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Production and partial characterization of a raw starch hydrolyzing enzyme from Aspergillus carbonarius S-CSR-0002.

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ABSTRACT

Conversion of raw starch by amylase is important in a way that some of the products could be used as industrial raw materials for value added products. This will reduce wastage and improve economic gain. This study was performed to isolate raw starch digesting microorganism from soil. This begins with the sample collection screened for amylase producers by observing the halo zone appeared around the colonies. Microorganism was characterized by saline wet mount and LPCB staining technique was Aspergillus carbonarius S-CSR-0002, gave the amylase yield (880 U/ml) in submerged fermentation process. Amylase from Aspergillus carbonarius S-CSR-0002, a fungus isolated from soil contaminated with canteen kitchen waste showed an ability to degrade tapioca, corn, arrow root, rice starches. Tapioca has the highest degree of hydrolysis followed by corn, rice, arrow root, consecutively. The crude amylase preparation had temperature and pH optimal activities at 30 °C and 7.0 respectively, optimal stabilities at 30 °C and 7.5 respectively. The optimum substrate concentration was 5%. The highest adsorption of crude enzyme was found with rice starch followed by arrow root, corn and tapioca. Crude enzyme was partially purified by 20-100% ammonium sulphate precipitation followed by dialysis, in which maximum amylolytic activity shown by 20% fraction was 1850 U/ml.

Key words: Amylase, Aspergillus carbonarius S-CSR-0002, raw starch, tapioca.

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INTRODUCTION

Starch is the form of storage polysaccharide present in plants and a vital source for production of sugar syrups, having a wide application in food industry [1]. Starch is the raw material required for the production of low molecular weight products such as glucose or dextrose, maltose, maltotriose and dextrin, which are applied widely in sugar, spirits, brewing and textile industries [2]. Because of the rising fossil fuel prices, energy derived from starch become the focus of attention [3]. Grain starch depolymerizes to glucose by the hydrolysis of α -1, 4 and α -1, 6 linkages between glucose monomers. Tapioca is starch extracted from cassava and has wide application in food, textile, chemical and pharmaceutical industry.

Conversion of starch to glucose, maltose and dextrin, involves gelatinization, liquefaction and sacharification process [4], which is achieved by either acid hydrolysis or enzymatic process, but dilute acid and high temperature corroded the equipment and it causes undesirable products limited yield and was costly. The use of enzyme has more advantages [5], but high temperature liquid phase enzymatic hydrolysis is used widely.

In view of energy costs, so direct hydrolysis of starch below gelatinization temperature is desirable, and there is reduced or no fermentation excess enthalpy and increases the net energy yield [6]. This strategy can be applied to raw starch which is produced by dry or wet milling. Thermally gelatinized starch have high viscosity and is very difficult to pump. When viscosity reduces the capacity of the equipment applied to the conversion also increases [7] and the absence of high temperature cooking minimises the reaction byproducts that decrease the yield [8]. This generated a worldwide interest in the discovery of several raw starch digesting amylases which does not require the gelatinisation and can directly hydrolyze the raw starch in a single step.

Raw starch digesting amylases (RSDAs) are enzymes which can catalyze the degradation of raw starch to sugars. RSDAs vary from other amylases in their special affinity and interaction with the microcrystalline structures of the raw starch molecule, through the starch binding domain, SBD [8], but other starch hydrolases can only act on gelatinized starch. For bioconversion of starch and starch based products raw starch digesting amylases from Aspergillus species have found important [9, 10]. Some of these enzymes are starch liquefying where as others are saccharifying. There are basically four groups of starch-converting enzymes: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; (iv) transferases.

Here, we report a new technique for selecting raw starch digesting enzyme producing fungal species on raw cassava starch and its identification, partial purification and characterization for the commercial utilization of the enzyme.

MATERIALS AND METHODS

PREPARATION OF RAW STARCH AS SUBSTRATE:

Raw starches such as Tapioca, corn, arrow root, rice were commercially purchased and washed thoroughly with distilled water, air dried, powdered, sterilized by 70% ethanol and dehydrated by acetone before use.

MATERIALS AND CHEMICALS:

Dialysis membrane,3 ,5-dinitrosalisylate reagent (DNS), were purchased from Sigma Chemicals Company, USA. Other analytical grade chemicals were purchased from E. Merck Ltd (India).

