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ORGINAL PAPER

Use of Potato Extract Broth for Culturing Root-Nodule Bacteria

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Abstract

Liquid media containing potato extract and 1% of glucose or sucrose were used to culture root-nodule bacteria (rhizobia) in shaken Erlenmeyer flasks. For comparison, these bacteria were also cultured in yeast extract-mannitol broth (YEMB) as a standard medium. Proliferation of rhizobia was monitored by measuring optical densities (OD_{550}) of the cultures and by plate counting of the viable cells (c.f.u) of the bacteria. In general, multiplication of the rhizobia in potato extract-glucose broth (PEGB) and potato extract-sucrose broth (PESB) was markedly faster, as indicated by higher values of OD_{550}, than in YEMB. The numbers of *R. leguminosarum* bv. *vicae* GGL and *S. meliloti* 330 in PEGB and PEGB were high and ranged from 1.2×10^{10} to 4.9×10^{10} mL^{-1} after 48 h of incubation at 28°C. *B. japonicum* B3S culture in PEGB contained 6.4×10^{9} c.f.u. ml^{-1} after 72 h of incubation. PEGB and YEMB cultures of the rhizobia were similar with respect to their beneficial effects on nodulation of the host-plants of these bacteria.

Key words: potato extract broth, proliferation, rhizobia

Introduction

Soil dwelling bacteria with the ability to form symbiotic relationships with leguminous plants (*Fabaceae*) belong to six genera: *Allorhizobium, Azorhizobium, Bradyrhizobium, Mezorhizobium, Rhizobium* and *Sinorhizobiumj* (*Ensifer*) and are commonly known as rhizobia or root-nodule bacteria (Vincent, 1981; Malek and Sajnaga, 1999; Willems, 2006). The most important feature of this symbiosis is fixation of atmospheric nitrogen by the bacteria, located inside root nodules, for the benefit of their host plants (Vincent, 1981; Kaminski *et al*., 1998). Inoculation of legume seeds or soils with commercial inoculants containing preselected strains of root-nodule bacteria is a common agriculture practice, which helps to ensure an effective symbiosis, particularly when natural soil populations of these bacteria are deficient, ineffective or only partially effective (Roughly, 1970; Stephens and Rask, 2000). The first step in the commercial production of rhizobial inoculants is mass culturing of the bacteria in a liquid medium, containing a cheap and complex source of nutrients. For example, yeast extract as a source of nitrogen, vitamins and other micronutrients is commonly used to prepare various media for culturing of rhizobia in research studies and for commercial purposes (Roughly, 1970; Vincent, 1981; Stephens and Rask, 2000). Many other substrates or industrial and agricultural by-product such as: proteolyzed pea husks (Gulati, 1979), corn steep liquor (Burton, 1979), malt extract (Bioardi and Ertola, 1985), cheese why (Bissonnette *et al*., 1986), or even wastewater sludge (Ben Rebah *et al.,* 2007) have been proposed as media for rhizobial biomass production.

The aim of this work was to examine if potato extract can be used as a basic source of nutrients for cultivation of root-nodule bacteria in liquid cultures.

Experimental

Material and Methods

Root-nodule bacteria. The Culture Collection of N_2-fixing Bacteria belonging to Department of Agricultural Microbiology of the Institute of Soil Sc. and Plant Cultivation in Pulawy was the source of the following species of rhizobia used in this work: *Bradyrhizobium japonicum* – strain B3S, *Bradyrhizobium* sp. (Lupinus) – strain LZ, *Rhizobium leguminosarium* bv. *viciae* – strain GGL and *Sinorhizobium meliloti* – strain 330. Stock cultures were maintained at 4°C on slants of yeast extract-mannitol agar (YEMA) supplemented with 3 g $CaCO_3$ L^{-1} (Vincent, 1981) and grown in yeast extract-mannitol broth (YEMB) as fresh starter cultures to inoculate experimental media.

