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Amylase production by *Aspergillus niger* through submerged fermentation using starchy food byproducts as substrate

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Abstract
Submerged fermentation holds tremendous fungal potentiality in high biomass yield of alpha-amylase. Isolation of fungi from bread sample and the rapid screening by plating on starch agar plates led to the finding of fungal strains capable of producing amylase. These strains were confirmed as *Aspergillus niger* by lacto phenol cotton blue staining. The effect of carbon sources of the medium for the activity of α- amylase from *Aspergillus niger* utilizing Coconut water, Tapioca water, Rice water and White Yam water were investigated. The maximum activity of α-amylase was recorded as 0.29 x 10^{-3} µmoles/sec. After 7 days of submerged fermentation on white Yam water at pH 7.0 and room temperature 28 °C. Among the three medium rice water recorded as second (0.09 x 10^{-3} µmoles/sec) and tapioca water (0.06 x 10^{-3} µmoles/sec) as third position. The enzyme produced by *Aspergillus niger* can be used in industrial process after characterization. The present study suggest that white yam water act as a potent substrate for industrial production of α-amylase and subjected for further explorations regarding industrial applications.

Keywords: Submerged fermentation, rice water, *Aspergillus niger*, tapioca water, amylase production

1. Introduction
Amylases are enzyme that breaks down starch or glycogen. The amylase can be derived from several sources such as plant, animal and microbes. The major advantage of using microorganism for production of amylase is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics [1]. The microbial amylases meet industrial demands; a large number of them are available commercially; and, they have almost completely replaced chemical hydrolysis of starch in starch processing industry [2]. Although many microorganisms produce this enzyme, the most commonly used for their industrial application are *Bacillus licheniformis*, *Bacillus amyloliquifaciens* and *Aspergillus niger*. Amylases stand out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries. They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetraose syrup. In detergents production, they are applied to improve cleaning effect and are also used for starch de-sizing in textile industry [3, 4].
The use of the submerged fermentation (SmF) is advantageous because of the ease of sterilization and process control is easier to engineer in these systems. Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns. Submerged fermentation has been defined as fermentation in the presence of excess water. Almost all the large-scale enzyme producing facilities are using the proven technology of SmF due to better monitoring and ease of handling [5]. To meet the growing demands in the industry it is necessary to improve the performance of the system and thus increase the conditions, particularly physical and chemical parameters are important in the development of fermentation processes due to their impact on the economy and practicability of the process [6]. The growth and enzyme production of the organism are strongly influenced by medium composition thus optimization of media components and cultural parameters is the primary task in a biological process [7].
Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques [8]. Selection of appropriate carbon and nitrogen sources or other nutrients is one of the most critical stages in the development of an efficient and economic process [9].

The processing of cassava and white Yam tubers for the production of nutrient enriched food products is usually accompanied with the production of stinking wastewater which usually constitute nuisance to both terrestrial and aquatic life. After boiling rice also produces a sticking water with high amount of starch.

The objectives of this work was to study the production of α-amylase by using *Aspergillus niger* through submerged fermentation using four different starchy substrates like Rice water, White Yam water and Tapioca water as carbon source and compare the activity of the amylase produced using these substrates. Enzymes are protein catalysts synthesised by living system and are important in synthesis as well as derivative process. Amylase are enzyme that breakdown starch or glycogen. The amylase can be derived from several sources such as plant, animal and microbes [10]. Alpha amylase (endo-1-4 dglucose-Dglucohydrolase 3.2.1.1) belong to the family of endo amylases. From an industrial point of view mostly bacterial and fungal source have been used for the production of alpha amylase. Properties of alpha amylase such as thermo stability, pH optimum and their other physicochemical properties are important in the development of most suitable fermentation process. Alpha amylase can produce by fungi in large amount but they are not usually heat stable beyond 40 °C. Bacterial species such as *Bacillus subtilis*, *B. megaterium*, *B. amyloliquefaciens* and *B. licheniformis* produces more heat stable enzymes. Bacterial species which produce alpha amylase enzyme, if it is often need to isolation of microorganism that can grow at high temperature and whose enzyme can function at temperature up to 95-100 °C.

Kirchhoff was the first scientist to report the discovery of alpha amylase in 1811.Starch is an abundant source of carbohydrate. It consists of amylopectin and amylose. Amylopectin is formed from linked alpha, 1-4 chain of glucose with linked (α, 1-6) branch points and amylase consists of chain of glucose α,1-4 linked. α amylase this enzyme breakdown α-1,4 glycoside linkage of starch and related products in an endo fashion and produce oligosaccharides. If the mode of action, properties and products of hydrolysis is depend upon the source of enzyme [11].

