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STUDY OF STARCH DEGRADING BACTERIA FROM KITCHEN WASTE SOIL IN THE PRODUCTION OF AMYLASE BY USING PADDY STRAW.

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Abstract

The starch degrading amylolytic enzymes are of crucial importance in biotechnology industries with huge application in food, fermentation, textile and paper production. They are universally distribution in bacteria and fungus. Present study aimed at production of pure form of α-amylase from kitchen waste soil with optimization of raw material such as carbon and nitrogen source of the culture media for it. **Objective:** To evaluate the kitchen waste soil for production of α-amylase with optimization of carbon and nitrogen source required for culture media. **Method:** The starch degrading bacteria was isolated from the kitchen waste soil environment and was used for production of α-amylase through submerged. The paddy straw extract was shown the best source of carbon and potassium nitrate as the best source of nitrogen with optimum pH 7.0 and temperature of 30ºC. The fermentor parameters were set with the agitator at speed 200rpm with 100% dissolved O_2 at fixed temperature and pH. After completion of the fermentation process, the activity of the enzyme was checked by the DNS method. **Results:** Enzyme produced and purified by this method, was found to have an enzymatic activity of 0.51 mg/ml after column chromatography by nanodrop spectrophotometer and coincide with standard in SDS-PAGE. Here, we have shown the cheap method of commercial production of economically valuable amylase by utilizing paddy straw.

Key Words: α-Amylase, Bacteria, Fungus, Optimization, Carbon, Nitrogen, Paddy straw, Fermentor, Agitator, Dissolved Oxygen, DNS method, Column Chromatography, Nanodrop Spectrophotometer, SDS-PAGE.

Introduction

Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized into exo-acting, endo-acting and debranching enzyme. Among the amylases, β-amylase is exo-acting whereas α-amylase is endo-acting enzyme. Unusual bacterial amylases are found in acidophilic, alkalophilic and thermoacidophilic bacteira[1]. Starch degrading amylolytic enzymes are most important in the biotechnlogy industries with huge application in food, fermentation, textile and paper production[2]. There are various reports on strach degrading micro-organisms from different sources and respective amylase activity[3,4]. α-Amylase is a hydrolytic enzyme and in recent years, interest in its large scale microbial production has increased dramatically due to its wide spread use in food, textile, baking and detergent industries[5]. Amylases are one of the most important industrial enzymes that have wide variety of applications ranging from conversion of starch to sugar syrups to the production of cyclodextrins for the pharmaceutical industry. These enzymes account for about 30 % of the world's total enzyme production [6].

Amylases have been reported to occur in microorganisms, although they are also found in plants and animals. Two major classes of amylases have been identified in microorganisms, namely α-amylase and glucoamylase. α-Amylase (endo-1,4-a-D-glucan glucohydrolase, E.C. 3.2.1.1) is extracellular enzymes that randomly cleaves 1,4-a-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Glucoamylase (exo-1, 4-a-D-glucan glucanohydrolase, E.C. 3.2.1.3) hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner[2]. Fungal α-amylases are produced by different fermentation techniques. Production of these α-amylases

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has been investigated through submerged (SmF) and solid-state fermentation (SSF)[7]. Almost all members of the *Bacillus* genus synthesized α-amylase, thus this genus has the potential to dominate the enzyme industry[8]. The industrially important Bacillus strains, which are extensively used to produces α-amylase, are *B. amyloliquefaciens*, *B. licheniformis*[9], *B. stearothermophilus*[10], *B. subtilis*[11], *and B. megaterium*[12] and *B. circulans*[13]. Natural fermentated media (foods, soils and waste) offers sources for isolation of micro organism strains producing amylases. Many strains used in food industry are isolated from fermented foods media[2, 14, 15].

Industrial conversion of starch with raw starch saccharifying amylase has been reported to represent an economically superior alternative to the conventional process which uses regelatinised starch as substrate based on energy utilization and process simplicity[16]. Only few micro-organism including *Aspergillus* species have been reported to posses ability to produce raw starch degrading amylase[17].

The present study deals with the production of amylase. Soil receiving the kitchen wastes is one of the rich sources of starch degrading micro organism as it contains mostly starchy substrate**.** Paddy straw is used for the production of amylase since it contains high amount of starch in it. It is widely avaiable. It is an cheap source that provides more benefits in the production of the amylase.

Materials and Methods

Collection of soil sample

Soil sample was collected from soil near the kitchen waste environment since this soil may contain high amount of starch. From this, the starch degrading bacteria *Bacillus sp* was isolated.

