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Enhancement of Amylase Production by *Bacillus* sp. Isolated from Marine Water

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Abstract: Twelve bacterial strains isolated from marine water were screened for amylase production and ten isolates of them showed amylolytic activity. Among them, five stains showed promising activity, which were then quantified. The isolate KUMB07 exhibiting the highest activity was identified as *Bacillus* sp. A further optimization study was done by solid state fermentations using wheat bran as substrate. The optimization conditions for highest amylase activity (62.81 U/g) were found to be incubation period 48 h, moisture content 40%, pH 7, temperature 35°C and inoculum concentration 2 ml.

Key words: Amylase · Marine microorganisms · Bacillus sp. solid state fermentation

INTRODUCTION

Amylases are the most important enzymes used in biotechnology, particularly in processes involving starch hydrolysis. Amylases stand out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries. They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetraose syrup. Amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands; a large number of them are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry [1]. The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics [2].

The majority of enzymes used to date have been obtained from mesophilic microorganisms. The applications of these enzymes are restricted because of their limited stability to extreme temperature, pH and ionic strength [3]. Therefore, efforts have been made on the enzymes of thermophilic and halophilic bacteria, which could be used in many harsh industrial processes where the concentrated salt solution and high temperatures used would inhibit many enzymatic conversions [4, 5].

Bacterial amylase is produced throughout the world by liquid surface or submerged fermentations. The presence of the product in low concentration and the consequent handling, reduction and disposal of large volume of water during down-stream processing in submerged fermentation (SmF) are cost intensive, highly problematic and poorly understood unit operations [6]. In recent years, however, the solid-state fermentation (SSF) processes have been increasingly applied for the production of this enzyme [7]. Compared to SmF, solid state fermentation is more simple, requires lower capital, has superior productivity, reduced energy requirement, simpler fermentation media and absence of rigorous control of fermentation parameters, uses less water and produces lower wastewater, has easier control of bacterial contamination and requires low cost for downstream processing [8, 9]. In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. Agro-industrial residues are generally considered the best substrates for the SSF processes and enzyme production. Amylase production and physico-chemical parameter optimization using wheat bran has been studied by SmF and SSF [10].

The purpose of this work was to study the production of amylase by marine *Bacillus* sp. in solid state fermentation and optimized the fermentation conditions for the production of amylase.

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MATERIALS AND METHODS

Microorganism: Bacterial species were isolated from marine water sample collected from Vizhinjam, Kerala, India and the isolated cultures were maintained on marine agar slants and subcultured for every 15 days.

Screenings of Amylase Producing Bacteria: Two stages of enzymatic screening were done. All isolates were subjected to primary screening, while secondary screening was performed for the total bacterial isolates showed enzymatic activities during the primary screening.

Preliminary Enzymatic Screening: All the isolates were tested for their amylolytic activities using starch agar media by conventional plate method [11].

Secondary Screening: Further studies for the screening were carried out by submerged fermentation method. The production medium for amylase consisted of (g/100 ml); glucose 2, yeast extract 0.3, peptone 0.5, NaCl 1.5, $Na_2HPO_4.2H_2O$ 1.1, $NaH_2PO_4.2H_2O$ 0.61, KCl 0.3 and $MgSO_4.7H_2O$ 0.01. The flask was then kept on shaker (120 rpm) at 37°C for 24 h. The enzyme was extracted by centrifuging the fermentation media at 5000 x g for 20 minutes at 4°C. The selected bacterium was identified according to Bergey's Manual of Systematic Bacteriology (1994).

Assay of Amylase: Amylase activity was determined by incubating a mixture of 0.5 ml of aliquots of each enzyme source and 0.5 ml of 1% soluble starch dissolved in 0.1 M phosphate buffer, pH =7, at 37°C for 15 min [12]. The reaction was stopped by adding 1 ml of 3, 5-dinitrosalicylic acid [13]. And then followed by boiling for 10 min. the final volume was made up to 12 ml with distilled water and the reducing sugar released was measured at 540 nm. One unit (U) of amylase activity was defined as the amount of µg of glucose equivalent liberated per min per ml of enzyme under the conditions of assay. The amount of glucose was determined from the glucose standard curve.

Solid State Fermentation for Amylase Production: Cultivation was achieved by solid-state fermentation (SSF) as previously reported by Ramesh and Lonsane [14]. The medium that was used for the cultivation of *Bacillus sp* under (SSF) had the following composition: 10 g of substrate were moistened with 10 ml of 0.01M phosphate buffer (pH 7.4) and placed in 250 ml Erlenmeyer flasks. The fermentation media were sterilized by autoclaving for 15 min at 121°C. The flasks were inoculated with 3 ml of the prepared bacterial suspension and incubated under static conditions at 37°C for 2 d. The extracellular crude enzyme was measured at the end of the fermentation period.

