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Dual Production of Amylase and Protease Using *Bacillus Subtilis*

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ABSTRACT

In the present study the dual production of amylase and protease is studied using *Bacillus subtilis* in a single medium. Microbial fermentation has been carried out in a batch reactor using *Bacillus subtilis* by submerged fermentation in a liquid media. The maximum time for production of amylase is 22 hours and protease is 48 hours. Among the different carbon sources used for the production, starch and dextrose showed maximum production of amylase and protease. The effect of enzyme activity was studied for amylase and protease at different temperatures (0^0C to 70^0C) and different pH (3.6 to 3.9). Maximum enzyme activity for amylase is at 6.2 pH at 55^0C and for protease is at 7.2 pH at 50^0C. After production of wild enzyme preliminary purification is done by dialysis.

Key words: *Bacillus subtilis,* Dual production, Enzymes, Amylase, Protease.

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INTRODUCTION

Bacteria belonging to the genus *Bacillus* are considered to be one of the most important sources of enzymes and other biomolecules of industrial interest, *bacillus* responsible for the supply of about 50% of the market for industrial enzymes [1]. The world market for enzymes is estimated at 1.6 billion dollars, 29% for the food industry, animal feed 15% and 56% in other applications [2]. Extracellular enzymes include proteases, amylases and lipolytic enzymes from *Bacillus subtilis* of great importance in industrial processes, such as pharmaceutical, leather, laundry, food and waste processing industries [3]. Among the different categories, the hydrolase enzymes are the largest of industrial application and, among these, the alpha-amylase and beta-galactosidase have received special attentions. The major amylase produced by Bacillus is heat resistant, which is commercially interesting because many processes require high temperatures, so the thermo sensibility ceases to be a limiting factor [4]. Members of the genus *Bacillus* produce a large variety of extracellular enzymes of which amylases are of particularly significant industrial importance [5]. Submerged fermentation has been defined as fermentation in the presence of excess water. Almost all the large-scale enzyme producing facilities are using the proven technology of SMF due to better monitoring and ease of handling. In the present study production of amylase and protease were studied in a batch reactor using *bacillus subtilis*. Different parameters like effect of carbon sources, effect of pH, effect of temperature on both the enzymes were studied. After production of enzymes preliminary purification of enzymes were done.

MATERIALS AND METHODS

Experimental

Collection of Culture:

The samples of *Bacillus subtilis* 1679 were collected and used for the experiment.

Bacillus subtilis Growth Medium

Bacillus subtilis from the agar slant the spores of were transferred to the liquid medium, which contains carbon, nitrogen and growth nutrients to grow the organisms. The various components of the starter medium are starch, 0.5gm, yeast extract 0.5 gm., ammonium sulphate 0.5 gm, magnesium sulphate 0.1gm. dissolved in 100 ml of distilled water. This medium was adjusted to the pH – 7.0 by using sodium hydroxide and dil. Hydrochloric acid and kept in a 250 ml conical flask. After inoculation of the medium by spores of *Bacillus subtilis* the organism was allowed to grow for overnight (20 hours) at 35^0C in an incubator. Then this growth culture was used to inoculate the enzyme production medium [7].

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Caesin hydrolysis

In the skimmed milk agar medium, after solidification of the medium single round streak inoculation was done from the organism growth culture at the two sides of the plate containing skimmed milk agar. The plate was incubated at 30^0C for 24 – 48 hours in an inverted position. After incubation the plate was observed for any clear zones around the growth of the organisms. If clear zones are seen it conforms that the organism has the ability to degrade protein, which breaks the peptide bond CO-NH into free amino acids. This conforms the presence of protease enzyme [8].

Mass production of enzyme

Mass production of enzyme was done in the submerged fermentation, the organisms was cultivated in the liquid media in the 500 ml conical flask.

The medium for enzyme production was starch, 3gms peptone, 0.15gms. ammonium sulphate, 0.036 gms. sodium chloride, 0.15gms. Di- potassium hydrogen phosphate, 0.006 gms. potassium dihydrogen phosphate, 0.9 gms. di-sodium hydrogen phosphate, 1.8gms. ammonium chloride 0.3gms. magnesium sulphate, 0.0734gms. thiamine hydrochloride, 0.006gms. in 300ml of distilled water. The medium constituents were mixed with 300ml of distilled water and the medium pH was adjusted to 7.0 by using 2N NaoH and dil. Hcl. After sterilization of the media, the media was inoculated by 3ml of solution from bacillus subtilis growth medium. To get proper mixing of components utilized by the organisms, the medium was kept in a continuous shaker at 250 rpm and this shaking would also give considerable supply of oxygen.

