

Screening and optimization of amylase from *Citrobacter freundii* isolated from the gut of *Carassius auratus*

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Abstract : Microbial amylases have various industrial applications. A large number of these amylolytic enzymes are available commercially and have almost replaced chemical hydrolysis of starch in starch processing industry. In this study, the production of extracellular amylase by *Citrobacter freundii* was screened. The process parameters were optimized to enhance amylase production in submerged fermentation. The maximum production of amylase was observed at 40°C (261 U/ml), at pH 7 (219 U/ml). Among the carbon sources, cellulose enhanced enzyme production (58 U/ml). Among the tested nitrogen sources, peptone recorded the highest influence on amylase production (212 U/ml) by *Citrobacter freundii*.

Keywords - Amylase, *Citrobacter freundii*, submerged fermentation.

I. INTRODUCTION

Enzymes are biological catalysts; they are catalytic proteins, which enhance the rate of thermodynamically favorable biological reaction to several thousands to million folds. Enzymes are highly specialized catalysts with extraordinary catalytic power and also with remarkable specificity catalyzing almost all cellular reactions. Therefore they are known as the basis of life. The use of enzyme mediated process can be traced to ancient civilizations. Today nearly 400 enzymes are known of about 200 are in commercial use. The majority of the industrial enzymes are microbial origin. Until 1960s the total sales of enzymes are only few million dollars annually, but the market has since grown spectacularly (Sharma *et al.*, 2001). Microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Chu, 2007). In order to meet the industrial requirements as well as increasing demand of global enzyme market, isolation and optimization of enzyme production conditions of new promising strains should be a continuous process.

Amylases are among the important industrial enzymes & also have great significance in biotechnological studies. Microbial production of amylase is more effective than that of other sources as the technique is easy, cost effective, and fast and can be modified to obtain enzymes of desired characteristics. The microbial amylase could be potentially useful in various pharmaceutical, fine-chemical industries etc., with the event of new frontiers in biotechnology, 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 (Souza and Magalhaes, 2010). These uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes. Many microorganisms are known to produce this enzyme which includes bacteria and fungi (Souza and Magalhaes, 2010). The one most commonly used for their industrial production are *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Aspergillus niger*. In order to meet the industrial requirements as well as increasing demand of global enzyme market, isolation and optimization of enzyme production conditions of new promising strains should be a continuous process. The main objective of the present study is optimize the process parameters for the production of amylase from *Citrobacter freundii*.

2.1. Screening of intestinal bacterial isolates for amylase production

The gut of *Carassius auratus* was dissected out and used for the isolation of amylase producing bacteria. All the isolates with apparently different morphological appearance were screened for the production of extra-cellular amylase. For screening of amylase producing strains, isolates were streaked on starch (1%) supplemented nutrient agar plates and incubated at 32°C for 48 h. The culture plates were then flooded with 1% Lugol's iodine solution (Jacob and Gerstein, 1960) to identify amylase activity.

2.2. Production of Amylase in submerged fermentation (SmF)

The culture medium used in this work for amylase production 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 and pH maintained to 7.0. 50 ml of production medium in 250 ml Erlenmeyer flask was inoculated with 5% inoculum of overnight grown culture *Citrobacter*

freundii and incubated at 30°C on rotary shaker at 100 rpm for 48 h. 2 ml of broth was harvested aseptically and centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant thus obtained was used for amylase assay.

2.3. Optimization of various parameters for enzyme production

2.3.1. Effect of different temperature on enzyme production

To determine the optimum temperature for maximum enzyme production, the test strain was inoculated in production media at different temperatures. 50 ml each of sterilized media were inoculated with 0.5 ml of inoculum in Erlenmeyer flask (250 ml) and incubated over a period of 2 days at different temperatures (25, 30, 35, 40 and 45°C). After incubation, the amylase production was estimated from the culture supernatant by spectrophotometric assay method.

2.3.2. Effect of different pH on enzyme production

The effect of different pH on enzyme production was determined by preparing production medium with different pH ranges of 5, 6, 7, 8 and 9 in Erlenmeyer flasks. Then these flasks were sterilized at 121°C for 15 minutes. After sterilization the flasks were allowed to cool and 0.5 ml inoculum was added in Erlenmeyer flask. It was incubated at 30°C for 2 days in a shaker at 150 rpm. After incubation, the production of amylase was determined in the culture supernatant by spectrophotometric assay method.

