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MODIFICATION OF YEM BROTH FOR MEDIUM SCALE PRODUCTION OF LEGUME INOCULANTS

MODIFICACIÓN DEL CALDO EXTRACTO DE LEVADURA - MANITOL PARA LA PRODUCCIÓN A MEDIANA ESCALA DE INOCULANTES PARA LEGUMINOSAS

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ABSTRACT

Yeast-extract-mannitol (YEM) broth, a widely used laboratory medium for the cultivation of rhizobia, was modified to reduce its cost and made it suitable for medium scale production of rhizobial legume inoculants. Yeast extract and mannitol, the most expensive ingredients, were reduced or substituted with more cheap substrates. With the addition of 1,1 g/L of glutamic acid or food grade sodium glutamate, yeast extract can be reduced to 0,05 g/L without affecting growth. Mannitol can be replaced with 12,5 g/L of pharmaceutical grade glycerin for *Bradyrhizobium* strains or with 10 g/L of food grade sugar for *Rhizobium* strains. The symbiotic properties of rhizobia grown on modified media were not affected.

Key words: *Rhizobium*, *Bradyrhizobium*, YEM, Legume Inoculant.

RESUMEN

El caldo extracto-de-levadura-manitol (LM), un medio ampliamente utilizado para el cultivo de rizobios, fue modificado para reducir su costo y utilizarlo en la producción a mediana escala de inoculantes para leguminosas. Los dos ingredientes más costosos, el extracto de levadura y el manitol, fueron reducidos o reemplazados con substratos más económicos. Se pudo reducir la concentración de extracto de levadura a 0,05 g/L sin afectar el crecimiento cuando se agregó 1,1 g/L de ácido glutámico o glutamato de sodio grado alimento. El manitol pudo ser substituido por 12,5 g/L de glicerina grado farmaceútico para las cepas de *Bradyrhizobium* o por 10 g/L de azúcar grado alimento para las cepas de *Rhizobium*. No se alteraron las propiedades simbióticas de las cepas cultivados en los medios modificados.

Palabras claves: *Rhizobium*, *Bradyrhizobium*, LM, YEM, Inoculantes para leguminosas.

INTRODUCTION

Bacteria of the genera *Rhizobium* Frank (1889) and *Bradyrhizobium* Jordan (1982), commonly known as rhizobia, form symbiotic relationships with legumes. As a result, the plant can satisfy its nitrogen requirements through biological nitrogen fixation carried out by the bacteria and thus avoiding the need for fertilizers. In this context, legume inoculants are defined as liquid or solid preparations of viable rhizobia designed for application to seeds or the soil to ensure the formation of a effective symbiosis (Thompson, 1991; Beck *et al.*, 1993).

The first step in the production of legume inoculants is massive growth of a selected rhizobial strain in liquid medium (Thompson, 1991). As is the case with many other industrial fermentations, the economy of such a process is largely governed by the price of the media utilized.

Many substrates have been proposed as media for large-scale rhizobial biomass production: proteolyzed pea husks (Gulati, 1979), malt sprouts (Boiardi & Ertola, 1985), and deproteinized leave extracts (Chanda *et al.*, 1987). Although these products have the advantage of being cheap residual by-products, their composition are extremely varied and consequently new batches of substrate have to be carefully evaluated in trial fermentations (Crueger & Crueger, 1984). Furthermore, some of them need to be pre-

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processed, e.g. filtrated and clarified, before they can be used in production. Although at large scale these inconveniences are largely compensated by the reduction in costs, at small or medium scale they discourage their use.

In order to evaluate an alternative scheme, the objective of this work was to modify the composition of yeast-extract-mannitol (YEM) broth, a widely used laboratory medium for rhizobial cultures, to make it suitable for the production of legume inoculants at a medium scale. We use cheap, easily available products of food and pharmaceutical grade to avoid the complications derived from the use of complex substrates.

