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Laterosporulin Biosynthesis from a newly isolated *Brevibacillus* sp. in liquid culture

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ABSTRACT

The present study deals with laterosporulin biosynthesis from newly isolated *Brevibacillus* sp. in liquid culture. Soil samples were collected from different habitats of Lahore(Pakistan) for the isolation of bacterial cultures. After primary and secondary screening, ISL-4 (0.53 IBU/ml) and ISL-7 (0.6 IBU/ml) were selected having better potential for bacteriocin. A significant ($p \leq 0.05$) increase in laterosporulin biosynthesis was observed when the parameters like medium volume, buffer pH (7.0) and incubation temperature (37°C) were optimized. Antimicrobial potential of laterosporulin was also exploited using various techniques. Minimum inhibitory concentration (MIC) and critical dilution assays (CDAs) were performed for exhibiting bacteriocin inhibitory potential against various indicator strains including *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Azotobacter* sp. It was concluded that *Brevibacillus* sp*.* possesses strong potential for laterosporulin biosynthesis. Further manipulation of physical and nutritional parameters improved its biosynthesis in liquid culture.

Keywords: *Brevibacillus*, laterosporulin biosynthesis, liquid culture, minimum inhibitory concentration, critical dilution assay

INTRODUCTION

All living forms produce antimicrobial agents as a source of enhancing their innate immunity. Bacteriocins are peptides which are ribosomally synthesized by many bacteria as component of their innate immunity (Jenssen *et al.,* 2006). These peptides are mostly produced by bacteria of *Bacillus* and *Lactobacillus* genera (Teixeira *et al.,* 2013). However, many other genera including *Brevibacillus* and *Paenibacillus* have also been explored for novel bacteriocins biosynthesis (Baindara *et al.,* 2015). *Brevebacillus brevis* and *B. laterosporus* have been observed to produce substances which possess inhibitory activity and resemble bacteriocin having stability at wide pH range and high temperature (Ren *et al.,* 2007). *B. laterosporus*, member of genus *Brevibacillus*, is particularly favorable for this quest of new bacteriocins as it is known to produce many toxins, thrombin inhibitors, polyketides, antimicrobial and antitumor agents (Kamiyama *et al.,*1994). Laterosporulin is a bacteriocin produced by *B. laterosporus* that belongs to class IId of bacteriocins. The unique feature of this peptide is that it resembles defensin of mammalian cells having structural and sequence similarity (Singh *et al.,* 2014). The antimicrobial peptides of strain *B. laterosporus* showed wide activity spectrum against many bacteria, fungi and even protozoa (Singh *et al.,* 2012). Studies on laterosporulin and its antimycobacterial potential have demonstrated that it is capable of inhibiting mycobacterial growth inside phagosomes of macrophages (Sosunov *et al.,* 2007).The laterosporulin is also destructive to cancerous cells. The bacteriocin was tested to demonstrate anticancer activity against cancerous cell lines as compared to normal cell lines. The bacteriocin exhibited cytotoxic effects against certain cancer cells but it did not show any activity against cells of prostate epithelium (Baindara *et al.,* 2017).

The fermentation technique which is used mostly for biosynthesis of such peptides is submerged fermentation (SmF) that utilizes substrates in presence of excess water. SmF is

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best suited for microbes that need high moisture content for their rapid growth like bacteria (Subramaniyam & Vimala, 2012). There exists a great scope for biosynthesis and characterization of laterosporulin from bacterial cultures inhabited in acidophilic soils. Therefore, there is an emergent need to develop the process in order to combat increasing resistance. Specific aims of this study include isolation of bacterial cultures and production of laterosporulin from selected isolates with exploitation of antimicrobial potential of characterized bacteriocin. In the present study, Bacterial cultures were isolated from acidophilic soil and screened for exhibiting laterosporulin potential. Physical and cultural parameters including medium volume, buffer pH and incubation temperature were optimized. Antimicrobial potential of laterosporulin was also exploited by determining minimum inhibitory concentration and performing critical dilution assays.

