RESEARCH ARTICLE

Encapsulation of Lactic acid bacteria in calcium alginate beads for higher bacteriocin production

Khandare SS[1] * and Patil SD[2]

[1] Department of Microbiology, J. B. College of Science, Wardha -442001, M. S. , India
[2]Department of Microbiology and Biotechnology, Shri Shivaji Science College, Amravati – 444 603, M. S. , India
*Corresponding author : Email : ksuhas21@gmail.com

Manuscript details:

Received: 04.10.2016
Accepted: 04.11.2016
Published : 01.02.2017

Editor: Dr. Arvind Chavhan

Cite this article as:
Khandare SS and Patil SD (2016) Encapsulation of Lactic acid bacteria in calcium alginate beads for higher bacteriocin production, *International J. of Life Sciences*, 4 (4):539-546.

ABSTRACT

Lactic acid bacteria (LAB) strains LAB -A, LAB -B and LAB-C isolated from batter of idli, very popular fermented food of south India and identified to species level using 16 S rRNA sequencing as *Pediococcus acidilactici* CSI29MX, *Pediococcus parvulus* MF 233 and *Pediococcus pentosaceus* QN1D respectively. All the three strains produced bacteriocins that inhibited Gram positive food borne pathogen *Staphylococcus aureus* and Gram negative *Pseudomonas aeruginosa.* LAB strains were encapsulated in calcium alginate for possible higher bacteriocin production. After 72 h encapsulated LAB demonstrated remarkable increase in bacteriocin production with 2600 and 2800AU/ml tested against *S.aureus* and *P.aeruginosa* respectively and viable cell number of encapsulated LAB increased from 4.5 x10^6 to 6.3 x10^6/ml during 24 to 72 h as compared to free cells with 1100 AU/ml and 1000AU/ml, against *S.aureus* and *P.aeruginosa* respectively and decreased remarkably with free cells from 4.2 x10^6 to 1.2 x10^6/ml after 24 h. Encapsulated *L. acidophilus* MTCC 10307 , standard strain exhibited 2000 AU/ml and 2200 AU/ml compared to free cells with 1100 and 900 AU/ml. The isolates *Pediococcus acidilactici* CSI29MX, *Pediococcus parvulus* MF 233 and *Pediococcus pentosaceus* QN1D showed higher potential for bacteriocin production than *L. acidophilus* MTCC 10307.

Key words*: P. acidilactici* CSI29MX, *P. parvulus* MF 233, *P. pentosaceus* QN1D, Encapsulation, Bacteriocin production, food borne pathogens

INTRODUCTION

The use of microorganisms and their natural products for the preservation of foods has been a common practice in the history of mankind (Ross *et al.,* 2002). In spite of modern advances in technology, the preservation of food is still a debated issue not only for developing countries but also for industrialized world. One of the concerns of food industry is the contamination by pathogens which are frequent cause of.

food borne diseases. Control of *S. aureus* and *P. aeruginosa* in foods is essential to protect human health. Foodborne illness is a major cause of concern throughout the world. *S.aureus* is responsible for staphylococcal food intoxication and is capable to survive and grow in a wide range of temperature and pH if food serves as better medium for growth. *P. aeruginosa* is another food spoilage organism which upon extended refrigeration spoils fresh meat (Frazier and Frazier 2008). Although steps have been implemented to eliminate and/or control bacterial populations in foods, bacterial foodborne illness and bacterial food spoilage are still major concerns. Hence, newer methods of control and elimination are required.

Bacterial antibiotic resistance has been considered an important problem due to extensive use of antibiotics in treatment of human and animal diseases and as a result multiple drug resistant strains developed (Roy 1997; Yoneyama and Katsumata, 2006). In order to control their abusive use in food and feed products one alternative is the application of some bacterial peptides as antimicrobial substances. Bacteriocins produced by lactic acid bacteria have no toxicity, and being protein in nature is rapidly digested by proteases in the human digestive tract (Saavedra *et al.,*2004).The lactic acid bacteria (LAB) produce an array of antimicrobial substances such as organic acids, diacetyl, acetoin, hydrogen peroxide, antifungal peptides and bacteriocins (Magnusson and Schnurer, 2001). Bacteriocin production could be considered as an advantage for food and feed producers since these peptides kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool. Many bacteriocins have a narrow host range and are likely to be most effective against related bacteria as well as food borne pathogens (Deegan *et al.,*2006).

