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Nattokinase For Biofilm Disruption

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ABSTRACT

Bacterial biofilm is a new field in which researchers are very attracted as well as interested because of their ability to evade human host innate immune response and these biofilms also possess strong resistance to antimicrobial agents. The enzyme Nattokinase is called as proteolytic enzyme because of its capability to cause breakdown of proteins to small peptides and even to amino acid residues. Nattokinase is a Serine protease i.e., it has serine residue at its catalytic site. Nattokinase possess strong fibrinolytic activity. It has been found that this enzyme is a potential antithrombotic agent and can prevent and treat cardiovascular diseases to a significant extent. It is the most active ingredient of a Japanese food known as 'Natto'. The current study is aimed to isolate the potential Nattokinase producer and perform various tests in order to find whether Nattokinase is a potential enzyme that can be used for developing novel agents for biofilm disruption. The current research was aimed to extract Nattokinase enzyme and test the extracted Nattokinase for protease and amylase activity in order to ensure for its biofilm disruption capability.

Keywords: Biofilm, Bacillus Subtilis Natto, Extracellular Polymeric Substances (EPS), Nattokinase, Staphylococcus Aureus

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INTRODUCTION

Bacterial biofilm

In nature, microorganisms survive usually by adhering to and growing upon animate and inanimate surfaces. These surfaces can be found anywhere, taking into consideration, surfaces found in/on soil aquatic systems, living tissues, etc. Bacterial cells present two types of cell growth i.e. freely flowing bacterial cells in suspension and immobile aggregate of cell. The cluster of immobile bacterial cell is known as Biofilm. Biofilm is colony of cells in which cells adhere to one another on any type of surface encapsulated within a matrix made primarily of Extracellular Polymeric Substances (EPS) originated by bacterial cells itself. Based on the nature of habitat where biofilm has developed, various other organic and inorganic matters such as clay particles, minerals particles of corrosion, slit particles or components of blood may also be present in biofilm matrix. Bacterial cells forming biofilm differ from freely floating cells with respect to transcription of genes. In a host, biofilm defend the bacterial cells from antimicrobial agents as well as from the immune cells of the host [1].

Composition of biofilm: Bacterial biofilm is an aggregate of bacterial cells wherein cells produce Extracellular Polymeric Substances (EPS) like various proteins, polysaccharide, enzymes, DNA, etc. Beside these components, water is the chief component of the biofilm upon which flow of nutrients rely, inside biofilm matrix. The structure of biofilm is made up of two parts i.e. channel for the transportation of nutrients through water and zone of closely and tightly packed cells that contain no illustrious pores in it. EPS alone contributes 50% -90% of entire organic carbon of bacterial biofilm and hence, regarded as the major component of the biofilm matrix. EPS differ in physiochemical properties depending on the bacterial species and also on the environment in which it has been formed.

In case of gram-negative bacteria, polysaccharides of biofilm matrix are mostly neutral or polyanionic. This property is significant as it enables the bonding of divalent cations like magnesium and calcium, which exhibit cross linking with polymer fiber and thus impart impressive binding force within a flourished biofilm. For gram-positive bacteria such as Staphylococcus aureus, the make-up of biofilm matrix is slightly different and may be principally polycationic. EPS may be possibly hydrophobic, yet majority of EPS have amphiphilic nature i.e. both hydrophilic and hydrophobic.

Primary conformation of EPS is determined by composition and structure of polysaccharides of the biofilm matrix. Metal ions, divalent cations, proteins and other macromolecules may also form association with EPS. Since EPS is hydrated, it prevents dehydration in some bacterial biofilm occurring naturally associating with these agents directly, thus contribute to antimicrobial resistance properties of bacterial biofilm [2], [3].

Biofilm of a gram-positive bacteria- staphylococcus aureus

Staphylococcus aureus is a gram-positive bacterium that can be easily found anywhere. These bacteria are most commonly found either in uppermost region of respiratory tract or on the skin of the infected person. It has been found that there is a co-relation between implant associated or hospital acquired infection and carriage by the nasal cavity. Invasion of nasal cavity by Staphylococcus aureus is the primary step for dissemination to other regions of the body. Once, after the breach of epithelial barrier of the body, it reaches the circulatory system, enhanced production of virulence factors occurs and bacterial cells follow planktonic growth mode. After this step, host innate immune response either removes the bacterial cells or the bacterial cells adhere to proteins in extracellular matrix and leads to formation of biofilm [2].