Optimization of Process Parameters: The fermentation condition for amylase prodcution by *Bacillus* sp. was studied. The experiments were carried out systematically in such a way that the parameter optimized in one experiment was maintained at its optimum level in the subsequent experiments. Various process parameters that enhance the yield of amylase under solid state fermentation were investigated by taking one factor at a time. The impact of incubation time (12-96 h), initial moisture content of the substrate (20-70% v/w), initial pH (5-9), incubation temperature (25-50°C) and inoculum concentration (1-6 ml) were evaluated. All the experiments were conducted in triplicate and the mean values were considered.

Enzyme Extraction: Amylase was extracted from SSF medium by a simple contact method [14]. After specified incubation period (in each case), 100 ml sodium phosphate buffer of pH 6.9 was added to each experimental flask. The flaks were shaken (150 rpm) for half an hour and the material was filtered through Whatmann No.1 filter paper. The filtrate was centrifuged at 1000 x g for 10 min at 10°C. The cell free supernatant was carefully collected and used as crude enzyme extract. Statistical Analysis: The SPSS software (10.0 versions) was used. All results were expressed as the mean ± SD.

RESULTS AND DISCUSSION

Isolation and Screening of Amylase Producing Bacteria: Of the 12 marine bacterial strains screened for amylase production, ten showed positive result. Of these, 5 isolates namely KUMB02, KUMB06, KUMB07, KUMB10 and KUMB11 showed high amylolytic activity for quantitative screening by submerged fermentation (Table 1). The isolate KUMB07 exhibiting maximum enzyme activity (4.60 U/ml) was selected for solid state fermentation. The selected culture was morphologically identified as *Bacillus* sp.

Table 1: Screening of the tested bacteria for amylase activity by submerged fermentation

No.	Bacteria	Amylase Acyivity(u/ml)
1	KUMB02	0.46 ± 0.28
2	KUMB06	2.00 ± 0.78
3	KUMB07	4.60 ± 1.06
4	KUMB10	2.94 ± 0.97
5	KUMB11	1.98 ± 0.56

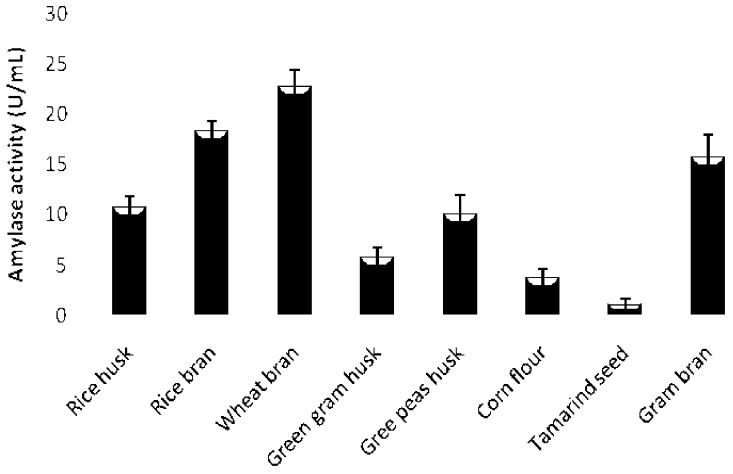

Fig. 1: Screening of substrates for amylase production by *Bacillus* sp

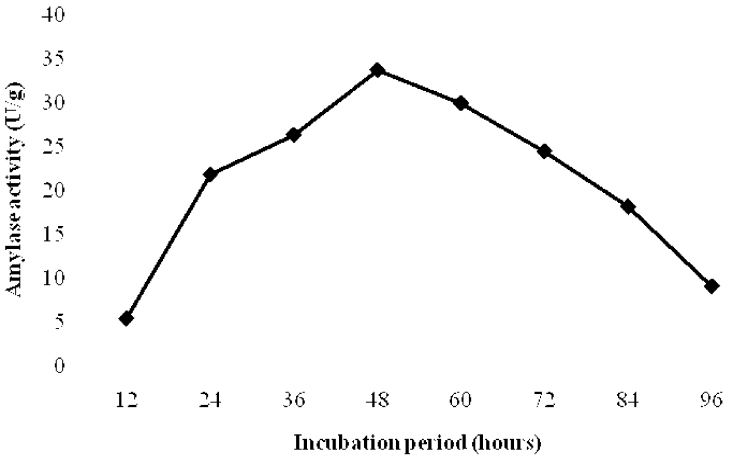

Fig. 2: Effect of incubation period on amylase production

Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries [15]. With increasing emphasis on environmental protection, the use of microbial enzymes particularly from extremophiles has gained considerable attention during the last several years in many industries, including manufacturing of chemicals, textiles; pharmaceuticals, paper, food and agriculture chemicals [16]. Alkaliphiles are reported to be a rich source of alkaline active enzymes, for example, amylase, protease, cellulase, xylanase and other enzymes that have numerous applications in many industrial processes due to an interest in their physiological adaptation to high pH [17].

