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Characterization of a partially purified bacteriocin of *Bacillus sp* MTCC 43 isolated from Rhizosphere of radish *(Raphanus sativus)* & its application as a potential food biopreservative

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This study presents bacteriocin, produced from *Bacillus* sp. MTCC 43 isolated from rhizosphere of root crop radish, *Raphanus sativus,* as inhibitor against serious food pathogens (*Staphylococcus aureus* and *Aeromonas hydrophila*). Bacteriocin of *Bacillus* sp. MTCC 43, partially purified by salt saturation method, exhibits very high activity (2.66 x 10^5 AU/ml). Viability of pathogens decreased drastically (up to 70%) within first 10 h of adding bacteriocin. Bacteriocin showed high thermostability (up to 100°C) for 10 min, expressed wide pH tolerance (4.0-10.0), and found sensitive to proteolytic enzyme trypsin.

Keywords: Bacteriocin, *Bacillus* sp. MTCC 43, Food pathogens, *Raphanus sativus*

Introduction

Biopreservation controls growth of pathogenic and spoilage causing organisms, thus enhancing shelf life of food products. Some bacteria produce bacteriocins (proteins or protein complexes), which have great potential as natural food biopreservatives.Bacteriocins possess bactericidal activity[1] against bacterial species, of which Gram positive bacteria of genus *Bacillus* and lactic acid bacteria (LAB) are main bacteriocin producing species[2-4]. This study partially purifies and characterizes bacteriocin, produced from *Bacillus* sp. MTCC 43 isolated from rhizosphere of radish (*Raphanus sativus)*, as a potential food biopreservative.

Experimental

Bacillus sp. MTCC 43 was isolated from rhizosphere of radish (*Raphanus sativus*) from local farm of University of Horticulture & Forestry, Nauni, Solan, India. Isolation was done by dilution series method (10^{-2}-10^{-8}) on nutrient agar plates at 37°C for 74 h[5] and identification was confirmed at Indian Institute of Microbial Technology, Chandigarh, India. Composition of nutrient agar (pH 6.8) is as follows: beef extract 3.0 g, peptone 5.0 g, NaCl 8.0 g, and agar 10 g/1000 ml. Culture (10 ml) of *Bacillus* sp. MTCC 43 (10^{-6} dilution) was added into nutrient broth (90 ml) and was incubated at 37°C for 72 h at 150 rpm in order to obtain bacterial culture of 1.0 OD.

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Ammonium Sulfate Precipitation

For partial purification, ammonium sulfate precipitation method was chosen[6]. Bacterial culture (1.0 OD) was saturated with different concentrations of ammonium sulfate (20, 40, 50, 70 and 80%) subsequently with constant stirring. At 80% saturation level, preparation was kept at room temperature (30°C) for 12 h. Centrifugation of supernatant was carried out at 20,000g at 4°C for 1 h. Pellet so obtained was dissolved in 100 ml of Tris-HCI Buffer (0.1 M, pH 7.0). Cell free extract of *Bacillus* sp. was dialyzed. After 24 h, dialyzed bacteriocin suspension was carefully removed from dialysis bags and was centrifuged at 10,000 g for 10 min at 4°C.

Calculation of activity Units (Arbitrary Units – AU ml) of Partially Purified Bacteriocin (PPB)

Serial Two-Fold Dilution Method

Activity units of PPB of *Bacillus* sp. were calculated by Serial Two-Fold Dilution Method[7]. PPB was diluted in saline water (10^{-2}, 10^{-4}……10^{-10}). Each dilution was used to estimate AU by Well Diffusion Method (WDA) and dilution corresponding to smallest detectable

Table 1. Increase in inhibition zone size of partially purified bacteriocin from Bacillus sp. MTCC 43

Pathogens	Bacteriocin		
	BCC[a] Mm	PBB[b] mm	Increase in zone size mm %
S. aureus	12	21	75
A .hydrophila	15	23	53

[a]Bacteriocin of cell culture; [b]Partially purified bacteriocin

zone was marked for further calculations. Under WDA, in nutrient agar plates, lawns of indicators were prepared by swabbing plates with cotton using indicator (1.0 OD). Wells (7 mm x 3 mm) were cut with a sharp borer in these plates and PPB (0.3 ml) was added in each well. Plates were incubated for 24 h at 37°C and results were noted in terms of zones of inhibition formed around wells.