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Preparation of potato extract. Glass bottle (2 L) containing 300 g of peeled potato tubers (cultivar Baszta), cut into smaller fragments, and 3 g of $CaCO_3$ was poured with 1 L of tap water and the content was boiled for 20 min. After boiling the mixture was filtered through cheese cloth and the volume of the extract was adjust to 1 L with distilled water. To prepare potato extract broth (PEB) supplemented C sources glucose or sucrose at the rate of 10 g L^{-1} of the filtered extract were added. For incubation experiments sets of 250 mL Erlenmeyer flasks containing 50 mL of PEB supplemented with glucose (PEGB) or sucrose (PESB) were prepared and autoclaved at 110°C for 20 min. After autoclaving these media had pH 6.7–6.8. When clear PEB media were needed, *e.g.* for measuring of optical densities (OD) of cultures larger volumes (*e.g.* 1.0 L) of PEGB and PESB were prepared and after autoclaving the media were left on a laboratory bench for 24–48 hours to allow particles to settle. Portions (50 mL) of clear liquid from the upper layer was than aseptically distributed into sterile 250 mL-Erlenmeyer flasks.

Incubation experiments. In the first experiment *R. leguminosarium* bv. *viciae* GGL and *S. meliloti* 330 were grown in potato extract – glucose broth (PEGB) and in potato extract-sucrose broth (PESB). *B. japonicum* B3S does not utilize sucrose (Jordan, 1984) and for this reason this species was cultured in PEGB only. For comparison yeast extract-glucose broth (YEMB) was also used to culture the bacteria. YEMB had the following composition (g L^{-1}): mannitol – 10; K_2HPO_4 – 0.5; $MgSO_4 \times 7\ H_2O$ – 0.2; NaCl – 0.1; yeast extract (Difco) – 0.5 (Vincent, 1981). Flasks containing these media were inoculated with 2 mL of starter cultures of the rhizobia (OD_{550} = 0.35–0.4) grown for 24 h in agitated YEMB at 28°C. There were three replicated flasks for each of the rhizobial species and for each medium tested. The flasks were incubated at 28°C on a rotary shaker (150 rev min^{-1}) and periodically 1 mL samples were withdrawn aseptically to measure optical densities of the cultures at λ = 550nm (OD_{550}).

In the second experiment the same media and incubation conditions was used but proliferation of rhizobia was monitored by determination of counts of viable rhizobial cells (colony forming units – c.f.u.) by dilution plating on Cong red-YEMA (Vincent, 1981). Colonies of the fast-growing and slow-growing rhizobia were counted after 3 and 7 days, respectively, of incubation at 28°C.

In the third experiment shortly-boiled potato extract-glucose broth (SB-PEGB) was used. This medium was prepared as described above with the exception of boiling time, which was shortened to 5 minutes. After autoclaving SB-PEGB had pH 6.8. Incubation and counting of rhizobial c.f.u were done as describe above.

Nodulation biotest with legumes. Rhizobial cultures obtained in the second experiment after 48 hours of incubation were used to inoculate seeds or seedlings of *Medicago sativa* L. (alfalfa) cv. Socza, *Pisum arvense* L. (field pea) cv. Grapis and *Glycine max* L. cv. Aldana, which were grown in sand pouches (Martyniuk *et al.*, 2000). Shortly, in this biotest seedlings were grown in polypropylene pouches filled with sterile sand moistened with sterile N-free nutrient solution to support plant growth. Before sowing the seeds were surface disinfected by soaking for 10 min. in 5% H_2O_2, followed by several rinses with sterile water. Two seeds of pea or soybean were sown into each pouch and inoculated with 1 mL of their respective rhizobial cultures grown on YEMB and PEGB (from Experiment 2). Disinfected seeds of alfalfa (lucerne) were pre-germinated on sterile 1% water agar at 25°C for 48 hours. Four uniform seedlings were than planted into each sand pouch and inoculated with 1 mL of *S. meliloti* 330 culture as above. Ten replicated sand pouches were prepared for each plant species and after emergence of seedling six pouches containing well developed and uniform seedlings were selected and placed in a growth chamber (Heresus HPS 1500–2000) running at 16 h/8 h day/night regime and temperature 22°C/15°C, respectively. After four weeks the experiment was terminated and numbers of nodules on the root system of nodulating seedlings were counted.