In recent years research is focussed on new approaches for increasing the cell concentration and bacteriocin production. One of the methods applied for maintaining high cell concentration and bacteriocin production is immobilization. (Idris and Suzana, 2006). Immobilization by cell encapsulation offers improvement of viability of cells and the plasmid stability (Kailasapathy, 2002). Alginates are widely used as immobilizing materials for cells. These are nontoxic linear heteropolysaccharides extracted from different types of algae (Melvik and Dornish 2004; Cook *et al.,*2012) and are naturally derived linear copolymers of 1, 4-linked β-D-mannuronic acid and α-L-guluronic acid residues (Martinsen *et al.,*1989). The alginate recovers the bacterial cells and forms a barrier, protecting them against environmental conditions (Anal and Singh, 2007). Calcium ions bind only between G blocks of more than 20 units, forming a polymer network, and at high calcium concentrations, multiple cross-linking among alginate chains is possible (Kong *et al.,* 2003). Alginates create matrixes when coupled with other naturally biodegradable or synthetic polymers, which provide scaffolds for cells, proteins, and genes for a variety of applications including encapsulation. Many lactic acid bacteria synthesize exo-polysaccharides, but they produce insufficient exo-polysaccharides to be able to encapsulate themselves fully (Shah, 2002).

Some studies have shown that entrapment of LAB in calcium alginate improves lactic acid production (Idris and Suzana, 2006;Rao *et al.,* 2008), but little is known on bacteriocin production by encapsulated LAB. The aim of this work was to study cell encapsulation of Lactic acid bacteria isolated from idli batter in calcium alginate beads for higher bacteriocin production. Thus, application of LAB capable to produce bacteriocins in situ or purified or semi-purified bacteriocins to foods, both free and entrapped in calcium alginate beads, may be an interesting technological aid to improve food safety.

MATERIAL AND METHODS

Bacterial strains and culture condition

The bacteriocin producing standard reference strain used in this study was *L. acidophilus* MTCC 10307 procured from Microbial Type Culture Collection, Chandigarh. Bacteriocinogenic LAB –A, LAB – B and LAB –C were isolated from idli batter and further identified to species level using 16 S rRNA sequencing as *P. acidilactici* CSI29MX, *P. parvulus* MF 233 and *P. pentosaceus* QN1D respectively. *S. aureus* and *P. aeruginosa* isolated from meat and identified on the basis of morphology, cultural characteristics and biochemical reactions. The LAB strains and *S.aureus*, *P.aeruginosa* were maintained at -70^0 C in De Man Rogosa Sharpe (MRS) broth and Brain Heart Infusion (BHI) broth (HI Media, Mumbai) respectively. Before use, the bacterial strains were cultured at least twice in the appropriate broths at 37 ^{0} C, for 24 h.

2) Encapsulation of LAB

L. acidophilus MTCC 10307, *P. acidilactici* CSI29MX, *P. parvulus* MF 233, *P. pentosaceus* QN1D were entrapped in calcium alginate according to Ivanova *et al.,* (2002) with some modifications. The strains were grown in MRS broth at 30^0 C for 24 h. Inoculated MRS broth was centrifuged at 10000 g, 15 min, 4 ^{0}C (Remi Model C-24 BL), washed with 0.85 % saline solution and resuspended in 3.5 ml peptone water (1g / l). For cell encapsulation equal part of cell suspension and sodium alginate (4 %) were mixed. The mixture was dripped in a solution of 100 mM $CaCl_2$, using a syringe. The droplets were collected in 100 mM $CaCl_2$ solution for formation of calcium alginate beads. Beads were kept for 30 min for gel strengthening and separated by sieve (2.00 mm) and washed three times with sterile distilled water. The diameter of the calcium alginate beads was determined. *L. acidophilus* MTCC 10307 was used as standard bacteriocinogenic LAB.

3) Bacteriocin Production

Bacteriocin production with encapsulated cells and free cells was carried out in Erlenmeyer flasks containing 250 ml of modified MRS broth (mMRS) at 30 ^{0}C and pH 6 without shaking. Bacteriocin production with free cells was used as control (Ivanova *et al.,* 2002).

