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 find a wide range of applications in the Pharmaceutical industry, health care and medicine. Nattokinase is a highly active fibrinolytic enzyme secreted by Bacillus subtilis and bacteriocins are proteaceous toxins produced by Lactococcus 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 °C 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. amyloquefaciens, 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 (Arokiamary et al., 2011). The health benefits offered by LAB can be therapeutic including production of vitamins, immunomodulation, reduction in the risk of diarrhea and mutagenicity 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 mannitol (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 presence or absence of growth.
**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

<table>
<thead>
<tr>
<th>Tests</th>
<th>Bacillus subtilis</th>
<th>Lactobacillus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MR</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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 °C in the optimized media. Peng et al., 2003 showed that the fibrinolytic enzyme produced by *Bacillus amyoliquefaciens* 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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Absorbance at 560 nm</th>
<th>Amount (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nattokinase (BS-1)</td>
<td>1.907</td>
<td>7</td>
</tr>
<tr>
<td>Bacteriocins (LB-C)</td>
<td>1.201</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Antagonistic activity

The bacteriocin produced by Lactic Acid Bacteria was checked for their Antagonistic activity against *Klebsiella pneumoniae, Salmonella typhi, E. coli, Verticillium 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 aeruginosa*. (Udhaayashree 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 cereus, 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

<table>
<thead>
<tr>
<th>Organisms</th>
<th>25 (µl)</th>
<th>50 (µl)</th>
<th>75 (µl)</th>
<th>100 (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>6 mm</td>
<td>8 mm</td>
<td>7 mm</td>
<td>10 mm</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>7 mm</td>
<td>6 mm</td>
<td>11 mm</td>
<td>9 mm</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>0 mm</td>
<td>0 mm</td>
<td>8 mm</td>
<td>10 mm</td>
</tr>
<tr>
<td><em>Verticillium lecanii</em></td>
<td>6 mm</td>
<td>5 mm</td>
<td>6 mm</td>
<td>8 mm</td>
</tr>
</tbody>
</table>
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.

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>
<thead>
<tr>
<th>Samples</th>
<th>RT (min)</th>
<th>Area (m)</th>
<th>Height (mAU)</th>
<th>Width (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nattokinase</td>
<td>10.847</td>
<td>1054.504</td>
<td>0.097</td>
<td>0.505</td>
</tr>
<tr>
<td>Bacteriocin</td>
<td>7.455</td>
<td>5386.817</td>
<td>0.518</td>
<td>0.535</td>
</tr>
</tbody>
</table>

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 °C, 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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REFERENCES