MATERIAL AND METHODS

Microorganisms

Three *Rhizobium* and four *Bradyrhizobium* strains were utilized. *Rhizobium* sp. PLC213 and PLA142a, and *Bradyrhizobium* sp. PLL113, PLL142a and TAL 22 were isolated from *Phaseolus lunatus* (Matos *et al.*, 1998; Somasegaran, 1993). *Rhizobium* sp. 9A and *Bradyrhizobium* sp. TAL 169 were isolated from *Phaseolus vulgaris* (Zúñiga & Carbajal, 1990) and *Vigna unguiculata* (Somasegaran, 1993), respectively. Cultures were routinely grown in YEM agar (Beck *et al.*, 1993). Stock cultures were maintained at 4°C on YEM agar slants.

Preparation of inoculum and growth conditions

Cultures were grown from single colonies inoculated in 16 × 100 mm test tubes with 4 mL of YEM broth devoid of mannitol and incubated at 28°C for 3 days in the case of *Rhizobium* strains and 5 days for the *Bradyrhizobium* strains. This culture was diluted to 10^{-2} with 0,85% saline solution and 0,1 mL of this dilution served as inoculum for tubes (10 × 75 mm) containing 1,6 mL of modified media. Tubes were vigorously shaken every 12 hours to minimize O_2 stress. Growth was determined by plate counts on YEM agar when cultures reached the late log phase.

Modifications to YEM broth

In the first three experiments we used *Bradyrhizobium* sp. PLL113 and *Rhizobium* sp. PLC213 strains. In the first experiment, yeast extract was reduced to 0,1, 0,05, 0,025, 0,005 and 0 g/L to determine the minimum concentration required to obtain similar growth to that of YEM broth. To compensate for this reduction, glutamic acid (1,1 g/L) was added as a nitrogen source. In a second

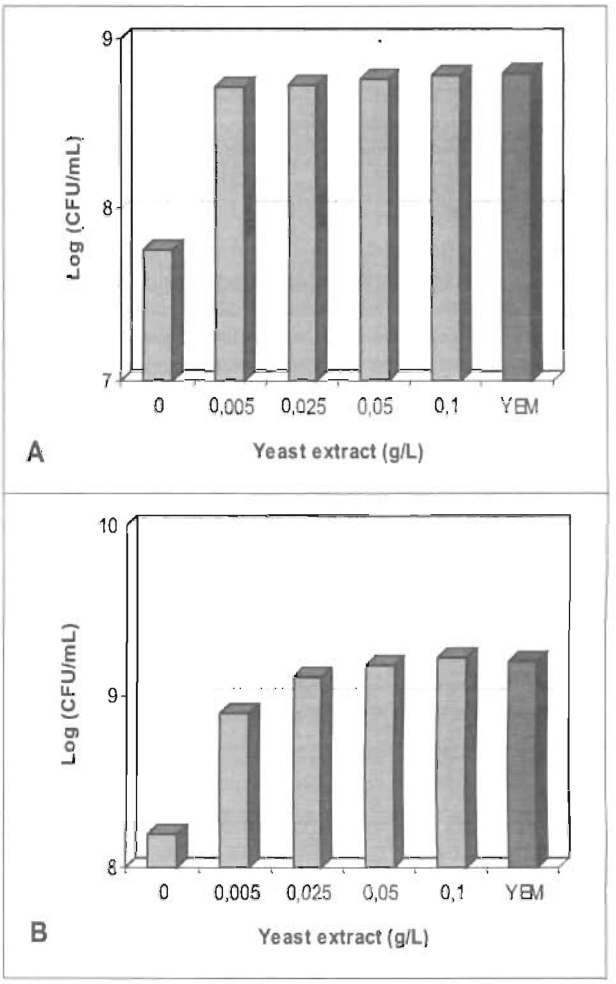

Figure 1. Growth of *Bradyrhizobium* sp. PLL113 (A) and *Rhizobium* sp. PLC213 (B) in media with different concentrations of yeast extract. Columns with asterisk do not differ significantly from YEM broth (Dunnet test, $P < 0,01$).

experiment, the growth on sucrose, glycerol and mannitol was evaluated. All carbohydrates were added at 10 g/L. In the third experiment, the growth was evaluated when glutamic acid was replaced with the same concentration of two brands of food grade sodium glutamate. Next, the growth of *Bradyrhizobium* sp. PLL113 was evaluated when reagent grade glycerol was replaced with three brands of pharmaceutical grade glycerin. All was added at a concentration equivalent to 10 g/L of pure glycerol. And finally, the growth of *Rhizobium* sp. PLC213 was evaluated when reagent grade sucrose was replaced by three different brands of refined and three unrefined food grade sugars. All was added at 10 g/L. In all experiments 4 or 6 replications (tubes) were used for each treatment (media) in a randomized complete design. Analysis of variance of transformed (Log_{10}) plate counts was performed and means were compared with Duncan or Dunnet tests at a 0,05 significance level (Sokal & Rohlf, 1979).

