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Optimization, production and partial purification of extracellular α -amylase from *Bacillus sp. marini*

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ABSTRACT

*The aim of the current study was to isolate amylase producing bacteria from the soil samples collected from marine environment of Andaman and Nicobar Islands, India. The isolation was performed by serial dilution and plating method. Total 35 bacteria were isolated from the collected soil samples. All isolates were screened for amylolytic activity by starch agar plate method, among 35 bacterial isolates only 4 isolates showed the amylolytic activity, one isolate was selected for further study. The isolate (potential strain) was identified as *Bacillus sp. marini* by microscopic, biochemical and molecular experiments. The best enzyme activity was observed at pH 7 and temperature 40°C, 5.5% NaCl concentration, starch as carbon source and yeast extract as nitrogen source. At the optimum conditions *B. sp. marini* produced 8000 U of amylase per litre of culture broth, which is 3 fold higher than before optimization.*

Keywords: Nicobar Islands, *Bacillus sp. marini*, optimization, amylase

INTRODUCTION

Amylases are among the most important industrial enzymes and also have great significance in biotechnological studies. Amylases are characterized by their ability to hydrolyze glucosidic linkages in polysaccharide. α -amylase (endo1, 4- α -D-glucan glucohydrolase, EC 3.2.1.1) is an extracellular enzyme acts on starch and degrade it in to disaccharide and trisaccharide [1], α -amylase can be derived from various sources such as plants, animals and microorganisms [2]. Microbial production of amylase is more effective than that of other sources as the technique is easy, cost effective, fast and can be modified to obtain enzymes of desired characteristics. The microbial amylases could be potentially useful in various pharmaceutical, fine-chemical industries etc., with the event of new frontiers in biotechnology, the use of amylase has widened in clinical research, medical chemistry and starch analytical chemistry. Amylases are also used in

baking, brewing, textile, detergent, paper and distilling industries. These uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes [3]. In previous studies, a variety of microorganisms have been reported to produce amylase such as *B. subtilis*, *B. licheniformis*, *B. brevis* MTCC 7521, *Streptomyces erumpens* MTCC 7317, *Aspergillus awamori* and *Penicillium fellutanum* [4-9].

Recently interest and demand for enzymes with novel properties are very high in various industries and it leads to the discovery of various types of the amylases with unique properties. Each application of amylases requires unique properties with respect to specificity. Currently microbes from marine sources are used for industrial production of enzymes, although the potential for synthesis of several novel enzymes by terrestrial microorganisms have been recognized. Screening of marine microorganisms with higher amylase activity could therefore facilitate the discovery of novel amylases suitable for new biotechnological and industrial applications [10]. Several physical and chemical parameters like pH, temperature, incubation time, carbon source and nitrogen source etc. affect the enzyme production and the enzyme activity [11].

In the present study, we report the isolation and characterization of novel amylase producing bacteria *B. sp. mirini* from the soil samples collected from marine environment of Andaman and Nicobar Islands, India. Production conditions were optimized (temperature, pH, carbon source, nitrogen source and NaCl concentration) to achieve high enzyme production and better enzyme activity.

MATERIALS AND METHOD

Sample

Soil sample were collected from marine environment of Andaman and Nicobar islands, India. Soil samples were collected from 3 to 4 cm depth with help of sterile spatula. Samples were transferred in to sterile plastic bags and brought to the Molecular and Microbiology Research Laboratory in aseptic conditions. The samples were stored in a refrigerator at 4°C up to further processing.

Isolation of bacterial cultures

Isolation of soil bacteria was performed by serial dilution and spread plate method. One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10^{-1} to 10^{-4} . A volume of 0.1 ml of each dilution was transferred aseptically to Nutrient agar, Zobell marine agar, and SYEP agar plates. The sample was spreaded uniformly using a glass rod. The plates were incubated at 37°C for 24 hr.

The bacterial isolates were further subcultured on the respective mediums in order to obtain pure culture. Pure isolates were maintained at 4°C in refrigerator for further studies.

Screening of potent amylase producing bacteria

Bacterial isolates were screened for amyolytic properties by starch hydrolysis test on starch agar plate. The microbial isolates were streaked as a line on the starch agar plate and plates were incubated at 37°C for 24 hr. After incubation 1 % iodine solution (freshly prepared) was flooded on the starch agar plate. Presence of blue color around the growth indicates negative result and a

clear zone of hydrolysis surround the growth indicates positive result. The isolates produced clear zones of hydrolysis were considered as amylase producers and further investigated [12].

