

Lactuca sativa biofertilization using biofilm from *Bacillus* with PGPR activity

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Abstract

Biofertilizers can be an alternative to chemical fertilizer as they increase sustainable soil fertility without causing pollution; however, their major problem is the poor survival of the free cells in the soil. A possible solution is the use of cells immobilized in biofilms; it provides a more suitable microenvironment for prolonged cell survival and allows the interaction of the bacterium and its metabolites with the plant. In this work, the planktonic *Bacillus subtilis* subsp. *spizizenii* showed a PGPR activity on *Lactuca sativa*, increasing the weight, 64 % the aerial part and 68 % the roots growth. This bacterium was able to produce a thick biofilm using glycerol as a sole carbon source. Different culture conditions were evaluated for biofilm production. The shear stress and the oxygenation during bacterial culture affected negatively the biofilm formation; a mechanically disrupted biofilm never recovered its integrity. The optimum temperature for biofilm production was between 30 °C and 37 °C. The presence of different divalent cations salts affected the biofilm formation; 2 mM MgSO₄ and 1 mM FeSO₄ in static growth culture increased the biofilm production 36 % and 72 % respectively, and CoSO₄ and CuSO₄ affected negatively its formation. The immobilized cells had a PGPR effect; it showed a higher benefit as a biofertilizer than the planktonic form, producing an increment of 39 % of the aerial part and 59 % the roots growth.

Key words: *Lactuca sativa*, biofertilizer, *Bacillus subtilis* subsp. *spizizenii*, PGPR activity, biofilm production, glycerol, culture conditions, immobilized cells, inoculant, sessile bacteria, planktonic bacteria, rhizosphere.

Introduction

The impact of mineral fertilizers on the environment is a world wide concern. Its use have resulted in polluted soils, with diminished fertility. Biofertilizers are a good alternative to chemical fertilizers; they increase soil fertility improving plant growth without causing pollution. The major problem of a bacterial biofertilizer as a free cell system is its poor survival in the soil (Seneviratne *et al.*, 2008; Malusá *et al.*, 2012). The cell immobilization represents a protective mean for microbial growth, offering survival advantages to cell compared to free cell system (Schoebitz *et al.*, 2013). The attachment of cell immobilized by a biofilm to plant roots may help in the absorption of nutrients and in the biocontrol of plant diseases (Vessey, 2003; Ashraf *et al.*, 2004).

B. subtilis, a spore forming Gram positive bacteria has become a model organism for cells immobilization because it is non-pathogenic and non-toxicogenic for human, animals or plants. It belongs to the group of bacteria called “plant growth promoting rhizobacteria” (PGPR). These are beneficial bacteria which have the ability to colonize roots and either promote plant growth through direct action or via biological control of plant diseases (Kloepper and Schroth, 1978). *B. subtilis* exhibits two growth models, the free-floating planktonic growth and the biofilm formation, where they remain sessile. Biofilm growth has been proposed to be the major bacterial life style in nature (Costerton *et al.*, 1995). This sessile bacterial growth is a microbial community embedded in a matrix of substances; mainly exo-polysaccharides, proteins and DNA (Branda *et al.*, 2005) that creates a regulated environment surrounding the bacterial cells, allowing the passage

of water, gases and nutrient while restricting the entrance of antibiotics, surfactants, bacteriophages, *etc.* (Costerton *et al.*, 1995; Vanderlinde *et al.*, 2010). Biofilm matrix composition of bacteria can vary for different environmental conditions, different strains of the same species may produce different matrix components (Danhorn and Fuqua, 2007).

Biofilm synthesis is governed by a number of physical, chemical and biological processes. Environmental factors such as pH, temperature, nutrients and osmolarity have also influence on biofilm development. The formation of a biofilm is dependent on the presence and reaction rates of the enzymes which control the development of many bacterial physiological and biochemical systems. Also metals might influence the biofilm development (Morikawa *et al.*, 2006). Microscopy studies have shown that biofilms have a complex and heterogenic architecture that depends on the type of bacteria and the growth conditions. The biofilm complexity is even higher for a biofilm formed on a liquid media (Branda *et al.*, 2005).

For the production of a low cost biofertilizer selection of a cheap carbon source is necessary and one alternative is glycerol, a by product of other industrial activity (da Silva *et al.*, 2009). The aim of the present study was to develop an immobilized cells system (biofilm) that can be used as a cheap biofertilizer, using *B. subtilis* subsp. *spizizenii*, and glycerol as sole carbon source; and determining the optimum conditions for its production.

