1	1
	Klebsiella pneumoniae	1	1
	Aeromonas hydrophila	0	2
	Hafnia alvei	0	1
Gram-negative non-fermenting bacilli	*Pseudomonas aeruginosa*	18	24
	Acinetobacter baumannii	2	2
	Stenotrophomonas maltophila	2	2
	Pseudomonas putida	1	0
	Acinetobacter haemolyticus	1	0
	Alcaligenes faecalis	1	0
Anaerobic bacteria	*Bacteroides fragilis*	6	6
	Bacteroides ovatus	0	1
	Prevotella oralis	2	0
	Prevotella denticola	0	1
Fungi	*Candida parapsilosis*	1	0
	Candida albicans	0	1

lis, and β-hemolytic streptococci (Table I). Analysis of antibiotic resistance revealed that among studied isolates, MRSA strains (Table I) and enterococci showing HLAR to gentamicin (3 isolates) were present. No vancomycin resistant enterococci (VRE) were isolated.

The most commonly isolated Gram-negative bacteria were non-fermenting bacilli, predominantly *P. aeruginosa*, *Acinetobacter baumanii* and *Stenotrophomonas maltophilia*. *Enterobacteriaceae* constituted large group of Gram negative bacteria isolated from ulcers. The most commonly isolated species were *Serratia marcescens*, *Morganella morganii*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella oxytoca* and *Enterobacter cloacae*. No ESBL(+) *Enterobacteriaceae* strains were isolated. Two isolates of *P. aeruginosa* strains showed resistance to impenem and meropenem. *B. fragilis* was the most commonly isolated anaerobic bacterial species. *Candida parapsilosis* was isolated in one case.

After PRP therapy, a total of 110 bacterial isolates were obtained from samples collected after a single, local application of platelet rich plasma. Multiple isolates were cultured for 32 of 34 subjects.

The bacterial species isolated after the treatment were very similar to those isolated previously, however the frequency of MSSA, *P. aeruginosa* or *E. coli* isolation increased (Table I). There were no significant changes

in antibiotic resistance profiles of isolated bacteria. Similarly to the initial cultures, no vancomycin resistant enterococci were isolated.

In this group of isolates, 5,1% strains were classified as ESBL positive and 8,3% of *P. aeruginosa* strains were resistant to impenem and meropenem. As previously, *B. fragilis* was the most commonly isolated anaerobic species. *Candida albicans* was isolated in a single case.

A comparison of the number of isolates in ulcer culture samples before and after PRP therapy showed an increase in the number of isolates containing multiple bacterial species. The maximum number of species in a single ulcer was five prior to PRP, it increased up to seven afterwards. The mean number of bacterial species isolated per subject before and after PRP was 2.44 +/– 0.22 and 3.24 +/– 0.29, respectively. Paired T-test comparing group of patients prior and after treatment shown that the increase in mean number of species is statistically significant (P = 0.035).

A comparison of the most commonly isolated bacteria in culture samples before and after PRP therapy showed a marked increase in the isolation ratio of MSSA and *E. coli*, with changes in the ratio of other strains being less pronounced (Table I). Although quantitative analysis was not performed, visual estimation of the colony density on plates with plated bacteria after PRP therapy shows that the use of platelet rich plasma decreased the amount of bacteria in about half of the patients. However, based only on semi-quantitative inspection, we cannot speculate if the observed decrease in number of bacteria was statistically significant.

The influence of platelets on wound healing is well known. Chronic ulcers show decreased activity of growth factors, which delays tissue repair mechanisms. In 1974, Ross *et al*. found in their *in vitro* studies that thrombin activated platelets may constitute a rich source of growth factors. Thrombocytes release over 30 growth factors to the wound. These include three isomers of platelet derived growth factor – PDGF (αα, ββ, αβ), vascular endothelial growth factor – VEGF, transforming growth factor β – TGF-β1, epithelial growth factor – EGF, insulin-like growth factor – IGF-1, angiopoietin 2 (Ang-2), interleukin 1β (IL-1β) and many others. Platelet rich plasma is also a source of proteins involved in cell adhesion, such as fibrin, fibronectin, vitronectin, osteocalcin or osteonectin (Everts *et al.*, 2006). The growth factors are released as a result of degranulation of thrombocyte granules soon after the coagulation cascade is initiated and bind the membrane receptors of adjacent cells, thus activating the intracellular signalling pathway (Anitua *et al.*, 2004; Eppley *et al.*, 2004). The growth factors attract the undifferentiated cells within the wound, stimulate cell division, trigger the capillary growth and improve epithelial repair mechanisms. Additionally, they inhibit cytokine expression, thus limiting the inflammatory response (Carter *et al.*, 2011; Scimeca *et al.*, 2010). Platelet rich plasma shows antibacterial properties, mainly due to PDGF, which activates macrophages and VEGF, which activates macrophages and monocytes. Furthermore, PRP contains leukocytes, which enhance its antibacterial properties. *In vitro* studies showed good efficacy of PRP against *S. aureus* (MSSA), which was comparable to the one of gentamycin and oxacillin (Cieślik-Bielecka *et al.*, 2012). Li *et al.* (2013) observed a significant antibacterial activity of PRP against MRSA in experimentally induced osteitis in rats. Also Moojen *et al.* (2008) confirmed the efficacy of PRP against *S. aureus*. Bielecki *et al.* (2007) who studied the *in vitro* effect of platelet rich plasma provided a broad analysis of antibacterial properties of PRP. They assessed the antibacterial effect of PRP by measuring the inhibition zones on agar plates with individual microbial strains. PRP was shown to inhibit the growth of *S. aureus*, both MSSA and MRSA, as well as *E. coli*. The effect of PRP on *K. pneumoniae, E. faecalis* and *P. aeruginosa* was not confirmed. In the case of *P. aeruginosa*, PRP may even induce microbial growth (ref). Chronic ulcers are typically colonised by *S. aureus* and *P. aeruginosa* (Lim *et al.*, 2006). As postulated by Gjodsbol *et al.* (2006), the presence of *P. aeruginosa* can particularly hinder ulcer healing and increase its surface area.

Our *in vivo* study shows that platelet rich plasma possibly inhibits growth of many bacterial species, including *S. aureus* and *P. aeruginosa*. However, based on a single study, it is difficult to determine whether this effect can be attributed to platelet rich plasma, microbial interactions, transfer of bacteria during wound dressing or other host factors. The use of PRP increased the number of microbial species in the healing wound. At the same time, the percentage of patients with culture-confirmed *P. aeruginosa* after PRP therapy has risen, which can be attributed to ulcer colonisation with nosocomial hospital strains. Nevertheless, these ulcerations tended to heal better despite the increased number of different microbial species. This may indicate colonisation rather than actual infection. Undoubtedly, the decreased colony count of most species was a beneficial effect likely to promote ulcer healing. This, however, requires further study.

In conclusion, local application of platelet rich plasma onto the surface of venous ulcers reduces the number of colonies, contributing to the increased variety of microbial flora at the same time.

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SHORT COMMUNICATION

In Search of the Antimicrobial Potential of Benzimidazole Derivatives

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Abstract

A broad series of 4,5,6,7-tetrahalogenated benzimidazoles and 4-(1*H*-benzimidazol-2-yl)-benzene-1,3-diol derivatives was tested against selected bacteria and fungi. For this study three plant pathogens *Colletotrichum* sp., *Fusarium* sp., and *Sclerotinia* sp., as well as *Staphylococcus* sp., *Enterococcus* sp., *Escherichia* sp., *Enterobacter* sp., *Klebsiella* spp. , and *Candida* spp. as human pathogens were used. MIC values and/or area of growth reduction method were applied in order to compare the activity of the synthesized compounds. From the presented set of 22 compounds, only 8, 16, 18 and 19 showed moderate to good inhibition against bacterial strains. Against *Candida* strains only compound 19 with three hydroxyl substituted benzene moiety presented high inhibition at nystatin level or lower.

Key words: antibacterial activity, antifungal activity, benzimidazole

Benzimidazole derivatives are of wide interest because of their diverse biological activity and clinical applications (Bansal and Silakari, 2012). This ring system is present in numerous antiparasitic, fungicidal anthelmintic, anti-inflamatory and antiviral drugs (Pedini *et al.*, 1994; Martin, 1997; Zacny *et al.*, 1999; Gaba *et al.*, 2014). Some benzimidazoles show also cytotoxicity against diverse cancer cell lines (Horton *et al.*, 2003; Padmavathi *et al.*, 2008; Karpińska *et al.*, 2012). Recently, we found that polyhalogenated benzimidazoles are potent inhibitors of casein kinase 2 (CK2), probably the most pleiotropic protein kinase in more than 300 known eukaryotic organisms (Pagano *et al.*, 2004; Gianoncelli *et al.*, 2009; Janeczko *et al.*, 2012). Interestingly, prokaryotes do not code for CK2 genes. The antimicrobial activity of benzimidazole derivatives is frequently studied with various microrganisms. Their antimycobacterial, antiprotozoal and antibacterial activity has been observed for variously modified benzimidazole derivatives (Andrzejewska *et al.*, 2004; Kazimierczuk *et al.*, 2005; Navarrete-Vázquez *et al.*, 2006; Laudy *et al.*, 2012). In addition to their antibacterial activity, benzimidazole derivatives possess antifungal activity. Benomyl, thiabendazole and thiophnate methyl are some main examples of this fungicide class. They are also used for the prevention of post-harvest rots and as soil-drench treatments (Kaplancikli *et al.*, 2004).

The major objective of the present study was examination of the antibacterial activity of established CK2 inhibitors possessing 4,5,6,7-tetrabromo- or 4,5,6,7-tetraiodobenzimidazole as a fragment of active structure (compounds 1–16) and with benzene-1,3-diole and benzene-1,2,3-triole substituted at the 2 position of the heterocyclic ring showing anticancer activity (compounds 17–22). Also, we present here the results of antifungal activity of the above mentioned compounds against three *Candida* species obtained as clinic isolates and three plant fungi: *Colletotrichum gloeosporioides*, *Sclerotinia sclerotiorum* and *Fusarium culmorum*.

The preliminary results presented below indicate that the biological potential of investigated benzimidazole derivatives is not limited to the previously reported features as CK2 inhibitors or anticancer potential.

For this study a series of benzimidazole derivatives were used. Compounds 1–16 were obtained as previously described (Janeczko *et al.*, 2012). 4,5,6,7-Tetrabromo- and 4,5,6,7-tetraiodobenzimidazoles and their respective 2-mercaptoderivatives were alkylated with ω-bro-

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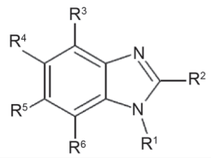
moalkyl esters in the presence of potassium carbonate in aprotic solvents to yield the desired compounds. The respective benzimidazoles containing ester moieties were hydrolyzed in alkaline medium to yield the corresponding carboxyalkyl compounds. The structures of the new compounds were confirmed by ^{1}H NMR and UV spectra and elemental analyses.

Compounds 17–22 were synthesized as previously described (Karpińka *et al.*, 2011; 2012; Los *et al.*, 2012). The compounds were formed by the reaction of *N*-alkyl(aryl)benzene-1,2-diamine derivatives with sulfinylbis[(2,4-dihydroxyphenyl)-methanethione] (STB) or its analogues in methanol under reflux (2.5–3.5 h) in moderate to good yields (63–77%). Structures of benzimidazoles used in this study are shown in Table I. The logP values were determined using the Molinspiration cheminformatics tool (http://www.molinspiration.com/cgi-bin/properties).

Antibacterial activity tests were carried out against the reference strains: *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus hominis* (PCM 2651), *Enterococcus faecalis* (PCM 2673), *Escherichia coli* (ATCC 8739), *Enterobacter cloacae* (PCM 2569), *Klebsiella oxytoca* (clinical isolate) and *Klebsiella pneumoniae* ssp. *pneumoniae* (PCM 1). Strains of bacteria were inoculated in Mueller-Hinton broth (Biocorp, Poland) for 24 h before performing the minimal inhibitory concentration (MIC) test, and incubated at 37°C with vigorous shaking (180 rpm). MIC was determined by the microbroth dilution method. Bacterial suspensions in Mueller-Hinton liquid medium at initial inoculums of 5×10^5 colony forming units per ml were added to polystyrene 96-well plates were exposed to the investigated benzimidazoles at adequate concentrations (range: 1–5 mg/ml) for 20 h at 37°C. MIC's were taken as the lowest drug concentration at which observable growth was inhibited. Tetracycline and chloramphenicol were used as reference compounds. Experiments were performed in triplicate.

The fungal strains tested were: 5 strains of *Candida albicans* (clinical isolates), 5 strains of *Candida glabrata* (clinical isolates) and 5 strains of *Candida tropicalis* (clinical isolates) and plant pathogens: *C. gloeosporioides*, *S. sclerotiorum* and *F. culmorum.* All clinical isolates were obtained from Paweł Kozak, Laboratory of Microbiology and Mycobacterium tuberculosis, The

Table I
Chemical structures of benzimidazole derivatives

Compound		MW	Log *P*
1	R^1 = H, R^2 = H, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	433.7	4.13
2	R^1 = CH_2COOH, R^2 = H, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	491.7	3.63
3	R^1 = H, R^2 = H, R^3 = I, R^4 = I, R^5 = I, R^6 = I	621.7	6.24
4	R^1 = CH_2COOH, R^2 = H, R^3 = I, R^4 = I, R^5 = I, R^6 = I	679.8	5.74
5	R^1 = H, R^2 = SCH_2COOH, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	523.8	4.81
6	R^1 = H, R^2 = $S(CH_2)_2COOH$, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	537.8	5.11
7	R^1 = H, R^2 = $S(CH_2)_3COOH$, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	551.9	5.39
8	R^1 = CH_3, R^2 = SCH_2COOH, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	537.8	5.05
9	R^1 = CH_3, R^2 = $S(CH_2)_2COOH$, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	551.9	5.34
10	R^1 = CH_3, R^2 = $S(CH_2)_3COOH$, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	551.9	5.62
11	R^1 = H, R^2 = SCH_2COOH, R^3 = I, R^4 = I, R^5 = I, R^6 = I	711.8	6.93
12	R^1 = H, R^2 = $N(CH_3)_2$, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	476.8	5.39
13	R^1 = CH_3, R^2 = $N(CH_3)_2$, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	490.8	5.63
14	R^1 = $(CH_2)_3COOH$, R^2 = H, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	519.8	4.20
15	R^1 = CH_2COOH, R^2 = $N(CH_3)_2$, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = Br	534.8	4.89
16	R^1 = $(CH_2)_3COOH$, R^2 = $N(CH_3)_2$, R^3 = Br, R^4 = Br, R^5 = Br, R^6 = B	562.9	5.47
17	R^1 = H, R^2 = 2,4-di-OH-5-Et-C_6H_2, R^3 = H, R^4 = CH_3, R^5 = CH_3, R^6 = H	282.3	3.96
18	R^1 = H, R^2 = 2,4-di-OH-5-Cl-C_6H_2, R^3 = H, R^4 = CH_3, R^5 = CH_3, R^6 = H	288.7	3.62
19	R^1 = H, R^2 = 2,3,4-tri-OH-C_6H_2, R^3 = H, R^4 = CH_3, R^5 = CH_3, R^6 = H	270.3	2.67
20	R^1 = CH_3, R^2 = 2,4-di-OH-C_6H_3, R^3 = H, R^4 = H, R^5 = H, R^6 = H	240.3	2.32
21	R^1 = C_6H_6, R^2 = 2,4-di-OH-5-Et-C_6H_2, R^3 = H, R^4 = H, R^5 = H, R^6 = H	330.4	4.89
22	R^1 = $(CH_2)_2OH$, R^2 = 2,4-di-OH-C_6H_2, R^3 = H, R^4 = NO_2, R^5 = H, R^6 = H	315.3	1.84

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Candida species were cultured in Sabouraud glucose liquid medium (Biocorp, Poland) for 48 h at the temperature 25°C and shaking (130 rpm). Activity of benzimidazoles against yeast was determined by the microbroth dilution method. Microbial cells suspensions at initial inoculums of 3×10^3 colony forming units per ml in Sabouraud glucose broth were exposed to the benzimidazoles at adequate concentrations (range: 0.001–5 mg/ml) for 48 h at 25°C. MIC was the lowest concentration of the compound that inhibited the visible growth of a microorganism after incubation. Nystatin was used as the control drug. Each experiment was replicated thrice.

