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Bacteriocin Production by a Marine Strain of *Bacills* sp. Sh10: Isolation, Screening and Optimization of Culture Condition

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Abstract: Antibacterial peptides have emerged as an alternative therapeutic to protect food and other products from deterioration and spoilage. In this study, bacteria were isolated from marine clams and screened for bacteriocin production using cross-streaking and spot-on-lawn methods. One of the isolates, identified as *Bacillus* species Sh10, produced proteinaceous bacteriocin with a broad spectrum of activity against human pathogens and food-spoilage bacteria. The effect of different media compositions and culture conditions namely, the type and concentration of carbon and nitrogen source, NaCl concentration, temperature, pH, aeration and incubation time on the production of bacteriocin by *Bacillus* sp. Sh10 was investigated. Optimum bacteriocin production was recorded when 2% tryptone and 1% glucose were added to a basal medium as nitrogen and carbon sources, respectively. The bacteria exhibited maximum bacteriocin production at 2% NaCl, pH 8, 30°C and 200 rev min^{-1} aeration. Bacteriocin was produced during the stationary phase, indicating it is synthesized as a secondary metabolite. *Bacillus* sp. Sh10 is able to produce bacteriocin at a wide pH and temperature range making it a good candidate for the production of bacteriocin on an industrial scale level.

Key words: *Bacillus* sp. Sh10, bacteriocin, optimization, marine clams

INTRODUCTION

The problem of antibiotic resistance of pathogenic bacteria has generated an urgent necessity for the development of new antimicrobial agents worldwide (Gauri *et al*., 2011). Recently, naturally produced antimicrobial substances have been considered (Al-Mahrous *et al*., 2010). Among these possibilities, bacteriocins are the most important and useful antibiotics against antibiotic-resistant bacteria (Gauri *et al*., 2011). Bacteriocins are antimicrobial peptides that are produced by bacteria isolated from diverse environments. They are ribosomally synthesized, hydrophobic or hydrophilic peptides of bacterial origin and inhibit the growth of closely related bacteria and are rapidly digested by proteolytic enzymes (Dominguez *et al*., 2007).

The production of bacteriocin is significantly influenced by different growth conditions and media compositions (Park *et al*., 2010). Reports on Lactic Acid Bacteria (LAB) indicated the influence of culture conditions and growth media on bacteriocin production (Zalan *et al*., 2005; Park *et al*., 2010). In spite of the intensive focus on LAB, *Bacillus* is another potential genus to exploit because this genus is able to produce a wide range of antimicrobial peptides with different basic chemical structures (Cladera-Olivera *et al*., 2004; Xie *et al*., 2009; Abriouel *et al*., 2011). The production of bacteriocin has been documented for *B. licheniformis* and *B. subtilis*, among others (Von Dohren, 1995; Stein, 2005). However, only a few reports have focused on the factors affected bacteriocin production by *Bacillus* species (Martinez-Cardenas *et al*., 2012). Lastly, several studies have assessed different antimicrobial substances from the marine environment (Selvin *et al*., 2004; Romanenko *et al*., 2008). Nevertheless, the search for their potential to produce bacteriocin is notably lacking. Hence, the present study was undertaken to determine the potential of isolated bacteria from marine clams to produce bacteriocin and to evaluate the optimum culture conditions for greater bacteriocin yields in the most promising bacteriocin-producing strain *Bacillus* sp. Sh10.

MATERIALS AND METHODS

Isolation of microorganisms: The carpet clam specimens (*Paphia textile*) were prepared from Bangi shop, in Selangor, Malaysia. They were washed with sterile seawater and shucked with a scalpel aseptically and

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homogenized with a sterile mortar and pestle. Aliquots of the diluted homogenates were added to the marine broth and incubated at 30°C for 24 h. After incubation, the bacterial culture obtained was serially diluted with saline solution and aliquots from each dilution were plated onto marine agar. The plates were incubated at 30°C. After 24 h of incubation, different colonies were picked and streaked on new marine agar plates to obtain a pure culture.

Indicator microorganisms: Gram-positive *Bacillus subtilis* ATCC 11774, *Staphylococcus aureus* ATCC 11632, Methicillin-Resistant *Staphylococcus aureus* (MRSA) N32064 and Gram-negative *Vibrio parahaemolyticus* ATCC 17802, *Aeromonas hydrophilia* bf314 and the yeast, *Candida albicans* were used in this study. All strains were kept as stock at -80°C in 40% glycerol.

