Research Article

# Production of Amylase by *Aspergillus niger* Using *Ziziphus* as Substrates Employing Liquid Fermentation

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**ABSTRACT**

Submerged fermentation of *Aspergillus niger* was carried out for enhanced production of α-Amylase using the seedless fruits of *Ziziphus jujuba* and *Ziziphus mauritiana* as substrate. These substrates are cheap raw materials for enzyme production. The process parameters influencing the production of α-Amylase were optimized. The optimum temperature and pH maintained for amylase activity was 30°C and 8 respectively. The amount of enzyme produced was also more in case of *Zyziphus jujuba* at 155.04 µg/ml min when compared to *Ziziphus mauritiana*. Thus these two fruits could be used for production of amylase enzyme commercially.

**Keywords:** *Ziziphus jujuba, Ziziphus mauritiana,* α-Amylase, *Aspergillus niger,* Fermentation

## INTRODUCTION

"White biotechnology" remains a challenge since new biocatalytic processes have to compete economically with the well-established chemical processes that have been optimized for years. Although many complicated chemical reactions can be efficiently performed by biocatalysts, industrial conditions are usually different from those in nature with respect to substrate concentrations, sheering forces, temperature and organic solvents.[1]

The use of conventional media for the production of industrial enzymes is very expensive. There is a need of searching more economically available substrates to reduce the cost. In this regard, residues from agricultural and food industry are generally considered as potential substrates for enzyme production. With a view to identify cost effective substrates which are easily available, two common fruits, namely, *Ziziphus jujuba* and *Ziziphus mauritiana* were identified for the production of industrial enzyme namely Amylase.

*Zizyphus jujuba* commonly called, Red date, Chinese date or Bera (Pushto), belongs to family *Rhamnaceae*. This family consists of 50 genera and more than 900 species. It is almost cosmopolitan and found mainly in subtropical to tropical areas. Many *Ziziphus* species yield edible fruits and among these we have chosen *Z. jujuba* (Chinese jujube) and *Ziziphus mauritiana* (Indian jujube), which are cultivated on a commercial scale.

Several extracellular enzymes are commercially available and widely used in industry. Among them, amylases (Alpha-Amylase (EC 3.2.1.1) also named as 4-α-D-glucan glucanohydrolase) are involved in the assimilation of starch based matter as substrates. Fungal amylases are used for hydrolyzing carbohydrate and other constituents of soy beans and wheat into simple sugar constituents. These enzymes find potential application in a number of industrial processes such as food processing, fermentation, textile, paper-industry and in biotechnology.[2] The spectrum of amylase applications has expanded into many new fields such as clinical, medicinal and analytical chemistry.[3]

The production of α-amylase by submerged fermentation using synthetic media has been reported by many workers[4-6]. The contents of such synthetic media are very expensive and uneconomical. Therefore, there is a need for replacing them by the more economically available substrates to reduce the cost. In this regard, agricultural residues and food-industry residues are generally considered as the best substrate for amylases production.[7-9] *Zizyphus* plants which are commercially cultivated yield good quantities of fruits. In the present study, *Zizyphus* fruits were tested as an economical source of carbohydrates for conversion to industrial enzymes.

Amylases are produced by a variety of micro-organisms such as *Bacillus subtilis, Rhizopus oryzae, Aspergillus niger*, *A. awamori*, *A. oryzae*.[10,11] *B. subtilis* and *R. oryzae* have been considered as the most important bacteria for industrial applications.[12-14] The selection of a particular strain, however, remains a tedious task, especially when commercially significant enzyme yields are to be achieved.

Fungi are preferred to bacteria for enzyme production because of its filamentous nature, which helps in its penetration through the submerged substrate. Amylase produced from the fungal cultures was found to be more stable than those of bacterial cultures, at a commercial scale. A lot of work has been carried out to optimize culture conditions and suitable strains of fungi.[15,16] Molds are capable of producing high amounts of amylase. *Aspergillus niger* is used for commercial production of amylase. Studies on fungal amylases especially in developing countries have concentrated mainly on

*Aspergillus niger*, probably because of their ubiquitous nature and non-fastidious nutritional requirements. Solid State Fermentation holds tremendous potentials for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as an enzyme source.[17-20]

Another advantage associated specially with *Aspergillus niger* is the control over bacterial contamination due to its capacity to grow in high range of acidic pH.