MATERIALS AND METHODS

Isolation and screening of bacterial cultures

Soil samples were collected from four different habitats i.e. garden area, irrigation area, industrial area and hilly area of Murree, Punjab, Pakistan for isolation of bacteria. The bacterial cultures were isolated from soil samples by sprinkling and serial dilution method on nutrient agar. Soil samples were sprinkled on plates and incubated at 37°C for 24 h. Preparation of stock solutions was done by mixing 1 g of each sample with 100 ml of water. They were diluted serially from 10^{-1} to 10^{-6} and 0.1 ml from 10^{-4}, 10^{-5}, 10^{-6}dilutions was spread uniformly onto plates. All plates were incubated at 37°C for 24 h.

Bacterial cultures were screened for their ability to produce laterosporulin by measuring diameter of inhibition zones after performing antimicrobial assay of fermented broth. The isolates with larger zones were selected for secondary screening. Twenty two various isolates were obtained and stored at 4°C. Secondary screening was done by measuring laterosporulin activity by spectrophotometer at 700 nm.

Inoculum preparation

Ten milliliter of sterile MOT was added to a slant culture of bacterial isolates having adequate growth. An inoculating wire-loop was used to disrupt the clumps of cells. The tube was swirled to obtain a homogenous cell suspension.

Fermentation technique

Laterosporulin biosynthesis was carried out aseptically using submerged fermentation. Twenty five milliliters of minimal medium containing 7.9 g/l $Na_2HPO_4.2H_2O$, 0.5 g/l NaCl, 3 g/l KH_2PO_4, 1 g/l NH_4Cl was transferred to each flask. The pH of medium was maintained at 7.2. The flasks were cotton plugged and autoclaved at 15 lbs/in^2 (121°C) for 15 min. They were seeded with 1 ml of inoculum and placed in shaking incubator at 160 rpm and 37°C for 72 h.

Analytical Techniques

Laterosporulin assay

The broth medium was inoculated with standardized culture of indicator strain. The indicator organism was obtained from a culture of *Alkalibacillus* sp. in nutrient agar slants. Tryptone glucose extract (TGE) medium was prepared in a flask by adding casein (0.25 g), beef extract (0.15 g), glucose (0.05 g) in 50 ml of distilled water. Flask containing TGE medium was seeded with loop full of *Alkalibacillus* sp. from slant and incubated in a shaking incubator at 160 rpm and 37°C for 6-10 h. Culture broth was transferred to two falcon tubes and tubes were centrifuged for 15 min at 3500 rpm. Supernatant was discarded and pellet was re-suspended in 9 ml of phosphate-NaOH buffer. Optical density (OD) of indicator suspension was measured against distilled water at wavelength of 700 nm and noted up to 0.2.Sample and indicator suspension were mixed in equal volumes with phosphate buffer phosphate–NaOH buffer (pH-6·0, 0·05 mol l/1) and incubated for 2 h. While in control tube, buffer and suspension were added. Absorbance of sample and the control was measured at 700 nm (Cabo *et al.,* 1999).

One bacteriocin unit (BU) can be defined in terms of amount of substance found active per unit volume of sample that can cause inhibition at rate of I=0.5, given specific conditions.

Minimum inhibitory concentration (MIC)

MIC of the bacteriocin was determined against indicator strains by using microplate Alamar blue assay (MABA) as described by Pettit *et al.* (2005). One hundred microliters of culture was added to each well of a polypropylene plate followed by serial dilution of the bacteriocin and incubated for 24 h at 37°C. Fifty microliter of 0.02 %

phenol red was added to each well and the plate was monitored from 12-24 h for color change.