Staphylococcus aureus form a biofilm of multiple layers fixed in glycocalyx with expression of miscellaneous proteins throughout. In Staphylococcus aureus, there is a locus of accessory gene regulator (agr) and a regulatory gene which functions to produce and retaliate to the signaling molecule for quorum-sensing called as Auto Inducing Peptide (AIP). AIP is a peptide made up of eight amino acid residues of which the last five residues form a 'Thiolactone' which remain restrained [3].

Signaling molecule is synthesized and secreted to the outside of cell during growth. As soon as the level of AIP touches the threshold concentration, AIP binds to histidine kinase receptors present bacterial cell surface. This leads to initiation of downstream signaling cascade that direct expression of genes responsible for toxin, proteases, haemolysin and several other virulence factors.

Extracellular Proteases of Staphylococcus aureus: All the proteases possessed by Staphylococcus aureus are orderly arranged in 4 different operons responsible for the synthesis of a total of 9 proteases. Out of 9, seven are serine proteases (SplA – F and SspA), which are organized into two separate operons, 1 is a metalloprotease (Aur) and remaining 2 are cysteine proteases (Staphopain ScpA and SspB). Out of the 7 serine proteases, 6 proteases encode by 'SplA-F' are secreted as active enzymes i.e. they do not need any proteolytic activation. The remaining one serine protease i.e. SspA, both cysteine proteases i.e. 'ScpA' and 'SspB' and metalloprotease i.e. 'Aur' are produced as pro-enzymes (zymogens) and thus require proteolytic activation [4].

Enzymes 'Aur' and 'ScpA' are converted to active enzymes outside the cell by auto-proteolytic cleavage and a subsequent proteolytic cascade leads to activation of 'SspA' and 'SspB'. This cascade commences with activation of 'SspA' via metalloprotease 'Aur' which subsequently activates 'SspB'.

All the proteases have a broad spectrum of activity i.e. they can deteriorate both 'self' and 'non self' proteins. These enzymes help in the survival under protein rich background by assisting in nutrient attainment and cleavage or degradation of peptides possessing antimicrobial properties [5]. The proteases present in Staphylococcus aureus are:

Cysteine proteases (Staphopains): Cysteine proteases are proteins or enzymes which are identified by the presence of two nucleophilic cysteine residues that are bonded through a thiol linkage. The 2 cysteine proteases secreted by S. aureus are ScpA and SspB, which are also termed as Staphopain A and Staphopain B respectively. The enzymes possess alike 3-dimensional crystal structure and belongs to 'Papain' family of proteases.

Serine proteases: These are the enzymes possessing serine as the nucleophilic amino acid residue at their active site. There are 7 serine proteases secreted by S. aureus i.e. 'SspA' and 'SplA-F'. SspA plays a key role in activation of SspB (a cysteine protease) and termed as V8 protease (a glutamyl endopeptidase). The other six 'Spl' proteases (SplA-F) are organized into polycistronic operon.

Metalloprotease (Aur): There is only a single metalloprotease released by S. aureus which is required for the activation of Serine proteases i.e. 'SspA'. This enzyme needs both Calcium (Ca^{2+}) and Zinc (Zn^{2+}) ions for its activity. 'Aur' also helps in cleavage or degradation of antimicrobial peptides.

Nattokinase

'Nattokinase' is named so due to its first producer bacterial strain i.e. Bacillus subtilis natto. This enzyme is naturally occurring and most commonly derived from a traditional food in Japan called 'Natto'. 'Natto' in manufactured by fermentation of soybeans viathe bacteria Bacillus subtilis natto. The temperature employed for fermentation is around 40°C i.e. 104°F for time approximately 15-18 hours. The end of fermentation process is marked by the appearance of viscous, sticky and string like material known as 'Poly-

Gamma Glutamic Acid'(ɣ-PGA) along with a characteristic odor [6].

Nattokinase belongs to the 'Subtilisin' family with nomenclature number 'EC 3.4.21.62'. Nattokinase in made up of 275 amino acids and there is no disulfide linkage in the protein chain. Asp-32, His-64 and Ser-22 are the three conserved residues present at catalytic center of this enzyme. The enzyme shows its activity up to 60°C temperature and within a broad range of pH i.e. 6-12. The gene sequence of Nattokinase is homologous to other members of the 'Subtilisin' family.

Medicine, health care and pharmaceutical industry are some of the areas where the enzyme Nattokinase finds applications. Nattokinase is able to degrade fibrin clots both directly as well as in directly. Indirectly, it supports the fibrinolytic activity of plasmin by activating tissue plasminogen activator (t-PA) and pro-urokinase [7], [8].