Materials and methods

Bacterial strains and growth conditions: *B. subtilis* subsp. *spizizenii* (common distribution) was stored in AGRAL culture

collection, FAUBA. For assays, bacterial inoculum was plated in nutrient agar overnight at 30 °C and resuspended in physiological solution.

Optimization of the biofilm formation conditions: In the optimization assays a basal salt medium (BSM) was used ($\text{g}\cdot\text{L}^{-1}$): K_2HPO_4 , 3.0; KH_2PO_4 , 1.0; NH_4Cl , 0.5; NH_4NO_3 , 0.1; Na_2SO_4 , 0.1; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.01; $\text{MnSO}_4\cdot 4\text{H}_2\text{O}$, 1.10^{-3} ; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 1.10^{-3} ; CaCl_2 , 5.10^{-4} ; EDTA, 0.01; pH 7; 1 % glycerol or 1 % glucose as carbon sources. Growth was measured at DO_{610} (Beckman spectrophotometer). Biofilm production was determined separating the pellicle from the liquid culture; the pellicle was washed twice with deionized water, dried and weighed. The effect of temperature (between 25 and 45 °C) and different concentrations of metals (iron as FeSO_4 , magnesium as MgSO_4 , cobalt as CoSO_4 and copper as CuSO_4 , at 30 °C) were assayed in BSM with 1 % glycerol and 55 mM of L-glutamic acid in static growth culture.

Biofilm assays: The effect of oxygen on biofilm production was studied by rotary shaking the culture medium at different speeds and by disruption of the biofilm for microaerobic condition (after 48 h of incubation the biofilm was broken allowing the passage of oxygen).

Plant growth assays: *L. sativa* seeds were surface-sterilized for 10 min with 1.5 % sodium hypochlorite, washed twice with sterile distilled water and incubated at 20 °C in dark conditions until radicle emergence (5 days).

The planktonic inoculum was obtained growing the bacteria in BSM supplemented with 1 % glycerol, 55 mM L-glutamic acid, 2 mM MgSO_4 and 1 mM FeSO_4 , until late log phase at 30 °C, $150 \text{ r}\cdot\text{min}^{-1}$. The biofilm inoculum was obtained after 96 h in the same medium in static growth culture.

Inoculation with *B. subtilis* in the planktonic form was made embedding the seeds for 30 min in a medium containing $1.10^8 \text{ CFU}\cdot\text{mL}^{-1}$. For inoculation with biofilm, it was used a pellicle with $1.10^8 \text{ CFU}\cdot\text{mL}^{-1}$ biofilm which was mixed with the seeds for 30 minutes.

Seeds were planted in pots (10 cm x 15 cm) in a tyndallized

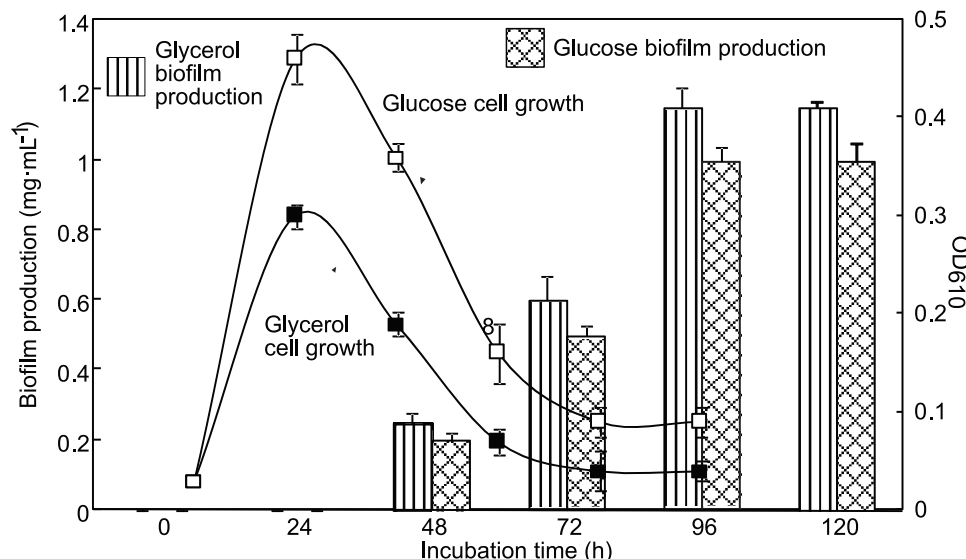


Fig. 1. *B. subtilis* subsp. *spizizenii* growth and biofilm production in BSM with 55 mM L-glutamic acid and 1 % glycerol or 1 % glucose in static growth culture at 30 °C.