Critical dilution assays (CDAs)

Serial two-fold dilutions were carried out in the medium which was previously used for growth of the indicator cultures (Pucci *et al.,* 1988). Ten microliter of each dilution was spread on the nutrient agar medium. Medium was inoculated with suspension of indicator cultures and was poured onto Petri plates. Plates were incubated for 16 h and observed for inhibition zones. The critical dilution was measured when clear zones turned into hazy ones. The bacteriocin titer was calculated by the relation, Bacteriocin titer = (1000/10) × DF, where DF = Dilution factor.

Statistical analysis

The statistical analysis for laterosporulin biosynthesis was based on level of significance and standard deviation from the mean values using software (SPSS ver. 20.16, USA).

RESULTS AND DISCUSSION

The data of Table I showed the isolation and screening of bacterial isolates for laterosporulin biosynthesis in liquid culture. Soil samples were collected from different habitats including garden area, irrigation area, industrial area and hilly area. Twenty two isolates were selected after primary screening. Morphological examination of isolates revealed cocci, bacilli, coccobacilli and mixed growth. Bacteriocin producing potential was observed in eight isolates and their diameters of zones were measured that ranged from 0.4 to 1.2 mm. The laterosporulin activity of eight isolates was detected ranging from 0.13 to 0.6 IBU/ml with better producer being ISL-4 and ISL-7.

Table I: Isolation and screening of acidophilic bacterial isolates for laterosporulin biosynthesis in liquid culture

Natural Habitats	Morphology	Strain coding	Zone dia.(mm)	Laterosporulin (IBU/ml)
Hilly area	Coccobacilli	ISL-1	0.7	0.22
		ISL-7	1.2	0.6
		ISL-15	0.9	0.5
Garden area	Bacilli	ISL-4	1.0	0.53
		ISL-5	0.8	0.38
		ISL-11	0.8	0.39
Agricultural area	Mix growth	ISL-16	0.6	0.18
Irrigated area	Mix growth	ISL-12	0.4	0.13

The effect of different medium volume was investigated on laterosporulin biosynthesis by *Brevibacillus* sp. as shown in Fig. 1. Composition of medium has strong influence on growth of bacterial strains and bacteriocin biosynthesis (Laulova, 1992). It was noticed that activity was increased by increasing volume of medium up to optimal volume at 75 ml with maximum activity of 1.65 IBU/ml for ISL-4 and 1.81 IBU/ml for ISL-7. Maximum activity at optimal volume was due to the fact that at this composition of medium, organism consumed sufficient amount of nutrients to achieve maximum growth and activity. It was observed that after obtaining the optimal medium volume, the laterosporulin biosynthesis started to decrease. The decline was presumably due to thickening of fermentation medium that might have resulted in poor aeration causing a decline in provision of oxygen inside medium for microorganisms.

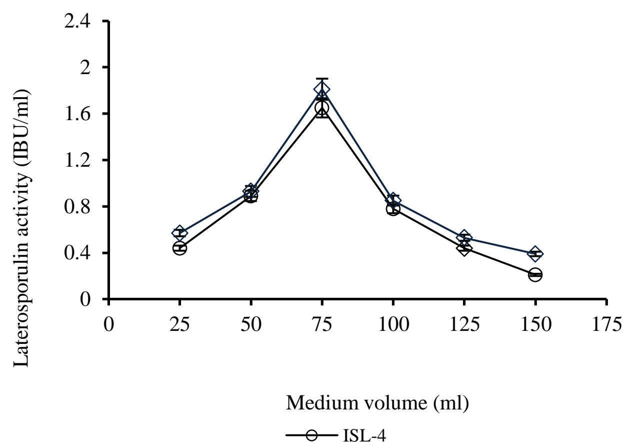

Fig. 1: Effect of different volumes of medium for enhanced laterosprulin biosynthesis by *Brevibacillus* spp. in liquid culture. Initial pH 7.2, temperature 37°C, time of incubation 72 h, size of inoculum 0.75 %. Y-error bars indicate standard deviation (±sd) among the three parallel replicates.