Selection of Solid Substrate for SSF: Seven different substrates were used to screen the amylase production by *Bacillus* sp. Among the seven substrates, wheat bran showed highest enzyme activity of 22.76 U/g which is about five times higher than from submerged fermentation. Results were shown in Figure 1. Recent evidences indicate that bacteria and fungi growing under SSF conditions are capable of supplying the global demand for various metabolites [18]. The product titers produced in SSF are many-fold higher than that from submerged culture, although the reasons for this are not clear [15].

Optimization of Fermentation Condition: In our study, the maximum amylase (33.76 U/g) production was found in 48 h. This might be that the *Bacillus* sp had entered in to its exponential phase. Thereafter, the enzyme production started decreasing (Figure 2). Krishna and Chandrasekaran [19] optimized solid state flask culture for production of α-amylase by *B. subtilis* using banana stalks as a substrate and incubation for 24 h.

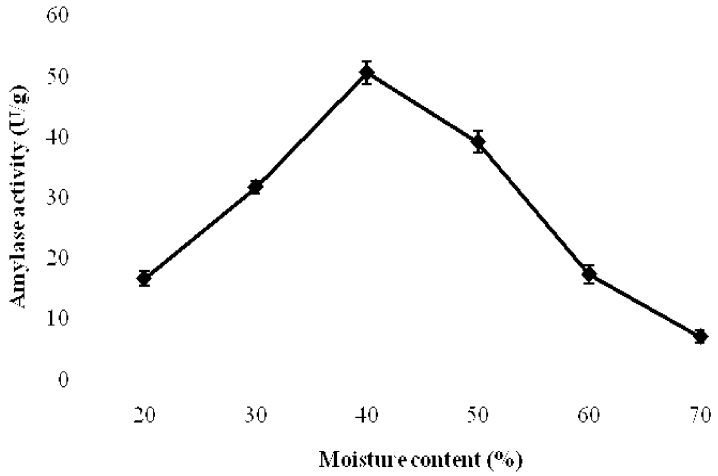

Fig. 3: Effect of initial moisture content on amylase production

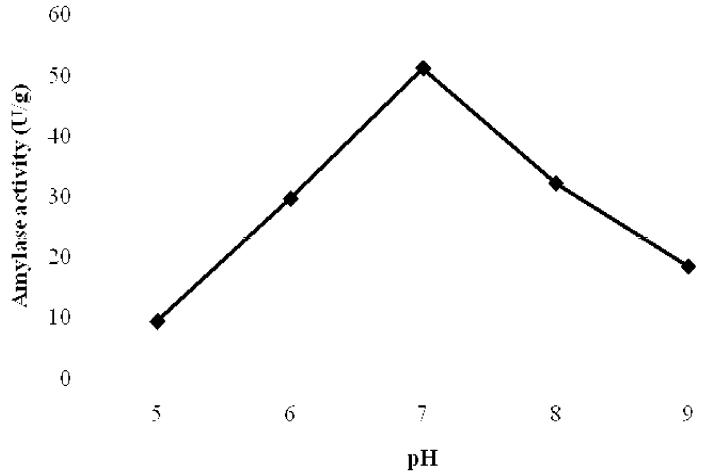

Fig. 4: Effect of pH on amylase production

The media optimization is an important aspect to be considered in the development of fermentation technology. The incubation period varies with enzyme productions [20]. Short incubation period offers potential for inexpensive production of enzymes. In the present study the amylase activity increased steadily and reached maximum at 48 h of incubation, as against a short duration of 24 h in the case of bacteria [21]. Tonkova *et al*. [22] produced α-amylase by *B. licheniformis* 44 MB 82-G, using glucose as carbon source and optimum enzyme activity of culture medium was recorded after 96 h.

The moisture content in wheat bran tested for maximum amylase production (50.62 U/g) indicated enhanced enzyme production with increase in the substrate moisture content up to 40%, beyond which it declined (Figure 3). The lowest enzyme activity of 7.04 U/g at 70% moisture level was observed.

Moisture content in SSF system can vary due to evaporation of the existing water through metabolic heat evolution, water consumption and liberation through fungal metabolism and also due to environmental factors. The moisture content in the substrate also depends on the type of microorganisms and the substrate used in the SSF. At the same time, the amount of moisture content also varied depending on the water binding characteristics of the substrates [23]. The maximum production of amylase was observed at 40% which indicated that the water binding capacity of wheat bran was high.

