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A novel α-amylase producing *Bacillus drentensis* isolated from coastal mud samples

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Abstract

Hydrolytic amylases produced from microorganisms are of high demand due to its larger industrial applications. In this study, α-amylase producing bacteria were isolated from the marine environment and produced enzyme was partially purified. Samples were collected from marine environment and bacterial isolates were screened and identified by 16S rRNA analysis as *Bacillus drentensis*, *Bacillus tequilensis* and *Bacillus subtilis*. Under optimized conditions such as pH, temperature and salt concentrations, higher enzyme activity was observed than normal conditions. In *Bacillus drentensis*, the enzyme activity of 16.73 U/mL was obtained in pH 5.0 at 40 °C with 1% salt concentration. Interestingly, this acidic amylase was stable even at larger salt concentrations. In fermentation period studies, *Bacillus drentensis* had maximum activity after 48 h incubation, with the cell mass of 2.5 (OD), protein content of 3.1 (mg/mL) and the enzyme activity was 26.33 U/mL. Partial purification by $(NH_4)_2SO_4$ precipitation at 40% saturation yielded 5.79% enzymes with the specific activity of 46.1 U/mL and the dialyzed extract yielded 14.01% enzyme with 293.1 U/mL of specific activity. SDS-PAGE results revealed that the molecular mass of isolated amylase was approximately 55kDa. Many *Bacillus* sp. were used to produce amylases but to the best of our knowledge it is the first report on production of α-amylase from *Bacillus drentensis* with the high specific activity of 293.1 U/mL.

Key words : Optimization, fermentation period, partial purification, protein concentration.

INTRODUCTION

Amylases are important industrial enzymes and its applications have widened in clinical, medicinal and analytical chemistry [1]. Amylase finds its main use in starch saccharification and has application in brewing, baking, detergent, textile, paper and distilling industry is common [2]. Production of purified enzymes and its characterization plays an important part in biotechnology. α- amylase is classified in the family 13 and they are glycoside hydrolases [3]. Properties of α-amylase such as pH spectra, thermo-stability, molecular weight and others can be very useful in its related industrial applications [4].

Demand for amylases with novel properties is very high in various industries and it requires unique properties with respect to specificity [5]. The enzymes produced from terrestrial microorganisms have been well documented. Marine sources are used to produce various novel enzymes that are used in industrial sectors [6-8]. Microorganisms that can produce amylases with some novel properties could be of huge importance in biotechnological industries [9]. Amylases produced by *Bacillus* sp. is found to show promising potential in number of industrial applications [10]. *Bacillus* species such as *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* are the important microbes mainly chosen for industrial enzyme production. A large quantity (20-30g/L) of extracellular enzymes is produced by various *Bacillus* strains so they are placed as the most significant industrial enzyme producers. Some *Bacillus* species produce the enzyme at exponential stage of growth and in some at the mid stationary stage [11]. Though, most of the *Bacillus* species have same growth pattern and similar enzyme profile, the optimum condition for enzyme production differs, depending on the type of strain and species [11]. In this study, *Bacillus drentensis* from coastal mud samples was optimized to produce acidic α-amylase and partial purification was done by ammonium sulphate precipitation and molecular weight of produced α-amylase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

METHODOLOGY

Sample collection

Mud samples from coastal environment were collected from Pudukkottai region, (10° 1' 12" North, 79° 13' 35" East) Tamil Nadu, India. Under sterile conditions, mud samples were collected at three different spots; one from the top layer (PDU-1), from 10 cm depth (PDU-2) and other near the trees (PDU-4). All the samples were brought to the laboratory and stored at -20° C until further processing.

Isolation and screening of amylase producing bacteria

Isolation was done by serial dilution and spread plate method. Initial selection was made on starch agar plates and further, tested for enzyme activity by starch hydrolysis method [12]. Screened isolates were spotted on to a starch agar plates and overnight incubated plates were flooded with 1% iodine solution. The zone of hydrolysis around the bacterial colonies indicated the positive result for amylase production. For well diffusion method, 25 µL of centrifuged overnight grown culture broth was used and tested for extracellular enzyme activity.