SAMPLE COLLECTION:

Sample were collected from soil contaminated with decaying material, including kitchen waste from college canteen.

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ISOLATION OF RAW STARCH HYDROLYZING FUNGUS FROM SOIL:

PROCEDURE OF ENRICHING THE SAMPLES:

Adequate quantity of soil sample was suspended in sterile distilled water (1g soil was suspended in 4 ml sterile distilled water) and mixed well. From this, 1 ml of supernatant was taken and inoculated in to conical flasks containing 20 ml of the enrichment medium. Kept for incubation at room temperature for 72 hr in a rotary shaker at 200 rpm. After 72 hr, 1 ml of the culture was transferred to 20 ml fresh sterile enrichment medium and incubated for 72 hr. This procedure was repeated for three times. After completing the third cycle of incubation, the broth cultures were taken for isolating microorganisms grown in it.

ISOLATION OF MICROORGANISMS FROM ENRICHMENT MEDIA:

Sample from enrichment broth cultures were spotted on to potato infusion agar plates. Incubated for 72 hr at room temperature. Isolated colonies grown on plates were selected and taken for confirming their RSHE production.

SCREENING OF ISOLATES FOR RSHE PRODUCTION:

Isolated colonies were cultured on potato infusion agar and incubated for 72 hr at room temperature. After incubation, a clear zone formed due to the production of RSHE by isolates.

PURIFICATION OF ISOLATE:

Colony of selected organism was isolated and purified by inoculating on potato infusion agar plate. This purified isolate was stored in refrigerator and maintained at monthly intervals.

IDENTIFICATION OF ISOLATE:

The selected organism was identified based on its various cultural, morphological and staining characteristics.

ENZYME PRODUCTION:

Enzyme production was done by submerged fermentation by using different raw starches as growth substrate (rice powder, corn powder, arrow root powder and tapioca powder)

SUBMERGED FERMENTATION:

A carbonarius was inoculated in potato infusion broth containing 0.5 % sterile raw starch (above mentioned) and incubated at room temperature for 3 days. The medium was then centrifuged and the supernatant was collected for separating extra cellular enzyme amylase. The enzyme activity was recorded. The growth substrate which gave maximum enzyme production was used for further studies [11].

ENZYME EXTRACTION:

The mixture was filtered through cheese cloth and centrifuged at 8,000 rpm at 4 °C for 15 min. The supernatant was filtered through cheese cloth and the filtrate was used as the crude enzyme preparation. Enzyme amylase was assayed by 3, 5- dinitro salicylic acid method [11].

AMYLASE ACTIVITY:

One unit of amylase activity is defined as the amount of enzyme required to catalyze the release of 1µg of glucose per ml under reaction conditions.

ENZYME ASSAY:

0.5 ml enzyme incubated for 10 min at 37 °C with 0.5 ml 1% raw tapioca starch solution in 0.2 M citrate phosphate buffer (pH 6), reaction stopped by adding 1 ml 3, 5 - DNS reagent. Tube containing mixture heated for 5 min in boiling water bath, cool in tap water, after diluting with the addition of 10 ml distilled water, the absorbance was determined at 540 nm using spectrophotometer [11].

OPTIMIZATION OF CULTURAL CONDITIONS:

For the RSHE production, three factors were selected, namely, substrate concentration, pH and temperature. The effect of three factors on amylase production was experimented.

EFFECT OF SUBSTRATE CONCENTRATION ON ENZYME PRODUCTION:

To study the effect of substrate concentration on enzyme production, One ml of uniformly prepared spore suspension (10^5 spores ml^{-1}) from 3 days old cultures was used as inoculums and incubated at different substrate concentrations such as 0.5% to 4%. Inoculated medium was incubated at 37 ºC in orbital shaker for 48 hr at 100 rpm and then estimated its enzyme activity.

EFFECT OF PH ON ENZYME PRODUCTION:

The production medium was adjusted at different pH levels such as pH 4.0 to 9.0 using 0.2 molar specific buffer solutions. One ml of uniformly prepared spore suspension (10^5 spores ml^{-1}) from 3 days old cultures was used as inoculums (*Aspergillus carbonarius*). Inoculated medium was incubated at 37 ºC in orbital shaker for 48 hr at 100 rpm and then estimated its enzyme activity.

