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Purification and Characterization of Bacteriocin Like Substance Produced from *Bacillus lentus* with Perspective of a New Biopreservative for Food Preservation

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Abstract. Molecular weight of bacteriocin like substance (BLIS) of a new strain of *Bacillus lentus* 121 was found to be approximately 11 kDa. Purification of BLIS was attained by single step gel exclusion chromatography. BLIS was characterized by studying the inhibitory spectrum. It was active at broad pH range, high temperature and high NaCl concentration and showed sensitivity to proteolytic enzymes like trypsin, α-chymotrypsin and papain, the characters desirable for food preservation. BLIS extended the shelf stability of milk upto 21 days as a biopreservative.

Keywords: bacteriocin like inhibitory substance, BLIS, *B. lentus,* antimicrobial activity, biopreservative

Introduction

Food products get easily contaminated with spoilage/ pathogenic bacteria, which not only causes public health problems but is also associated with great economical losses (Sharma and Kapoor, 2004). Several processes have been developed to enhance the safety and shelf life of perishable foods with chemical preservatives. However, experiencing the adverse side effects of chemical preservatives on human health, efforts are currently underway on the search for safe products of natural origin; ultimately, biopreservation has emerged as an attractive approach for food preservation.

Biopreservatives are biologically derived antimicrobial substances which arrest/retard growth of spoilage-causing microorganisms thus contributing preservative potential to foods (Marekova *et al.,* 2007). Among biopreservatives, bacteriocins are catching rapid attention and believed to be safe for human consumption since these become inactive when treated with protease. These are mostly heat stable and wider pH tolerant, thus can withstand heat,acidity/alkalinity of food during storage conditions. In recent years, several new bacteriocins have been obtained from different species of *Bacillus* and purified (Sharma and Gautam, 2008). Important bacteriocins, purified so far, are nisin, diplococcin, acidophilin, bulgaricin, helviticin, enterocin, lacticin and plantaricin (Xiaomei *et al.,* 2006; Ogunbanwo *et al.,* 2003).Out of all bacteriocins, only nisin has got the GRAS (Generally Recognized as Safe) status and is used commercially to preserve various dairy products (Jeevaratham, *et al.,* 2005) while others are still in the process for commercialization. Nisin, though very effective, has limitations in its use due to narrow pH specificity. Thus, consistent efforts are being made for isolating bacteria capable of producing novel bacteriocin-like-substances (BLIS) for food preservation at wider scale. *Bacillus lentus* isolated from fermented dough was reported for the first time to produce BLIS having characteristics desirable for food preservation (Sharma *et al*., 2006). The BLIS of *B.lentus* has been found to withstand heat and acidity/alkalinity of foods during storage conditions, to be stable for longer period and to express inhibition against a broad range of selected food pathogens. Thus, keeping in mind these attributes, an effort has been made to purify and characterize it with the aim of using it as a high profile food biopreservative.

Materials and Methods

Isolate used. *Bacillus lentus,* BLIS producer, showing very high activity units was isolated from dough, a traditional food of Himalayan states of India (Sharma *et al*., 2006), and used in the study.

Test indicators. Test indicators, used for analyzing the pattern of antimicrobial activity, were: *Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Aeromonas hydrophila, Listeria monocytogens* MTCC 1143, *Leuconostoc mesenteroides* MTCC107, *Enterococcus faecalis* MTCC 2729, *Pseudomonas aeruginosa* and *Lactobacillus* sp.

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Well diffusion method. The antimicrobial activity of BLIS was tested by well diffusion method (Kimura *et al*., 1998) as follows. One ml of inoculum of each indicator bacteria (1.0 OD) was swabbed properly on pre-poured sterilized nutrient agar plates with the help of sterilized cotton bud. Wells of 7 mm diameter and 5mm depth were cut on the lawns of indicators and 300 µl of BLIS was poured into each well. The plates were incubated at 37 °C for 24 h and zones of clearance formed around the wells were measured.

Production of BLIS. The culture of *B. lentus* (1.0 OD_{540}) was added to 1000 ml of nutrient broth @10%. The inoculated flasks were kept at 37 °C at 150 rpm for 72 h.

