

EFFECT OF DIFFERENT PARAMETERS ON OPTIMUM PRODUCTION OF MICROBIAL ALPHA AMYLASE PRODUCTION FROM *BACILLUS SUBTILLUS*

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Abstract

This paper covers the progress made in research on microbial alpha-amylase, a highly demanded industrial enzyme in various sectors such as food, pharmaceuticals, textiles, detergents, etc. Amylases are of ubiquitous occurrence and hold the maximum market share of enzyme sales. The article surveys the α -amylase family and the major characteristics, microbial sources, production aspects, downstream processing, salient biochemical properties, industrial applications, enzyme engineering and some recent research developments.

*The present study was concerned with the production of α amylase by *Bacillus Subtilis*. The fermentation was carried out by 250 mL Erlenmeyer flask. The maximum production of enzyme optimized at the pH 7.5 (561 IU/mL/min), while the incubation temperature investigated and the maximum production optimized at 40°C (553 IU/mL/min), The production of the enzyme was obtained maximum at 48 hours after incubation (542 IU/mL/min). The nitrogen sources tested NaNO_3 where the best inorganic sources respectively.*

INTRODUCTION

Alpha (α)amylase, an extracellular enzyme degrades α , 14 glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose a. Alpha amylase activity is frequently determined by an amyloclastic method which is based on the rate of enzymatic hydrolysis of a soluble starch substrate. This method depends on the fact that as sample alpha amylase fragments the substrate, there is a progressive decrease of the blue color formed when iodine is added. A typical amyloclastic procedure is that of the AOAC (1960) which with certain modifications (Briggs, 1961) is used extensively. The first enzyme produced industrially was an amylase from a fungal source in 1894, which was used for the treatment of digestive disorders (7). α -Amylases are ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. Amylases from plant and microbial sources have been employed for centuries as food additives. Barley amylases have been used in the brewing industry.

Fungal amylases have been widely used for the preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization (Burhan et al., 2003). The liquid culture in submerged fermentation is preferable since it allows supplementation of specific growth components such as carbon, nitrogen or inorganic sources that affect microbial growth and product formation (Prescott, 1987). However, the mutant strain of *B. Subtilis* was found to be the best for biosynthesis of α amylase. The production of amylase is dependent on the strains, composition of media, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, time of incubation and thermostability. The effect of temperature on the relative activity of α amylase from *B. subtilis* was detected and temperature was optimized between 60°C- 70°C, for maximum

activity (Kim *et al.*, 1995). These uses have placed greater stress on increasing indigenous alpha amylase production and search for more efficient processes. The production of amylase is dependent on the strain, composition of media, methods of cultivation, cell growth, nutrient requirements, metal ions, pH, temperature, time of incubation and thermostability (Chang *et al.*, 1995). Highly active alpha amylase is very essential for the conversion of starches into oligosaccharides. So, it is worthwhile to isolate a potent strain of microorganism for the production of alpha amylase.

Using enrichment techniques a large number of microorganisms like fungi and bacteria have been isolated from soil and extensively screened for alpha amylase production (Shin *et al.* 1991). Among them *Bacillus* species such as *Bacillus subtilis* and *Bacillus licheniformis*, remain the organisms of choice for better production of alpha amylase (Sunkyoung and Kyeokeun, 1996). In the present work, the influence of different carbon, nitrogen sources, surfactants, soluble starch and yeast extract on αamylase production in *Bacillus subtilis* was examined.

MATERIALS AND METHODS

Isolation of Organisms

The bacterial cultures of *Bacillus* species was isolated from different soil samples by serial dilution method (Clark *et al.*, 1958). The nutrient broth-starch- agar medium (Table 1) were used for the isolation of bacteria.

Table1: Composition of Nutrient broth Starch Agar Medium.

Ingredients (g/1)	Concentrations
Nutrient broth	8.0
Starch	10.0
Agar agar	20.0
1000 ml	Distilled water

One gram of the soil sample was dissolved in 100 ml of sterilized saline water and suitable dilution was made. Ten ml of the diluted suspension was taken in a test tube and given heat shock at 90°C for 15 minutes and then cooled at room temperature.