Table 1. Effect of *B. subtilis* subsp. *spizizenii* planktonic form on *L. sativa* growth

Treatment	Aerial biomass ^a (mg/plant)	Root (mg/plant)
Uninoculated	72 ± 9	2.5 ± 0.3
Inoculated-Planktonic	118 ± 15	4.2 ± 0.4

Fresh weight of aerial part and root were determined after 15 days of growth. ^aEach value is the mean ± SE.

mixture 2:1 of soil (Escobar, Buenos Aires) and compost (C / N ratio 19). Plants were grown in a culture chamber thermostated for 15 days at 20 °C with 70 % relative humidity and 16/8 light/dark photoperiod. The weight of aerial part and root of plants were measured (30 plants per treatment). Cells densities of the rhizosphere were estimated by counting the number of CFU (Alef and Nannipieri, 1995). Experiments were performed in triplicate for each treatment.

Statistics: The significance was assessed by the Anova test, using Infostat Software (Di Rienzo *et al.*, 2008).

Results

Effect of the inoculation of *L. sativa* with *B. subtilis* subsp. *spizizenii*: Table 1 shows that the inoculation of *Lactuca sativa* seeds with *B. subtilis* subsp. *spizizenii* produced an increased weight of 64 % of the aerial part and 68 % on root growth. These results showed that this strain had a PGPR effect on the plant.

Biofilm production in static growth culture with glycerol or glucose: In static conditions the growth curves were similar for both carbon sources. The maximum optic densities were 0.46 for glucose and 0.30 for glycerol at 24 h followed by a sharp decline until 72 h. From 96 h onward the optic densities were constant for each medium (0.09 for glucose and 0.04 for glycerol).

In both cases, biofilm production in static condition began at 48 h, reaching the highest production at 96 h remaining constant up to 120 h, coinciding with the late stationary phase. For glycerol and glucose as carbon sources there was no direct relationship between biofilm synthesis and biomass formation; biofilm production was higher with glycerol while biomass was higher with glucose (Fig. 1).

Numerous studies have claimed that glucose is the best carbon source for planktonic growth of *B. subtilis* (Stülke and Hillen, 2000; Singh *et al.*, 2008). This was also true for the *B. subtilis* subsp. *spizizenii*, however for this bacterium, glycerol proved to be a better carbon source for biofilm development.

Effect of shear stress on biofilm formation: For both carbon sources, cell densities in the biofilm were higher at $80 \text{ r}\cdot\text{min}^{-1}$, the low shaking speed might allow a higher interaction between cells and nutrients in the liquid culture and the developing biofilm, enabling the presence of a higher number of cells in the biofilm. The cell growth in the liquid culture was higher for glucose than for glycerol.

