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IDENTIFICATION, PRODUCTION AND CHARACTERIZATION OF NATTOKINASE, BACTERIOCIN FROM BACTERIAL SPECIES

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ABSTRACT

The study was aimed at identification, production and characterization of nattokinase, bacteriocin from bacterial species. Nattokinase and bacteriocins finds a wide range of applications in Pharmaceutical industry, health care and medicine. Nattokinase is a highly active fibrinolytic enzyme secreted by *Bacillus subtilis* and bacteriocins are proteinaceous toxins produced by *Lactobacillus* to inhibit the growth of closely related bacterial strains. *Bacillus subtilis* and *Lactobacillus* isolates shown positive results to microscopic, biochemical analysis. The nattokinase and bacteriocins were produced by optimizing the media. The enzymes were purified by ammonium sulfate precipitation and HPLC. The enzyme activity for nattokinase was found at 7 mg/ml, pH 8.0 and temperature 48 ºC and the enzyme activity for bacteriocin was found at 3.9 mg/ml, pH 6.5 and temperature 30 °C. Bacteriocins from *Lactobacillus* showed good antagonistic activity against pathogenic bacteria. Nattokinase from *Bacillus subtilis* played a significant role in thrombolytic and anti-coagulation at *in vitro*. The results indicated that the pure enzyme has a potential in dissolving blood clot.

Keywords: Fibrinolytic, Thrombolytic, Anti-coagulation, Bacteriocins, Nattokinase, Ammonium sulfate precipitation

INTRODUCTION

Nattokinase is a fibrinolytic enzyme that belongs to the second wide family of serine protease. It hydrolyses fibrin and is the major protein constituent of blood clots, an insoluble white protein formed by the conversion of fibrinogen through thrombin. Nattokinases was initially discovered and extracted from Japanese conventional fermented soybean foods that were of certain importance due to their efficient biological thrombolysis of fibrin and clots of blood in blood vessels **(Sumaya Ali Hmood *et al.*, 2016)**. Previous studies observed that these enzymes could also be purified from brewing rice wine and Indonesian fermented soybean **(Liu *et al.*, 2005)**. Most of the nattokinases are secreted by different *Bacillus* spp. including *B. subtilis, B. amyloliquefaciens, B. amylosacchariticus* and *B. licheniformis* **(Wang *et al.*, 2006)**. A mild and frequent enhancement of the fibrinolytic activity in the plasma is seen by oral administration and stability in the gastrointestinal tract of Nattokinase **(Tai *et al.*, 2006).** The fibrinolytic activity of Nattokinase can be retained in the blood for more than 3 hours, which makes it probable for clinical use and now it is widely considered as a promising oral medicine for thrombolytic therapy **(Vignesh H *et al.*, 2014).** It has been reported that rich foods is very much useful for prevention of disease as well as aging process. Enzymes are in use as digestion aids but mostly ignored as drug **(Patil R *et al.*, 2018).** Bacteriocins from lactic acid bacteria (LAB) are natural antimicrobial peptides or proteins with interesting potential applications in food preservation and health care **(Savita Jandaik *et al.*, 2013).** Bacteriocins are small proteins with bactericidal or bacteriostatic activity. This organism prevents the growth of pathogenic bacteria in different ecosystems by production of antimicrobial substance such as organic acids and hydrogen peroxide **(Arokiyamary *et al.*, 2011).** The health benefits offered by LAB can be therapeutic including production of vitamins, immunomodulation, reduction in the risk of diarrhoea and mutagenic activity and a decrease in serum cholesterol (**Anwar A. Abdulla *et al.*, 2014**). Several bacteriocins from *Lactobacillus spp*. has been characterized with respect to their protein sequence, molecular mass, biochemical properties and antimicrobial activity spectrum. Bacteriocins that have all D-amino acids have antibacterial activity but exhibit more resistance to proteolytic enzymes and are less cytotoxic compared with bacteriocins that have all L-amino acids **(Pangsomboon *et al.*, 2006).** Genes that encode bacteriocins are found exclusively on the chromosome, and it is bacterial ribosomal synthesized peptides or proteins with antimicrobial activity **(Sano *et al.*, 2002).** Depending on the producer organism bacteriocins can be classified into several groups as Class I, II and III **(Ashok kumar *et al.*, 2011).** LAB bacteriocins are divided into three main groups, based on their amino acid sequence, mode of action, heat tolerance, biological activity, presence of modified amino acids, and secretion mechanism **(Hoda Mahrous *et al.*, 2013).**

MATERIALS AND METHODS

Collection of samples

The milk and fermented curd were collected from local dairy, Hosur, Tamil Nadu, India. The *Bacillus subtilis* MTCC 5981 was obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India.