Results showing the effect of pH on amylase production by *Bacillus* sp in SSF of wheat bran are presented in Figure 4. The maximum activity of α-amylase (51.2 U/g) was observed in the fermentation medium adjusted at pH 7. Different organisms have different pH optima and decrease or increase in pH on either side of

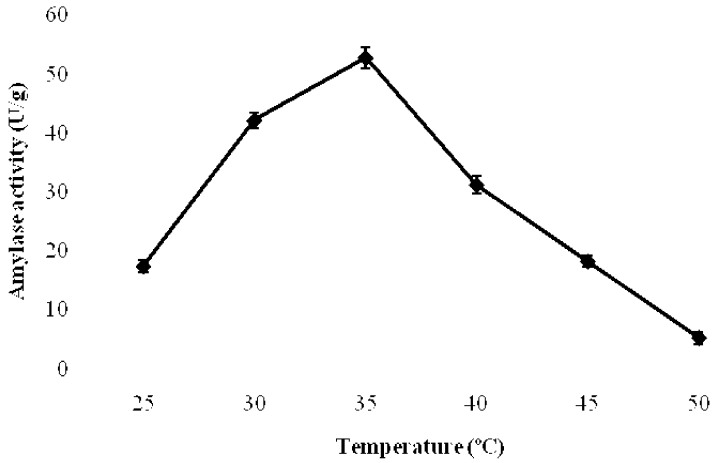

Fig. 5: Effect of incubating temperature on amylase production

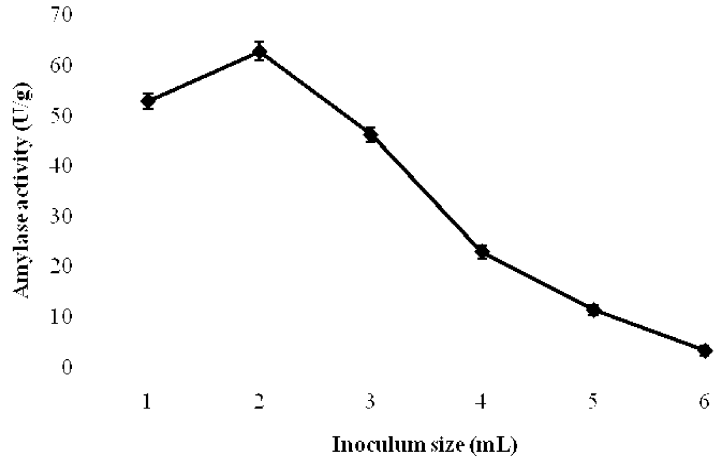

Fig. 6: Effcet of inoculum size on amylase production

the optimum value results in poor microbial growth [24]. Similar result was observed with Terui [25] who reported 6.8 as the optimum pH for the production of α-amylase by *B. subtilis*.

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium [26]. Most of the earlier studies revealed an optimum pH range between 6.0 and 7.0 for the growth of bacterial strains and enzyme production [26-28].

Results for the effect of different incubation temperatures on the production of amylase by *Bacillus* sp. are showed in figure 5. The maximum production of amylase (52.76 U/g) was obtained at 35°C. Kokab *et al*. [29] reported production of amylase by *Bacillus subtilis* using banana peel at 35°C. Increase in incubation temperature, decreased the production of enzyme. The production of the enzyme was greatly inhibited at 50°C. It might be due to the fact that at high temperature, the growth of the bacteria was greatly inhibited and hence, enzyme formation was also prohibited [15, 30].

Temperature also plays an important role in activation and inactivation of enzymes. Each enzyme has an optimum temperature for maximum enzyme activity. Sayem *et al*. [31] demonstrated that at temperatures of 60 and 70°C, the enzyme lost its activity rapidly with the optimum at 40°C.

Inoculum size also affects the maximum amylase enzyme production. Varying of the inoculum size of bacterial cells during the fermentation indicated that 2 ml of inoculum was optimum for the enzyme production (Figure 6). Maximum activity observed was 62.81 U/g. Increase in inoculum size was found to adversely affect the enzyme production. Inoculum size also influences the enzyme production. Lower inoculum size requires longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzyme. An increase in the number of spores in inoculum would ensure a rapid

proliferation and biomass synthesis. After a certain limit, enzyme production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in a decrease in metabolic activity [32]. A balance between the proliferating biomass and available nutrient would yield an optimum at which the enzyme synthesis would be maximum [33]. The present investigation has been directed toward exploring marine bacteria as a source for compounds of industrial interest, such as enzymes. The isolated *Bacillus* sp. showed a good amylolytic activity.

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