Cultures of *C. gloeosporioides*, *S. sclerotiorum* and *F. culmorum* were carried out in solid Potato Dextrose Agar medium (Biocorp, Poland) at room temperature for 7 days. Antifungal activity was determined by the method of serial twofold dilutions. The dilution series of the tested compounds, ranging from 0.0005 to 2 mg/ml, were prepared in molten Potato Dextrose Agar medium. Identical mycelial fragments were applied to the Petri plates. The inoculated plates were incubated at room temperature for 7 days. The MIC values were determined as the lowest concentration of the test sample inhibiting visible fungal growth. Metconazole and tebukonazole were used as control compounds. The experiments were carried out in triplicate.

Antibiotic resistance is one of the most important issues not only in microbiology and clinical medicine, but is also a major public health problem. In the last 25 years the incidence of microbial infections has risen in the world, which generally is associated with the formation and expansion of antibiotic resistance in primary sensitive microorganisms. This phenomenon is due to genetic mutations of organisms that produce defense mechanisms to respond to environmental factors against their life processes. One way to counteract these difficulties is the conscious use of the currently available antibiotics. On the other hand, it is necessary to develop and synthesize new compounds, distinctly different from the existing ones, but synthesized on the basis of structural elements with known biological activity. Here we present a set of benzimidazole derivatives possessing antimicrobial potential. All compounds from Table I were tested against a set of bacterial and fungal strains using microbroth dilution method for bacterial and *Candida* strains as well as serial twofold dilutions method for *Colletotrichum*, *Sclerotinia* and *Fusarium* strains.

From the presented set of 22 compounds, only 8, 16, 18 and 19 showed moderate to good inhibition against bacterial strains. The antibacterial screening indicated that among the tested bacterial strains, good inhibitory results were obtained only against *Staphylococcus* species (Table II). This strain is an opportunistic pathogen that forms the natural microflora of the human organism, but under certain conditions can lead to infections. *Staphylococcus* species may occasionally cause generally hospital-acquired infections in patients whose immune system is impaired, for example by chemotherapy or predisposing illness. The activity of our compound against *Staphylococcus* was observed in case of tetrabromoderivatives (7–9 and 16) substituted with bromine atoms at R^3-R^6 positions as well as benzene derivatives (18, 19) substituted with benzene ring at R^2 position. MIC values were found in the range of 0.6–5.0 mg/ml. Other compounds didn't show any influence on the tested strains in the used concentrantion range. Similar results were obtained by Vinodkumar *et al.* (2008) for 2 – (4-phenylethynyl-phenyl)-benzimidazoles and their derivatives. The MIC's for *S. aureus* and *S. typhimurium* in this case were in the range of 0.2–0.5 mg/ml. In the study of Shingalapur *et al.* (2009) the effect of 5 – (nitro/bromo)-styryl-benzimidazole-2 on the growth of *S. aureus*, *E. faecalis*, *E. coli* and *K. pneumoniae* was tested. MIC values obtained (1–4 μg/ml) suggest the positive effect of nitro- and bromo- substituents on antibacterial activity of such compounds. However, the introduction of bromo substituents on the aromatic ring has highly increased the activity compared to the nitro group. Moreover, the growth of *S. aureus* was also effectively inhibited by trichloro-derivatives. 5,6-dichloro-2-amine and 5-chloro-2-4-benzyloxyphenyl benzimidazole gave MIC values equal to 3.12 μg/ml (Tunçbilek *et al.*, 2009).

In recent years, an increase in the incidence of fungal infections is clearly visible. Paradoxically, it is correlated with the progress of the medicine. Particularly affected are people who are undergoing treatment with immunosuppressive antibiotics with a broad spectrum of activity and transplant patients who are in neutropenia. Fungal infections are caused primarily by strains of *Candida*, especially *C. albicans* and *C. tropicalis*, although recently increases the frequency of isolation of species such as *C. parapsilosis*, *C. glabrata* and *Candida krusei.* Thus, the verification of the antifungal activity of the presented compounds was obligatory. For determination of antifungal activity, all compounds were tested against fifteen clinical isolates of *C. albicans*, *C. glabrata* and *C. tropicalis*. Five clinical isolates from the vagina and urine were used in the case of *C. albicans*. Compounds 16, 19 and 21 showed activity below 5 mg/ml in case of at least one isolate (Table II). In the case of *C. glabrata* isolated from urine, sputum and vagina, only compounds 17–20, and 22 showed good inhibition of growth with MIC values below 1 mg/ml (Table II). *C. tropicalis* on the other hand was found to be the most resistant among all tested human pathogens from the derived fungi (Table II). In this case only two

Table II
MIC values (mg/ml) of benzimidazole derivatives against bacterial, Candida, and phytopathogen strains.

Compound	*S. aureus*	*S. epidermidis*	*S. hominis*	*E. faecalis*	*E. coli*	*K. pneumoniae*	*K. oxytoca*	*C. albicans*§					*C. glabrata*§					*C. tropicalis*§					*C. gloeospo-rioides*	*F. culmorum*	*S. sclerotio-rum*
								A	B	C	D	E	F	G	H	I	J	K	L	M	N	O			
1	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.5	0.5	0.25
2	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.5	2–5	0.5
3	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	1.25	5–10	5–10	5–10	2.5	5–10	5–10	5–10	2.5	0.125	0.25	0.125
4	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	1	1	0.25
5	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.5	0.5	0.25
6	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.5	0.5	0.5
7	2.5	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.5	1	0.125
8	5	0.6	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.5	2–5	0.125
9	5	5–10	1.25	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.5	2–5	0.5
10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	2–5	2–5	0.125
11	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	2.5	5–10	5–10	1	1	0.5
12	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	1	1	0.5
13	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	2–5	2–5	1
14	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	2–5	2–5	0.5
15	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	2–5	2–5	1
16	1.25	1.25	0,6	5–10	5–10	5–10	5–10	5–10	2.5	2.5	5–10	5–10	5–10	5–10	5–10	5–10	5–10	1.25	5–10	5–10	5–10	5–10	0.25	0.25	0.031
17	5–10	5–10	0.15	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	2.5	5–10	5–10	0.3	5–10	5–10	5–10	5–10	5–10	5–10	0.25	0.125	0.125
18	1.25	2.5	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.6	0.3	0.3	5–10	0.3	1.25	1.25	2.5	5–10	2.5	0.25	0.25	0.125
19	0.6	1.25	1.25	5–10	5–10	5–10	5–10	0.02	0.3	0.6	0.3	0.08	0.6	0.3	0.3	0.6	0.08	0.6	1.25	1.25	2.5	0.6	0.25	0.125	0.06
20	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	2.5	0.6	5–10	5–10	0.6	5–10	5–10	2.5	5–10	5–10	0.125	0.125	0.06
21	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	2.5	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.5	0.25	0.125
22	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.3	2.5	5–10	5–10	5–10	5–10	5–10	5–10	5–10	5–10	0.5	0.25	0.25
Chloram**phenicol**	0.005	0.005	0.005	0.005	0.0012	0.005	0.0012																		
Tetracycline	0.005	0.04	0.01	0.005	0.0012	0.005	0.0012																		
Nystatin								0.6	0.04	0.125	0.6	0.04	0.02	0.15	0.08	0.08	0.08	0.3	0.15	0.6	0.6	0.6			
Ketoconazole								0.04	0.04	0.04	0.01	0.04	0.04	0.15	0.02	0.04	0.01	0.04	0.04	0.04	0.04	0.04			
Amphotericin B								0.04	0.02	0.02	0.02	0.01	0.04	0.04	0.04	0.04	0.02	0.04	0.04	0.04	0.04	0.02			
Metconazole																							0.031	0.007	0.007
Tebuconazole																							0.015	0.0017	0.007

§ A, C, D, E, H, I, J, O – isolates from vagina; B, F, L, N – isolates from urine; G, M – isolates from sputum; K – isolate from throat

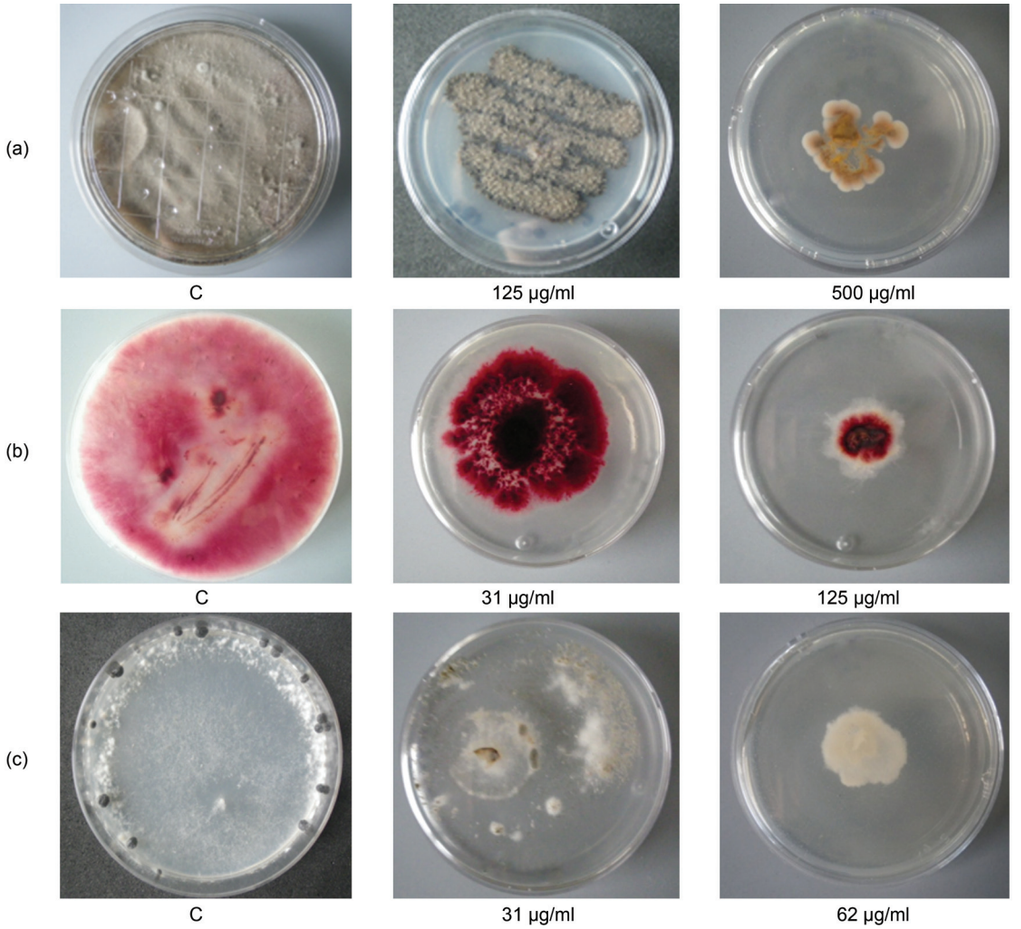

Fig. 1. Influence of presented compounds on spawn development of phytopathogens.
(a) *C. gloeosporioides*, (b) *F. culmorum*, (c) *S. sclerotiorum* were grown in the presence of compound 4, 16 and 22 respectively.

compounds (18 and 19) inhibited the growth of this strain with MIC values in the range of 0.6–1.25 mg/ml making them promising hits for future development. Growth of certain *C. tropicalis* strains was also inhibited by compounds 3, 11, 16, 18, and 20 with MIC's in the range of 1.25–2.5 mg/ml. Although, only compound 19 with three hydroxyl substituted benzene moiety at the diazole ring and two methyl substituents on the aromatic ring was able to effectively inhibit all strains of *Candida* used in this study (MIC = 0.6–2.5 mg/ml). The fact that compound 19 successfully inhibits growth of all tested strains is of great importance because it can lead in the future to design agents with broad antifungal activity. Similar activities were obtained in the case of bis-5-methylbenzimidazoles (Küçükbay *et al.*, 2003a) and electron-rich olefins derived benzimidazoles (Küçükbay *et al.*, 2003b) against *C. albicans* and *C. tropicalis*. When nitro- and halogen-benzimidazoles were substituted with long-chain alkyl acids they gained higher potency up to 37.5 µg/ml of MIC value (Sharma *et al.*, 2009). The spectrum of infections caused by *Candida* is very extensive and includes both superficial and deep infections. Superficial infection in favorable conditions can develop in the skin, the epidermis and mucous membranes. An important clinical problem is the deep infections that may take the form of organ, systemic or disseminated infetions, and at the same time have high, over 70% mortality. *Candida* species are the fourth most common pathogen isolated from the blood of hospitalized patients. Thus, it is justified to design and synthesize new antifungal agents.

Interesting results were obtained in the case of plant pathogens. All tested benzimidazoles showed good inhibitory properties against *C. gloeosporioides*, *F. culmorum* and *S. sclerotiorum*. MIC values were found in the range of 0.031–2 mg/ml (Table II). The inhibition of growth of these species is very important from the agriculture point of view as they are characterized by high non-specificity and polifagism and inflict a lot of damage in crop breeding. *S. sclerotiorum* was found to be the most sensitive strain especially to compounds 16–21 with MICs below 0.125 mg/ml. This fact makes those compounds promising output agents as this pathogen is attacking more than 400 species of plants around

the world, including many important crops (oilseeds, pulses, fodder plants, vegetables and ornamental plants). Additionally, there was an evident effect of the presented benzimidazoles on the macroscopic look of the studied fungi colonies. On control plates, abundant, fluffy spawns developed whereas spawns that grown on plates with presence of benzimidazoles were clearly poorer developed (Fig. 1.) what suggests that the presented compounds can effectively inhibit the expansion of fungi spawns. Only compound 15 didn't show such influence.