Partial purification of BLIS. Partial purification of BLIS was carried out through ammonium sulphate precipitation using salt saturation method (Sharma *et al.*, 2006). *B. lentus* culture (1.0 OD_{540}) was saturated with different concentrations of ammonium sulphate i.e. 10, 20, 40, 50, 60, 70, and 80% with subsequent constant stirring. Precipitation occurred at 80% saturation level of ammonium sulphate in culture supernatant of *B. lentus*. This preparation was kept at 4 °C for 24 h. After 24 h, centrifugation was repeated at 20,000 × g at 4 °C for 30 min. This led to separation of pellets and supernatant. The pellets were suspended in 0.1 M PO_4 buffer, pH 7.0 and stored at 4 °C.

Purification of BLIS. ***Column chromatography (Gel exclusion)*.** For carrying out gel exclusion column chromatography, BLIS sample (5 ml) was loaded on Sephadex G-100 column which was packed according to the recommended procedure of Kumar and Vadhera (1980) as follows: Sephadex G-100 (5 g) was weighed and suspended in 500 ml of phosphate buffer for overnight. It was swollen for 5 h in boiling water bath, deaerated next day for 1h and brought to room temperature before packing the column of dimension 75x1.5 cm. Packing was done to avoid entrapment of any air bubbles in the gel bed. Column was then eluted with phosphate buffer (pH 7.0, 0.1M) and 3 ml fractions of the sample were collected in each of a total of 30 tubes. A flow rate of 15 ml/h of the sample was maintained. The fractions were analyzed for protein contents by taking OD at 280 nm.

Protein estimation. Protein concentration of BLIS of *B. lentus* was measured using the Lowry's method (Lowry *et al.*,1951). Following reagents were used for protein estimation:

A. Alkaline sodium carbonate (2 % Na_2CO_3 in 0.1 N NaOH),
B. 1 % copper sulphate,
C. 2 % sodium potassium tartarate,
D. Alkaline copper reagent (prepared afresh by mixing A + B + C in the ratio of 100 : 1 : 1) and
E. 1 N folin-ciocalteu phenol reagent, and prepared afresh.

0.1 ml BLIS sample was added to 0.9 ml of distilled water in the test tubes. 3 ml of reagent D was added and the tubes were allowed to stand at room temperature for 10 min. Then 0.3 ml of 1N folin's reagent was added to each test tube. The test tubes were incubated at 37 °C for 30 min and OD was measured at 670 nm against the reagent blank. The blank reagent contained all the reagents except for the BLIS. The amount of protein samples were worked out from standard curve prepared by using bovine serum albumin (10-100 µg/ml) and the estimated protein was expressed in mg/ml.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS PAGE was performed following the method of Laemmli (1970). Purification of BLIS was monitored, using 12% acrylamide gel. Standard molecular weight marker (Sigma) was run along with the sample.

Staining of gel. The gel was stained using silver staining method of Merril *et al*. (1981). Gel was kept in fixing solution for overnight, then put in 30% (50 ml) ethanol for 30 min and later kept in Farmers reagent for 5 min. Three washings of 10 min each were given with autoclaved distilled water ; 0.1% $AgNO_3$ solution (100 ml) was added and the gel was kept in dark for 30 min. Three washings were given again with distilled water for 20 sec each. Developing solution (100 ml) was added and gel was gently shaken in gel rocker until brown colour bands appeared in it. The reaction was brought to an end with 1% acetic acid and the gel was stored in plastic bags at refrigerated temperature.

Calculation of activity units (arbitrary units-AU/ml) of BLIS. The activity units of culture supernatant, partially purified and purified BLIS of *B. lentus* were calculated by serial two fold dilution method of Barefoot and Klaenhammer (1983).The culture supernatant, partially purified and purified BLIS were serially diluted and the reciprocal of smallest detectable zone of inhibition was marked for calculation of AU/ml.

Arbitrary units (AU/ml) were calculated as follow:

a) for culture supernatant of BLIS
0.3 µl = 200
1 ml = 6.6 x 10^5 AU/ml

b) for partially purified bacteriocin of BLIS
0.3 µl = 600
1 ml = 20 x 10^5 AU/ml

c) for purified bacteriocin of BLIS
0.3 µl = 1200
1 ml = 4 x 10^6 AU/ml

Characterization of purified BLIS. ***Effect of temperature on activity of BLIS.*** BLIS (0.5 ml) aliquot was added to 4.5 ml of nutrient broth in a test tube. Each test tube was then overlaid with paraffin oil to prevent evaporation and then kept for 10 min at different temperatures *viz.* 40, 50, 60, 70, 80, 90, and 100 °C on a water bath and moist heat treatment at 121°C was given in autoclave for the same time. Activity of heat treated BLIS was analyzed by the following methods.

