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# Simultaneous Production of Protease and Alpha Amylase by *Bacillus Subtilis* in a Microprocessor Controlled Fermentor

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**Abstract**

The simultaneous production of *Bacillus subtilis* based protease and alpha amylase using a computer controlled laboratory scale 7.5 $dm^3$ batch bioreactor was described in this study. The growth and sporulation of *Bacillus subtilis* was monitored and maximum production of protease and alpha amylase was found to coincide with the maximum sporulation. Two types of proteases were detected in the fermentation broth; neutral and alkaline protease most active in a pH range of 7.0-8.0 and 8.0-10 respectively. Maximum production of proteases was observed at an incubation temperature of 37°C while that of alpha amylase was observed at 40°C. Similarly the optimum aeration and agitation for proteases production were 0.6 l/l/m and 200rpm respectively and for that of alpha amylase were also 0.6 l/l/m and 150 rpm respectively. The kinetic parameters $Y_{p/x}$ and $q_p$ were also found highly significant at above fermentation conditions.

*Keywords: Simultaneous, sporulation, protease, amylase, fermentation, agitation.*

## 1.0 Introduction

Proteases and alpha amylase are the two most important industrial enzymes representing more than 70 % [1] of the total worldwide sales of the enzyme market. Proteases are the enzymes, which catalyze the cleavage of peptide bonds and cause the total hydrolysis of proteins while alpha amylase degrades α 1-4 linkage of starch and other substrates in an endo fashion producing maltose and glucose [2 & 3]. Both the enzymes are physiologically necessary for microorganisms so they are widely distributed in a wide number of microorganisms and are successfully produced in large quantities by these organisms through fermentation process.

Both fungi and bacteria are employed for the production of proteases and alpha amylase but the choice of the organism mainly depends upon the application of the enzyme produced. For example, the proteases to be used in food industry are mainly produced by fungi; on the other hand the proteases to be used in leather or detergent industries are mainly produced by bacteria. Similarly the alpha amylase produced by fungi is more stable than the bacterial enzyme due to high thermostability and heat sensitivity [4]. The most commonly used microorganisms for protease production includes *Bacillus* spp., *Streptomyces* spp., *Aspergillus* spp., *Rhizopus* spp. and *Penicillium* spp. [5, 6 & 7] and for alpha amylase production includes *Bacillus* spp., *Steptomyces* spp., *Themomyces lanuginosus, Rhizopus* spp., *Trichoderma* spp., *Penicillium* spp. *Fusarium* spp. and *Aspergillus* spp. [8, 9, 10 & 2].

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Proteases and alpha amylase occupy a pivotal position with respect to their applications in industrial fields and hence acquired a great significance in present day biotechnology. Although they can be derived from several sources such as plants, animals and microorganisms, yet the enzyme from microbial sources generally meet the industrial demands. The proteases are widely used in brewing, baking, tenderization of meat, dairy industry, synthesis of aspartame, developing effective therapeutic agents, treatment of wounds, detergent and leather industries [11, 12, 13 & 1]. The alpha amylase is extensively used in many industries including paper, textile, pharmaceuticals [9 & 10], starch liquefaction, brewing, food and sugar industries [14, 15, & 16].

Both enzymes can be produced economically and profitably in a large scale through a fermentation process using a specific and potent strain. Submerged as well as solid-state fermentation techniques are used for the production of proteases and alpha amylase however, bacteria usually give higher yields through submerged fermentation. A new trend in the fermentation technology has arrived which is the simultaneous production of two or more than two products. In this technique, more than one product is produced by a single microorganism in a single fermentation batch under same or different conditions. Several workers have used this technique and have successfully produced more than one product simultaneously in a single batch operation [17, 18 & 19]. Such fermentation technique can save time, labor and energy can be saved and fermentation batches can be economized for maximum outputs from minimum inputs.

Keeping in view the economics of batch fermentation, the said work was undertaken to produce two most important commercial enzymes simultaneously by a single strain of *Bacillus subtilis* using submerged fermentation technique.

## 2.0 Materials & Method

### *2.1 Organism, Growth and Maintenance*

A potent strain of *Bacillus subtilis* was taken from the culture bank of Institute of Industrial Biotechnology, GC University, Lahore. The culture was grown and maintained on nutrient agar medium. The bacterial slopes were incubated at 37 °C for 48 hours and then stored at 4°C. However, the strain was weekly transferred onto fresh slopes.