Identification of amylase producing bacteria

Selected isolates were identified by Gram staining, biochemical analysis and by 16S rRNA analysis. DNA was extracted from the bacterial isolates by boiling method. Shortly, overnight grown 500µL bacteria were centrifuged at 10,000 rpm for 5 min, and to the pellet 100µL of sterile distilled water was added and heated for 10 min at 95°C and supernatant was used as a template. Primer sequences for 16S rRNA amplification were 27F: 5'-AGAGTTTGATC MTGGCTCAG- 3' and 1492R: 5' -

CGGTTACCTTGTTACGACTT- 3'. Sequence identities were determined using nucleotide blast at www.ncbi.nlm.nih .gov/BLAST/ and species level identification was made. In BLAST nucleotide sequence similarity search, sequences having identity of >98% was deemed as sufficient for species identification.

Determination of amylase activity

For activity determination, 0.5 mL enzyme solution and 0.5 mL soluble starch (1g/100mL) was used. Soluble starch was prepared using 0.01M phosphate buffer. This mixed liquor was incubated for 3 min at boiling conditions and reducing sugar was measured by DNS method [13]. Control was maintained without addition of enzyme solution. One unit of amylase activity is defined as the amount of enzyme that produces one micromole of reducing sugar in one minute at a constant temperature using soluble starch as substrate.

Optimization conditions

Different pH, temperature and salt concentration were optimized for maximum yield of the enzyme and also maximum enzyme activity was measured at different conditions. Effect of pH on enzyme production was measured at pH-5, 6, 7, 8, 9 and for temperature optimization culture conditions were maintained at 35, 40, 45, 50, 55 (°C). Dependency of salt concentration towards amylase production was analyzed with varying concentrations of NaCl (1% to 5%) in the medium. Optimal fermentation period to produce maximum enzyme was assessed at conditions; pH 7.0 at 40 °C with 1% salt concentration, and from incubated larger volume of culture 1 mL was aliquoted at every 12 h intervals to assay the enzyme activity and cell mass.

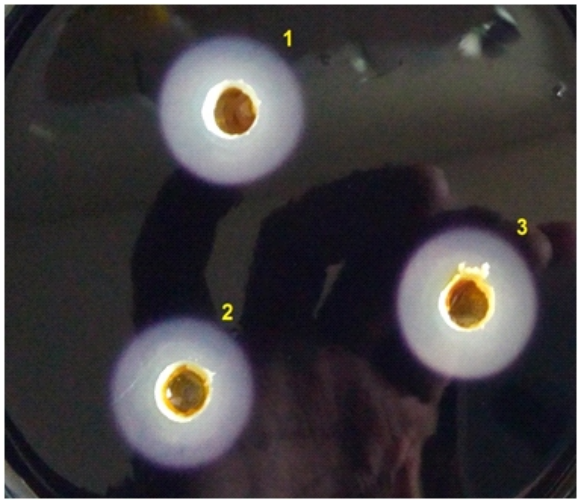

Figure 1: Representation of zone of hydrolysis in starch agar plates stained with iodine. Clear white circles (1,2,3) indicates hydrolysis of starch by three selected isolates.

Protein estimation

The total protein content of the extract was determined following Lowry's method [14].

Partial purification of α-amylase

Partially purified enzyme was obtained by ammonium sulphate precipitation. Different saturation (30%, 40%, 50%, 60% and 70%) conditions were used. To the crude culture supernatant, respective saturations were obtained with the addition of ammonium sulphate and left undisturbed for 4 h at 4 °C. Supernatant was recovered, and dissolved in 50mM phosphate buffer (pH 7.4) and dialyzed against 10mM phosphate buffer for 18 h.

The dialyzed enzyme was separated using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and molecular weight of the partially purified α-amylase was analysed.

RESULTS

Isolation of potential α-amylase producer

Initial growth was made on the nutrient agar plates with different concentrations of NaCl and it was found that 1-3% salt concentration was ideal for the growth of bacteria isolated from coastal mud samples. All the three samples were processed to isolate amylase producer and the initial screening on starch agar plates (containing 1% NaCl) showed that, 7 isolates produced clear zone of hydrolysis (Fig.1). Three isolates were screened as potential amylase producers that were showing higher zone of hydrolysis compared to the other isolates screened in this study (Table 1). All the three selected isolates were assayed for α-amylase production in pH 7.0 at 37 °C for 24 h. P3I2 (PDU-4) isolate was found to have the higher enzyme activity than the other two selected isolates. Isolate that was found to have maximum enzyme activity was further studied.