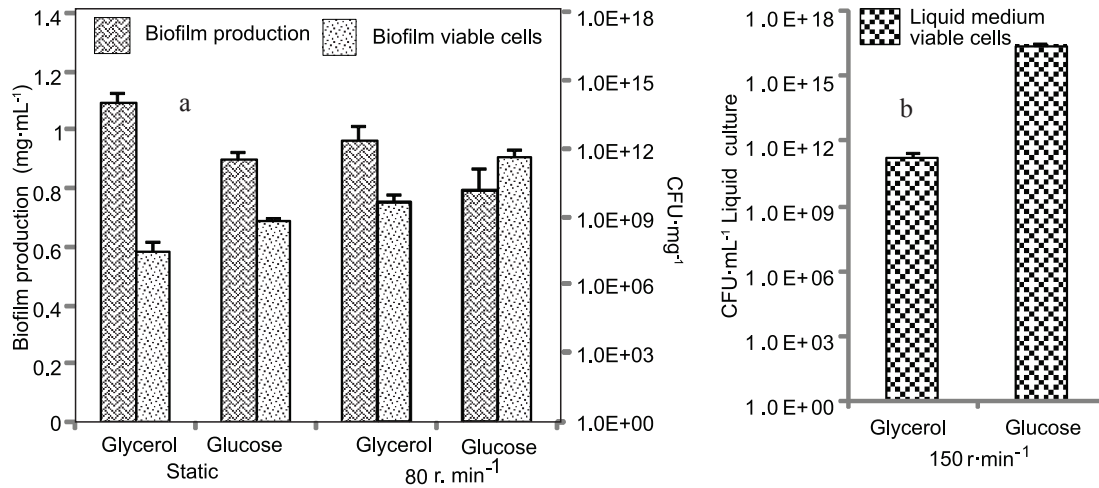


Fig. 2. Growth and biofilm formation of *B. subtilis* subsp. *spizizenii* in BSM, 55 mM L-glutamic acid and 1 % glycerol or 1 % glucose as carbon sources at 96 h, 30 °C. a: growth in the biofilm under static conditions or 80 r·min⁻¹. b: growth in the liquid culture under 150 r·min⁻¹.

The biofilm formation at low shaking speed (80 r·min⁻¹) was slightly lower than in static conditions, 13 % for each carbon source (Fig. 2a). There was no biofilm formation with high shear stress (150 r·min⁻¹) (Fig. 2b). Other authors have shown that the matrix cohesion in biofilm might be strong enough to support low shear stress (Lemos *et al.*, 2015).

Biofilm production in static and microaerobic static culture: Intact biofilm matrix decrease oxygen diffusion into the culture, disruption of the biofilm surface might allow air diffusion to the medium during incubation. For both carbon sources, the disrupted biofilms stayed broken and never recovered their integrity during the culture (data not shown).

Biofilm production, in both glycerol and glucose culture, was higher when the biofilm was intact, with lower oxygen availability for the bacterium. At 96h in microaerophilic condition (biofilm disrupted), the biofilm production decreased 29 % for glycerol and 22 % for glucose (Fig. 3 a, b). The optic densities of the medium in contact with the biofilm at 96h were higher in the disrupted biofilms (0.09 for glycerol and 0.17 for glucose). The presence of oxygen facilitates the bacterial growth in the liquid medium. Morikawa *et al.* (2006) observed that *B. subtilis* B1 culture fully

aerated with a microporous Teflon tube did not produce biofilm.

On the other hand, in static growth culture with glycerol as carbon source the medium pH decreased, with a thick biofilm development. In microaerophilic condition the pH did not decrease and the biofilm structure was not stable (data not shown).

Effect of temperature on biofilm production in glycerol medium: Biofilm production was highly dependent on growth temperature, being the maximum between 30 °C and 37°C. The biofilm production at 45 °C was approximately 53 % lower; however, at this temperature the biofilm had the highest cell density (3 10¹⁰ CFU·mg⁻¹ biofilm); at lower temperatures the cell densities were around 1 10⁸ CFU·mg⁻¹. These results are different from other *Bacillus*, where the temperatures of maximum biofilm production and of maximum cell growth were in the same range (Elhariry, 2008).

Effect of metals on biofilm formation: The addition up to 2 mM FeSO₄ benefitted the biofilm formation with an increase of 36 %, however, higher concentrations inhibited bacterial growth. Kalantari (2008) observed this inhibitory effect on growth for *B.*

