

## Starch Waste as a Substrate for Amylase Production by Sago Effluent Isolates *Bacillus subtilis* and *Aspergillus niger*

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**Abstract:** Sago effluent contains large amount of organic material which has a potential to affect the fertility of soil in agricultural lands when disposed off. In order to reduce this problem and make it beneficial, an experiment was conducted using sago effluent isolates *Bacillus subtilis* and *Aspergillus niger*. In present study, an attempt was made to convert the biodegradable sago into useful product by microbial degradation. During the process sago waste with *Bacillus subtilis* or *Aspergillus niger*, showed the maximum amylase production of 9.95 mg/ml/min at a pH of 7 and constant temperature of 37°C and maximum amylase production of 9.7 mg/ml/min at a pH of 5 and constant temperature of 20°C. From the result, this study concluded sago effluent as cheap substrate for amylase production, compared to wheat.

**Key words:** Sago effluent • *Bacillus subtilis* • *Aspergillus niger* • Biodegradable • Amylase

### INTRODUCTION

Sago and starch production from the tapioca root crops is one of the major food industries in Southeast Asia. There are nearly about 1000 sago and starch processing factories operation in Salem and Namakkal district of Tamil Nadu, India. These sago industries release large amount of wastewater containing organic and inorganic solid wastes. This wastewater commonly referred as effluent which has obnoxious odour, irritating colour, lower pH, higher BOD and COD. It affects the health of soil, natural ecosystem, animals, plants and human beings [1]. Rajannan and Oblisami [2] report that among various strains *Bacillus* sp. was found to be more tolerant followed by *Pseudomonas* sp. at high concentration of the effluent. This suggests the toxic nature of the industry effluent on soil microbial population.

Enzymes, commonly known biocatalysts are unique and highly specific proteinaeous substance. Enzymes are produced by cells and have enormous ability to catalyze virtually all of the chemical reactions or activities of living system in a highly and very effective manner.

The catalytic efficiency of enzymes is extremely the transformations of as many as 10000 to 100000 moles of substrates per minute per mole of enzyme [3]. Modern definition states that enzymes are catalysts of biological origin, which accelerate the various cellular reactions [4]. Enzymes that participate in hydrolysis of starch are referred to as amylolytic enzymes or amylases. Specific enzymes classified within the group include glucoamylase (also known as amyloglycosidase), pullulanase and isoamylase [5]. Enzymatic degradation of starch yields glucose, maltose and other low molecular weight sugars and their degradation products serve as important substances for food and pharmaceutical industries. The present investigation aims at the evaluation of sago effluent substrate for analysis formation.

### MATERIALS AND METHODS

Two different starch wastes (sago waste and wheat bran) were tested for amylase production using bacterial isolate (*Bacillus subtilis*) and fungal isolate (*Aspergillus niger*) as amalyase producers.

**Sample Collection:** Sago waste was collected from sago serve industries limited, Kaveri, Namakkal district in a clean polythene bag. The wet sample was then sun-dried for two days and ground to make it as powder, which is then transferred to laboratory for analysis. Wheat bran was collected from cattle feed shop in clean polythene bags and transferred to laboratory for analysis.

**Isolation of Amylase Producing Organisms:** The amylase producing bacteria and fungi were isolated from the sago effluent irrigated soil. The soil was collected in a sterile container and processed potato dextrose agar (PDA) plates by Bertrand *et al.* [6]. The isolates having amylase producing ability were selected using starch agar plates (Starch; 1 gm, peptone; 0.5 gm, yie agar; 0.3 gm, nacl; 0.3 gm, agar agar; 1.5 gm, distilled water; 100 ml) method.

**Identification of Bacteria:** The isolates were screened by phenotypic characters and initially identified using Bergey's manual of determinative bacteriology [7].

#### **Production of Amylase by Submerged Fermentation**

**Seed Culture:** The effluent isolates, *Bacillus subtilis* and *Aspergillus niger* stored in refrigerator were respectively cultured in nutrient agar slant (pH 7.4+/-0.2) for *B. subtilis* and potato dextrose agar (pH 3.5) for *Aspergillus niger* to obtain respective seed culture.

#### **Demonstration of Amylase Production on Starch Agar Plates:**

Starch agar (pH 7.4+/-0.2) plates were inoculated with the seed culture of *Bacillus subtilis* and seed culture of *Aspergillus niger* separately. After sufficient incubation period (24 hours for bacterium and 5 days for fungus), the plates were flooded with iodine. After 5 minutes, excess iodine was poured out. The production of amylase was visualized by appearance of clear zone surrounding the organisms [8].