Identification of amylase producing bacteria

Cultural characterization

The isolates were observed under the microscope, the colony morphology was noted with respect to color, shape, size, nature of colony and pigmentation.

Microscopic observation

The bacterial isolates were Gram stained and observed under a high power magnifying lens in light microscope. Endospore staining, capsule staining and motility test were performed to observe the morphology and motility of the cells [13].

Biochemical characterization

The bacterial isolates were characterized biochemically by indole test, methyl red test, voges proskauer test, Simmons citrate test, catalase test, oxidase test, urease test, nitrate reduction test, gelatin hydrolysis test, Starch hydrolysis test, H₂S production [13].

Molecular characterization

The strain was screened based on the above traits and the efficient isolate was sent for molecular characterization based on 16S rDNA sequencing. The 16S rDNA gene sequencing analysis was done using the primer (5'- AGA GTT TGA TCC TGG CTC AG-3') and ABI3730XL capillary DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan) was used to determine 16S rDNA sequence. The phylogenetic tree was constructed and the genus and species were successfully identified. The restriction sites in the 16s rDNA sequence of the isolate *Bacillus* sp. *marini* were predicted using online software Genebee.

Amylase production

Freshly prepared inoculum was used to inoculate the production medium. For the preparation of inoculum a loop full of bacterial isolate was transferred in 50 ml of inoculum medium contained (g/L) starch 10, peptone 10, yeast extract 20, KH₂PO₄ 0.05, MnCl₂.4H₂O 0.015, MgSO₄.7H₂O 0.25, CaCl₂.2H₂O 0.05 and FeSO₄.7H₂O 0.01. The flask was loaded on a rotary shaker incubator (Sanyo Gallen Kamp, UK) at a speed of 2000 rpm at 37°C for 24 hours.

Amylase production was carried out by submerged fermentation. 500 ml of the production medium (same as inoculation medium) was inoculated with 10 ml of bacterial inoculum. The flask was loaded on a rotary shaker incubator at a speed of 2000 rpm at 37°C for 24 hours. After incubation, fermented broth was centrifuged at 7000 rpm for 15 min in a cooling centrifuge. Supernatant was collected and used for the estimation of amylase.

Optimization of temperature, pH, carbon source, nitrogen source and NaCl concentration on amylase enzyme productivity and enzyme activity

Effect of temperature on enzyme production and enzyme activity was studied by adjusting the incubation temperature at 30, 35, 40, 45 and 50°C and production medium pH 6.5, 7.0, 7.5, 8.0 and 8.5. Similarly, effect of carbon source, nitrogen source and NaCl concentration was studied

by adjusting different carbon sources (Sucrose, fructose, dextrose, starch and lactose), nitrogen sources (Meat extract, beef extract, yeast extract, nutrient broth and urea, casein) and NaCl concentrations (0.5, 1.5, 2.5, 3.5 and 4.5 %) in the production medium [14, 15].

Partial purification of amylase enzyme

Partial purification of amylase enzyme was achieved by ammonium sulphate precipitation followed by dialysis. 100 ml of cell free extract was saturated with ammonium sulphate up to 80%. The content was incubated over night and centrifuged at 5000 rpm for 20 min. Supernatant was collected and saturated up to 90% with ammonium sulphate. Then the content was centrifuged at 5000 rpm for 20 min and pellet was collected for further analysis.

The enzyme mixture was transferred in a dialysis bag and immersed in phosphate buffer at 4°C for 24 hr. buffer was continuously stirred using a magnetic stirrer throughout the process. Buffer was changed three times during the process in order to obtain proper purification.

Enzyme assay for amylase enzyme

Amylase was determined by spectrophotometric method as described by Fisher and Stein [13]. According to procedure 1.0 ml of culture broth was taken in test tube in duplicate and 1.0 ml of substrate (starch) was added in test tube. The test tubes were covered and incubate at 35°C for 15 minutes in water bath. Then 2.0 ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 5 minutes. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer. A unit of amylase activity was defined as the amount of amylase required to catalyze the liberation of reducing sugar equivalent to one μ mol of D-glucose per minute under the assay conditions [16].

Estimation of protein content

The total protein was calculated using Lowry's method [17]. Bovine serum albumin (BSA) was used as a standard.

