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ORIGINAL ARTICLE

Production of novel antimicrobial protein from *Bacillus licheniformis* strain JS and its application against antibiotic-resistant pathogens

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ABSTRACT

Bacillus licheniformis strain JS has been shown to produce antimicrobial protein (AMP) using wheat bran as carbon and nitrogen source. Monitoring of AMP production revealed maximum after 72 h and extracted optimally at 70% saturation of ammonium sulphate. The extracted protein was further purified by ion exchange column chromatography which was eluted at 0.3 M NaCl concentration. The 16 kDa purified antimicrobial protein shows more activity against Gram-positive bacteria *Bacillus cereus* as compared to other bacteria. Further, combinatorial effect of AMP with antibiotics showed increased efficiency of Kanamycin, Neomycin and Streptomycin. So, the AMP extracted from *B. licheniformis* strain JS could be promising to increase the efficiency of present antibiotics against antibiotic-resistant pathogens. Thus, this study could be useful to design new therapeutic strategies to treat infectious diseases.

Keywords Anionic · Antimicrobial protein · *B. licheniformis* · DEAE-Cellulose · Antibiotic resistance

Introduction

The alarming increase in antibiotic-resistant bacterial infections is a serious threat to humans and animals worldwide. In this respect, antimicrobial peptides (AMPs) are potential therapeutic tools, because of their rapid and specific killing activity against pathogens. So far, more than 800 AMPs have been isolated from different sources such as humans, animals, plants, insects and bacteria (Reddy et al. 2004). The AMPs are classified as ribosomal and non-ribosomal peptides. The ribosomal AMPs are also called as bacteriocins consist of only 19 to 37 amino acids, where as large peptide with molecular weight 90,000 Da (Joerger 2003). So far, the peptides exhibiting antimicrobial properties are small, amphipathic and cationic in nature, whereas very few reports on anionic peptides are found (Lai et al. 2002; Cytrynska et al. 2007; Akeel et al. 2017). Recently, several reports have suggested alternative mechanisms of AMPs on multiple targets, but specifically known for its interactions with bacterial cell membrane for permeabilization (Zhang et al. 2001; Jenssen et al. 2006). Anionic antibacterial peptides kill bacterial cells by causing precipitation of cytoplasmic proteins and intracellular content flocculation (Brogden et al. 1996, 2003).

The *Bacillus* genus has been known for its diversified characteristics. It has potential to produce antibiotics since for 50 years. The production of antimicrobial peptides has been described for many *Bacillus* species like *B. thuringiensis* (Paik et al. 1997), *B. subtilis* (Zheng et al. 1999), *B. cereus* (Bizani and Brandelli 2002) and *B. licheniformis* (Cladera-Olivera et al. 2004). Among the different species of *Bacillus,* the *B. licheniformis* has been widely accepted for its industrial applications (de Boer et al. 1994).

The novel antimicrobial peptides could be an option for the control of infectious diseases caused by the antibiotic resistant microorganisms. Thus, to understand its effectiveness, it is very important to isolate microorganisms producing AMPs and study its characteristics, and its mechanism of action against particular bacteria. Present work deals with the purification and characterization of antimicrobial peptide from *B. licheniformis* strain JS.

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Materials and methods

Screening of AMP producers

The isolation of antimicrobial peptide producer organism was carried out by screening of various available cultures such as *Bacillus licheniformis* strain JS, *Bacillus megaterium*, *Bacillus thuringiensis*, and *Bacillus subtilis*. The *B. licheniformis* strain JS was found to produce antimicrobial compound, so it was selected for further studies.

Production of antimicrobial protein

The Czapek dox medium containing $NaNO_3$ 0.3 gm, K_2HPO_4 0.1 gm, $MgSO_4.7H_2O$ and KCl 0.05 gm, $FeSO_4.7H_2O$ 0.001 gm in 100 ml distilled water with Wheat bran as carbon source was used for production of antimicrobial peptide from *B. licheniformis* strain JS. The fresh culture of *B. licheniformis* strain JS was inoculated into production media. The flask was then incubated at 37 °C for 96 h in static condition. The antimicrobial activity of AMP was determined by antimicrobial assay.