Bacillus subtilis natto- nattokinase producing bacteria

Bacteria that have the ability to produce Nattokinase are isolated from various sources around the globe. Amongst all the possible sources for isolating the Nattokinase producing bacteria, the most profitable sources are fermented foods. In 1968, Nattokinase was first time isolated from traditional Japanese food known as 'Natto'. Another fermented food is Korean soybean sauce known as 'Chung Kook-Jang' that is very popular for Nattokinase production.

Bovine milk, cow dung and rust are various vibrant sources which were found to produce Nattokinase. Although there are various sources for Nattokinase production, the best way is using fermented food like the Japanese food 'Natto'. Nattokinase is formed by fermentation of soybean with bacteria Bacillus subtilis natto. It is the starter strain that is used for production of fermented food 'Natto'. Before the isolation of Bacillus subtilis natto, fermentation was initiated using dried rice straw because dried rice straw is the natural habitat of this particular strain of bacterial strain [9]–[11].

LITERATURE REVIEW

Srijan Aggarwa et al. concluded that a modern approach for calculating the total tensile strength of intact bacterial biofilms has been published recently. In this context, analyses are reported of video files from testing the elastic modulation, resilience and failure strain of the Staphylococcus epidermidis biofilm. In the context of previously informed values, the elastic module was 1270±280 Pa (17–6000 Pa). The significant ductility of bacterial biofilms can be seen from the high failure strains (240±16 percent). Further, from the region under the stress-strain plot (2.8±0.44 k J-m-3), the toughness of the biofilm sample was calculated. It was therefore demonstrated that the video check micro-scanner files would, in addition to the overall tensile power, be used to evaluate certain mechanical property parameters [12].

Ashrafudoulla et al. concluded that pathogenic microorganisms can easily contaminate food and give it a stigmatized toxin that might inflict possible harm to the user. To date, there have been 31 identified and numerous unidentified foodborne diseases pathogens worldwide reported. Bacterial species live close together, share matrix components & build in nature 3-D stiff biofilm constructions. Due to its strong phenotypic heterogeneity & natural resistance to common hygiene products, mono bacterial and mixed biofilms may be kept after the sanitizing process in foods and on food processor surfaces. The ability and efficacy of numerous microbial anti-biofilm agents have been shown through comprehensive studies. The authors isolated *Vibrio parahaemolyticus* which was investigated for evaluation of antibiotic sensitivity and pathogenic level. The isolated microorganism was examined for 17 virulence factors and examined against a variety of antibiotics including vancomycin, tetracyclin and penicillin [13].

The present study discusses the issues linked both to mono and mixed-bacterial biofilms and their application in various food sectors in the management of organic anti-biofilm agents. Moreover, the analysis addresses the recent developments, the current limitations and potential technological solutions for designing microbe based anti-agents for biofilm in the food industry with a broad variety of applications.

METHODOLOGY

Design

The experiment was carried out in such a manner that the results can be as accurate as possible. For carrying out the experiment, Bacillus subtilis was chosen as it can be easily available and can be grown easily in the laboratory. The microorganism was isolated from dried rice straw. Next, for the confirmation that isolated microorganism is none other than bacillus subtilis natto, gram staining, methyl-red test, Voges-Proskauer test, indole test and test for production of poly-gamma glutamic acid (ɣ-PGA) were performed. Further, the nattokinase enzyme was isolated using basal medium containing soya peptone. Nattokinase was confirmed using blood clot analysis. For confirmation of amylase activity and protease activity of nattokinase, amylase assay and protease assay were carried out, respectively.

Sample and instrument

The study was carried out on Bacillus subtilis natto to check its potential for secreting Nattokinase enzyme. Bacillus subtilis natto is a spore forming bacteria. Biotin is required for the growth of bacteria under natural conditions. It is a natural producer of enzyme Nattokinase. Nattokinase is known for its fibrinolytic activity. The enzyme was analyzed for its amylase activity and protease activity to determine whether it could be a good source for making novel agents for counteracting biofilm production.

Data collection

Isolation and Confirmation of Bacillus subtilis natto (Isolation): Dried rice straw was suspended in sterile water and heated at 95oC for 5 minutes. Above formed solution was added with tryptic soy broth and incubated overnight at room temperature. After incubation, aliquots were taken and spread on glutamate starch phenol red (GSP) agar plate and incubated overnight at room temperature.

