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Development of thermostable amylase enzyme from *Bacillus cereus* for potential antibiofilm activity

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ABSTRACT

The marine bacterial strain *Bacillus cereus* was used to produce amylase enzyme and has excellent alkali-stable and thermostable enzymatic activity. The combined effects of pH, temperature and incubation time on amylase activity were studied using response surface methodology. The amylase enzyme activity was also determined in the presence of various metal ions, chelating agents, detergents and the results showed that the maximum enzyme activity was observed in the presence of calcium chloride (96.1%), EDTA (63.4%) and surf excel (90.6%). The amylase enzyme exhibited excellent antibiofilm activity against marine derived biofilm forming bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* in microtiter plate assay and congo red assay. Light and confocal laser scanning microscopic (CLSM) analysis were also used to confirm the potential biofilm activity of amylase enzyme. The CLSM analysis showed the inhibition of complete biofilm formation on amylase enzyme treated glass surface. Further *in vivo* toxicity analysis of amylase enzyme was determined against marine organisms *Dioithona rigida* and *Artemia salina*. The results showed that there is no morphological changes were observed due to the minimal toxicity of amylase enzyme. Overall these findings suggested that marine bacterial derived amylase enzyme could be developed as potential antibiofilm agent.

1. Introduction

Amylase enzyme is one of the most important industrial enzymes which hold the supreme market share of enzyme sales with major applications in starch industry, baking, analytical chemistry, detergents, textile desizing, medicine, pulp and paper industries [1]. These amylase enzymes originate from different sources like plants, animals and microorganisms; among them, microbial amylases are the most produced and used in industries due to their high productivity and thermostability [2]. Because of the industrial importance of amylase, there is ongoing interest in the isolation of new bacterial strains producing amylase that suitable for new industrial applications [3].

Studies on bacterial amylase in developing countries are concentrating mainly on *Bacillus* sp. probably because of simple nature and cheaper nutritional requirements of these organisms [4]. However, *Bacillus* sp. produces a large variety of extracellular enzymes such as amylases, proteases, and lipases which have significant industrial importance [5]. The amylases produced by different *Bacillus* species vary not only in their types (saccharifying or liquefying) but also in the varying range of pH and temperature [6]. The bacterial source of the enzyme is usually either from *Bacillus amyloliquefaciens or Bacillus licheniformis*, the latter now being of greater industrial importance [7].

The optimal production of a microbial enzyme and its growth depends on the nature of strain involved as well as various environmental parameters such as dissolved oxygen, agitation, temperature, pH, substrate and nutrients [8]. Although production of amylase has been implemented on agricultural and industrial wastes and its co-products such as starch materials to solve pollution problems and obtain a low cost medium [9]. Moreover, the rice husk, wheat bran and starchy potato wastes were used as low-cost carbon substrates for amylase activity by *B. subtilis* [10]. However, the optimum conditions to produce the enzyme or metabolite are not always the same as that for growth [11]. Therefore, statistical and experimental factorial designs combined with Response Surface Methodology (RSM) could have much impact

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on experimental designs which tests one factor at a time and time consuming as it requires many experiments [8].

On other hand, amylase enzymes have potential applications other than industries such as pharmaceuticals, medical, clinical applications etc. Recently α-amylase enzyme has been found as a good antibiofilm agent against biofilm forming clinical pathogens *Vibrio cholera, S. aureus* and *P. aeruginosa* [12]. Biofilms are less susceptible to host inflammatory and immune responses and have higher antibiotic resistance than free-living planktonic cells [13]. Production of new antibiofilm agents against biofilms requires an understanding of bacterial biofilm-specific functional characters. Several studies have been initiated to clarify the complicated mechanisms underlying biofilm development and many aspects of these mechanisms are still poorly understood [14]. There are very less studies were reported that the antibiofilm activity of amylase enzyme with inadequate methods including but limited with spectrometric and agar plate method. In the present study, we dealt with optimization and characterization of amylase enzyme produced by *Bacillus cereus* isolated from deep sea water sample. Further, the potential antibiofilm efficiency of amylase enzyme against marine biofilm forming bacteria and its toxicity against marine copepod *Dioithona rigida* and brine shrimp *Artemia salina* was evaluated.