The effect of buffer pH was observed on laterosporulin activity as shown in Fig.2. The pH was varied from 5.5 to 8.0 with activity being 1.23 and 1.5 IBU/ml for ISL-4 and ISL-7, respectively, at pH 5.5. The optimization of buffer pH is necessary to establish an assay method with high sensitivity and accuracy because values close to neutral, favor the adsorption of bacteriocins on the cell surface requiring an extraction process with a high degree of recovery (Cabo *et al.,* 1999). The activity increased sharply with increasing pH from 5.5 to 6.0 and then there was steady increase in activity up to pH 7.0 where it became maximum i.e. 3.43 for ISL-4 and 3.74 IBU/ml for ISL-7. Activity started declining afterwards and became insignificant at pH 8.0.

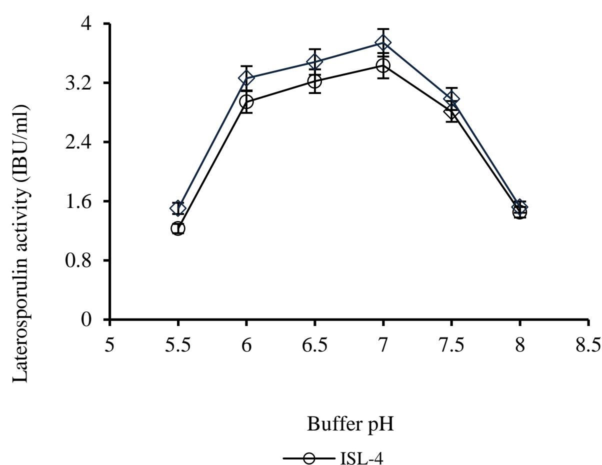

Fig. 2: Effect of different buffer pH on enhanced laterosporulin activity by *Brevibacillus* sp. Temperature 37°C, Time of incubation 2 h, wavelength 700 nm. Y-error bars indicate standard deviation (±sd) among the three parallel replicates.

The data of Fig. 3 highlight the effect of incubation temperature (31°C, 34°C, 37°C, 40°C, 43°C, 46°C) on bacteriocin activity. The incubation temperature is critical parameter for laterosporulin activity as low temperature causes decrease in kinetic energy resulting in fewer chances of collisions while higher temperatures can conclude in denaturation of peptide (De Vuyst *et al.,* 1996). As temperature was increased to 37°C, significant increase in activity was noticed for both ISL-4 (3.48 IBU/ml) and ISL-7 (3.72 IBU/ml).

When temperature was further enhanced beyond 37°C, steady decline was observed at 40°C. The activity of both isolates was 3.12 for ISL-4 and 3.39 for ISL-7 at 40°C. Sharp decline in activity was measured as temperature reached to 43°C. This reduction was due to decreased moisture content and nutrient depletion resulting in increased death rate of microbes.

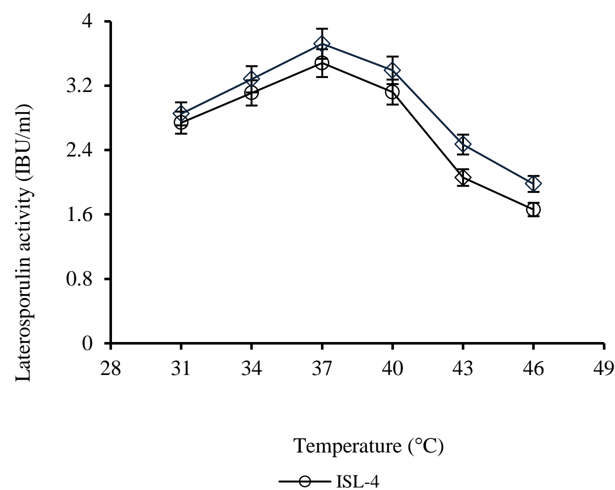

Fig. 3: Effect of different temperatures on enhanced laterosporulin activity by *Brevibacillus* sp. Buffer pH 7.0, Time of incubation 2 h, wavelength 700 nm. Y-error bars indicate standard deviation (±sd) among the three parallel replicates.