4) Determination of cell concentration in the beads

One ml of calcium alginate beads measured (by removing water) and suspended in 9 ml of 0.1M potassium phosphate buffer (PPB) pH 7.4 with gentle shaking for 30 min for destruction of the beads. The suspension was submitted to decimal serial dilutions using 0.1% sterile peptone water. One hundred µl for each dilution was plated in duplicate on MRS agar and incubated at 30^0 C for 24, 48, 72 and 96 h. The number of released lactobacilli was expressed as cfu/ml (Ivanova *et al.,* 2002).

5) Determination of bacteriocin activity

Bacteriocin assays were performed in cell free supernatants (CFS) of free and encapsulated *L. acidophilus* MTCC 10307, *P. acidilactici* CSI29MX, *P. parvulus* MF 233 and *P. Pentosaceus* QN1D. CFS was prepared by centrifugation 6000 g, for 15 min, at 4 ^{0}C of the MRS broth culture incubated at 30 ^{0}C for 24 h. LAB produces inhibitory compounds lactic acid or hydrogen peroxide. To eliminate the possible inhibitory effect of either lactic acid or hydrogen peroxide, pH was neutralized and catalase treated supernatant was used. CFS was filter-sterilized through a 0.22 µm membrane filter and was submitted to serial dilutions using sterile 0.1 M PPB (pH 7.0). Agar well diffusion assay was used for testing the antimicrobial activity. Ten µl from each dilution were transferred to the wells containing two layers of media, constituted of 12 ml of 15% (w/v) bacteriological agar (HI Media) overlaid with 5 ml of BHI supplemented with 0.85% (w/v) bacteriological agar, containing food borne pathogen *S. aureus* and spoilage organism *P. aeruginosa* ,6 x 10^5 cfu/ ml and 7.4 x 10^5cfu/ ml respectively. The plates were incubated at 37 ^{0}C for 12 h and observed for inhibition zones. One arbitrary unit (AU) of the bacteriocin in the CFS was defined as the reciprocal of the highest dilution having a detectable zone of inhibition and were expressed as arbitrary units (AU) per millilitre (Kaiser and Montville, 1996).

6) Influence of entrapment on cell viability and bacteriocin production

The cell viability and bacteriocin production by *L. acidophilus* MTCC 10307, *P. acidilactici* CSI29MX, *P. parvulus* MF233 and *P. pentosaceus* QN1D were evaluated before and after entrapment in calcium alginate. Before entrapment, 1 ml of the bacterial suspensions prepared as described previously was submitted to serial decimal dilutions, plated on MRS agar and incubated at 30^0 C for 24, 48, 72 and 96 h for counts of viable cells. For assay of bacteriocin production 1 ml of the same bacterial suspension was added to 10 ml MRS broth, incubated at 30^0 C for 24, 48, 72 and 96 h and tested for antimicrobial activity. 1 ml of entrapped cells was treated for release of cells, plated on MRS agar and incubated 30 ^{0}C for 24, 48, 72 and 96 h for determination of viable cells. For assay of bacteriocin production, 1 ml of entrapped cells were added to 10 ml of MRS broth and incubated at 30^0 C for 24, 48, 72 and 96 h then the suspension was tested for antimicrobial activity, as described previously (Barbosa *et al.,* 2015).

RESULTS AND DISCUSSION

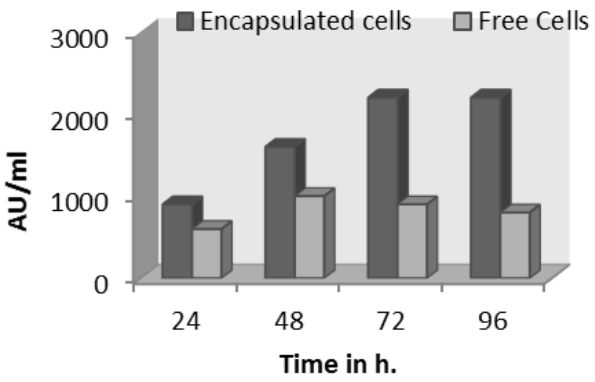
Results of cell viability (Table 1) reveal better survival of entrapped *L. acidophilus* MTCC 10307, *P. acidilactici* CSI29MX, *P. parvulus* MF 233 and *P. pentosaceus* QN1D in calcium alginate beads than the free cells. Entrapped cells did not show decrease in cell viability after 72 h