Mode of Action of PPB

To determine mode of action, PPB of *Bacillus* sp. was mixed with its test indicator (1:1). Preparation was kept for incubation at 37°C for different time intervals (1,2….9, 10 h). Controls (indicator without bacteriocin) were run in parallel. After every time interval, preparation (0.1 ml) was mounted on nutrient agar plates by spread plate methods. After incubation of petri plates at 37°C for 24 h, results were obtained by counting number of colonies (cfu/ml) of bacteriocin treated and bacteriocin untreated cells on plates.

Characterization of PPB

Effect of Temperature on Activity of PPB

Using WDA, bacteriocin (0.5 ml) was added to nutrient broth (4.5 ml) in a test tube. Each test tube was then overlaid with paraffin oil to prevent evaporation and then treated at different temperatures (40, 50, 60°C…90 and 100°C), each for 10 min and 20 min, respectively. WDA was performed with heat-treated bacteriocin to detect inhibition zones. Using Optical Density Method (ODM), heat treated bacteriocin (40-100°C for 20 min) was mixed with its antagonistic strains, *Staphlococcus aureus* and *Aeromonas hydrophila*, separately in the ratio of 1:1 in test tubes. Their OD was measured at 540 nm after incubating samples at 37°C for 24 h.

Effect of pH on Activity of PPB

Using WDA, an aliquot (0.5 ml) of each bacteriocin was added to nutrient broth (4.5 ml) and this preparation in each test tube was adjusted at different pH (3, 4, 5…..10, 11) and incubated for 30 min at 37°C. Each pH treated bacteriocin was assayed using WDA. Using ODM, bacteriocin at different pH (3-11) was mixed with its respective indicators in the ratio of 1:1 in different test tubes and their OD was measured at 540 nm after incubation of samples at 37°C for 24 h.

Effect of proteolytic Enzyme- Trypsin on Activity of PPB

Lawns of test indicators were prepared in nutrient agar petri plates and effect of proteolytic enzyme on activity of PPB was studied[8]. For enzyme activity, enzyme control I (EC 1) (0.3 ml phosphate buffer) and enzyme control II (EC 2) (0.15 ml bacteriocin of each isolate +0.15 phosphate buffer) were prepared. Under enzyme reaction (ER), 0.5 mg of enzyme trypsin (sigma) was dissolved in1 ml of 0.1 M phosphate buffer (pH 7) and then added to bacteriocin of *Bacillus*. sp. in the ratio of 1:1. ER and ECI and ECII were assayed by WDA on indicator plates.

Application of Bacteriocin as Biopreservative in Milk

Fresh pasteurized cow's milk, (200 ml) was taken in each of two sterilized bottles. PPB of *Bacillus. sp.* (1 ml-2.66 x 10^5 AU/ml) was added in 1st bottle while 2nd bottle was kept as control. Bottles were corked with tin lid by crown corking machine in canning unit of UHF, Nauni, Solan (India) and kept for storage in refrigerator at low temp. Morphological and biochemical changes were observed in samples on day-to-day basis for one month.

Results and Discussion

PPB (2.66×10^5 AU/ml), secreted from *Bacillus* sp. MTCC 43, expressed same inhibition spectrum as that of bacteriocin originally secreted by the organism i.e. it was antagonistic to serious pathogens like *S. aureus* and *A. hydrophila*. Similar record[9] of maintaining exact antimicrobial pattern of bacteriocin against its sensitive indicators after purification steps has been noted down. Crude extracts and partially purified cultures of *Streptococcus bovis* HC5 inhibit *S. bovis* 333187 and *S. bovis* 15351[10]. An increase was observed in size of inhibition zones in PPB of *Bacillus* sp. against its corresponding sensitive test strains indicating higher potency of bacteriocin after its partial purification. Increased size of inhibition zones after partial purification was recorded (Table 1) against