Microorganism associated with α-amylase production. Industrial enzymes have been produced from plant, animal and micro-organism, but the plant and animal source is rather because of several reasons. If the concentration of enzyme in plant source is generally low but starch processing in industrially required as large quantities of enzyme. On the other hand if the enzyme from animal source origin is from the byproduct of meat industry and so it is supply is limited. However the α-amylase from microbial source can be produced in abundant quantities. Microorganism utilized different nitrogen, carbon sources for the production of α-amylase nitrogen source such as yeast extract, peptone, ammonium sulphate casein, ammonium nitrate, chicken feathers, carbon source such as corn starch, potato starch, cane sugar etc.

α-amylase is produced by bacterial species of Bacillus such as *B. subtilis*, [12, 13] *B. licheniformis* etc. Are generally preferred for the property of thermo stabilities the enzyme α-amylase utilized in various fermentation processes extreme thermophillic bacteria such as *Rhodothermus marinus* and mesophilic bacteria such as *B. megaterium*, *B. macerans* and *B. coagulans* are generally utilized while in the case of most thermo stable α-amylase utilized in industry is produced from *B. licheniformis* [14, 15]. And highly thermo stable α- amylase are also obtained in hyper thermophilic and thermophilic archea such as *Pyrococcus furiosus*, *Thermococcus hydrothermalis* [16, 17].

Fungi as a source of material for α-amylase production. α-amylase producing strain of yeast; fungi and actinomycetes were isolated. Especially aspergillus species are also source of α-amylase. It has gained more attention because of the easy availability and high productivity of the fungi, which are also suitable for genetic manipulation. Different species of aspergillus such as *A. niger*, *A. oryzae*, *A. flavous*, *A. tamarie*, *A. fumigatus* have frequently used for the production of α-amylase [18-27]. Pencillium species such *P. chrysogenum* and *P. camemberti* also used for the production of α- amylase and also in cheese production of α-amylase was obtained from thermophilic fungi species such as *Hemicola insolen*, *H. lanaginosa*, *H. stellata* etc. From the industrial point of view some species of yeast such as *Candida tsukubaensis*, *Filobasidium capsuligeum*, *Lipomyas kononenkoae*, *Saccharmycopsis capularis*, *Saccharomyces cerevisiae* have been used for the production of α-amylase [18, 4].

Industrial uses of α-amylase: Bacterial amylase plays an important role in industrial production process. Many industrial processes involving manufacturing such as industrial, environment process and food biotechnology utilizes the enzyme.

Glucose And Fructose Industry: Many industry use α-amylase for the production of glucose. This enzyme hydrolysis starch and convert it into maltose and glucose. Alpha amylase is widely used in many starch processing industries for the production of glucose [38, 19].

In bakery industry the α-amylase play an important role in improvement of quantity, aroma, taste and porosity of the product. This enzyme is the major part of bread used in Russia, USA and the European countries. α-amylase can also affect anti-salting in baking bread and help to improve the softness of bread [34]. Enzyme has significant role in the improvement of detergent quality by affecting bleaching. The addition of enzyme increases the stability and effectiveness of the bleach in laundry's detergent and soap bar composition [19].

Fermentable sugar is produced by the conversion of starch with the help of alpha amylase. Starch such as grains, potatoes etc. are required for the production of ethyl alcohol [33]. Starches increase the stiffness of the finished products after washing out the cloths. Alpha amylase is used as a resizing agent. It improves paper quality, protect against mechanical injury and increase the stiffness and strength in paper. It readily hydrolysis the starch polymer into fructose and glucose which increase the digestibility of carbohydrates [39, 40].

Amylase are enzymes that breakdown starch or glycogen. The amylase can be derived from several sources such as plant, animal, and microbes. The major advantage of using microorganism for production of amylase is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristic [1]. The microbial amylase meet industrial demands: a large number of them are available commercially and they have almost completely replaced chemical hydrolysis of starch processing industry [2]. They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixture, high fructose syrup etc.

The use of the submerged culture is advantage because of the ease of sterilization and process control is easier to engineer in these system depending on the strain and the culture condition. Most of the enzyme are produced by submerged

fermentation is becoming popular for producing enzyme due to it is inherent advantage example higher yield, improved oxygen circulation, less energy requirement, minimum effort in downstream processing, effect of process variables, namely incubation period, temperature, initial moisture content, pH of the medium, supplementary carbon source, supplementary nitrogen source, and inoculums level on production of α-amylase have been studied.