RESULT AND DISCUSSION

Isolation of amylase producing bacteria

Total four marine sediments were collected and processed by serial dilution and spread plate method for the isolation of marine bacteria. A total 35 different bacterial strains were isolated on the bases of colony morphology. All isolates were primarily screened for amylase production by starch plate method. Among 35 isolates, 3 isolates hydrolysed the starch on starch agar. The strain showing maximum inhibitor zone were selected and further study of amylase production, enzyme activity and optimization.

Identification of amylase producing bacteria

The fungal isolates were characterized on the basis of colony characteristics, microscopic appearance and biochemical tests. Considering the colony characteristics (Table 1), microscopic appearance and biochemical tests the isolate was identified as *Bacillus* sp. Taxonomical identification of the bacterial isolate was performed by 16S rDNA analysis. The 16S rDNA sequence of the bacteria was blasted using online tool blast of NCBI gene bank and the phylogenetic tree was constructed with other homologous sequences (Figure 1).

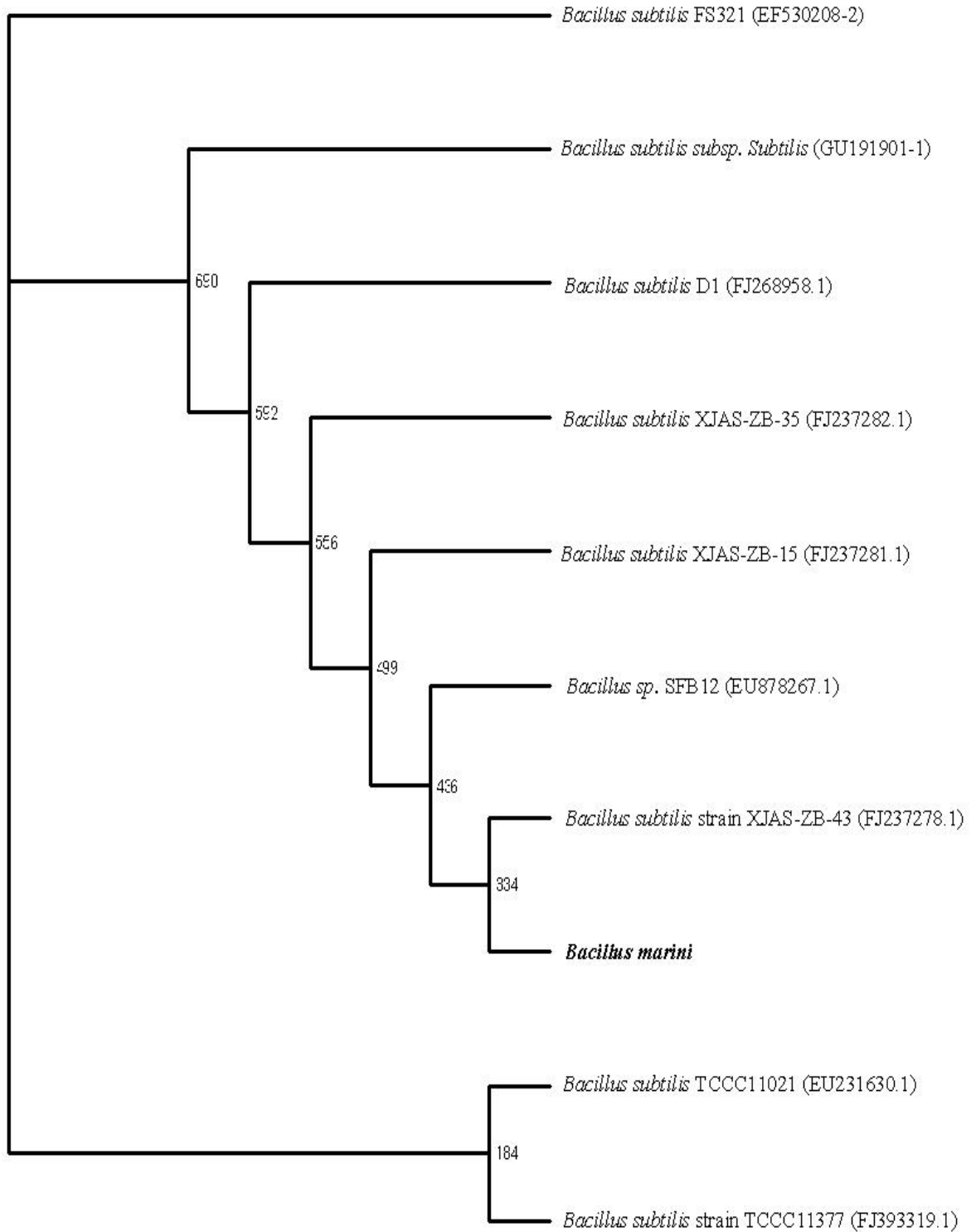


Figure 1: Phylogentic tree of *Bacillus marnii*

The sequence was submitted to NCBI gene bank (accession number: HM454198). Based on the morphological, physiological, and biochemical characteristics and taxonomical identification, the isolate was identified as *B. sp. marini*.

