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Degradation of Natural Rubber by *Achromobacter* sp. NRB and Evaluation of Culture Conditions

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Abstract

A natural rubber degrading candidate was isolated from a soil sample from Aswan, Egypt. The strain was able to grow on natural rubber as a sole source for carbon and energy. According to its degradation behavior, it grew adhesively and in direct contact with the rubber substrate and led to disintegration of the material during cultivation. Furthermore, this strain was not able to form a clear zone (translucent halos) around bacterial colonies after cultivation on NR latex plates. Taxonomic analysis of the strain based on partial 16S rRNA similarity examinations indicated that bacterial candidate belongs to genus *Achromobacter* sp. Schiff's reagent staining tests performed during cultivation of the strain on NR latex gloves of different sizes, treated or nontreated, revealed that the strain was able to colonize the rubber surface. Formation of bacterial films and occurrence of compounds containing aldehyde groups during cultivation was observed. The tested strain showed a higher colonization efficiency on small or treated pieces of NR latex gloves, while a lower colonization efficiency was recognized when grown on large or nontreated NR latex gloves. Plackett-Burman experimental design, based on numerical modeling, was applied to evaluate the significance of culture conditions affecting natural rubber degradation by the bacterial candidate. Eleven variables through fourteen trials were studied simultaneously. Based on rubber mineralization data, the highest positive variables affecting rubber degradation were NR granules, K_2HPO_4, Na-succinate and NH_4Cl, while $MgSO_4 \times 7H_2O$ and KH_2PO_4 were the lowest significant variables.

K e y w o r d s: rubber degradation, colonization of NR latex gloves, Plackett-Burman design

Introduction

Natural rubber (NR) is a biopolymer (poly-1,4-*cis*-isoprene) that is synthesized by many plants mostly belonging to the Euphorbiaceae or Compositae and by some fungi. NR has been commercially produced from more than a century by cultivating and tapping the rubber tree (*Hevea brasiliensis*) at a level of several million tons per year. Due to the superior properties of NR in comparison to (chemo-) synthetic rubber, the natural rubber product is still used as a basic material for tires, latex gloves, condoms, seals and many other items.

As a natural product, NR is subjected to biological mineralization cycles, and many reports on the biodegradability of natural rubbers have been published (Rook, 1955; Leeflang, 1963; Tsuchii *et al*., 1985; Heisey and Papadatos, 1995; Subramananiam, 1995; Jendrossek *et al*., 1997; Linos and Steinbuchel, 1998; Berekaa *et al*., 2000; Linos *et al*., 2000 and Arenskotter *et al*., 2001). Two different strategies towards utilization of NR are present among these microorganisms. One group of bacteria shows adhesive growth on rubber material in direct contact to the rubber. A second group of bacteria employed a different strategy and forms clearing zones or halo-formation on latex overlay plates. This indicates an exo-enzyme activity in these actinomycete (Tsuchii *et al.*, 1985). However, the biochemical and molecular basis of rubber degradation is poorly understood. It is assumed that degradation of the rubber backbone is initiated by oxidative cleavage of the double bond resulting into molecular mass oligo (cis-1,4-isoprene) derivatives with aldehyde and

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keto groups at their respective ends that persumably are degraded by reaction involving β-oxidation (Linos *et al.*, 2000, Bode *et al*., 2001; Tsuchii *et al.*, 1985 and Tsuchii and Takeda, 1990). Enrichement techniques with solid (natural or cross-linked) rubber recently led to the isolation of several new rubber-degrading bacteria that had high rubber degrading activity but do not necessarily form clear zones on latex agar (Jendrossek *et al.*, 1997). In 1989, Tsuchii and his co-workers found that the degradation of cis-1,4-polyisoprene rubber is by oxygenative attach of the polymer. This was evident by staining NR latex gloves with Schiff's reagent (Ehrlich *et al*., 1948). Recently, the similar mechanism of degradation was suggested by Bode *et al.*, (2001) after analysis and identification of the degradation product by HPTLC and GPC during growth of gram-positives (*Nocardia* sp. DSMZ 43191 and *Streptomyces coelicolor* 1A) and gram-negative (*A. colcoaceticus* and *Xanthomonas* sp.) bacteria on natural and synthetic rubber.