EFFECT OF TEMPERATURE ON ENZYME PRODUCTION:

To study the effect of incubation temperature for maximum amylase enzyme production, solution was incubated at different temperatures such as 20 °C to 60 °C. One ml of uniformly prepared spore suspension (10^5 spores ml^{-1}) from 3 days old cultures was used as inoculums. Inoculated medium was incubated at 37 ºC in orbital shaker for 48 hr at 100 rpm and then estimated its enzyme activity.

PARTIAL CHARACTERIZATION OF RSHE:

EFFECT OF SUBSTRATE CONCENTRATION ON ENZYME ACTIVITY:

Effect of substrate concentration (%) on the enzyme activity was measured by conducting amylase assay at various substrate concentration ranges 0.25-4%. Activity of the enzyme was measured as described previously [12].

EFFECT OF PH ON ENZYME ACTIVITY:

Effect of pH on the enzyme activity was measured by conducting amylase assay at various pH ranges (2.0-9.0) using 0.2 M buffers. Activity of the enzyme was measured as described previously [13].

EFFECT OF TEMPERATURE ON ENZYME ACTIVITY:

The activity of the enzyme was determined by incubating the reaction mixture in the amylase assay at different temperatures (°C) ranging from 20-55 °C were studied. The activity of the enzyme was measured as described previously [13].

ENZYME STABILITY:

Enzyme stability is determined to find out at what conditions the enzyme can be stored by maintaining its maximum activity and at those conditions, the enzyme remains most stable [12].

TEMPERATURE STABILITY:

Mixed 0.5 ml of supernatant with 0.5 ml of 0.2 M Citrate phosphate buffer (pH 6.0) in different test tubes. Each tube is incubated at different temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 °C) for 24 hr. Added 0.5 ml of raw tapioca starch solution to each tube. Incubated at 37 °C for 10 min. Added 1 ml DNS reagent to each tubes, filtered and filtrate was collected. Tube containing mixture were heated for 5 min in boiling water bath and cooled in running tap water. Diluted by adding 10 ml of distilled water. Absorbance was determined at 540 nm.

PH STABILITY:

Mixed 0.5 ml of supernatant with 0.5 ml of 0.1M buffers at different pH ranges in different test tubes (pH: 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0). Each tube was incubated at 37 °C for 24 hr. Added 0.5 ml of raw tapioca starch solution to each tube. Incubated at 37 °C for 10 min. Added 1 ml DNS reagent to each tubes, filtered and filtrate was collected. Tube containing mixture were heated for 5 min in boiling water bath and cooled in running tap water. Diluted by adding 10 ml of distilled water. Absorbance was determined at 540 nm.

DETERMINATION OF RAW STARCH ADSORBABILITY:

Affinity of enzyme towards raw tapioca, corn, rice arrow root starch was studied by incubating 0.5 % of each raw starch with 0.5 ml of enzyme at 37 ^{o}C for 10 min. After centrifugation, amylase activity of the supernatant was measured and the adsorption percentage was calculated by the following equation:

$$\text{Adsorption (\%)} = [(B-A)/B] \times 100$$

Where, A indicates the residual activity after adsorption on raw starch and B represents the activity on the original enzyme solution [14].

PARTIAL PURIFICATION OF ENZYME USING AMMONIUM SULFATE PRECIPITATION METHOD:

ENZYME ASSAY:

Amylase assay was conducted before and after partial purification of enzyme with the above mentioned procedure.

AMMONIUM SALT PRECIPITATION METHOD:

The crude enzyme (100 ml) was placed in an ice bath. Ammonium sulphate was added at varying concentration of 20%, 40%, 60%, 80% and 100% saturation with constant stirring under ice for 1hr. Precipitated protein was removed by centrifugation at 10,000 rpm for 20 min at 4 °C. Then the supernatant was discarded. The precipitate was dissolved in minimum volume of 50 mM Tris - HCl of pH 8 [12], assayed for determining enzyme activity [15].