Table 1: Characterization of keratinolytic bacteria (H5 isolate)

| | Characterization of bacteria | Result |
|------------------------|------------------------------------|---|
| Cultural characters | Colony morphology on nutrient agar | Small, round, regular, mucoid, creamy yellow, fast growing colonies |
| Microscopic characters | Spore staining | Spore forming |
| | Gram staining | Gram positive rods in chains |
| | Motility | Non motile |
| Biochemical characters | Indole | Negative |
| | Methyl Red | Negative |
| | Voges Proskauer | Positive |
| | Citrate utilization | Positive |
| | Catalase | Positive |
| | Oxidase | Positive |
| | Urease | Negative |
| | Nitrate reduction | Positive |
| | Gelatin liquefaction | Positive |
| | Starch hydrolysis | Positive |
| | Hydrogen sulphide | Negative |

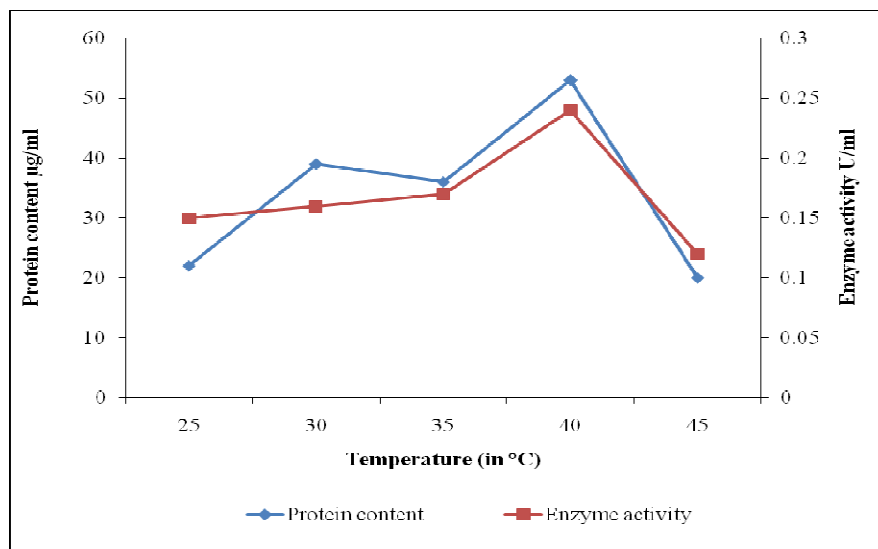


Figure 2: Effect of temperature on enzyme activity and protein content

Effect of temperature on enzyme activity and protein content

Production of amylase by the *B. sp. marini* was optimum at 40°C temperature and as the temperature increased or decreased, there was gradual decrease in the protein content and enzyme activity (Fig 2). At 50°C, the production of amylase and protein content was extremely low. Results are supported by the earlier studies carried out for production of amylase by sponge associated marine bacterium *Halobacterium salinarum* MMD047 [14].

Effect of pH on enzyme activity and protein content

Optimum pH for the amylase production was found at 7 at 40°C, as the pH was increased, there was gradual decrease in growth of the organism, protein content and enzyme activity (Fig 3). The organism did not grow in production medium adjusted to pH below 5 and above 10.

