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Characterization of Bacteriocin Produced by *Bacillus atrophaeus* Strain JS-2

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ABSTRACT

The isolate of *Bacillus atrophaeus* JS-2 (GeneBank accession number JN409601) was found to produce a bacteriocin which showed antimicrobial properties against *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Escherichia coli*, *Salmonella typhimurium* and *Aspergillus niger*. There was prominent proteolytic activity with some lipolytic activity. The antimicrobial activities were seen between pH 5-8 but optimally at pH 5 and when the organism was grown at 40°C. During purification of the compound by precipitating with 80% ammonium sulfate, it was found to be associated with other proteins which were seen in the DEAE cellulose ion exchange chromatography. Characterization by SDS-PAGE showed single band at 43 kDa which was both proteolytic and lipolytic.

Key words: Bacteriocin, lipolysis, proteolysis, thermotroph, *Bacillus*

INTRODUCTION

The emergence of multidrug resistant pathogenic bacteria has intensified the search for possible alternatives to the present chemotherapeutic practices. Amongst the various alternatives like using plant extracts (taken from different parts of plants), better hygienic and sanitary practices to avoid the infection, improved nutritional foods to strengthen immunity etc., certain materials of microbial origin too are being investigated. It is well known that antimicrobial substances like antibiotics (metabiotic agents) which were considered as miracle drugs have been in practice since long time and the indiscriminate use of these has led to development of drug resistant pathogens. The other types of antimicrobials, distributed widely in the prokaryotic world, are bacteriocins (Xie *et al.*, 2009). Its antimicrobial properties were aimed at stimulating the immune system and were used as an aid treatment of gastrointestinal and urinary tract diseases. These are peptide synthesized by prokaryotes (Torkar and Matijasi, 2003) and have varying molecular weight, with antimicrobial action including bactericidal, fungicidal, immunomodulatory activity (Xie *et al.*, 2009). Bacteriocin was first detected in 1925 by Gratia, who observed *E. coli* was inhibited by the presence of a compound called colicin V. This was observed to be released into the medium by *E. coli* V (Shelburne *et al.*, 2007; Sutyak *et al.*, 2008; Desriac *et al.*, 2010). Similar antibiotics were observed to be released other bacteria like antibiotics from lactic acid bacteria, like plantaricin produced by *Lactobacillus plantarum*.

Bacteriocins are of different types differing in the primary amino acid sequence and exhibiting and inhibiting a large population of bacteria from different genera (Nes and Holo, 2000). The data of sequenced bacteriocins can be found in UniProtKB/Swiss-Prot database along with BACTIBASE, BAGEL, APD2 and CyBase (Kawulka *et al.*, 2004; Hammami *et al.*, 2007; Desriac *et al.*, 2010). Bacteriocins are not only heat stable (Lisboa *et al.*, 2006; Javed *et al.*, 2010) but active over a wide range of pH (Martirani *et al.*, 2002). Some of the bacteriocins are circular peptides, where the N and C termini of the peptides linked by an amide bond (Martin-Visscher *et al.*, 2009; Maqueda *et al.*, 2008). There are different bacteriocidal mechanisms like by forming ion channels in the cytoplasmic membrane; by non specific degradation of cellular DNA; by inhibition of protein synthesis or by cell lysis (Riley and Wertz, 2002).

In recent years, groups of antibacterial protein produced by gram positive bacteria have attracted great interest in their potential use as food preservatives. Bacteriocins such as nisin used in food preservation and is active against food borne pathogens such as *C. botulinum*, *B. cereus* etc. These are also used as shelf life extender and adjuncts in novel food processing technology.

This investigation aims to produce, isolate and characterize bacteriocin produced by *B. atrophaeus* JS-2. The organism is like *B. subtilis* which is a gram positive, rod shaped, catalase positive bacterium commonly found in soil (Madigan and Martinko, 2005).

MATERIALS AND METHODS

The organism used as the most potential producer of bacteriocin was *B. atrophaeus* JS-2 (isolated in this laboratory), The culture was tested against different test organisms by agar well diffusion method using nutrient agar medium. The identification was confirmed by 16 rDNA analysis.