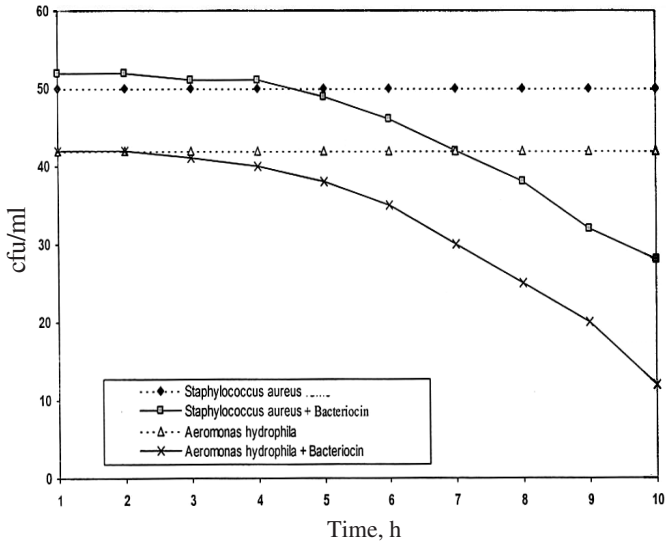

Fig. 1—Bacteriocidal effects of partially purified bacteriocin of *Bacillus* sp. against *S. aureus* and *A. hydrophila*

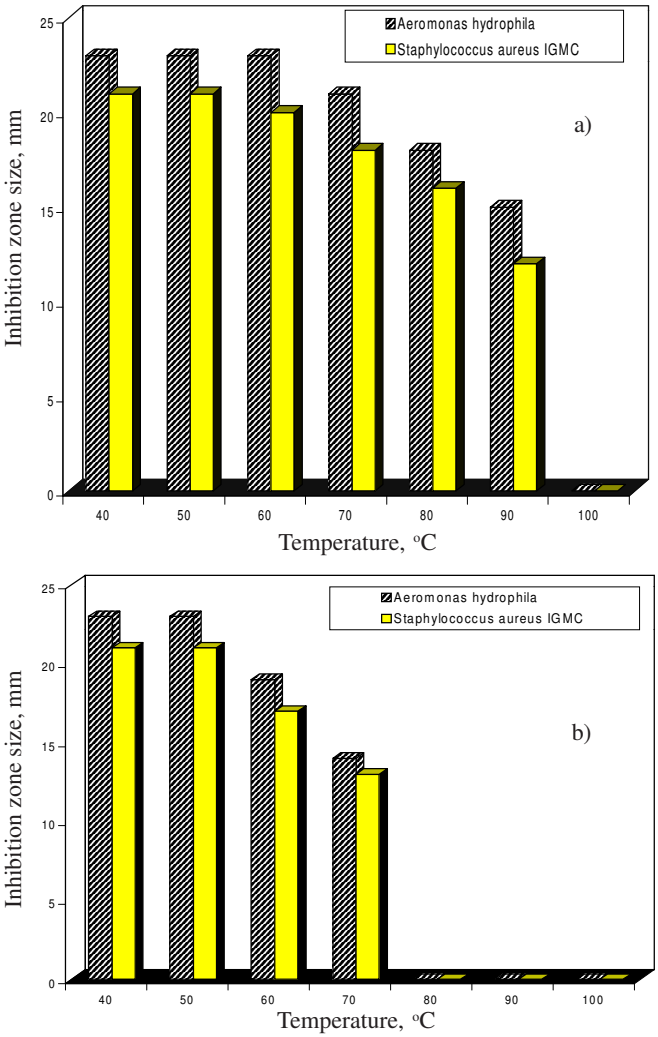

Fig. 2—Effect of temperature on activity of partially purified bacteriocin of Bacillus sp. in term of inhibition zone at: a) 10 min; b) 20 min

S. aureus (75%) and *A. hydrophila* respectively (53.3%) with zone size of 21 mm and 23 mm of PPB as compared to 12 mm and 23 mm of BCC respectively. Thus bacteriocin had retained its original antagonistic properties along with increase in potency of bacteriocin after partial purification. Bacteriocin exhibits increase in activity after every step of purification. Partially purified bacteriocin Plantaricin S and Plantaricin T of *L. plantarum* LPC 010 inhibited their sensitive strains, *Propionibacterium sp, Clostridium* sp., and *Enterococcus faecalis*, more strongly than cell culture of same organism[11]. Similarly, PPB of *B. mycoides* also showed 500% increase in zone size against *L. mesenteroides* and 233.3% against *L. monocytogenes* over cell culture[9].