On the other hand, experimental design techniques present a more balanced alternative to one-factor-at-a-time approach. Indeed, Plackett and Burman design comprise one type of two-level screening and can be constructed on the basis of fractional replication of a full factorial design (Plackett and Burman, 1947). This design allows to obtain an unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations, the accuracy being the same for all effects (Akhnazarova and Kafarov, 1982).

In this study, the isolation of a rubber-degrading bacteria able to utilize natural rubber and tire rubber as a sole carbon source was reported. Furthermore, the degradation and colonization of the bacterial isolate on treated as well as nontreated NR latex gloves and the effect of size of rubber substrate on colonization effeciency were investigated. Special emphasis is given to the influence of different cultivation conditions on the degradation efficiency by applying fractional experimental design for the first time.

Experimental

Materials and Methods

Rubber products. NR latex concentrate was obtained from Alexandria Company for Chemical and Drug Production, Alexandria, Egypt. Raw NR (SMR10) was obtained from Al-Nessr Tire Company, Alexandria, Egypt. NR latex gloves pieces as well as the rubber tire pieces were extracted before use with chloroform in order to remove chemicals with microbicidal activities.

Bacterial isolate identification. The rubber-degrading strain used in this work was enriched and isolated during a screening strategy for isolation of local rubber-degrading bacteria from soil sample from Aswan. Beside morphological classification, molecular characterization of the bacterial isolate was carried out by the analysis of the 16S rRNA gene. For this purpose, DNA was isolated and purified according to Sambrook *et al.* (1989). Amplification of the 16S rDNA gene was performed by PCR-technique using primers designed to amplify 16S rRNA gene. The forward primer was 5'-AGAGTTTGATCMTGGCTCAG-3' and the reverse primer was 5'-TACGGYTACCTTGTTACGACTT-3'. The reaction was performed with 100 ng of genomic DNA in a final volume of 50 µl, including a reaction buffer 1 x, 30 pmole of each primer, and 2 units of taq polymerase. Thermocycling consisted of an initial denaturation for 5 minutes at 94°C and 30 cycles for 1 minute at 94°C (denaturation), 1 minute at 55°C (primer annealing), and 1.5 minutes at 72°C (extention). The PCR product was checked on 1% agarose gel by electrophoresis (Ausubel *et al.*, 1999), stained with ethidium bromide (0.5 mg/ml) and visualized using ultraviolet transillumination.

DNA sequencing. DNA was sequenced by dideoxy chain termination method according to Sanger *et al*. (1977). Sequencing was performed on an Applied Biosystems 3100 genetic analyzer (Applied Biosystems) using BigDye terminator cycle sequencing ready reaction mix according to manufacturer's instructions (Applied Biosystems). The nucleotide sequence produced was compared with the 16S rDNA sequences available from NCBI data base using nucleotide BLAST search program.

Cultivation. Cultivation was carried out in Erlenmeyer flasks containing mineral salts medium, (MSM), with rubber as sole carbon source (Linos and Steinbuchel, 1998). The rubber materials were added at a concentration of 0.5% (w/v). NR latex concentrate was added directly and the entire media was autoclaved. All cultures were inoculated with cells obtained from 3–6 days preculture in Luria-Bertani complex medium which were washed twice with sterile saline before use. During incubation at 30°C, the cultures were agitated at 150 rpm on a rotary shaker.

Enrichment from garden soil. Samples of garden soils from Aswan were suspended in a saline solution and one ml of soil was used to inoculate 50 ml MSM with natural rubber granules (NRG) as a sole C-source in 250 ml Erlenmeyer flasks that were incubated under shaked conditions. Growth was monitored by increase in turbidity and the change in substrate nature and color. For enrichement, 1 ml of the culture was transferred to a fresh medium containing the rubber material and left for incubation. This process was repeated several times. At the end, serial dilutions were made and inoculated on NB plates. The resulted colonies were further subcultured on NB agar medium for purification. Single pure colonies were further tested for growth on MSM with Latex or NRG as a sole C-source.

Mineralization of rubber substrate. Degradation and mineralization of rubber substrate, carried out in screw capped erlenmyer flasks with internal glass container, was obtained by determination of the amount of CO_2 released during cultivation of cells on the rubber substrate. The released CO_2 was trapped in $Ba(OH)_2$ solution resulting in precipitation of CO_2 as $BaCO_2$. The decrease in alkalinity was determined by titration with 0.25 N HCl and compared to a non-inoculated control as described previously (Linos and Steinbuchel, 1998).

