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Antimicrobial Activity and Characterization of Bacteriocin of *Acetobacter tropicalis* S3O1: A Novel Methionine Producing Bacteria Isolated from Sago Industrial Waste

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A B S T R A C T

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria. Four methionine producing microorganisms isolated from sago industrial waste namely *Candida tropicalis* S1O1, *Kluyveromyces marxianus* S2O1, *Acetobacter tropicalis* S3O1, and *Lactobacillus paracasei* subsp. *tolerans* S6O2 were tested for bacteriocin activity against pathogenic bacterial strains, *Bacillus cereus* (MTCC 1272), *Escherichia coli* (MTCC 2622), *Listeria monocytogenes* (MTCC 1143), *Staphylococcus aureuss* (MTCC 1144). Among the four methionine secreting microorganisms, *Acetobacter tropicalis* S3O1 exhibited good bacteriocin activity against *Bacillus cereus* (MTCC 1272) and *Staphylococcus aureus* (MTCC 1144). The sensitivity of the bacteriocin towards temperature, pH and various enzymes were also investigated.

Introduction

Increased consumption of food formulated with chemical preservatives has increased consumer concern and created a demand for more natural and minimally processed food and led to the interest towards naturally produced antimicrobial agents (Cleveland *et al.*, 2001). Bacterial strains with antimicrobial activity play an important role in the food industry, agriculture and pharmaceutical industry (Tolinacki *et al.*, 2010). Bacteriocins are ribosomally synthesized antimicrobial peptides that are active against other bacteria, either of the same species, or across genera. Some bacteriocins are small peptides consisting of only 19 to 37 amino acids, whereas others are large peptides with molecular weights of up to 90,000 (Shin *et al.*, 2008). Bacteriocin are produced by bacteria to reduce the competition against other bacteria which are present in the same ecological niche (Barefoot and Grinstead, 1993).

Bacteriocins are produced by both Gram-positive (mostly LAB) and Gram-negative bacteria (*Serratia marcesens, Pseudomonas aeruginosa, Escherichia coli*) and the bacteriocins from Gram-positive bacteria

seem to possess a broader range of susceptible organisms (Ingolf *et al.,* 2007). Apart from prokaryotes certain eukaryotic fungi such as *Tricoderma* is reported to produce bacteriocins (Goulard *et al.,* 1995). Bacteriocins are hydrophobic and heat stable possessing bacteriocidal activity that are rapidly digested by proteases in the human digestive tract and relatively hydrophobic and heat stable. Bacteriocins can be classified into 4 groups (lantibiotics, non-modified heat-stable bacteriocins, large heat-labile bacteriocins and cyclic bacteriocins) on the basis of their molecular mass, thermo stability, enzymatic, sensitivity, and presence of posttranslational modified amino acids and mode of action Klaenhammer (1993).

Bacteriocins differ greatly with respect to sensitivity towards pH and temperature. Certain bacteriocins from *Lactobacillu*s strains are considerably more tolerant of acid than alkaline pH values (Tagg *et al.,* 1976). There are also reports that the supernatant of bacteriocin producing strains are resistant to autoclaving conditions and to heat treatments. It has also been reported that some bacteriocins produced by *Lactobacillu*s strains were inactivated by heat treatments of 60º -100 ºC (Soumya *et al.,* 2012).

Sensitivity of bacteriocins towards enzymes such as catalase and chemicals such as chloroform has also been reported in various studies to ensure the stability of the bacteriocins for better utilization Soumya *et al.,* (2012).

Proteolytic enzymes present in the human digestive tract inactivate and digest the bacteriocins like any other proteins in the diet (Tolinacki *et al.,* 2010). The present study focus on the capability of bacteriocin production by methionine synthesizing microorganisms and their sensitivity towards temperature, pH and various enzymes.

Materials and Methods

Methionine producing microorganisms

Methionine producing microorganisms were previously isolated from sago industrial waste and screened for methionine production in this lab (Arunkumar *et al.,* 2014). Molecular characterization of the screened organisms revealed that the organisms were *Candida tropicalis*S1O1, *Kluyveromyces marxianus* S2O1, *Acetobacter tropicalis* S3O1, and *Lactobacillus paracasei* subsp. *tolerans* S6O1.

The Methionine producing microorganisms were grown in 100 ml Erlenmeyer flasks containing 25 ml of modified MRS broth (glucose 15.0g, agar 13.5g, peptone 10.0g, beef extract 8.0g, sodium acetate.H_2O 5.0g, K_2HPO_4 2.0g, triammonium citrate 2.0g, $MgSO_4.7H_2O$ 0.2g, $MnSO_4.4H_2O$ 0.05g, tween80 1.0 ml, pH -6.2 ± 0.2) at 28^oC on rotary shaker at 120 rpm for 24 h.