Isolation of bacteria

Lactobacillus was isolated by streak plate method from the collected milk and curd samples were streaked on MRS (De Man Rogosa & Sharpe) agar plate and incubated at 37 °C anaerobically for 48 hrs. At the end of 48 hrs, when the colonies became predominant, morphologically distinct and well developed colonies were picked and transferred to MRS broth. *Bacillus subtilis* was isolated by streak plate method from the obtained sample into nutrient agar plates and the plates were incubated at 37 °C for 24 hrs. The isolated colonies were picked and transferred to nutrient broth.

Identification of bacteria

The isolates were identified on the basis of Morphological and Biochemical Characteristics as given in the Bergey's Manual of Systematic Bacteriology **(Holt *et al.*, 1994)**. The tests were performed for identification of bacteria by Grams staining, Motility, Catalase, Methyl red (MR), Voges proskaur (VP), Urease, Citrate utilization, Fermentation test- lactose, glucose, sucrose.

NaCl tolerance test

The media was adjusted with different concentration (5, 7 & 9%) of NaCl with composition of nutrient agar, phenol red and manitol (1%). After sterilization, each plate was inoculated with 1% fresh overnight culture of *Lactobacillus and Bacillus subtilis* and incubated at 37 °C for 24 – 48 hr. The culture plates were observed for the presence or absence of growth.

pH tolerance test

The media of MRS agar and nutrient agar was adjusted with different pH (3, 4, 5 for *Lactobacillus* & 5.5, 6.5, 7.5, 8.5 for *Bacillus subtilis*). pH was determined by a digital electrode pH meter. After sterilization, each plate was inoculated with 1% fresh overnight culture of *Lactobacillus and Bacillus subtilis* and incubated at 37 ℃ for 24 – 48 hr. The culture plates were observed for the presence or absence of growth.

Enzyme production

The media used for the optimal production of nattokinase was composed of Soy peptone- 0.5%, NaCl- 0.25%, Na_2HPO_4- 0.1%, $MgSO_4$- 0.02, $MnCl_2$- 0.05%, Glucose- 1% and Casein- 1%. The pure culture was inoculated into a media and was incubated at 37 ºC in an incubator for 72 hrs and inoculum used for the production of the nattokinase enzyme. The media was prepared on MRS broth for the optimal production of bacteriocins. The pure culture was inoculated into a media and was incubated at 37 ºC in an incubator for 72 hrs and inoculum used for the production of the Bacteriocin.

Partial purification

Ammonium sulfate precipitation

Solid ammonium sulfate was added to the culture filtrate at 70% saturation for NK and Bacteriocins and stirred for 24 hrs at 4 ºC **(Deepika *et al.*, 2017).**The precipitate obtained was separated by centrifugation at 10000 rpm for 20 mins at 4 ºC. The resultant supernatant was solubilized in 1N NaOH.

Enzyme assay

The protein is estimated by Lowry's method for both enzymes. 0.5 ml of sample was mixed with 1.125 ml of Lowry's reagent. The tubes were incubated at room temperature for 10mins. 0.5 ml of Folin's - Phenol reagent was added and incubated at dark in room temperature for 30 mins and the OD was observed at 560 nm.

Blood clot analysis

Human and chicken blood drawn for analyzing nattokinase and were transferred to different pre-weighed sterile micro-centrifuge tubes and incubated at 37 ºC for 45 mins **(Deepika *et al.*, 2017).** After clot formation, serum was completely removed and each tube with clot was again weighed to determine the clot weight.

Clot weight = [Weight of clot containing tube] - [Weight of tube alone]

Each microfuge tube, containing clot and 100 µl of crude enzyme and ammonium sulfate precipitated enzyme was added to the clots. All the tubes were incubated at 37 ºC for 90 mins and observed for clot lysis. After incubation, the obtained fluid was removed and the tubes were again weighed to observe the difference in weight after clot disruption. The obtained difference in weight taken before and after clot lysis was expressed as percentage of clot lysis.

Antagonistic activity

The antagonistic activity of bacteriocins for *Lactobacillus* spp. isolates was measured by the agar well diffusion assay and tested against *Klebsiella pneumoniae, Salmonella typhi, Escherichia coli, Verticillum lecanii.* It was measured by the diameter of zone of inhibition.