Screening of bacteriocin production: Bacteriocin activity was detected by the cross-streaking method (Lai *et al*., 1983) and confirmed by the spot-on-lawn method (Ko and Ahn, 2000). For the cross-streaking method, isolated bacteria from the marine clam were inoculated in a 1 cm streak onto marine agar plates and were incubated at 30°C. After 24 h, plates were exposed to chloroform vapor for 30 min to kill the bacteria. The plates were then exposed to air in a laminar flow cabinet for 1 h to allow evaporation of the remaining chloroform. Cultures of the test strains which contained 1×10^8 CFU mL^{-1} cells, were streaked perpendicular to the original line of growth on each producer plate. All plates were again incubated at 30°C overnight to observe the clear zone around the producer cultures. For the spot-on-lawn method, all the bacteria were grown in marine broth at 30°C. After 24 h, the cultures were centrifuged at 12,000 rpm for 30 min at 4°C and then filtered across a cellulose acetate filter (0.22 μm) to remove residual cells. Then, 10 μL of cell free supernatant was spotted on the surface of Muller Hintone Agar overlaid with 1×10^8 CFU mL^{-1} of cells of the indicator organism and incubated at 30°C for 24 h. The amount of bacteriocin production was calculated as an Arbitrary Unit (AU) per milliliter defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the indicator strain (Hoover and Harlander, 1993).

Phenotypic characterization of isolates: Phenotypic characterization of the isolate Sh10 was done by different tests referring to Bergey's manual of determinative bacteriology and agriculture handbook (Holt *et al*., 1994).

Effect of different media compositions and culture conditions: The selected strain (Sh10) was subjected to different media compositions and culture conditions for bacteriocin production. Growth (OD at 600 nm) and bacteriocin production (using the spot-on-lawn method as mention above) were evaluated at each step. Samples were collected after 24 h except for the incubation period. *C. albicans* was used as an indicator organism. Each experiment was carried out at least three times.

Basal medium and inoculum preparation: In order to determine the optimal media composition and cultural conditions for growth and bacteriocin production, Pridham and Gottlieb (1948) used inorganic salts medium [(g L^{-1}) $(NH4)_2SO_4$-2.64, KH_2PO_4-2.38, $MgSO_4.7H_2O$-1.00, $CuSO_4.5H_2O$-0.0064, $FeSO_4.7H_2O$-0.0011, $MnCl_2$ $.4H_2O$-0.0079, $ZnSO_4.7H_2O$-0.0015], this composite used as the basal medium. Three percent NaCl was added to basal medium and the pH was adjusted to 8. Strain Sh10 was grown in basal medium and incubated at 30°C for 24 h at 200 rev min^{-1} in an incubator shaker (INFORS AG CH-4103). A 2% (v/v) inoculum from this culture containing OD_{600} = 1 was used for inoculations in each optimization experiment.

Effect of different carbon and nitrogen sources: Different carbon and nitrogen sources were used for the optimization of the growth and bacteriocin production. The carbon and nitrogen sources were sterilized separately and added before inoculation. Glucose, starch, lactose, mannitol, sucrose, arabinose, galactose, fructose, maltose and sorbitol were added separately as carbon sources into the sterile basal medium at 1% concentration. Various nitrogen sources such as casein, yeast extract, beef extract, tryptone, peptone, soya bean meal, ammonium chloride, sodium nitrate, ammonium nitrate and urea were provided separately into the sterile basal medium at 1% concentration. The cultures were incubated in an incubatore shaker (200 rev min^{-1}, 30°C). The basal medium served as the control.

Effect of different concentrations of glucose and tryptone: The effect of different glucose and yeast extract concentration ranging from 0-3% on the bacteriocin production was studied separately.

Effect of different concentrations of NaCl: The effect of salinity on growth and bacteriocin produced by the strain Sh10 was carried out by cultivating in various NaCl concentrations, ranging from 0-5%, into the basal medium enriched with 2% tryptone and 1% glucose as nitrogen and carbon sources respectively.

Effect of different temperature, initial pH and levels of aeration: The effect of different culture conditions such as different temperatures (20, 25, 30, 35, 40 and 45°C), initial pH (4-11) and aeration (0, 50, 100, 150 and 200 rev min^{-1}) on the production of antimicrobial compounds was studied separately by inoculating the seed culture into the minimal medium (2% v/v) supplemented with optimum NaCl concentration, glucose (1%) and tryptone (2%).