Effect of Carbon sources for Amylase and Protease production

Different carbon sources were given to the enzyme production medium, each at a time. The various carbon sources are glucose, starch, fructose, dextrose and sucrose.

Effect of pH for Amylase

One ml of the sample from the enzyme production medium was taken and centrifuged. Then the 0.5 ml of supernatant was transferred to different test tubes. The supernatant containing enzyme was mixed with different buffers at different pH. Acetate buffer was used for pH 3.6, 4.2, 5.0. Phosphate buffer was used for pH 6.2, 7.2, 8.0. Tris Hcl buffer was used for pH 9.0. and incubated at 37^0c for 10 minutes. Then the enzyme activity was measured by DNS method.

Effect of pH for protease:

One ml of the sample from the enzyme production medium was taken and centrifuged. Then the 0.5 ml of supernatant was transferred to different test tubes. The supernatant

containing enzyme was mixed with different buffers at different pH. Acetate buffer was used for pH 3.6, 4.2, 5.0. Phosphate buffer was used for pH 6.2, 7.2, 8.0. Tris Hcl buffer was used for pH 9.0. and incubated at 37^0c for 10 minutes. Then the enzyme activity was measured by Anson's method.

Effect of Temperature for Amylase and Protease

One ml of the sample from the enzyme production medium was taken and centrifuged. Then the 0.5 ml of supernatant was transferred to different test tubes. For amylase the supernatant containing enzyme was mixed with 0.5ml of 0.1M – phosphate buffer at pH 6.6 to the test tubes. For protease the supernatant containing enzyme was mixed with 0.2 M boric acid and borax buffer at pH 8.0 to the test tubes. Then the mixtures were kept in different temperatures and their enzyme activity was found [9].

Preliminary purification of enzymes by Dialysis

The volumes of the crude enzyme extract from *Bacillus subtilis* were taken and the ammonium sulphate to be added was obtained from the solubility data. The crude enzyme solution was successively precipitated at 30% and 70% saturation of ammonium sulphate in the magnetic stirrer. Precipitate was collected by centrifugation at 10000 rpm for 10 min at 40c. Then the precipitate was dissolved in 10 ml of 50mM tris Hcl and subjected to dialysis. About 10 cm size of dialysis bag was successively boiled in 100ml of distilled water, 2% sodium bicarbonate and 1mM EDTA solution and again 100ml of distilled water for 10 min at 100^0C. Then the dialysis bag was cooled to room temperature and kept in refrigerator for 30 min. The precipitate which is dissolved in 10 ml of 50mM Tris Hcl were tramnsfered in to the dialysis bag and dialysed against same buffer overnight at 40^0C [10].

Protease Enzyme Assay

Protease Enzyme Assay Casein solution of 2% (1ml) was incubated with 1ml of enzyme solution and 1ml of sodium phosphate buffer (pH 7) for 20 minutes at 40℃. The reaction was stopped using 10% Tricholoroacetic acid solution. After 20 minutes, the mixture was centrifuged at 10,000rpm for 5minutes. After centrifugation the supernatant was developed with Bradford reagent and read at 580nM [11].

Amylase Enzyme Assay

Amylase activity was assayed as described by Bertrand et al. (2004) by taking 1ml of the crude enzyme into each of the test tubes and 1ml of 1% soluble starch in sodium phosphate buffer having a pH of 6.4.

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RESULTS AND DISCUSSIONS

In the study *Bacillus subtilis* were characterized for protease production using skim milk agar. The proteolytic activity was detected by the presence of clear zones.

