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Amylase Production by *Aspergillus Niger* using Agroindustrial Residues under Temperature Mediated Solid State Fermentation

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Abstract

Plant residues contain nutrients required for the growth of bacteria and fungi. Use of agro industrial wastes or residues to cultivate fungi for enzymes production is an economical method combined with waste recycling. In this study, agro industrial residues were used as fermentation medium for amylase production by *Aspergillus niger.* Cultivation temperature in the range of 20°C, 25°C, 30°C, 35°C and 40°C was considered as the fermentation factor to optimize the enzyme production. The wastes used were one of the following materials at a substrate concentration of 100g: wheat bran, ragi husk, coconut shell, bagasse, paddy straw and ground nut shell and the fermentation was carried out for a period of 14 days. The results revealed that all the substrates promoted the growth of *A. niger* and ragi husk was proved to be the best growth medium that secured the highest amylase activity. The highest enzyme production of 213U ml-1 was observed at 25°C and the enzyme activity was consistent in fermentation media containing ragi husk and paddy straw as substrates. Most of the substrates tested in this study were utilized by A. niger within 7 days of solid state fermentation for the production of amylase. However, utilization of ragi husk as the substrate needed more fermentation time and at the same time it has resulted in highest enzyme activity. In consideration of the results, agro industrial residues could be used as economical medium for the production of fungal amylases by solid state fermentation.

Keywords: *Aspergillus niger*; Agro industrial residues; Ragi husk; Coconut husk

Introduction

Agricultural residues pose a serious disposal problem Rodriguez-Couto [1] however with the advent of biotechnological innovations, many new areas have opened for their utilization as raw material. The residues contain high amount of proteins, sugars, and minerals which offers appropriate environments for growth of microorganisms. Both bacteria and fungi have the ability to utilize the agricultural residues through fermentation process hence the residues can be used as raw materials for other product formation and development. Agricultural residues are commonly used as substrates in solid state fermentation (SSF) and molds are frequently used in SSF than bacteria and yeasts, which require comparatively higher moisture content for efficient fermentation. The growth rate of fungi are enhanced by use of these substrates which resulted into the conversion of lignocellulosic substrate into less complicated ones by degrading action of several enzymes. Amylase are the glycoside hydrolases produced by many animals, plants, bacteria and fungi and the amylase family comprises of three major groups, namely α-amylase, β-amylase and glucoamylase. The main stream applications of α-amylase in many modern biotechnological purposes are basically of bacterial and fungal origin. Alpha amylases are synthesized as extracellular enzymes by bacteria and fungi which hydrolyze starch into maltose, glucose and maltotriose. They cleave the 1,4 D-glucosidic linkages between adjacent glucose units in the linear amylase chain. Amylases are employed in the starch processing industries for hydrolysis of starch into simple sugars [2]. The application of amylases in food industries such as baking, brewing and fruit juices requires their large scale production. Industrial demand for amylase is limited with specific applications as in the food industry, where fungal amylases are preferred over other microbial sources mainly because of their more accepted GRAS status. Industrial production of amylases is achieved by solid state or submerged fermentation. Fungal amylases are commonly produced by solid state fermentation due to low capital investment, less water output, superior productivity and better product recovery.

Agroindustrial wastes are considered as the best substrates for the production of α-amylase by solid state fermentation [3,4]. *Aspergillus* species biosynthesize a large variety of extracellular enzymes of which α-amylases are of world-wide interest in fermentation, food, pharmaceutical, textile and paper industries [5,6]. *Aspergillus* was widely used to produce α-amylase employing agro industrial wastes in solid state fermentation Selvakumar, et al., 1 Akpan et al., Francis et al., Ramachandran et al., Negi, et al., Suganthi et al., Kumar et al., [7-13] and submerged fermentation [14]. In the present investigation, the comparison of fungal amylase production using agro industrial residues through solid state fermentation was performed. In addition temperature was considered as one of the fermentation factors to optimize the enzyme production.