Production of bacteriocin: The organism was directly inoculated into 100 mL sterile Brain Heart Infusion (BHI) medium (1% peptic digest of animal tissues, 1.25% calf brain infusion, 0.5% beef heart infusion, 0.2% dextrose, 0.5% NaCl, 0.25% disodium phosphate). The flask was incubated at 30°C under shaking conditions (110 rpm) for 16 h. The medium having the growth was centrifuged at 6000 x g at 4°C for 20 min. The residue along with cells was discarded.

The cell free medium containing the bacteriocin was first brought to 80% (w/v) saturation with ammonium sulfate according to Gomori (1955). This was refrigerated overnight. The precipitated proteins were regimented by centrifugation at 6000 x g for 20 min at 4°C. The final residue was dissolved in 5 mL of 20 mM phosphate buffer (pH 6.8) and was dialyzed against same buffer. This was the crude bacteriocin preparation. This was taken to determine protease and lipase activity using casein and egg yolk lecithin, respectively.

Further it was passed through column containing DEAE cellulose resin and fractions were collected using NaCl gradients of 40-200 mM with step of 40 mM. The protein content of these fractions were checked by absorbance at 280 nm and confirmed by Lowry method (Lowry *et al.*, 1951). The antibacterial activity was also checked against *B. licheniformis* (NCIM 5339).

Molecular weight was determined by SDS-PAGE. Molecular weight markers ranging from 20-43 kDa (oval albumin 43 kDa, carbonic anhydrase 29 kDa, trypsin inhibitor 20 kDa) were used. Procedure was carried out on 16% polyacrylamide gel. One hundred microliter sample was loaded along with markers and electrophoresis was carried out at 100 volts. Then gel was subjected to silver staining.

Test microorganisms: The effect of the bacteriocin produced by the organism was tested against: *B. cereus* (NCIM 2156), *B. megaterium* (NCIM 2087), *B. licheniformis* (NCIM 5339), *M. aureus*

(NCIM 5121), *E. coli* (NCIM 2066), *S. typhimurium* (NCIM 2501), *P. aeruginosa* (NCIM 2036), *S. flexneri* (NCIM 5265), *P. vulgaris* (NCIM 2027) and *A. niger* (NCIM 545).

The above mentioned test organisms were grown on sterile BHI agar. The medium was seeded with respective organism. On solidification of the medium, 5 and 8 mm wells were prepared and filled with 50, 100 µL of crude bacteriocin. The tests were carried out in triplicates. The plates were incubated at 25°C for 24 h. The diameter of zone of inhibition was measured and recorded.

Effect of pH on bacteriocin production: The pH of BHI medium was adjusted to 5, 6 and 8 with 0.1 N HCl and 1 N NaOH and then inoculated with fresh culture of the isolate. Flasks were incubated at 30°C on shaker (110 rpm) for 16 h. The medium was then centrifuged by method as mentioned above and precipitated by Ammonium sulfate precipitation (80% saturation). This precipitate was dissolved in the same buffer and was tested for antimicrobial activity by agar well diffusion method.

Effect of temperature on bacteriocin production: BHI broth was prepared, inoculated with fresh culture of *B. subtilis* and incubated at different temperatures like 8, 30 and 40°C for 16 h. The cell free medium was precipitated by ammonium sulfate precipitation method. This precipitate after redissolution in the buffer was used for determination of antimicrobial activity by agar well diffusion method.

RESULTS AND DISCUSSION

The organism *B. atropheus* JS-2 was identified by 16rDNA analysis as given in Fig. 1 and deposited with GeneBank (accession No. JN409601) showed maximum zones of inhibition against the test organisms. The results of antibacterial activity by agar well diffusion method are as shown in Fig. 2.

This strain was deposited with NCIM, NCL. Pune (India) with the identification number NCIM 2010. Bacteriocins from *Bacillus* have attracted little attention. A plasmid linked antilisterial