Extraction of antimicrobial protein

After incubation the broth was centrifuged at 8000 rpm for 15 min at 4 °C and the supernatant was collected. Further the supernatant was used for extraction of Antimicrobial peptides by the following methods.

Method I—70% Ammonium sulphate was added to supernatant with constant stirring and kept overnight in refrigerator at 4 °C. Then, the mixture was centrifuged at 8000 rpm for 20 min at 4 °C and the precipitate was collected. Then, this precipitate was dissolved in 25 mM phosphate buffer having pH 7.0 and dialysis was carried out in same buffer.

Method II—after incubation period, the broth was centrifuged and collected supernatant was subjected to cold acetone precipitation (60%). In this, cold supernatant and chilled acetone were mixed with constant stirring and kept in deep freeze at − 15 °C for overnight. After overnight precipitation, the mixture was centrifuged at 8000 rpm for 20 min at 4 °C and precipitate was collected. The collected precipitate was then dissolved in 25 mM phosphate buffer and dialysis was carried out in same buffer.

Purification of antimicrobial protein

The column used for chromatography was packed by activated DEAE cellulose. Then, the flow rate was adjusted as 5 ml min^{-1} and dialyzed sample loaded on to the column. The protein was eluted by 0.1–1.0 M NaCl gradient solutions. The eluted fractions were checked for the protein content by using Lowry method and standard graph was obtained by Bovine Serum Albumin as standard protein. Then high protein containing fractions were dialyzed and used for antimicrobial activity.

Antimicrobial assay

Test organism such as Gram-positive *B. subtilis* and *B. cereus*, and Gram-negative *Salmonella typhimurium* and *Shigella dysenteriae* were spread on the nutrient agar plates. The wells were prepared, and in each well, 50 µl sample was added. The plates were kept for diffusion in refrigerator for 10 min. After diffusion, plates were incubated for 24 h at 37° and observed for the zone of inhibition.

Effect of temperature and trypsin digestion on antimicrobial activity

To study the effect of temperature, AMP was kept at different ranges of temperature such as 10–100 °C for 60 min. The effect of trypsin digestion on antimicrobial peptide was studied by adding 1 ml protein into 0.5 ml digestion buffer containing trypsin with concentration 1 µg/µl. Then, this mixture was incubated for 3 h at 37 °C and then kept at 95 °C for 5 min for inhibition of trypsin. After trypsin action, AMP was checked for antimicrobial activity.

Molecular weight determination

The fraction collected after ion exchange column chromatography showing antimicrobial activity was checked for purity by SDS-PAGE. The antimicrobial peptide band was visualized by Coomassie brilliant blue staining. The molecular weight of antimicrobial peptide was determined by comparison with standard molecular marker proteins (Phosphorylase b 98 kDa, Bovine Serum Albumin 66 kDa, Ovalalbumin 43 kDa, Carbonic Anhydrase 29 kDa, Soyabean Trypsin Inhibitor 20 kDa).

Combinatorial effect of AMP and Antibiotics

The combinatorial effect of AMP and different class of antibiotics (Penicillin, Kanamycin, Neomycin, Gentamicin and Streptomycin) were studied. The agar diffusion method was used to check efficiency of combined effect of AMP with different antibiotics. In this, test organisms such as *B. cereus* and *S. typhimurium* were spread on the nutrient agar plates and three wells on each plate were prepared. Then these three wells were added with AMP, respective antibiotic and mixture of AMP + antibiotic and plates were kept for diffusion at 4 °C for 10 min. After diffusion, plates were incubated at 37 °C for 24 h and observed for zone of inhibition.

Statistical Analysis

Statistical analysis was carried out using Graph Pad software (GraphPad InStat version 3.00, GraphPad Software, San Diego California USA). Results obtained were the mean of three or more determinants. Analysis of variance was carried out on all data at $p < 0.05$.

Results

Screening and production of AMP

The different species of *Bacillus* were screened for the production of antimicrobial peptide. It was seen that among *B. licheniformis* strain JS, *B. megaterium*, *B. thuringiensis*, and *B. subtilis,* the *B. licheniformis* has the ability to produce AMP. So, it was selected for the further studies on AMP. *B. licheniformis* strain JS was found to produce antimicrobial peptide in Czapek dox medium with wheat bran as sole source of carbon. From Fig. 1, the maximum antimicrobial activity was found after 72 h in broth and further incubation leads to decrease in antimicrobial activity. AMP production was found enhanced at 37 °C at static condition than at shaking conditions, whereas most of reports suggest more production at shaking conditions.