Evaluation of culture conditions using Plackett-Burman design. For the screening purpose, various medium components as well as environmental factors have been evaluated. The different factors were prepared in two levels: –1 for low level and +1 for

high level, based on Plackett-Burman statistical design (Plackett and Burman, 1947). Eleven independent variables were screened in 14 combinations according to the design shown in the Results and Discussion section. All trials were performed in triplicate and the average of observations was considered as the final result. The main effect of each variable was calculated simply as the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at low setting (–1) of that factor.

Plackett-Burman experimental design is based on the first order model: $\Psi = \beta_0 + \Sigma\beta_i\Xi_i$, where Y is the response (mineralization), β_0 is the model intercept and β_i is the variables'estimates. The model describes interaction among factors and it is used to screen and evaluate the important factors that influence rubber degradation. The variables whose confidence levels were higher than 95% were considered to significantly influence the measured response.

Staining of rubber-degrading colonies. The actively growing colonies of *Achromobacter* sp. on the rubber surface were visualized clearly by staining with Schiff's reagent (Ehrlich *et al*., 1948). The purple color produced by the reagent was evidence that isoprene oloigomers containing aldehyde group were produced and accumulated during the microbial degradation of rubber (Tsuchii *et al*., 1985).

Results

Enrichment, isolation, and molecular characterization of microorganism. The rubber-degrading isolate was enriched and isolated during a screening strategy for isolation of local rubber-degrading bacteria from Aswan (Material and methods). Axenic culture of the rubber-degrading isolate revealed a gram-positive and oxidase-negative and non-motile rods that often forming complex cell aggregates. Based on cell morphology, colony morphology, growth on nutrient broth as well as several biochemical tests, the bacterial isolate was identified as *Achromobacter* sp. To confirm the biochemical test results for bacterial identification, 16S rRNA sequencing was carried out for validation of bacterial classification (Rainy *et al.*, 1994). The sequence was deposited in GenBank sequence database, and given the accession number AY590430. The closest known relative of the submitted nucloetide sequence, with 94% similarities was found to be the corresponding sequence of *Achromobacter ruhlandii* (AF205370) and *Achromobacter xylosoxidans* subsp. *xylosoxidans* strain TERI 1009 (AY269219), thus the isolated rubber-degrading strain was given the name *Achromobacter* sp. NRB.

Mineralization of the rubber substrates. The ability of the *Achromobacter* NBR to degrade raw natural rubber granules as well as some forms of tire rubber was investigated in this experiment. For this purpose, 250 ml Erlenmeyer mineralization flasks with screws and containing 50 ml MSM were prepared. As carbon source, raw solid natural rubber materials or tire rubber was added at concentration of 0.5% (w/v). Natural rubber substrate was cutted in the form of small pieces of 1 cm^2 in diameter and tire rubber was added as large piece of 3 cm^3 before addition to the MSM and autoclaving. The flasks were inoculated with 2 ml of a 3-days old preculture of *Achromobacter* sp. NRB cells that were washed with sterile saline solution 0.9 % (w/v). Mineralization was estimated at different time intervals.

The results presented in Figure (1a, b) showed that *Achromobacter* sp. NRB was able to degrade and mineralize raw natural rubber and tire rubber. It was also recognized that the optimum mineralization level of raw natural rubber granules was approximately 10 and was reached after 4 weeks of incubation (Figure 1a). Indeed, results in Figure 1b showed that the strain recorded 5.9 and 7.2% CO_2 release during growth for 60 days on treated and nontreated tires, respectively.