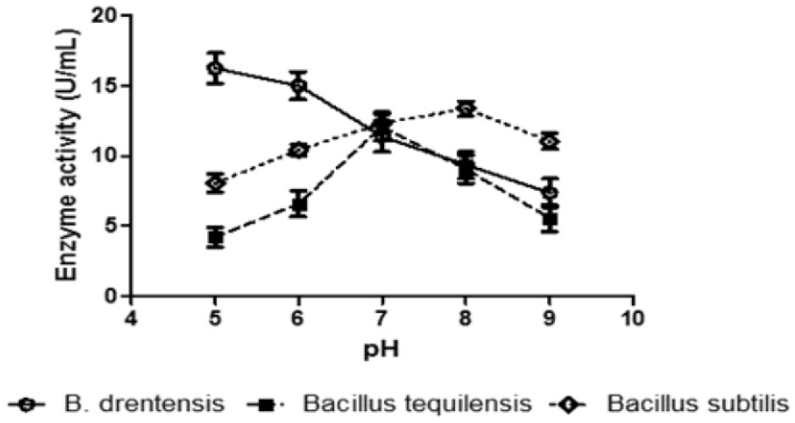

Figure 2: Effect of pH on enzyme activity. *B. drentensis* was found to have higher activity at acidic pH 5.0 after 48 h of incubation period. Error bars represent the mean-standard deviation for each experimental result.

Table 1: Comparison of zone of hydrolysis in starch agar plates and representation of the enzyme activities of three selected strains.

S.No.	Sample	Zone of hydrolysis (mm)	Enzyme activity at pH 7.0 at 37 °C for 24 h. (U/mL)
1.	P3I2	4.3	16.73
2.	P2I4	2.5	12.3
3.	P3I7	3.5	13.5

Table 2: Influence of fermentation period on enzyme activity from *Bacillus drentensis* corresponding to its cell mass and concentration of protein.

Fermentation period	Cell mass (O.D.)	Protein Concentration (mg/mL)	Enzyme activity (U/mL)
12 hr	0.9	0.5	13.2
24 hr	1.6	1.1	16.73
36 hr	2.5	2.6	19.1
48 hr	2.5	3.1	26.33
60 hr	2.0	3.5	17.8
72 hr	1.7	3.8	14.9

Identification of isolates

Three isolates that showed clear zone of hydrolysis were taken for identification. The isolates were identified to be rod shaped gram positive, catalase negative, indole negative, oxidase positive and colourless in Eosin methylene blue. 16S rRNA analysis was done and the obtained sequences were aligned. The nearest neighbour was retrieved using BLAST programme and confirmed to be *Bacillus drentensis* (KT760403), *Bacillus tequilensis* and *Bacillus subtilis* respectively. These three strains were further taken for enzyme optimization studies.

Determination of enzyme activity

Effect of pH, temperature and salt concentration

Optimum process control parameters found to have influence on enzyme activity, so optimization of varying conditions was analyzed. The obtained data showed that, *Bacillus drentensis* had maximum activity of 16.73 U/mL at pH 5.0 at 40 °C, *Bacillus tequilensis* had 12.3 U/mL of activity at pH 7.0 at 40 °C and *Bacillus subtilis* with 13.5 U/mL of activity at pH 8.0 at 40 °C. Either increase or decrease in pH or temperature reduced the activity of enzyme, explaining the ability of amylase to be active

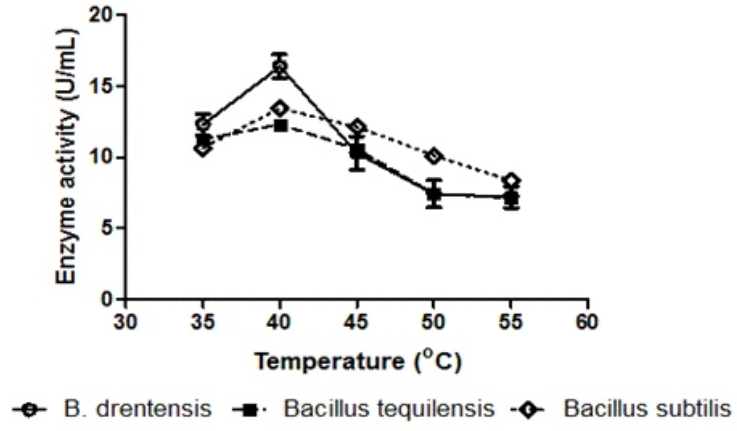

Figure 3: Effect of temperature on enzyme activity. *B. drentensis* was found to have higher activity at 40 °C after 48 h of incubation period. Error bars represent the mean-standard deviation for each experimental result.