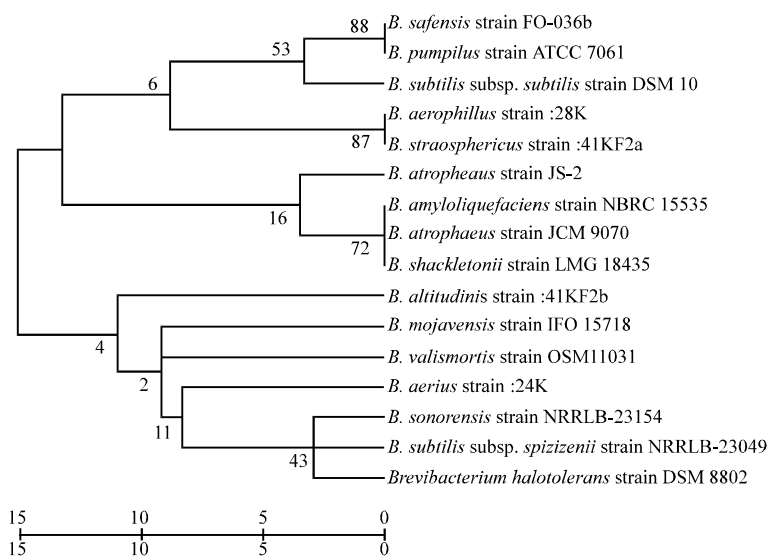


Fig. 1: Phylogenetic tree for the isolate *B. atropheus* JS-2 using MEGA 4 software

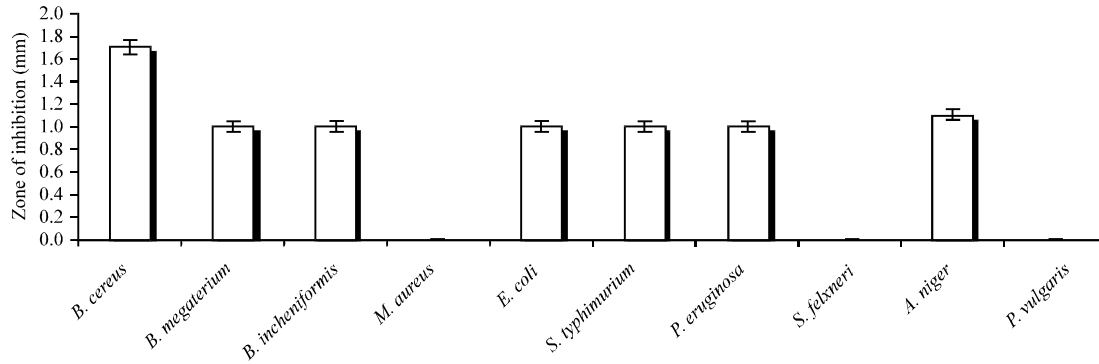


Fig. 2: Antibacterial activity of dialyzed ammonium sulfate precipitate of cell free medium of *B. atrophaeus* JS-2

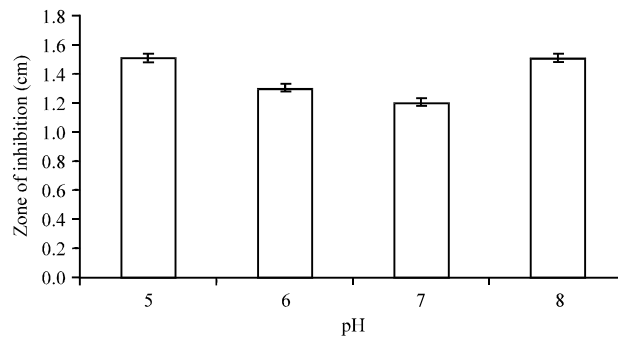


Fig. 3: Effect of pH on the antimicrobial activity against *B. licheniformis*

bacteriocin produced by *B. subtilis* 22 A, coagulin produced by *B. coagulans*, Thruicin 7 produced by *B. thuringiensis* (Torkar and Matijasi, 2003). *B. subtilis* is one of the major produces subtilisin which is a proteolytic enzyme having similar inhibitory activity to Morricin 269 (De la Fuente-Salcido *et al.*, 2008), subtilisin A, (Shelburne *et al.*, 2007), bacteriocin produced by *B. licheniformis* MKU3 (Kayalvizhi and Gunasekaran, 2008) and that produced by a *B. brevis* strain (Abriouel *et al.*, 2011).

Bacteriocin produced by *B. atrophaeus* JS-2 was active over wide range of pH values 5-8. However, optimum activity was observed at pH 5 (Fig. 3).

These results were similar to a bacteriocin produced by a *Bacillus* species (Adetunji and Olaoye, 2011) and bacteriocin produced by *B. subtilis* LFB112 (Xie *et al.*, 2009).

The substance was relatively heat stable with good antimicrobial activity at 40°C (Fig. 4).