Referring the obtained results to the chemical structure of active compounds some common characteristics that determine biological activity should be indicated. Such activity is undoubtedly connected with the permeability of membranes and cell walls of microorganisms. Better anti-microbial properties are mainly demonstrated by benzimidazoles with greater hydrophilicity, that is lower logP (compounds 17–22). Such tendency is also seen regarding the molecular weight, where smaller compounds (MW < 300 kDa) present better activity. Reference of the obtained results to the chemical structure of the tested compounds broadens the knowledge of the practical use of new biologically active substances containing benzimidazole ring and gives rise to possible chemical modification of compounds in order to achieve greater effectiveness in inhibiting the growth of undesirable microorganisms.

In view of the above findings, the presented candidates may be valuable in designing more potent and selective anti-microbial agents serving as promising starting templates.

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SHORT COMMUNICATION

Pexophagy in Penicillin G Secretion by *Penicillium chrysogenum* PQ-96

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Abstract

Penicillin G oversecretion by *Penicillium chrysogenum* PQ-96 is associated with a strictly adjusted cellular organization of the mature and senescent mycelial cells. Abundant vacuolar phagy and extended cellular vacuolization combined with vacuolar budding resulting in the formation of vacuolar vesicles that fuse with the cell membrane are the most important characteristic features of those cells. We suggest as follows: if the peroxisomes are integrated into vacuoles, the penicillin G formed in peroxisomes might be transferred to vacuoles and later secreted out of the cells by an exocytosis process. The peroxisomal cells of the mycelium are privileged in penicillin G secretion.

Key words: *Penicillium chrysogenum*, penicillin G, secretion

The last two steps in penicillin G biosynthesis are located in the peroxisomes. Secretion of this antibiotic from the peroxisomes across the plasma membrane of *Penicillium chrysogenum* is poorly understood (Weber *et al*., 2012). This experimental study was designed to provide details supporting the hypothesis that pexophagy (autophagy of peroxisomes) is involved in the large-scale secretion of penicillin G from the mycelial cell of *P. chrysogenum* PQ-96.

In this experimental program the high penicillin-producing strain *P. chrysogenum* PQ-96 was examined. Activity of penicillin G produced by this strain is described in Fig. 1. For comparative ultra-structural analyses the low-penicillin-producing strain *P. chrysogenum* Q-176 was investigated (Fig. 2). The examined strains were grown in complex media (Kurzątkowski *et al*., 2014a) and the antibiotic assay was carried out as described previously (Garcia-Estrada *et al*., 2007). The preparation for transmission electron microscopy and immunoelectron microscopy was performed as described previously (Kurzątkowski *et al*., 2014a). The ultrathin sections were examined under a transmission electron microscope JEOL, JEM 1220 (Tokyo, Japan).

The lack of clear involvement of any of these ABC transporters (van den Berg, 2001; Patent description number WO 2001/32904) in secretion of penicillin G is intriguing and may indicate that the secretion of this antibiotic in the overproducing strains does not proceed through the classical ABC pumps. At present, new secretion pathways, *e.g.* the secretion by exocytosis may have been implemented in the high-penicillin producing strains (Martín *et al*., 2010). Although, fusion of the vacuoles to the plasma membrane by an exocytosis process is possible, there is currently no evidence in the literature to support that this might be a major mechanism of penicillin G secretion (Martin *et al*., 2010).

In fed-bath cultures the industrial strains secrete 40–55 g of penicillin G per liter of the liquid fermentation medium. The knowledge concerned with the cellular arrangements in penicillin G overproduction is important for further strain improvement, which is of great economical importance. Compartmentalization in penicillin G biosynthesis by *P. chrysogenum* PQ-96 was described previously (Kurzątkowski *et al*., 2014a; 2014b). Our studies have proven that overproduction of penicillin G is associated with strongly adjusted mycelial and cellular organization (Fig. 1a-d, Fig. 2i-h, Fig. 3e-h). The productive mycelial cells of the high-yielding strain exhibited numerous large peroxisomes frequently arranged at the periphery of the cytoplasm and around the vacuoles including vacuolar invaginations. The surveys of a large number of hyphal sections

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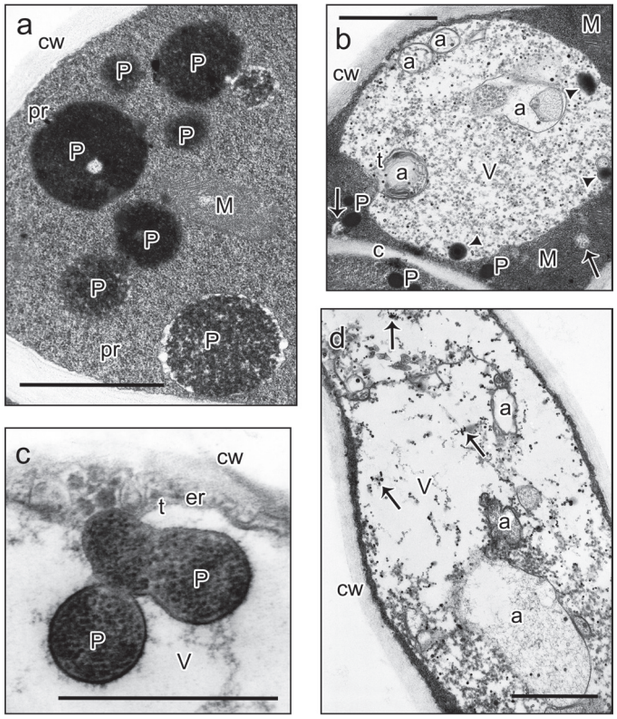

Fig. 1. *P. chrysogenum* PQ-96, 72 h culture, high-penicillin-producing strain, activity of penicillin G biosynthesis (U/ml): total yield at 72 h of cultivation – 8300 (4.98 g/1000 ml), increase of yield between 48 h and 72 h of fermentation – 4850.

(a-d) Transmission electron microscopy. **(a)** In the sub-apical non-growing productive cell numerous electron opaque peroxisomes (P) up to 1.0 µm in diameter can be observed. **(b)** Sub-apical productive cell of the mycelium is visible. Note the degrading organelle located in invaginations of the tonoplast (t) into a vacuole (V). Vacuolar engulfed pexophagy (arrow heads) is seen. It is a characteristic feature of the hyphal cell at the highest activity of penicillin G secretion. At the cell wall (cw) and the cross wall (c) vacuolar vesicles packed with organelle debris are visible (arrows). Some peroxisomes (P) and mitochondria (M) are located at the vacuole beginning the process of autophagy (a). **(c)** Late sub-apical degrading highly vacuolated hyphal cell. In invaginations of the tonoplast (t) into the vacuole (V) the pexophagy (P) is seen. **(d)** Late-apical cell of the mycelium is seen. In the interior of an extended vacuole (V) the products of organelle-autophagy (a) can be seen (arrows). Scale bar = 1 µm.

led us to the conclusion that the immuno-gold marker of isopenicillin N synthase is abundantly arranged at polyribosomes surrounding the peroxisomes. Such a cellular accumulation of isopenicillin N synthase may enhance the selective, continuous and sufficient substrate supply in penicillin G biosynthesis. It was recently found that δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine tri-peptide is present in the cytoplasm and accumulates in the fermentation medium to concentrations of up to 2 mM (for review see Kurzątkowski *et al.*, 2014a). The affinity of isopenicillin N synthase for this tri-peptide is in the sub-mM range. The high concentration of the tri-peptide in the fermentation broth might explain our unexpected results concerning the localization of isopenicillin N synthase at the periphery of the cytoplasm and in channel-like structures of the cell wall. This location might be a precisely adopted structural arrangement enabling the withdrawal of the tri-peptide from the fermentation broth and from the cytoplasm for the peripherally located isopenicillin N synthase to increase the efficacy and yield in penicillin G biosynthesis. On the contrary, in the mature non-growing hyphal cells of the low penicillin-producing strain *P. chrysogenum* Q-176 the total lack of peroxisomes about 0.1 µm in diameter were visible.

Peroxisomes play a crucial role in the production of penicillin G and cephalosporin C by industrial strains (Kurzątkowski and Gębska-Kuczerowska, 2015). High penicillin G producing strains show increasing numbers of large peroxisomes mainly at the period of the intensive antibiotic biosynthesis (van den Berg *et al.*, 2008; Meijer *et al.*, 2010; Weber *et al.*, 2012). The presence of functional peroxisomes remarkably affects the efficiency of penicillin G biosynthesis (van den Berg *et al.*, 2008; Meijer *et al.*, 2010; Bartoszewska *et al.*, 2011). Mutants blocked in the biosynthesis of peroxisomes exhibit a significantly reduced activity of penicillin biosynthesis (Weber *et al.*, 2012).

The results of our experiments exhibit that the abundant vacuolar pexophagy of large peroxisomes combined with vacuolar budding and the presence of numerous vacuolar vesicles which fuse with the plasma membrane are the most important structural features characterizing the non-growing productive cells as well as the late-apical degenerating highly vacuolated cells of the tested industrial strain. This structural arrangement is closely combined with the period of large-scale secretion of penicillin G. Such a cellular organization was not visible in the mature cells of the low-penicillin-producing strain Q-176.

We suggest that the abundant pexophagy of large peroxisomes as well as the vacuolar budding observed in the productive and senescent cells of *P. chrysogenum* PQ-96 might be directly involved in large-scale secretion of penicillin G. In these cellular arrangements the penicillin G formed in peroxisomes might be transferred to vacuoles and late secreted out of the cells by an exocytosis process. The vacuolar pH of about 5 is suitable for the stability of penicillin G. Our discoveries are consistent with the reported positive cor-

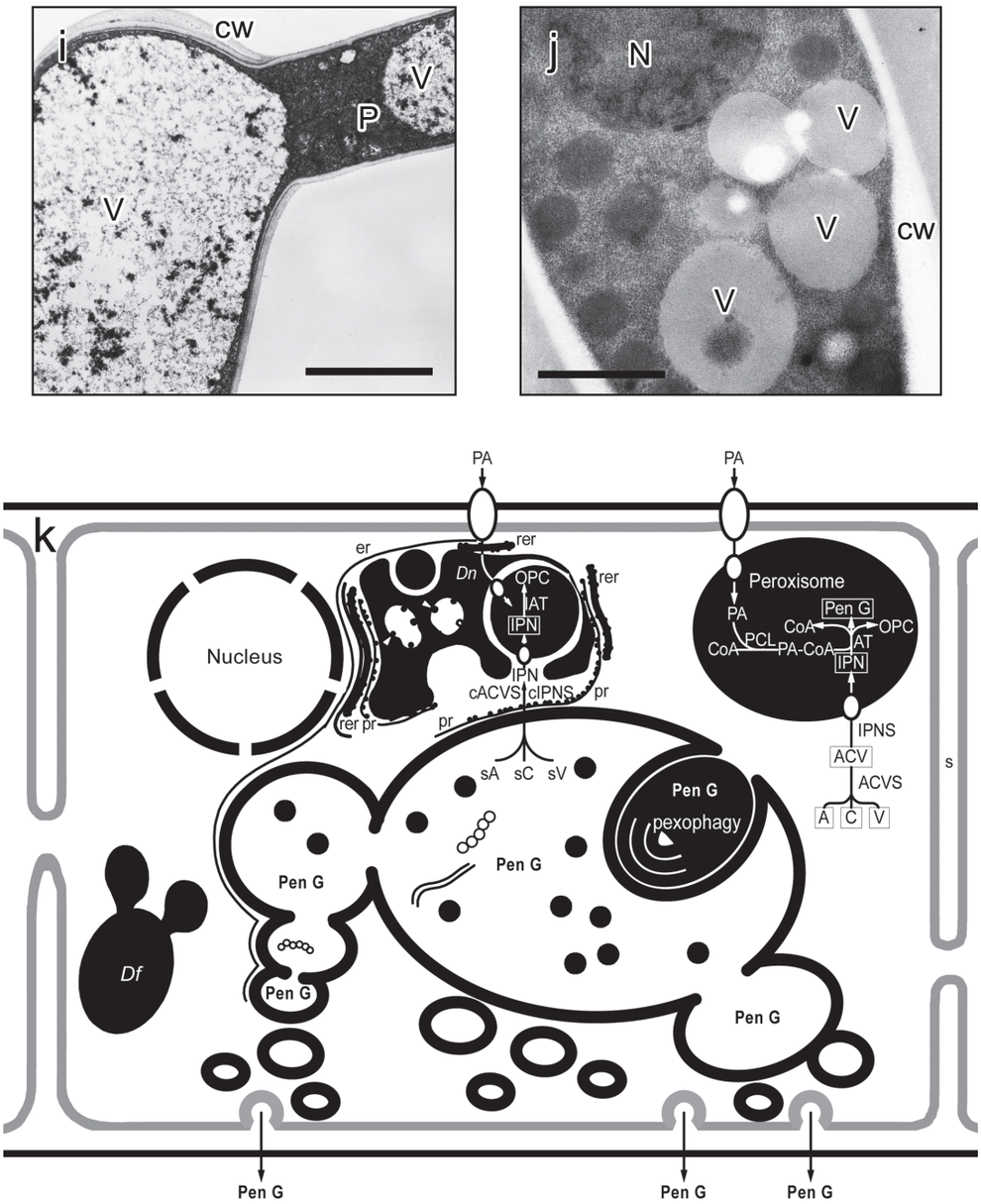

Fig. 2. *P. chrysogenum* Q-176 (low-yielding strain), 72 h culture, activity of penicillin G secretion 70 U/ml.
(i) Transmission electron microscopy. In the sub-apical non-growing branching cell of the hyphae only one small peroxisome is visible. **(j)** Ultrathin sections treated with rabbit immunoserum to isopenicillin N synthase followed by goat anti-rabbit IgG-15 nm gold conjugate showed exceptionally few gold grains. Scale bar = 1 μm. **(k)** A hypothetical overview of penicillin G secretion from sub-apical non-growing vacuolated mycelial cells of *P. chrysogenum* PQ-96 is depictured. The final steps of penicillin G biosynthesis are located in peroxisomes where isopenicillin N is converted to penicillin G. In the process of vacuolar pexophagy penicillin G is transported to the interior of vacuoles. Finally penicillin G is secreted from the vacuoles to the fermentation medium in the process of vacuolar budding followed by fusion of vacuolar vesicles with the cell membrane (exocytosis). *Abbreviations*: A – α-aminoadipic acid; C – L-cysteine; V – L-valine; sA, sC, sV – vacuole sequestered pool of the precursor amino acids; ACV – δ--(L-α-aminoadipyl)-L-cysteinyl-D-valine tri-peptide; ACVS – ACV synthetase; IPN – Isopenicillin N; IPNS – Isopenicillin N synthase; PA – phenylacetic acid; CoA – coenzyme A; PA-CoA – phenylacetyl-coenzyme A; PCL – PA-CoA ligase; AT – acyl-CoA:isopenicillin N acyltransferase; OPC – 6-oxopiperidine-2-carboxilic acid; Pen G – penicillin G; er – endoplasmic reticulum; rer – rough endoplasmic reticulum; pr – polyribosomal membranes; *Dn* – *de novo* synthesis of peroxisomes; *Df* – fission of pre-existing peroxisomes.

relation between the number of large peroxisomes and penicillin G secretion (Meijer *et al.*, 2010), as well as between the extended vacuolization and antibiotic secretion (Sakai *et al.*, 2006).