Methodology

Agro Industrial Residues

Wheat bran, ragi husk, coconut shell, bagasse, paddy straw and groundnut shell were purchased from local market of Bangalore. The residues were washed in tap water and cut into small pieces followed by homogenization in blender before autoclaved to remove microbial contamination.

Microorganism Used

A fungal strain of *Aspergillus niger* was used in this study. One milliliter of the spore suspension was poured onto sterile Petri-plates, containing sterile potato dextrose agar (PDA) medium and spread uniformly and incubated at 25°C for 7 days. The pure culture was maintained in PDA slants at 4°C.

Inoculum Preparation

Ten milliliter of distilled water containing 0.1% Tween-80 was transferred to a fully sporulated (7 days old) *A. niger* slant culture. The spores were dislodged using the inoculation needle under aseptic conditions and the appropriate dilution was used as inoculum.

Substrate Preparation

100 g of agroindustrial residues (wheat bran, ragi husk, coconut shell, bagasse, paddy straw and groundnut shell) were taken separately in 250 ml Erlenmeyer flasks and 100 ml of basal salt solution [$(NH_4)_2SO_4$ – 0.2g, KH_2PO_4 -0.1g, $MgSO_4.7H_2O$ - 0.05g, ZnSO4 - 0.01g, Distilled water - 100 ml] was added (1:1 w/v). The moisture content was maintained at optimum levels and the contents of the flasks were autoclaved at 121°C for 20 mins.

Solid state fermentation

After autoclaving, the contents of the flasks were inoculated with 10^7 spores/ml of A. niger and incubated under static conditions. Varying incubation temperatures (20°C, 25°C, 30°C, 35°C and 40°C) was considered to determine its effect on enzyme activity after 7th and 14th day of fermentation from 100 g of each substrate. Each experiment was done in triplicates.

Enzyme Extraction

Crude enzyme was extracted from the fermentation medium by adding 25 ml of 0.1M phosphate buffered saline (pH 7) to each of the inoculated substrate beds and was kept in a rotary shaker for 15 minutes at 120 rpm. The suspension was filtered through cheese cloth and centrifuged at 8000 rpm at 4°C for 15 min. The supernatant was used for amylase activity.

Enzyme Assay

Alpha-amylase activity was determined by mixing 1.25 ml of 1% soluble starch, 0.25 ml of 0.1 M acetate buffer (pH 5.0), 0.25 ml of distilled water, and 0.25 ml of crude enzyme extract [15]. After 10 min of incubation at 30°C, the liberated reducing sugars were estimated by the dinitrosalicylic acid (DNS) method Miller [16] using glucose as standard. One unit (IU) of α-amylase is defined as the amount of enzyme releasing 1 µmol glucose equivalent per minute under the assay conditions.

Results

Through the analysis of fermentation process, it was observed that agro industrial residues were alternate feedstock for *A. niger* cultivation. Results obtained showed that ragi husk followed by paddy straw served as good substrates, enabling the growth of *A. niger,* which produced a higher amounts of the enzyme than the other substrates used. Solid state fermentation was carried out with spore suspension of *A. niger* using various agro industrial residues for a period of 14 days to evaluate the effect of incubation temperatures on the enzyme production. Temperatures in the range between 20°C and 40°C were used. Among the substrates tested, ragi husk promoted the highest enzyme activity (213.15 U ml^{-1}) at 25°C at the end of 14 days fermentation (Figure 1). It was also observed that the enzyme activity was neither constantly increased nor decreased irrespective of the temperature and fermentation period tested while using ragi husk as the substrate. There was a sudden increase in enzyme activity (186.51 U ml^{-1}) when the incubation temperature was 25°C but was reduced drastically at 30°C and 35°C. Again there was an increase in enzyme activity at 45°C (150.98 U ml^{-1}). The trend was similar at the end of 14 days fermentation period. It was observed that the highest enzyme activity of 37.75 U ml^{-1} at 30°C was produced in media containing coconut husk as the substrate after 7 days of fermentation. However, 14th day of fermentation had resulted in constant increase of enzyme activity with increasing temperature and the maximum activity was observed as 45.52 U ml-1 at 35°C (Figure 1, Figure 2).

