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Research Article

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Isolation, screening and optimization of amylase production by a marine bacterium *Bacillus subtilis* SJ33

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Abstract

Enzymes are among the most important products obtained for human needs mainly through microbial sources. The aim of the present study was isolation, screening and optimization of the enzyme production by using the marine bacterium *Bacillus subtilis* SJ33. In the present study, the growth parameters showed the profound influence on the amylase production on the candidate species such as the maximum production of the enzyme obtained at 48 hrs of incubation and the optimum was pH 7. The temperature also influenced on the maximum production of the amylase, the optimum was 35°C for higher production of the enzyme. The optimum salinity of 2.0% showed to maximum production of the amylase. In the present study, the starch and peptone was found to the best carbon and nitrogen sources to maximum production of the amylase. With optimized parameters in the mass scale medium maximum growth of 2.89 OD and enzyme activity of 64U/ml/min was obtained at 48 hrs. of incubation.

Keywords: Amylase, *Bacillus subtilis*, Vellar estuary, sediment, enzyme production.

Introduction

Enzymes are very proficient catalysts for biochemical reactions. They speed up reactions by providing substitute reaction pathway of lower activation energy. The molecules at the beginning of the process upon which enzymes may act are called substrates and the enzyme converts these into different molecules, called products, which initiate and accelerate thousands of biochemical reactions in living cells. Enzymes are among the most important products obtained for human needs through microbial sources. Microbial enzymes account the major volume. However, about less than 50 species are actually used to produce the entire list of microbial enzymes of commercial importance. The potential obviously exists to search for the species producing novel enzymes or enzymes with better properties and yield (Sing and Kumari, 2016).

A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or the other**.** Industrial enzyme market is an oligopoly with few strong players, and amylases are one among them. Amylases are glycosidase which catalyze the hydrolysis of glycosidic linkage in starch to generate smaller sugars useful to bioindustry. In biotechnology, amylase are the most important enzymes used (Sing and Kumari, 2016). Although amylase can be derived from several sources - such as plants, animals and microorganisms - the enzymes from microbial sources generally meet industrial demands. The amylase family of enzymes has been well characterized through the study of various microorganisms, especially bacteria and fungi. Microbial production of amylase is more beneficial than other sources because it is economical.

Production rate is high and can be engineered to obtain enzymes of desired characteristics. The microbial amylases could be potentially useful in various pharmaceutical, fine-chemical industries, paper industries etc. With the emergence of biotechnology, the use of amylase has widened in clinical research, medical chemistry and starch analytical chemistry. These increased uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes (Aiyer, 2005). The major advantages of using microorganisms for production of amylases are the ability to produce in bulk and ease at which it can be manipulated for desired products (Lonsane and Ramesh, 1990).

Materials and Methods

Sample collection

Sediment samples were collected from Vellar estuary (Latitude 11 29'N, Longitude 76 46'E), Tamil Nadu. Samples were transported to the laboratory and kept at 4ºC until further analysis.

Isolation and screening of bacterial strains for amylase production

Isolation and primary screening for amylase producers was done by using starch agar (containing 1% starch and 2% agar) plate method. Sediment samples were serially diluted up to 10^{-4} with sterilized 50% aged sea water and 0.1 ml the diluted samples were spread over the surface of starch agar medium. Plates were incubated at 30°C for 24 hrs. Morphologically different colonies were selected for the secondary screening. In secondary screening, 50 µl of cell free culture was inoculated in the wells made in starch agar medium. The plates were incubated at 30°C for 48 hrs. After incubation, the plates were flooded with 1% of iodine solution for 5 min and washed with water to remove the excess color (Bahadure *et al.,* 2010). Based on the highest size of zone of clearance around the well the potential strain was selected and maintained on starch agar slant.

Identification of potential bacterial strain

The potential bacterial strain was biochemically identified using Bergey's manual of determinative bacteriology (Holt *et al.,* 1994).