The novelty of this experimental program is the discovery of essential cellular features associated with the large scale secretion of penicillin G from the mycelium of *P. chrysogenum* PQ-96 to the fermentation

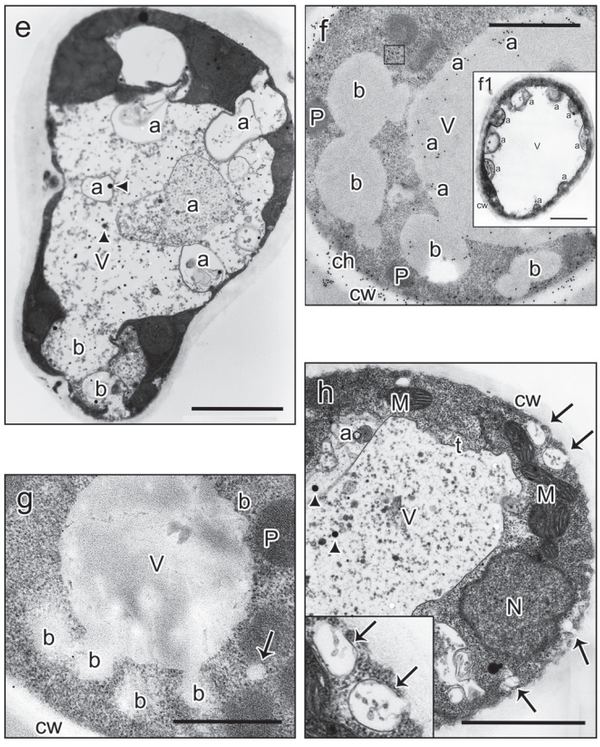

Fig. 3. *P. chrysogenum* PQ-96, 72 h culture. **(e, f1, h)** Transmission electron microscopy.

(e) A sub-apical non-growing productive vacuolated cell of the mycelium. The autophagy (a) of organelles (arrow heads) and budding (b) of a large vacuole (V) is correlated with the highest activity of penicillin G secretion. **(f, g)** Immuno-gold electron microscopy of cytosolic isopenicillin N synthase. **(f)** The marker of the enzyme is associated with peroxisomes (P) including the membranes involved in vacuolar (V) budding (square) and is located in numerous vacuolar invaginations exhibiting places of pexophagy (a) as well as at the periphery of the cytoplasm where the vacuolar vesicles fuse with the plasma membrane and in channels (ch) of the cell wall (cw). Abundant vacuolar budding (b) is seen. **(f1)** At the periphery of a large vacuole (V) numerous structures are visible (a) corresponding to the places of pexophagy shown in position **(f). (g)**. Control sample – ultrathin sections treated with rabbit pre-immunoserum, followed by goat anti-rabbit IgG-15 nm gold conjugate. Immuno-gold localization of not specifically bound antibody showed exceptionally few gold grains. Abundant budding (b) of a vacuole (V) is characteristic for intensive penicillin G secretion. At the vacuolar periphery a peroxisome (P) is visible. A vacuolar vesicle (arrow) of about 100 nm in diameter is seen. **(h)** Section through the sub-apical non-growing vacuolated cell is visible. In the cell at intensive penicillin G secretion, fusion of numerous vacuolar vesicles with the cytoplasm membrane can be observed (arrows). In the interior of these vesicles organelle debris similar to that located in the vacuole (V) are visible. It suggests that after vacuolar pexophagy the vacuolar vesicles are involved in penicillin G secretion. Note the organelle debris (arrow heads) located in the vacuole. Autophagosomes (a) in the vacuole area are visible. Note the mitochondria (M) and the nucleus (N) closely arranged at the massive cell wall (cw). Scale bar = 1 μm.

medium, *i.e.*: abundant vacuolar pexophagy of large peroxisomes, intensive cellular vacuolization, budding of vacuoles, fusion of vacuolar vesicles with the plasma membrane. We have come to the conclusion that in large-scale secretion of penicillin G the pexophagy phenomenon and exocytosis should be currently considered as a putative alternative for active secretion by the ABC transporters.

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SHORT COMMUNICATION

Endophytic Detection in Selected European Herbal Plants

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Abstract

A total of 181 cultivable endophytic bacterial isolates were collected from stems of 13 species of herbs inhabiting Europe (Poland): *Chelidonium majus* L., *Elymus repens* L., *Erigeron annuus* L., *Euphrasia rostkoviana* Hayne, *Foeniculum vulgare* L., *Geranium pratense* L., *Humulus lupulus* L., *Matricaria chamomilla* L., *Mentha arvensis* L., *Papaver rhoeas* L., *Rosmarinus officinalis* L., *Solidago gigantea* L. and *Vinca minor* L. The isolates were screened for their antifungal activity and fifty three were found to inhibit fungal growth. Of these, five had strong antifungal properties. These selected isolates were identified as: *Pseudomonas azotoformans*, *P. cedrina*, *Bacillus subtilis* group and *Erwinia persicina*.

Key words: *Alternaria alternate*, antifungal activity, endophytic bacteria, herbs

It is widely believed that all plants are colonized by an endophytic microflora composed of microscopic fungi and bacteria that live inside plant tissues without causing them any harm. Endophytes have been isolated from above-ground parts of plants (stems, flowers, leaves and fruits), from roots and from seeds (Reinhold-Hurek and Hurek, 1998; Tan and Zou, 2001). Numerous studies confirmed that endophytes have a great applicable potential. They have found uses in three main fields connected with crop yield enhancement and growth promotion (1), industrial and medical applications (2) and environmental pollution control (3).

Numerous studies reported that endophytes control plant pathogens through synthesis of different antimicrobial compounds (1). Miller *et al*. (1998) described endophytic bacteria *Pseudomonas viridiflava* isolated from grasses and producing ecomycins B and C. It was investigated that these lipopeptides inhibit the human pathogens *Candida albicans*. Similar studies were conducted by Guan *et al*. (2005), who reported antimicrobial agent producing strain *Streptomyces griseus* inhabiting *Kandelia candel*. Most research has focused on the antifungal properties of isolated endophytes. Strobel *et al*. (2004) for example isolated oocydin A producing endophytes classified as *Serratia marcescens*. Antifungal compound producing endophytes (*Paenibacillus polymyxa*) were also reported by Beck *et al*. (2003). Endophytic-induced plant growth promotion is also achieved through fixation of atmospheric nitrogen, production of iron-chelating siderophores, solubilisation of minerals and production of phytohormones.

Endophytes can act as mini-factories and often produce novel compounds (2). Researchers reported endophytes producing novel antibiotics, anticancer and antiviral compounds, volatile organic compounds, insecticidal agents, immunosuppressive compounds and antioxidants (Strobel and Daisy, 2003; Owen and Hundley, 2004). Castillo *et al*. (2002) for example isolated from *Kennedia nigricans* endophytes classified as *Streptomyces* strain NRRL 30562 that produces antibiotic and antimalarial agents – munumbicins. Antimalarial compounds were also reported by Ezra *et al*. (2004), who obtained coronamycin producing *Streptomyces* sp. isolated from *Monstera* sp. Few studies have been published describing anticancer compounds producing endophytes. The first one was by Stierle *et al*. (1993), who reported taxol producing endophytic fungus *Taxomyces andreane*, isolated from *Taxus brevifolia*.

Some reports confirmed that endophytes enhance phytoremediation (3). These endophytes inhabit plants grown in xenobiotic contaminated soil and express different mechanisms necessary to degradation of contaminants (Germaine *et al*., 2004; 2006).

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In the past few years the search for endophytes inhabiting medicinal plants intensified. It is now recognized that herbs are a very rich source of microorganisms with different biochemical properties. Numerous recent studies have been devoted to the identification of endophytes colonizing herbs from Asian countries. One study isolated 18 endophytic bacteria from herbal plants in Indonesia, such as citrus, turmeric, *Andrographis paniculata* and *Piper crocatum* (Soka *et al*., 2012). Another study obtained 19 bacterial endophytes and 113 fungal endophytes from plants grown in India: *Digitalis lanata*, *Digitalis purpurea*, *Plantago ovata* and *Dioscorea bulbifera* (Ahmed *et al*., 2012). Indian herbs were also investigated by Amirita and colleagues, who managed to isolate 334 fungal strains inhabiting the internal tissues of *Adhatoda vasica*, *Costus igneus*, *Coleus aromaticus* and *Lawsonia* (Amirita *et al*., 2012). Another study of medicinal plants grown in Taiwan isolated 156 fungal endophytes from 20 species from the *Lauraceae* and *Rutaceae* family (Ho *et al*., 2012). In 2014 our team presented endophytic microflora of *Hypericum perforatum* (Rekosz-Burlaga *et al*., 2014). From stems and leaves of the tested plants four bacterial strains were isolated.

The aim of the present study was to describe the endophytic microflora of selected medicinal plants inhabiting European countries. Isolated bacteria were tested for their antifungal properties against a plant pathogenic fungi and strains displaying the greatest antifungal activity have been classified according to their morphological, physiological and molecular characteristics.

Plant samples were collected from two areas in central Poland, near Kozienice town (51.575°N, 21.750°E) and in Warsaw city (52.259°N, 21.020°E), during the vegetative seasons of 2007 and 2008. Bacterial endophytes were isolated from stems of 13 native growing herbal plant species: *Chelidonium majus* L., *Elymes repens* L., *Erigeron annuus* L., *Euphrasia rostkoviana* Hayne, *Foeniculum vulgare* L., *Geranium pratense* L., *Humulus lupulus* L., *Matricaria chamomilla* L., *Mentla arvensis* L., *Papaver rhoeas*L., *Rosmarinus officinalis* L. *Solidago gigantea* L. and *Vinca minor* L. Bacterial isolation from plant material was performed according to the procedure of Hung and Annapurna (2004). Five plants of each species were tested. Samples were first washed in distilled water for 5 minutes and the surfaces were sterilized by bathing in a mixture of 0,1% mercuric chloride ($HgCl_2$) and 70% ethanol. The samples were then rinsed four times in sterile distilled water and dried on sterile filter paper. To verify the efficiency of sterilization, samples were placed on nutrient agar. The water from the fourth rinse was also plated on nutrient agar to confirm sterilization. To isolate endophytes, the samples were cut longitudinally and placed cut side down on nonselective media, including water agar, nutrient agar, potato dextrose agar and tryptic soya agar. All plates were then incubated at 28°C for 2–7 days. The produced colonies were sub-cultured several times to obtain pure cultures. To confirm that the colonies originated from a single cell and were not mixed, their morphology was recorded and the bacteria were Gram-stained and observed under light microscope. Pure isolates were maintained at –20°C.

The antifungal properties of the bacterial isolates were assayed using an *in vitro* test with the following fungal strains: *Alternaria alternata* ES11, *Paecilomyces variotti* ES23, *Chaetomium* sp. E13, *Byssochlamys* sp. E9, *Aureobasidium* sp. E4 and *Fusarium* sp. E23. Fungal strains were obtained from Department of Microbial Biology, Warsaw University of Life Sciences in Poland. Fungal spores were spread onto the surface of potato-dextrose agar (PDA) and then three 20 µl drops of each bacterial culture (grown in nutrient broth at 30°C for 24 h with shaking at 180 rpm) were spotted onto the surface of the inoculated plate. All plates were then incubated for 7 days at room temperature (Goryluk *et al*., 2009). If bacteria inhibited fungal growth, a zone of inhibition appeared around the colonies. For the isolates with the strongest antifungal properties, the diameters of the hyaline inhibition zones were measured (mm). This test was repeated five times for each bacterial isolate. Data were analyzed using one-way analysis of variance. Homogenous groups of means were determined with the Tukey's procedure of multiple comparisons at the significance level 0.05. The analyses were performed using Statgraphics 4.1 statistical package. The isolates with the strongest antifungal activity were selected for further identification.

The identification of selected isolates was made based on morphological observations (1) and the biochemical properties (2) of the bacteria. The results were analysed according to Bergey's Manual of Systematic Bacteriology (Brenner *et al*., 2005; Vos *et al*., 2009). To confirm the species identification, molecular analysis of 16S rRNA gene sequences (3 and 4) was performed.

1) For colony characterization of the selected strains, the nature of the colonies, their pigmentation and shape were recorded. To describe the properties of the bacterial cells, microscopic observations were made to determine their shape and size, their ability to move and form spores as well as their Gram-staining characteristics.
2) The biochemical properties of the bacterial isolates were tested following standard protocols. Seven different carbon compounds (lactose, saccharose, glucose, arabinose, mannitol, rahmnose, citrate) were used to check the ability to substrate utilization, provided as the sole carbon source. The ability to gelatin, starch, urea and arginine hydrolysis was

monitored. Mixed-acid fermentation test (Methyl-Red test) was performed. The activities of catalase, sulfhydrolase and lysine decarboxylase were determined. Production of fluorescent pigments, indole and acetoine was evaluated. The ability to grow at extreme temperatures (4°C and 55°C) was tested.

3) Molecular characterization
Genomic DNA was isolated from the selected strains as described by Hung and Annapurna (2004). Using these DNAs as templates and primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') (Johri and Johri, 2004), fragments of the 16S rRNA genes were amplified by PCR. The amplicons were analyzed by electrophoresis on a 1% agarose gel, purified using a commercial kit (Clean up; A&A Biotechnology) and then sequenced using an automated DNA sequencer (454 GS FLX Titanium, Roche). The gene sequences were submitted to NCBI GenBank (accession numbers KJ130483-KJ130486). Bacterial strains were deposited in collection of Department of Microbial Biology, Warsaw University of Life Sciences in Poland.
4) Bioinformatics analysis
The 16S rRNA gene sequences from the bacterial isolates were compared with 16S ribosomal RNA sequences (Bacteria and Archaea) in the NCBI database using Standard Nucleotide BLAST with the default settings, to identify the most similar sequences. For each strain, 11 nucleotide sequences (unknown sequence and the 10 most similar) were aligned using CLUSTAL W2 (Larkin *et al*., 2007). The multiple sequence alignments were then used to create phylogenetic trees by the Neighbor Joining method with complete deletion of gaps, implemented in MEGA5 software (Saitou and Nei, 1987; Tamura *et al*., 2004; 2011).