Colonization on NR-latex gloves and staining with Schiff's reagent. Colonization of the rubber degrading strain *Achromobacter* sp. NRB on the surface of pretreated and non-treated NR-latex glove was examined by Schiff's reagent (Ehrlich *et al.*, 1948). In this experiment, *Achromobacter* sp. NRB cells were cultivated in 250 ml Erlnmeyer flasks with 50 ml MSM and pretreated or non-treated NR-latex gloves (0.5% w/v) as a sole source for carbon. At the end of incubation period, NR-latex glove was removed from the flask, washed several times with saline solution (0.9% w/v) and then tested by Schiff's reagent. Results in Figure 2 shows that actively growing colonies on the rubber surface were clearly visualized by staining with this reagent and gave a clear purple color thus providing evidence for the occurrence of degradation products containing aldehyde groups during the degradation. Further investigations showed that the colonization of rubber-degrading bacteria on the surface of NR-latex gloves increased during the experiment. Indeed, colonization efficiency was enhanced after pretreatment and removal of antimicrobicidial agents fron NR-latex gloves.

Size of NR-latex gloves and colonization efficiency. Staining with Schiff's reagent was also used to examine the effect of size of rubber substrate on colonization efficiency. In this experiment, *Achromobacter* sp. NRB cells were cultivated in MSM and pretreated NR-latex gloves as a sole source for carbon. Two different sizes of NR latex gloves (with diameter of 1 cm^2 and 4 cm^2 in length and width, for each of the

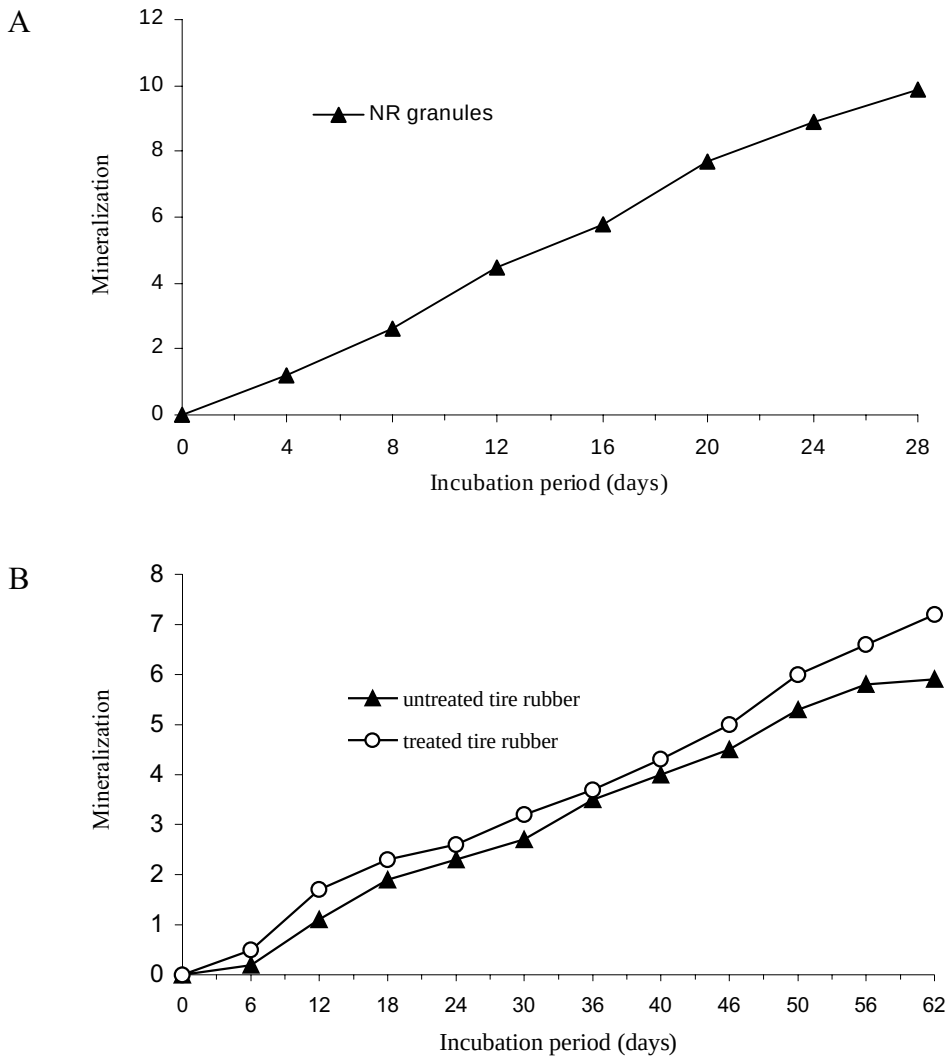

Fig. 1. Mineralization (expressed as: % CO_2 release) of raw natural rubber (A) and tire rubber (B) by *Achromobacter* sp. NRB.