Data were subjected to the analysis of variance using Anova test.

Results

In the first experiment two fast-growing rhizobial species: *Rhizobium leguminosarum* bv. *viciae* GGL and *S. meliloti* 330, and one slow-growing *Bradyrhizobium japonicum* B3S were used. Figure 1 shows that starting from 24 hours of incubation proliferation of *S. meliloti* 330 in potato-extract media (PEGB and PESB) was markedly faster, as indicated by higher values of OD_{550}, than in YEMB. Similar results were obtained for *R. leguminosarum* bv. *viciae* GGL, though during the first 24 hours of incubation all cultures had comparable optical densities (Fig. 1). Slow-growing *B. japonicum* B3S does not metabolize sucrose, therefore growth of this rhizobial species was monitored for 72 hours only in potato medium enriched with glucose (PEGB) and compared with that in YEMB. As Figure 1 shows all cultures of *B. japonicum* B3S in PEGB, except that at 6 h of incubation, had significantly higher densities than those in YEMB.

This experiment was repeated, but since some rhizobial strains, *e.g.* GGL strain of *R. leguminosarum* bv. *viciae* tested in the first experiment, gave clumpy

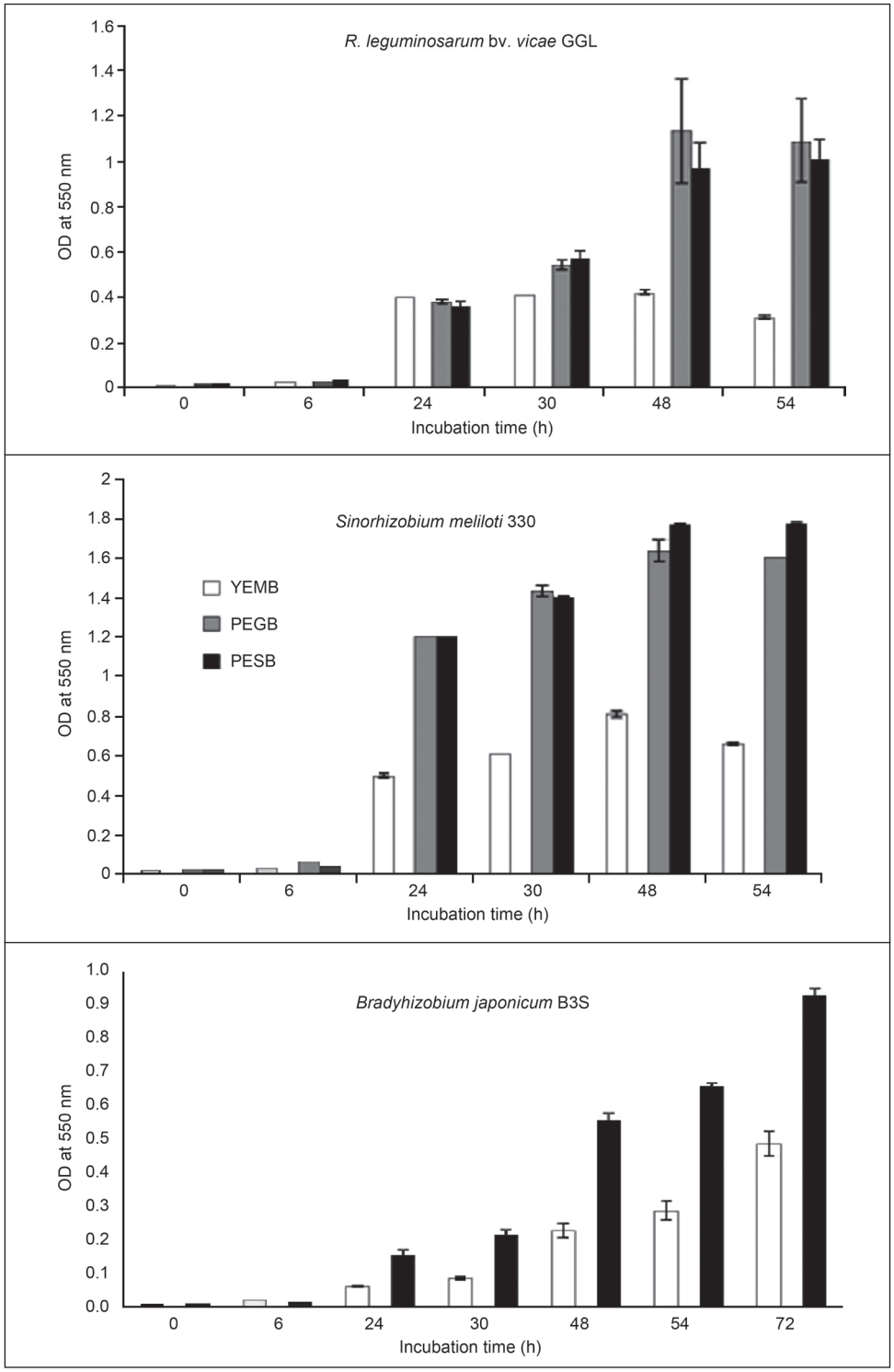

Fig. 1. Optical densities (OD_{550}) of rhizobial cultures grown in yeast extract-mannitol broth (YEMB), potato extract-glucose broth (PEGB) and in potato extract-sucrose broth (PESB) at various samplings during incubation at 28°C.
Bars represent standard deviations of three measurements.