1) Well diffusion method: as described earlier.

2) Optical density (OD) method: BLIS, treated at various temperatures for 10 min, was mixed with 0.5 ml indicator strains (*L. monocytogenes*, *S. aureus* and *A. hydrophila*) and incubated at 37 °C for 24 h. Their OD was measured at 540 nm. Minimum OD exhibited maximum inhibition of the sensitive strain by that particular fraction of heat treated BLIS.

Effect of pH on activity of BLIS. BLIS (0.5 ml) was added to 4.5 ml of nutrient broth, at pH, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. The test tubes were incubated for 30 min at 37 °C. Each pH treated BLIS was assayed by well diffusion and optical density methods as already mentioned.

Effect of salt (NaCl) concentration on activity of BLIS. To check the effect of salt concentration on activity of BLIS, different salt concentration viz., 0.1, 0.5, 1.5, 2.5, 3.5, 4.5, 5.5% were prepared in distilled water (100 ml). BLIS (0.5 ml) was added to 0.5 ml of each NaCl concentration; controls were run in parallel. Effect of various salt concentrations on BLIS activity was assayed using well diffusion assay and optical density methods.

Effect of stability on activity of BLIS. The potency of purified BLIS was checked at regular intervals *viz*. 0.5, 1.0, 1.5, 2.0, 3.0, 4.5, 5.0 and 5.5 months. Purified BLIS was stored in a clean sterilized glass bottle in frozen state. BLIS activity was recorded against three indicator strains *viz. L. mono-cytogenes*, *S. aureus* and *A. hydrophila* by well diffusion method, noting the size of individual zones.

Effect of proteolytic enzymes on activity of BLIS. Proteolytic enzymes namely trypsin, α-chymotrypsin, and papain were selected for the study. Lawns of *L. monocytogenes*, *S. aureus* and *A. hydrophila* were prepared.

EC_1 i.e. Enzyme control I as 0.3 ml phosphate buffer, EC_2 i.e. Enzyme control II as 0.15 ml bacteriocin of each isolate + 0.15 ml of phosphate buffer and ER i.e. enzyme reaction as 0.25 mg of enzyme trypsin/α chymotrypsin or papain were dissolved separately in 1ml each of 0.1 M phosphate buffer and then added to BLIS in the ratio of 1:1.

The preparations EC_1, EC_2 and ER were incubated for 1 h at 37 °C. Enzyme reaction and both enzyme control were assayed by well diffusion method against corresponding indicators.

Application as biopreservative for milk. Fresh cows milk procured locally was taken in sterilized glass flask and boiled at 100 °C for 2 min, followed by cooling at room temperature. Milk sample, 10 ml each, were put in three clean and sterilized bottles. Mixed inoculum of *L. monocytogens* and *S. aureus* in the ratio of 1:1 (8.80 log cfu/ml, 1OD) was used for inoculating the sample.

To one set of milk, BLIS was added @ 2000 ppm (Lucke,2000) while the standard chemical preservative, benzoic acid, was added to another set of milk at the same concentration for comparative study. The third set of milk was kept as control without any addition. These tubes, after proper plugging and sealing with hot wax, were kept in refrigerator (4 °C). Colony count was made by pour plate method and log cfu/ml was calculated on day 0, 7, 14, 21 and 28 for mixed inoculum. Morphological changes were also observed simultaneously. Experiments were conducted in triplicate and results were analyzed statistically; factorial CRD was used for comparative study of the biopreservative with the chemical preservative, against indicators, to enhance storage of milk.