### *2.2 Inoculum preparation*

The inoculum of *Bacillus subtilis* was prepared in 500 ml Erlenmeyer flasks containing 100 ml of nutrient broth medium. The flasks were sterilized in an autoclave at 121 degrees C (15 psi) for 15 minutes. After cooling, the medium was aseptically inoculated with bacteria from a 48 hours old slope. The flasks after inoculation were incubated for 24 hours on a rotary incubator shaker (Gallenkamp, UK) at 37°C and 200 rpm.

### *2.3 Fermentation Experiments*

The fermentation experiments for the simultaneous production of alkaline protease and alpha amylase from *Bacillus subtilis* were carried out in a laboratory scale 7.5 $dm^3$ batch bioreactor (New Bruinswick Scientific, USA) with a working volume of 5.0 $dm^3$. The bioreactor was equipped with a microprocessor controlled monitor, which was used to measure and control

foam, temperature, pH, stirring rate and dissolved oxygen. The vessel of the bioreactor was equipped with a four-blade turbine.

Peristaltic pumps were attached to control the foam and pH by automatic addition of an antifoam silicon agent or an acid/base respectively. Five liters of the fermentation medium containing (g/l) soybean meal, 20; starch, 10; glucose, 5.0; polypeptone, 10; $KH_2PO_4$, 1.0; $(NH_4)_2SO_4$, 1.0 and $Na_2CO_3$, 5.0 (pH 8.0) was added to fermentor vessel and was sterilized in an autoclave. After cooling, the medium was inoculated with already prepared 200 ml of the inoculum containing 3.5 x $10^8$ CFU/ml. The fermentor was run for a batch operation for the said interval of time. After a fixed period of incubation, the fermented broth was centrifuged at 5,000 rpm for 10 minutes and the supernatant was assayed for protease and alpha amylase activity.During all the experiments chemicals of analytical grade and calibrated lab ware were used.

### *2.4 Assay of Protease*

The method of McDonald and Chen [20] was used for the assay of protease. Casein (1% solution in 0.1 M Phosphate buffer of pH 8.0) was incubated with one ml of enzyme sample at 30°C for one hour. The reaction was initiated by the addition of five ml of 5% trichloroacetic acid (TCA) solution. The mixture was then centrifuged at 5000 rpm for 10 min. and one ml of supernatant was mixed with five ml of alkaline reagent. One ml of 1N NaOH was added to this mixture to make the contents of the tube alkaline. After 10 min., 0.5 ml of Folin and Ciocalteau reagent was added to the test tubes and mixed. The blue colour developed was measured with UV spectrophotometer (CECIL, CE 7200, Cambridge, England) at 700 nm after 30 min. One unit of protease activity is defined as the amount of enzyme required to produce an increase of 0.1 in optical density at 700 nm under the defined conditions.

### *2.5 Assay of alpha amylase*

Alpha amylase activity was estimated according to the method of Rick and Stegbauer [21]. One milliliter of enzyme sample was incubated with 1 ml of 1% soluble starch solution (in 0.1 M phosphate buffer pH 7.0) at 40°C for 10 min. The reducing sugars were measured by adding 3,5-dinitro salicylic acid reagent to the reaction mixture. The tubes were boiled for 5 min, cooled and measured for O.D at 546nm in the spectrophotometer. One unit activity is defined as the amount of enzyme that releases 1 µ mole of maltose per minute.

### *2.6 Kinetic analysis*

Kinetic parameters for batch fermentation experiments were determined according to the methods described by Pirt [22] and Lawford and Rouseau [23]. The following parameters of kinetics were studied:

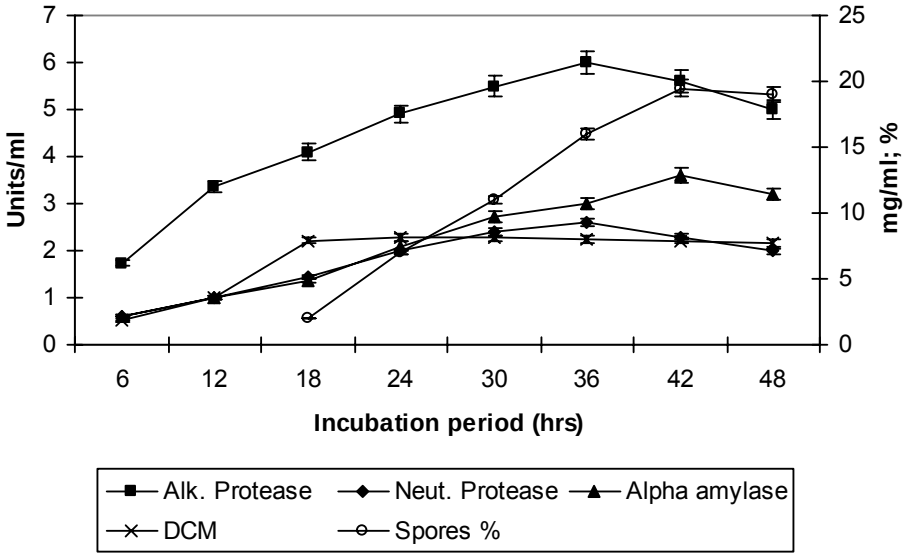
i. Maximum specific growth rate $(\mu)_{max}$ per hour - The value of $(\mu)_{max}$ was calculated from plot of ln x vs. time of fermentation.
ii. Product yield coefficient (Y p/x) U/ml/mg - The value of Y p/x was determined by the equation: $Y\ p/x = dp/dx$
iii. Specific product yield coefficient ($q_p$) U/ml/h - The value of $q_p$ was determined by the equation: $Yp/x\ .\ (\mu)_{max}$

## 3.0 Results and Discussion

### *3.1 Relationship of growth and sporulation with enzyme production*

The correlation of growth and sporulation of *Bacillus subtilis* with the enzyme production was studied and a set of typical enzyme production curves is presented in Figure 1 along with growth and sporulation curves. Figure 1 shows that bacterial growth reached its maximum value after 24 h of cultivation while maximum sporulation occurred after 42 h of incubation. It is also clear from the results that the production rate of alpha amylase is linear up to about 36 h and maximum alpha amylase production coincides with maximum sporulation. In fact, the curves for alpha amylase production and sporulation in *B. subtilis* are almost identical. During the present studies, it was found that two types of proteases i,e; alkaline and neutral protease were secreted in the fermentation broth by the microorganism as already reported by other workers [24 & 25]. The maximum production of alkaline protease also corresponded with the point of maximum sporulation, but the maximum production of neutral protease occurred somewhat earlier at about 36 h of cultivation. Alkaline protease is an absolute requirement for sporulation [26] in *B. subtilis* hence the name "sporulation protease".

Under most growth conditions, *Bacillus* species produce extracellular protease during the post-exponential growth phase [27]. Mandelstam [26] attributed this behavior to an increased need for turnover of cell proteins at a slower growth rate. Similarly the role of alpha amylase in sporulation is to liberate energy for bacteria by hydrolysis of materials present in the substrate. Over production of some extracellular enzymes is a reflection of very high demands of energy and nitrogen compounds for sporulation.




*Inc. temperature: 35 ℃; Agitation: 200rpm; Aeration: 0.6 l/l/m*

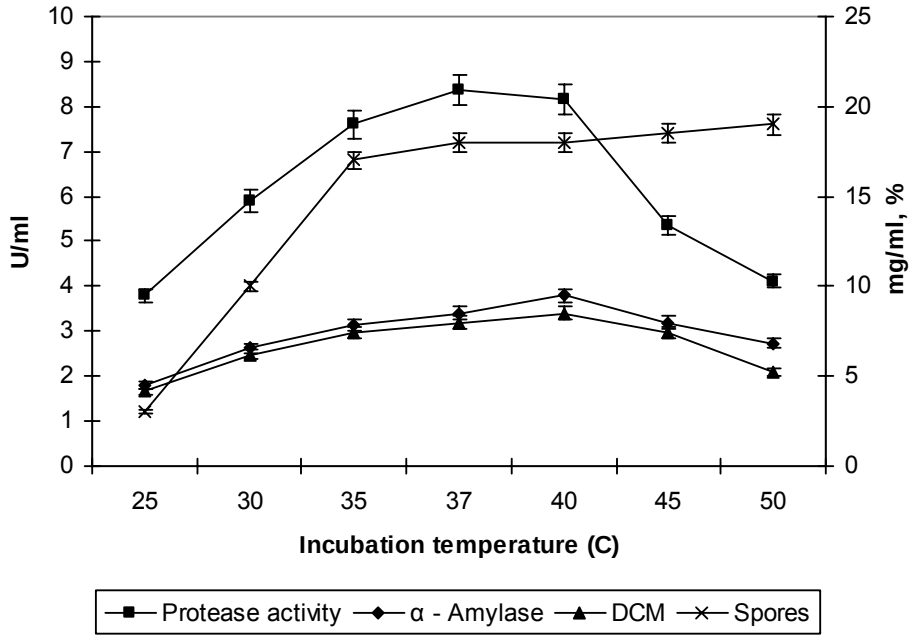
Figure 1 Relationship of growth and sporulation with the production of proteases and alpha amylase by *Bacillus subtilis*