Mode of Action of PPB

On treating strains of *S. aureus* and *A. hydrophila* with bacteriocin (1:1) at time interval of 1 h, 2 h,----------10 h, it was observed that with increasing time there was a decrease in the number of bacteriocin treated viable cells as compared to control (Fig. 1). At 1 h, bacteriocin treated *A. hydrophila* and *S. aureus* had 42 cfu/ml and 52 cfu/ml respectively, which decreased to 38 cfu/ml and 49 cfu/ml respectively after 5 h of treatment, followed by further reduction to 12 cfu/ml for *A. hydrophila* and 28 cfu/ml for *S. aureus* at 10 h. Cells of control showed no decrease throughout the time interval. These results strongly indicated bactericidal mode of action of bacteriocin of B*acillus* sp. Bactericidal effect of bacteriocin of *Lactobocillus helveticus* CNR2328 on its indicators has also been shown[12]. Bacteriocin treated cells of indicators were reduced from 1000000 cfu/ml at 1 h to 29999 cfu/ml at 7 h. Bactericidal mode of action of bacteriocin of *B. thuringiensis* has also been reported for indicators where viability of sensitive cells reduced from 5.5 log cfu/ml before 50 min to 25 log cfu/ml with 200AU/ml of bacteriocin[13].

Characterization of PPB

Effect of Temperature on Activity of Bacteriocin

Bacteriocin of *Bacillus* sp. was heat stable (40-90°C) for 10 min while a rapid decline in its activity was noted after same treatment for 20 min. When bacteriocin was heat treated for 10 min (Fig. 2a), zone size for *A. hydrophila* was 23 mm at 40-60°C, 20 mm at 70°C, 18 mm at 80°C and 15 mm at 90°C, while for *S. aureus*, it was 21 mm at 40° and 50°C, 20 mm at 60°C, 18 mm at 70°C, 16 mm at 80°C and 12 mm at 90°C. At 100°C, no zone of inhibition was formed and hence no

bacteriocin activity. Bacteriocin of *Bacillus* sp. was thermostable (40-70°C) when it was treated between 40-100°C for 20 min (Fig. 2b). Nil activity was observed at 80°, 90° and 100°C, as no zone was formed. Thus, smaller the size of inhibition zone, lesser the bacteriocin activity. At 10 min, bacteriocin has stood more thermostable as compared to its heating for 20 min.

Same results were shown in terms of optical density by heat treated bacteriocin of *Bacillus* sp. between 40-100°C for 20 min and then mixed in ratio of 1:1 with strains *A. hydrophila* and *S. aureus*. At 40°C and 50°C heat-treated bacteriocin, OD was 0.10 (minimum) for *A. hydrophila* and 0.21 for *S. aureus*. Increase in OD was observed after 60°C heat-treated bacteriocin (0.40 for *A. hydrophila* and 0.50 for *S. aureus*) and indicators attained maximum OD with 100°C heat-treated bacteriocin (1.0 for *A. hydrophila* and 1.2 for *S. aureus*), thereby indicating complete loss of bacteriocin activity of *Bacillus* sp. at 100°C (Fig. 3). PPB has overall shown thermostability, thus it falls under class II (heat stable) bacteriocins[14,15].

Effect of pH on Activity of Bacteriocin

Maximum activity (Fig 4) was observed at pH 7 (zone formed of 22 mm for *A. hydrophila* and 21 mm for *S. aureus*), while bacteriocin treated at pH 3 and pH 11 formed no zone of inhibition, hence nil activity; decline in bacteriocin activity was noted when pH was increased from 8.0 to 10.0 and also when it was lowered to 6.0, 5.0 and 4.0. At pH 7, OD was minimum (Fig. 5) showing highest inhibition of test indicators; as pH shifted to acidic (6.0-3.0) and alkaline (8.0-11.0), there was a constant increase in OD reaching to maximum (1.1) in both extremes for *A. hydrophila* and *S.aureus*. Lactococcin R of *Lactococcus lactis* subsp. *cremoris* is reported active in pH range (2-9) with maximum activity (1600 AU/ml) at pH 6.5-7.0[16]. Tochicin from *B. thuringiensis* subsp. *tochigiensis* was stable in pH range of 3.0-9.0, while activity of thermoleovorins S_2 and N_9 of *B. thermoleovorans* was not lost over wide pH range (3.0-10.0)[8,17]. Bacteriocin activity of *Bacillus* sp. strain 8A was exhibited in pH 5.0-8.0, with maximum activity (100% residual activity) at pH 7.0[18]. Activity of bacteriocin of *Bacillus*. sp. in broad pH range suggests its use in acidic, neutral as well as alkaline foods for biopresesvation.