Results and Discussion

BLIS of *B. lentus* showed a broad range antagonistic spectrum against *L. monocytogens*, *S. aureus*, *A. hydrophila*, *Lactobacillus* sp., *P. aeroginosa* and *E. coli*. BLIS was found to be effective against gm +ve as well as gram -ve bacteria whereas generally other BLIS were found active against only closely related gram +ve strains (Chen and Williams, 2003; Osmangaoglu *et al.*, 2001). However, there are a few reports of bacteriocin of LAB isolated from different food sources capable of inhibiting the growth of both gram +ve and gram -ve bacteria (Sharma and Gautam, 2008; Ivanova *et al.*, 2000). BLIS secreted by *B. lentus* were partially purified by salt saturation method. Final purification of BLIS was attained by column chromatography. Single gel exclusion chromatography step was used subsequently after ammonium sulphate precipitation. So far, this is the first time that any BLIS has been purified through only one chromatographic step. Other BLIS have been purified in 2 or 3 steps. Protein activity was observed in fractions 12-20 at 280 nm (Fig.1a), which was found to be 0.45, 0.62, 0.70, 0.91, 1.20, 0.92, 0.81, 0.65 and 0.32 OD, respectively. Fraction 16 showed the highest activity of OD 1.20, confirmed by well diffusion method as well. Fractions 2-20 were pooled for further studies. Inhibitory activity of pooled fractions were observed against the three tested strains i.e. *L. monocyto-genes*,

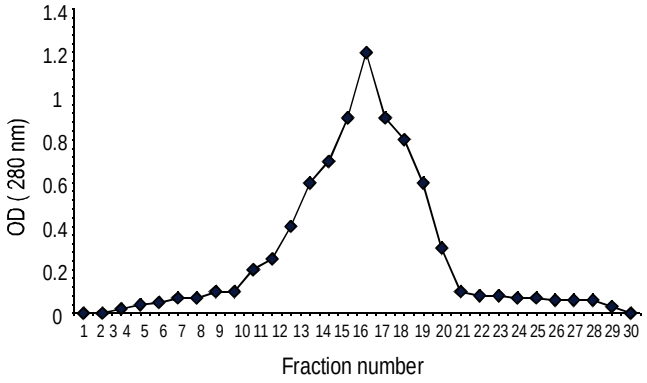

Fig. 1(a). Protein activity of BLIS fractions in terms of OD at 280 nm.

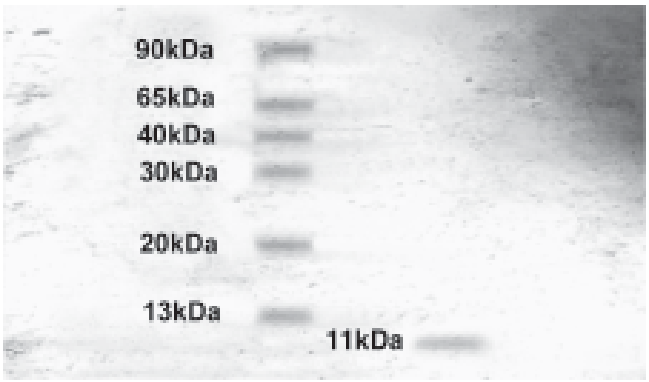

Fig. 1(b). SDS-polyacrylamide gel electrophoresis of purified BLIS of *B. lentus.*

S. aureus and *A. hydrophila,* and zones of 36 mm, 32 mm and 30 mm, respectively, were measured. The purity of bioactive fraction was checked by SDS PAGE and the molecular weight of BLIS was found to be approximately 11 kDa (Fig 1b).

BLIS exhibited very high activity ($4x10^6$ AU/ml) which reflected its strong potential against the tested spoilage-causing and food-borne pathogens thus proving its efficacy as preservative for enhancing the shelf life of food items. There was a consistent increase in activity of BLIS at each step of purification ranging from $6.6 x10^5$ AU/ml for culture supernatant, $20 x 10^5$ AU/ml for partially purified BLIS to $4x10^6$ AU/ml for purified BLIS (Table 1). The purified preservative showed 50 fold activity as compared to the crude BLIS. After purification, a spectacular increase in inhibition zone size was noticed as compared to the zone size of partially purified BLIS. There was 50% increase in zone size of purified BLIS against *L. monocytogenes,* and *A. hydrophila* over partially purified BLIS while 45.5% increase against *S. aureus* was observed.

Further characterization was done on the basis of temperature and pH in terms of inhibition zone and OD. BLIS was found active at 121°C against *L. monocytogenes* and *S. aureus* while it lost activity against *A. hydrophila* at 121°C. It showed high thermostability w.e.f. 40 to 100 °C for all the three indicators tested though inhibition zone size decreased with the increase in temperature (Fig. 2). It is clear from Fig 3 that optical density was low at 40 °C and 50 °C for the three test strains and as the temperature increased, there was an increase in OD indicating lesser activity of the preservative. Similar studies have been reported about bacteriocin of *L. brevis, L. plantarum* and *L. lactis* to be heat stable at high temperature *viz.*, 121°C (Ogunbanwo *et al*., 2003) while others lost their activity even at 50 °C (Gharairi *et al.*, 2005; Vanghan *et al*., 1992).