0.5 ml of each diluted suspension will be transferred to the Petri plates containing nutrient broth-starch-agar medium. The Petri plates were gently rotated clockwise and anticlockwise to facilitate a uniform spreading of diluted suspension on nutrient broth starch agar medium. The Petri plates were incubated at 40°C in the incubator for 18-48 hours. Independent colonies showing clear zones of starch hydrolysis will be picked up and transferred to nutrient starch agar slants. The slants were incubated at 40°C for maximum growth. The slants were then stored at 5°C in the cold cabinet.

Screening and Identification of *Bacillus* Strain

The isolated *Bacillus* strains were screened for alpha amylase synthesis by shake flask fermentation. The selected *Bacillus* strain was identified, on the basis of standard morphological and biochemical tests (Buchanan and Gibbons, 1974).

Culture Maintenance

Ten ml of nutrient starch agar medium (Table 1) were transferred to Mc-Cartny bottles. The bottles was sterilized in an autoclave at 151b/inch² pressure (121°C) for 15 minutes and placed in slanting position at room temperature and allowed to solidify. A loop full of bacteria from the slants was aseptically transferred to each Mc-Conckey bottle. The bottles were incubated at 40°C for 24-48 h for maximum growth. After sufficient growth, sterilized paraffin oil was added to each bottle to cover the whole surface area of the bacterial growth and placed in the cold cabinet at 5°C for culture maintenance.

Fermentation Technique

Alpha amylase fermentation was carried out by submerged fermentation. The submerged fermentation was used for production of alpha amylase.

Basal medium for fermentation

Inoculums preparation

Submerged fermentation was carried out in shake flask and stirred fermentor.

ShakeFlaskTechnique

Fifty ml of the fermentation medium (Table 2) were transferred to each of 250 ml cotton

plugged conical flask. The flasks were sterilized in the autoclave and cooled at room temperature. Each flask was inoculated with one ml of bacterial inoculum. The flasks were then placed in the rotary shaking incubator "200•rpm"•at•40°C•for•48•hours. After 48 h, the fermented broth was centrifuged at 7000 rpm for 15 min. The supernatant were used for the estimation alpha amylase. All the experiments were carried out triplicates.

Table 2: Composition of Inoculum Medium

Ingredients (g/l)	concentration
Peptone	1.5
Beef extract	1.0
Soluble starch	5.0
Phosphate buffer (0.02M)	500ml
pH=7.5	

Optimization of various parameters for alpha Amylase production in shake flask

i) Effect of Temperature

The effect of temperature on the production of alpha amylase from *Bacillus Subtilis* was carried out. The fermentation was carried out at 20, 25, 30, 35, 40, 45, 50, 55 °C.

ii) Effect of pH

The effect of pH on the production of alpha amylase from *Bacillus Subtilis* was carried out. The initial pH of the fermentation medium was varied from 5.0, 5.5 6.0, 6.5 7.0, 7.5, 8, 8.5 and 9. The incubation temperature of the fermentation medium was maintained at 40°C.

iii) Effect of Incubation Period

Growth of *Bacillus Subtilis* and production of enzyme were carried out for 72 h at 40°C. The sample will be collected after every 6hrs to observe the production of alpha amylase.

iv) Effect of Different Nitrogen Sources

Different inorganic nitrogen sources such as ammonium nitrate, ammonium sulphate, ammonium chloride, urea and ammonium carbonate (containing 0.2% nitrogen) were evaluated for the production of alpha amylase by parental and mutant strain of *Bacillus Subtilis*.