Effect of Proteolytic Enzyme on Activity of Bacteriocin

Bacteriocin of *Bacillus* sp. exhibited zone of 21 mm for *S. aureus* and 23 mm for *A. hydrophila* when

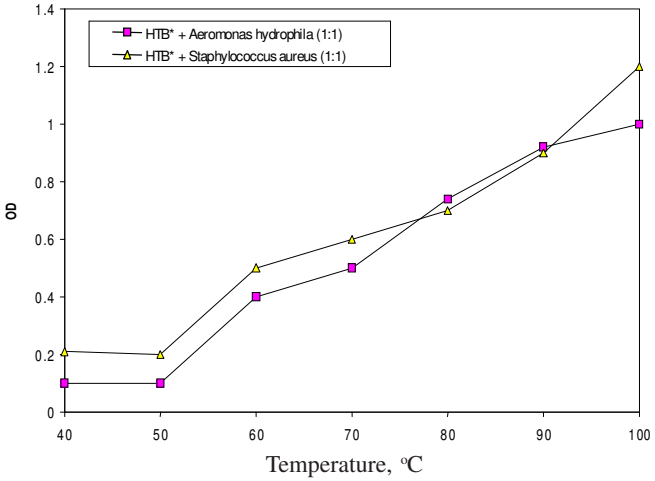

Fig. 3—Effect temperature on activity of partially purified bacteriocin of Bacillus sp. in terms of OD540; *HTB, Heat treated bacteriocin

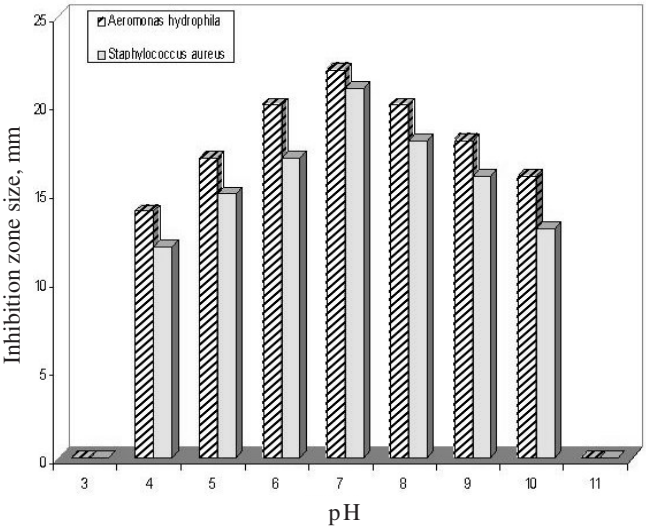

Fig. 4—Effect pH on activity of partially purified bacteriocin of *Bacillus* sp. (in terms of inhibition zone)

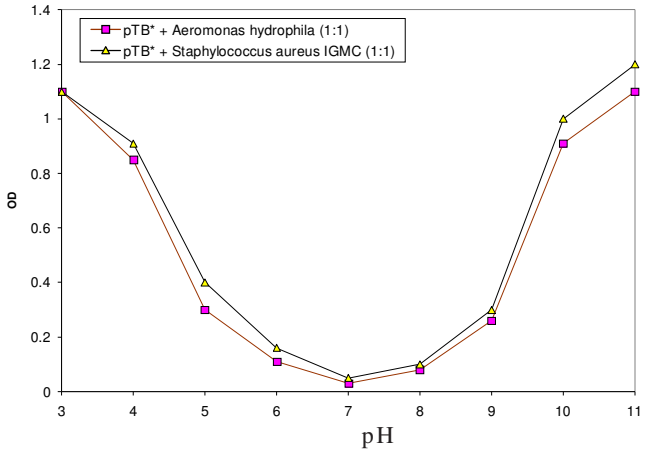

Fig. 5—Effect of temperature on activity of partially purified bacteriocin of *Bacillus* sp. (in terms of OD540) (*PTB, pH treated bacteriocin)