For studying the effect of pH, purified BLIS was exposed to pH ranging from 2 to 11. Well diffusion assay was used with test indicators revealing maximum inhibition zone of 36, 32 and 30 mm against *L. monocytogens, S. aureus* and *A. hydrophila,* respectively, at pH 7.0 thus showing its highest activity at neutral pH. BLIS was also found active on shift of pH towards acidic (pH 3.0) as well alkaline side (pH 10/11) though partial loss in its activity was indicated by decreasing zone sizes against the specific test strains (Fig. 4). OD_{540} for

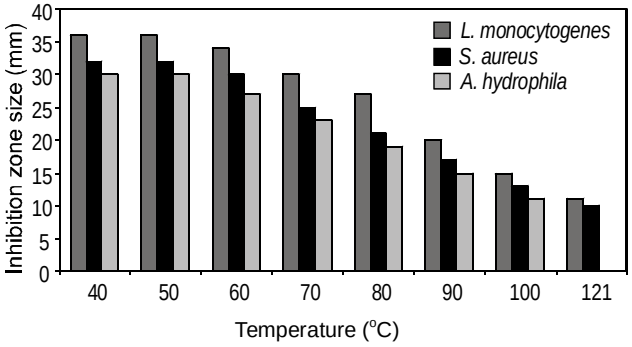

Fig. 2. Effect of temperature on activity of purified BLIS (in terms of inhibition zone).

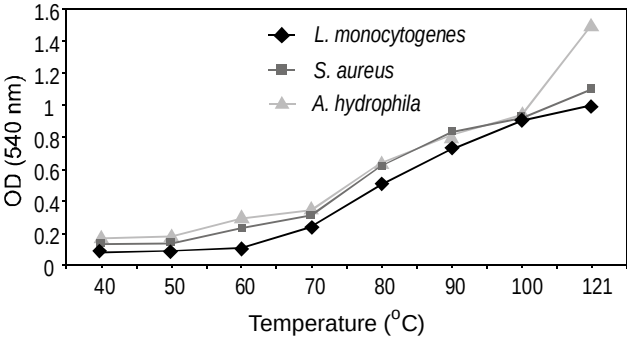

Fig. 3. Effect of temperature on activity of purified BLIS (in terms of OD at 540 nm).

Table 1. Profile of culture supernatant, partially and wholly purified BLIS produced by *B. lentus.*

Purification status	Volume (ml)	Activity unit (AU/ml)	Total activity	Protein (mg/ml)	Specific activity (AU/mg)	Purification fold	Recovery (%)
Culture supernatant	500	6.6×10^5	3.3×10^8	13.2	5×10^4	1	100
Partially purified BLIS (ammonium sulphate ppt.)	20	20×10^5	4.0×10^7	8.9	2.3×10^5	4.5	67
Purified BLIS	10	4.0×10^6	4.0×10^7	1.6	2.5×10^6	50	12

Total activity = volume x activity; protein concentration was determined by Lowry's method; specific activity = the activity unit/protein concentration; purification fold is increased in specific activity; recovery (%) = concentration of the remaining protein as % of the initial concentration of protein.

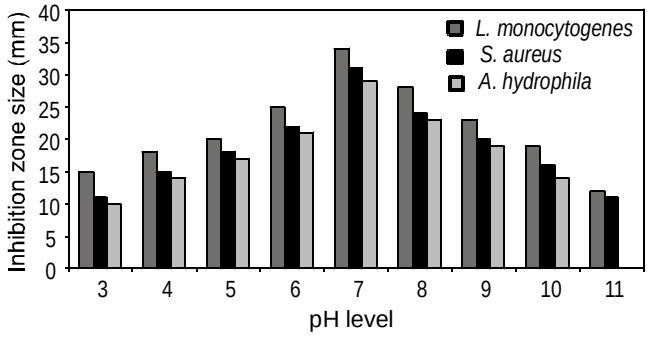

Fig. 4. Effect of pH on activity of purified BLIS (in terms of inhibition zones).