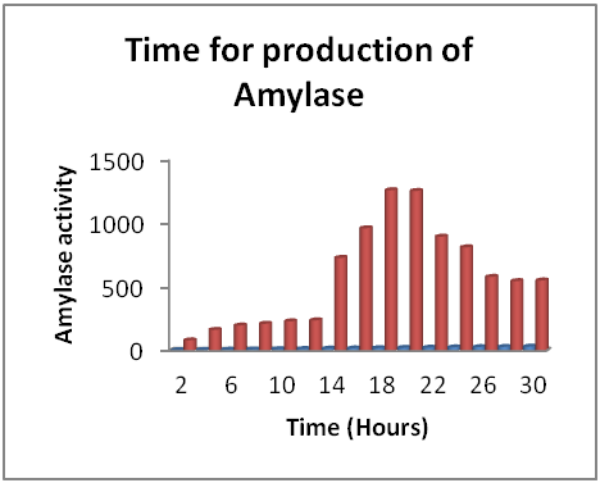

Figure 1

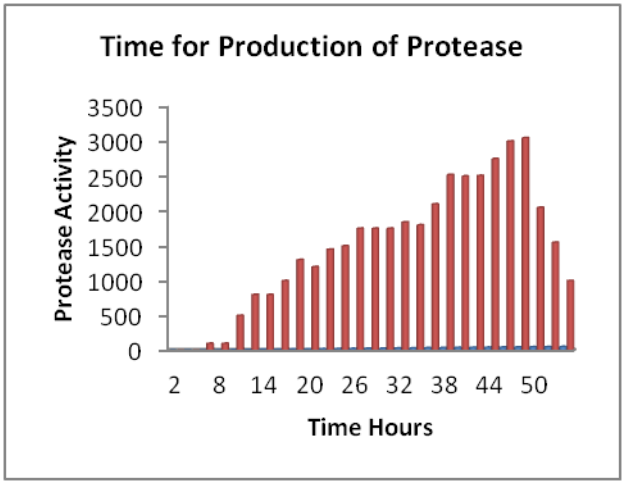

Figure 2

The maximum time for the production of enzyme was carried out in the enzyme production medium stated in materials & methods. The medium is used for the production of amylase and protease. The fermentation was carried out in a shaker at 250 rpm. With a pH of 7.0 maintained at 37^0C. The maximum enzyme production of 1266.6 µg/ml.min of amylase is found at 22 hours. The maximum enzyme production of protease is 3050 µg/ml.min at 48 hours.

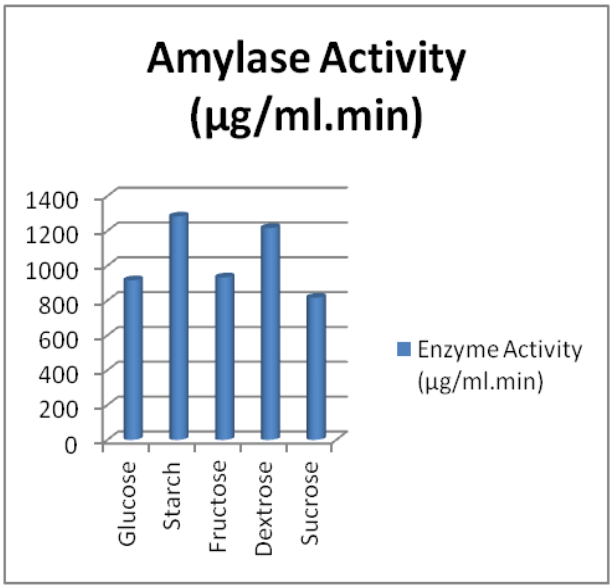

Figure 3

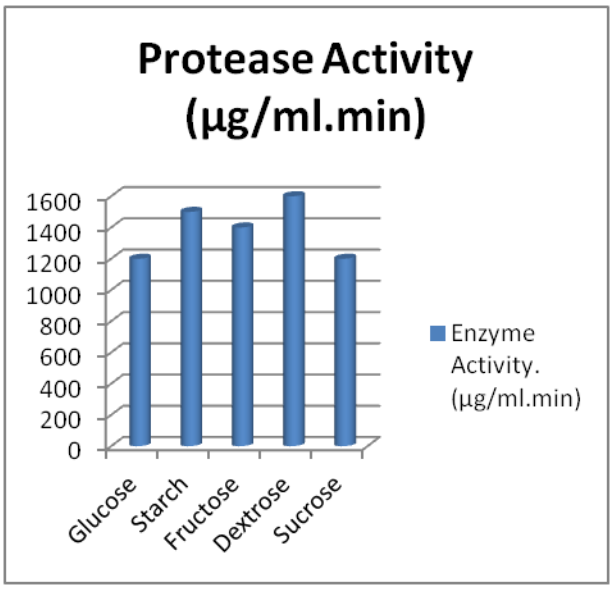

Figure 4

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Effect of carbon sources

Effect of different carbon sources were studied for the production of amylase and protease shown in figure 3 & 4. Among glucose, starch, fructose, dextrose and sucrose, starch showed the maximum production of amylase of 1283.3 µg/ml.min. Dextrose showed the maximum production of protease of 1600 µg/ml.min.

Effect of pH

The effect of pH on the production of amylase and protease was studied at different pH. On pH 6.2 it showed maximum enzyme activity of amylase. On pH 7.2 it showed maximum enzyme activity of protease shown in figure 5 & 6.