Microplate Alamar blue assay (MABA) for the determination of minimum inhibitory concentration (MIC) of *B. laterosporus* bacteriocin broth against selected pathogenic bacterial cultures using rifampicin as positive control was done and shown in Table II. The concentration of bacteriocin broth was changed from 2.5 to 10µM where rifampicin was used as control. The inhibition of indicator cultures i.e. *S. aureus*, *E. coli*, *P. aeruignosa* and *Azotobacter* sp. was compared and highlighted in Fig. 4. It was revealed that both isolates showed best inhibitory activity against *S. aureus* at 10µM which was 85% for ISL-4 and 90% for ISL-7. Inhibition for *E. coli* was not so prominent.

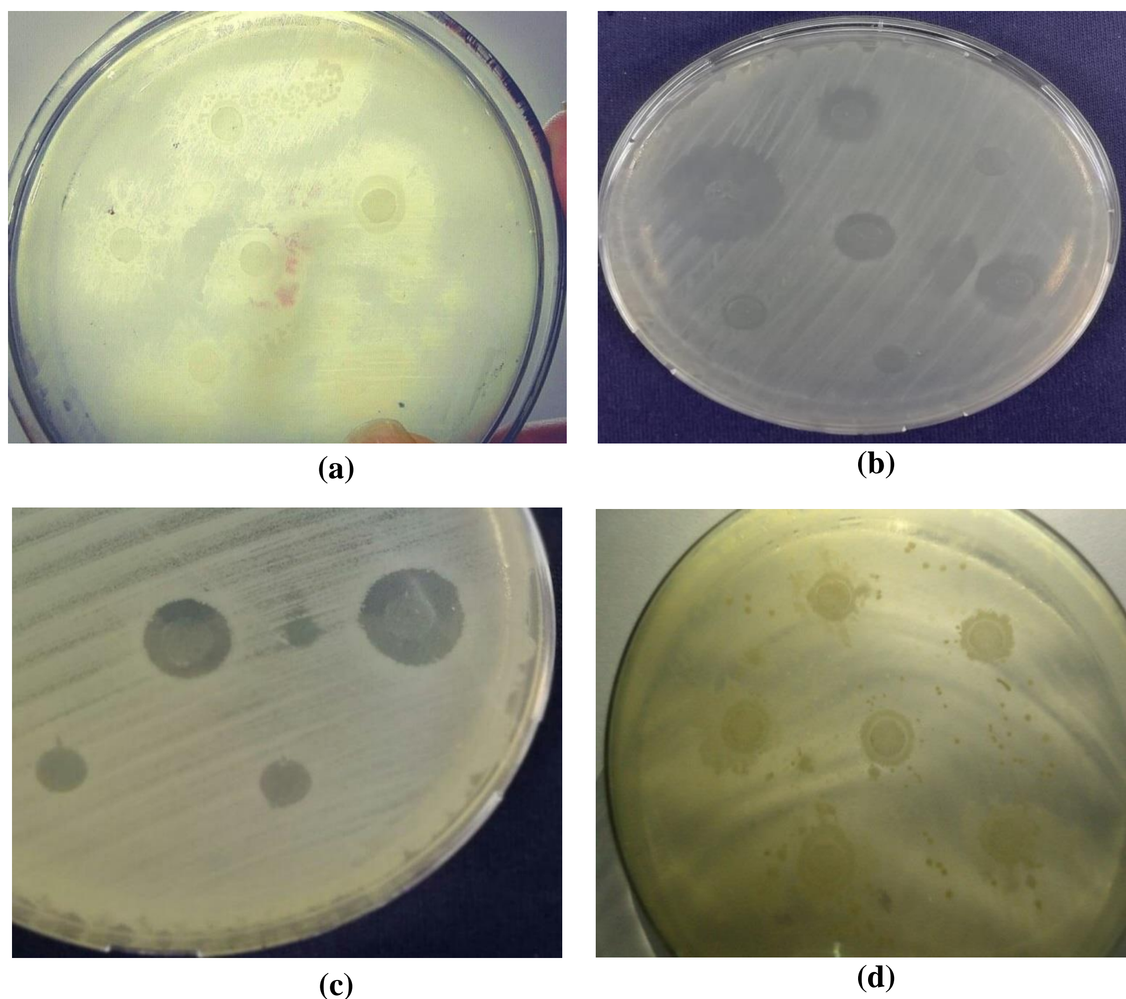

Fig. 4: Antimicrobial potential of laterosporulin against various indicator strains including (a) *E. coli,* (b)*S. aureus,* (c) *P. aeruginosa and* (d) *Azotobacter* sp.

Table II: Microplate Alamar blue assay (MABA) for the determination of minimum inhibitory concentration (MIC) of *B. laterosporus* bacteriocin broth against selected pathogenic bacterial cultures using rifampicin as positive control

Bacteriocin broth (µM)	Inhibition (%)							
	***B. laterosporus* (ISL-4)**				***B. laterosporus* (ISL-7)**			
	E. coli	***S. aureus***	***Azotobacte sp.***	***P. aeruginosa***	***E. coli***	***S. aureus***	***Azotobacter sp.***	***P. aeruginosa***
Rifampicin	100	100	100	100	100	100	100	100
10	68	85	55	72	62	90	82	50

7.5	41	65	39	49	45	78	70	32
5	27	40	23	34	30	55	51	20
2.5	12	15	8	22	18	34	30	14

Broths (100 µl) were loaded to each well of a nutrient agar plate followed by serial dilutions. The plates were incubated at 37°C for 24 h.

Table II: Microplate Alamar blue assay (MABA) for the determination of minimum inhibitory concentration (MIC) of *B. laterosporus* bacteriocin broth against selected pathogenic bacterial cultures using rifampicin as positive control