RESULTS AND DISCUSSION

Effect of Incubation Temperature

The effect of temperature on bacterial growth and αamylase production from *Bacillus* strain was studied. The production of enzyme and bacterial growth was determined at different temperatures ranging from 30°C to 60°C. The data of Ttable.1 shows the effect of different incubation temperatures on the production of αamylase by *B. Subtilis*. The fermentation was carried out at 20, 25, 30, 35, 40, 45, 50, 55 °C in rotary incubator shaker. The maximum production of αamylase was obtained at 40°C (553 IU/mL/min). As the incubation temperature was increased, the production of the enzyme was decreased. The production of the enzyme was greatly inhibited at 30°C (296 IU/ml/min). Thus the incubation temperature 40°C was selected for maximum production of enzyme.

Effect of different temperatures on the production of alpha amylase by *Bacillus Subtilis* (pH=7.5, Incubation time period =48 h)

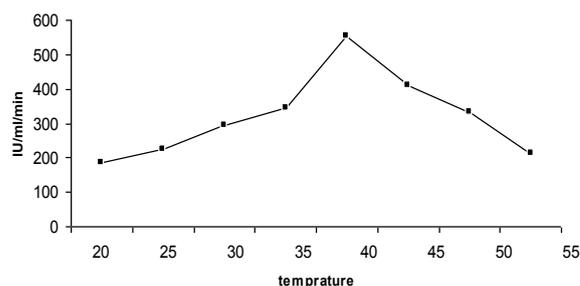


Figure 2: Effect of incubation temperature.

Effect of pH on the Activity of Enzyme

The effect of initial pH of reaction mixture (enzyme substrate complex) for the activity of αamylase. The enzyme activity was extremely low at pH 5.0 (117 IU/mL/min). The activity of enzyme was gradually increased and found maximum at pH 7.5 (561 IU/mL/min). Further increase in the initial pH resulted decrease in the activity of αamylase. However, the pH of reaction mixture for the hydrolysis of starch

was found to be optimum at 7.5.

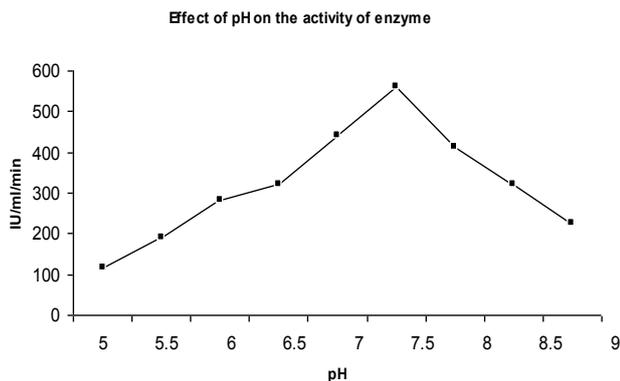


Figure 2: Effect of pH on the activity of enzyme.

Rate of α amylase Fermentation

Table 2 shows the time course of α amylase fermentation by *Bacillus Subtilis* in shake flask. The culture was incubated at 40°C for different intervals of time (0-72 h). The production of enzyme was reached maximum (542 IU/mL/min) at 48 h after inoculation. Further increase in incubation period however, did not show any significant increase in enzyme production rather it was decreased. Thus optimum time of enzyme synthesis was found to be 48 h after inoculation.

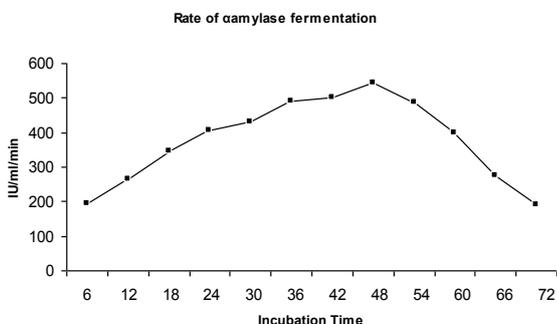


Figure 3: Rate of α amylase fermentation.

Effect of Different Nitrogen Source

The inorganic salts on the basis of 0.2% nitrogen were added in the fermentation medium. The maximum production of alpha amylase was achieved in the culture medium containing sodium nitrate. Other nitrogen sources such as ammonium nitrate, ammonium sulphate, ammonium chloride, urea and ammonium carbonate gave 1779, 2187, 2107, 1254, and 1072 U/ml respectively (table 5). The different concentration of sodium nitrate was also evaluated for the production of

alpha amylase (table 5). 0.2% sodium nitrate on the basis of nitrogen was found to be optimum for the production of alpha amylase. Further increase in the nitrogen resulted decrease in the production of alpha amylase, however 0.2% nitrogen in the form of sodium nitrate was selected.