Indicator microorganisms preparation

Bacillus cereus (MTCC 1272), *Escherichia coli* (MTCC 2622), *Listeria monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 1144) were obtained from Microbial Type Culture Collection, Chandigarh.

The test bacteria were cultured on Nutrient agar at 28^oC for 24 h. The cultures were subcultured regularly (every 30 days) and stored at 4^oC. Ten millilitre of distilled water was taken into the screw cap tube and pure colony of freshly cultured pathogenic bacteria was added into the tube and mixed.

The OD (optical density) was measured with the colorimeter. And microbial population was confirmed to be 10^8 CFU ml^{-1}. This suspension is used as inoculum.

Detection of bacteriocin activity

The antibacterial spectrum of the bacteriocin from *Candida tropicalis* S1O1, *Kluyveromyces marxianus* S2O1, *Acetobacter tropicalis* S3O1, and *Lactobacillus paracasei* subsp. *tolerans* S6O2 was determined using the agar well diffusion method by Tagg and McGiven, (1971).

The sterile supernatant from a 24h culture of the methionine producing microorganisms was obtained by centrifugation at 12000 rpm for 20 min at 4^{o}C, followed by heat treatment of the supernatant at 90^{o}C for 3 min in a water bath. About 50 µl of the supernatant were poured into 5 mm diameter wells that had been cut in hardened nutrient agar plates previously seeded with indicator bacteria (10^{8} CFU ml^{-1}). After 24 h of incubation, the diameters of the zones of growth inhibition were measured.

Kinetics of bacteriocin production

The cells of the overnight culture of *Acetobacter tropicalis* S3O1 were washed two times with modified MRS broth in order to remove previously synthesized bacteriocin. The culture was incubated at 28ºC in modified MRS broth and samples were taken every 6 h to determine bacteriocin production (AU/ml) and simultaneously the growth pattern of the bacterial isolate was measured following the turbidimetric method of Brown (1980).

In order to quantify the yield of bacteriocin, cell-free supernatants were serially diluted in modified MRS broth before loading 5 µl of each dilution onto the indicator strain *Bacillus cereus* (MTCC 1272). One arbitrary unit (AU) of bacteriocin was defined as the reciprocal of the highest dilution yielding a zone of growth inhibition on the indicator lawn Mayar-harting *et al.,* (1972).

Effect of temperature, pH and enzymes on bacteriocin activity

To study the thermostability of the bacteriocin, aliquots of 50 µl of cell free supernatant of the overnight culture of *Acetobacter tropicalis* S3O1 were incubated for 15 min at temperatures ranging from 30ºC to 100ºC with 10 degrees increments. After the heat treatment, the samples were cooled to room temperature and the remaining bacteriocin activities were determined by agar well diffusion assay. The pathogenic microorganisms *Bacillus cereus* (MTCC 1272) was used as the indicator organisms in characterization studies. To determine the effect of pH on bacteriocin activity, the pH of the bacteriocin samples was adjusted stepwise from 1 to 12, in steps of one pH unit using either 1N HCl or 1N NaOH. The samples were incubated for 1 h at 37°C and bacteriocin activity was determined by the agar well diffusion assay. The effect of the enzymes on bacteriocin activity was done as described by Kojic *et al.,* 1995. The following enzymes (final concentration 1 mg/ml) and buffers were used: pronase E and proteinase K in 10 mM Tris−HCl (pH 8), trypsin and α-chymotrypsin in 50 mM Tris−HCl (pH 8); pepsin in 20mM HCl (pH 2); catalase, and lipase. The reaction mixtures were incubated at 37ºC for 1 h. The remaining bacteriocin activity was tested by the agar well diffusion assay. Enzyme-free buffers and supernatants with buffers, incubated at 37°C for 1h, were used as controls.

Results and Discussion

The results obtained from the study clearly showed among the four methionine secreting strains only *Acetobacter tropicalis* S3O1 exhibited good bacteriocin activity against *Bacillus cereus* (MTCC 1272) with the inhibition zone above 8 mm (Table 1; Fig. 1). *Staphylococcus aureus* (MTCC 1144) was

also inhibited by *Acetobacter tropicalis* S3O1 with a zone of about 8 mm. Bacterial strains belonging to the genera *Acetobacter* capable of producing bacteriocins have been reported earlier Puttalingamma, (2013).