DIALYSIS OF THE PURIFIED ENZYME:

8 cm of the dialysis bag was cut and placed in 100 ml of 2 % w/v sodium bicarbonate. 1 mM EDTA was added to chelate any metal ions. It was boiled for 10 min and then washed with boiling distilled water for 10 min. The boiling process was repeated with distilled water again. The activated dialysis bag was filled with the enzymes and sealed from the both sides without any air bubbles using closure clips. The bag was kept in 500 ml of 50 mM Tris-HCl (pH 8) solution on a magnetic stirrer in ice cold condition for 8 hr. The buffer was changed frequently for every hour to avoid equilibration.

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RESULTS

ISOLATION OF RAW STARCH DIGESTING FUNGUS:

Based on amylolytic activity which was reflected by wide clear zone formation around the colony on the solid potato infusion agar media, was selected for further screening (Fig. 1). A new strain of Aspergillus carbonarius S-CSR-0002 isolated from soil sample showed a high raw starch digesting activity towards tapioca starch.

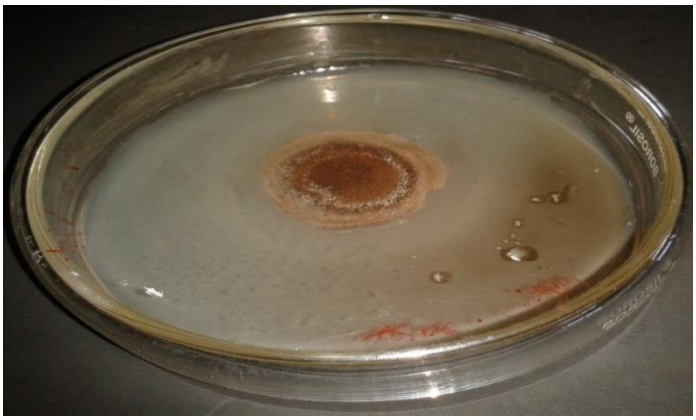

Fig. 1: Isolates on potato infusion agar with tapioca as substrate

ENZYME PRODUCTION:

SUBMERGED FERMENTATION USING DIFFERENT GROWTH SUBSTRATE:

The ability of the crude amylase of Aspergillus carbonarius S-CSR-0002 to digest different raw starches (Tapioca, corn, arrow root, rice) was studied. Tapioca starch was used as the standard. The results are presented in Fig. 2 in terms of the amount glucose (µg/ml) produced.

Results indicated that at 10 min incubation the crude enzyme was able to hydrolyse the raw starches tested. Tapioca and corn powder were rapidly hydrolysed to give 400 U/ml and 300 U/ml glucose respectively. Arrow root was least hydrolysed to give 40 U/ml of glucose.

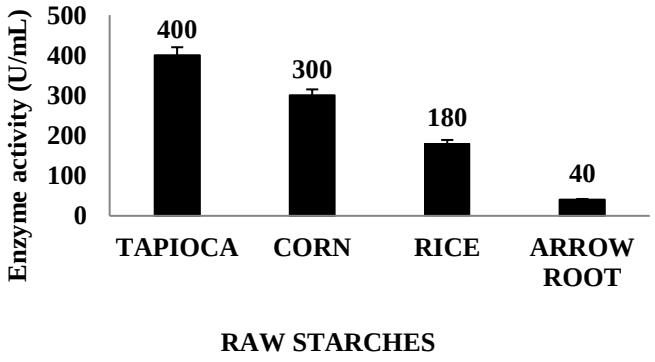

Fig. 2: Production of enzyme using different raw starches

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OPTIMIZATION OF CULTURAL CONDITIONS:

Effect of three factors on amylase enzyme production was experimented and the results are shown in (Fig. 3, 4 and 5).