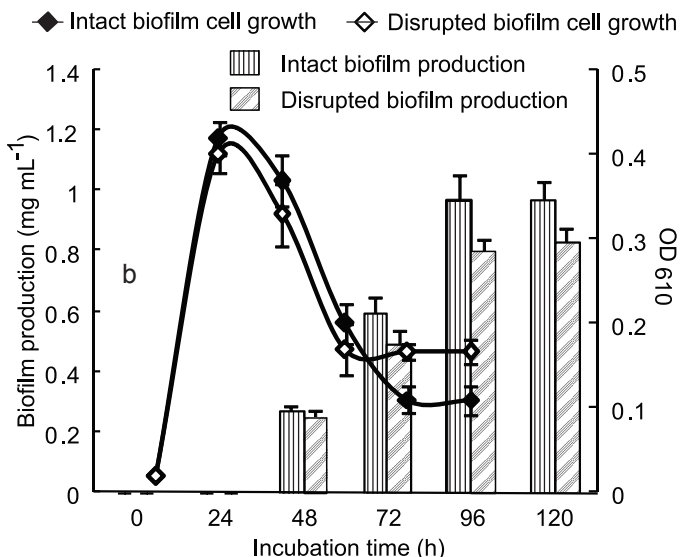
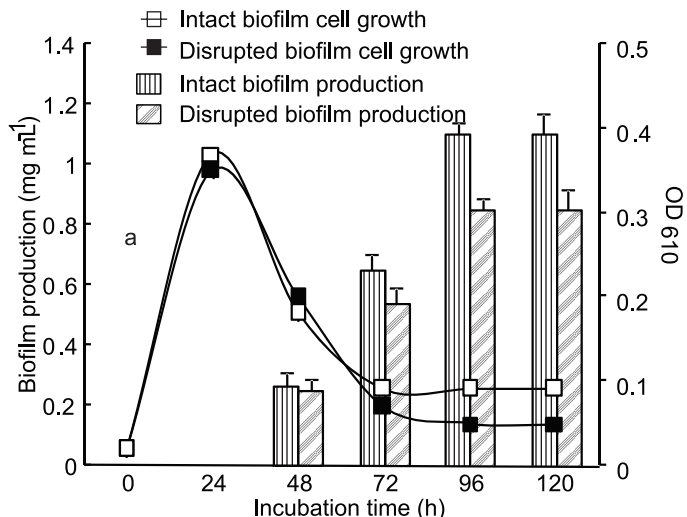


Fig. 3. Growth and biofilm formation by *B. subtilis* subsp. *spizizenii* in BSM, 55 mM L-glutamic acid and 1 % glycerol (a) glucose 1 % (b) in static culture and microaerobic static culture system at 30 °C.

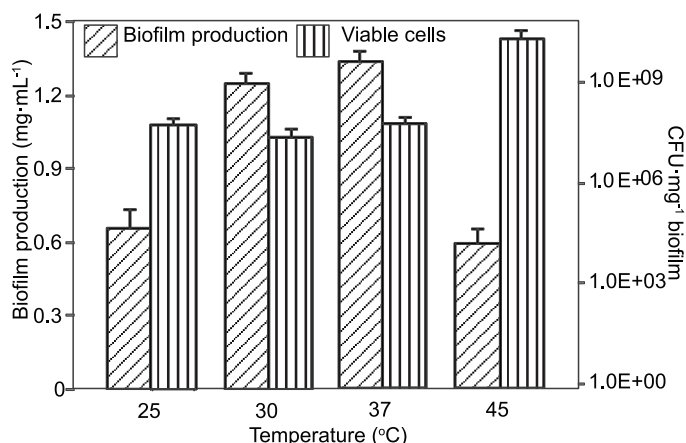


Fig. 4. Effect of temperature on growth and biofilm production by *B. subtilis* subsp. *spizizenii* grown in BSM, 55 mM L-glutamic acid and 1 % glycerol in static growth culture at 96 h.

cereus when the iron concentration was higher than 1 mM.

Among the assayed metal salts, 2 mM MgSO₄ had the highest effect on the biofilm development with an increase of 72 % over control. Divalent cations, such as Mg²⁺, can influence biofilm formation directly through their effect on electrostatic interactions in the bonding processes and indirectly via physiology-dependent pathways by acting as important cellular cations and enzyme cofactors.

The addition of CoSO₄ or CuSO₄ to the medium inhibited the biofilm formation; from 6 mM CuSO₄ cell growth inhibition was also observed. The deleterious effect of transition metals such as cobalt and copper on *Bacillus* can be compared with the negative effect of these salts on other bacteria such as *Streptococcus pneumoniae* and *Klebsiella pneumoniae* B5055 (Chhibber *et al.*, 2013; Honsa *et al.*, 2013).

Effect of *B. subtilis* subsp. *spizizenii* inoculation in planktonic form or biofilm on *L. sativa* plant growth: The biofilm used in this assay was produced in the optimum yield conditions determined in this work: 1 % glycerol, 55 mM L-glutamic acid, 2 mM MgSO₄, 1 mM FeSO₄, at 30 °C in a static growth culture, 96 h, with a yield of 1,9 g·L⁻¹ and a 1.10⁸ CFU.mg⁻¹ biofilm.

Comparing inoculation forms of *B. subtilis* subsp. *spizizenii*, in their planktonic form or as immobilized cells in the biofilm on *L. sativa*, the application of the biofilm directly on seeds favored the plant development. The planktonic form caused a weight increase of 57 % on the aerial part and 76 % on the root with respect to non-inoculated plant. The PGPR effect was higher for the immobilized cells system in the biofilm compared to the planktonic inoculum, with an increment of 39 % of the aerial part and 59 % on the root growth.