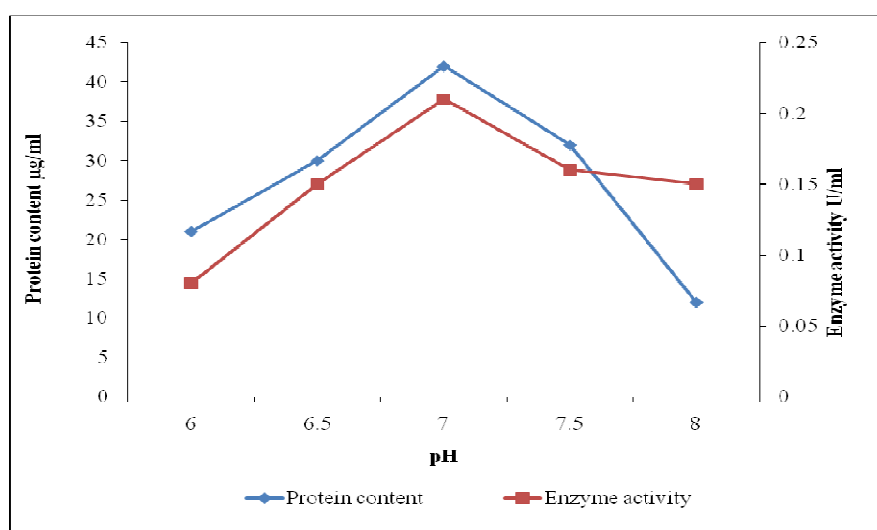


Figure 3: Effect of pH on enzyme activity and protein content

Effect of carbon sources on enzyme activity and protein content

The effect of Carbon source on amylase production was characterized using five different sugars at 1 % (w/v) concentration. *B. mirini* showed the maximum enzyme activity in the presence of starch as carbon source, whereas, the minimum enzyme activity and protein content was observed in the presence of dextrose (Figure 4). Earlier in some studies starch was used as substrate for the production of amylase from *Bacillus* sp. [18], however, the effect of carbon source changes with the production strain and other conditions [19].

Effect of nitrogen source on enzyme activity and protein content

Production of amylase by *Bacillus* sp. *marini* has been studied in presence of five different organic and inorganic nitrogen sources. Among all, yeast extract showed maximum enzyme activity (Figure 5). Similarly Teodoro et al. (16) reported the use of yeast extract as nitrogen source with combination in peptone [20].