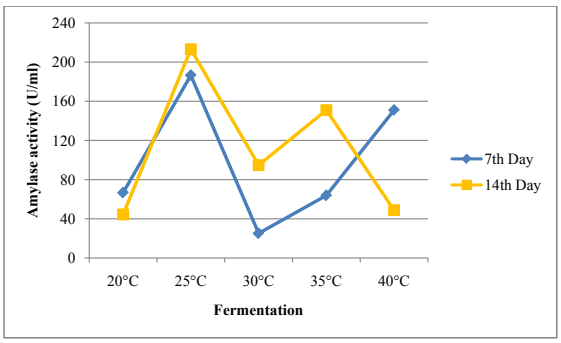

Figure 1: Effect of fermentation temperature on amylase activity by *A. niger* with ragi husk as substrate

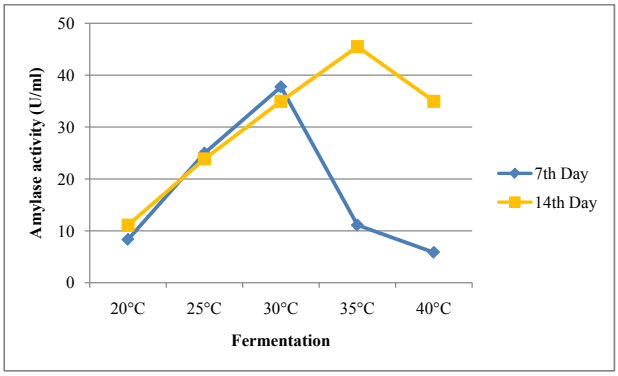

Figure 2: Effect of fermentation temperature on amylase activity by *A. niger* with coconut husk as substrate

The use of groundnut shell for the amylase production was highly temperature dependent throughout fermentation period. It was found that 30°C was optimal to produce the highest enzyme activity of 35.52 and 21.61 U ml^{-1} at 7^{th} and 14^{th} day of fermentation respectively (Figure 3). Similar results were obtained in media containing paddy straw as the substrate for *A. niger* but 25°C was optimum for maximum enzyme activity. At the end of 7*th* and 14*th* day, the recorded enzyme activities were 81.6 and 68.11 U ml-1 (Figure 3, Figure 4).

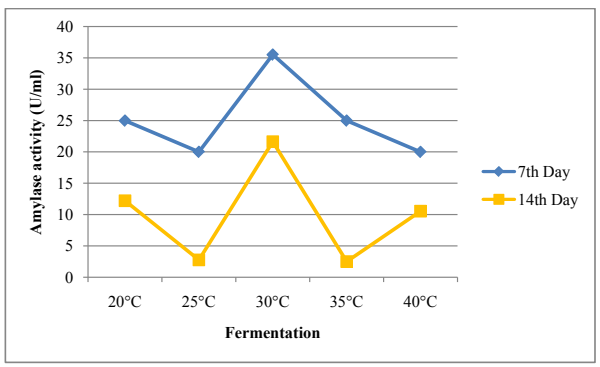

Figure 3: Effect of fermentation temperature on amylase activity by A. niger with groundnut shell as substrate

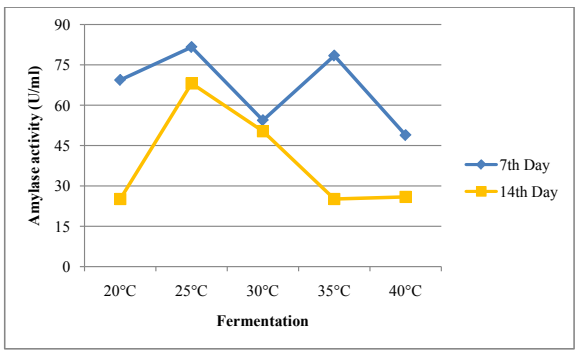

Figure 4: Effect of fermentation temperature on amylase activity by A. niger with paddy straw as substrate