Table 3: Comparison of partially purified α-amylase from *Bacillus drentensis.*

Sample	Amylase activity (U/mL)	Total activity (U/mL)	Total protein (mg)	Specific activity (U/mL)	Purification fold	Yield %
Crude	16.7	1673	79	21.1	1	1
$(NH_4)_2SO_4$ precipitation (40% saturation)	32.3	96.9	2.1	46.1	2.1	5.79
Dialyzed	46.9	234.5	0.8	293.1	13.8	14.01

at particular conditions that should be taken under consideration for scale-up processing. Amylases from *B. drentensis* were found to be active at acidic pH 5.0 and 6.0 but showed lower activity at alkaline conditions (Fig.2). Maximum enzyme activity was observed at 40 °C (Fig.3) and significant variation in enzyme activity was found at temperatures above 40 °C. Varying the salt concentration in the starch medium found that, the enzyme activity was very constant for the amylase produced from *Bacillus drentensis* up to 5% NaCl. In case of, *Bacillus tequilensis* and *Bacillus subtilis* 2% NaCl concentration was found to be optimal, and at the higher salt concentrations enzyme activity was found to be lowered (Fig.4). The result for enzyme activity was irrespective of the growth of isolates at different salt concentrations.

Effect of fermentation period

Optimized fermentation conditions for maximum amylase production was analysed and having highest production rate than any other tested *Bacillus* sp., *Bacillus drentensis* was chosen for further studies. After 48 h of incubation time, 26.33 U/mL of enzyme activity was assayed with 3.1 mg/mL of protein content. When compared with 24 h activity rate of 16.73 U/mL and at 72 h 14.9 U/mL; 48 h incubation period was found to be the optimum period for maximum enzyme activity. Our result clearly indicates the influence of fermentation time on enzyme activity (Table 2). Lower yield after 24 h is due to shorter incubation time than need for enzyme production and at 72 h enzyme activity reduced constantly may be due to non-availability of nutrients or synthesis of some toxic products after longer incubation period.

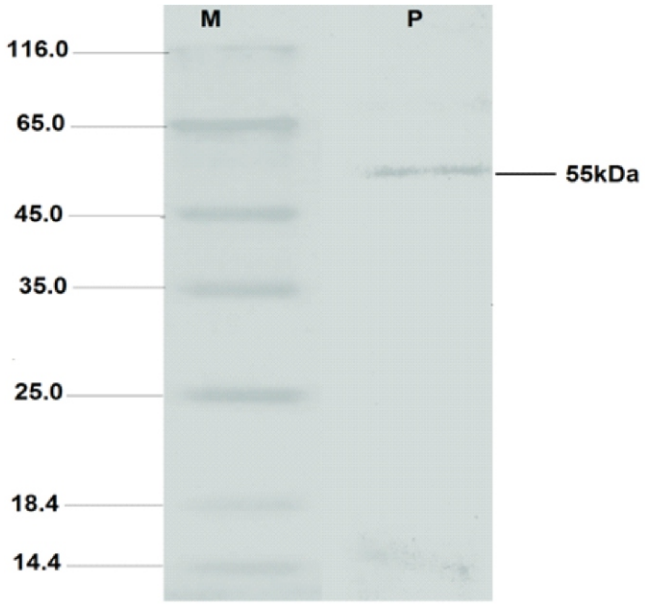

Figure 5: SDS-PAGE results for amylase produced from *Bacillus drentensis*. M- Protein marker, P-partially purified amylase from Bacillus drentensis. Molecular weights were represented in kDa and produced amylase was ~55.0 kDa.