Optimization studies for amylase production

To study the effect of growth and enzyme production, submerged fermentation in a basal medium containing (g/100ml): peptone - 0.5g, yeast extract - 0.3g, NaCl - 0.3g, K_2HPO_4 - 0.1g, $MgSO_4$ - 0.02 g and soluble starch - 1g (Madigan *et al.,* 2011) was used. The medium was prepared by using 50% aged sea water and medium pH was maintained at 7. Different growth parameters like incubation period (0-72 hrs), pH (6, 7, 8, 9, 10 and 11),u temperature (25ºC, 30ºC, 35ºC and 40ºC), salinity (0.5%, 1.0%, 1.5%, 2.0% and 2.5%), different carbon (maltose, sucrose, glucose, starch and cellulose) and nitrogen sources (peptone, ammonium nitrate, beef extract and yeast extract). Enzyme activity was assessed for every 12 hrs and it was expressed as U/ml/min.

Amylase assay

Amylase activity was determined as described by Palanivelu *et al.,* 2001. The reaction mixture consisting of 1.25 ml 1% (w/v) soluble starch (Merck) solution, 0.25 ml, 0.1 M Sodium acetate buffer (pH 5.0), 0.25 ml of distilled water, and 0.25 ml of properly diluted crude enzyme extract was taken and after 10 minutes of incubation at 50ºC, the liberated reducing sugars (glucose equivalents) were estimated by the dinitro salicylic acid method (Miller, 1959). One unit (U) of α-amylase is defined as the amount of enzyme releasing 1µmol glucose equivalent per minute under the assay conditions. The amount of enzyme produced was expressed as U/ml/min.

Mass scale production

Based on the optimization results, the potential strain *Bacillus subtilis* was cultivated in the basal medium with the optimized parameters such as 48 hrs of incubation period, pH 7, temperature 35°C, 2% salinity, starch as the carbon source and peptone as the nitrogen source in a 1000ml of conical flasks. After sterilization at 121°C for 15 min. the medium was inoculated with full loop of *Bacilllus subtilis* and incubated at 35°C for 48 hrs. In the mass scale production medium biomass and the enzyme activity were assessed at the 48 hrs of incubation. Growth was assessed by measuring the OD at 600 nm with a UV spectrophotometer and the enzyme activity was assessed as previously.

Results and Discussion

The study has proved that amylase producers are abundantly present in the sediment sample collected from Vellar estuary. The observed density of amylase producers was 1.6 X 10^6 CFU/g (Fig. 1).

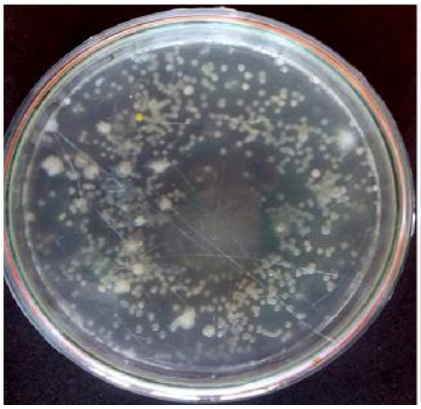

Fig. 1: Amylase producers

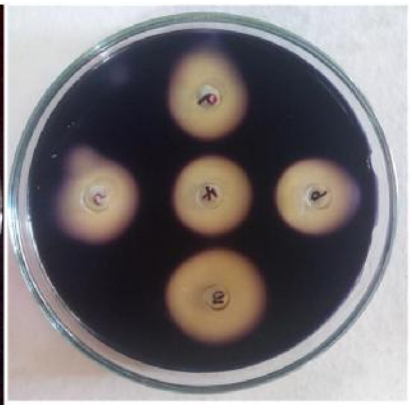

Fig. 2: Well assay screening in starch agar

A total of 8 morphologically different strains were selected for amylase production screening. Based on the primary screening, only 5 isolates were selected for the secondary screening for amylase production. The secondary screening using well assay (Fig. 2 and Table 1) showed Strain 10 was the most potential strain and it was biochemically identified as *Bacillus subtilis* (Table 2). This strain was used for further study.