2. Materials and methods

2.1. Isolation and identification of microorganisms

The deep seawater samples were collected from Indian Ocean Equator region (1°0. 1370 N 82°59.9040 E) at 1000 m depth during the onboard cruise of Sagar Kanya 289 (SK 289, NCAOR, Goa, India). One ml of water sample was taken and transferred to an Erlenmeyer's flask containing 9 ml of sterile 50% seawater and the sample suspension was further diluted up to 10 folds. One ml of the diluted suspension was spread over the surface of nutrient agar medium prepared in 50% seawater to enhance the isolation of microorganisms. The colonies found on the plates were transferred onto agar plates and incubated at 28 °C. The colonies were observed from the second day and purified by repeated streak on agar plates. The purified colonies were stored at −80 °C in nutrient broth containing 30% glycerol. For taxonomic identification, the isolates were subjected to a series of morphology characterization [15] and biochemical tests [16] such as indole test, nitrate reduction, Voges-Proskauer (VP), and degradation of starch, The amylase-producing bacterial colonies were selected after flooding the plates with iodine solution. The strain (88DSB10) produced the high level of amylase enzyme was selected for further experiments. Further, the isolated strain was identified by amplification of 16S rDNA and sequencing, the sequences were submitted to NCBI database. The sequences were aligned using ClustalX 2.0 software and the phylogenetic tree was constructed using neighbor joining method (Mega 6.0 software) with bootstrap confidence of 1000 replicates.

2.2. Production and partial purification of amylase enzyme

The selected strain (88DSB10) was cultivated in minimal medium (M9) containing 10% NaCl and 1% soluble starch and pH of the medium was adjusted to 10.5 after autoclaving with 10% Na_2CO_3. Cultures were grown for 20 h at 37 °C with shaking at 200*g*. After the removal of cells by centrifugation at 10,000*g* for 20 min. at 4 °C and the supernatant was used for further work [17]. Previously chilled (−20 °C) ethanol was added dropwise to the clear supernatant for continuous stirring to the final concentration of 75% and the solution was stored at −20 °C for 24 h. The precipitate was recovered by centrifugation at 13,000*g* for 20 min. at 4 °C and resuspended in 100 mM phosphate buffer solution (pH 7) [18]. Further 1 μg/ml concentration of enzyme was dialyzed with molecular weight cut-off of 30 kDa. The concentrated enzyme solution was precipitated by drop-wise addition of two volumes of chilled absolute ethanol. The precipitate was recovered in 100 mM phosphate buffer solution (pH 7) and dialyzed overnight against the same buffer at 4 °C.

2.3. SDS page

The molecular weight of the crude and partially purified amylase was estimated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined according to Lowry's method with bovine serum albumin as standard. The purified enzyme (1 μg) was loaded onto 1 mm thick 10% polyacrylamide gel together with molecular size markers. The relative molecular mass of amylase was determined by comparing with known standard molecular weight markers (97.4–29.0 kDa). After completion of electrophoresis, the protein gel was stained with Coomassie Brilliant Blue R-250.

2.4. Enzyme assay

The activity of the amylase enzyme was assayed by adding 0.5 ml of the enzyme to 0.5 ml soluble starch (1% *v*/*v*) in 100 mM glycine-NaOH buffer at pH 10.5 and incubated at 37 °C for 60 min. The 10 ml of 0.1 N HCl was added to stop the reaction and A_{540} was measured [2] in double beam spectrophotometer (Shimadzu model 1800). The amount of reducing sugars released during the starch hydrolysis was measured and using the glucose standard curve. A unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of sugar reduced per minute under enzyme assay conditions by dinitrosalicylic acid (DNS) method.

2.5. Effects of pH and temperature on activity and stability

The enzyme activity at different temperature was determined by incubating the enzyme with glycine-NaOH buffer between 40 and 100 °C for 30 min [19]. The effect of different pH on amylase activity was carried out by assessing the enzyme in citrate-phosphate buffer (pH 4–6), Sodium-phosphate buffer (pH 6.5–8), Glycine-NaOH buffer (pH 8.5–10.5) and Borax-NaOH buffer (pH 11–13) for 20 min [20]. The stability of the enzyme at different temperature was determined by incubating the enzyme between 50 °C and 100 °C and for the measurement of pH stability, the enzyme was pre-incubated at 37 °C for 8 h [21–23].

2.6. Optimization of amylase activity

To study the effect of four main variables such as starch, pH, temperature and NaCl on enzyme activity was obtained through optimization; based on five levels (−2, −1, 0, +1, +2) a second-order quadratic model was used [24]. The design consisted of 30 experiments with four factorial points, four axial points to make a central composite design and five center points for replication in order to determine the experimental error. The enzyme activity under the optimized conditions was obtained from the Central Composite Design (CCD). Design Expert 7.1.5 (State-Ease, MN, USA) was used for regression analysis and plotting the response surface.

A reduced cubic polynomial model was calculated to estimate the response of the dependent variable for the production of amylase enzyme and its activity.