To evaluate the antifungal properties of the selected bacterial isolates, their influence on the growth and development of *A. alternata* ES11 was assayed *in vitro*. A fungal spore suspension was prepared and added to 300 ml of PDB containing a suspension of bacterial cells. Control cultures were also prepared: PDB with fungal spores or PDB with bacteria. All cultures were incubated for 10 days at 25°C with shaking (1500 rpm). The numbers of bacteria and fungi were then evaluated using the plate count method and by microscopic analysis of the cultures. To prepare the fungal spore suspension *A. alternata* was cultured on PDA for one week at 25°C and the spores were harvested aseptically and suspended in sterile distilled water. To prepare the suspension of bacteria, the strains were cultured in nutrient broth for 12 h at 30°C with shaking (1500 rpm) and then 0.5 ml of these cultures were used to inoculate PDB. The plate count method was used to determine the concentration of fungal spores and bacterial cells.

Our results presented in this paper revealed that 12 tested herbs – *Chelidonium majus* L., *Elymus repens* L., *Erigeron annuus* L., *Euphrasia rostkoviana* Hayne, *Foeniculum vulgare* L., *Geranium pratense* L., *Humulus lupulus* L., *Matricaria chamomilla* L., *Mentha arvensis* L., *Rosmarinus officinalis* L., *Solidago gigantea* L. and *Vinca minor* L. are inhabited by bacterial endophytes. This is the first examination of endophytic microflora of the tested herbs, except *C. majus* L. which was tested for the first time by our team in 2007 (Goryluk *et al*., 2009). From the tested plants 181 bacterial isolates were obtained. The highest number of bacterial isolates came from *C. majus* (48 isolates) and from *G. pratense* (30 isolates) and the smallest number (6 isolates) from: *F. vulgare*, *S. gigantea* and *Matricaria chamomilla*. No isolates were obtained from *Papaver rhoeas*. This is not the first time, that a studied plant has apparently lacked endophytes. Soka *et al.* (2012) failed to isolate any endophytes from a specimen of the herb *Nothopanax scuttelarium*. It is not known whether the inability to isolate endophytic microorganisms from some species of plants is because they are naturally sterile, or if there is some methodological problem. All of the plants examined in our work share some common properties: they are very expansive, they grow in poor environments and have medicinal properties like anti-inflammatory, antibacterial, antifungal or anticancer activities. Plant extracts have frequently been used to treat medical conditions, and of the plants studied here, *C. majus* L. has been the source of numerous medicines: Ukrain (anti-cancer activity), Chelifungin (anti-fungal properties) and Di-Sancor (anti-HIV properties). In addition, infusions of *C. majus* have been shown to have a positive influence on the nervous and digestive systems (Ożarowski, 1976). This herb contains a number of active substances such as alkaloids. One outstanding issue is the contribution made by endophytic microorganisms to the medicinal properties of such plants. There are some examples where endophytes produce biologically active substances. One report presented in 1993 showed that *Taxomyces andreanae*, an endophytic fungus isolated from the yew tree *Taxus brevifolia*, produced paclitaxel (Stierle *et al*., 1993). This compound is used as an anticancer medicine (Taxol®) and previously had to be isolated from yew trees by a process that was long and costly due to low yield. The discovery that the endophyte is responsible for synthesizing paclitaxel has made the production of this medicine much easier. Another example is the endophytic fungus *Chaetomium globosum*, which produces hypericin isolated from the herb *Hypericum perforatum* (Kusari *et al*., 2008).

Endophytic bacteria isolated from tested plants were screened for antifungal properties against plant pathogenic fungi – *A. alternata*, *Paecilomyces variotti*,

Chaetomium sp., *Byssochlamys* sp., *Aureobasidium* sp. and *Fusarium* sp. These facultative fungal pathogens causes diseases of different plants all over the world, including various vegetables, fruits and cereal crops. Some of them, for example *A. alternata*, produces mycotoxins, which can be transferred from the infected plant to the tissues used as food. *Fusarium* species also produce mycotoxins, like zearalenone or fumonisins. *Fusarium* spp. they are important pathogens of many agricultural plants, like corn, wheat or soybean (Muthomi and Mutitu, 2003). Antifungal activity of bacteria has been detected in many genera and one of the significance of this kind of researches is the possibility to use of these bacteria as plant protection agents. Of the 181 endophytic isolates tested in our study, 53 displayed antifungal properties (29%). The highest number of bacteria with such activity was obtained from *C. majus* (22 isolates, 45%). 10 out of 18 isolates from *Erigeron annuus* L. (55%) and almost half of the isolates from *Mentha arvensis* L. (6 out of 13, 46%) had antifungal properties. Only one isolate with antifungal properties was obtained from *G. pratense* L. (3%), *Humulus lupulus* L. (12%), *S. gigantea* L. (16%) and *R. officinalis* L. (12%). Among the fungal species tested, *A. alternata* was the most sensitive to bacterial influence. The growth of this fungus was inhibited by 39 of the bacterial isolates. On the other hand, the least sensitive was *Fusarium* sp., which was inhibited by only one isolate: 30B. The growth of *Byssochlamys* species was inhibited by 7 isolates. The other fungi tested, *P. variotti*, *Chaetomium* sp. and *Aureobasidium* sp., were inhibited by 25, 26 and 27 of the bacterial isolates, respectively. The obtained results are in accordance with those achieved by our team earlier (Goryluk *et al.*, 2009) when isolates obtained from *C. majus* exhibited antifungal activity against *A. alternata*, *Chaetomium* sp. and *P. variotti*. In another study, Sgroy *et al.* (2009) used *A. alternata* to show that two endophytic bacteria (*Brevibacterium halotolerans*, *Bacillus pumilus*) isolated from *Prosopis strombulifera*, can inhibit fungal growth. Hui *et al.* (2012) isolated one endophytic species (*Bacillus subtilis*) from *Prunus mume* with antifungal properties against *A. alternata* and *Fusarium* sp.

Endophytic bacteria with the strongest antifungal properties were selected for further researches – 2–5b and 30B isolate from *C. majus*, P2 and P3 from *E. repens* and N2-1a from *S. gigantea*. First, taxonomic classification was conducted based on morphological, physiological and molecular characteristics. Afterwards, the diameter of fungal growth inhibition zones produced by these isolates and their influence on mycelium development of the fungus *A. alternata* was examined.

Only one of the isolates produced spores and was Gram-positive (30B). This isolate, in contrast to the remaining isolates, produced bigger cells (2.5×1.2 μm), formed chains and was not able to move. Gram-negative isolates formed smaller single cells (1.6×1.1 μm) and were able to move but very weakly. Some of the four Gram-negative isolates could be differentiated by the nature of their colonies. Isolate 2–5b produced round pink colonies with a regular shape, and a glossy and smooth appearance. Colonies of isolates P2 and P3 were similar in shape and appearance to those of 2–5b, but they produced fluorescent pigments. Isolate N2-1a also produced similar colonies, but without any pigmentation. In contrast, colonies of isolate 30B had an irregular crater-like shape and matt appearance.

All of the isolates could utilize lactose, sucrose, glucose, arabinose and mannitol, they were indole negative and catalase positive (Table I). Based on the

Table I
Biochemical characteristics of selected endophytic isolates.

Substrate utilization	Isolate				
	2–5b	30B	P2	P3	N2-1a
Lactose/saccharose	+/+	+/+	+/+	+/+	+/+
Glucose/arabinose	+/+	+/+	+/+	+/+	+/+
Mannitol/ rhamnose	+/+	+/–	+/–	+/–	+/+
Citrate/gelatin	+/–	–/+	+/+	+/+	+/–
Starch/urea	–/–	+/–	–/–	–/–	–/–
Arginine/lysine	–/–	+/–	+/+	+/+	–/+
Methyl-Red test	–	–	–	–	–
Metabolite production					
Fluoresceine/Pyocyanine	–/–	–/–	+/–	+/–	–/–
Hydrogen sulfide	–	+	–	–	+
Indole/catalase	–/+	–/+	–/+	–/+	–/+
Acetoine	+	+	–	–	+
Growth temperature 4°C/55°C	–/–	–/–	+/–	+/–	–/–

Table II
Phylogenetic affiliation of isolates based on the analysis of 16S rDNA fragments.

Isolate[a] (GenBank acc. No.)	Best match with database[b] (GenBank acc. No.)	Microbial affiliation
2–5b (KJ130483)	*E. persicina* (NR026049)	*E. persicina*
P2 (KJ130484)	*P. azotoformans* (NR037092)	*P. azotoformans*
P3 (KJ130485)	*P. cedrina* (NR042147)	
N2-1a (KJ130486)	*P. cedrina* (NR024912)	*P. cedrina*

[a] The sequence of isolate
[b] The sequences of isolates were compared with nucleotide sequences from database (similitude in 99%) and the phylogenetic tree was constructed; the best match was selected as the closest sequence from the phylogenetic tree

morphological and biochemical properties of the isolates, they were identified as *Bacillus* sp. (30B), *Erwinia* sp. (2–5b) and *Pseudomonas* spp. (P2, P3, N2-1a). The PCR amplification, sequencing and bioinformatic analysis of 16S rRNA gene sequences from each of the isolates enabled the identification of four of them to species level (Table II). Their phylogenetic affiliation was estimated based on the phylogenetic tree constructed to visualize the relationship between the sequences of isolates and related organisms from the GenBank database. Isolate 2–5b was determined as *Erwinia persicina* (GenBank accession number KJ130483), isolate P2 and P3 as *Pseudomonas azotoformans* (GenBank accession numbers KJ130484 and KJ130485, respectively) and isolate N2-1a as *Pseudomonas cedrina* (GenBank accession number KJ130486). Isolate 30B was placed within the *Bacillus subtilis* group, but further investigations are required to verify this species identification.

Endophytes classified as *Bacillus* and *Pseudomonas* species are very often isolated by researchers (Goryluk *et al*., 2009; Lodewyckx *et al*., 2001; Narayan *et al*., 2013; Rekosz-Burlaga *et al*., 2014). Liu *et al*. (2014) for example isolated *Bacillus* sp. strain and *P. azotoformans* strain as an endophytes from xerophilous moss *Grimmia montana*. *P. cedrina* was isolated by Behrendt *et al*. (2003) as plant associated bacteria inhabiting phyllosphere of *Solanum tuberosum* L. *Bacillus* and *Pseudomonas* genera are well-known for its production of diverse secondary metabolic products. Our results are in accordance with this statement because the strongest antifungal activity against *A. alternata* was reported for *Bacillus* sp. strain 30B (Table III). Fungal growth inhibition zone had 24 mm. Smaller inhibition zones were observed for *E. persicina* strain 2–5b and *P. cedrina* strain N2-1a, while the narrowest inhibition zones were produced by *P. azotoformans* strains P2 and P3. In contrast, these strains had the strongest activity against *Chaetomium* sp. while other strains produced smaller inhibition zones. Only one strain, *Bacillus* sp. 30B, inhibit the growth of all tested fungi, even *Fusarium* sp. strain. Similar results were obtained by Narayan *et al*. (2013) who isolated *B. tequilensis* endophytic strains with strong and broad spectrum of antifungal activity against all tested pathogenic fungi, like *Alternaria panax* and *Fusarium oxysporum*. This strain produced fungal inhibition zones wider than 8 mm. Other researchers, Tschen and Tseng (1989) isolated *Bacillus* sp. strains active against *Fusarium* sp. and *Paecilomyces* sp. which produced bacereutin. Axelrood *et al*. (1996) in turn, obtained *B. amyloliquefaciens* strains with antifungal activity against *Fusarium oxysporum*. *B. amyloliquefaciens strains* with strong activity against *Fusarium* sp.

Table III
Antifungal activities of bacterial endophytes (named 2–5b, 30B, P2, P3, N2-1a) against six fungal strains.

Isolates	Fungal growth inhibition zone (mm)					
	A. alternata	*Chaetomium* sp.	*P. variotti*	*Byssochlamys* sp.	*Aureobasidium* sp.	*Fusarium* sp.
2–5b	18.6 (b)	10.6 (a)	n	n	n	n
30B	24.0 (c)	11.2 (a)	19.0	6.2	7.0	14.4
P2	8.4 (a)	16.4 (bc)	n	n	n	n
P3	N9.4 (a)	18.0 (c)	n	n	n	n
N2-1a	15.2 (b)	12.4 (ab)	n	n	n	n

n – no inhibition zone
Letters in parenthesis indicate homogenous groups of means, which do not differ significantly at $\alpha = 0.05$. Analysis were prepared separately for each fungal strain.

were also isolated by Cuijuan *et al*. (2014). To confirm the antifungal activity against *A. alternata*, dual-culture experiment was performed. After 10 days of incubation of fungal spores with the bacteria, no fungal colonies were detected in any of the cultures, while the bacterial number were slightly reduced (*E. persicina* strain 2–5b and *Bacillus* sp. strain 30B) or unchanged (*Pseudomonas* spp. strains P2, P3 and N2-1a). Microscopic observations showed that fungal spores incubated in presence of bacteria were deformed and unable to grow and form mycelium.

Presented researches revealed that tested herbs: *C. majus* L., *E. repens* L., *E. annuus* L., *Euphrasia rostkoviana* Hayne, *F. vulgare* L., *G. pratense* L., *H. lupulus* L., *Matricaria chamomilla* L., *Mentha arvensis* L., *Rosmarinus officinalis* L., *Solidago gigantea* L. and *Vinca minor* L. are inhabited by endophytic bacteria. 29% of the isolates displayed antifungal properties against plant pathogens. The highest number of endophytes with antifungal activity was obtained from *C. majus* L., *E. annuus* L. and *Mentha arvensis* L. (45%, 55% and 46%, respectively). The highest antifungal activity was recorded for *Bacillus* sp. strain 30B which was isolated from *C. majus* L.

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SHORT COMMUNICATION

Effects of Selected Herbicides on Growth and Nitrogen Fixing Activity of *Stenotrophomonas maltophilia* (Sb16)

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Abstract

A study was carried out to determine the effects of paraquat, pretilachlor and 2, 4-D on growth and nitrogen fixing activity of *Stenotrophomonas maltophilia* (Sb16) and pH of Jensen's N-free medium. The growth of Sb16 and pH of medium were significantly reduced with full (X) and double (2X) doses of tested herbicides, but nitrogen fixing activity was decreased by 2X doses. The nitrogenase activity had the highest value in samples treated with 1/2X of 2, 4-D on fifth incubation day, but 2X of 2, 4-D had the most adverse effect. An inhibition in the growth and nitrogenase activity was recovered on the last days of incubation.