Fig. 2. Enhanced degradation of NR-latex gloves by *Achromobacter* sp. NRB.
A nontreated, B treated.

tested sizes) were used. At the end of incubation period, the glove samples were removed from the flask, washed several times with saline solution and then tested by Schiff's reagent. Results in Figure 3 showed that colonization efficiency was influenced by the size of rubber substrate. Indeed, the colonization efficiency was increased when rubber substrate was applied in small pieces (diameter; 1 cm^2) while lower colonization efficiency was recorded in case of large size substrate (diameter; 4 cm^2).

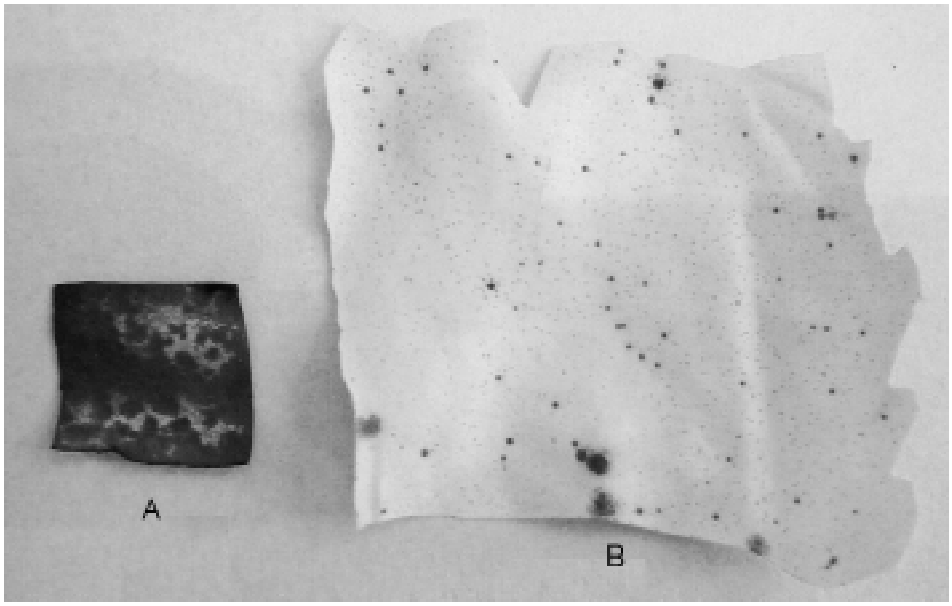

Fig. 3. Enhanced colonization and degradation of small sized NR-latex gloves (A) and large sized NR-latex gloves (B) by *Achromobacter* sp. NRB.

Evaluation of cultivation conditions. A statistical experimental design was applied for evaluation of cultivation conditions during degradation of rubber substrate by *Achromobacter* sp. NRB. Eleven factors were studied through the application of Plackett Burman design (Table I). The independent variables examined and their setting are shown in Table II. Each factor was tested in both low (–1) and high (+1) levels and the estimate of each factor was determined. Results in Table I showed that the highest natural rubber mineralization of 36% was obtained in the combination number 3, while the lowest mineralization was obtained in combination numbers 1 and 6, with a value of 8% for each. Statistical analysis of these data revealed that the value of determination coefficient R2, that measures precision of the model fitting, is >0.85. This indicates that less than 1.5% of the total variations is not explained by the model, which ensures the good adjustment of the model to experimental results.

Table I
Plackett-Burman experiemental design for evaluating the effect of different nutritional and environmental conditions on mineralization of natural rubber by *Achromobacter* sp. NRB