2.3.3. Effect of different carbon sources on enzyme production

To test the effect of different carbon sources on enzyme production by *Citrobacter freundii* five different carbon sources were screened. They were glucose, fructose, maltose, starch and cellulose. They were supplied individually in addition to the production media at a concentration of 1% taken in conical flasks. Then the flasks were sterilized at 121°C for 15 minutes and cooled. Then 0.5 ml inoculum was added in Erlenmeyer flask and incubated at 30°C for 2 days in a shaker at 150 rpm. Then, amylase production was estimated in the culture supernatant by spectrometric assay method.

2.3.4. Effect of different nitrogen sources on enzyme production

The effect of different nitrogen sources on enzyme production by *Citrobacter freundii* was determined. Five different nitrogen sources such as yeast extract, peptone, glycine, ammonium sulphate and ammonium nitrate were individually tested. They were added in addition to the production medium for amylase production. The flasks were sterilized at 121°C for 15 minutes and cooled. Then, 0.5 ml inoculum was added in each flask and incubated at 30°C for 2 days in a shaker at 150 rpm. Finally the enzyme production was determined in the culture supernatant by spectrophotometric assay method.

3. RESULTS AND DISCUSSION

3.1. Effect of temperature on amylase production

The production of amylase increases with the increase in incubation period up to 40°C. The maximum production of amylase was observed at 40°C (261 U/ml) by the strain *Citrobacter freundii* (Fig. 1). Temperature is an effective parameter for production of amylase by bacteria. Results are supported by previous studies carried out for maximum production of amylase at temperature 37°C (Mishra *et al.*, 2005). Ashwini *et al.* (2011) also reported that *Bacillus* sp. was not capable of producing the enzyme at temperature below 25°C on other hand, a progressive decline in enzyme production was observed at 45°C and no enzyme production was observed at 50°C.

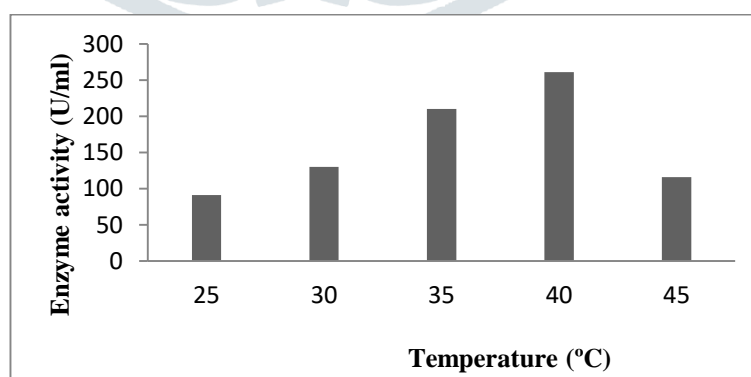


Fig. 1. Effect of different temperature on amylase production

3.2. Effect of different pH on amylase production

The production of amylase increases with the increase in pH up to 7. The maximum production of amylase was observed at pH 7 (219 U/ml) by the strain *Citrobacter freundii* (Fig. 2). Similar results have been reported by earlier studies carried out for production of amylase by Vidyalakshmi *et al.* (2009). Earlier studies have revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth. The pH is known to affect the synthesis and secretion of α -amylase like its stability. Similarly, in various *Bacillus* sp. maximum amylase production was observed in pH range of 6.5 to 7.5 (Deb *et al.*, 2013).

