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Encapsulation of Biosurfactant-Producing *Bacillus licheniformis* (PTCC 1320) in Alginate Beads

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Abstract: In the present study, biosurfactant production by immobilized *Bacillus licheniformis* PTCC 1320 in alginate beads has been studied. Alginate solution and bacterial suspension were mixed and stirred to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into a stirring calcium chloride solution from a 5 cm height to form spherical beads. The beads were kept under agitation for about 2 h to harden and were then washed. Equal numbers of beads were charged to 250 mL erlenmeyer flasks each containing 50 mL of nutrient broth medium. Beads containing *B. licheniformis* placed in the nutrient broth medium were incubated in a shaker incubator at 30°C. Samples were collected at 24 h intervals and the parameters of biosurfactant production, such as surface tension, emulsification activity and foam production were studied. Results showed that *B. licheniformis* entrapped in calcium alginate beads is able to preserve its viability and produce biosurfactant as a secondary metabolite. Maximum biosurfactant production was achieved during the first 24 h.

Key words: *Bacillus licheniformis*, bacterial immobilization, biosurfactant, alginate

INTRODUCTION

Biosurfactants (microbial surfactants) belong to a structurally diverse group of surface-active molecules that are produced by a variety of microorganisms (bacteria, fungi and yeasts) (Lu *et al*., 2007; Abouseoud *et al*., 2008).

These molecules have gained attention because of lower toxicity, higher biodegradability, better environmental compatibility and specific activity at different temperatures, pH ranges and salinities (Nitschke *et al*., 2004). Interests in the production of biosurfactants have steadily increased during the past decades and their applications have been greatly extended in the past five decades as an improved alternative to their chemically synthesized counterpart'sespecially in food, pharmaceutical and oil industry (Desai and Banat, 1997; Banat *et al*., 2000). Microbial surfactants are known to occur in a variety of chemical structures, such as glycolipids, lipoproteins-lipopeptides, fatty acids, neutral lipids, phospholipids and lipopolysaccharides, polymeric and particulate structures (Muthusamy *et al.*, 2008; Arima *et al*., 1968; Kaeppeli and Finnerty, 1979). *Bacillus licheniformis* produces several biosurfacants, including appreciable amounts of four slightly different lipopeptides which the lichenysin is the most potent surface active lipopeptide among them (Rahman and Gakpe, 2008). These molecules act synergistically and exhibit excellent temperature, pH and salt stability. These are also similar in structural and physio-chemical properties to surfactant (McInerney *et al*., 1990) one of the most powerful biosurfactants composed of a seven amino-acid ring structure coupled to a fatty-acid chain via lactone linkage (Arima *et al*., 1968). Several strains belonging to genus *Bcillus* including *B. licheniformis* have been reported effective for the biocontrol of multiple plant diseases caused by soil borne or post-harvest pathogens. Members of this group often possess antibiotic activity as well. In particular different *Bacillus* species excrete a broad spectrum of bioactive peptides with great potential for biotechnological and biopharmaceutical applications (Banat *et al*., 2000). Cell encapsulation represents one of the current leading methodologies aimed at the delivery of

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biological products and alginate is the most frequently employed material for this purpose. Because, of its mild gelling, biocompatibility and biodegradability properties, alginate has long been used in food and pharmaceutical industries (Orive *et al*., 2006). In 2007, Ca-alginate entrapment was defined as a promising immobilization method of *B. licheniformis* KBR6 for repeated tannase production (Mohapatra *et al*., 2007). In the present study, biosurfactant production by immobilized *Bacillus licheniformis* PTCC 1320 in alginate beads has been studied. Some of the properties of produced biosurfactant such as surface tension, emulsification activity and foam production were determined.

MATERIALS AND METHODS

Test organism: *Bacillus licheniformis* PTCC 1320 was purchased from the Persian Type Culture Collection, Tehran, Iran.

Cultivation of organism: *Bacillus licheniformis* PTCC 1320 was grown in 250 mL erlenmeyer flasks, each containing 50 mL nutrient broth medium. The flasks were incubated at 30°C in a shaker incubator (DK-S1020) at 250 rpm. Samples were withdrawn every 24 h to analyze the surface activity, emulsification index and foam activity. This process was continued up to 120 h. All measurements were done on the supernatant obtained after centrifuging liquid culture (Makkar and Cameotra, 1997; Desai and Banat, 1997).