HPLC analysis

The crude sample of nattokinase and bacteriocins was analyzed by high pressure liquid chromatography (HPLC) using C 18 column containing 5 mm sized particles (250 mm and 4.5 mm ID Lichrosper m 100) and a 20 µl loop injector. Mobile phase was used as a mixture of acetonitirile (A) and water acidified to pH 3 with o- phosphoric acid and methanol (B) in the ratio of 1.1 with flow rate of 1.2 ml/min. Detection was carried out by UV detector at 248 nm.

RESULTS AND DISCUSSION

Isolation of Bacteria

MRS Agar was reported to be the best suitable selective media for the isolation of *Lactobacillus spp.* by (**Gad G.F.M *et al.*, 2014)**. Selective media is used to cultivate specific type of microorganisms. Thus, the ability of bacterial species to be cultured on specific media is regarded as an important characteristic in identification of the microorganisms. For *Bacillus subtilis* bacterial colonies were produced as the result of pour plate method which was done on nutrient agar plates which were incubated at 37 ˚C overnight.

Morphological Characteristics

Lactobacillus was identified on the basis of characteristic morphology, gram positive, rod shaped bacteria. However, another report showed that *Lactobacilli* were physiologically diverse group of rod-shaped, gram-positive **(Hoque *et al.*, 1994)**. *Bacillus subtilis* identified on the basis of morphology and showed gram positive, rod shaped bacteria. Previously, *Bacillus subtilis* were identified and confirmed by gram positive **(Debajitborah *et al.*, 2012)**.

Biochemical Characteristics

Catalase Test

Enzymes that decompose hydrogen peroxide into water and oxygen. Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. If this is allowed to accumulate in the bacterial cells it becomes lethal to the bacteria. *Lactobacillus* showed negative and *Bacillus subtilis* showed positive results.

Methyl Red Test

This test is the ability of the organism to produce acid end product from glucose fermentation, this is a qualitative test for acid production. Both *Lactobacillus* and *Bacillus subtilis* showed negative results as absence of red colour was observed.

Voges proskaur (VP) Test

This test is used to determine the ability of the organisms to produce neutral end red colour product from glucose fermentation. Both *Lactobacillus* and *Bacillus subtilis* showed negative results.

Citrate utilization Test

This test based on the ability of an organism to use citrate as its only sole source of carbon and ammonia as its only source of nitrogen. *Lactobacillus* showed negative and *Bacillus subtilis* showed positive results thus growth on the plate and colour changes from green to blue.

Fermentation Test

Glucose

This test was done to help differentiate species of family *Enterobacteriaceae* and for the bacteria's ability to ferment glucose, produce gas/an acid end product. *Lactobacillus* and *Bacillus subtilis* showed positive result by appearance of yellow colour and indicating the glucose fermentation.

Sucrose

This test was done to help differentiate species of family *Enterobacteriaceae* and for the bacteria's ability to ferment glucose, produce gas/an acid end product. *Lactobacillus* and *Bacillus subtilis* showed positive result by appearance of yellow colour and indicating the sucrose fermentation.

Lactose

This test for the bacteria ability to ferment lactose. *Lactobacillus* and *Bacillus subtilis* showed positive result by appearance of yellow colour and indicating the lactose fermentation.

Urease Test

This test is performed to determine the bacteria's ability to hydrolyze urea to make ammonia using the enzyme urease. Formation of pink colour showed the presence of urease in *Bacillus subtilis* and absence in *Lactobacillus.*

Motility Test

This test used to help differentiate the species of bacteria that are motile from non-motile. Both *Lactobacillus* and *Bacillus subtilis* showed non-motile, there will only be growth along the stab line.

NaCl tolerance Test

The isolated *Lactobacillus* and *Bacillus subtilis* were able to tolerate 1-10% NaCl. The growth of *Lactobacillus* was observed at a 7 % NaCl while *Bacillus subtilis* showed a growth at a 9 % NaCl. Thus *Lactobacillus* and *Bacillus subtilis* are salt tolerant bacteria. The other physiological parameter for growth of a cell is the requirement of sodium chloride as the physiological saline prevents the cell from osmotic shock **(Kavitha *et al.*, 2016).**

Table 1 Biochemical test results

Tests	*Bacillus subtilis*	*Lactobacillus*
Catalase	+	-
Urease	+	-
MR	-	-
VP	-	-
Citrate	+	-
Lactose	+	+
Sucrose	+	+
Glucose	+	+

Where**,** + positive, - negative

pH tolerance Test

The isolated *Lactobacillus* and *Bacillus subtilis* were able to tolerate pH. There was a growth in the *Lactobacillus* at pH 4 while *Bacillus subtilis* showed growth at pH 7.5**. Marwa A *et al.*, 2015** reported to the effect of pH values on the activity of the bacteriocins extracted from L. acidophilus, the activity of bacteriocins were very stable over a wide range of pH (2, 4, 6 and 8).