Effect of incubation period: The effect of the incubation period (0-48 h) on antimicrobial compound production was studied by keeping other parameters at optimum levels.

RESULTS

Isolation and screening of bacteriocin-producing bacteria: From 30 bacterial isolates from marine clams, three were found to produce bacteriocin by the cross-streaking and spot-on-lawn methods (Table 1). One of the isolates (strain Sh10) with broad inhibitory activity against gram-negative and gram-positive bacteria and *C. albicans* was selected for further studies. Treatment of culture supernatants of this strain with trypsin (1 mg mL^{-1}) completely inactivated the bacteriocin. This showed the proteinaceous nature of this antimicrobial substrate which is considered as bacteriocin. This isolate was gram positive, rod shaped, an endospore former, motile, able to hydrolyze starch with positive catalase and Voges Proskaur (VP) reactions but negative in indole production. Based on the tests, the isolate Sh10 was identified as a *Bacillus* sp. and called *Bacillus* sp. Sh10.

Table 1: Antimicrobial spectrum of bacteriocin producing bacteria isolated from marine clam

	Bacteriocin producing bacteria					
	Cross-streaking method			Spot-on-lawn method		
Indicator strains	Sh8	Sh10	Sh23	Sh8	Sh10	Sh23
S. aureus ATCC 11632	+	+	+	+	+	+
MRSA N32064	-	+	+	-	+	+
B. subtilis ATCC 11774	-	-	-	-	-	-
A. hydrophilia wbf314	-	+	-	-	+	-
V. parahaemolyticus ATCC 17802	+	+	-	+	+	-
C. albicans	-	+	-	-	+	-

Positive result (+), Negative result (-)

Effect of different media compositions and culture conditions: The influence of culture media components and culture conditions on the bacteriocin produced by strain Sh10 was investigated using *C. albicans* as an indicator organism. The influence of culture medium components on bacterial growth and bacteriocin production was examined using various carbon and nitrogen sources. Optimum bacteriocin activity was noted when glucose (400 AU mL^{-1}) and tryptone (400 AU mL^{-1}) were used as carbon and nitrogen sources, respectively. Whereas, other carbon and nitrogen sources were used in this study, they were associated with less bacteriocin activity. Bacteriocin activity was not observed when using lactose, starch and sorbitol as carbon sources (Fig. 1a-b).

Different concentrations of glucose and tryptone had a considerable influence on bacteriocin production (Fig. 2a-b). Optimum glucose and tryptone concentrations for the production of bacteriocin were noted to be