L. monocytogens, *S. aureus* and *A. hydrophila* was the lowest at neutral pH indicating highest activity of BLIS. pH range of 3.0-4.0 and 10.0-11.0 showed maximum OD expressing minimum activity of BLIS (Fig 5). In this respect, BLIS has an edge over nisin for food preservation since it can remain stable in all acidic, alkaline or neutral types of foods (Periago and Moezelaar, 2001).

The bacteriocin pediocin PA1 when used against *L. monocytogens* was found effective at a pH range of 5.5 to 6.5

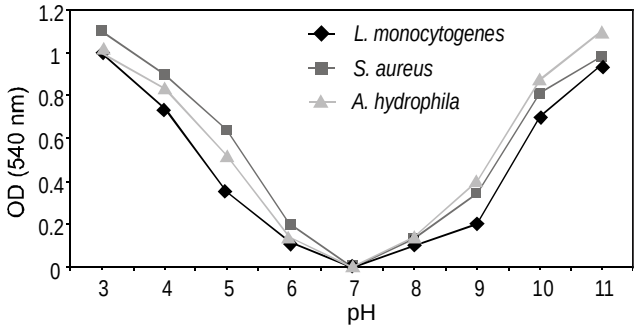

Fig. 5. Effect of pH on activity of purified BLIS (in terms of OD at 540 nm).

(Gravesen *et al.*, 2002). Nisin, the only commercially available bacteriocin, has its use limited to acidic foods only. Bacteriocin of *Bacillus cereus* was reported to be active at pH 3.0 to 12.0 (Naclerio *et al.*, 1993).

Effect of salt (NaCl) concentration *viz* 0.5% to 5.5% on BLIS activity was observed by well diffusion assay (inhibition zone) and OD method against *L. monocytogenes*, *S. aureus* and *A. hydrophila* while controls were run in parallel. BLIS retained its activity till 3.0% concentration of salt but as the concentration increased to 3.5% there was a slight decrease in zone size and increase in OD, which further enhanced with increase in concentration of salt upto 4.5% and 5.5%. The controls run in parallel showed no zone formation against the indicators. The results reveal that a very high salt concentration partially inhibit the BLIS activity (Fig. 6 & 7). Bacteriocin activity in some LAB was enhanced in the presence of low NaCl concentration of 1-2 percent but was lost at a slight higher concentration of 3 percent or more. Bacteriocin of *L. curvatus* LTH 1174 showed decrease in activity at low salt concentration of 2 per cent (Vignolo *et al.*, 1995) while that of *Carnobacterium piscicola* A9b was not affected by 0-10

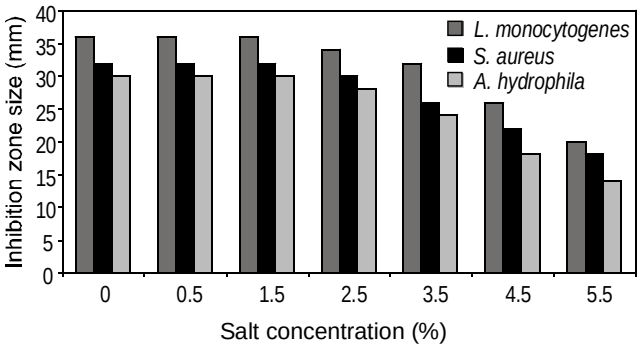

Fig. 6. Effect of salt (NaCl) concentration on activity of purified BLIS (in terms of inhibition zone).

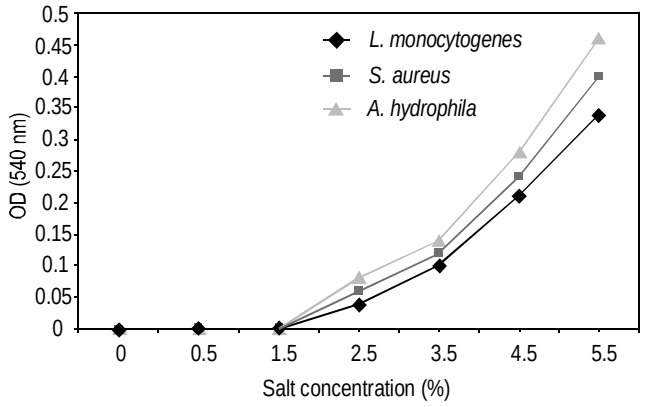

Fig. 7. Effect of salt (NaCl) concentration on activity of purified BLIS (in terms of inhibition zone).

per cent NaCl against *L. monocytogenes.* Himelbloom *et al*. (2001) found that bacteriocion stability remained unaltered for one month at 4 °C at low salt concentration.