Table 1: Screening of isolates for amylase production

S.No.	Number of the strains	Zone of clearance
1	Strain 6	1.7cm
2	Strain 7	1.5 cm
3	Strain 8	1.8 cm
4	Strain 9	2.0 cm
5	Strain 10	2.7 cm

Table 2: Morphological and biochemical characterization of the potential strain *B. subtilis*

Characteristics	**Results**
Colony shape	Irregular
Gram staining	+
Starch hydrolysis	+
Nitrate reduction	+
Citrate utilization	+
Indloe	_
Glucose	+
Sucrose	+
Methyl red	+
Oxidase	_
Catalase	+

In the present study maximum amylase production by the selected isolate was done after optimized to various factors such as incubation period, pH, temperature, salinity, agitation, carbon source and nitrogen source (Figs. 3-8).

Effect of incubation period on amylase production was showed that 48 hrs was the optimum incubation period for maximum amylase production (Fig. 3). Sonia Sethi *et al.,* 2013 reported the increase of incubation period above the optimum, the cells may reach the decline phase and displayed low amylase production. Similarly *Bacillus sp*. showed that the amylase production was detected from 48-72 hours and maximum production was obtained at 48 hours (Prabakaran and Hewitt, 2009)

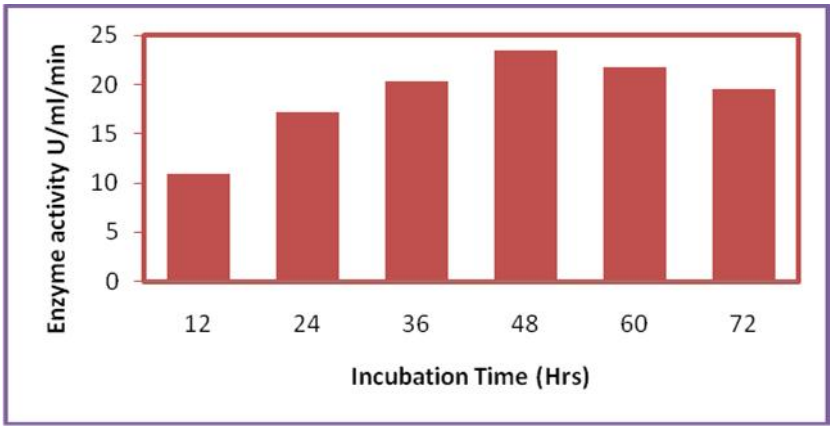

Fig. 3: Effect of incubation period on enzyme production

In the present study the amylase producing bacterial strain was subjected to different pH for maximum enzyme production. The maximum amylase production was obtained at pH 7.0 (20.32U/ml/min). When pH is altered below or above to the optimum, the amylase production was found to be decreased (Fig. 4). Aqeel and Umar (2010) reported the maximum amylase production was obtained at pH 7.0.