The enzyme activity in bagasse containing medium was higher at 14th day than 7th day. In other words, both 25°C and 35°C resulted in 105.4 and 111.01 U ml-1 of amylase activity after 14 days. Whereas it was15.54 and 0 U ml-1 at the end of 7 days (Figure 5). The results obtained with wheat bran medium was not conclusive as there most of the temperature led to no enzyme activity but there was an exclusion at 30°C after 7 days of fermentation (Figure 5, Figure 6).

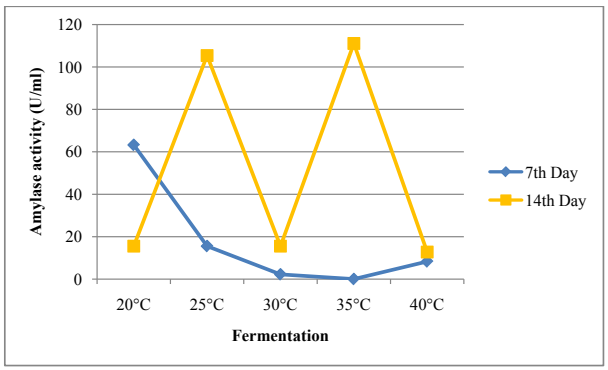

Figure 5: Effect of fermentation temperature on amylase activity by *A. niger* with bagasse as substrate

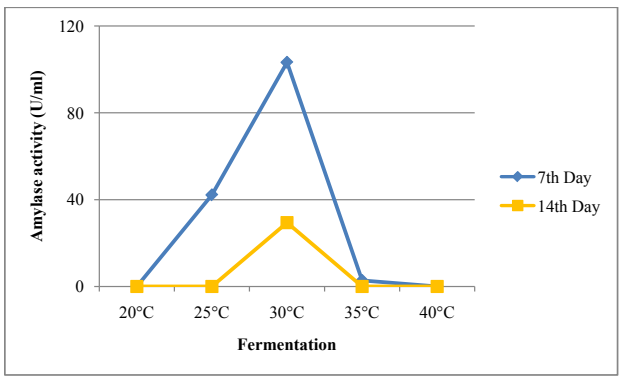

Figure 6: Effect of fermentation temperature on amylase activity by *A. niger* with wheat bran as substrate

As the enzyme activity was varying with each substrate tested and fermentation period used, the effect of temperature on the other hand was evaluated. The results summarized that 20°C, 30°C, 30°C, 25°C, 30°C have resulted in highest enzyme activity for bagasse, coconut husk, groundnut shell, paddy straw, ragi husk and wheat bran respectively for 7 days fermentation period. However the temperatures of 35°C, 35°C, 30°C, 25°C, 25°C and 30°C were optimum to obtain the maximum enzyme activity while using bagasse, coconut husk, groundnut shell, paddy straw, ragi husk and wheat bran respectively after 14 days fermentation period. In general, variation of the temperature brought about a change in metabolic pattern of the microorganism; it exhibited its best enzyme production in the mesophilic range. Temperature is one of the important factors, which strongly affect the SSF process [5]. In the present study, both 25°C and 30°C were proved to be the best temperature for the enzyme synthesis independent of the substrates tested. On the other hand, incubation at higher temperature affected the fungus harmfully, which reflected on the reduced enzyme synthesis.