Confirmation (Gram-staining): Some bacterial colonies from GSP agar plate were transferred on a glass slide using a sterile inoculating loop and smear was prepared by passing the slide through the flames of a Bunsen burner. After cooling down, the smear was drowned with crystal violet stain & held for 1 minute. Next the smear was rinsed softly under tap water. Next, the smear was flooded with Gram's Iodine, held for 1 minute and rinsed gently under tap water. Now, 95% ethyl alcohol was applied on the smear for 5-10 seconds and the smear was again rinsed gently under tap water. Further, the smear was stained using safranin and held for 30 seconds and rinsed gently under tap water. At last the smear was observed under light microscope.

Methyl-Red (MR) and Voges-Proskauer (VP) Test

Preparation of MR-VP broth: 30mL distilled water, 0.21gm peptone, 0.15gm glucose and 0.15gm dipotassium phosphate were added in a glass bottle, mixed well and sterilized in an autoclave at 121oC and 15 psi for 15 minutes.

Methyl-Red test: 15mL MR-VP broth was taken in a sterile test tube, inoculated with the bacterial cells from GSP agar plate with the help of a sterile inoculating loop and incubated at 37oC for 24 hours. Now 5mL of aliquot from the test tube was taken in a fresh test tube and a few drops of Methyl red indicator was supplemented to it.

Voges-Proskauer test: 15mL of MR-VP broth was taken in another test tube, inoculated with bacteria from the GSP agar plate using a sterile inoculating loop and incubated at 37oC for 24 hours. Next, 5mL aliquot from the broth in transferred to a fresh test tube and added with 0.6 mL VP-1 reagent and 0.2mL VP-2 reagent (40% KOH in distilled water). The test tube was vigorously shaken for 1-2 min. & allowed to stand for about 30 min.

Indole test

For making tryptone broth, in a clean test tube 15mL of distilled water, 0.15g of tryptone and 0.075g of sodium chloride were added and mixed well. The tryptone broth was sterilized in an autoclave at 121oC and 15psi pressure for 15 minutes. Next, the tryptone broth was inoculated with bacterial cells from GSP agar plate using a sterile inoculating loop and incubated at 37oC for 24 hours. Further, 5mL of the broth was transferred in a fresh test tube and added with 0.5mL of Kovac's reagent.

Test for Production of Poly-Gamma Glutamic Acid (ɣ-PGA)

Differential medium for screening was prepared using glucose, $MgSO_4.7H_2O$, yeast extract, K_2HPO_4, KH_2PO_4, L-Glutamic acid, agar and neutral red, and sterilized in an autoclave at 121°C for 15 minutes. The medium was then transferred to a petri plate. Further, some bacterial colonies from GSP agar plate was transferred with help of a sterilized inoculating loop to the freshly prepared differential medium and the medium was incubated overnight at 37°C.

Extraction and Confirmation of Nattokinase (Extraction of Crude enzyme): 1L basal media was prepared using soya peptone, yeast extract, K_2HPO_4, $MgSO_4$, maltose, glucose, NaOH and acetic acid and sterilized in an autoclave at 121°C for 15 minute. Next, the medium was inoculated with some bacterial colonies from GSP agar plate and incubated at 37°C & 160 rpm in an incubator shaker. After 7 days, the cell debris was removed using a centrifuge and the supernatant containing crude enzyme was collected and stored for further evaluations.

Confirmation: Blood sample was collected, transferred to a centrifuge tube and incubate at 37°C for 45 minutes. After the formation of clot, the serum was completely removed. Now 100 µl of crude enzyme was added to the centrifuge tube containing the blot clot and the centrifuge tube was again incubated for 90 minutes at 37°C.

Amylase Assay: Starch Casein agar medium was prepared by adding soluble starch, Casein (Vitamin Free), KNO_3, $MgSO_4.7H_2O$, K_2HPO_4, NaCl, $CaCO_3$ and $FeSO_4.7H_2O$, sterilized in an autoclave at 121°C for 15 minutes and transferred to a petri plate. Next, some bacterial colonies from GSP agar plate were transferred using a sterilized inoculating loop and the plate was incubated at 37°C overnight letting the bacteria to grow. Further, the petri plate was flooded with Iodine – Potassium Iodide solution.

Protease Assay: 0.65% Casein solution (g / ml) was prepared in a test tube by dissolving casein in potassium phosphate buffer and incubated for 5 minutes at 37°C. Next, crude enzyme was added and test tube was again incubated at 67°C for 10 minutes. Further, the reaction was stopped by adding trichloroacetic acid & the test tube was incubated at 37°C for 30 minutes. At last, sodium carbonate and Folin's phenol reagent was added to the test tube solution and the test tube was incubated at 37°C for 30 minutes.

Data analysis

Isolation and confirmation of Bacillus subtilis natto (Isolation): Referring to Figure 1, after the incubation of GSP agar plate with tryptic soy broth, growth of bacteria was seen on the agar plate.