Key words: *Stenotrophomonas maltophilia* (Sb16), nitrogenase activity, paraquat, pretilachlor, 2, 4-D

Microorganisms employed to enhance the availability of nitrogen by fixing atmospheric N_2 to the crops are called N_2 fixing bacteria. There is a natural association between rice plant and N_2 fixing bacteria. *Stenotrophomonas maltophilia* is broadly found on or in plants and has a worldwide distribution (Denton and Kerr, 1998). Diazotrophic *S. Maltophilia* strain Sb16 isolated from Tanjong Karang, a rice (Oryza sativa L.) growing area in Selangor, Malaysia, has been proven to improve rice production (Naher *et al*., 2009). The Sb16 strain used in this study was obtained from the Soil Microbiology Laboratory, Department of Land Management, Universiti Putra Malaysia (UPM), Serdang, Selangor. Herbicides have been the most efficient chemical weed management approach since they were introduced to agriculture. They may cause undesirable effects when applied at high concentrations. Various herbicides act on various types of plant species, on various processes of plant metabolism, and at various periods in plant growth cycles. Translocated herbicides are taken up into the plant's vascular system, while contact herbicides only affect the part of the plant contacted by the spray. Some herbicides represent a risk of vapourising to other sites, while others stay effective for a long term in the soil, harming planted crops at a later time. If they come into contact, herbicides applied to soil or plants might interfere with the microbial biofertiliser inoculated to crop plants. The herbicides commonly used for rice production are paraquat, glyphosate, oxadiazon, propanil, pretilachlor, 2, 4-D, *etc*. Paraquat, pretilachlor and 2, 4-D have been chosen for the present study. The effect of herbicides on N_2 fixing activity of diazotrophs is a major concern among researchers as it is vital to the soil fertility of rice fields. Meanwhile, studies on the effects of herbicides on symbiotic N_2 fixation have concentrated on *Rhizobium* sp. under *in vitro* conditions (Moorman, 1986). However, there are insufficient studies on the effects of herbicides on other species of N_2 fixing bacteria. Besides, as attributing of N_2 fixation to the specific bacterium in the plant system is impossible, the effects of herbicides on N_2 fixation ability of the specific bacterium need to be determined in laboratory conditions to predict the role of strain in N_2 fixation process in the plant system under natural soil condition. The objective of the present investigation was to determine the effect of paraquat, pretilachlor and 2, 4-D at concentrations corresponding to 1/2, 1 and 2 times of their recommended field application rate (X) on the growth and N_2 fixing

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activity of Sb16 and pH of Jensen's N-free medium (Jensen, 1951) within a determined incubation time.

Medium was inoculated with a colony of Sb16 strain and incubated at 35°C on a rotary shaker at 150 rpm for 3 days. Aliquots of 1 ml of approximately 10^8 cfu/ml of live cells, with adjusted optical density $(OD)_{600}$ of ≈ 0.1, was inoculated to each culture flask.

The herbicides used in this study were paraquat dichloride (13% w/w) Syngenta Capayam, pretilachlor (28.7% w/w) Syngenta Sofit N300 EC and 2, 4-D isopropylamine (28% equivalent) (35.5 % w/w) Kompressor Ancom Cropcare. The herbicides solutions were prepared by mixing the required amount of active ingredient in sterilised distilled water to obtain concentrations corresponding to 0, 1/2, 1 and 2 times of the recommended field application rate. Therefore, four rates of 0, 0.78, 1.56 and 3.12 mg/ml of paraquat at the rate of 12 g/l; 0, 0.72, 1.44 and 2.87 mg/ml of pretilachlor at 5 g/l; 0, 1.42, 2.84 and 5.68 mg/ml of 2, 4-D at 8 g/l were prepared to get the concentrations of 0, 1/2, 1 and 2 times of the recommended field application rate, respectively. Thereafter, herbicides were sterilised by filtration (Millipore filter, 0.22 µm) aseptically in a laminar flow cabinet.

The prepared herbicides solutions were added to each sterilised flask (250 ml) containing 75 ml Jensen's N-free broth medium. Control flasks were without herbicides. Before inoculum application, optical density (OD_{600}) of inoculum was checked and regulated to approximately 0.1 and the drop plate method for cell count on N-free agar was employed to confirm the population (Somasegaran and Hoben, 1985). Aliquots of 1 ml of the desired inoculum (approximately 10^8 cfu/ml) of live cells was transferred to each culture flask using sterilised pipette. The flasks were incubated at 28°C on a rotary shaker for 7 days till the end of the experiment.

At each sampling period, 1 ml of the culture was sampled and 10-fold serial dilutions were made up to 10^{-8}. The mixture in each test tube was shaken vigorously to suspend bacterial cells. Aliquots of 0.1 ml of appropriate dilutions was placed on each Jensen's agar plate. The plates were then incubated at 32°C. The population was determined using the drop plate method at 24 h intervals within 7 days.

The N_2 fixing activity of Sb16 strain in Jensen's N-free broth, amended with paraquat, pretilachlor and 2, 4-D, was determined using acetylene reduction assay (ARA) based on the methods by Hardy *et al.* (1968) and Somasegaran and Hoben (1985). At every 24 h interval, 1 ml of the suspension was taken from each flask and transferred to a 10 ml air-tight Syringe. A sample of 10% of air was extracted from each syringe and pure acetylene gas (99.8%) was injected with a gas-tight syringe. The syringes containing bacterial suspensions were allowed to incubate on incubatory shaker for 1 h. Thereafter, 1 ml of the air sample from each incubated syringe was injected into a Gas Chromatography (HP 6890) equipped with Hydrogen Flame Ionisation Detector (FID) with a temperature of 120°C, injector temperature 150°C with Column (Agilent J&W GC Column, HP-PLOT/Q, 30 M, ID 0.53, Film Thickness 40 µm) and carrier gas (nitrogen) 70–80 kPa for lighting the FID, Hydrogen 100 kPa and air 10 kPa. The produced ethylene was determined based on the transformation rate of acetylene to ethylene (% v/v).

Changes in the pH of Jensen's N-free broth, inoculated with Sb16 strain and amended with herbicides, were determined using a standard pH meter (pHM 210, MeterLab®) equipped with a glass electrode at every 24 h interval within 7 days.

The study was conducted as factorial complete randomised design (CRD) with four replications. The factors were 3 types of herbicides with 4 different concentrations and 7 incubation periods. The data were subjected to analysis of variance (ANOVA) and analysed using SAS (version 9.3). The treatment means were compared by using Duncan's multiple range test (DMRT). The number of bacteria was $\log_{10}$ transformed before statistical analysis.

Results of our study shown that population of bacteria in the presence of each of the herbicides exhibited a similar trend. The population significantly increased from day 1 – day 3 of the incubation period. However, it decreased on the fourth day, followed by a significant increase on the fifth day. The population significantly declined from the sixth-seventh day. Meanwhile, the population in the samples treated with double dose of paraquat reached that of the control sample on the last day of incubation (Fig. 1A). In particular, Sb16 had the lowest population (6.25 $\log_{10}$ cfu/ml) in the samples amended with double dose of pretilachlor on the seventh day (Fig. 1B). However, the highest population (7.93 $\log_{10}$ cfu/ml) was obtained in the samples

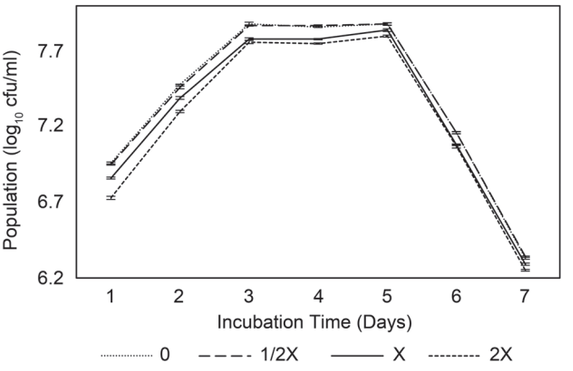

Fig. 1A. Effect of different concentrations of paraquat on population of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

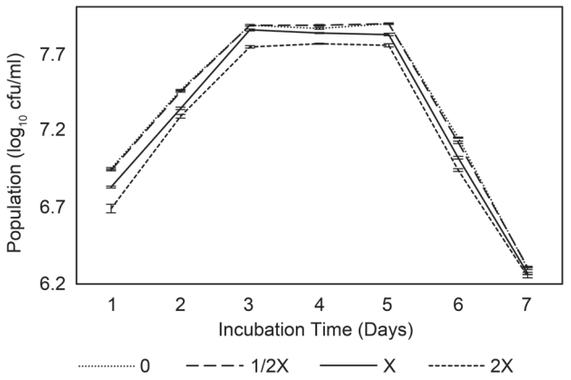

Fig. 1B. Effect of different concentrations of pretilachlor on population of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

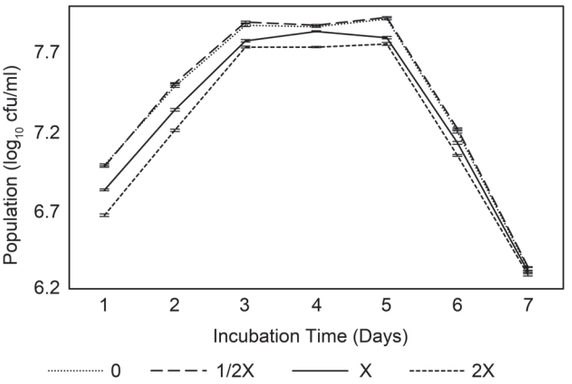

Fig. 1C. Effect of different concentrations of 2, 4-D on population of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

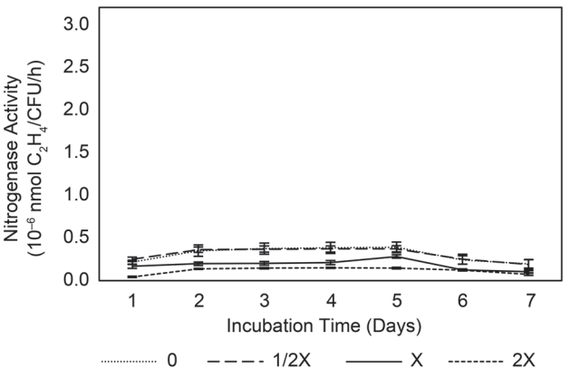

Fig. 2A. Effect of different concentrations of paraquat on nitrogenase activity of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

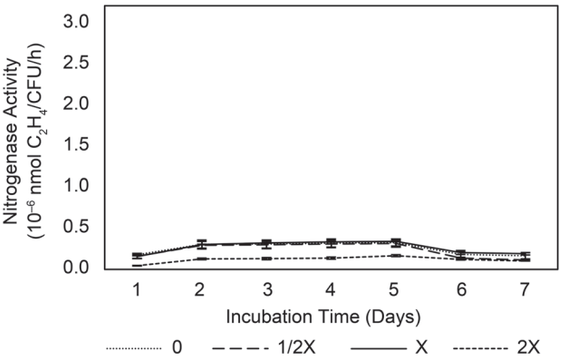

Fig. 2B. Effect of different concentrations of pretilachlor on nitrogenase activity of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

amended with half dose of 2, 4-D on the fifth day (Fig. 1C). Based on the results of statistical analysis, the population was significantly influenced by the type of herbicides. The population decreased significantly upon treatment with herbicides at full and double doses when compared with the control. There was a significant three-way interaction effect between the herbicides, concentrations and incubation time on the Sb16 population.

The nitrogenase activity in the presence of each of the herbicides increased from the first to fifth incubation day, followed by a decline up to day 7. The nitrogenase activity in the samples amended with double dose of paraquat started to decline from the fifth incubation day, but those with the half and full doses decreased from the sixth day (Fig. 2A). The nitrogenase activity in the samples amended with full dose of pretilachlor reached to that treated with half dose on the second day (Fig. 2B.). The highest nitrogenase activity (3.1×10^{-6} nmol C_2H_4/cfu/h) was obtained in the samples amended with half dose of 2, 4-D on

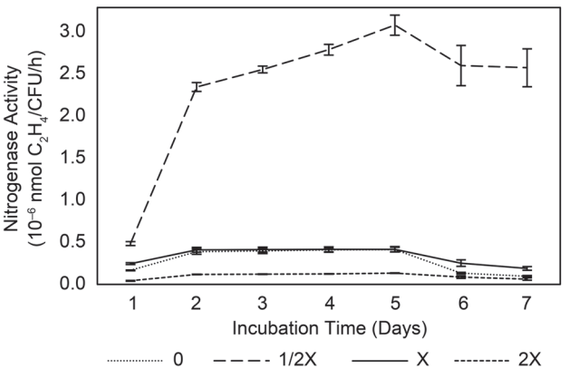

Fig. 2C. Effect of different concentrations of 2, 4-D on nitrogenase activity of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

the fifth day. However, the lowest nitrogenase activity (4.2×10^{-8} nmol C_2H_4/cfu/h) was recorded in the samples amended with double dose of 2, 4-D on the first day (Fig. 2C). Results of the statistical analysis

showed no significant differences in nitrogenase activity between samples amended with paraquat and pretilachlor. Addition of half dose of herbicides resulted in a significant increase in nitrogenase activity compared to the control, while nitrogenase activity decreased significantly in the presence of double dose of herbicides. Herbicides, concentrations and incubation time showed a significant three-way interaction effect on the nitrogenase activity of Sb16.

Acidity (pH) in the presence of each of the herbicides significantly increased from days 1–5 of incubation, followed by a significant decline till day 7. Acidity in the samples amended with half, full and double doses of paraquat reached the value of the control sample on the seventh day (Fig. 3A). The highest pH in herbicide amended samples was recorded by 7.46 with half dose of pretilachlor on the fifth day (Fig. 3B). However, the lowest pH (6.8) was obtained in the samples amended with double dose of 2, 4-D on the first day (Fig. 3C). The statistical study of the results showed that pH did not differ significantly between samples amended with paraquat and pretilachlor. Similarly, pH of Jensen's medium was not affected significantly by half dose of herbicides but significantly decreased in the presence of full and double doses of herbicides. There was a significant three-way interaction effect between herbicides, concentrations and incubation time on the pH of Jensen's medium.

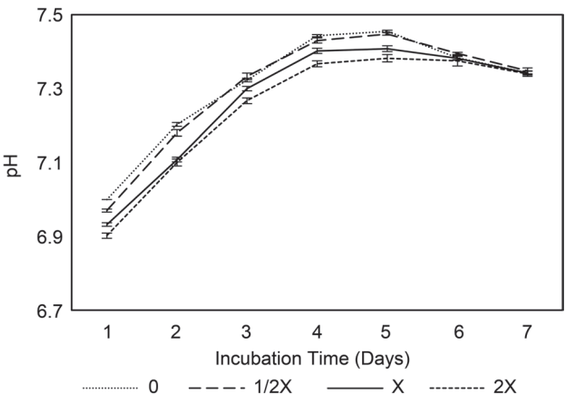

Fig. 3A. Effect of different concentrations of paraquat on pH of Jensen's N-free medium inoculated with diazotrophic Sb16 during 7 days of incubation period; Bars indicate standard error (n = 4).