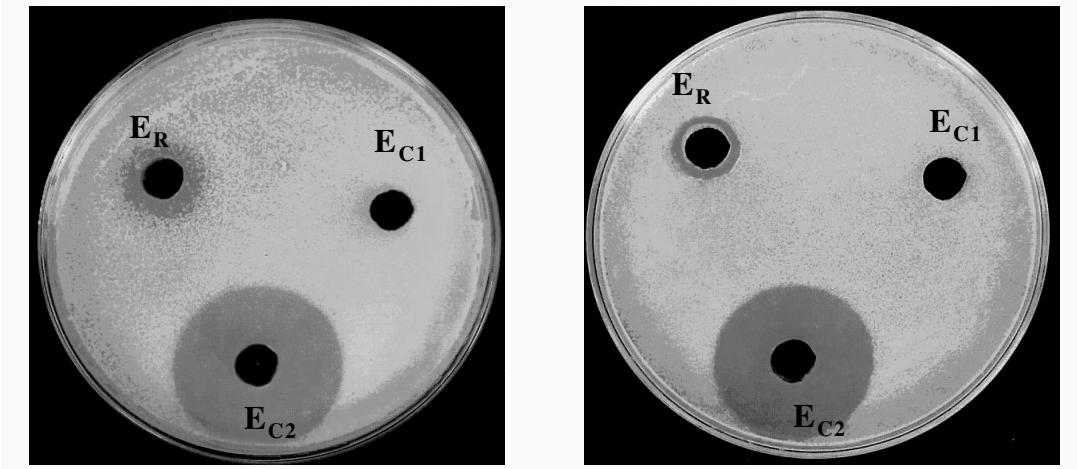

Plate 1—Effect of proteolytic enzyme—trypsin of partially purified bacteriocin of *Bacillus* sp. against: a) *A. hydrophila*; and b) *S. aureus* [EC1, Enzyme control I (phosphate buffer); EC2, Enzyme control II (phosphate buffer + bacteriocin (1:1); and ER, Enzyme reaction (Enzyme + bacteriocin (1:1)]

E_{C1}: Enzyme control I: Phosphate buffer
E_{C2}: Enzyme control II: Phosphate buffer + bacteriocin (1:1)
E_R: Enzyme reaction: Enzyme + bacteriocin (1:1)

treated with phosphate buffer (EC II), whereas buffer alone (EC I) treated with respective indicators formed no inhibition zone. However, bacteriocin of *Bacillus* sp., treated with trypsin (ER), formed zone of 2 mm against *S. aureus* and 4 mm against *A. hydrophila,* thereby indicating sensitivity of bacteriocin for 0.5 mg/ml trypsin (Plate 1). Similar results were given when bacteriocin of *B. thuringiensis* subsp. *tochigiensis* named as tochicin was found sensitive to trypsin[8]. Antibacterial activity of bacteriocin was lost after treatment with 1 mg/ml of enzyme. Cerein 7 of *B. cereus* was also reported trypsin sensitive[19]. Inhibitory activity of bacteriocin of *B. cereus* lost completely after treatment with trypsin, chymotrypsin and proteinase K[20].

Application of Bacteriocin Biopreservative in Milk

Morphological and biochemical changes were observed in milk bottles containing PPB and control. After 20th day of preservation, curdling and fermentation noticed in milk bottle with PPB as compared to control, in which same changes occurred on 7th day. No colour change was observed in both bottles. Thus, bacteriocin of *Bacillus sp*. can act as a potential biopreservative to check growth of major spoilage causing and milk borne pathogens and is useful to extend its shelf life significantly (Plate 2.). Bacteriocin of *Loctococcus lactis*, added in a model cultured milk, showed no spoilage up to 6 days against *Listeria monocytogens*[21]. Combined effect of

Plate 2—Effect of partially purified bacteriocin of *Bacillus* sp. in milk and compared to control after 18 days

bacteriocin with heat also extended storage life of pasteurized milk by suppressing growth of different micro-organisms[22].

Conclusions

Unique combination of various properties of bacteriocin [high thermo-stability, wider pH tolerance, proteinaceous nature, bactericidal mode of action and its strong antimicrobial activity against serious food pathogens (*S. aureus* and *A. hydrophila)*] places it as an attractive biopreservative having potential for safe preservation of food items in food processing industry.

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