Isolation of starch degrading bacteria

Serial dilution of soil sample was performed using the distilled water. This was cultured in nutrient agar medium (Peptone 5.0g, beef extract 3.0g, NaCl 5.0g, Distilled water 1000ml & pH 7.0) by spread plate technique and incubated at 37°C for 24hours.

Screening for starch degrading bacteria

The random individual colonies were picked and cultured separately on nutrient agar medium and further starch hydrolysis test was performed after the incubation period. Iodine was used as an indicator here. The colonies were isolated based on their cultural, microbial and biochemical characteristics.

Maintenance of the isolated culture

The isolated culture was maintained in nutrient agar slants and plants by streak plate technique. This culture was stored at 4°C in refrigerator and was used for further process in the production of amylase.

Optimization of media components and various parameters

(A) Optimization of carbon and nitrogen sources

The amylase production media was prepared with various carbon sources (glucose, maltose, dextrose, fructose and starch) and the culture was inoculated. This was incubated at 37°C for 24hrs. Then the enzyme activity was checked by DNS test. Similarly to optimize the nitrogen sources (urea, potassium nitrate, peptone, yeast extract and tryptone), and similarly the enzyme activity was checked as for carbon source.

(B) Optimization of pH and Temperature

The production media was prepared with all the nutritional components with the alteration in the pH alone varying from (6.0,6.5,7.0,7.5 & 8.0) also with temperature ranging from (4°C,30°C,37°C,42°C & 52°C) and was inoculated with the culture and incubated at 37°C for 24hrs. After incubation the activity was checked by DNS method.

(C) Optimization of raw material as carbon source

The raw material extract was utilized as the carbon source. The raw material of about 10gm was weighed and boiled with 200ml of distilled water and the content was reduced about 100ml. This extract was utilized for the preparation of media.

The production media was prepared using the raw materials such as molasses, coconut oil cake, wheat bran, rice bran and paddy straw. The culture was inoculated and incubated at 37°C for 24hrs. After incubation the activity was checked using the DNS test.

Production of the Enzyme

(A) Preparation of production media

The production media was prepared using all the optimized sources such as Paddy straw extract as a natural carbon source, potassium nitrate as nitrogen source, with the pH 7.0.

(B) Enzyme production by submerged fermentation

The production media of about 1litre was prepared and this was poured into the media tank in 2L Sartorius B-Lite fermentor. Then the set up was autoclaved for about 3hrs and was allowed to cool. After cooling, this was inoculated with the 5ml of broth culture using a sterile syringe. Then all the parameters such as temperature 30°C, pH 7.0, Dissolved oxygen 100%, agitator speed 200rpm was kept constant. To control the pH Acid 0.1N HCl and Alkali – 0.1N NaOH was used. Then the fermentor was allowed to run for about 24hrs. After 24hrs, the enzyme source was taken along with the media for downstreaming processes.

Downstreaming

(A) Centrifugation and ammonium sulphate precipitation

The crude source of the enzyme was collected by centrifugation process. Leaving out the pellet, the supernatant was considered as the crude source of the enzyme. Then further, this supernatant was subjected to ammonium sulphate precipitation. This phenomenon was used to precipitate the enzyme.

(B) Dialysis

The precipitated enzyme was transferred to dialysis bag and the enzyme along with the bag was kept immersed in the beaker containing 500ml of phosphate buffered saline. By the process of osmosis, the enzyme was purified here.

(C) Ion exchange chromatography

About 0.5ml of 10mM Tris buffer was taken into four different tubes. About 5ml of each of 5mM, 10mM, 15mM and 20mM was added to the test tubes and the volume was made upto 10ml with distilled water. Then the sample was added into the column and eluted. Then 10ml of 5mM NaCl solution was added and eluted, this was followed by 10mM and 15mM respectively.

After each step in the downstreaming process the activity of the enzyme was checked by using the DNS test.

SDS-PAGE

The SDS-PAGE apparatus was set up and then the sample was loaded in it along with the marker.