Trial	NR granules	Na-succinate	NH_4-sulphate	NH_4-chloride	Trace elements	Yeast extract	$MgSO_4$	K_2HPO_4	KH_2PO_4	Temp.	Agitation	Mineralization (% CO_2 release)
1	1	1	1	–1	1	1	–1	1	–1	–1	–1	36
2	–1	1	–1	–1	–1	1	1	1	–1	1	1	17
3	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	8
4	1	1	–1	1	1	–1	1	–1	–1	–1	1	19
5	1	–1	1	–1	–1	–1	1	1	1	–1	1	19
6	1	–1	–1	–1	1	1	1	–1	1	1	–1	8
7	1	–1	1	1	–1	1	–1	–1	–1	1	1	29
8	–1	–1	1	1	1	–1	1	1	–1	1	–1	16
9	–1	–1	–1	1	1	1	–1	1	1	–1	1	20
10	0	0	0	0	0	0	0	0	0	0	0	10
11	1	1	–1	1	–1	–1	–1	1	1	1	–1	31
12	0	0	0	0	0	0	0	0	0	0	0	10
13	–1	1	1	–1	1	–1	–1	–1	1	1	1	13
14	–1	1	1	1	–1	1	1	–1	1	–1	–1	15

Table II
Variables and their levels employed in Plackett-Burman design for screening of culture conditions affecting natural rubber degradation by *Achromobacter* sp. NRB

Factor	Variable	Values	
		–1	+1
F1	NR granules (g/L)	5	16
F2	Na-succinate (g/L)	0.5	2
F3	Ammonium sulphate	4	10
F4	Ammonium chloride (g/L)	0.5	2
F5	Trace elements (ml/L)	0.5	4
F6	Yeast extract (g/L)	0.25	1
F7	$MgSO_4 \times 7H_2O$ (g/L)	0.2	0.8
F8	K_2HPO_4 (g/L)	1	4
F9	KH_2PO_4 (g/L)	0.1	2
F10	Temperature (°C)	30	37
F11	Agitation (rpm)	100	150

Moreover, the main effects of the examined variables on mineralization of natural rubber were calculated and illustrated graphically in Figure 4a. It was clear that the degradation of rubber by the bacteria was positively affected by the amount of NR granules, Na-succinate, NH_4Cl and K_2HPO_4 concentration. Agitation, temperature and concentration of trace element solution had no effect. $MgSO_4 \times 7H_2O$ and KH_2PO_4

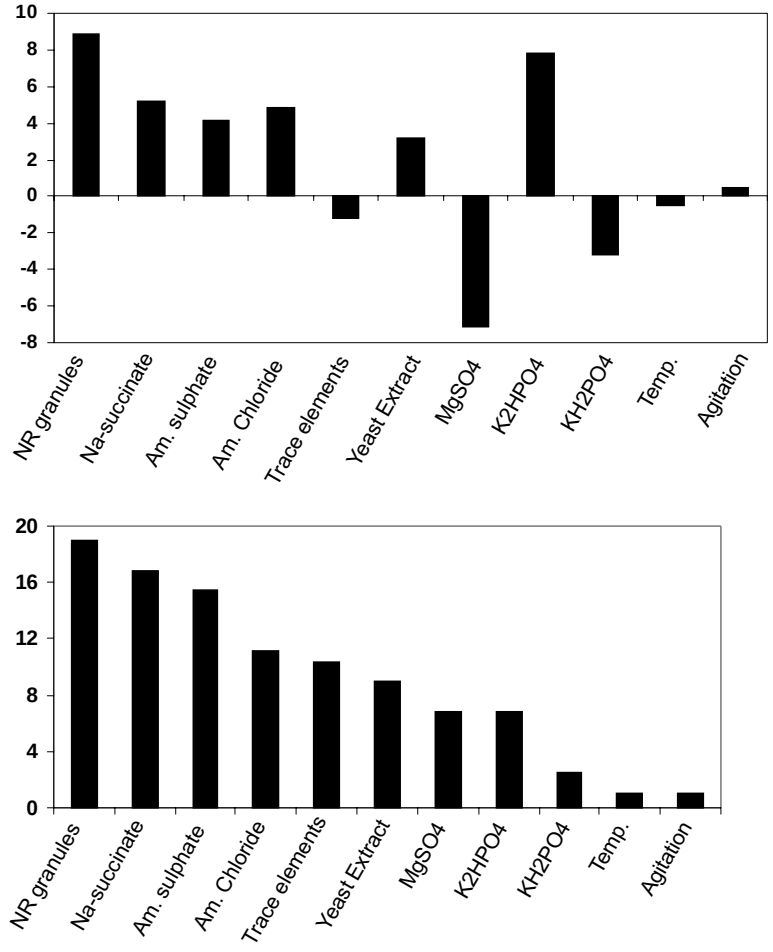

Fig. 4. Effect of environmental and nutritional factors on degradation of natural rubber by *Achromobacter* sp. NRB based on results of Plackett-Burman design

were the lowest significant variables effecting rubber degradation. Furthermore, the ranking of each factor estimate was clearly shown in Pareto chart (Figure 4b). This chart displays the magnitude of each factor estimation of each variable affecting rubber degradation.