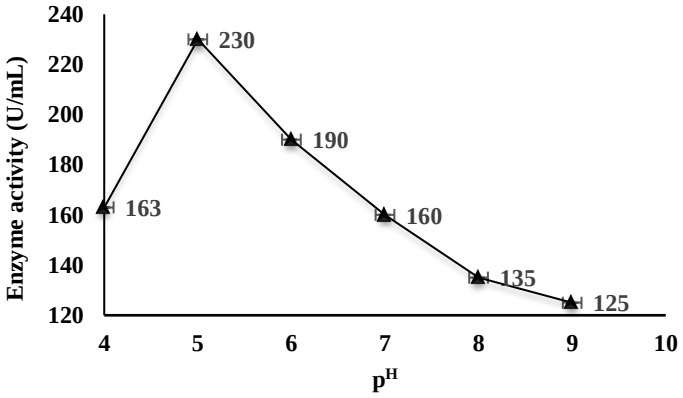

Fig. 3: Effect of p^H on enzyme production

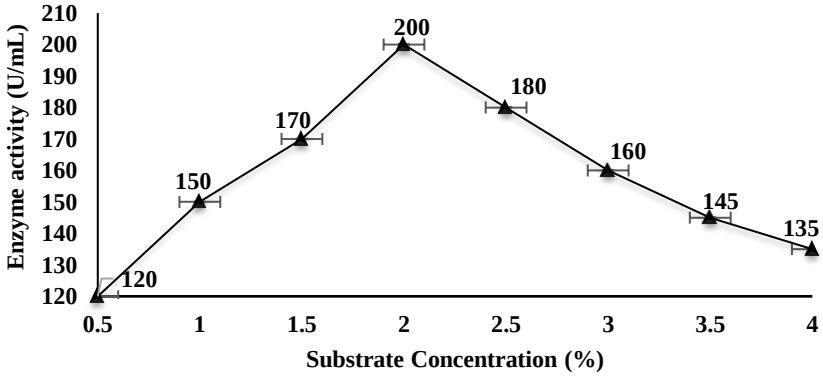

Fig. 4: Effect of substrate concentration on enzyme production

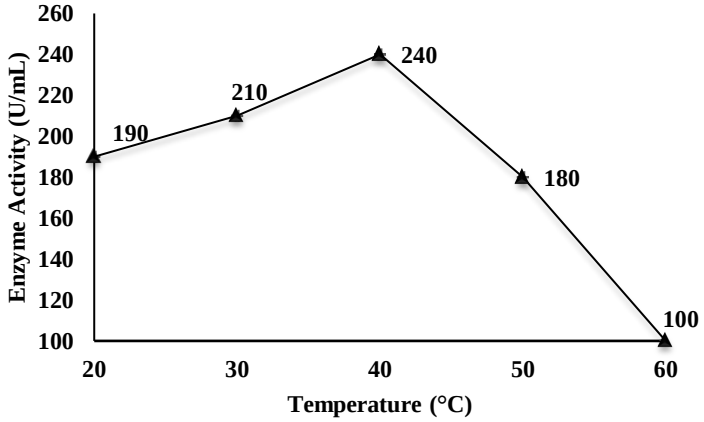

Fig. 5: Effect of temperature on enzyme production

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PARTIAL CHARACTERIZATION OF RSHE:

EFFECT OF SUBSTRATE CONCENTRATION ON ENZYME ACTIVITY:

Aspergillus carbonarius S-CSR- 0002 is very effective on the 5% starch solution. Amylase activity increased with increase in the starch concentration from 1% to 5%. Beyond 5% there was a decline in amylase activity (Fig. 6).

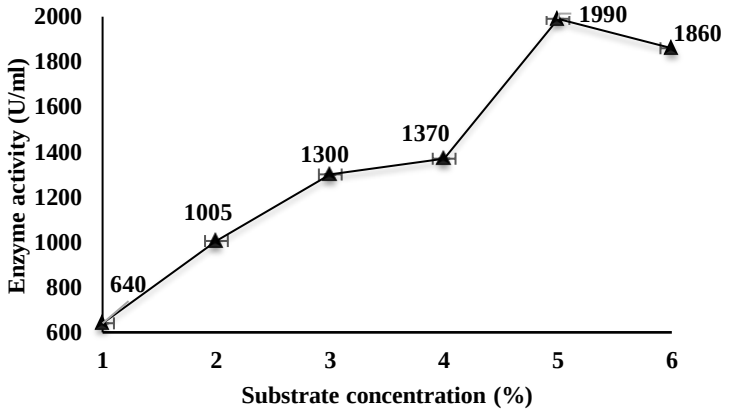

Fig. 6: Enzyme assay at different substrate concentration

EFFECT OF PH ON ENZYME ACTIVITY:

The influence of pH on the activity of crude enzyme was determined using buffers at various pH values (Fig. 7). Aspergillus carbonarius S-CSR-0002 showed optimum amylase activity at pH 6.0 was 1130 U/ml. A gradual decrease in pH is observed after pH 6.0.