The most effective amylase are those that are thermo stable they are generally preferred as their application minimize contamination risk and reduce reaction time thus enabling considerable energy save. Amylase has potential application in a wide range, number of industrial processes such as food, fermentation, textile, paper, detergent and pharmaceutical industries. Starch is an important storage product of many economically important crops such as wheat, rice, maize, tapioca, coconut water and potato. Starch converting enzyme is used in the production of maltodextrin, modified starch or glucose and fructose syrups. Amylase are a group of hydrolyses which can specifically cleave glycosidic bond in starch. There are two important group of amylase which includes glucoamylase and α-amylase. Microbial amylase has successfully replaced chemical hydrolysis of starch in starch processing industries. Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzyme from fungal and bacterial source have dominated application in industrial sectors. Major advantage of using fungi for amylase production is the economical bulk production capacity.

The amylase derived from several species, source such as plant, animal and microbes. However the enzyme from fungal source has dominant application in industrial sectors. The major advantage of using microorganism has economical bulk production. The production process of α-amylase mainly they can be two major methods for large scale production of α-amylase are solid state fermentation and submerged fermentation.

Many factors involved in the production and optimization of α-amylase such as nitrogen and carbon source supplied, metal ions, pH, and temperature. Nitrogen source used for the production of α-Amylase: Various nitrogen source including corn steep liquor, casein, yeast extract, tryptone, ammonium nitrate, sodium nitrate and ammonium chloride are utilized for the production of α-amylase in basal medium. Organic nitrogen source like peptone, yeast extract, usually having stimulating effect peptone is the best for the production α-amylase [41-43, 26]. Mainly some organism including such as bacillus species and aspergillus species such as *A. flavus*, *A. niger* and bacillus species including *B. subtilis*, *B. licheniformis*

Carbon source used for the production of α-Amylase: α-Amylase is produced from many source of carbon such as fructose, glucose, maltose, galactose, sucrose, lactose, dextrose industrial waste like syrup and molasses, agriculture waste involving sugarcane and rice husk [26]. Microorganism for the production of α-amylase such as *B. subtilis*, *B. licheniformis*, *A. oryzae* etc. Metal Ion: Metal ion play an important role for the production of α- amylase because of most α-amylase are metallo enzyme.

Effect of temperature on α-Amylase activity: Action of enzymes is time dependent process. Increase in temperature will lead to an increase in activity of kinetic reaction. High temperature can affect enzyme activity because enzyme proteinaceous molecule. Thermo stable α-amylases have been isolated from organisms such as, *B. amyloliquefaciens B. licheniformis, B. subtilis* and *A. niger*. The effect of temperature on α-amylase action has been reported previously in such studies. Optimum temperature was noted to be 65 °c at low substrate concentration and 75 °C at high substrate concentration. If the temperature range of Bacillus species up to about 30-70 °C and Aspergillus species about 30-40 °C [18, 30, 31].

Effect of pH on α-Amylase activity: The pH on enzyme stability and activity is also depending on time and temperature. In general, enzymes are less stable at high temperature over time at pH value near the limit of the optimum. α-Amylase are stable a pH range of 4-11 [15].

Rice water, tapioca water and white yam water has been used as a potent substrate for the production of amylase by *A. niger* in submerged fermentation. The cheap starchy agro-by products have been reported to be a good substrate for the cost effective production of alpha amylase. The synthetic media are used for the production of amylase fungal species have been studied a lot for the production of alpha amylase [16].

Coconut water (Thenga vellam in Malayalam) is the suspension of starch, sugars and minerals obtained by draining ripe coconut (*Cocos nucifera*) for the extraction of oil.

Rice water (Kanji vellam in Malayalam) is the suspension of starch obtained by draining boiled rice (*Oryza sative*) or by boiling rice. Rice water is also a milky liquid which contain vitamin B, E and mineral. Rice water is relatively containing good source of carbohydrate, calcium, iron, vitamin. The protein content is about 36-58% and presence fat is 16-25%.

Tapioca water (Kappa vellam in Malayalam) (*Manihot esculenta*) is a suspension of starch is obtained by boiling pieces of tapioca with water. The use of cassava in submerged fermentation such as the microorganism have a very fast growth rate, they can be easily modified genetically for growth on a particular substrate under particular cultural condition, the protein content is quiet high varying from 35-60%.

White Yam water (Kachil vellam in malayalam) (*Dioscorea rotundata*) have an average crude protein content is 4-7% and the starch content about 75.6-84.5% and which contain high carbohydrate content more than 85% and fat contain 0.17g.

Given lacking qualitative and quantitative data on various starchy substrates in Kerala, objective of this study were to screens a variety of easily available and inexpensive starchy plant materials as substrate for the production of α-amylase using *Aspergillus niger* through sumerged fermentation.