Discussion

The microbial suceptibility of NR, either in the raw or in the vulcanized state, was sufficiently examined and reviewed (Leeflang, 1963; Tsuchii, 1995; Heisey and Papadatos, 1995; Jendrossek *et al.*, 1997 and Linos *et al*., 2000). In this study, rubber-degrading bacterial isolate was enriched from soil sample. According to biochemical and molecular characterization analysis, it was identified as *Achromobacter* sp. NRB. Interestingly, several *Achromobacter* sp. were isolated during degradation of a number of environmental contaminants such as polychlorinated biophenyles, PCB (Chang *et al.*, 1992), polyaromatic hydrocarbons, PAH (Ivashchenko and Semenchuk, 2001), alkanosulfonate degradation (Erdlenbruch *et al.*, 2000) and toluidine (Hinteregger and Streichsbier, 2001). The obtained strain was able to degrade and mineralize raw natural rubber granules as well as some forms of tire rubber and to use it as a sole source for carbon.

In contrast to the growth in common media where carbon source is dissolved, the growth of microorganisms on natural rubber products is very slow and the effect of culture conditions upon microbial growth on the insoluble solid substrate like rubber has not been well characterized. Therefore, colonization of *Achromobacter* sp. NRB on rubber substrates was intensively investigated.

Furthermore, colonization of rubber degrading strain *Achromobacter* sp. NRB on the surface of pretreated and non-treated NR-latex glove was examined by Schiff's reagent. The actively growing colonies on the rubber surface were clearly visualized and gave a clear purple color, thus providing evidence for the occurrence of degradation products containing aldehyde groups during the degradation (Tsuchii *et al.*, 1996). Further investigations showed that the colonization of rubber-degrading bacteria on the surface of NR-latex gloves increased during the time course of the experiment. Indeed, colonization efficiency was enhanced after preatreatment and removal of antimicrobicidial agents from NR-latex gloves (Berekaa *et al.*, 2000). The degradation of rubbers after removal of antimicrobial substances that were added for different purposes by the manufacturing companies will provide a new approaches for a future microbial treatment of rubber waste by combining chemical and biological methods.

Interestingly, staining with Schiff's reagent indicated that the colonization efficiency was influenced with the size of rubber substrate. The colonization efficiency was increased when rubber substrate was applied in small pieces (diameter 1 cm^2) while; lower colonization efficiency was recorded in case of large size substrate (diameter 4 cm^2). A similar finding was done by Tsuchii and co-workers (1996) during the growth of *Nocardia* sp. 835A on latex gloves pieces.

In screening program to evaluate the factors affecting the degradation of rubber materials, several physical and chemical factors affecting biodegradation process were tested by application of Plackett-Burman design. This design offers a good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component.

It was clear that the degradation of rubber was positively affected by the amount of NR granules. It is well known that increase in amount of rubber granules leads to increase in surface area of rubber substrate and hence to higher mineralization rate. A similar finding was reported by Tsuchii and his co-workers (1996). Furthermore, Na-succinate positively affected the rate of rubber degradation. Tsuchii *et al.* (1996) reported a significant increase of rubber degradation in presence of other co-substrate. The presence of NH_4Cl and K_2HPO_4 were essential in degradation of rubber substrate where the first was used as N-source and the second serves as a buffer and phosphate source. On the other hand, agitation, temperature and concentration of trace element solution had no effect. $MgSO_4 \times 7H_2O$ and KH_2PO_4 were the lowest significant variables effecting rubber degradation. These factors may be dropped in all further optimization experiments.

The results of this study will help to improve degradation effeciency by *Achromobacter* sp. NRB through treatment of rubber material and applying substarte in small pieces. Further improvement of the degradation condition will apply fractional experimental design. Close study on molecular mechnism of rubber degradation by this isolate demands search for induced protein(s) through comparative protein analysis. N-terminal sequence of interesting protein(s) and search for homology with similar protein in databases.

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