Table 2. Effect of metal salts on biofilm production (mg·mL⁻¹) by *B. subtilis* subsp. *spizizenii* grown in BSM, 55 mM L-glutamic acid, 1 % glycerol, with different concentrations of salts in static growth culture at 30 °C

Metal salt (nM)	Biofilm production (mg·mL ⁻¹)						
	Control ^a	0.5	1	2	4	6	8
FeSO ₄	1.1±0.1	1.1±0.1	1.5±0.1	1.5±0.1	—*	—*	—*
MgSO ₄	1.1±0.1	1.3±0.1	1.8±0.2	1.9±0.2	1.3±0.1	1.2±0.1	1.2±0.1
CoSO ₄	1.1±0.1	—	—	—	—	—	—
CuSO ₄	1.1±0.1	—	—	—	—	—*	—*

- : no biofilm. * : no growth. ^aEach value is the mean ± SE.

Cultivable bacteria in *Lactuca sativa* rhizosphere: The bacteria aerobic count, CFU·g⁻¹ rhizosphere, was two orders higher in root of plants inoculated with the planktonic state compared to those inoculated with biofilm immobilized cells (Fig. 6). In spite of the lesser bacterial growth on the root inoculated with biofilm, the later had a higher beneficial effect on plant growth (Fig. 5).

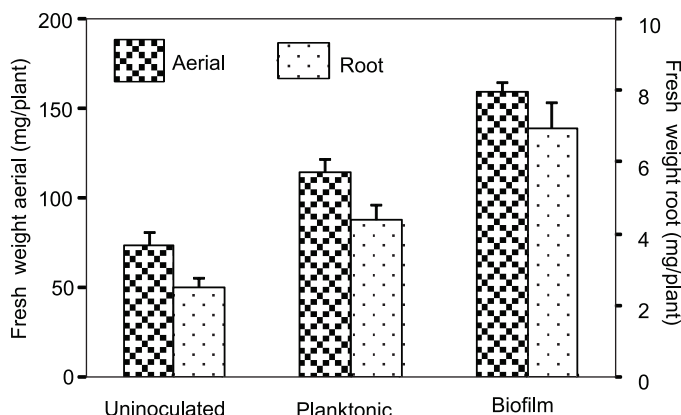


Fig. 5. Effect on plant growth of *L. sativa* inoculated with *B. subtilis* subsp. *spizizenii* planktonic or biofilm form. Fresh weight of aerial part and root were determined after 15 days of growth.

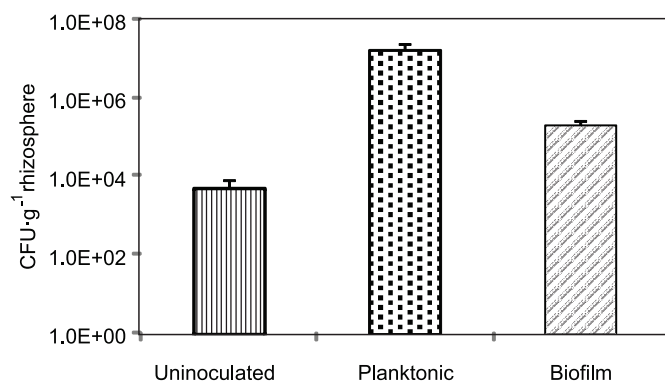


Fig. 6. The soil of the plant root rhizosphere was separated and the aerobic microflora was counted after 15 days of plant growth.

Discussion

Each year more crop production is needed to provide the growing demand for food. In order to maintain the productivity, more and more chemicals are released into the environment. These chemicals enter the food chain resulting in serious harmful effects on human health (Townsend, 2003). The demand for chemical-free products by consumers and the cost of agrochemicals prompted the development of new, more environment friendly technologies (Compant *et al.*, 2005). Several biofertilizers based on plant growth-promoting

microorganisms have been introduced. In this sense, *B. subtilis* has a PGPR activity in crop ecosystems (Choudhary and Johri, 2009; Kumar *et al.*, 2011).

In general, the bacteria in the inoculants formulation are in the free-floating planktonic growth. An innovative approach to enhance the beneficial effect on the plant would be the increase of the intimate contact between the bacteria and the plant. This can be achieved using a cell immobilization system, a biofilm.