Bacteriocin broth (µM)	Inhibition (%)							
	***B. laterosporus* (ISL-4)**				***B. laterosporus* (ISL-7)**			
	E. coli	***S. aureus***	***Azotobacter* sp.**	***P. aeruginosa***	***E. coli***	***S. aureus***	***Azotobacter* sp.**	***P. aeruginosa***
Rifampicin	100	100	100	100	100	100	100	100
10	68	85	55	72	62	90	82	50
7.5	41	65	39	49	45	78	70	32
5	27	40	23	34	30	55	51	20
2.5	12	15	8	22	18	34	30	14

Broths (100 µl) were loaded to each well of a nutrient agar plate followed by serial dilutions. The plates were incubated at 37°C for 24 h.

Critical dilution assays (CDAs) of *B. laterosporus* bacteriocin broth was performed by inoculating with a standardized suspension of the indicator strain and results are shown in Table III. The critical dilutions of laterosporulin broth were prepared from 1×10^{-1} to 1×10^{-6}. Laterosporulin titres (AU/ml) were calculated as 1,000/10 × D, where 'D' is the dilution factor. Maximum titre was obtained with dilution of 1×10^{-5} where it was 56 AU/ml for ISL-4 and 72 AU/ml for ISL-7. It was noticed that further increase in dilution reduced titer to 45AU/ml for ISL-4 and 56 AU/ml for ISL-7. Therefore, dilution of 1×10^{-5} was found to be critical dilution for laterosporulin. The critical dilution assay for bacteriocins was performed in a similar study done by Parente *et al.* (1995).

CONCLUSION

In the present study, *Brevibacillus* isolates were isolated from acidophilic soil samples. The isolates ISL-4 and ISL-7 were assessed for laterosporulin biosynthesis in liquid culture. The optimization of cultural conditions particularly medium volume, initial pH and temperature boosted laterosporulin activity to a maximum of 3.72 IBU/ml. Overall,ISL-7 revealed 9.2% enhancement in yield compared toISL-4. MIC and CDAs were performed which confirmed laterosporulin inhibitory potential against various indicator strains.

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