while free cells demonstrated a decrease in cell viability after 24 h. The results agreed with (Ivanova *et al.,* 2002) who earlier reported that cell viability decrease significantly after 72 h of fermentation while the viability of immobilized cells remain almost stable up to 120 h. This could be explained by the limited immunity of the free cells to their own bacteriocin in the medium. The cells which have already stopped bacteriocin production are sensitive to their own bacteriocin. During the fermentation with immobilized cells the space separation between cells and bacteriocin gave the possibilities to keep the viability of entrapped cells (Ivanova *et al.,* 2002).

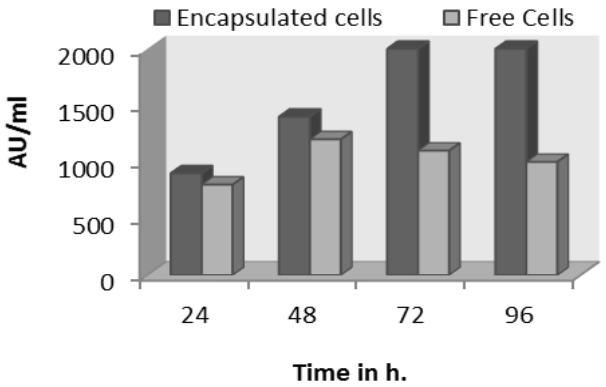
The maximum bacteriocin production in MRS broth (2800 AU/ml) occurred with the bead size 2.5 ± 3 mm by *P. parvulus* MF 233 on 72 h of fermentation whereas *L. acidophilus* MTCC 10307, *P. acidilactici* CSI29MX, and *P. pentosaceus* QN1D produced 2200, 2400 and 2600 AU/ml of bacteriocin respectively in 72 h (Fig 1,2,3,4). Bacteriocin production was bead size dependent. The larger the diameter of the beads the largest the amount of bacteriocins produced. The influence of the size of alginate beads on production of microbial metabolites was also reported by Zain *et al.*, (2011) for ethanol production, who showed that yeast ST1 produce more ethanol when encapsulated in alginate beads of size of 0.5 cm than in beads of size of 0.3 cm. Idris and Suzana (2006) reported that production of lactic acid by *Lactobacillus delbrueckii* subsp. *delbrueckii* ATCC 9646 was much higher when encapsulated in calcium alginate beads of size 1.0 mm using 2.0% sodium alginate.

Table 1: Cell viability of LAB (Entrapped and free cells x10⁶/ml)

Time in h	*L. acidophilus* MTCC 10307		*P. acidilactici* CSI29MX		*P. Parvulus* MF233		*P.pentosaceus* QN1D	
	Entrapped	free cells	Entrapped	free cells	Entrapped	free cells	Entrapped	free cells
24	4.0	3.9	4.5	4.0	4.6	4.0	5.0	4.2
48	4.9	3.0	5.0	3.0	5.1	3.2	5.6	3.8
72	5.3	1.2	5.4	1.8	5.3	2.0	6.3	2.2
96	4.9	0.6	5.1	0.8	5.0	0.9	5.2	0.8



A



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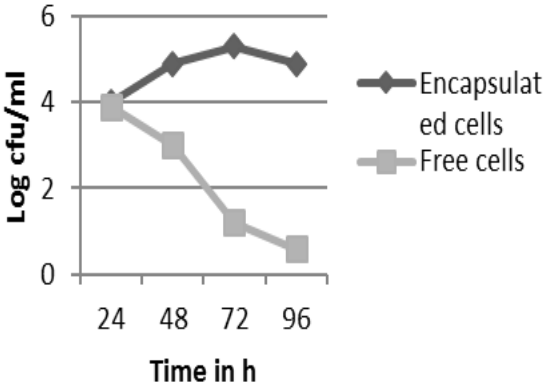
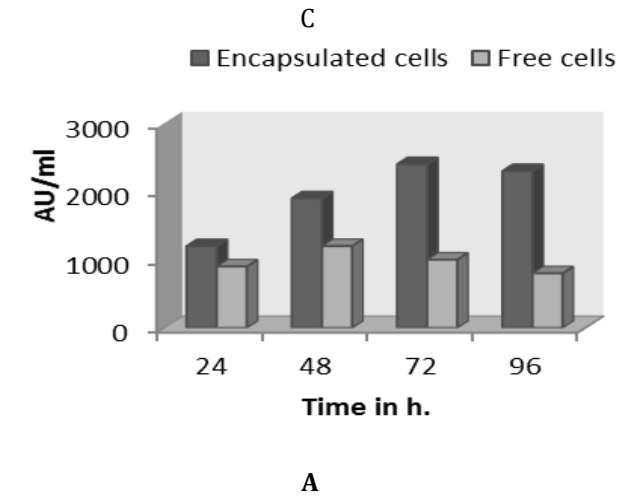