Enzyme Production

The composition of medium influencing the production of bacteriocin by *Lactobacillus* isolates. It showed that maximum activity was noted at pH 6.5, temperature 30 °C. Bacteriocin production was frequently regulated by pH and growth temperature, as has been shown in several studies involving the pediocin **(Ibrahim Khalil *et al.*, 2017).** Thus variation in the concentration of constituents of cultivation media might have an influence on the amount of bacteriocin produced by microorganisms. Nattokinase media optimization revealed that addition of soy peptone enhanced the production of enzyme. And maximum enzyme activity was observed at pH 8.0 and temperature 48 ºC in the optimized media. **Peng *et al.*, 2003** showed that the fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* had an optimal activity at 48 °C and pH 9.0.Nattokinase yield varied in different optimized fermentation media. However, comparison of these fibrinolytic activities is difficult due to different activity assay methods and the absence of specific activity.

Ammonium sulfate precipitation

The crude extract produced was initially subjected to precipitation with ammonium sulfate. In the present study, 70% ammonium sulfate saturation ratio was selected to determine the best ratio for nattokinase and bacteriocins precipitation. Solid ammonium sulfate was selected to precipitate the nattokinase and bacteriocin due to its high solubility, availability, low cost and proteins stabilization that occurs **(Deepika *et al.*, 2017).**

Estimation of protein

The nattokinase and bacteriocins showed the presence of protein content. Previously, **Debajitborah *et al.*, 2012** found that amount of protein in nattokinase was to be 5.4 mg/ml and **Ibrahim Khalil *et al.*, 2017** showed the protein concentration in bacteriocins was found to be 1.32 mg/ml. Proteins are compared in the collected sample extracts to know the amount of proteins present using BSA (Bovine Serum albumin) as standard. The amount of protein present in the nattokinase and bacteriocins was found to be 7 and 3.9 mg/ml.

Table 2 Protein Estimation

Samples	Absorbance at 560 nm	Amount of protein (mg/ml)
Nattokinase (BS-1)	1.907	7
Bacteriocins (LB-C)	1.201	3.9

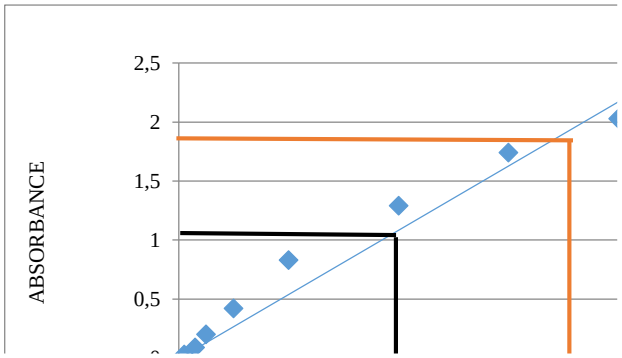

Figure 1 Graph shows protein content with absorbance at 560 nm

Antagonistic activity

The bacteriocin produced by Lactic Acid Bacteria was checked for their Antagonistic activity against *Klebsiella pneumoniae, Salmonella typhi, E. coli, Verticillum lecanii*. From this it was found that bacteriocins from probiotic seem to be most active against bacterial species. The supernatant of *L. acidophilus* strains showed variable degree of antagonistic activity with respect to inhibition zones. It was found that isolates from curd was most inhibitory against the tested bacterial pathogens. *Lactobacillus fermentum* showed inhibitory activity against *Escherichia coli, Salmonella typhi, Bacillus cereus, Staphylococcus aureus, Pseudomonas mirabilis, Klebsiella pneumonia* and *Pseudomonas aeuroginosa*. **(Udhayashree *et al.*, 2012)**. **Chumchalova *et al.*, 2004** observed the antagonistic activity of four strains of *Lactobacillus plantarum* against *Salmonella typhi, E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella paratyphi, Bacillus cerus, Bacillus subtilis, and Bacillus megaterium.* The probiotic bacteria may also have competed for nutrients, simultaneously produced hydrogen peroxide and bacteriocins that acted as antibiotic agents **(Ibrahim Khalil *et al.*, 2017).** Thus bacteriocin showed the antimicrobial activities against the pathogens and the results showed in table 3.