2. Materials and Methods

2.1 Sample collection/Substrate collection and sterilization

Rice water, Tapioca water and White Yam water were obtained by draining boiled small pieces of fresh tuber or by boiling small pieces fresh tuber until it completely dissolves into the water. They are stored in sterile bottles under aseptic condition until use.

2.2 Isolation of *Aspergillus niger*

A piece of bread was kept in a moist condition at room temperature in dark for 2 days. The bread sample was serially diluted and different dilutions were inoculated on potato dextrose agar (PDA) medium. The slants were incubated at 30 °C 4 days. Fungal cultures were observed on PDA medium. The fungal strain was subjected to lactophenol cotton blue staining for studying the morphology. The fungal culture was confirmed as *Aspergillus niger* by studying the morphology and the spore colour.

2.3 Lacto phenol cotton blue staining
Place a drop of Lacto phenol Cotton Blue Solution on a slide. Using an inoculating needle carefully spread the fungal culture into a thin preparation. Place a cover slip edge on the drop and slowly lower it. Observe under low to high power objectives of microscope. Lactic acid acts as a preservative for fungi. The phenol portion kills the fungi. The cotton blue stains the fungal elements. Fungal elements are stained a deep blue; background is pale blue.

2.4 Determination of Amylase activity
The *Aspergillus niger* isolate was tested for amylase production by starch hydrolysis. When starch agar medium was inoculated with the organism and subsequently flooded with iodine solution, the zone of clearance around the microbial growth indicated the production of amylase and the fungal isolate was taken for amylase production.

2.5 Enzyme production by Solid State Fermentation
The *Aspergillus niger* was subjected to solid state fermentation in different substrates like rice water, tapioca water and white yam water, replicated four times each; which was used as liquid substrates for submerged fermentation. Each substrate was taken in about half in all the bottles 1% of inoculums was inoculated after sterilization and incubated at room temperature for six days.

2.6 Enzyme extraction
25 ml of 0.1M phosphate buffer saline (pH 7) was added to each of the inoculated substrate beds and was vigorously shaken in rotary shaker for 15 min at 120 rpm. The mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4 °C for 15 min. The supernatant was filtered through cheesecloth and the filtrate was used as the crude enzyme preparation. Enzyme amylase was assayed by Dinitrosalicyclic acid method.

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

The culture supernatants were collected separately. 4 test tubes were taken and marked sample, pure blank (PB), substrate blank (SB) and enzyme blank (EB). With the help of a pipette, 2 ml of phosphate buffer was transferred to all the tubes. 1ml of starch was added to all tubes except PB & SB. 1% Sodium Chloride was added to all the test tubes. 1ml of distilled water was added to PB & SB. The contents of the test tubes were mixed well and then incubated for 5mins at 37 °C. After incubation crude enzyme was added to all the test tubes except PB & EB, and distilled water is added to PB & EB. The contents of the test tubes were mixed well and incubated for 10 mins at 37 °C. After incubation 1ml of 2N NaOH were added to all the test tubes. The reducing sugars liberated were assayed calorimetrically by the addition of 1ml Dinitrosalicyclic acid (DNS) reagent. The contents of the test tubes were mixed well and incubated in boiling water bath for 10 mins. Intensity of the colour developed was read at 520 nm using a calorimeter. A standard graph was plotted and the enzyme activity was calculated. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µmol of sugar per minute under the standard assay conditions and enzyme activity is expressed in terms of IU per gram fermented substrates.

2.8 Statistical analysis
The survey results were analyzed and descriptive statistics were done using SPSS 12.0 (SPSS Inc., an IBM Company, Chicago, USA) and graphs were generated using Sigma Plot 7 (Systat Software Inc., Chicago, USA).

Table 1: Details of substrates used in submerged fermentation

Substrate	Common Name	Source Plant
Tapioca water	Kappa vellam	*Manihot esculenta*
Rice water	Kanji vellam	*Oryza sative*
White Yam water	Kachill vellam	*Dioscorea rotundata*
Coconut water	Thenga vellam	*Cocos nucifera*

Fig 1: Production of amylase using submerged fermentation using various substrates, coconut water (top left); white yam water (top right); rice water (bottom left); tapioca water (bottom right).

Fig 2: Amylase produced by submerged fermentation using various substrates and their enzymatic activity, coconut water (top left); white yam water (top right); rice water (middle left); tapioca water (middle right).

3. Results and discussion

3.1. Isolation of Fungi

Four different fungal isolates differentiated on the basis of colony morphology were obtained after spreading. All the four isolates were subcultures by point inoculation and used for further studies.