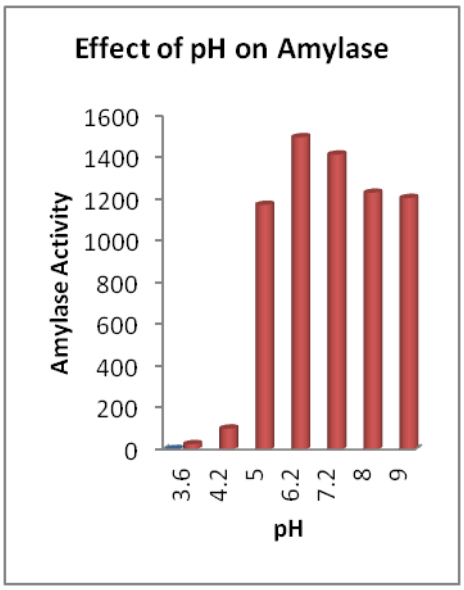

Figure 5

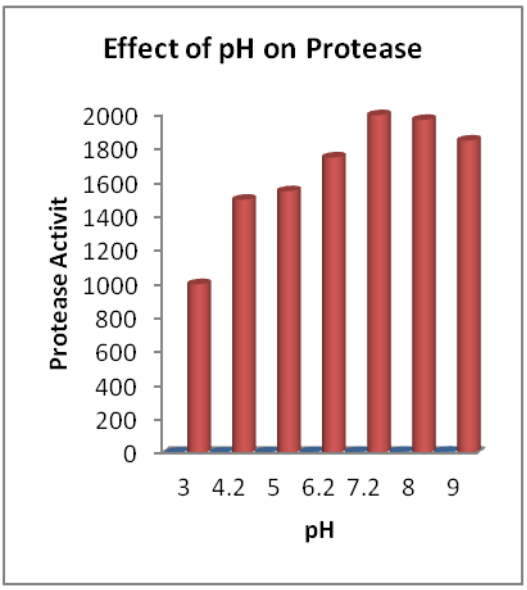

Figure 6

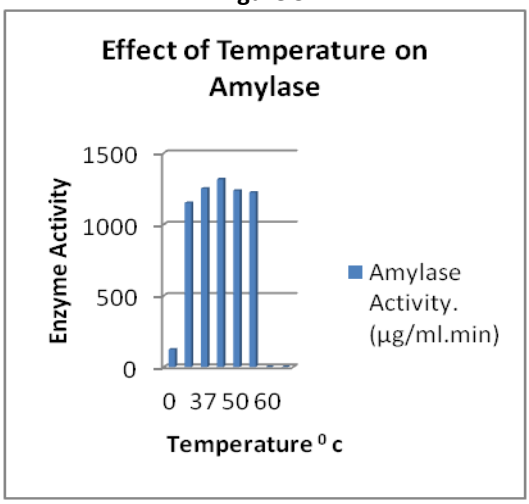

Figure 7

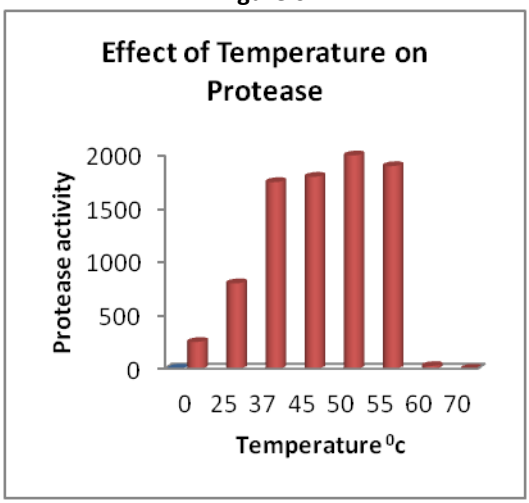

Figure 8

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Effect of Temperature

The effect of temperature on the production of amylase and protease was studied at different temperatures. When the temperature is 45^0C amylase showed maximum enzyme activity. When the temperature is 50^0C protease showed maximum enzyme activity shown in figure 7 & 8**.**

Table: 1. Preliminary Purification by Dialysis

Enzymes	Enzyme Activity(µg/ml.min) Before Purification	EnzymeActivity(µg/ml.min) After Purification
Amylase	2256.12	4066.23
Protease	2012.03	3216.20

In preliminary purification of enzymes by dialysis amylase showed maximum enzyme activity of 4066.23µg/ml.min. and protease showed 3216.20 µg/ml.min shown in table: 1.

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