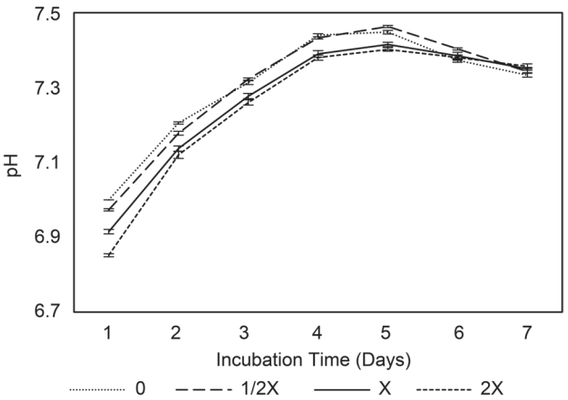

Fig. 3B. Effect of different concentrations of pretilachlor on pH of Jensen's N-free medium inoculated with diazotrophic Sb16 during 7 days of incubation period; Bars indicate standard error (n = 4).

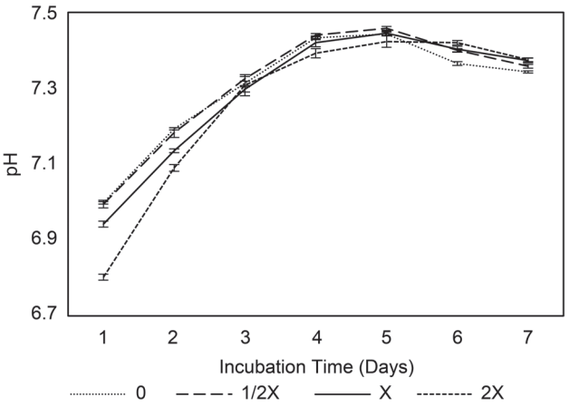

Fig. 3C. Effect of different concentrations of 2, 4-D on pH of Jensen's N-free medium inoculated with diazotrophic Sb16 during 7 days of incubation period; Bars indicate standard error (n = 4).

An inhibition in the growth of Sb16 resulting from the addition of higher concentrations of herbicides was recovered on the last incubation days. This finding concurs with the study of Latha and Gopal (2010) which revealed an initial decrease in the growth of *Azospirillum* lipoferum with herbicides 2, 4-D, butachlor, pretilachlor and pyrazosulfuron compared to the control treatment after 24 h of incubation, followed by an increase over time. The increase in bacterial population over time can be due to the mineralisation of the herbicides by the bacteria as energy and carbon sources. Soil microorganisms use many herbicides as good carbon and/or nitrogen sources (Qiu *et al.*, 2009). The insignificant effect of half dose of herbicides on growth of Sb16 and pH of medium can be explained by alterations in the medium nutrient, chemical structure and herbicide degradation by the bacteria. Ayansina and Oso (2006) found lower microbial counts in higher concentrations of herbicides compared to the recommended doses.

Adeleye *et al.* (2004) studied the toxicity of 2, 4-D amine to *Bacillus subtilis* and found that a decrease in the survival percentage occurred at higher concentrations. Smith and Beadle (2008) also reported the toxicity of 2, 4-D and its metabolic intermediates on *Burkholderia cepacia.* The significant stimulation in the nitrogenase activity of Sb16 in lower concentrations of 2, 4-D in the present study corresponds to the findings of Saikia *et al.* (2014) who reported a higher rate of acetylene reduction in seedling roots of citronella ino-

culated with *Azospirillum brasilense* and treated with 2, 4-D than with *A. brasilense* alone. 2, 4-D does not only seem to affect several distinct metabolic pathways in a variety of organisms, but shows a direct biphasic effect depending on its initial concentration (Toyoshiba *et al.*, 2006). At low concentrations, 2, 4-D might stimulate growth of the organism by cell division and elongation; however, it may induce abnormalities at high concentrations. Non-targeted organisms exposed to 2, 4-D might respond by starting a metabolic chain-reaction that is commonly associated with changes in the cellular membrane integrity and fluidity.

Based on the data obtained on growth and N_2 fixing activity in the present study, paraquat showed moderate toxicity on Sb16 strain. Drouin *et al.* (2010) found that paraquat applied in a range between 0.367–367 kg/ha had no bactericidal effect on *Sinorhizobium* strains, but it inhibited three strains of *Bradyrhizobium* at 36.7 kg/ha. Paraquat toxicity is thought to be mediated by the superoxide anion (O_2^-), a reactive species generated by the reoxidation of reduced paraquat by molecular oxygen (Bus *et al.*, 1974).

According to the results of the present study, pretilachlor had the highest adverse effect on the growth and nitrogenase activity of Sb16. However, as research on the effect of pretilachlor on pure cultures of bacterial strains *in vitro* condition is scarce, the exact mechanism of the action of this chemical in microorganisms remains unknown. In general, the microbial degradation of chloroacetanilide herbicides can be initiated by two reactions; formation of glutathione conjugate (Stamper and Tuovinen, 1998) or N-dealkylation (Li *et al.,* 2013).

Meanwhile, during the incubation period, a variety of factors can influence the growth and nitrogenase activity of bacteria. These factors include O_2, CO_2, nutrients and inorganic salts of the medium, as well as some environmental factors such as light, pH and temperature. Thus, changes in any one of these factors, within the seven-day culture, affect the growth and nitrogenase activity of Sb16.

In the present study, the decrease of pH in the Jensen's medium amended with herbicides could be due to the decrease in the growth and nitrogenase activity of Sb16 following the contact with herbicides, leading to a more acidic medium. Optimal pH for N_2 fixation is 5–8 (Leigh, 2002). Through N_2 fixation by Sb16, ammonium ion (NH_4^+) can be produced. The presence of high NH_4^+ concentration increases pH of the medium. The increase in the pH of the medium on the last incubation days after the initial inhibition could be related to an increase in the growth and activity of Sb16 and also adaptability to stressful conditions.

The study showed that the tested herbicides at half dose had a positive effect on the growth and nitrogenase activity of Sb16. However, double dose significantly decreased the growth and nitrogenase activity of Sb16 and pH of the Jensen's medium. An increase in the activity and growth of Sb16 and pH of the Jensen's medium amended with double doses was found to occur on the last incubation days. It can be concluded that at their recommended doses, the tested herbicides might have insignificant effects on the growth and nitrogenase activity of Sb16 under natural field conditions. Further studies involving other strains of *S. maltophilia* need to be carried out so as to better clarify the susceptibility of these diazotrophic strains to the herbicides.

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SHORT COMMUNICATION

Basidiospore and Protoplast Regeneration from Raised Fruiting Bodies of Pathogenic *Ganoderma boninense*

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Abstract

Ganoderma boninense, a phytopathogenic white rot fungus had sought minimal genetic characterizations despite huge biotechnological potentials. Thus, efficient collection of fruiting body, basidiospore and protoplast of *G. boninense* is described. Matured basidiocarp raised under the glasshouse conditions yielded a total of 8.3×10^4 basidiospores/ml using the low speed centrifugation technique. Mycelium aged 3-day-old treated under an incubation period of 3 h in lysing enzyme from *Trichoderma harzianum* (10 mg/ml) suspended in osmotic stabilizer (0.6 M potassium chloride and 20 mM dipotassium phosphate buffer) yielded the highest number of viable protoplasts (8.9×10^6 single colonies) among all possible combinations tested (regeneration media, age of mycelium, osmotic stabilizer, digestive enzyme and incubation period).

Key words: basidiocarp, basidiospore, incubation, lytic enzyme

Ganoderma boninense, a phytopathogenic fungus causes basal stem rot (BSR) disease in oil palm and thus, is deemed a prime candidate for genetic, molecular and transformation studies. Very little studies have documented the biology and molecular genetics of *G. boninense* (Shi *et al*., 2012). The studies of fungal functional genomics are becoming more prevalent with the availability of fully sequenced genomes (Michielse *et al*., 2005; Wang *et al*., 2010). Currently, genome sequencing of *G. boninense* is underway (Malaysian Palm Oil Board) (http://www.genomesonline.org), hence an efficient protoplast and basidiospore production system would prepare the species to functional studies related to BSR disease. The objective of this study is to develop a highly efficient method for protoplast regeneration from basidiospores collected from raised fruiting bodies under the glasshouse conditions. A morphological description of the ideal fruiting structure which gives the best sporulating activity was determined. In order to determine the optimal condition desired for *G. boninense* protoplast isolation and regeneration, factors such as age of mycelia, type of osmoticum, lytic enzymes and enzyme incubation period were investigated.

The *G. boninense* strain T10 was provided by Applied Agricultural Resources, Sdn Bhd (AAR), Sungai Buloh. The strain was verified positive *G. boninense* as indicated by visible brown ring formation under the Ganoderma Selective Medium (GSM) amendment. The fungus was maintained on maltose extract agar (MEA; Merck) prior to usage. A rubber wood block (RWB) measuring $6 \times 6 \times 6$ cm was soaked in distilled water overnight prior to sterilization at 121°C for 30 min. The RWB was supplemented with 100 ml of molten MEA, swirled to uniform distribution. Three pieces of mycelia cubes (1×1 cm) were inoculated at random sides of the RWB, thereafter incubated in darkness at room temperature for six weeks. Sterilized soil mixture prepared at 3:2:1 topsoil/peat/sand was filled in flower pot (40×30 cm) covering 3:4 parts of the total volume. The RWB inoculum with an attached oil palm seedling (3-month-old) on its side was carefully planted into soil, at about 1–2 cm from the soil surface, positioned vertically to expose the adaxial axis

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of the RWB to the open air. Soil was maintained moist under regular watering. Development of the *G. boninense* fruiting body was monitored for a period of five months throughout a hot and wet climate. The resultant fruiting body harvested at varying maturity periods were subjected to basidiospore isolation. Fruiting body was immersed into 10% Chlorox solution for 30 min followed by 70% ethanol for 10 min. A sterile razor blade was used to cut the hymenium region into small cubes of 5 × 5 mm each. The excised cubes (0.5 g) were dropped into 100 ml of deionized water. The mixture was subjected to shaking for 30 min at 85 × *g* at room temperature. The mixture was filtered using a small amount of glass wool fitted into a glass tunnel. The filtrate was collected as aliquots of 2 ml. The basidiospore suspension was subjected to low speed centrifugation at 1000 × *g* for 1 min. The concentration of the basidiospore suspension harvested from the supernatant region was determined using a haemocytometer. Direct inoculation onto PDA (39 g/l), MEA (48 g/l) and Ganoderma Complete Medium (GCM) plates using a sterile cotton swab was performed to evaluate presence/ absence of contaminants. The GCM medium was prepare following Choi *et al*. (1987) with slight modifications; glucose 30 g/l, sucrose 20 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, K_2HPO_4 1 g/l, KH_2PO_4 0.5 g/l, yeast extract agar 10 g/l, casein hydrolysate 5 g/l. Basidiospore suspensions (10^5–10^6 ml^{-1}) were subjected to serial dilution to yield 3–5 mycelial colonies in each 100 ml PD-broth. Mycelia were harvested from the liquid culture at day 3, 4 and 5 to investigate the effect of age on protoplast release. The osmotic stabilizer was prepared to constitute either sucrose or potassium chloride at 0.6 M in presence of 20 mM dipotassium hydrogen phosphate. Lytic enzyme fixed at 10 mg/ml, was diluted into the osmotic stabilizer. Commercial lytic enzymes utilized were β-(1–3) D-glucanase, lysing enzyme from *Trichoderma harzianum* and Driselase (Sigma-Adrich, USA). The mixture containing mycelia and osmotic stabilizer (1:5 weight/vol) was incubated in a water bath at 30°C for 5 h. The production of protoplast was examined at 1, 3 and 5 h throughout the incubation period. The resultant protoplasts were spread onto regeneration media consist of PDA and GCM, supplemented with 0.6 M sucrose and 20 mM phosphate buffer. The number of viable protoplasts released was observed as the number of single colonies (mycelial pellets). The number of colonies appeared on the surface of the regeneration plates at day 10 after inoculation was recorded.

At week-3 after the artificial set-up for development of *G. boninense* fruiting bodies, visible hyphal knots were observed on the surface of the potting media, suggesting the initiation of photomorphogenesis. The rates of differentiation among the pots were highly variable. This in turn suggests presence of individual niches among the pots (Kamada, 2002) affecting polarisation of hyphae into fruiting structure. Fruiting body initials were observed protruding out from the surface of RWB at week-4 onwards. The sporophore was about 1 cm, white in colour, and round in shape with no visible hymenium (Fig. 1A). Next, the single-structured sporophore budded to form two of more structures round in shape with white surface, affixed on a deep red-brownish coloured stunted stipe (Fig. 1B). At this stage, visible hymenium was observed on the underside (white surface) with poor distribution. The sporophore differentiates into primordium/basidiocarp with characteristic cap and stipe formation. The cap takes up a bracket shape with reddish to orange colour on its outer surface, white coloured on its terminal region and confluent formation of visible hymenium on its underside (Fig. 1C). As the basidiocarp continues to develop, the cap showed deep red colouration entirely with orange coloration at the sub-terminal region and white coloration at the terminal region of the bracketed shape. On the underside, even distribution of visible hymenium was observed (Fig. 1D). Finally, the bracketed shape showed a completely deep red coloration and shrunk over time. The underside was found covered with visible hymenium (Fig. 1E).