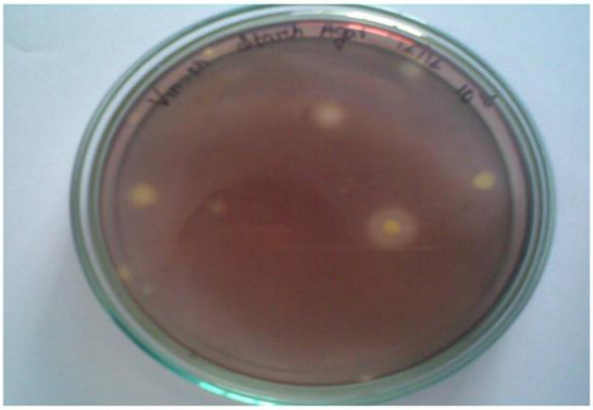
Enzyme Assay

Using the Nano-Drop Software at first the wavelength was set to 280nm and blank was measured using 3 microlitre distilled water. Then the sample was loaded of the same quality and the protein was measured.

Results and Discussion

The food industries, including brewing, baking and jam making, are the main users of starch. Starch hydrolyzing enzymes include the α-amylases (EC 3.2.1.1), ß-amylases (EC 3.2.1.2), glucoamylases (EC 3.2.1.3), isoamylases (EC 3.2.1.68), pullulanases (EC 3.2.1.41) and cyclodextrin glucanotransferases (EC 2.4.1.19)[18]. The genus *Bacillus* produces a large variety of extracellular enzymes, some of which are of industrial importance. Some strains have been developed already for the massive production of a particular amylase for industrial purposes[19]. The screening for a single amylase is difficult because one strain can produce different amylases with different specificities or the amount of amylase produced may be very low. Thus, the cloning of one gene directing the synthesis of the desired amylase in a well characterized host like *E. coli* or *B. subtilis* should help greatly in the characterization of new amylases and also will allow a significant increase in yield[20,21]. To improve production of α-amylase, as well as to construct a recombinant *Saccharomyces cerevisiae* strain for the baking industry, the α-amylase gene of *B. subtilis* RSKK246 was cloned and expressed in both *E. coli* and *B. subtilis*.

Fig 1: Isolation of amylase producing organism



The bacterium from cassava steep water was isolated. Cassava is a good amylase producer. Hence cassava steep water was used for the production of amylase since it has got potential industrial applications[22]. Characteristics of the isolated strain showed positive responses for Gram's staining, motility and utilization of carbohydrates such as glucose and maltose[22]. The kitchen waste soil contains high amount of starch and that was utilized for the isolation of starch degrading bacteria.

Figure 1 represents the isolated colonies. The colonies were isolated by serial dilution and the colonies were counted. The colony characteristics of the isolated culture were studied**.** On adding the iodine solution to starch agar plates, the colonies that had the ability to hydrolyze the starch only formed the zones **(Figure 1**). The various morphological characteristics of the isolated culture are shown in **Table 1.** Depending upon the zone of clearance, colonies were selected and the biochemical tests **(Table 2**) were performed.

Table 1: Morphology characteristics of isolated *Bacillus* sp

S.No	TEST	RESPONSE OF SPECIES
1	Grams staining	Positive
2	Shape	Short rod
3	Motility	Motile
4	Spore staining	Endospore

Table 2: Biochemical characteristics for *Bacillus* Sp

Sl. No	TEST	RESPONSE OF SPECIES
1	Indole	Negative
2	Methyl Red test	Negative
3	Voges prauskeur test	**Positive**
4	Citrate test	**Positive**
5	Catalase test	**Positive**
6	Urease test	Negative
7	Carbohydrate ferementation	
	1. **Glucose**	**Positive**
	2. **Fructose**	Negative
	3. **Maltose**	**Positive**
	4. **starch**	**Positive**

From the results of biochemical tests, the culture was selected for the further studies. By performing biochemical tests, *Bacillus sp.* were isolated and identified for the further studies. On exposure to different pH and temperature, the colony produced the best growth at pH **7.0** and the temperature **30°C.** The conventional method of optimization involves changing one independent variable while maintaining all others at a fixed level. The present investigation was aimed at optimization of medium components **(starch, potassium nitrate and paddy straw)** which has the significant role in the maximum production of amylase **(Fig 2-6)**.