### *3.2 Effect of incubation temperature on enzyme production*

The effect of incubation temperature (30-50°C) on enzyme production, cell growth and sporulation was also studied and the results were presented in Figure 2. At low temperature

(25°C), cell mass was less (4.14 mg/ml), whereas at 40 °C cell mass was maximum i,e; 8.5 mg/ml. Sporulation shows a progressive increase from 25 to 35°C (3-17 %) and remains almost constant at 17-19 % within the temperature range of 35°C and 50°C, in which the maximum yields of alpha amylase was also found. However, production of proteases appeared to be maximum at a cultivation temperature of 37°C.

For alpha amylase production, it appears that a temperature of 40°C is optimal whilst for protease the optimum was around 37°C. There was a very sharp increase for proteolytic activity near 37°C, whilst the maximum for alpha amylase was observed at 40°C and the increment was gradual. The kinetic parameters also revealed the same results as depicted in the experiments. The product yield coefficient (Yp/x) and specific product yield coefficient ($q_p$) for protease production were maximum at 37°C showing the optimum temperature for protease production while in the case of alpha amylase production the kinetic parameters showed maximum values at an incubation temperature of 40°C.




*Inc. period: 42hrs; Agitation: 200rpm; Aeration: 0.6 l/l/m*

Figure 2 Effect of temperature on the production of proteases and alpha amylase by *Bacillus subtilis*

### *3.3 Effect of aeration and agitation on enzyme production*

In the experiments concerned with variation in aeration (0.2-1.0 l/l/m), the maximum cell mass was found at an aeration rate of 0.6 l/l/m, in conjunction with a sporulation rate of 18.5 % (Figure 3). These properties of growth and sporulation corresponded to the maximum production of both the enzymes tested.

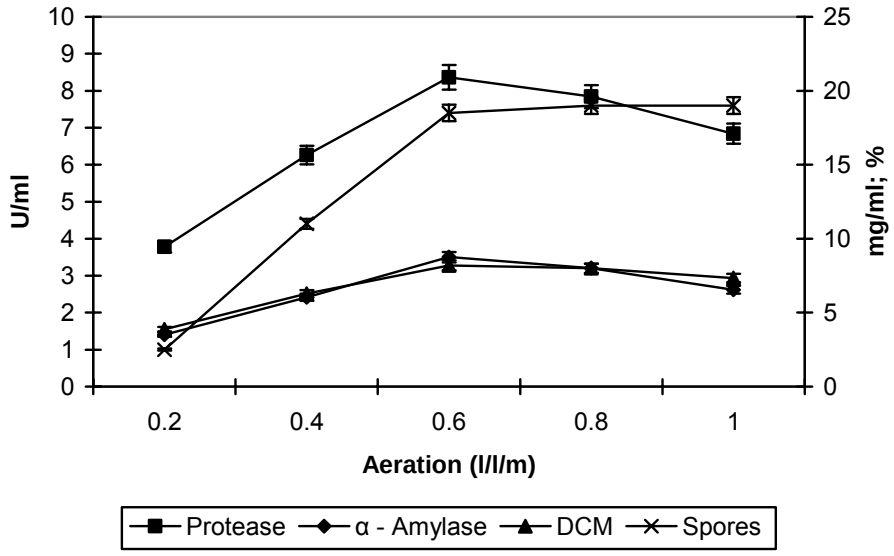
In the agitation experiments, variable stirring speeds ranging from 100 – 300 rpm were evaluated. The stirring speeds lower than 200 rpm were not sufficient to keep the solids in the medium in homogeneous suspension and secondly to break the clumps of cells. On the other hand, at speeds higher than 250 rpm, excessive foaming occurred thus necessitating the

automatic addition of such large quantities of antifoam agent (silicon oil) and thus impairing the yielded enzyme. In addition, the lower speed (150 rpm) was found to be suitable for alpha amylase production, but for proteases the higher speed (200 rpm) produced significantly better results.