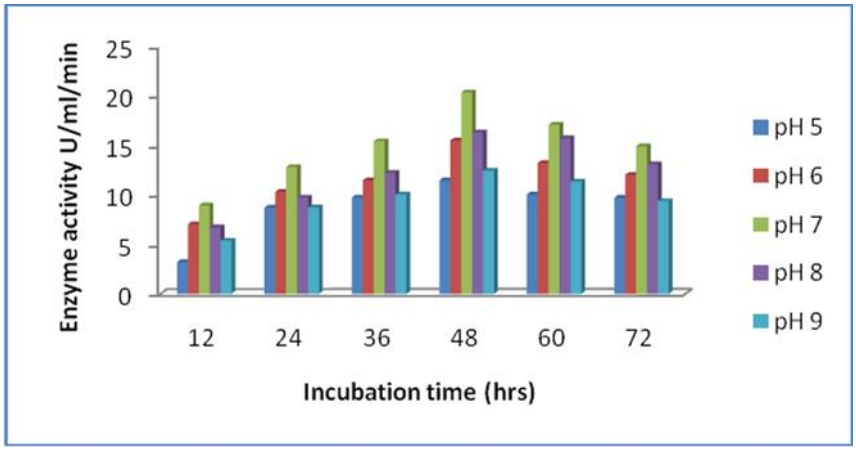

Fig. 4: Effect of pH on enzyme production

The effect of different temperature on amylase production was optimized. In this study temperature influenced the amylase production (18.08U/ml/min) and optimum temperature for maximum production was obtained at 35^{o}C as showed in Fig. 5. Further increased in temperature, the amylase production also decreased. (Suman and Ramesh, 2010).

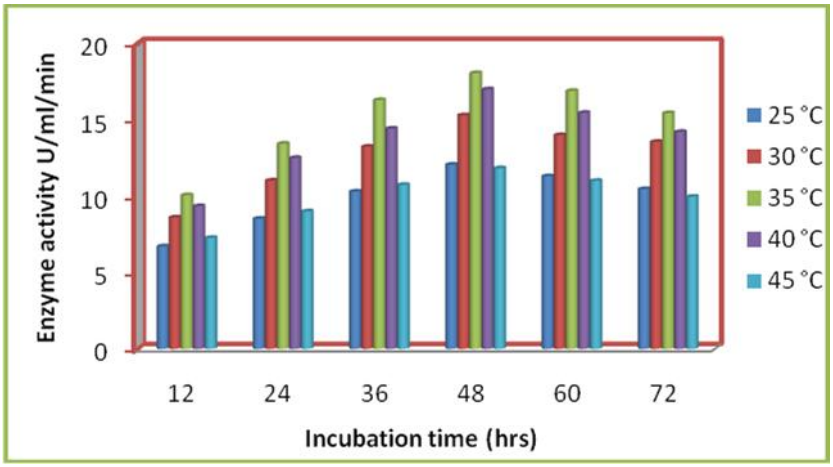

Fig. 5: Effect of temperature on enzyme production

In the present study, different concentration of NaCl such as 0.5%, 1.0%, 1.5%, 2.0% and 2.5% were optimized. The results showed that 2% of NaCl concentration was found to be optimum concentration for maximum amylase production. The effect of different NaCl concentration included in the production medium and the maximum production was observed at 2.0% concentration (Fig. 6).

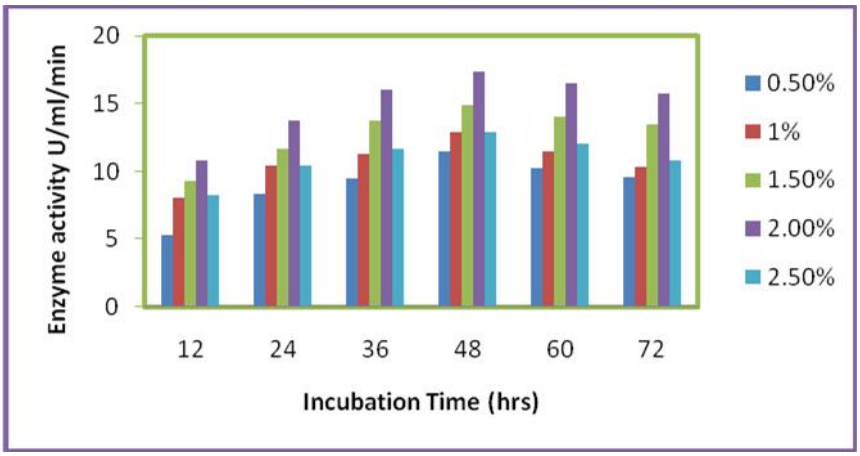

Fig. 6: Effect of salinity on enzyme production

Carbon sources greatly influence amylase production and the most commonly used substrate is starch (Bajpai, 1989). Different sources of carbon such as maltose, sucrose, glucose, starch and cellulose were optimized for maximum production of amylase. The obtained results showed that, starch was found to be best carbon source which produced maximum enzyme production (19.08 U/ml/min) compared to other carbon sources (Fig. 7). Similarly Lin *et al.*, 1998 and Asghar (2007) also obtained the same results. Suman and Ramesh (2010) suggested that starch is a generally accepted source and best component for the production of amylolytic enzyme.