Fig 2: Optimization of PH

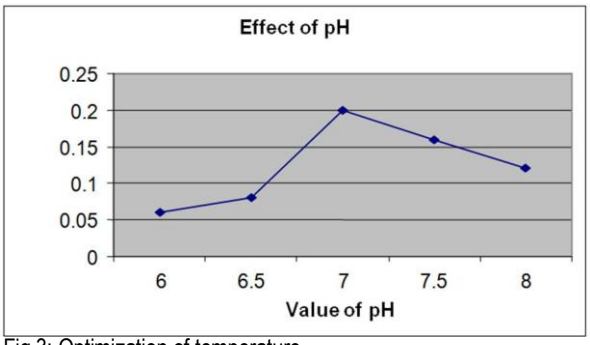


Fig 3: Optimization of temperature

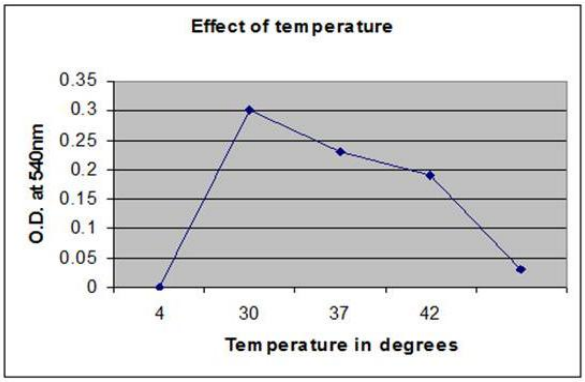


Fig 4: Optimization of carbon source

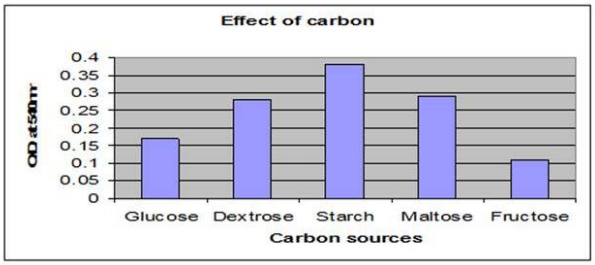


Fig 5: Optimization of nitrogen source

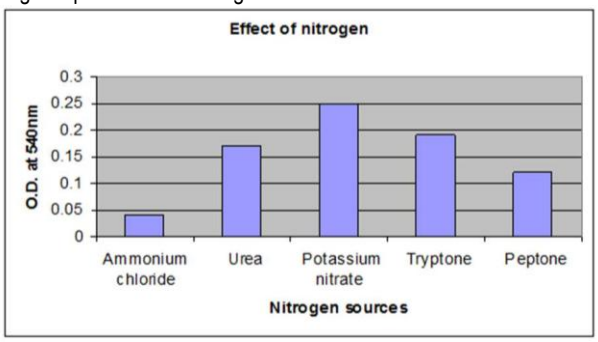


Fig 6: Optimization of raw materials

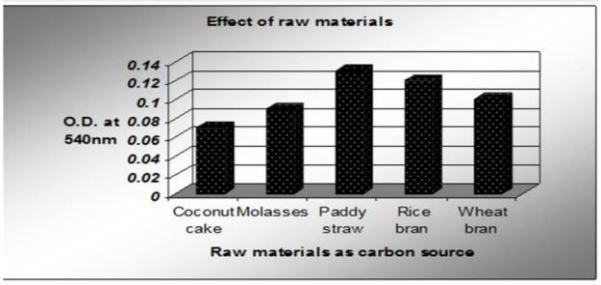
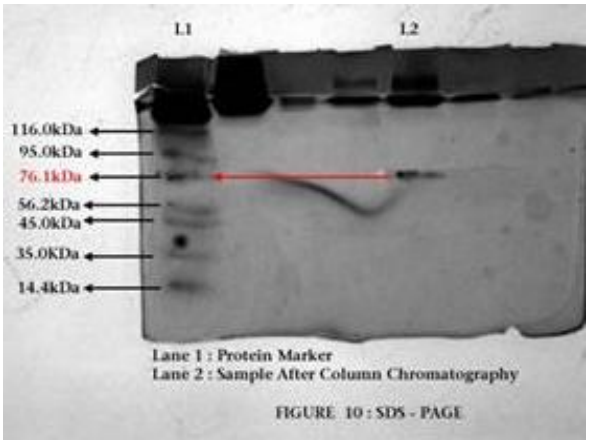


FIG 7: SDS-PAGE



The isolated bacterial colonies showed positive response towards Gram's staining, spore staining and motility. In biochemical characterization, the bacteria showed positive response towards starch, glucose, maltose

in carbohydrate fermentation and Voges-Proskauer, citrate, catalase and nitrate in other tests and this is represented in **Table 1 and 2.** *Bacillus thermooleovorans* is reported to prefer glucose as a good carbon source and caesin as a good nitrogen source. The activity was found to be optimum at pH 7.0 and temperature 55°C. On using raw materials, wheat bran showed the highest production[23]. The bacteria showed optimum activity at pH 7.0 and temperature 30°C. The carbon sources used were glucose, maltose, starch, fructose, dextrose and the nitrogen sources used were urea, peptone, ammonium chloride, potassium nitrate and tryptone. Here starch and potassium nitrate showed better utilization than the other sources. On using the raw materials such as wheat bran, rice bran, paddy straw, molasses and coconut oil cake, the bacteria showed the optimum activity on utilizing paddy straw. This was represented in **Fig 5-9.**