Figure 1 : Bacteriocin production by *L. acidophilus* MTCC 10307 with encapsulated and free cells (A) AU/ml of bacteriocin against *P.aeruginosa* and (B) *S.aureus.* (C) Cell viability of encapsulated and free cells (Log cfu/ml).



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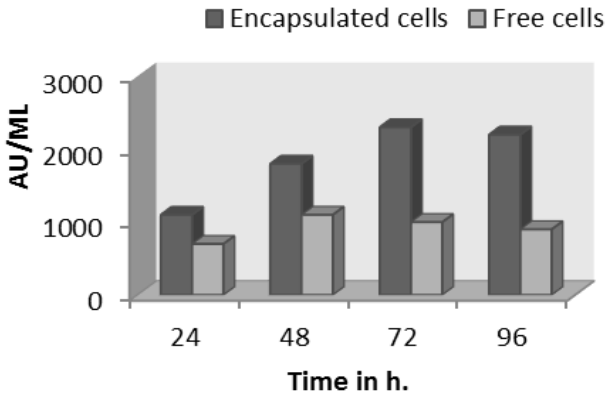

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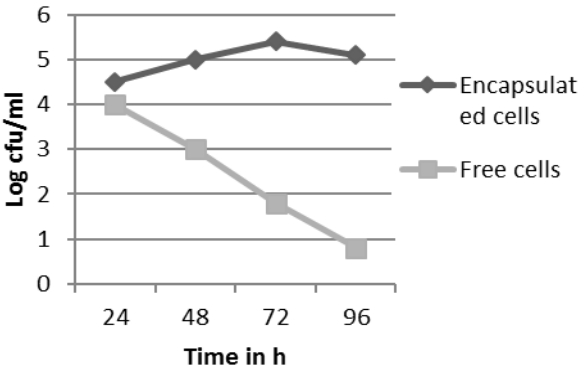
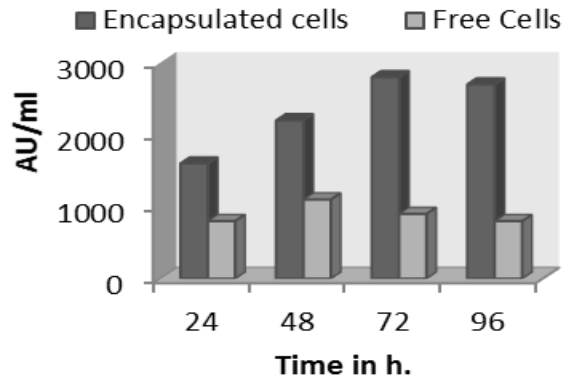


C

Fig 2 : Bacteriocin production by *P. acidilactici* CSI29MX by encapsulated and free cells
(A) AU/ml of bacteriocin against *P.aeruginosa* and (B) *S.aureus*
(C) Cell viability of encapsulated and free cells (Log cfu/ml)



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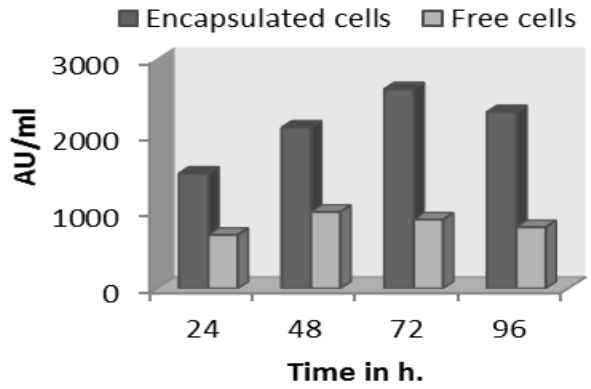