Bacteriocin production

Bacterial kinetics of the isolate *Acetobacter tropicalis* S3O1clearly established that the bacteriocin production was dependent on the growth phase. Tolinacki *et al.,* (2010) reported that bacteriocin production was growth dependent in case of *Lactobacillus paracasei* subsp. *paracasei*BGUB9. The bacteriocin production was detected only after 6h of incubation, even though the turbidimetric results showed the multiplication of the bacterial cells in modified MRS broth from 2 h of incubation.

The bacteriocin production by *Acetobacter tropicalis* S3O1 showed a plateau after 12 h of incubation and reached a maximum after 32 h (Fig. 2). There after the bacteriocin production halted and started decreasing at the end of the stationary phase.

Table.1 Antimicrobial activity of methionine producing microorganisms against pathogenic bacterial strains

Methionine producing isolates	Strain	Source	Bacteriocin activity			
			Bacillus cereus **(MTCC 1272)**	***Escherichia coli*** **(MTCC 2622)**	***Listeria monocytogenes*** **(MTCC 1143)**	***Staphylococcus aureus*** **supsp. *aureus* (MTCC 1144)**
Candida tropicalis	S1O1	Lab collection	–	–	–	–
Kluyveromyces marxianus	S2O1	Lab collection	–	–	–	–
Acetobacter tropicalis	S3O1	Lab collection	++	–	–	+
Lactobacillus paracasei subsp. *tolerans*	S6O2	Lab collection	–	–	–	–

+ - inhibion zone below and upto 8 mm; ++ - inhibion zone above 8 mm; − - no inhibition zone

Table.2 Effect of temperature, pH and various enzymes on bacteriocin activity of *Acetobacter tropicalis* S3O1

Temperature (^{o}C)	Sensitivity of bacteriocin	pH	Sensitivity of bacteriocin	Effect of enzymes	Sensitivity of bacteriocin
30	R	3	R	Catalase	R
40	R	4	R	Lysozyme	R
50	R	5	R	Pepsin	S
60	R	6	R	Trypsin	S
70	R	7	R	Pronose E	S
80	S	8	R	Proteinase K	S
90	S	9	R	α-Chymotrypsin	S
100	S	10	S		

R – Resistant; S – Sensitive

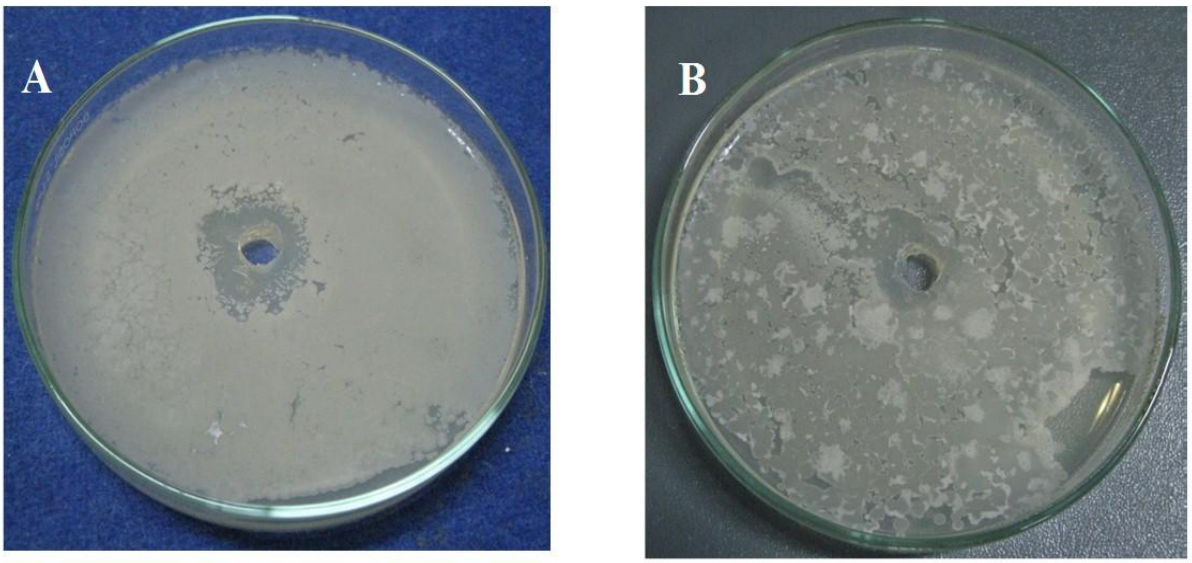

Figure 1. Antimicrobial activity of the bacteriocin from *Acetobacter tropicalis* S3O1 against *Bacillus cereus* (MTCC 1272) (A); *Staphylococcus aureus* supsp. *aureus* (MTCC 1144) (B).