#### **Fermentation with Respect to Bacteria and Fungi:**

Loopful of *Bacillus subtilis* was transferred from slant culture into nutrient broth (Peptone; 10.0 gm, Meat extract; 10.0 gm, Sodium chloride 05.0gm, Agar; 5.0gm, Distilled water; 1000 mL) and incubated over night. From this, 1% of the inoculum was transferred to 100 ml of production medium. The submerged fermentation was performed at pH 6 and temperature 30°C. *Aspergillus niger* was cultured in potato dextrose broth at 20°C for 3-5 days. The submerged fermentation was performed at pH 5.

#### **Effect of pH and Temperature on Enzyme Production:**

The effect of pH value and temperature on amylase production was carried out using the following pH range (pH 4-9) and incubation temperature (30°C, 37°C and 39°C) for *Bacillus subtilis* and for *Aspergillus niger* the pH levels, as 2-7 and temperature viz., 18°C, 20°C and 25°C.

## **RESULTS AND DISCUSSION**

**Inoculation Period:** Amylase is a number of extracellular enzymes that bacteria excrete into environment. At different inoculation periods (12, 24, 36 or 48 hours) during the growth of *Bacillus subtilis*, amylase production on starch wastes was determined. The enzyme activity with uninoculated sterile medium was found to be 0.265mg/ml/min for starch, 0.417 mg/ml/min for sago and 0.503 mg/ml/min for wheat bran. The amylase activity is expressed as milligram of starch hydrolyzed in 1 minute by 1 ml of the enzyme preparation. The optimum time, for enzyme production by *Bacillus subtilis* was determined as 24 hours and for *A. niger* was determined after 5 days.

On overnight broth culture suspension of *Bacillus subtilis* was transferred to the production media with different pH range. Among the different pH ranges (pH 4-9) and constant temperature of 30°C, maximum amylase yield obtained from pure starch was 9.77 mg/ml/min at a pH of 6. Sago waste and wheat bran respectively provided maximum amylase yield of 9.83 and 8.05 mg/ml/min at a pH of 7 (Figure 1).

In liquid state fermentation, highest production of enzyme was achieved after 24 hours of growth of *Bacillus subtilis* among the two starch wastes tested (sago waste and wheat bran), sago waste was found to be most preferred cheap substrate followed by wheat bran for enzyme (amylase) synthesis.

#### **Effect of Incubation Temperature:**

*A. niger* showed highest amylase enzyme activity at temperature range from 18-20°C. The optimum temperature was attained at 20°C with a concentration of 9.70 mg/ml/min. Gitishree *et al.* [10] reported maximum amylase production at 20°C. The effect of temperature on the activity of amylase enzyme produced by *A. niger* revealed that there was a sudden increase in protease production from 18-20°C with a concentration of 9.48 mg/ml/min and 9.48 mg/ml/min. However, there was sudden decrease in the activity of enzyme produced by *A. niger* when the temperature was increased above 20°C. This result agrees with