cultures resulting in a marked variability of OD measurements, particularly in the case of potato-extract media (Fig. 1), in the second experiment growth of the rhizobia was monitored by plate counting of viable cells (c.f.u) after 6, 24, 48 and 72 hours (in the case of *B. japonicum* B3S) of incubation. Results of this experiment, shown in Table I, agree well with those obtained in the previous experiment and indicate that the final cultures (after 48 of 72 h of incubation) of the rhizobia grown in PEGB and PESB contained, in general, significantly higher numbers of viable cells than those in YEMB. The numbers of viable cells (c.f.u) of the rhizobia in PEGB and PESB were high and ranged from 1.2×10^{10} to 4.9×10^{10} mL^{-1} in the case of the fast-growing

Table I
Numbers of c.f.u. of rhizobia in yeast extract-mannitol broth (YEMB), potato extract-glucose broth (PEGB) and in potato extract-sucrose broth (PESB) after 6 h, 24 h and 48 h of incubation at 28°C

Medium	Incubation time (h)			
	6	24	48	72
Rhizobium leguminosarum bv. *viciae* GGL				
YEMB	2.3×10^8 a*	$3{,}3\times10^9$ a	1.6×10^{10} a	n.a**
PEGB	2.5×10^8 a	3.0×10^9 a	4.9×10^{10} b	n.a
PESB	3.0×10^8 a	3.7×10^9 a	1.2×10^{10} a	n.a.
Sinorhizobium meliloti 330				
YEMB	4.5×10^8 a	1.6×10^9 a	1.9×10^9 a	n.a.
PEGB	4.4×10^8 a	2.5×10^{10} b	1.5×10^{10} b	n.a.
PESB	3.0×10^8 a	2.1×10^{10} b	3.3×10^{10} c	n.a.
Bradyrhizobium japonicum B3S				
YEMB	2.7×10^7 a	1.4×10^8 b	1.4×10^9 a	3.2×10^9 a
PEGB	2.7×10^7 a	1.3×10^8 b	1.3×10^9 a	6.4×10^9 b

* numbers of particular rhizobial species within any one incubation time with the same letters are not significantly different (p = 0.05)
** not analyzed

rhizobia after 48 h of incubation (Table I). PEGB culture of *B. japonicum* B3S contained 6.4×10^9 c.f.u. mL^{-1} after 72 h of incubation (Table I).