Purification of antimicrobial peptide

The extracellular AMP produced by *B. licheniformis* strain JS in medium was precipitated by ammonium sulphate and acetone precipitation methods. The 70% saturation of ammonium sulphate was found optimum for the precipitation of AMP. However, AMP extracted by 60% acetone precipitation was less active as compared to ammonium sulphate. Hence, extracted AMP by ammonium sulphate precipitation was further purified by the ion exchange column chromatography. Various proteins were eluted from the column at NaCl gradient between 0.1 and 1.0 M, but AMP was eluted at 0.3 M NaCl concentration.

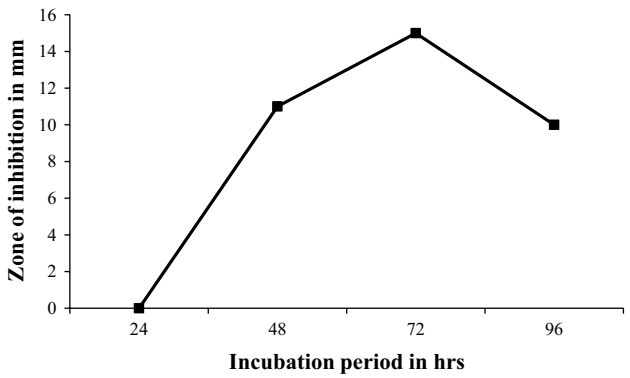

Fig. 1 Production of antimicrobial protein by *B. licheniformis* strain JS. The fresh culture was inoculated in Czapeck dox broth containing wheat bran at 37 °C and after 24 h of interval aliquot was taken to check antimicrobial activity

Antimicrobial activity of AMP

The antimicrobial peptide produced by *B. licheniformis* in crude form was tested against variety of Gram-positive (*B. cereus, B. subtilis*) and Gram-negative (*S. dysenteriae, S. typhimurium*) bacteria. The extracted AMP showed significant activity against Gram-positive and Gram-negative pathogens. The purified AMP was found more active than the crude AMP. The dilution study revealed that the AMP at 50 µl (100 µg/ml) shows maximum zone of inhibition as compared to 75 µl (100 µg/ml) and 100 µl (100 µg/ml) against Gram-positive (*B. cereus*) organisms (Fig. 2). In case of Gram-negative (*S. dysenteriae*) organism, the zone of inhibition was maximum at 100 µl (100 µg/ml).

Effect of temperature and trypsin digestion

In the study of effect of temperature on the antimicrobial activity of AMP, it was seen that the antimicrobial activity was 100% at temperature range between 10 and 90 °C (Table 1). This confirms that the antimicrobial peptide produced by the *B. licheniformis* strain JS is having thermotolerant activity. The trypsin digestion study reveals that the AMP retains its 100% activity.

Molecular weight determination

The AMP purified by ion exchange column chromatography analyzed by SDS-PAGE is shown in Fig. 3. The molecular mass of purified antimicrobial peptide was found as 16 kDa. The presence of single band indicates the complete purification by ion exchange column chromatography.

Combinatorial effect of AMP and antibiotics

The AMP extracted from *B. licheniformis* strain JS has shown very good inhibitory effect against different pathogenic bacteria. It was also found that when this AMP was mixed with antibiotics such as Kanamycin, Neomycin and Streptomycin, the efficiency of these antibiotics increased up to twofold, whereas no effect was observed in case of Penicillin antibiotic (Table 2).