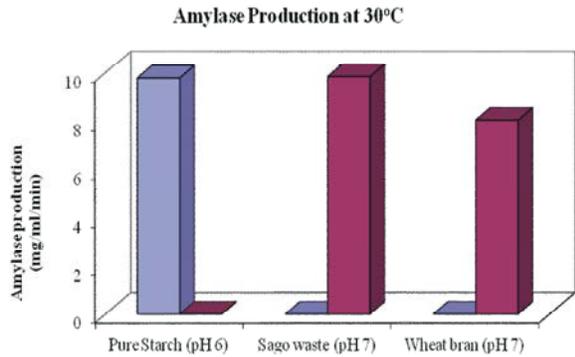


Fig. 1: Amylase Production at different pH at 30°C by *Bacillus subtilis*

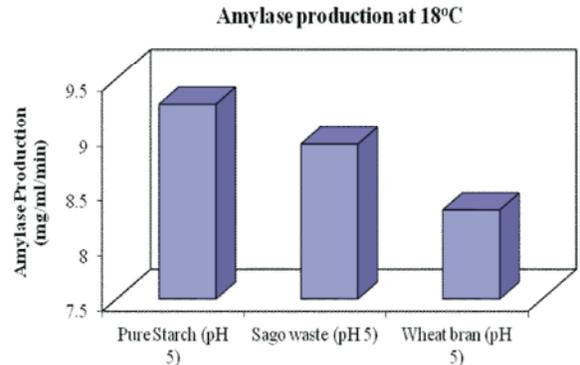


Fig. 4: Amylase Production at different pH at 18°C by *Aspergillus niger*

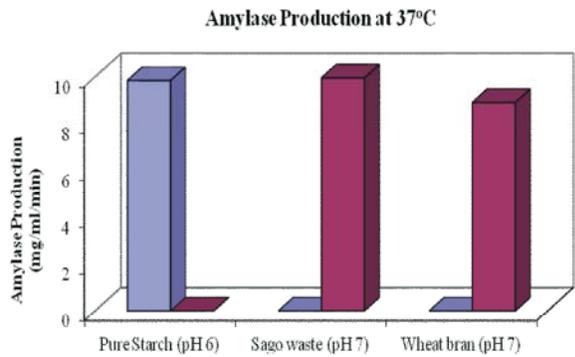


Fig. 2: Amylase Production at different pH at 37°C by *Bacillus subtilis*

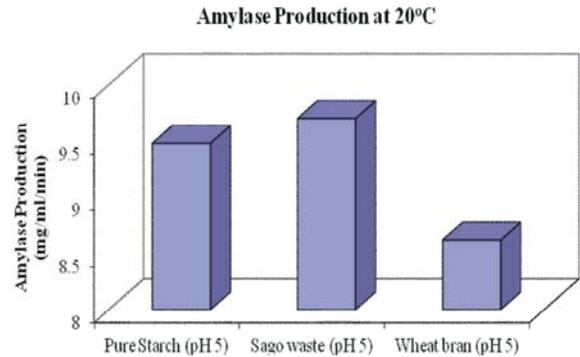


Fig. 5: Amylase Production at different pH at 20°C by *Aspergillus niger*

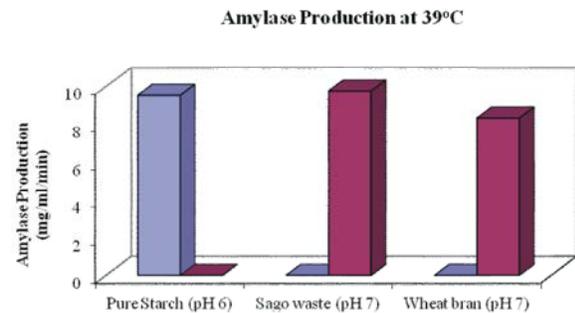


Fig. 3: Amylase Production at different pH at 39°C by *Bacillus subtilis*

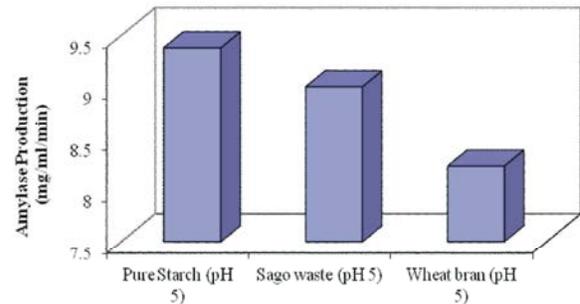


Fig. 6: Amylase Production at different pH at 25°C by *Aspergillus niger*

Kalpana *et al.* [8], that amylase production decrease with increasing temperature from 25-30°C and that amylase production come to an end at higher temperature. Oyeleke *et al.* [9] reported maximum amylase yield for *A. flavus* and *A. fumigatus* at 20°C.

Among the different pH ranges (pH4-9) and constant temperature of 37°C, maximum amylase yield obtained from pure starch was 9.84 mg/ml/min at a pH of 6. Sago waste and wheat bran respectively provided maximum amylase yield of 9.95 and 8.90 mg/ml/min at a pH of 7

(Figure 2) and also among the different pH ranges of (pH4-9) and constant temperature of 39°C, maximum amylase yield obtained from pure starch was 9.54 mg/ml/min at a pH of 6. Sago waste and wheat bran respectively provided maximum amylase yield of 9.76 and 8.34 mg/ml/min at a pH of 7 (Figure 3).