In this work, the inoculation of *L. sativa* seeds with *B. subtilis* subsp. *spizizenii* in planktonic growth showed PGPR effect. Cells immobilized in a biofilm had a higher effect on both, the aerial part and the root. The inoculation form had a different effect on the aerobic bacteria count in the rhizosphere, being higher in the case of the planktonic inoculum. The lower aerobic bacterial number found in the rhizosphere and the higher PGPR caused by biofilm inoculation supports the idea that the phenotype developed in the biofilm might produce metabolites different from those of the planktonic state, being more effective or concentrated for the promotion of the plant growth. Even more, in the biofilm there are oxygen gradients that cause different metabolisms, producing intermediate metabolites that may favor the plant growth (Vilain and Brözel, 2006; Stewart and Franklin, 2008).

A fundamental aspect for the introduction of a new biofertilizer in the global market is a low cost production, which implies the use of an economic carbon source for the biofilm synthesis. An option could be glycerol, a byproduct of biodiesel industry, which is currently an environmental problem (da Silva *et al.*, 2009). Our results showed that *B. subtilis* subsp. *spizizenii* was able to synthesize a robust biofilm using glycerol as the sole source of carbon. Even more, the amount of biofilm synthesized was higher with glycerol than produced with glucose, a carbon source commonly used. We found that there was no direct relationship between the biofilm synthesis and the cell growth in the culture media; biofilm production was higher with glycerol while biomass was higher with glucose. This lack of relationship was previously reported for other bacteria with different pair of carbon sources. Gorret *et al.* (2001) reported that cellular growth and biofilm production have different nutritional requirements. Another difference observed using this carbon sources was the final pH value of the culture medium, which remained constant in the case of glucose but it diminished to 5.5 for glycerol (data not shown). The pH variation using glycerol as carbon source may be the result of its different metabolism products.

The biofilm synthesis was slightly affected by low rotary shaking, being inhibited at 150 r·min⁻¹. The stirring of the media may have two effects: the shear stress and the increment of the interaction between cells and nutrients present in the liquid phase. Shear stress may interfere with biofilm formation by preventing the aggregation of the polymers that form the biofilm matrix. Other factor may be the effect of oxygen on biofilm formation. To study this effect a system was assayed in which the biofilm was disrupted mechanically as soon as it was formed (48 h), allowing the diffusion of oxygen to the culture in static conditions. In this system the final biofilm production was minor than in intact biofilm. This suggests that the oxygen depletion is a key factor in the biofilm production. Morikawa *et al.* (2006) had similar results, observing no biofilm formation using a microporous Teflon tube

Poreflon to aerate the culture without visible air bubbling in static conditions.

Another important factor for biofilm synthesis is the achievement of the optimal culture conditions. There is plenty of information describing the effect of temperature on bacterial growth in culture; however, the effect of temperature on biofilm formation is not so well documented. In this study, the optimum temperature for biofilm production was different from the temperature for maximum cell density in the biofilm. Mendoza-Hernández *et al.* (2008) reported a maximal biofilm production by *Klebsiella pneumoniae* ATCC 10031 at 35 °C, showing a marked decrease in biofilm production when the temperature was 10 °C below or above that temperature.

The addition of divalent cations to the culture media had different effects on biofilm production. Mg²⁺ and Fe²⁺ caused an increase of the biofilm production, while Cu²⁺ and Co²⁺ inhibited the synthesis of biofilm, indicating that Cu²⁺ is toxic at high concentration. Metal salts may interact with biofilm components, changing its architecture and modifying the transport of water and nutrient inside the biofilm. Some components of biofilm matrix such as exopolysaccharides may interact with metal ions producing more stable biofilms (Sutherland, 2001).

The optimized culture conditions determined in this study for an economical biofilm production using waste glycerol as a sole carbon was achieved using: 1 % glycerol, 55 mM L-glutamic acid, in a medium of minimal salt supplemented with 2 mM MgSO₄ and 1 mM FeSO₄, at 30 °C in static growth culture, during 96 h.

Whole cell immobilization is a good alternative as a biofertilizer, enhancing the interaction between a PGPR bacterium, *B. subtilis* subsp. *spizizenii*, and its metabolites with the cell root, making them more available for the plant benefit. This system has a potential application in hydroponic crops.

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