There was slight loss of activity when stored at 8°C for 7 days. These reports are similar to bacteriocin produced by *B. subtilis* LFB112 (Xie *et al.*, 2009). Unlike the previous reports, the substance showed not only proteolytic activity against casein but also significant lipolytic activity against egg yolk lecithin.

In ion exchange chromatography (Fig. 5) fraction number 7 having protein content 12 µg mL⁻¹, showed antimicrobial activity against *B. licheniformis*. Specific activity of fraction number 7 for lecithinase is 0.83 µg/min/g of lecithin and for protease it is 1.04 µg/min/g of casein. SDS-PAGE showed single unit band with molecular weight of 43 kDa. These findings are similar to serracin P having two major subunits of 23 and 43 kDa (Jabrane *et al.*, 2002).

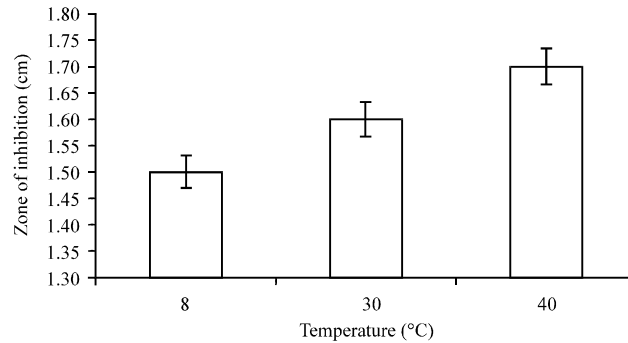


Fig. 4: Effect of temperature on the effect of bacteriocin from the organisms. It was tested against *B. licheniformis*

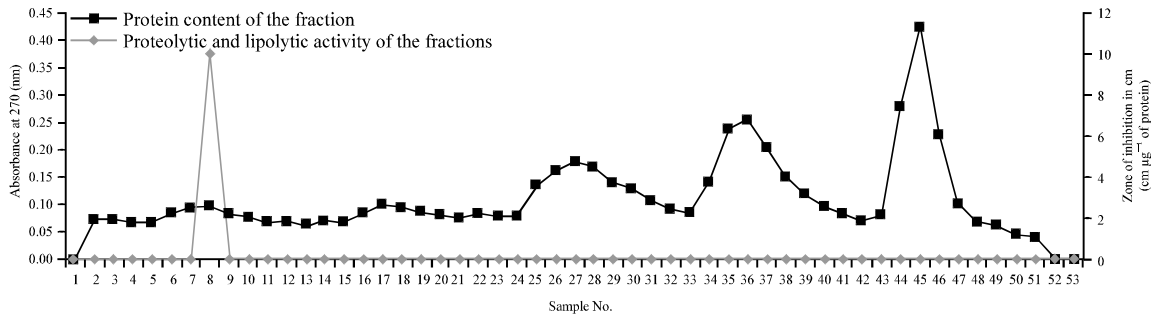


Fig. 5: Ion exchange chromatography of the dialyzed ammonium sulfate precipitate

CONCLUSION

B. atrophaeus JS-2 is not reported as producer of many antimicrobial compound. This bacteriocin produced by *B. atrophaeus* JS-2 strain presents a broad spectrum of antimicrobial activity. The reports available to date speak of the bacteriocin of *B. subtilis* as a serine protease with a high molecular weight of 43 kDa (Lee *et al.*, 2002), unlike trypsin which has a molecular weight of 23 kDa. However, in this study it has also been found to be a strong phospholipase which increases its ability to perforate the cell membranes and bring about microbicidal action. This could be an explanation as to its antifungal activity against the most common spoilage fungi- *A. niger* which causes black mold of onions, disease in peanuts and in grapes. Its inhibitory action against *B. cereus* NCIM 2156, a potent food poisoning organism and *B. licheniformis* NCIM 5339 which sometime causes neutropenic leukemia due to high lecithinase activity of its exotoxin, intestinal pathogens like *E. coli* and *S. typhimurium* suggest that the bacteriocin could be useful with respect to both animal and human health. It also inhibited opportunistic pathogens like *P. aeruginosa*. It is therefore, not only a potent antimicrobial agent capable of combating antibiotic resistance but also a biocontrol agent of bacterial and fungal plant pathogen causing mostly postharvest spoilage of fruits and vegetables. The thermal stability and pH stability of the bacteriocin will not only be an excellent alternative to the limitations of LAB bacteriocins but will be important in food preservation and food processing.

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