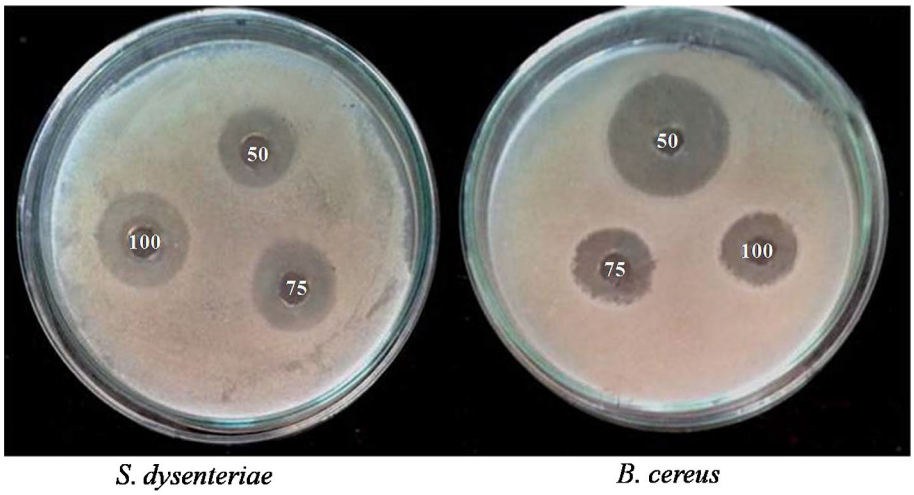

Fig. 2 Antimicrobial activity of purified AMPs produced by the *B. licheniformis strain JS*. The protein purified by DEAE-cellulose column chromatography subjected to antimicrobial activity against *B. cereus* and *S. dysenteriae*

Table 1 Effect of temperature and trypsin digestion on antimicrobial activity

Temperature in ° C	Zone of inhibition against test organism in mm	
	B. cereus	*S. dysenteriae*
Control	21.3 ± 0.33	14.0 ± 0.57
10	20.0 ± 1.15	14.0 ± 0.57
20	20.0 ± 1.52	14.0 ± 1.00
30	21.0 ± 0.57	14.0 ± 0.57
40	19.6 ± 0.88	14.3 ± 0.88
50	20.6 ± 0.33	14.3 ± 0.33
60	19.3 ± 0.88	13.0 ± 0.57
70	20.3 ± 0.33	13.3 ± 0.66
80	20.0 ± 0.57	13.6 ± 0.66
90	19.3 ± 0.88	13.0 ± 0.57
100	19.0 ± 0.57	13.3 ± 0.88
After trypsin digestion	20.0 ± 0.57	13.3 ± 0.33

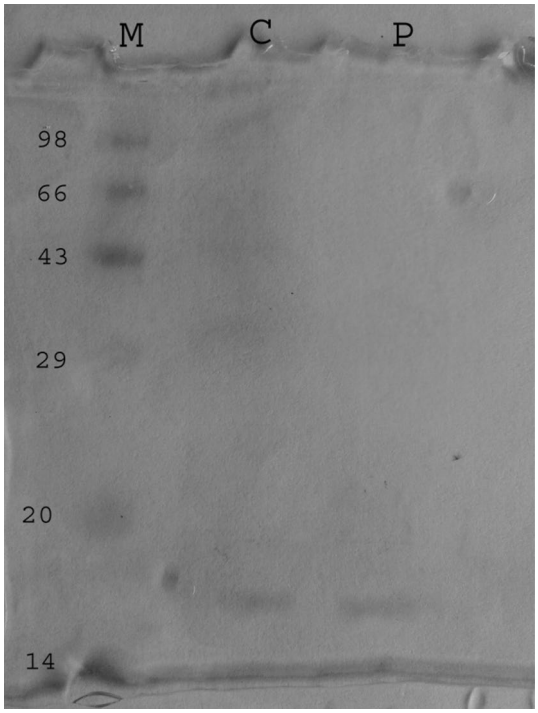

Fig. 3 SDS PAGE analysis of the purified AMPs. C-crude protein, P-Purified AMPs, M-molecular weight markers

Discussion

The *B. licheniformis* strain JS isolated from mushroom bed left over after the production of oyster mushroom (*Pleurotus sajor-caju*) which has been reported earlier for production of thermophilic chitinase (Waghmare and Ghosh 2010). In this study, we had first time reported the production of antimicrobial protein from *B. licheniformis* strain JS. So far, different strains of *B. licheniformis* have been reported for the production of antimicrobial compounds (Cladera-Olivera et al. 2004; Callow and Work 1952; Prave et al. 1972). It is essential that organism must be provided with optimal growth conditions to increase AMPs production. The *B. licheniformis* strain JS has produced maximum AMPs after 72 h of incubation period using wheat bran, whereas Cladera-Olivera et al.2004 reported the maximum production of AMPs after 15 h from *B. licheniformis* strain P40 using Brain Heart infusion medium. Thus, our study shows that the production of AMP is economically feasible from *B. licheniformis* strain JS when wheat bran is used as a sole source of carbon.