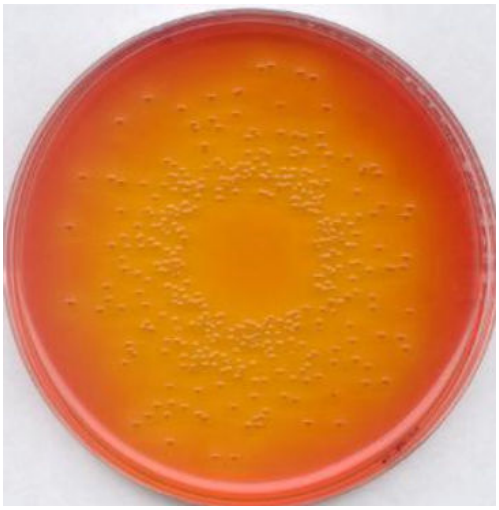

Figure 1: Growth of Bacterial cells on GSP agar medium.

Confirmation (Gram Staining): Gram-staining procedure was carried on isolated bacteria. Further, the smear was observed under light microscope to find whether the isolated bacterium is gram positive or gram negative. Figure 2 is an image of bacterial cells under light microscope. Violet-Purple colored cells were observed under microscope confirming that the isolated bacterium is gram positive.

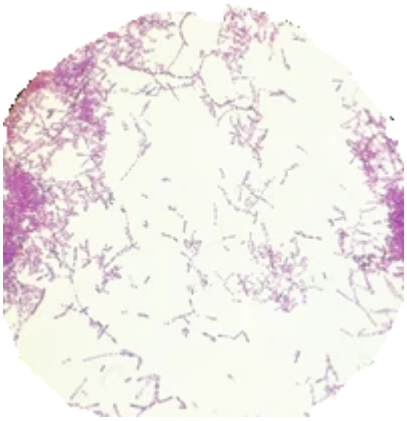

Figure 2: Microscopic image of bacterial following gram staining.

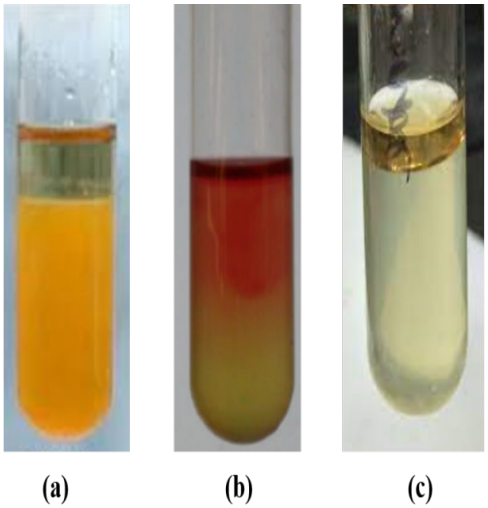

Figure 3: (a) Observation of Methyl-red test (b) Voges-Proskauer test (c) Indole test.

Confirmatory test for Nattokinase

Blood clot analysis was performed for the confirmation that the extracted enzyme is Nattokinase. Figure 4 is an image of blood clot test of extracted enzyme.

No blood clot was observed after incubation of test tube containing blood sample with extracted enzyme. Thus, the enzyme lysed all the blood clots.

Methyl-red, Voges-Proskauer, Indole test and test for production of Poly-gamma glutamic acid

Methyl red, Voges-Proskauer, Indole test and test for production of Poly-gamma glutamic acid were carried out for confirmation that the isolated bacterium is none other than Bacillus subtilis natto. Figure 3a, 3b and 3c illustrates the observation for Methyl-red, Voges-Proskauer and Indole test, respectively. Observations and inference of Methyl-red, Voges-Proskauer, Indole test and test for synthesis of Poly-gamma glutamic acid are listed in Table 1 given below.

Figure 4: Observation of Blood clot analysis of extracted enzyme.

Amylase Assay

Amylase assay of Nattokinase was carried out for checking the amylase activity of Nattokinase. Figure 5 illustrates the observation of the amylase assay.

After the addition of Iodine – Potassium Iodide solution, a white and clear hydrolytic zone is observed on the petri plate near the growth of bacteria.

Figure 5: Observation of Amylase assay of Nattokinase.

Protease Assay

Protease assay was performed to check the proteolytic activity of Nattokinase. Figure 6 illustrates observation of the protease assay. Blue color was formed in the test tube after addition of sodium carbonate and Folin's phenol reagent to the test tube.

Figure 6: Observation of Protease Assay of Nattokinase.

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