Cultivation on blood agar: *Bacillus licheniformis* PTCC 1320 was inoculated on blood agar plates containing 5% v/v blood and incubated at 37°C for 48 h. Haemolytic activity was detected as the presence of a definite clear zone around colonies (Worth, 1919). This was judged as possibility of biosurfactant production (Salandari, 2009).

Preparation of calcium alginate beads: Sodium alginate solution 2% w/v was prepared. The solution was taken into a 10 mL sterile syringe, with a 23-gauge stainless steel needle and added drop wise into a stirring calcium chloride 2% w/v solution from a 5 cm height (Abouseoud *et al*., 2008; Orive *et al*., 2006).

Calcium alginate beads preparation was carried out with various concentrations of sodium alginate and calcium chloride. A sum of formed beads were moved to erlenmeyer flasks containing nutrient broth medium. The flasks were incubated at 37°C were shaken at 250 rpm for 120 h. At definite time intervals; the size, shape and stability of beads were evaluated visually.

Preparation of microbial suspension: Microbial cells were harvested from medium by centrifugation at 8000 rpm, for 8 min at 30°C (Eppendorf 5810 R) and the pelleted cells were washed with sterile normal saline and recentrifugation at 8000 rpm for 8 min (Corvey *et al*., 2003). Multiple microbial suspensions at different dilutions were prepared from the microbial pellets of previous step, in order to evaluate the concentration suitable for encapsulation.

Cell immobilization: Both autoclaved alginate solution 4% and cell suspension were mixed and stirred for 10 min to obtain a uniform mixture. The slurry was taken into a sterile syringe and added drop-wise into a stirring 2% calcium chloride solution from 5 cm height. The spherical formed beads were kept under agitation for about 2 h to harden and then were washed (Worth, 1919). Equal numbers of beads were transformed to 250 mL erlenmeyer flasks each containing 50 mL nutrient broth medium. The flasks were incubated at 37°C in a shaker incubator at 250 rpm and every 24 h, surface activity, emulsification index and foam activity were analyzed. This process was continued up to 120 h and all measurements were done on the supernatant obtained from liquid culture containing beads.

Surface activity measurement: Biosurfactant concentration (critical micelle dilution or CMD) was calculated by measuring the surface tension for different concentrations with a duNouy Tensiometer (KRUSS-K100). Twenty five milliliters of bead-free supernatant was put into a glass beaker (38 mL) and placed onto the tensiometer platform. Between each measurement, the platinum-iridium ring was rinsed three times with water, three times with acetone and was then allowed to air-dry (Wei *et al*., 2005). All measurements were made on bead-free broth. CMD^{-1} (Critical Micelle Dilution)$^{-1}$ and CMD^{-2} (Critical Micelle Dilution)$^{-2}$ measurements were performed by measuring the surface tension of 10 and 100 times diluted cell-free broth (Carrillo *et al*., 1996). Uninoculated culture medium was used as negative control.

Emulsification measurement: Emulsifier activity was measured by adding 5 mL of mineral oil (liquid paraffin) to 5 mL of supernatant in a graduated tube and vortexing at high speed (3000 rpm) for 2 min. The emulsion stability was determined after 24 h. The emulsification index (E24) was calculated by dividing the height of the emulsified layer (mm) on total height of the liquid column (mm) (Wei *et al*., 2005; Cooper and Goldenberg, 1987).

Foam forming activity measurement: Supernatant samples were withdrawn every 24 h. The 5 mL of supernatant in a graduated tube was vortexing at high speed 3000 rpm for 1 min. Foaming activity was detected as the duration of foam and foam height in the graduated cylinder) (Razafindralambo *et al*., 1998; Heerklotz and Seelig, 2001).

Loading measurement: Twenty *Bacillus* loaded beads were treated with 20 mL of sodium citrate 55 mm solution and stirred magnetically for 5 min at room temperature. Beads were de-gelled via the exchange of calcium ions by sodium ions. A triplet set of nutrient agar plates were inoculated each with 20 μL of de-gelled mixture and incubated at 37°C for 24 h (Orive *et al*., 2006).

RESULTS

Detection of biosurfactant production: In the present study, hemolytic activity was evaluated as potential predictor of surfactant-producing bacteria. *Bacillus licheniformis* PTCC 1320 was isolated from nutrient agar cultures and tested by hemolytic method. The strain showed hemolytic activity.