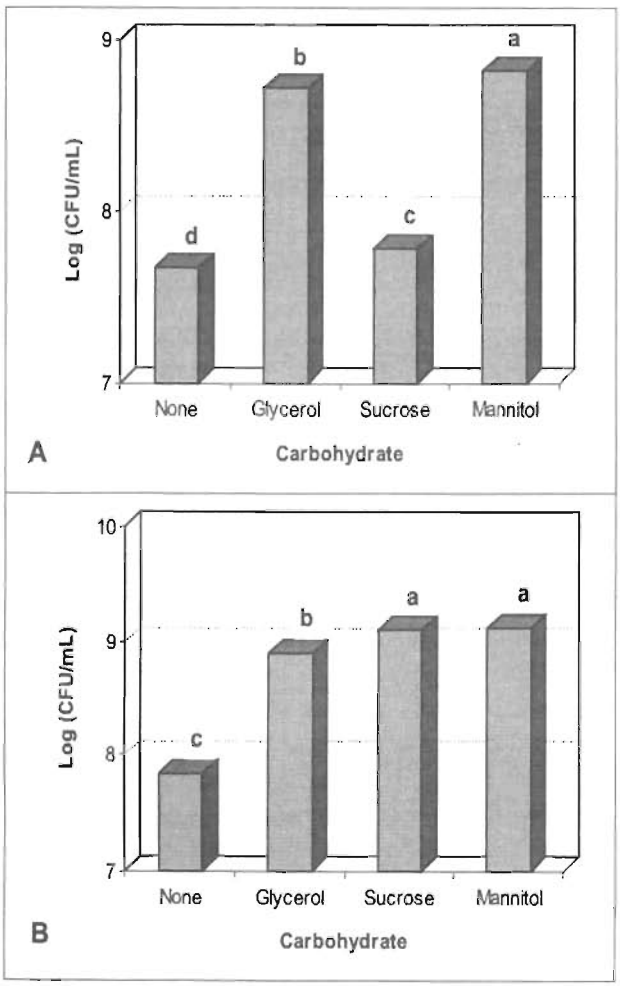

Figure 2. Growth of *Bradyrhizobium* sp. PLL113 (A) and *Rhizobium* sp. PLC213 (B) in media with different carbohydrates. Columns with the same letter do not differ significantly (Duncan test, $P < 0,01$).

Plant inoculation tests

The symbiotic properties of *Bradyrhizobium* sp. PLL113 and *Rhizobium* sp. PLC213 strains grown on modified media were evaluated by inoculating them on plants of lima bean (*Phaseolus lunatus* L.) cv. Sieva. Plants were grown in test tubes (150 x 25 mm) with Broughton & Dillworth (BD) nutrient agar (Beck *et al.*, 1993). For each strain, three tubes were inoculated with 1 mL of liquid culture the same day of transplantation. Plants were maintained in a growth chamber with 12 h photoperiod and irrigated every three days with diluted (1:4) BD nutrient solution. Presence and interior color of nodules was recorded 21 days after transplantation.

RESULTS AND DISCUSSION

Most researchers today produce rhizobial cultures in YEM broth which contains mannitol as a carbon source and yeast extract as a source of both nitrogen and growth factors. Both reactives are the most expensive ingredients of that medium making it not suitable for commercial production of inoculants. Therefore our efforts were focused on the reduction or substitution of them with more economy products.

Figure 1 shows the growth of *Bradyrhizobium* sp. PLL113 and *Rhizobium* sp. PLC213 in media with different concentrations of yeast extract. There was a significant effect ($P < 0,01$) of this ingredient in the growth of both strains. Similar results have been obtained by Gulati & Seth (1978). Growth of *Bradyrhizobium* sp. PLL113 in

Table 1. Growth of *Bradyrhizobium* sp. PLL113 and *Rhizobium* sp. PLC213 in media with two brands of food grade sodium glutamate (GLU) and reagent grade glutamic acid.