Discussion

Micro-organisms utilize various substrates as nutrient source for growth and metabolic activities and subsequently produce metabolism-related products. However, fine-tuning of nutrient concentrations regulate the microbial metabolism and associated metabolic product production. Balancing of nutrient concentration with minimum experimentation to optimize the enzyme production is a fine art in microbial metabolism. Enzymes are relatively expensive reagents and 50% of the cost of production is associated with capital investment, while the cost of raw materials accounts for almost one third of such costs. Cost of substrates on which enzyme-producing microbes can be cultivated has always been an important factor in production. Substitution of feed stocks with natural resources can result in an increased return on investment. Starch is the major constituent of fermentation medium for α-amylase production which is too expensive. Agricultural wastes lessen the final fermentation media cost and have gained importance for economic biosynthesis of amylase under solid state fermentation. Keeping in view, various agro-wastes were tried and evaluated for the α-amylase biosynthesis in this study. To identify the potential substrate for biosynthesis of α- amylase, experiments were performed by constituting the medium with each of the agro industrial residues as fermentation medium ingredients. Fungi represent primary organisms for production of enzymes and secondary metabolites and amongst all *Aspergillus* is the most prevailing amylolytic genus of the nature [17]. *A. niger* is suitable for solid state fermentation because its morphology facilitates colonization and penetration into solid substrates [18]. Agro-industrial residues were used to produce amylase in previous studies [19-22]. Wheat bran was reported as the best substrate for amylase production by *Rhizopus microsporus* var. *oligosporus Nunez* et al., [23] and *A. niger* Khan et al., [24]. In a study by Kumar et al., [25], rice bran was found to be the best substrate for amylase production by A. niger. In this study, the substrates of choice were wheat bran, ragi husk, coconut shell, bagasse, paddy straw and groundnut shell. Each experimental flask received 100 g of substrate and basal medium to carry out solid state fermentation for a period of 14 days. Both ragi husk and paddy straw were proved to be the best substrates for amylase production.

The enzyme biosynthesis by filamentous fungi is reported to be influenced by numerous factors, such as pH, temperature Ferreira Costa, et al. [26] carbon and nitrogen sources George et al., 1999. Media formulations based on agricultural wastes are heterogeneous by nature and it is important to optimize the fermentation conditions to improve enzyme production at a low production cost [27-28]. Higher temperatures during the fermentation may lead to enzymatic inactivation and suppression of cell viability. Similarly, low temperature values may reduce the metabolism of the microorganism Mazutti et al., Francis et al. [9,29] and consequently, the enzyme synthesis. A temperature of 30°C was found optimum for amylase production by *A. niger* Gupta et al., Roses et al., Wang et al., [30-32] and *A. oryzae* [10]. Singh et al., [33] has reported 35°C as optimum incubation temperature for amylase productivity of 341.7 U/ml by Aspergillus fumigatus. In a study by Prakasham et al., [35] using *A. awamori,* 31°C found optimum for acid amylase production. The present investigation involved determination of temperature influenced enzyme activity in samples in triplicate by quantifying the reducing sugar (glucose). It was also noted that the enzyme production was not increased/decreased with increasing/decreasing temperature. In the case of bagasse as substrate when culti vating A. niger, the enzyme activity was 105.4 U ml^{-1} at 25°C and was dropped drastically to 15.54 U ml^{-1} followed by increasing upto 111 U ml^{-1}.

Similarly, the enzyme activity was increased when the temperature was increased from 25°C to 30°C and 30°C to 35°C while using groundnut shell and ragi husk as substrates respectively. The reason for this could be the optimal temperatures for the enzyme production by *A. niger* under the experimental conditions was varied depending on the substrates tested. Besides, the breakdown of substrates at higher temperatures might have influenced the enzyme production which had resulted in the increasing activity. However, the reason for fluctuations in enzyme activity at different temperatures needs to be explored further so that appropriate fermentation temperature for a particular substrate can be used to get the maximum enzyme activity. The highest enzyme production was observed at mesophilic temperature and the enzyme activity was consistent in fermentation media containing ragi husk and paddy straw as substrates. Another finding of the study is that most of the substrates tested were utilized by *Aspergillus niger* within 7 days of solid state fermentation for the production of amylase. However, utilization of ragi husk as the substrate needed more fermentation time and at the same time it has resulted in highest enzyme activity.

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