Preparation of calcium alginate beads and cell immobilization: Bead formation was practiced with different concentrations of sodium alginate and calcium chloride. Further work was carried out using 2% as chosen concentration, resulted in spherical and uniform calcium alginate bodies and proper stability. Ideal concentration of microbial suspension was achieved when a concentration equal to 0.5 McFarland standard was prepared. Also under incubation conditions in the shaker incubator (30°C, 250 rpm, 120 h) selected, beads were stable and no rupture was seen over 120 h.

Surface tension studies: Maximum biosurfactant production was achieved during 24 h incubation and CMD values followed this pattern (Fig. 1). It can be supposed that while surface tension was minimal, biosurfactant production by the microorganism was maximal. As a result the microbial suspension achieved from 24 h incubation was used for encapsulation.

After encapsulation, beads containing *B. licheniformis* were cultured in nutrient broth and surface tension lowering continued until 24 h of growth. Maximum of bioemulsifier production was achieved in 24 h of incubation and CMD values were minimum at this point. According to the surface tension profile of supernatant and CMD values shown in (Fig. 2), it can be supposed that while surface tension was minimal, biosurfactant production by the microorganism was maximal. Hence, this time was chosen as optimum for biosurfactant production by the microorganism for further experiments.

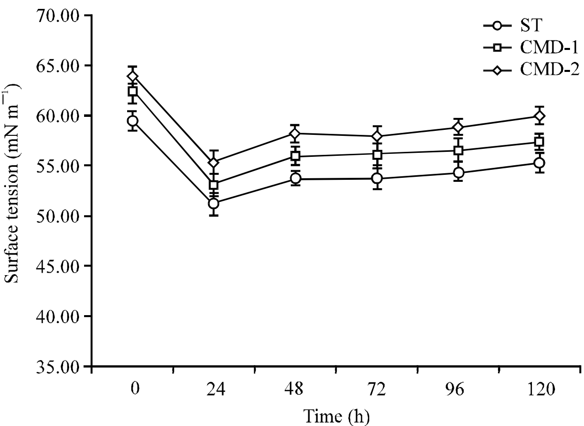

Fig. 1: Surface tension profile of *B. licheniformis* before encapsulation

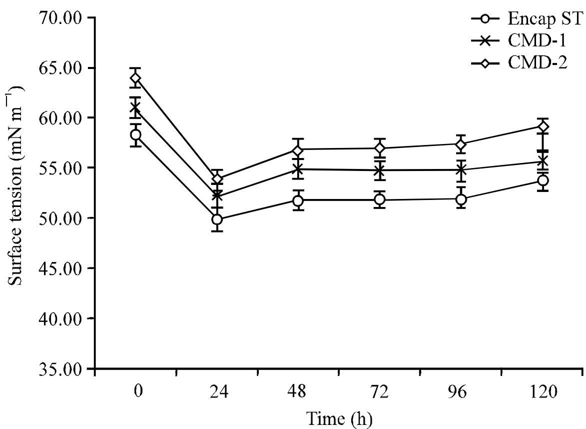

Fig. 2: Surface tension profile of encapsulated *B. licheniformis*

Emulsification index measurement of supernatant: Emulsification index of supernatant of *B. licheniformis* in nutrient broth was measured and as it is seen maximum E_{24} value was observed after 24 h of incubation (Fig. 3).

Effect of immobilization on emulsification index supernatant of beads containing *B. licheniformis* in nutrient broth is shown (Fig. 4). As it is shown, increasing in incubation time causes an improvement in E24 and at the first 24 h of incubation, this index was maximum and has a descending process until 120 h. Results also show that there is a rational correlation between surface activity (Fig. 2) and emulsification index (Fig. 3) and while the surface tension of supernatant was lowest, emulsification

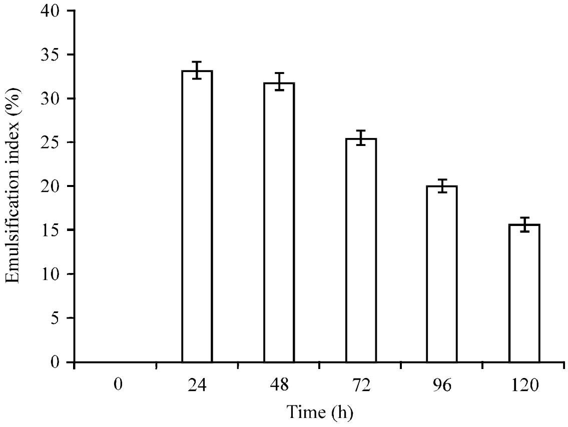

Fig. 3: Emulsification index profile of *Bacillus licheniformis* before encapsulation