Aminoacid (1,1 g/L)	Growth (Log10 CFU/mL)	
	Bradyrhizobium sp. PLL113	Rhizobium sp. PLC213
GLU-1	8,79	9,07
GLU-2	8,86	9,06
Glutamic acid	8,81	9,05

medium with 0,05 g/L of yeast extract did not significantly differ ($P < 0,05$) from that in YEM broth. For *Rhizobium* sp. PLC213 this minimum concentration was 0,025 g/L. At the concentrations tested here, yeast extract is acting mainly as a source of growth factors, specially hydrosoluble vitamins of the B complex (Crueger & Crueger, 1984). Here both strains grew relatively well at low levels of yeast extract and therefore of vitamins. This observation is in contradiction with the generally accepted assumption that *Rhizobium* strains are very exigent in their vitamin requirements while *Bradyrhizobium* strains are more tolerant to low levels (Graham, 1963; Sierra *et al.*, 1996).

In order to find a substitute for mannitol, we evaluated the growth of *Bradyrhizobium* sp. PLL113 and *Rhizobium* sp. PLC213 in media with different carbohydrates (Figure 2). The growth

Table 2. Growth of *Bradyrhizobium* sp. PLL113 in media with three brands of pharmaceutical grade glycerin (GLI) and reagent grade glycerol.

Carbohydrat e (12,5 g/L)*	Growth (Log10 CFU/mL)
GLI-1	8,79
GLI-2	8,79
GLI-3	8,81
Glycerol	8,78

* Of pure glycerol

of the *Bradyrhizobium* strain was different in all the tested carbon sources ($P < 0,01$), while *Rhizobium* sp. PLL113 grew similarly well on mannitol and sucrose and poorly on glycerol ($P < 0,05$). Both strains can grow without carbohydrate utilizing glutamic acid as a solely source of carbon and nitrogen but the growth was very limited because the low concentration of that aminoacid. In general, the growth patterns observed agree with the characteristics of both strains as reported in the literature. *Rhizobium* sp. PLC213 grew well on sucrose in agreement with reports that state that this genera have uptake mechanisms and

Table 3. Growth of *Rhizobium* sp. PLC213 in media with refined sugar (RS), unrefined sugar (US) and reagent grade sucrose.

Carbohydrat e (10 g/L)*	Growth (Log10 CFU/mL)
RS-1	9,19
RS-2	9,19
RS-3	9,18
US-1	9,23
US-2	9,22
US-3	9,20
Sucrose	9,19

catabolite enzymes for the metabolism of disaccharides (Jordan, 1984; Stowers, 1985). On the other hand, Jordan (1984) states that the genus *Bradyrhizobium* rarely utilized sucrose, while Hamdi (1985) affirms that the slow growing rhizobia (*Bradyrhizobium*) do not metabolize this carbohydrate. In this study, the bradyrhizobia strain showed a poor growth on sucrose in comparison with mannitol or glycerol. Similar results were observed by Stowers & Elkan (1984). Interestingly, the latter authors did not find significant invertase activity in the 20 strains that showed a limited growth on sucrose. Although we did not measure invertase activity, we can conclude either that the invertase synthesized by *Bradyrhizobium* sp. PLL113 have a very low activity or that some of the sucrose was partially

Table 4. Composition of YEM and modified media.

Ingredient	Composition (g/L)		
	YEM	*Bradyrhizobium*	*Rhizobium*
Potassium phospate	0,5	0,5	0,5
Magnesium sulphate	0,2	0,2	0,2
Sodium chloride	0,1	0,1	0,1
Yeast extract	0,5	0,05	0,05
Sodium glutamate	-	1,1	1,1
Mannitol	10,0	-	-
Glycerol	-	12,5	-
Sugar*	-	-	10,0

* Refined or unrefined

hydrolyzed in the sterilization process and the strain was metabolizing the fructose and glucose produced. In spite of the bad growth of bradyrhizobia in sucrose, many manufactures of commercial inoculants use this carbohydrate to minimize production costs (Williams, 1984). Our results with *Bradyrhizobium* sp. PLL113 indicate that this practice would be a wasteful of the ingredient.