3.2. Screening of Fungal Isolates for Alpha Amylase Production

All the four fungal isolates were subjected to screening procedure and after completion of incubation period plates were flooded with iodine solution and observed for zone of hydrolysis.

3.3. Identification of the Isolate Showing Maximum Hydrolysis

Based on morphological studies, and lactophenol cotton blue staining characteristics the isolate was identified as *Aspergillus niger*.

3.4. Evaluation of starchy food as Substrates for SSF

Enzyme activity in the extracted enzymes from different substrates was determined by DNS assay. The average activity of enzyme produced by *Aspergillus niger* from tapioca water as substrate was 0.06 x 10^{-3} µmoles/sec. An average activity of 0.09 x 10^{-3} µmoles/sec obtained for enzyme produced from rice water. The average activity of enzyme produced from white yam water were 0.29 x 10^{-3} µmoles/sec. From the above observations, among the three starchy substrates used white yam water was found be more efficient for the production of amylase enzyme.

This indicates that even the percentage of starch is higher in tapioca (29%) water there is a reduction in the activity of amylase than other substrates used, white Yam (21%) and rice water (22%). Higher activity was recorded in the cause of white Yam water and then rice water. It can be seen that maximum amylase activity was seen when *Dioscorea alata* water was used as substrate followed by rice water, tapioca water and coconut water. The enzyme activity was maximum in the box containing *Dioscorea alata* water as substrate and it was found to be (0.29 x 10^{-3} µmols/s) followed by rice soup (0.09 x 10^{-3} µmols/s), Tapioca water (0.06 x 10^{-3} µmols/s) and coconut water (0.02 x 10^{-3} µmols/s). *Dioscorea alata* is the most efficient substrate which produced amylase under the culture condition.

The production process of α-amylase, there are two major method for large scale production of α-amylase are solid state fermentation and submerged fermentation [28, 29]. Plant products has been reported to be good substrate for the cost effective production of alpha amylase. Many factors are involved in the production and optimization of α- amylase such as nitrogen and carbon source. If the metal ions play an important role for the production of α-amylase are metaloenzyme. Ca^{2+} and $CaCl_2$ ions are significantly important for the production of this enzyme.

Effect of temperature and pH on α- amylase activity. Increase in temperature will lead to an increase in activity reaction of kinetics, but also accelerate the denaturation induced by higher physiological temperature. If soluble enzyme is used in manufacturing process, it is beneficial to operate at the maximum temperature. The effect of temperature on α-amylase action has been studied if optimum temperature was noted to be 65 °C at low substrate concentration [18, 30-32].

The effect of pH on the enzyme activity is depending on the time and temperature. In general enzymes are less stable at high temperature over time at pH value near the limit of the optimum. The optimum pH should be determined to be under certain conditions. In such case it is important to choose an enzyme with a pH range from 4 to 11 [15, 33-37]. If the Bacillus species have pH range between 7-8 and the Aspergillus species is about the pH range between 3-5.

Among four substrates screened White Yam water gave highest enzyme production 0.29 x10^{-3} µmols/s.), which was almost two times higher than that produced by other substrates. *Dioscorea alata* has been a highly reported substrate producing promising results, among the various agriculture byproducts substrates used. Widespread suitability of *Dioscorea alata* may be due to the presence of sufficient nutrients. *Dioscorea alata* especially *Dioscorea alata* water are rich source of starch. *Dioscorea alta* are rich in fiber, protein and energy contents. These agriculture byproducts residues are cheap raw materials for amylase production

Table 2: Activity of enzyme produced by *Aspergillus niger* from various substrates; tapioca water, rice water and white yam water.

Substrate	Trial 1	Trial 2	Trial 3	Trial 4	Average
Tapioca water	0.05 x10^{-3}	0.05 x10^{-3}	0.60 x10^{-3}	0.70 x10^{-3}	0.06 x10^{-3}
Rice water	0.07 x10^{-3}	0.09 x10^{-3}	0.09 x10^{-3}	0.12 x10^{-3}	0.09 x10^{-3}
White yam water	0.27 x10^{-3}	0.27 x10^{-3}	0.30 x10^{-3}	0.30 x10^{-3}	0.29 x10^{-3}
Coconut water	0.02 x10^{-3}	0.02 x10^{-3}	0.03 x10^{-3}	0.03 x10^{-3}	0.02 x10^{-3}

4. Conclusions

The results obtained in the present study suggest that the white yam water may act as a potent substrate for industrial production of α-amylase and subjected for further explorations regarding industrial applications.

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