Therefore, we have chosen *A. niger* for the production of Amylase enzymes.

The aim of the present study was to investigate the potential of two *Ziziphus* species as substrate for the production of α-amylase using strains of the fungus, *A. niger* and to the determination of optimum production conditions.

## MATERIALS AND METHODS

### Source of organism

The standard strain of *Aspergillus niger* was procured from the Jayagen Biologics Analytical Laboratory, Chennai.

### Classification of *Apsargillus niger*

**Domain – Eukaryota**

**Kingdom -- Fungi**

**Phylum — Ascomycota**

**Subphylum – Pezizomycotina**

**Class – Eurotiomycetes**

**Order – Eurotiates**

**Genus – *Aspergillus***

**Species – *Aspergillus niger***

### Subculturing

Frozen stocks on agar slants were activated periodically (fortnightly) and maintained on PDA-agar slants. The mother culture has been subcultured for three times. Figure 1 shows the agar slant prepared from the strain of *Aspergillus niger*.

**Figure 1:** Agar slants of *Aspergillus niger*

### Collection of samples

*Z. mauritiana and Z. jujuba* fruits were collected from local market at Chennai. The fruits were sun-dried to concentrate the sugars in them (Figure 2, and 3).

**Figure 2:** *Zizyphus mauritiana*

**Figure 3:** *Zizyphus jujuba*

### Sample preparations

The fruits were sun dried for 8 to 10 days and were gently crushed using mortar and pestle to separate the pulp from the seed. The pulp was stored in a cool dry place till further use.

### Preparation of culture medium or *Ziziphus* juice

The juice is produced by heating the powdered fruit in water at 85°C for 45 min with continuous stirring. The extract is filtered, decanted, further clarified and sterilized at 120°C for 20 min. (Figure 4 depicts the growth of *A. niger* in both fruit media)

**Figure 4:** *Aspergillus niger* growth in *Zizyphus jujuba and Zizyphus mauritiana*

### Fermentation process

The strains of *A. niger* were inoculated in sterile 250 ml Erlenmeyer flasks containing 20 ml of culture medium composed by (g/L): glucose, 20.0, yeast extract, 5.0, $KH_2PO_4$, 5.0, at pH 7.0 (Lagzouli). The cultures were developed in 100 ml Erlenmeyer flasks containing 10ml of fruit syrup inoculated with 2% (w/v) inoculums level and incubated at 30°C for 36 h at shaker incubator (Zaldivar Aguero).[18]

### Analytical methods

The culture was homogenised lightly with mortar and pestle and it was centrifuged at 20,000 rpm for 30mins at 4°C and the supernatant was used as an enzyme source. All processes were carried out under standard conditions (Hernandez).[4]

### Enzyme assay of Crude Enzyme

Enzyme assay was carried out by DNS method of Miller, 1954, in which 0.5ml enzyme was allowed to react with 0.5 ml of substrate (0.5 % starch in 100mM Tris buffer) under standard reaction conditions and the reaction was stopped by adding DNS reagent (Miller, 1954).[21] The amount of maltose released was determined by comparing the absorbance reading of the test enzyme at 540 nm with the standard graph plotted by the known concentration of maltose ranging from 0.05mg/ml to 0.8 mg/ml. One unit amylase activity was defined as amount of enzyme that releases 1 micromoles of maltose per minute under standard reaction conditions.