In broth culture suspension of *Aspergillus niger* was transferred to the production media with different pH range and incubated at three different temperatures (18, 20 and 25°C). Among the different pH ranges tested (2-7)

and constant temperature of 18°C, maximum amylase yield obtained from pure starch was 9.27 mg/ml/min at a pH of 5 (Figure 4). Sago waste and wheat bran respectively provided maximum amylase yield of 8.91 and 8.31 mg/ml/min at a pH of 5 and constant temperature of 20°C maximum amylase yield obtained from pure starch was 9.48 mg/ml/min at a pH of 5 (Figure 5). Sago waste and wheat bran respectively provided maximum amylase yield of 9.70 and 8.62 mg/ml/min at a pH of 5 (Figure 6). The constant temperature of 25°C maximum amylase yield obtained from pure starch was 9.38 mg/ml/min at a pH of 5. Sago waste and wheat bran respectively provided maximum amylase yield of 9.0 and 8.23 mg/ml/min at a pH of 5.

At different time periods (4-7 days) during the growth of *Aspergillus niger*, *Bacillus subtilis* amylase production on starch wastes was determined. The optimum pH for the enzyme produced by *A. niger* for this study was recorded at pH 5 with pH range of 5-6 also supporting enzyme activity. Kalpana *et al.* [9] reported maximum amylase production at for *A. niger* at pH 5-6 and recorded maximum amylase production at pH 5. In a similar finding, Oyeleke *et al.* [9] reported the optimum pH for the activity of amylase produced by *A. flavus* and *A. fumigatus* as pH 6 and pH 5 respectively. The optimum pH for amylase production by *Bacillus subtilis* in this study was recorded at pH 7 with a concentration of 9.95 mg/ml/min. In a similar findings Gitishree *et al.* [10] reported maximum amylase production at pH 8. *Bacillus subtilis* showed highest amylase enzyme activity at temperature range from 37-39°C. The optimum temperature was attained at 37°C with a concentration of 9.95 mg/ml/min. Temperature and pH are the most important factor affecting enzyme activity. The effect of temperature and pH was studied for the production of amylase enzyme by *A. niger* and *B. subtilis*. This is similar to the work of Oyeleke and Oduwale, [11]; Daniel *et al.* [12] that during isomerisation, temperature is preferably maintained within the range 20-40°C and the best activity is obtained with 20-37°C. Maximum amylase yield was obtained at pH 5 for *A. niger*. This result suggests the slightly acid nature of the amylase produced. Maximum amylase yield for *B. subtilis* was recorded at pH 7, although, pH 7-10 supported amylase production. This result suggests that maximum amylase production was obtained at alkaline pH. The vast activity of amylase enzyme at various pH suggest the wide application nature of the amylase enzyme identified. Oyeleke and Oduwale [11] reported the similar findings between pH ranges of 6-8. This result suggests that the *B. subtilis* used for this work functions best at alkaline pH.

The absorbance of assay mixture with uninoculated sterile medium in the place of enzyme was found to be 0.097 mg/ml/min for starch, 0.101 mg/ml/min for sago and 0.232 mg/ml/min for wheat bran. The optimum time period, for enzyme production by bacterial isolates *Aspergillus niger* was determined as 5 days. The amylase producing bacteria isolated from sago waste were subjected to submerged fermentation using starch medium and the waste starch. The submerged fermentation carried out in an autoclavable auto-controlled fermentor provided high yield of enzyme. When pure starch was used, it gave maximum yield of 10.7 mg/ml/min. But, when sago waste and wheat bran were used, it respectively gave maximum production of 11.3 and 9.8 mg/ml/min comparatively sago waste shows higher yield. The submerged fermentation of filamentous fungi is more preferred for production of industrially important enzymes.

Amylase production on pure starch media did not boost up the production of bacterial amylase but the low-grade cheap impure substrate sago waste and wheat brain in the present study produced very high amount of amylase when compared to pure starch. This can be correlated to the previous studies done by Ray *et al.* [13]. Wheat bran also produces bacterial amylase of 8.9 mg/ml/min which is the maximum yield obtained at temperature of 37°C and pH 7. Since sago waste produces bacterial amylase of 9.95 mg/ml/min, wheat bran is considered to be less important. Effective substrate for amylase production when compared to the enzyme yield obtained from sago waste as substrate. The same experimental parameters were repeated for *Aspergillus niger*. The highest production of amylase was achieved on 5<sup>th</sup> day of fungal growth. Apparently, there was good correlation between cell growth and enzyme synthesis, which was similar to the results obtained by Panday *et al.* [14]. From the pedestal study on the effect of these two substrates, sago waste and wheat bran, sago waste is said to be most economical substrate for *B. subtilis* and *A. niger*. Thus, sago waste can be considered as the best starch wastes for amylase production (both using bacteria and fungi).

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