The growth of *Bradyrhizobium* sp. PLL113 and *Rhizobium* sp. PLC213 on glycerol was significantly lower ($P < 0,01$) than that on mannitol at the same concentration (Figures 1 and 2). These results were not totally unexpected taking into account that glycerol is a gluconeogenic substrate and similar findings obtained by Bissonnette *et al.* (1986). When we calculate the percentage of growth in medium with glycerol with respect to mannitol, we found that the *Bradyrhizobium* strain showed a better growth (79%) on glycerol than the *Rhizobium* strain (61%). Day (1991) mentions that a British inoculant company uses 10 mL/L of glycerol to grow *B. japonicum*. Taking into account this report and that the bradyrhizobia strain did not

Table 5. Growth of seven rhizobial strains in YEM and modified media.

Strain	Growth (Log10 CFU/mL)	
	Modified	YEM
Bradyrhizobium sp. PLL113	8,80	8,78
B. sp. PLL142a	8,70	8,72
B. sp. TAL169	8,68	8,66
B. sp. TAL22	8,78	8,81
Rhizobium sp. PLC213	9,14	9,17
R. sp. PLA142a	9,24	9,25
R. sp. 9A	9,41	9,38

Table 6. Costs of YEM and modified media for rhizobial strain culture.

	Composition (g/L)		
Ingredient	**YEM**	***Bradyrhizobium***	***Rhizobium***
Potassium phospate	0,030	0,030	0,030
Magnesium sulphate	0,015	0,015	0,015
Sodium chloride	0,003	0,003	0,003
Yeast extract	0,083	0,008	0,008
Sodium glutamate	-	0,004	0,004
Mannitol	1,145	-	-
Glycerol	-	0,220	-
Sugar*	-	-	0,006
TOTAL	**1,276**	**0,28**	**0,066**
REDUCTION		**78%**	**95%**

*Unrefined

grow well on sucrose, we performed an additional experiment to test its growth at 10 mL/L of glycerol (equivalent to 12,5 g/L). At this concentration there was not significant difference ($P = 0,8$) with mannitol.

Table 1 shows the growth of *Bradyrhizobium* sp. PLL113 and *Rhizobium* sp. PLC213 in media with food grade sodium glutamate. Analysis of variance did not reveal significant differences in growth compared to media with reagent grade glutamic acid. Similar results were obtained when reagent grade glycerol or sucrose were replaced with pharmaceutical grade glycerin or food grade refined and unrefined sugar (Tables 2 and 3). These results demonstrate that food and/or pharmaceutical grade equivalents of glutamic acid, glycerol or sucrose are sufficiently pure, and free of growth inhibitors to rhizobia. Although crystals of unrefined sugar are covered by a fine pellicle of meal which contains little amounts of nitrogen compounds, organic acids, vitamins and reducing sugars that can act as nutrients (Spencer, 1932; Skrabonja, 1970), our results shown that their presence do not significantly promote the growth of rhizobia.

With the results obtained with *Bradyrhizobium* sp. PLL113 and *Rhizobium* sp. PLC213, two modified media were determined (Table 4). When five other strains were grown in these media, no significant differences were observed as compared to YEM broth (Duncan test, $P < 0,05$) (Table 5). These results demonstrate that the new media can be satisfactorily used with other rhizobia.

In the production of rhizobial biomass it is very important to demonstrate that the media do not alter the symbiotic properties of the bacteria making it useless as an inoculant strain (Thompson, 1991). *Bradyrhizobium* sp. PLL113 and *Rhizobium* sp. PLC213 grown on the modified media elicited the production of normal nodules (Nod+) of pink or red interiors (Fix+) confirming that they did not lose their nodulation and nitrogen fixing capabilities.

The results obtained in this work demonstrate that is possible to use cheap, easily available products of food and pharmaceutical grade to substitute the most expensive ingredients of YEM broth. These modifications permitted a 78%

reduction in cost with the *Bradyrhizobium* modified media over YEM. In the case of the *Rhizobium* modified media the reduction is even larger, 95% (Table 6).

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