In the third incubation experiment shortly-boiled potato extract enriched with glucose (SB-PEGB) was used to grow *S. meliloti* 330 and two species of *Bradyrhizobium*, *B. japonicum* B3S and *Bradyrhizobium* sp. (Lupinus), strain LZ. All the rhizobia tested in this experiment grew in the shortly-boiled PEGB medium equally well, or even better, than in PEGB used in the

Table II
Numbers of c.f.u. of rhizobia in shortly-boiled potato extract-glucose broth (SB-PEGB) after 6 h, 24 h, 48 h and 72 h of incubation at 28°C

Incubation time (h)			
6	24	48	72
Sinorhizobium meliloti 330			
$3.0\pm0.1\times10^{9*}$	$3.6\pm0.2\times10^{10}$	$3.2\pm0.7\times10^{11}$	$2.8\pm0.4\times10^{11}$
Bradyrhizobium japonicum B3S			
$1.1\pm0.1\times10^8$	$3.4\pm0.3\times10^9$	$3.2\pm0.4\times10^{10}$	$4.6\pm0.5\times10^{10}$
Bradyrhizobium sp. (Lupine) LZ			
$4.3\pm0.2\times10^8$	$7.9\pm0.8\times10^9$	$4.1\pm0.2\times10^{10}$	$2.0\pm0.4\times10^{10}$

* Numbers of rhizobia ±SD (standard deviation) of three measurements

first two experiments. For example, SB-PEGB cultures of *S. meliloti* 330 and *B. japonicum* B3S after 48 h of incubation contained about 10-fold higher numbers of c.f.u. than the cultures of these bacteria in PEGB used in the first experiment (Tables I and II). Proliferation of *Bradyrhizobium* sp. (Lupine) LZ in SB-PEGB was slower than *B. japonicum* B3S, particularly after 24 h and 48 h of incubation, but in the final culture (after 72 h) a high population (2.0×10^{10}) of the lupine microsymbiont c.f.u. was found (Table II). In shortly-boiled PEGB the highest numbers of *S. meliloti* 330, 3.2×10^{11} c.f.u. mL^{-1}, were detected after 48 h of incubation. The SB-PEGB cultures of the slow-growing rhizobia (*R. japnicum* B3S and *B.* sp (Lupinus) LZ) contained $2–4.6\times10^{10}$ c.f.u. mL^{-1} after 72 h of growth (Tables II).

In the nodulation biotest seedlings of alfalfa, pea and soybean were inoculated with cultures of their specific symbiotic bacteria, *S. meliloti* 330, *R. legumi-*

Table III
Nodulation of legumes 4 weeks after their seed inoculation with 48 h old cultures of respective rhizobia (Experiment 2) grown in yeast extract-glucose broth (YEGB) and in potato extract-glucose broth (PEGB)

Seed treatment	Percentage of plants with nodules	Number of nodules per plant
Alfalfa (*Medicago sativa* L.)		
Non-inoculated seeds	80	1.2 a*
Inoculated with YEMB culture of *S. meliloti*	100	2.3 b
Inoculated with PEGB culture of *S. meliloti*	100	2.1 b
Pea (*Pisum arvense* L.)		
Non-inoculated seeds	50	7.9 a
Inoculated with YEMB culture of *R. l.* bv. *viciae*	100	23.7 b
Inoculated with PEGB culture of *R. l.* bv. *viciae*	100	23.9 b
Soybean (*Glycine max* L.)		
Non-inoculated seeds	0	0.0 a
Inoculated with YEMB culture of *B. japonicum*	100	6.5 b
Inoculated with PEGB culture of *B. japonicum*	100	5.6 b