Table 1 Product yield coefficient (Yp/x) and specific rate of product formation ($q_p$) of *Bacillus subtilis* at different incubation temperatures

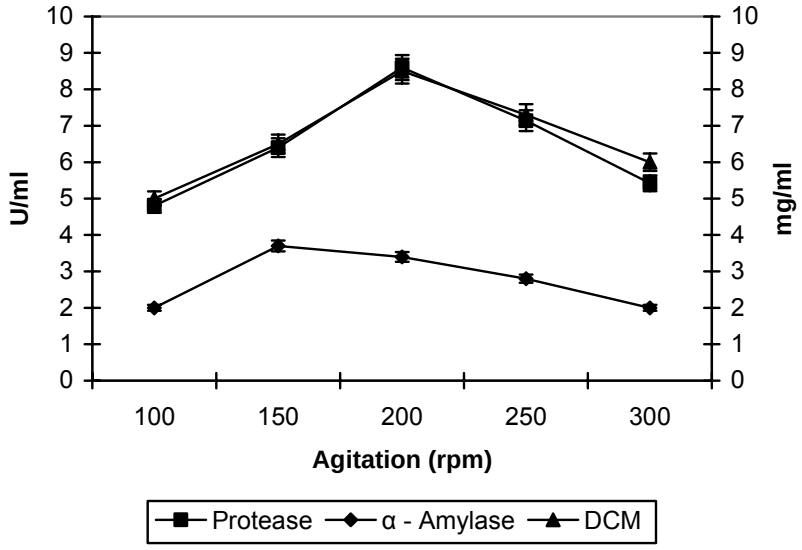
| **Incubation temperature (C°)** | **Y p/x for protease (U/ml/mg)** | **$q_p$ for protease (U/ml/h)** | **Y p/x for alpha amylase (U/ml/mg)** | **$q_p$ for alpha amylase (U/ml/h)** |
|---|---|---|---|---|
| 25 | 0.917 | 0.121 | 0.434 | 0.057 |
| 30 | 0.951 | 0.126 | 0.422 | 0.056 |
| 35 | 1.02 | 0.135 | 0.437 | 0.058 |
| 37 | **1.047** | **0.139** | 0.425 | 0.056 |
| 40 | 0.96 | 0.127 | **0.45** | **0.059** |
| 45 | 0.722 | 0.096 | 0.431 | 0.057 |
| 50 | 0.686 | 0.091 | 0.430 | 0.057 |

The values of Y p/x and qp obtained after kinetic analysis of results revealed that the best aeration and agitation for protease production were 0.6 l/l/m and 250 rpm respectively while for alpha amylase production the same aeration rate but a different agitation rate i,e; 150 rpm was found to be significant.




*Inc. temperature: 35 ℃; Agitation: 200rpm; Inc. period: 42hrs*

Figure 3 Effect of aeration on the production of proteases and alpha amylase by *Bacillus subtilis*




*Inc. temperature: 35 ℃; Inc. period: 42hrs; Aeration; 0.6 l/l/m*

Figure 4 Effect of agitation on the production of proteases and alpha amylase by *Bacillus subtilis*

Table 2 Product yield coefficient (Y p/x) and specific rate of product formation ($q_p$) of *Bacillus subtilis* at different aeration levels

| **Aeration (l/l/m)** | **Y p/x for protease (U/ml/mg)** | **$q_p$ for protease (U/ml/h)** | **Y p/x for alpha amylase (U/ml/mg)** | **$q_p$ for alpha amylase (U/ml/h)** |
|---|---|---|---|---|
| 0.2 | 0.97 | 0.129 | 0.36 | 0.047 |
| 0.4 | 0.99 | 0.131 | 0.38 | 0.050 |
| 0.6 | **1.02** | **0.135** | **0.42** | **0.055** |
| 0.8 | 0.98 | 0.130 | 0.40 | 0.053 |
| 1.0 | 0.93 | 0.123 | 0.35 | 0.046 |

Table 3 Product yield coefficient (Y p/x) and specific rate of product formation ($q_p$) of *Bacillus subtilis* at different agitation rates

| **Agitation (rpm)** | **Y p/x for protease (U/ml/mg)** | **$q_p$ for protease (U/ml/h)** | **Y p/x for alpha amylase (U/ml/mg)** | **$q_p$ for alpha amylase (U/ml/h)** |
|---|---|---|---|---|
| 100 | 0.96 | 0.127 | 0.4 | 0.053 |
| 150 | 0.98 | 0.130 | **0.56** | **0.074** |
| 200 | **1.01** | **0.134** | 0.4 | 0.053 |
| 250 | 0.97 | 0.129 | 0.38 | 0.050 |
| 300 | 0.90 | 0.119 | 0.33 | 0.043 |

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