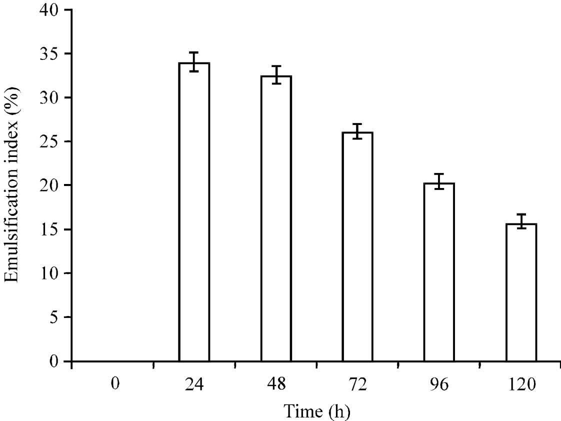

Fig. 4: Emulsification index profile of encapsulated *Bacillus licheniformis*

index raised to maximum. As a result, 24 h of incubation was considered as the best time for biosurfactant production.

Foam stability and foam height test: Maximum foam stability and foam height were achieved in 24 h of incubation (Fig. 5, 6). As shown these factors decreased over time beyond 24 h.

Foam stability and foam height of supernatant of beads containing *B. licheniformis* in nutrient broth, showed maximum foam height and foam stability in 24 h of incubation (Fig. 7, 8).

Loading measurement: After 24 h incubation of bead lysate on nutrient agar plates, growth of typical bacillial colonies of *B. licheniformis* was observed and a count of 200-250 CFU per bead was calculated.

This study showed that *B. licheniformis* entrapped in calcium alginate beads is able to preserve its viability

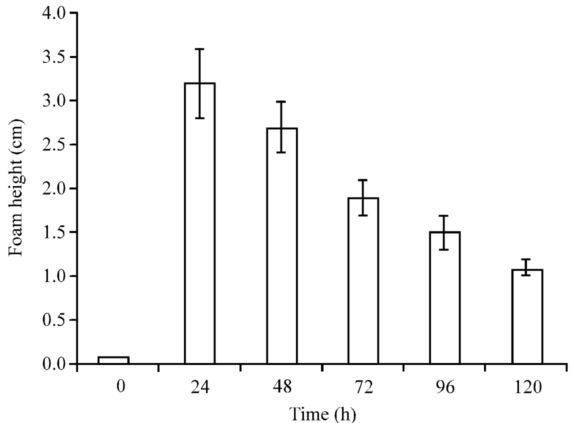

Fig. 5: Foam height profile of *Bacillus licheniformis* in nutrient broth before encapsulation

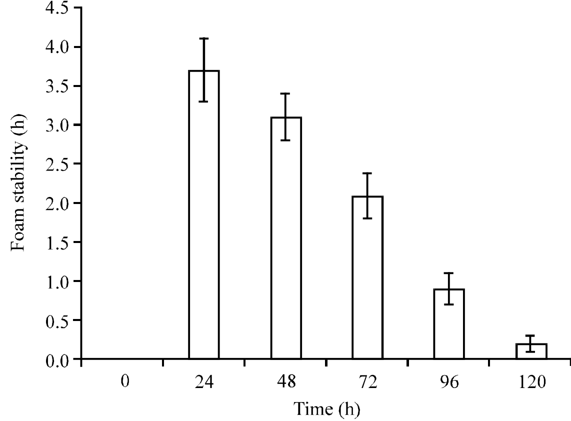

Fig. 6: Foam stability profile of *Bacillus licheniformis* in nutrient broth before encapsulation

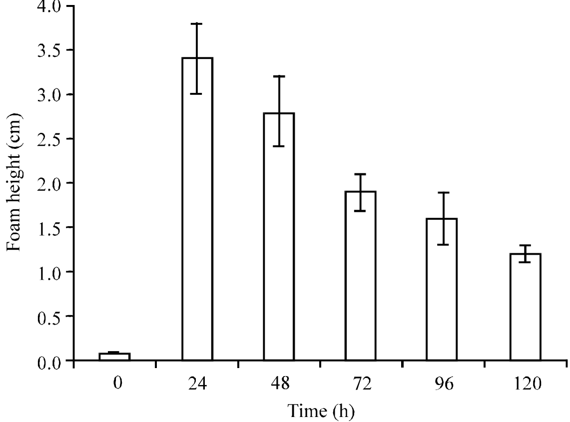

Fig. 7: Foam height profile of encapsulated *Bacillus licheniformis*

and produce biosurfactant as a secondary metabolite. Maximum biosurfactant production was achieved during the first 24 h.