Amylase was assayed by adding 1 ml of enzyme fermented broth supernatant to 1 ml of 0.5% soluble starch and incubated for 30 min at 37°C. The reaction was stopped by adding 3 ml of 1-dinitro-salicylic acid, followed by boiling for 10 min. The final volume was made to 10 ml with distilled water and the absorbance measured at 540 nm with colorimeter. A calibration curve of absorbance and concentration of D-glucose was established with known amount of glucose (Bernfeld).[22] One unit of amylase activity was defined as the amount of enzyme that releases 1µmol of reducing sugar as D-glucose per min under the assay conditions. The results are presented as specific activity (µmol/ml/min).

## RESULTS AND DISCUSSION

It is found that the enzyme produced was more in case of produced by *Ziziphus jujube* is more when compared to *Zizyphus mauritiana as is evident in Table 1 and Table 2. The amount of enzyme produced was also more, i.e.* 155.04 µg/ml min in *Ziziphus jujube as compared to Zizyphus mauritiana as was* found calorimetrically. (Figure 5)

**Table 1:** Amylase enzyme OD values of *Ziziphus jujuba*

| S. No | Enzyme solution | 0.5% Starch solution | | DNS solution | | Rochelle salt | OD value at 540 nm |
|---|---|---|---|---|---|---|---|
| 1 | 1ml | 1ml | Incubation for 15 minutes | 3ml | Boiling for 5 minutes | 1ml | 0.71 |
| 2 | 1ml | 1.5ml | | 3ml | | 1ml | 0.50 |
| 3 | 1ml | 2ml | | 3ml | | 1ml | 0.38 |
| 4 | Blank | 1ml | | 3ml | | 1ml | 0.24 |

**Table 2:** Amylase enzyme OD values of *Ziziphus mauritiana*

| S. No | Enzyme solution(ml) | 0.5% Starch solution (ml) | | DNS solution(ml) | | Rochelle salt(ml) | OD value at 540 nm |
|---|---|---|---|---|---|---|---|
| 1. | 1 | 1 | Incubation for 15 minutes | 3 | Boiling for 5 minutes | 1 | 0.10 |
| 2. | 1 | 1.5 | | 3 | | 1 | 0.31 |
| 3. | 1 | 2 | | 3 | | 1 | 0.54 |
| 4. | Blank | 1 | | 3 | | 1 | 0.05 |

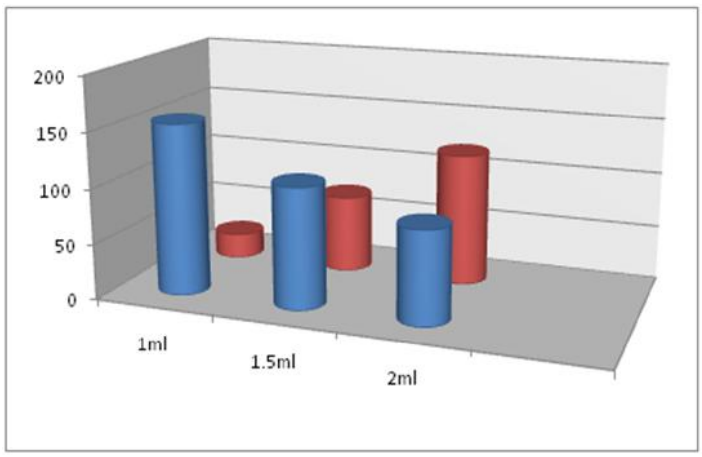


**Figure 5:** Effect of substrate on enzyme Amylase (X axis: substrate in ml, Y axis: µg/ml min glucose) *Ziziphus jujuba and Ziziphus mauritiana*

From the above results it is evident that *Aspergillus niger* is able to produce α-amylase enzyme using both *Ziziphus jujuba and Ziziphus mauritiana* as substrate at optimum conditions. The results also show that using *Ziziphus jujuba,* the enzyme (α amylase) produced is more active when the substrate concentration was low. From this we conclude that the substrate has an inhibiting effect on the enzyme activity. In case of *Ziziphus mauritiana,* on the other hand, the enzyme (α amylase) produced was more active when the substrate concentration is more, thus showing that the increase in substrate concentration increases the enzyme activity. Therefore these two fruits can be used as cheap resource for production of amylase enzyme effectively.

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