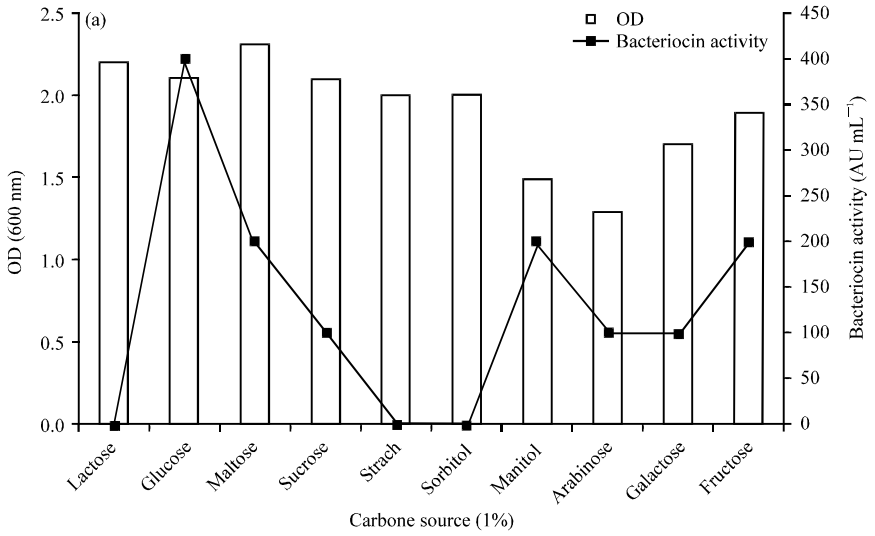

Fig. 1(a-b): Continue

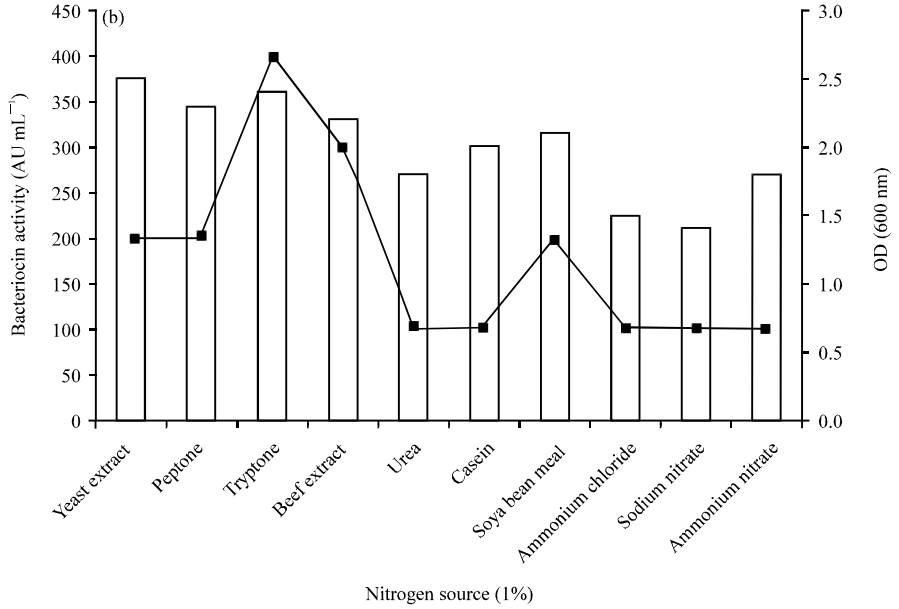

Fig. 1(a-b): Effect of different (a) carbon sources and (b) nitrogen sources on growth and bacteriocin production by *Bacillus* sp. Sh10

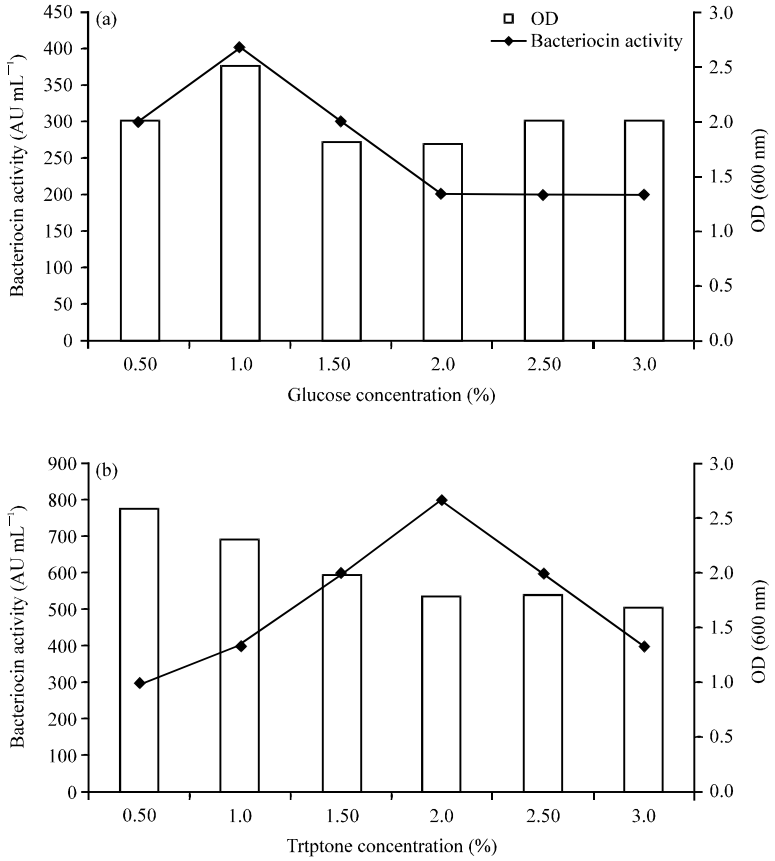

Fig. 2(a-b): Effect of different concentration of (a) glucose and (b) tryptone on growth and bacteriocin production by *Bacillus* sp. Sh10