The 70% saturation of ammonium sulphate was found optimum for the precipitation of AMP, whereas Sirtori et al. (2006) reported that even 50% saturation of ammonium sulphate is also suitable for extraction. Among ammonium

Table 2 Combinatorial effect of AMP and antibiotics

	Zone of inhibition against test microorganisms in mm			
	B. cereus		*S. typhimurium*	
	Antibiotic	Antibiotic + AMP	Antibiotic	Antibiotic + AMP
Kanamycin	16.0 ± 0.57	28.0 ± 0.57	15.0 ± 1.0	23.0 ± 0.57
Streptomycin	20.0 ± 1.15	29.3 ± 0.88	11.0 ± 1.0	23.3 ± 0.33
Gentamicin	19.0 ± 0.57	20.6 ± 0.33	16.3 ± 1.5	17.0 ± 0.57
Neomycin	11.0 ± 0.57	27.3 ± 0.33	18.0 ± 1.0	23.0 ± 0.33
Penicillin	–	–	–	–

sulphate and acetone precipitation, AMP extracted by ammonium sulphate showed more antimicrobial activity. This suggests that ammonium sulphate extraction method is more significant method than the acetone precipitation. The extracted AMP purified by DEAE-Cellulose in the present study produced by *Bacillus* sp. Similarly, Motta et al. (2007) also reported the purification of antimicrobial peptide by DEAE-Sepharose. This result shows the binding affinity of purified peptide with DEAE indicating anionic nature of peptide. The single band on the SDS-PAGE indicates that the antimicrobial protein secreted by the *B. licheniformis* strain JS could be purified by subsequent steps such as ammonium sulphate extraction and DEAE-Cellulose ion exchange chromatography. AMP produced by *B. licheniformis* strain JS is more active in less concentration against Gram positive same as that of antibacterial peptide extracted from different species of *Bacillus*. The broad spectrum nature of AMP isolated from *B. licheniformis* strain JS showed antimicrobial activity against Gram-negative organism but at lesser extent than the Gram positive similar to the antimicrobial peptide extracted from *B. licheniformis* strain P40 (Teixeira et al. 2009).

AMP produced from *B. licheniformis* strain JS is thermolerant similar to earlier report on *Bacillus* sp. (Cladera-Olivera et al. 2004; Teixeira et al. 2009). The antimicrobial activity of AMP did not affect even after the action of trypsin at higher temperature. It indicates that AMP is resistant to action of trypsin. So, this property of AMP makes it applicable for the administration through digestive system. Generally, the AMPs produced by the bacteria are < 10 kDa (Reddy et al. 2004), but the AMP produced by *B. licheniformis* strain JS having large molecular weight, i.e., 16 kDa. Similarly, few reports suggest that certain bacterial genus have ability to synthesize antimicrobial proteins of high molecular weight such as 30 kDa from *P. aeruginosa* JU-Ch 1 (Grewal et al. 2014), *Lactobacillus rhamnosus* 231 (Ambalam et al. 2009), *Clavibacter michiganensis* subsp. *michiganensis* (Liu et al. 2013) and *B. subtilis* ATCC 21331 (Aishah et al. 2014). The combinatorial effect of AMP with antibiotics showed increase in the efficiency of Kanamycin, Neomycin and Streptomycin antibiotics. So, it could be because of AMPs produced by *B. licheniformis* may facilitate entry of these antibiotics inside the pathogens and increase their efficiency. This suggests that the AMPs extracted from *B. licheniformis* formulated with Kanamycin, Neomycin and Streptomycin could be useful to control antibiotic resistant pathogens.

Conclusion

The present work concludes the ability of *B. licheniformis* strain JS to produce a novel 16 kDa antimicrobial protein. It shows stability in the presence of trypsin at high temperature. The purified peptide also increases the efficiency of Kanamycin, Neomycin and Streptomycin. Hence, the isolated AMP would be promising for the development of new therapeutics in the treatment of infectious diseases.

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Compliance with ethical standards

Conflict of interest All authors have declared that they have no conflict of interest.

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