When the effect of three proteolytic enzymes *viz*. trypsin, α-chymotrypsin and papain on the activity of purified BLIS of *B. lentus* was studied, no zones were found against *L. monocytogenes*, *S. aureus* and *A. hydrophila*. However, when BLIS was treated with phosphate buffer (EC_2) and then reacted with their respective sensitive indicators, zones of 36 mm, 32 mm were formed against *L. monocytogenes*, *S. aureus* and *A. hydrophila*, respectively. Phosphate buffer in which trypsin, α-chymotrypsin and papain were diluted had nil effect towards activity of BLIS. The inactivation of BLIS with proteolytic enzymes proved that BLIS is proteinaceous in nature. This further adds to the fact that BLIS can be broken down by the proteolytic digestive juices when consumed thus rendering it completely harmless in human system. Ivanova *et al.* (2000) showed that bozacin 14 produced by *Lactococcus lactis* subsp. *lactis* was sensitive to proteinase K, pronase E and pepsin indicating it to be proteinaceous in nature and thus it may be considered as a bacteriocin. Ogunbanwo *et al.* (2003) showed that bacteriocin produced by *Lactobacillus plantarum* F1 lost its activity after treatment with all the proteolytic enzymes. Bacteriocin lactococcin R 9/2, lactococcin R 10/1 were inactivated by only proteinase K and α-chymotrypsin. Lactococcin R 9/2 was inactivated by α-amylase as well (Elotmani *et al*., 2002).

Effect of storage on the activity of purified BLIS. Purified-BLIS showing activity of 4 x 10^6 AU/ml was stored at freezing temperature and periodically checked at interval of 0.5 months, i.e. after 0.5, 1.0, 1.5, 2.0, 3.0, 4.5, 5.0 and 5.5 months. The zone size of 36 mm, 32 mm and 30 mm against *L. monocytogenes, S. aureus* and *A. hydrophila* was maintained, retaining the full potency, till one month after purification. After 1.5 months of storage, there was a slight decrease in zone size being 32 mm for *L. monocytogenes,* 28 mm for *S. aureus* and 26 mm for *A. hydrophila* which was maintained for 2.0 months. There was further decrease in zone size *viz.* 30 mm, 26 mm and 24 mm for the three strains, respectively, at the end of 3.0 months. After 4.5 months of storage of purified BLIS the zones of inhibition showed further decrease being 22 mm, 18 mm and 16 mm for the respective indicators showing partial loss of activity of the bio-preservative. After 5.0 months of storage of BLIS in frozen state, the zone size reduced to 18 mm, 14 mm and 12 mm respectively, and after 5.5 months of storage, to 14 mm, 12 mm and 10 mm, respectively, for *L. monocytogenes, S. aureus* and *A. hydrophila*.

BLIS was fully stable for all the three hosts for a month after its purification while there was a slight decrease in the activity at three months of storage at refrigerated temperature. After 4.5 months of storage there was 61.1%, 56.3% and 53.3% decrease in the activity against the three microbes in the same order, while the loss of stability of BLIS reached to 38.9%,37.5% and 33.3% after 5.5 months of storage. This study proved that BLIS of *B.lentus* could be stored as purified fraction for approximately three months at refrigeration temperature with minimal loss in its activity.

Yildrim and Johnson (1998) observed that bacteriocin lactococcin R produced by *Lactococcus lactis* subsp. *cremoris* remained active at -20 °C and -70 °C for 3 months after storage. A bacteriocin isolated from group B-*Streptococcus* was found extremely stable when stored in its crude form at 4 °C for at least 6 months with no loss of activity while there was loss of activity in purified bacteriocin after 1 week at 4 °C (Tagg *et al*., 1975). A bacteriocin JF426, isolated from *Serratia marcescens* having molecular weight of 6.4 kDa, was stable when stored in frozen condition showing biological activity for at least 3 months (Foulds, 1972).