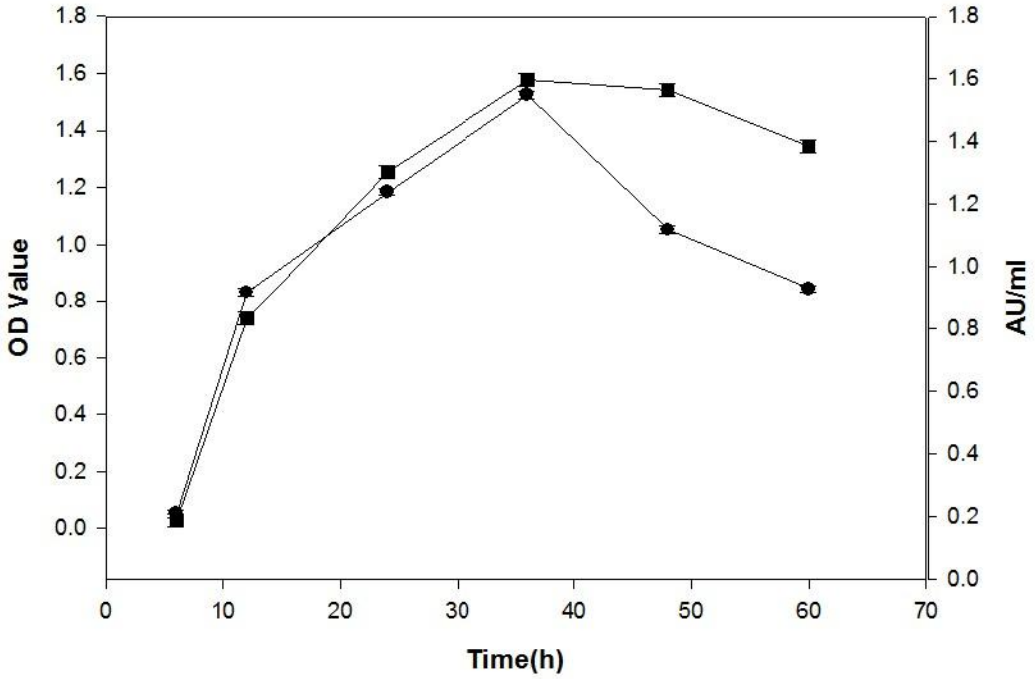

Figure 2. Kinetics of bacteriocin production by *Acetobacter tropicalis* S3O1 in modified MRS broth at 28°C. Growth of the strain *Acetobacter tropicalis* S3O1 was followed by using the turbidimetric method (■). Bacteriocin concentration is expressed as arbitrary units per milliliter (AU/ml) (●).

Biochemical characterization of bacteriocin

The antimicrobial activity of the bacteriocin from *Acetobacter tropicalis* S3O1remained unaffected upto 70^{o}C and heat treatment above completely abolished the bacteriocin activity (Table 2). Bizani and Brandelli, (2012) have reported bacteriocins of *Bacillus* sp. Strain 8A was thermostable upto 80^{o}C for 30 min.

Similarly, bacteriocins resistant to high temperature have been identified in *Enterococcus feacalis*, *E. faecium* and *Lactococcus lactis* (Elotmani *et al.*, 2002).

The cell-free supernatant from *Acetobacter tropicalis* S3O1 retained its activity within the pH range from 3 to 9 and the antimicrobial activity was lost at pH 10. Similar wide range pH activity was reported in other bacteria such as *Cornobacterium piscicola* C5526 and *Streptococcus macedonicus* ACA-DC 198 (Georgalaki *et al.*, 2002).

The bacteriocin from *Acetobacter tropicalis* S3O1 was sensitive to the activity of the proteolytic enzymes such as pronase E, proteinase K, pepsin, trypsin and α-chymotrypsin. The inhibitory action on the growth of sensitive indicator cells was not affected by treatment with enzymes lysozyme and catalase.

Treatment of bacteriocins with proteolytic enzymes will abolish the antimicrobial activity, whereas enzymes like lipase, catalase, lyzozyme, phospholipase do not affect its activity Sharma and Gautham, (2008).

Hence the biochemical characterization of the bacteriocin from *Acetobacter tropicalis* S3O1 clearly revealed the protein nature of the bacteriocin. The bacteriocin from *Acetobacter tropicalis* S3O1 exhibit strong inhibitory action towards food borne pathogens such as *Bacillus cereus* (MTCC 1272) and *Staphylococcus aureus* ssp. *aureus* (MTCC 1144) and its thermostability, pH tolerance and protein nature proves that it may be used as a natural preservative in different processed food products.

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