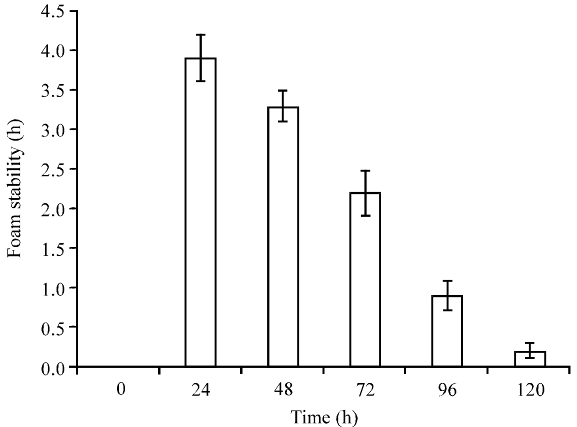

Fig. 8: Foam stability profile of encapsulated *Bacillus licheniformis*

DISCUSSION

Under certain conditions many microorganisms are able to produce secondary metabolites, like biosurfactants. These materials present great potential for biotechnological and biopharmaceutical applications due to their biological properties. This will make biosurfactants highly sought biomolecules for present and future applications as fine specialty chemicals, biological control agents and new generation molecules for pharmaceutical, cosmetic and health care industries (Nitschke *et al*., 2004). Although, a large number of biosurfactant producers have been reported in the literature of biosurfactant research, attempts particularly related to production enhancement and economics has been confined mostly to a narrow selection of bacterial species of genera *Bacillus*, *Pseudomonas* and *Candida* (Lotfabad *et al*., 2009; Arima *et al*., 1968; Wei *et al*., 2005; Abouseoud *et al*., 2008; Cooper and Goldenberg, 1987). *Bacillus* is a recognized and well-studied genus. A lipopetide produced by *B. licheniformis* has hemolytic activity which is directly related to biosurfactant properties (Arima *et al*., 1968).

Successful commercialization of every biotechnological product depends largely on its bioprocess economics. For the production of commercially viable biosurfactants, process optimization at the biological and engineering level needs to be improved. Cell encapsulation represents one of the current leading methodologies aimed at bioprocess development. In addition to its various benefits, beading bacteria in a diffusible polymeric matrix avoids the need for frequent biocatalyst preparation and paves the way for continuous fermentation and facilitates downstream processing of bio-products. In addition, by the means of immobilizing microorganism, it would be possible to have a better study on factors that affect the desired product. Immobilization of microorganisms can therefore bring forth many advantages. This include: No need for inoculum preparation, convenience of product refining and saving time and money (Orive *et al*., 2006; Adinarayana *et al*., 2005; Kuyukina *et al*., 2006). Alginate is the most frequently employed material for the entrapment of eukaryotic and prokaryotic cells due to its mild gelling and biocompatibility and biodegradability properties (Orive *et al*., 2006).

In this study we investigated biosurfactant production from *B. licheniformis* PTCC 1320 in its immobilized format within alginate beads. At the end, results of this trial showed practicability of this method and possibility of a continuous culture system of biosurfactant production. At laboratory scale, if entrapment of viable cells is successful, cells survive and preserve their productivity and the desired product is detected from cell-free supernatant, it could be claimed that a constructive step towards process development of that product has been taken. This is what the current study aimed at and what was achieved. In future, complementary approaches should be taken by researchers to further improve the biosurfactant production via process optimization changes in culture conditions and ingredients. Moreover, genetic engineering of biosurfactant producer microorganisms can enhance the level of this product.

The prime aim of the present study was evaluating achieve ability of immobilizing *B. licheniformis* in calcium alginate beads to provide a concrete base for further process developments of its biosurfactant production. The project was also aimed at providing the practical basis for widespread studies on many other immobilized microorganisms.

CONCLUSION

This study showed that *B. licheniformis* entrapped in calcium alginate beads is able to preserve its viability and produce biosurfactant as a secondary metabolite. Maximum biosurfactant production was achieved during the first 24 h.

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