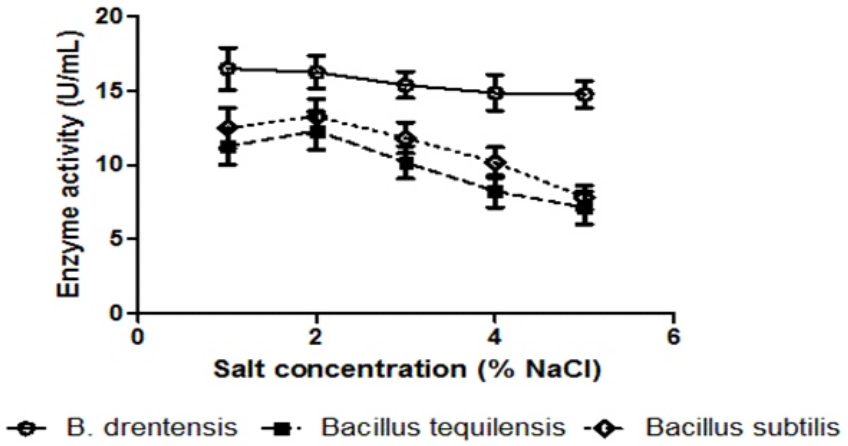

Figure 4: EEffect of salt concentration on enzyme activity. *B. drentensis* was found to have higher activity even at high salt concentrations after 48 h of incubation period. Error bars represent the mean-standard deviation for each experimental result.

Partial purification of α-amylase

Ammonium sulphate precipitation and dialysis had achieved partially purified enzyme that had maximum enzyme activity. It was found that, at each stage of purification the activity of extracellular amylase increases gradually (Table 3). Localization of amylase activity was seen at 40% saturation fraction and by dialysis, highest purification fold of 13.8 was obtained with 14% yield. Molecular mass of amylase produced by *Bacillus drentensis* was approximately 55kDa.

DISCUSSION

The genus *Bacillus* is found to produce a large variety of extracellular enzymes, which has potential in industrial applications. Microbial enzymes are cost effective, can produce larger quantity, have chemical stability, environmental production and vast availability so they are widely used in industries [15, 16]. Amylases produced from *Bacillus* species are particularly have considerable industrial applications [17]. Amylases are among the most important enzymes used in great significance for biotechnology, constituting industries such as, detergents, textiles, starch, and in baking [18]. Present study deals with the production and condition optimization of crude extracellular amylase produced from *Bacillus drentensis*. Isolated *B. drentensis* were able to hydrolyze starch showing clear zone of hydrolysis in medium supplemented with soluble starch. To enhance the production of enzyme various parameters associated with amylase production were studied. It is very important to optimize the culture conditions for the maximum microbial growth and enzyme production [19]. Among the physical parameters, pH of the growth medium plays an important role. Most of the earlier studies on bacterial strains revealed that optimum pH for bacterial growth and enzyme production was pH 6.0 and 7.0 [20]. The maximum amylase produced from *B. drentensis* in this study was observed at slightly acidic condition, pH 5.0. A study conducted by O. El-Tayeb (2007) showed that *B. amyloliquefaciens* 267CH produced maximum alpha amylase at pH 6.0 [21]. Bacterial growth and production of metabolites are controlled by environmental factors such as temperature. *Bacillus* species are known to produce amylases at much wider range of temperatures especially *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus* are reported to produce α-amylase at temperatures 37-60°C [22, 23]. In present study, for the determination of optimum temperature for amylase production, the fermentation was carried out at different temperatures (35 to 55°C) and maximum enzyme production was observed to be 40°C. It was reported that the optimum range for enzyme production was 40-42°C [24]. Incubation time was found to have strong influence on enzyme production and maximum activity reached after 48 h incubation. A similar result was also found in studies on *B. subtilis*, and other *Bacillus* species [25, 26, 27]. It might be due to the accumulation of other by products in the medium when incubation period is increased and also the efficient induction might not occur until the stationary phase is reached with the reduced nutrient sources. Some reported that the production of α-amylase by *Bacillus* species was reached maximum after 24 h, at the beginning of the stationary phase [28, 29]. Molecular mass of α-amylase produced from *Bacillus* species ranged from 50-60kDa [30]. Similar results were obtained in our study with molecular weight of amylase produced from *B. drentensis* was approximately 55.0 kDa. In the present study we reported the culture conditions and media composition for optimal production of amylase by *B. drentensis*. The optimum enzyme production by the bacterium was found at 40°C. Importantly, α-amylase produced from *B. drentensis* was stable at acidic pH 5.0 that can be more suitable for future use in various industries.

CONCLUSION

To the best of our knowledge, this is the first report of *Bacillus drentensis* producing acidic alpha amylase. The potential of *Bacillus drentensis* to synthesize alpha amylase that retains its activity at acidic pH and high salt concentration could find its application in industries. It can be concluded that, *B. drentensis* can be a potential producer of extracellular amylase that could be studied further to find its industrial applications. Due to the importance of these findings, studies can be carried on it order to commercialize the production process.

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