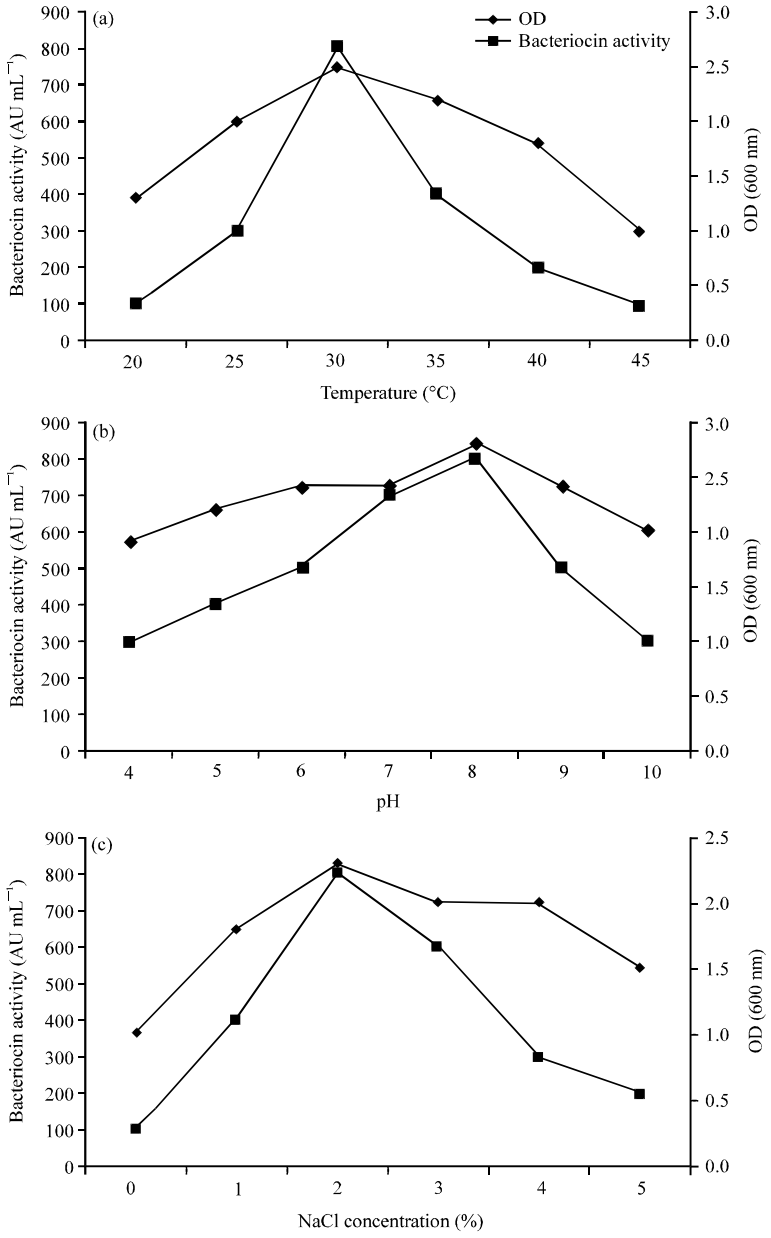

Fig. 3(a-c): Effect of different (a) incubation temperature, (b) pH values and (c) sodium chloride on growth and bacteriocin production by *Bacillus* sp. Sh10

1% (400 AU mL^{-1}) and 2% (800 AU mL^{-1}), respectively. Whereas, optimal growth was recorded at 1% glucose and 0.5% tryptone. No significant relationship was observed between growth and bacteriocin production (Fig. 1-2).

Bacteriocin production was greatly affected by temperature. Maximum bacteriocin production (800 AU mL^{-1}) was achieved at 30°C followed by optimum growth (Fig. 3a). However, increasing the temperature from 30-50°C led to reduced bacteriocin activity and cell mass. There was a gradual increase in bacteriocin activity and cell growth with an increase in pH from 4-8 but pH 9 and 10 led to reduced activity and cell growth (Fig. 3b). The optimum pH for growth and bacteriocin activity was observed at pH 8. Regarding various concentrations of sodium chloride tested from 0-5%, the optimum level of bacteriocin activity was recorded as 800 AU mL^{-1} at 2% NaCl (Fig. 3c). An increase in the level of aeration led to a gradual increase in growth and bacteriocin activity. Optimum aeration for bacteriocin activity (800 AU mL^{-1}) and cell mass was obtained at 200 rev min^{-1} (Fig. 4).