Table 3 Antagonistic activity results

Organisms	25 (µl)	50 (µl)	75 (µl)	100 (µl)
Klebsiella pneumonia	6 mm	8 mm	7 mm	10 mm
Salmonella typhi	7 mm	6 mm	11 mm	9 mm
Escherichia coli	0 mm	0 mm	8 mm	10 mm
Verticillum leccani	6 mm	5 mm	6 mm	8 mm

Blood clot analysis

The blood clot lysis was also assayed in the microfuge tubes. Human and chick blood clot was incubated with the enzyme, and clot degradation was analyzed. Previously, **Sumaya Ali Hmood, *et al.*, 2016** concluded that, the nattokinase purified from *Bacillus spp.* B24 using wheat bran as substrate displays excellent fibrinolytic activities *in vitro*. **Subathra *et al.*, 2015** showed 94% clot lysis was visually observed after 10 minutes in the tube that received 70% precipitate of mutant UV60 strain. The clot was weighed to determine the clot weight and results showed in table 4.

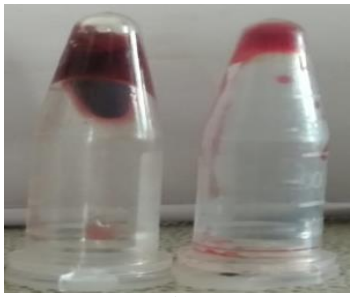

Figure 2 Before treatment

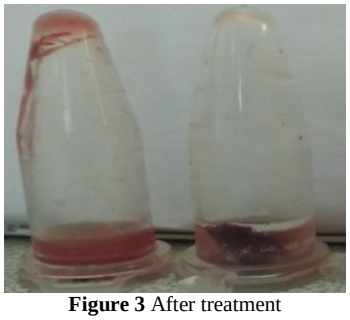

Figure 3 After treatment

Table 4 Blood clot analysis results

Samples	Weight of the tube alone	Before Treatment	After Treatment	% of clot lysis
Human blood	1.025	1.288	1.118	26.3 %
Chicken blood	1.026	1.428	1.332	30.6%

HPLC analysis

The high performance liquid chromatography was performed for nattokinase and bacteriocins. The sample was made to run for 10 min and the volume of sample taken for analysis is 20 µl and the analysis was made at 260 nm. Methanol was used as the solvent system for the study. After the analysis, peak was obtained as shown in table 5.

Table 5 HPLC Results

Samples	RT (min)	Area (m)	Height (mAU)	Width (min)
Nattokinase	10.847	1054.504	0.097	0.505
Bacteriocin	7.455	5386.817	0.518	0.535

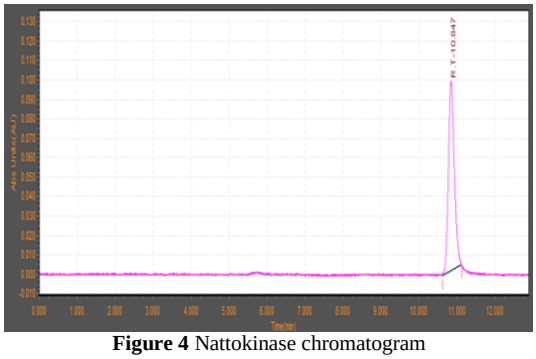

Figure 4 Nattokinase chromatogram

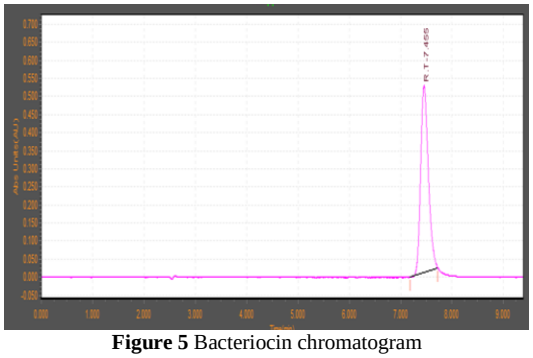

Figure 5 Bacteriocin chromatogram

CONCLUSION

The isolates *Bacillus subtilis and Lactobacillus* showed positive results to microscopic, biochemical analysis. For Nattokinase, enzyme activity was found at pH 8.0 and temperature 48 ºC, for bacteriocin, enzyme activity was found at pH 6.5, temperature 30 °C. Bacteriocins from *Lactobacillus* showed good activity against pathogenic bacteria. This makes a good antagonistic agent. Nattokinase from *Bacillus subtilis* played a significant role in thrombolytic and anti-coagulation in vitro. The results indicated that the pure enzyme has a potential in dissolving blood clot.

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