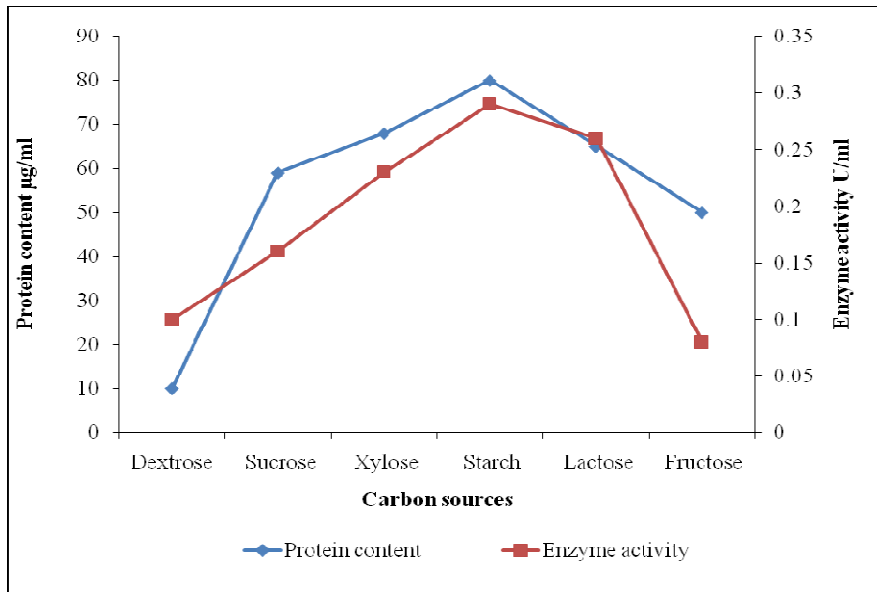


Figure 4: Effect of carbon sources on enzyme activity and protein content

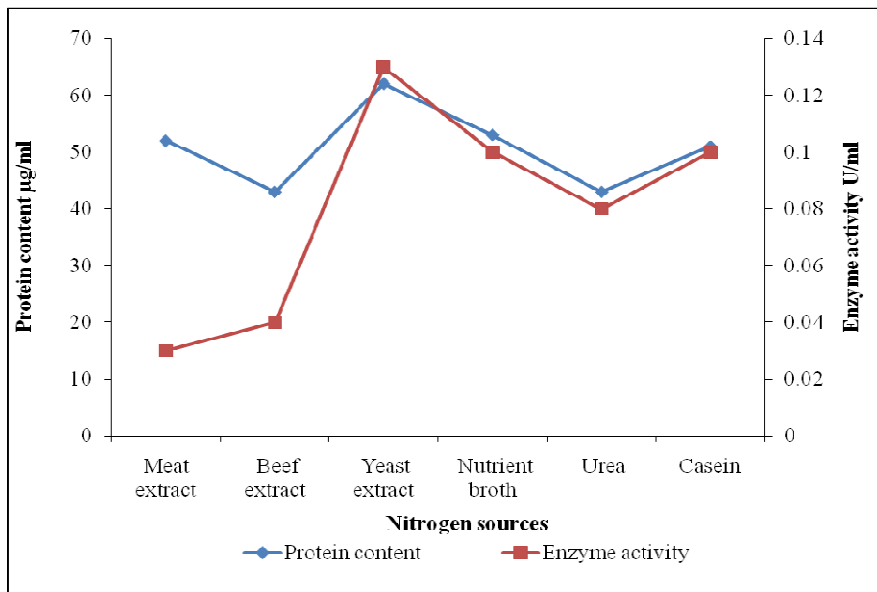


Figure 5: Effect of nitrogen source on enzyme activity and protein content

Effect of NaCl concentration on enzyme activity and protein content

At 4.5 % NaCl concentration the enzyme activity was find maximum, as the NaCl concentration was increased or decreased there was gradual decrease in enzyme production, enzyme activity and growth of organism (Fig 6).

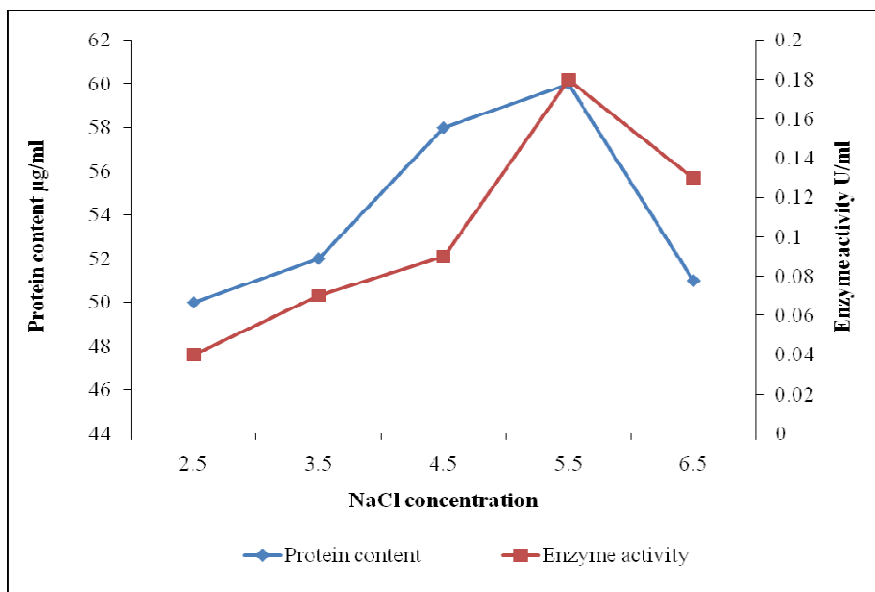


Figure 6: Effect of NaCl concentration on enzyme activity and protein content

Partial purification of amylase

Partial purification of amylase enzyme was performed by Ammonium sulphate precipitation followed by dialysis. Partially purified amylase exhibited specific activity of 0.035 U/ml/mg which corresponds to 5.80 purification fold and 60.0 % Yield (Table 2).

Table 2: Partial purification of amylase from *Bacillus sp. Marini*

| Purification steps | Enzyme activity (mg) | Protein activity (U/ml/mg) | Specific activity (U/ml/mg) | Purification fold | Yield % |
|------------------------|----------------------|----------------------------|-----------------------------|-------------------|---------|
| Crude extract | 0.580 | 80 | 0.006 | 1 | 100 |
| Ammonium precipitation | 0.409 | 30 | 0.016 | 2.66 | 70.5 |
| Dialysis | 0.350 | 10 | 0.035 | 5.8 | 60 |

CONCLUSION

Amylase is one of the most important enzymes known and is of great significance having approximately 25 % of enzyme market. They find potential applications in food, pharmaceutical and fine chemical industries. Earlier several studies reported the biological production of amylase from microorganisms. This study reports biological production of amylase by marine bacteria *B. sp. marini*. Enzyme production was found maximum in presence of starch as carbon source, yeast extract as nitrogen source, 6.5 % NaCl concentration, temperature 40°C and pH 7.0. At the optimum conditions *B. sp. marini* produced 8000 U of amylase per litre of culture broth, which is 3 fold higher than before optimization.

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