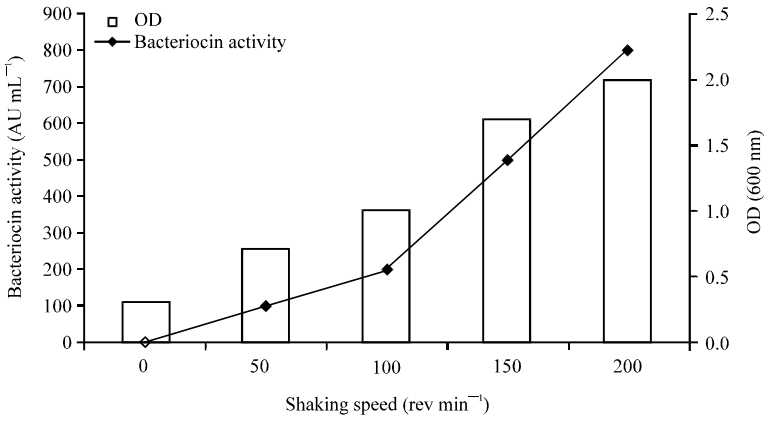

Fig. 4: Effect of different shaking speed on growth and bacteriocin production by *Bacillus* sp. Sh10

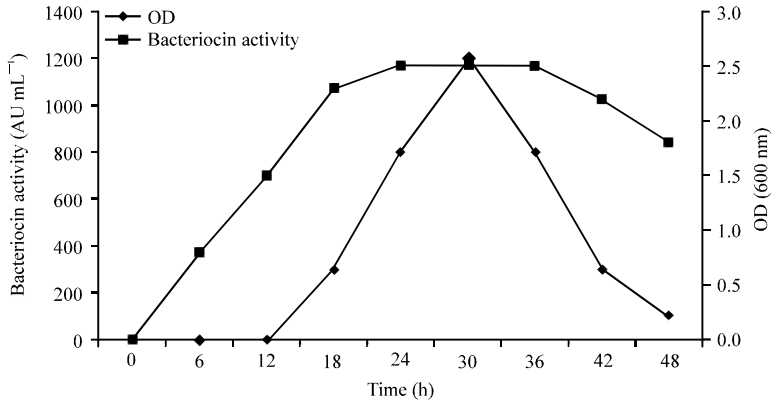

Fig. 5: Effect of different time periods on growth and bacteriocin production by *Bacillus* sp. Sh10

Studies on the growth kinetic and bacteriocin production were conducted by measurement of the inhibition activity of the producer strain Sh10 at various time periods against *C. albicans* as the indicator organism (Fig. 5). Bacteriocin activity was not detected during the exponential growth phase but it was detected at the end of this phase and reached a maximum (1200 AU mL^{-1}) during the mid-stationary phase and decreased at the end of this phase.

DISCUSSION

Bacteriocin screening from marine clam-associated bacteria shows that the marine environment has a great potential for bacteriocin-producing bacteria. Strain Sh10 was identified as a *Bacillus* sp. based on biochemical tests and was designated as *Bacillus* sp. Sh10. A bacteriocin produced by this strain inhibited the growth of human and foodborne pathogens. Recently, due to the antimicrobial activity of bacteriocin against spoilage and foodborne pathogenic bacteria, there has been a hightened interest in their application in food industries (Galvez *et al*., 2008).

Bacteriocin activity and cell mass depend on environmental factors and the composition of the growth medium. Therefore, optimization of medium components is important for the enhancement of bacteriocin and viable cell production (Preetha *et al*., 2007; Kayalvizhi and Gunasekaran, 2008). Based on the results obtained in this study, bacteriocin production by *Bacillus* sp. Sh10 is also strongly influenced by the medium composition and growth conditions. Several studies have shown that the type and level of different carbon sources affect bacteriocin production (Mataragas *et al*., 2004; Drosinos *et al*., 2005, 2006; Kanmani *et al*., 2011). In this study, the maximum inhibitory level of bacteriocin was observed in minimal medium supplemented with 1% glucose as the carbon source. Most workers have assumed that high bacteriocin production in association with the presence of glucose in the medium (Mulders *et al*., 1991; Matsusaki *et al*., 1996; Kanmani *et al*., 2011) and the absence of glucose is one of the limiting factors for the production of bacteriocin (Russell and Mantovani, 2002).