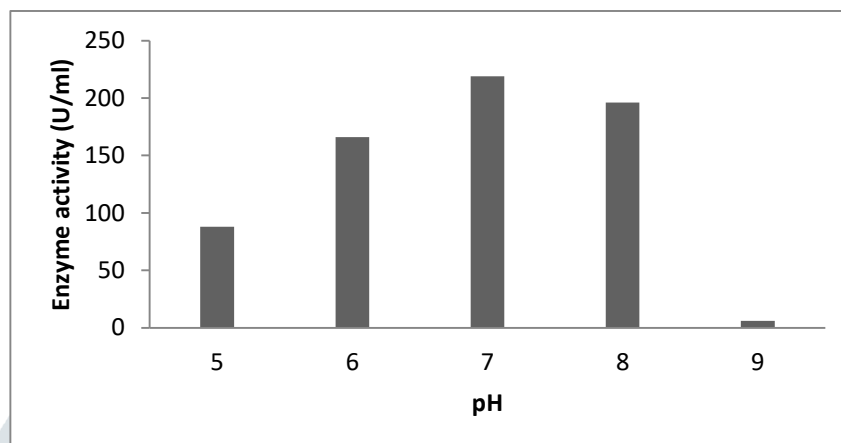


Fig. 2. Effect of different pH on amylase production

3.3. Effect of different carbon sources on amylase production

The effect of carbon sources at 1% (w/v) level was determined on amylase production. The result on the ability of *Citrobacter freundii* on production of amylase by utilizing the carbon sources is given in Figure 3. Similar results were also been reported for the best growth and amylase activity in the presence of starch as carbon source at 37°C for *Citrobacter* sp. (Kar and Ghosh, 2008). Results are supported by earlier studies carried out for production of amylase with starch as carbon source for *Citrobacter* sp. (Ashwini *et al.*, 2011).

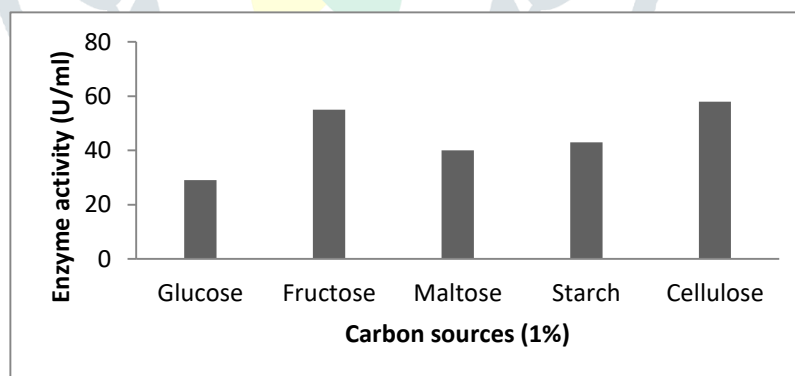


Fig. 3. Effect of different carbon sources on amylase production

3.4. Effect of different nitrogen sources on amylase production

The result on the ability of *Citrobacter freundii* on production of amylase by utilizing the nitrogen sources is given in Figure 4. Among the tested nitrogen sources, yeast extract recorded the highest influence on amylase (212 U/ml) by *Citrobacter freundii*. Amylase biosynthesis by microorganisms has been correlated to the presence or absence of several amino acids and complex nitrogen sources in the medium (Singh *et al.*, 2011).

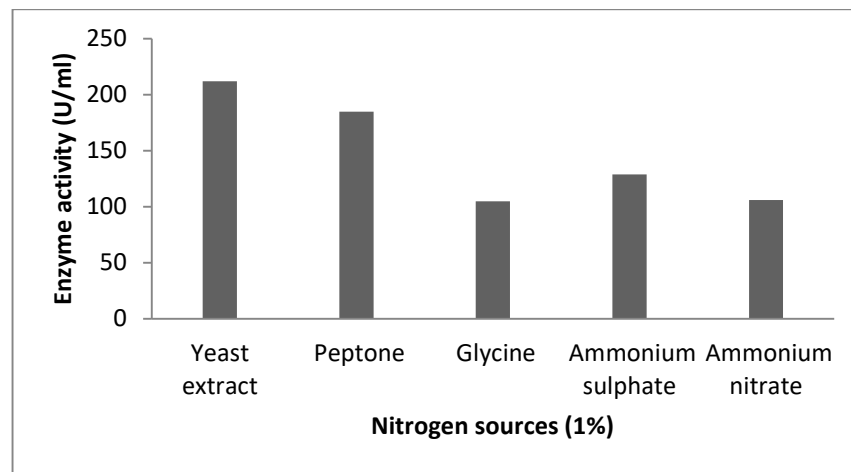


Fig.4. Effect of different nitrogen sources on amylase production

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