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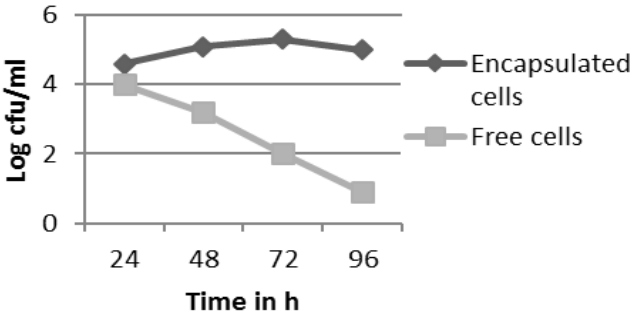

C

Fig 3 : Bacteriocin production by *P. parvulus* MF 233 encapsulated and free cells
(A) AU/ml of bacteriocin against *P. aeruginosa* and (B) *S.aureus*
(C) Cell viability of encapsulated and free cells (Log cfu/ml).

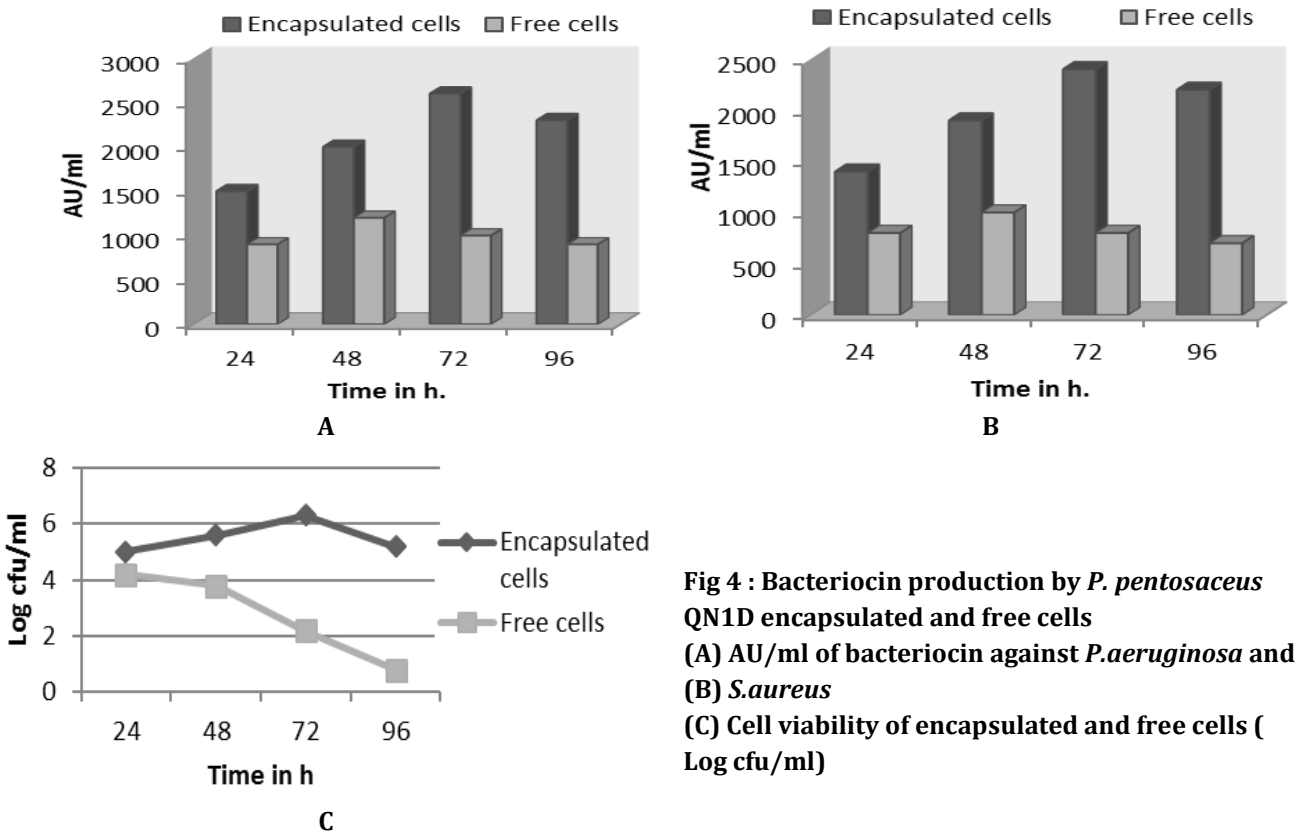

Fig 4 : Bacteriocin production by *P. pentosaceus* QN1D encapsulated and free cells
(A) AU/ml of bacteriocin against *P.aeruginosa* and (B) *S.aureus*
(C) Cell viability of encapsulated and free cells (Log cfu/ml)