In order to determine the effect of fruiting body's maturity on production of basidiospores, five distinct sizes of fruiting bodies were harvested. Fruiting bodies with a basidiocarp diameter in a range from 4–6 cm (Fig. 1D) showed highest rate of basidiospore production with a yield of 83×10^3 ml^{-1} mycelial pellets. Sporophore (less than 1 cm) with no visible formation of hymenium failed to sporulate even in the presence of a distinct fruiting structure. It appeared that the best way to determine a sporulating fruiting body would be to harvest fruiting bodies with a well-developed basidiocarp (diameter >3 cm), visible hymenium and brightly coloured (Fig. 1C-1E). Sporophore with invisible hymenium showed poor rate of sporulation in comparison to well-developed basidiocarp, which showed visible hymenium (Fig. 1F) and large pore size. Basidiocarp with a pale colouration showed poor sporulation activity with a total of 35×10^3 ml^{-1} mycelia pellets, indicates senescence period of the fungus life cycle (Fig. 1E). Amongst the regeneration media selected, PDA showed the best regeneration capability followed by GCM (Fig. 2A) whilst MEA (data not shown) showed poor ability to regenerate basidiospores. This protocol recruited a combination of both forcible spore discharge method and the density gradient method which are widely employed for basidiomycetes basidiospore isolation (An, 2005). Hymenium was selectively harvested for basidiospore isolation as this region enclosed within the hard fruiting structure, renders a highly sterile environment. The tissues were dis-

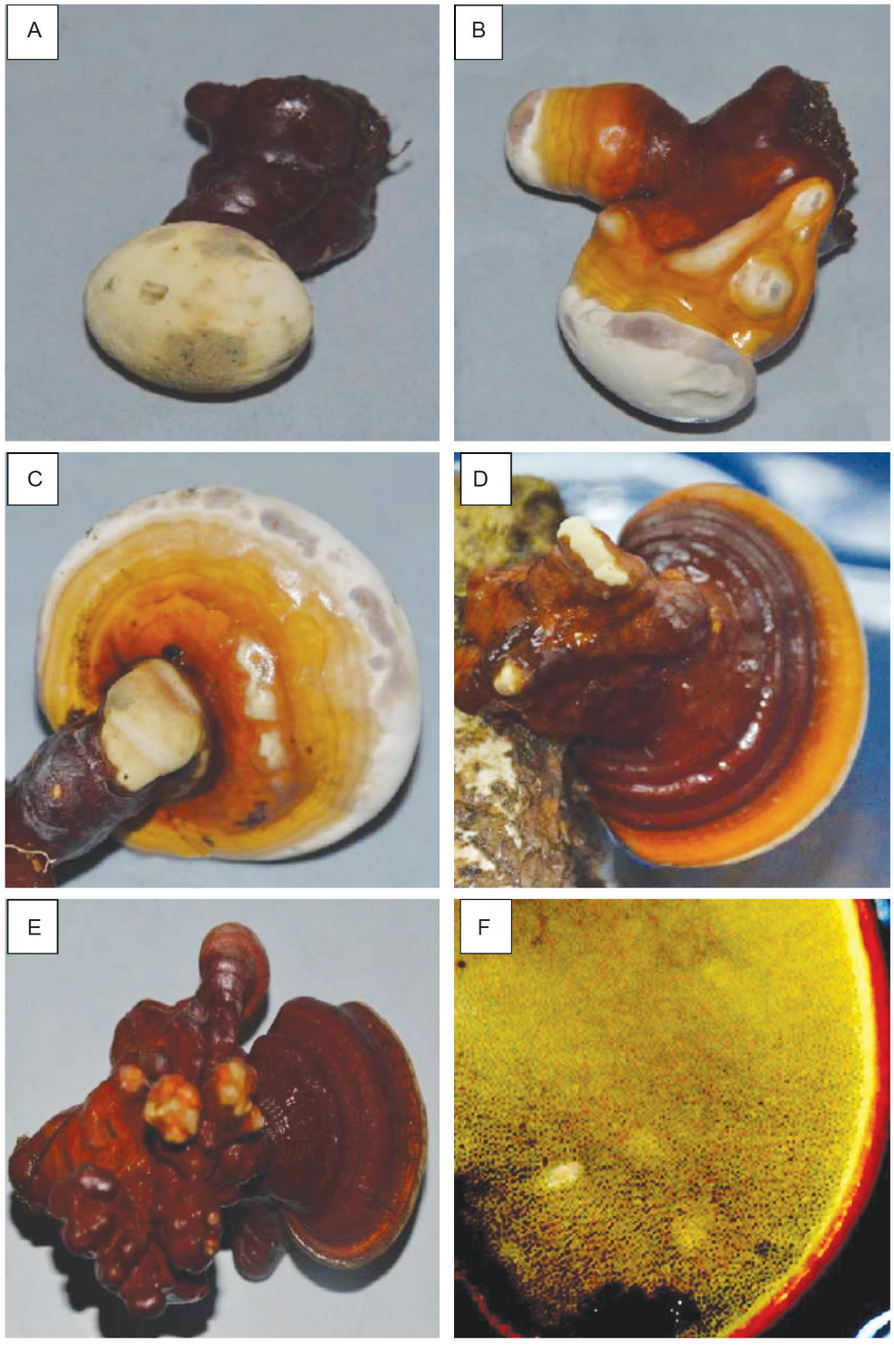

Fig. 1. Fruiting bodies of *G. boninense* harvested at different developmental stages. A) Sporophore; B) Budding sporophores; C) Basidiocarp; D) Matured basidiocarp; E) Senescent basidiocarp; F) Hymenium observed at underside of matured basidiocarp.

sected into small pieces to increase the surface required to allow the adhering basidiospores to escape into liquid solution during the shaking process. Ethanol based cleaning facilitated removal of coloured compound present on the fruiting structure. A prolonged pre-treatment of basidiospores under 10% Chlorox solution explained the resistant nature of the *G. boninense* basidiospores, which showed no adverse effect over the regeneration process. Centrifugation at relatively low speed enables large particles (contaminants) to be easily separated from basidiospores which are much smaller in size, yielding a virtually contaminant free basidiospore suspension as evident during basidiospore regeneration into mycelial colonies (Fig. 2A).

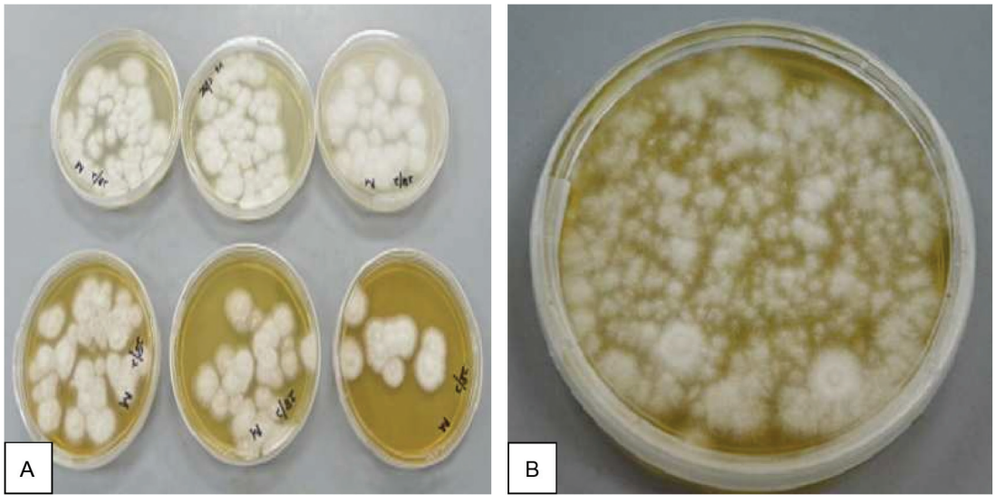

Fig. 2. A) Basidiospores (*G. boninense*) regenerated on PDA (above) and GCM (below) in absence of antibiotics supplementation on day 14 after inoculation.

B) Single colonies regenerated on PDA suspended in phosphate buffer and 0.6 M sucrose from protoplast suspension after 3 h enzymatic digestion using 10 mg/ml lytic enzyme from *Trichoderma harzianum* and 0.6 M KCl as osmotic stabilizer at day 10 after inoculation.

The number of protoplasts regenerated under all possible combinations tested in this study ranged from $2–89 \times 10^5$ single colonies ml^{-1} (Table I). To assign the best possible combination for efficient protoplast regeneration, treatments with mycelial colonies ranging at $10^6–10^5$ were designated as high, $10^4–10^2$ being moderate and below 10^2 as poor yield. Each treatment was evaluated using mycelium at different ages subjected to varying lytic enzymes, osmotic stabilizers and growth media under selected incubation periods. Mycelia were harvested at day 3, 4 and 5 to identify optimal age for protoplast production. Day 1 and 2 were not utilized since no visible mycelium mass were observed. Mycelia aged 3-day-old produced the highest viable protoplast yield (89×10^5 single colonies ml^{-1}) on PDA when treated with lytic enzyme from *T. harzianum* at an incubation period of 3 h (Fig. 2B) followed by mycelia aged 4-day-old (14×10^3 single colonies ml^{-1}) under a similar treatment conditions. Moderate protoplast yield was observed on mycelia aged 5-day-old (35×10^2 single colonies ml^{-1}) with peaked protoplast yield at an incubation period of 5 h. The protoplast production observed during 3 h incubation period was relatively moderate to high for 3 and 4-day-old mycelia ($3 \times 10^2–78 \times 10^5$ single colonies ml^{-1}). In contrast, the 5-day-old mycelia ($5 \times 10^2–84 \times 10^3$ single colonies ml^{-1}) recruited a prolonged incubation period, 5 h for an optimal digestion of a more intricate cell wall structure. The structure of fungal cell wall remodels throughout its growth. Newly synthesized cell walls are generally more thin and smooth in comparison to older tissues which comprise of primary and secondary layers which are heavily deposited with amorphous matrix materials (Annamalai and Lalithakumari, 1991; Lalithakumari, 1996).

Incubation period was observed as limiting factor throughout the three intervals. Mycelial age was found to affect the protoplast productions. Generally, a similar trend was observed on the incubation period tested among all treatment combinations. Poor to moderate protoplast production was observed in the first hour ($2–76 \times 10^2$ single colonies ml^{-1}), however, protoplast yield increased rapidly and peaked at 3 h ($8–89 \times 10^5$ single colonies ml^{-1}). At 5 h, protoplast yield declined ($2 \times 10^2–84 \times 10^3$ single colonies ml^{-1}) probably indicates the proteolytic activity of the pre-existing protoplast which affects the production of newly-formed protoplast (Robinson and Deacon, 2001). Enzyme incubation period of 3 h was found effective for maximal protoplast production from *G. boninense* mycelia of 3-day-old (89×10^5 single colonies ml^{-1}) and 4-day-old (14×10^3 single colonies ml^{-1}). In basidiomycetes, the fungal cell wall constitutes α-1-3-glucan, xylose and hemicelluloses (Adams, 2004). Three different commercial lytic enzymes from different origin exhibited variable enzymatic enzyme-cell wall digestibility of *G. boninense*. The lytic enzyme from *T. harzianum* contains β-glucanase, chitinase, cellulase and protease activities. This preparation has been predominantly employed for protoplasing filamentous fungi (Liu *et al.*, 2010; Rivera *et al.*, 2014), basidiomycetes (Shi *et al.*, 2012) and yeast (Kelly and Nurse, 2011). Driselase originates from basidiomycetes sp. containing laminerase, xylanase and cellulase activities. It has also shown efficient digestibility on other fungal species (Souza *et al.*, 2014). The β-(1–3) D-glucanase

Table I
Effect of incubation time and lytic enzymes (10 mg/ml) on protoplast yield/ml from *G. boninense* mycelia treated in different osmotic stabilizer and regeneration media

Mycelial age	Lytic enzyme/ Source	Media	Osmotic stabilizer	Protoplast regenerated as single colonies /ml		
				1 h	3 h	5 h
3-day culture	Driselase	PDA	Sucrose	50	56×10^5	23×10^2
			KCl	80	78×10^5	40×10^2
		GCM	Sucrose	40	34×10^5	11×10^2
			KCl	20	46×10^5	7×10^2
	β-(1-3) D-glucanase	PDA	Sucrose	21×10^2	22×10^4	3×10^2
			KCl	34×10^2	34×10^4	4×10^2
		GCM	Sucrose	18×10^2	17×10^4	2×10^2
			KCl	26×10^2	17×10^4	5×10^2
	T. harzianum	PDA	Sucrose	3×10^2	72×10^5	11×10^2
			KCl	13×10^2	89×10^5	28×10^2
		GCM	Sucrose	10×10^2	65×10^5	14×10^2
			KCl	15×10^2	69×10^5	21×10^2
4-day culture	Driselase	PDA	Sucrose	81×10	17×10^2	17×10^2
			KCl	74×10	34×10^2	21×10^2
		GCM	Sucrose	68×10	18×10^2	34×10^2
			KCl	76×10	26×10^2	18×10^2
	β-(1-3) D-glucanase	PDA	Sucrose	23×10	3×10^2	26×10^2
			KCl	17×10	13×10^2	3×10^2
		GCM	Sucrose	9×10	10×10^2	13×10^2
			KCl	15×10	15×10^2	10×10^2
	T. harzianum	PDA	Sucrose	58×10^2	11×10^3	15×10^2
			KCl	76×10^2	14×10^3	16×10^2
		GCM	Sucrose	67×10^2	12×10^3	17×10^2
			KCl	65×10^2	12×10^3	15×10^2
5-day culture	Driselase	PDA	Sucrose	23	93	8×10^2
			KCl	17	88	6×10^2
		GCM	Sucrose	18	45	7×10^2
			KCl	12	82	5×10^2
	β-(1-3) D-glucanase	PDA	Sucrose	5	9	13×10^2
			KCl	6	2	19×10^2
		GCM	Sucrose	2	8	27×10^2
			KCl	3	8	35×10^2
	T. harzianum	PDA	Sucrose	45	23×10^2	76×10^3
			KCl	46	35×10^2	84×10^3
		GCM	Sucrose	38	11×10^2	57×10^3
			KCl	42	29×10^2	63×10^3

was utilized to study the effect of single enzyme treatment over the *G. boninense* cell wall digestion. The lytic enzyme from *T. harzianum* showed the best efficiency (89×10^5 single colonies ml^{-1}) for *G. boninense* protoplast production indicated β-1, 3 glucanase, protease and chitinase are synergistically effective in degradation of *G. boninense* cell wall. The finding complements with the fungi polysaccharides cell wall compositions which constitute mainly cellulose and chitin (Yan *et al*., 2004; Wang *et al*., 2010; Souza *et al*., 2014). This is followed by Driselase (78×10^5 single colonies ml^{-1}), which showed better enzyme activity in comparison to β-(1–3) D-glucanase (34×10^4 single colonies ml^{-1}). Driselase acted selectively on both xylan hemicellulose and glucose polymers which constitute *G. boninense* cell wall (Mendoza, 1992). The results demonstrated stable integration of several compositions in fungi cell wall, thus, the usage of only one lytic enzyme is not

favourable for protoplast release of *G. boninense*. Protoplasts release was fairly high on both potassium chloride and sucrose supplemented osmotic stabilizers under most treatments. Potassium chloride treated protoplasts regenerated ($3–89 \times 10^5$ single colonies ml^{-1}) at a higher rate than sucrose treated protoplasts ($2–72 \times 10^5$ single colonies ml^{-1}) under all treatment combinations (Table I). The findings, however, contradicted with protoplast production from *G. lucidum* which demonstrated sucrose as optimal osmotic stabilizer (Choi *et al*., 1987). Osmotic stabilizer composed of inorganic sugar KCl was consistently more efficient than the organic sugar, sucrose. PDA was the most suitable regeneration medium for protoplast as number of single colonies regenerated in PDA ($5–89 \times 10^5$ single colonies ml^{-1}) was consistently higher than GCM ($2–69 \times 10^5$ single colonies ml^{-1}) in all treatment combinations (Table I). Medium used for regeneration of protoplast was consistent with basidiospore regeneration which evident PDA as the highly efficient medium for reversion into filamentous morphology.

In conclusion, usage of inorganic sugar for lysing procedure and organic sugar for selection facilitates isolation and regeneration of *G. boninense* protoplast respectively. Next, raised fruiting body for collection of actively growing mycelia (exponential phase) is a fundamental key attribute to protoplast regeneration procedure. Our study is the first to demonstrate isolation of contaminant free *G. boninense* basidiospore and its subsequent regeneration into mycelium, which was directly used for protoplast regeneration. The method described here detailing on the protoplast and basidiospore preparation would render *G. boninense* strain receptive to advanced technologies which ultimately creates avenue towards high-throughput researches.

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