Table 3. Enzyme assay by Nano Drop Spectrophotometer

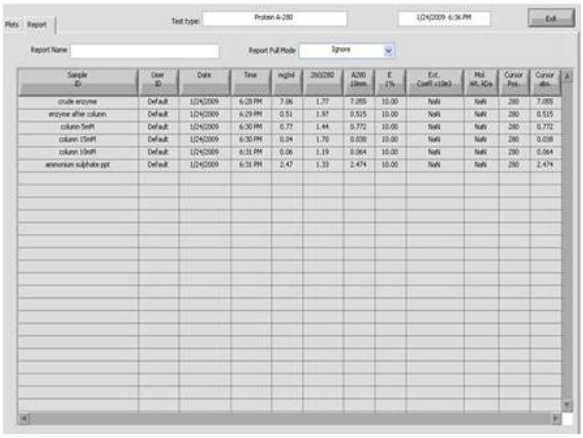
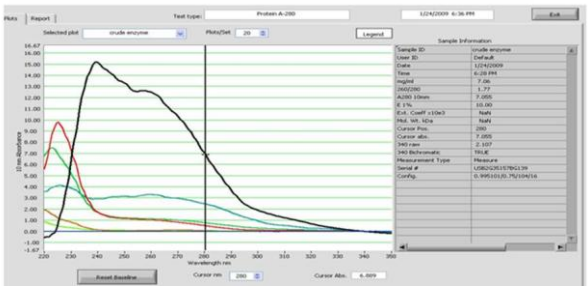


Fig 8: Enzyme assay



α-Amylase activity was determined by procedure of Bernfeld (1955) using soluble starch as a substrate[24]. The amylase activity was determined by using Miller (1959) DNS test using glucose as a substrate.

The elution profiles on DEAE cellulose and BioGelP100 were determined. There were more than one peak of amylase activity but the one with the highest activity was purified further. The purified sample yielded α-amylase with a specific activity of 854.8U/mg of protein with a yield of 43.17%[22].

Though many earlier studies have been done on production of α-amylase, this study involved the submerged state fermentation. Purposefully this method was used since it gives high nourishment than that of the solid-state fermentation. After fermentation, the produced enzyme was purified by centrifugation. This method was preferred since the macro molecules and the cell debris get settle down forming the pellet in the tube, so that the crude source of the enzyme can be obtained. Then ammonium sulphate precipitation was performed. In this, by the process of salting in the enzyme, the salt forms a bond with enzyme and gets precipitated. Since enzymes do not readily form any bonds with water, salt solution is preferred and the enzyme is precipitated out. Then the sample was subjected to dialysis with phosphate buffered saline, on the principle of osmosis The salt solution alone migrate towards the PBS leaving the enzyme in the dialysis bag, which was possible due to the porosity of the bag which allowed only salt solution to leak out of the bag and not the enzyme. In the column chromatography, the enzyme sample after dialysis was used and eluted. After the final step of the down streaming process, in the SDS PAGE, the sample was loaded along with the marker, using which the molecular weight of the protein can be found. The enzyme activity at each purification method was measured to be 7.06mg/ml after centrifugation, 2.47mg/ml after ammonium sulphate precipitation, 0.51mg/ml after column chromatography by nanodrop spectrophotometer and this was represented in **Table 3 & Figure 8.** The finally purified enzyme separated by SDS PAGE are shown in **(Figure 7)** which were separated and compared to the standard marker in Lane 1, of 116.0 kDa, 95.0 kDa, 76.1 kDa, 56.2 kDa, 45.0 kDa, 35.0 kDa and 14.4 kDa kDa and purified sample in Lane 2. Standard of 76.0 kDa was shown to coincide with the purified enzyme. Thus the amylase, isolated from kitchen waste soil bacteria *Bacillus sps,* optimized using starch, potassium nitrate and cheap raw material paddy straw. Paddy straw was mainly used because it is a very cheap source of carbon and also easily available. Due to all the above reasons, the economically valuable amylase can be commercially produced on large scale by utilizing paddy straw in a cheaper way.

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