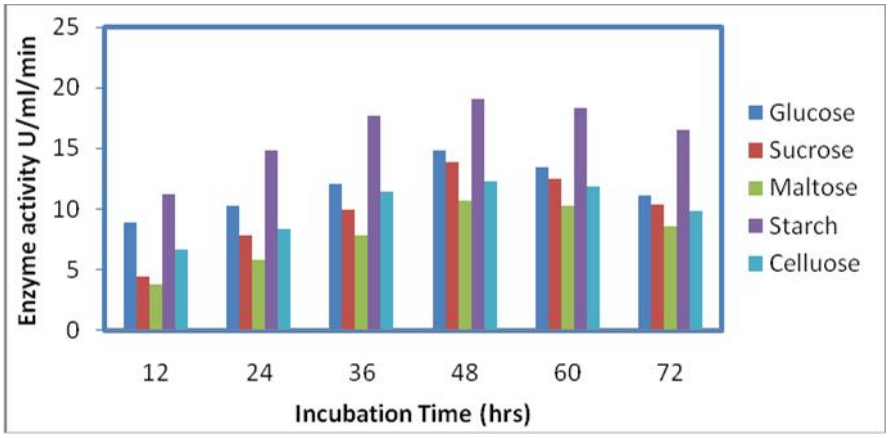

Fig. 7: Effect of carbon sources on enzyme production

The influence of different nitrogen sources like peptone, ammonium nitrate, beef extract and yeast extract were optimized It has been found that peptone was found to the best nitrogen sources for optimum growth and amylase production of *Bacillus subtilis* (Fig. 8). Generally the organic nitrogen sources such as peptone and yeast extract are frequently enhanced the amylase production in the medium (Hewitt and Solomons, 1996).

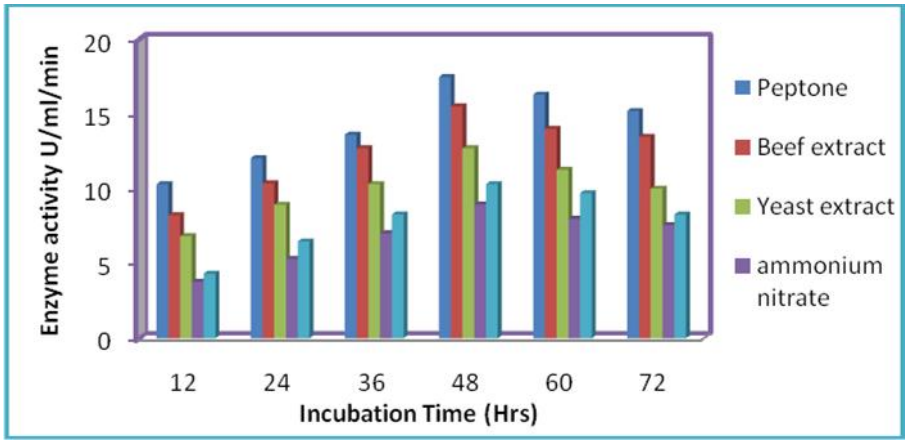

Fig. 8: Effect of nitrogen sources on enzyme production

The optimized growth parameters for *Bacillus subtilis* were used for large quantity of crude protein production in a 1L flask and kept for mass scale production. After mass cultivation the culture broth was centrifuged at 10,000 rpm for 5min. The cell pellet and supernatant was separated and assessed for biomass and enzyme activity. Surprisingly it coincided with the maximum growth of 2.89 OD and enzyme activity of 64U/ml/min was obtained at 48 hrs.

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