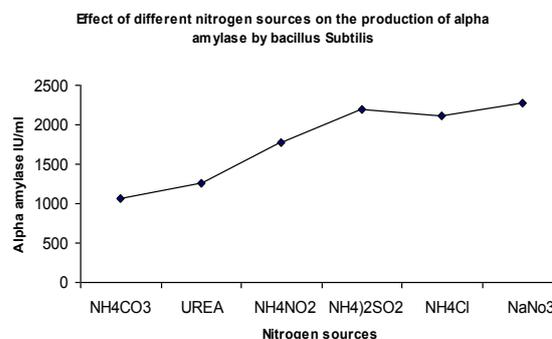


Figure 4: Effect of different Nitrogen source.

The production and stability of α amylase depends upon temperature. In present study, the fermentation was carried out at different incubation temperatures. The maximum production of enzyme was observed at 40°C. Biosynthesis of α amylase was significantly decreased with the increase in the incubation temperature beyond 40°C. It might be due to that at high temperature, the growth of the bacteria was greatly inhibited and hence, enzyme formation was also prohibited (Haq *et al.*, 1997; Chengyi *et al.*, 1999) Optimization of the volume of fermentation medium is very necessary for air supply, nutrient supply, growth of microorganisms and production, of enzyme (Ivanova *et al.*, 2001). In present study, the rate of enzyme was increased with the increase in the fermentation period and reached maximum 48 h after inoculation. It might be due to the organism entered in the incubation period resulted in the decreased production of α amylase. It may be due to the accumulation of other by products in the fermentation medium. In present study, the rate of enzyme was carried out after every 6hr of incubation. The production of enzyme was increased with the increase in the fermentation period and reached maximum 48 hr after inoculation further increase in the incubation period result in the decrease production of alpha amylase. It might be due to depletion of the nutrients and death phase of the bacteria due to production of protease in the

fermentation medium. At the death phase of the bacterium the production of alpha amylase in the fermentation medium was greatly inhibited.

The hydrolytic action of α amylase is greatly affected by pH. In present study, the different pH (4-9) of starch solution was tested for the activity of α amylase. The maximum activity of the enzyme was obtained at slightly alkaline pH 7.5. At acidic pH the results were extremely low. It might be due to the enzyme was inactive in the acidic medium (Anyangwa *et al.*, 1993; Castro *et al.*, 1993). In microbial production of enzyme, nitrogen can be an important limiting factor (Park *et al.*, 1997). The effects of different organic nitrogen source on the production of alpha amylase by parental strain of *Bacillus Subtilis* have been investigated. The nutrient broth provide adequate concentration of nitrogen for the growth of the microorganism and hence enzyme formation (Nielson, 2000).

Inorganic nitrogen sources have industry effect on the production of alpha amylase (Felix *et al.*, 1996). The different inorganic nitrogen sources (containing 0.2% nitrogen) were tested for the production of alpha amylase by

parental strain of *Bacillus Subtilis*. The parental strain gave optimum result in the presence of NaNO_3 .

CONCLUSION

Alpha amylases are one of the most widely used enzymes required for the preparation of fermented foods. Apart from food and starch industries, in which demand for them is increasing continuously, they are also used in various other industries such as paper and pulp, textile, *etc.* With increase in its application spectrum, the demand is for the enzyme with specificity. Research is focused on developing thermo tolerant and pH tolerant α -amylase from microbes, modifying them genetically or applying site-directed mutagenesis to acquire desired properties in the enzyme. Commercially most of the production of α -amylase is carried out in submerged fermentation, but solid-state fermentation is being looked at as a potential tool for its production, especially applying agro industrial residues as substrate.

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