The nitrogen source also significantly affected bacteriocin production (Mataragas *et al*., 2004). From 10

different nitrogen sources used in the present study, tryptone was the most effective nitrogen source that highly accelerated the production of bacteriocin. Kanmani *et al.* (2011) also noted that maximum bacteriocin production by *Enterococcus faecium* MC13 was achieved when tryptone and glucose was used as nitrogen and carbon sources, respectively. Tryptone contains a high amount of free amino acids and short peptides that accelerate bacteriocin production. No evident relationship was obtained between growth and bacteriocin production while using different concentrations of carbon and nitrogen sources. In agreement, Messens *et al.* (2003) and Motta and Brandelli (2008) did not observe a relationship between optimum growth and bacteriocin production by *Bacillus* sp. P34 and *Lactobacillus curvatus* LTH1174, respectively. Kim *et al.* (1997) also noted that optimum growth does not always lead to a high yield of bacteriocin production.

Strain Sh10 is able to grow and produce bacteriocin at all pH values, temperatures and NaCl concentrations. This finding demonstrated that this strain has a wide adaptability to the environment. Salt concentration has a significant effect in the release of bacteriocin from different bacteriocin-producing bacteria (Verluyten *et al.*, 2004; Delgado *et al.*, 2007). The present study showed that the addition of 2% NaCl improved the maximum cell mass and bacteriocin production by *Bacillus* sp. Sh10 and reduced these factors at lower or higher NaCl concentrations. The negative effect of NaCl on bacteriocin production could be due to its interference with the inducer receptor interaction (Nelsen *et al.*, 1998). However, Delgado *et al.* (2005) reported that addition of NaCl improved plantaricin S production by *Lactobacillus pentosus* B96.

The bacteriocin produced by *Bacillus* sp. Sh10 was studied at different pH values ranging from 4-10, showing inhibitory activity in the acidic and alkaline pH ranges with optimal activity at pH 8. Bacteriocin production in alkaline conditions are now gaining more attention in many food industries because the pH of several food products vary from natural to alkaline. It has been reported that nisin is the only commercial bacteriocin used as a food supplement at acidic pH while it is instable at alkaline pH (Liu and Hansen, 1990). In agreement, bacillocin 490 from *B. licheniformis* showed antimicrobial activity between acidic and alkaline pH values (Martirani *et al.*, 2002).

Strain Sh10 is also capable of producing bacteriocin in a broad range of temperatures from 20-40°C with optimum bacteriocin production at 30°C. This property of bacteriocin can be used as a preservative in food industries to protect from food spoilage even at low temperatures. In agreement, Bizani and Brandelli (2004) and Sarika *et al.* (2010) also reported that the amount of bacteriocin production at 30°C was more significant than at higher temperatures. At higher temperatures and pH values, the production of bacteriocin is lower because the energy used for maintenance purposes is much higher when the temperature or pH increases (Leroy and De Vuyst, 1999; Drosinos *et al.*, 2005). On the other hand, there was a gradual increase in cell growth and bacteriocin production with an increase in cell growth and bacteriocin activity while the aeration increased from 0-200 rev min^{-1}. The ability of cells to synthesize bacteriocin significantly depended on the oxygenation of the culture medium (Kamoun *et al.*, 2009).

This study demonstrated that incubation time has a significant role in bacteriocin production. Bacteriocin concentration increased to a maximum at the mid-stationary growth phase and started declining at the end of this phase indicating it is synthesized as a secondary metabolite. Production of bacteriocin is generally associated with primary metabolite kinetics (De Vuyst *et al.*, 1996; Moretro *et al.*, 2000; Cladera-Olivera *et al.*, 2004) however, bacteriocin production is recorded as a secondary metabolite in *Lactobacillus plantarum* LPCO10 (Jimenez-Diaz *et al.*, 1993), *B. licheniformis* 26 L-10/ 3RA (Pattnaik *et al.*, 2001), *Lactococcus lactis* ssp. *Lactis* (Cheigh *et al.*, 2002), *L. pentosus* B96 (Delgado *et al.*, 2005) and *Vibrio mediterranei* 1 (Carraturo *et al.*, 2006).

CONCLUSION

This study is based on the screening, isolation and optimization of media composition and culture conditions for bacteriocin production from *Bacillus* sp. Sh10 isolated from marine clams. This investigation showed that media and culture conditions have a significant effect on bacteriocin production by *Bacillus* sp. Sh10. The strong antimicrobial activity of this bacteriocin against food spoilage pathogens revealed its application in the food processing industry as a preservative. For this purpose, complete purification, characterization in terms of stability and the influence of various physiochemical factors requires further investigation.

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