The immobilization of *L. acidophilus* MTCC 10307 and *P. acidilactici* CSI29MX, *P parvulus* MF233 and *P. pentosaceus* QN1D in calcium alginate beads resulted in the increased production of bacteriocin. The fermentation was performed in mMRS broth (without phosphate) because phosphate in the medium weaken the cross links in alginate capsules causing disruption of beads (Ivanova *et al.,* 2002)). As a result of immobilization the bacteriocin concentration was increased to many folds. Immobilized *L. acidophilus* MTCC 10307 after 72 hrs of fermentation recorded 120% increase in bacteriocin production whereas *P. acidilactici* CSI29MX with 120 % , *P parvulus* MF233 with 154 % and *P. pentosaceus* QN1D shows 116 % increase in bacteriocin production respectively compared with the free cells.

Most studies with encapsulated LAB focused on improving resistance of LAB to hostile environmental conditions (Brachkova *et al.,* 2010; Ortakci and Sert 2012; Shamekhi *et al.,* 2013; Todorov *et al.,* 2012) or enhancement of lactic acid production (Rao *et al.,* 2008; Narita *et al.,* 2004). Scannell *et al.,*(2000) have shown that production of bacteriocins by *Lactococcus lactis* subsp. *Lactis* DPC 3147 and *L. lactis* DPC 496, entrapped in calcium alginate, in culture medium under controlled temperature 30^0C and pH 6.5 was more effective than production by non encapsulated cells. Ivanova *et al.,* (2002) and Sarika *et al.,* (2012) reported similar results for encapsulated *Enterococcus faecium* A2000, *Lactobacillus plantarum* MTCC B1746 and *L. lactis* MTCCB440. Nilsang (2010) reported that the lactic acid production with immobilization of lactic acid bacteria fermentation was 2.92 g /l /h whereas, lactic acid production rate of free cell without agitation and free cell with agitation were 1.22 g /l/h and 1.01 g /l /h respectively. Similar results have also been reported by Rao *et al.,*(2008) with immobilized *Lactobacillus delbrueckii* cells in calcium alginate beads for lactic acid production. The results showed that immobilized cells can increase lactic acid production when compared with free cells. Muthukumarasamy and Holley (2006) reported that counts of *Lactobacillus reuteri* entrapped in calcium alginate beads presented a slight reduction while counts of non-entrapped cells showed more reduction in number during manufacture of salami.

Some studies have shown that instead of bacteriocin producing LAB, encapsulation of semi-purified bacteriocins in vesicles composed by one or more phospholipid bilayers (liposomes) is more effective

than entrapment in alginate (Mills *et al.*,2011;Zou *et al.*,2012). These materials should be considered as an interesting technological alternative for the control of *S.aureus,* and *P.aeruginosa* in food products.

CONCLUSION

The results of this study showed that three strains of LAB, *P. acidilactici* CSI29MX, *P parvulus* MF233 and *P. pentosaceus* QN1D isolated from idli batter were found to possess potential for production of antimicrobial peptide, bacteriocin. The immobilization of these strains in Ca-alginate beads made possible the increasing bacteriocin production with increase in the biomass. As a result of the immobilization, the bacteriocin concentration was increased by 116 % to 154 % compared with the free cells. The three strains showed 27 to 30 % increase in AU/ml, than the standard reference strain L*. acidophilus* MTCC 10307**.** The bacteriocin inhibit the growth of food borne pathogenic and spoilage organisms, Gram positive *S.aureus* as well as Gram negative *P.aeruginosa* and can be used for prevention of spoilage of foods from these organisms in food industry. These strains are good candidates for further investigation as novel bacteriocinogenic starters.

Conflicts of interest: The authors stated that no conflicts of interest.

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