The storage studies of milk inoculated with mixed inoculum of *L. monocytogens* and *S. aureus* with addition of lenticin, benzoic acid and control showed initial log cfu/ml of 8.80 and initial pH value of 6.8. There was a decrease in log cfu/ml of BLIS samples till the 7th day. The log cfu/ml for benzoic acid and control was 8.40 and 8.90, respectively. On the 14th day, values of log cfu/ml of BLIS, chemical preservative and control were respectively 6.90, 7.50 and 9.00. Mean value was 7.80 while log values changed to 7.40, 8.10 and 10.80 for BLIS benzoic acid and control, respectively, on the 21st day onward. The mean value was 8.77 which increased further on 28 days of preservation being 8.0, 9.8 and 9.92 in the same

order and the final pH was 6.7,5.9 and 5.5. The calculated mean for BLIS was 7.58, for benzoic acid 8.52 and for control, 9.92. CD value was 0.05, T value was 0.07 and D value was 0.09. The T×D value was calculated to be 0.16 (Table 2). Graphical representation of log cfu/ml against days showd that BLIS is a better preservative than benzoic acid for milk inoculated with *L.monocytogenes* and *S.aureus* (Fig 8). The results show higher stability of the sample till day 21 and better activity of BLIS as compared to benzoic acid when used commercially for preservation of milk. Morphological changes were also recorded which showed no curdling, colour change or offensive smell of milk in case of the biopreservative and the chemical preservative containing samples while control showed curdling from the 1st week onwards. Concentration of BLIS was kept below 5000 ppm in milk sample which is the permissible limit for food (Ray, 2001). Xiaomei *et al*. (2006) reported that *Bacillus subtilis fmbJ* was found to produce an antimicrobial substance, which exhibited inhibitory activity against food spoilage microbes and pathogens. The inhibitory effect of antimicrobial extracts against two gram +ve, seven gram -ve and five moulds was identified.

Table 2. Comparative study of activity of BLIS and chemical preservative (sodium benzoate) against mixed inoculum *(L. monocytogenes + S. aureus)* for storage of milk at 4 °C.

Days	Biopreservative (BLIS) (log cfu/ml)	Chemical preservative (sodium benzoate) (log cfu/ml)	Control (no preservative) (log cfu/ml)	Mean
0	8.80	8.80	8.80	8.80
7	6.80	8.40	8.90	8.03
14	6.90	7.50	9.00	7.80
21	7.40	8.10	10.80	8.77
28	8.00	9.80	12.10	9.97
Mean	7.58	8.52	9.92	

$CD_{0.050}$; treatment (T) = 0.07; days (D) = 0.09; T x D = 0.16.

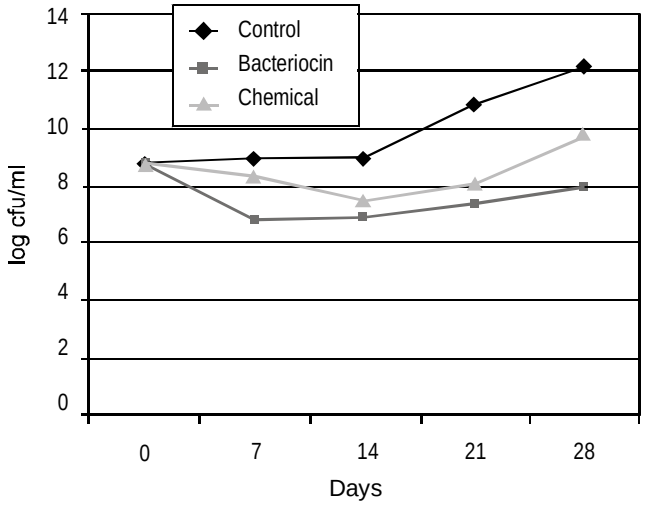

Fig. 8. Comparative study of activity of BLIS and chemical preservative (sodium benzoate) against mixed inoculum (*L. monocytogenes* + *S. aureus*) to enhance storage of milk at 4 °C.

Conclusion

Purified BLIS obtained from *B. lentus*, an isolate of sour dough, a traditional fermented food, had low molecular weight, high thermostability and salt tolerance. It had activity over a broad pH range, long storage life and was proteinaceous in nature. Considering these characteristics, desirable for a food preservation, BLIS of *B.lentus* is recommended for use as a potential biopreservative in different food items. It could enhance the shelf stability of milk upto 21 days and could delay spoilage better than other standard chemical preservatives.

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