* Numbers of nodules on roots of particular plant species with the same letter are not significantly different ($\alpha = 0.05$)

nosarum bv. *viciae* GGL and *B. japonicum* B3S, respectively, grown for 48 h in YEMB and PEMB (Experiment 2) in order to compare nodulating ability of these cultures. It was found that both cultures were similar with respect to their beneficial effects on nodulation of all the host-plants of the rhizobia used in this experiments (Table III).

Discussion

Commercially available potato extract-dextrose agar (PDA) medium is commonly applied to culture various saprophytic and plant pathogenic fungi (Hawksworth *et al.*, 1995; Sharma and Pandey, 2010) and the results of our experiments indicate that potato extract based broth can also be used for successful cultivation root-nodule bacteria, as the numbers of all the rhizobial species cultured in PEGB or PESB were, in general, significantly higher than those grown in the reference YEMB, particularly at the end of the incubation period (Tables I and II). Cultures of the fast-growing species of the rhizobia, *R. leguminosarum* bv. *viciae* GGL and *S. meliloti* 330, that we tested in our experiments, reached their late log phase (the highest OD_{550} values) usually after 48–54 h of incubation (Fig. 1), but the slow-growing *Bradyrhizobium* species required a longer incubation period, similarly to results reported earlier (Roughly, 1970; Burton, 1979; Ben Rebah *et al.*, 2007). The numbers of the viable cells (c.f.u) of the rhizobia in PEGB and PESB accounted for 1.2×10^{10} to 4.9×10^{10} mL^{-1} in the case of the fast-growing rhizobia and 6.4×10^{9} c.f.u ml^{-1} in the case of *B. japonicum* B3S, (Table I) were comparable or higher than those obtained in other studies on culturing of the root-nodule bacteria in various liquid media (Gulati, 1979; Bioardi and Ertola, 1985; Bissonnette *et al.*, 1986; Ben Rebah *et al.*, 20007). It has been found that the composition of growth media can have a significant effect on some characteristics of rhizobia. For instance, high concentrations of yeast extract ($> 3.5\ g\ L^{-1}$) in liquid media can decrease nodulation potential of root-nodule bacteria grown in such media (Bissonnette *et al.*, 1986; Stephens and Rask, 2000). For this reason nodulation of alfalfa, pea and soybean seedlings inoculated with the cultures of their specific rhizobia grown in PEGB and in YEMB was compared (Table III). This experiment has shown that both cultures of the rhizobia had similar effectiveness with respect to their effect on nodulation of the legumes. Thus, the use of potato extract based media for the proliferation root-nodule bacteria seems to be safe with respect to the preservation of their infectivity and ability to induce nodules on roots of the host plants. Relatively high percentages of nodulating alfalfa and pea seedlings found in the control (non-inoculated) treatment (Table III) were connected with rather a mild seed sterilizing agent (5% H_2O_2) that we used in this experiment. Some legume seeds, *e.g.* alfalfa, are particularly difficult to disinfect (Vincent, 1981).

In the shortly-boiled PEGB the numbers of the tested rhizobial strains were markedly higher than in PEGB, reaching 3.2×10^{11} c.f.u. mL^{-1} in the case of *S. meliloti* 330 and $2–4.6\times10^{10}$ c.f.u. mL^{-1} in the case of the slow-growing rhizobia (Tables II), indicating that short boiling (5 min) is not only an energy-saving treatment but also is probably less destructive for some growth factors, *e.g.* vitamins, than longer boiling (20 min). Moreover, it seems that, after separating the extract for culturing of microorganisms, shortly boiled (blanched) potato fragments could be used for other purposes, *e.g.* as frozen consumption product.

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