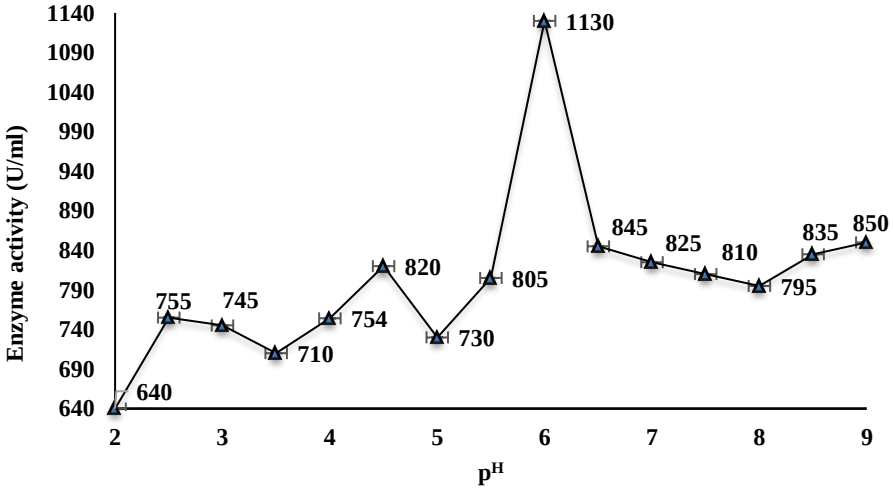

Fig. 7: Enzyme assay at different pH

EFFECT OF TEMPERATURE ON ENZYME ACTIVITY :

The influence of temperature on amylase activity of the crude enzyme showed that enzyme activity increased progressively with increase in temperature from 20 °C reaching a maximum at 30 °C (Fig. 8). Above 30 °C, there was a reduction in the amylase activity.

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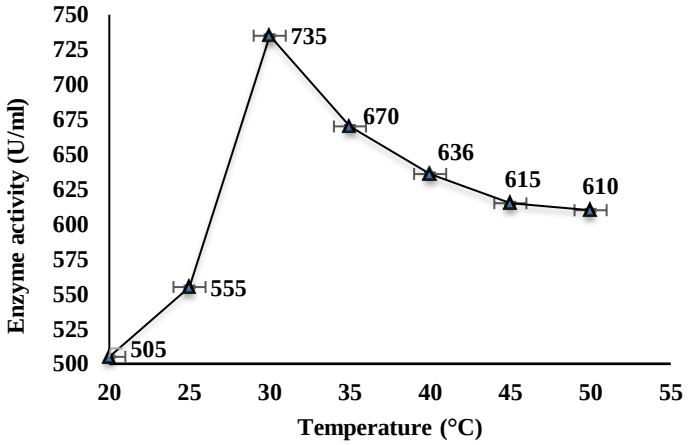

Fig. 8: Enzyme activity at different temperatures

ENZYME STABILITY:

TEMPERATURE STABILITY:

Enzyme stability was determined to find out at what conditions the enzyme can be stored by maintaining its maximum activity. At these conditions, the enzyme remains most stable.

The thermal stability of the enzyme was measured for 10 min at pH 4.0.The enzyme was stable in the temperature range of 20-35 °C and rapid decrease in activity was observed at temperature above 35 °C (Fig. 9).

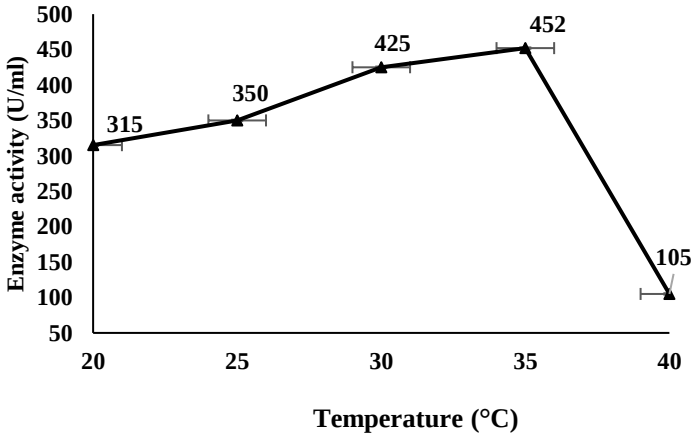

Fig. 9: Enzyme stability at different temperatures

PH STABILITY:

pH stability of Aspergillus carbonarius S-CSR- 0002 amylase was evaluated by assaying the enzyme after incubation of the enzyme in different buffer systems of pH value 2.0 to 10.5 for 20 min. The enzyme was stable at pH 6.5-7.5 and a rapid decrease in activity was observed in pH 8.0 (Fig. 10).

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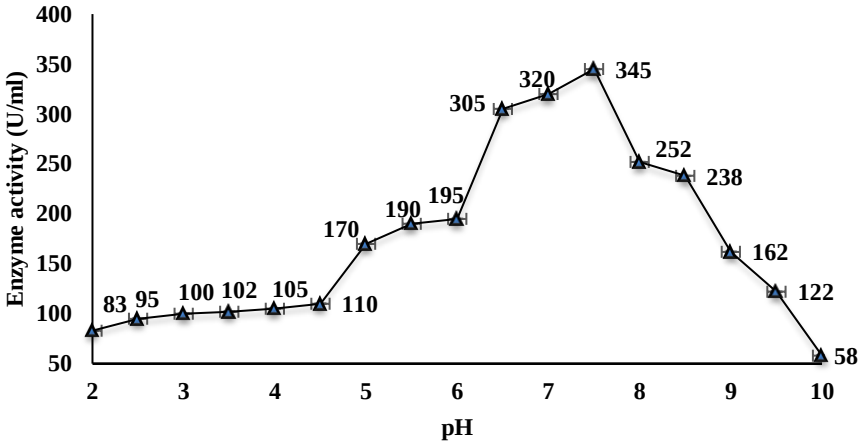

Fig. 10: Enzyme stability at different pH

DETERMINATION OF RAW STARCH ADSORBABILITY:

The adsorption of amylase on various types of raw starch was determined. The highest adsorption was found with rice starch followed by arrow root, corn and tapioca (Fig.11).

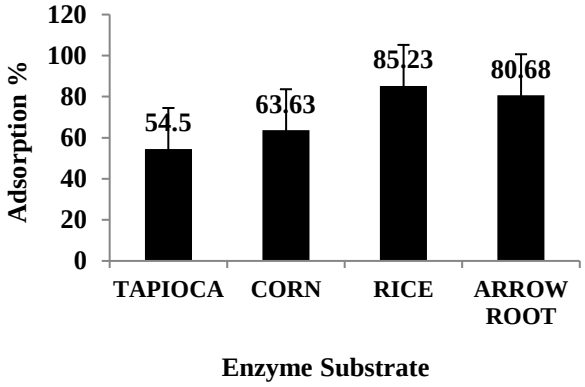

Fig. 11: Adsorption percentage of enzyme on different substrates

PARTIAL PURIFICATION OF ENZYME:

ENZYME ASSAY:

The activity of enzyme achieved was about 880 U/ml.

PROTEIN ESTIMATION:

Protein content was estimated using BSA [15] and the amount of protein present was 0.1 mg/ml.

AMMONIUM SULPHATE PRECIPITATION METHOD FOLLOWED BY DIALYSIS:

The culture supernatant of Aspergillus carbonarius S-CSR-0002 containing an initial enzyme activity of 880 U/ml was concentrated by varying concentrations of ammonium sulphate precipitation followed by dialysis. Varying concentrations of ammonium sulphate precipitation screening was carried out to determine the best percentage of ammonium sulphate saturation which gave the highest amylolytic activity. The result

for ammonium sulphate saturation on the precipitation of enzyme from Aspergillus carbonarius S-CSR-0002 was shown in Table 1. The optimum ammonium sulphate saturation was 20 % saturation.

Table 1: Enzyme assay in different fractions

Percentage of ammonium sulphate saturation	Enzyme activity (U/ml)	Protein content (mg/ml)
20%	1850	0.175
40%	1725	0.165
60%	1400	0.135
80%	1500	0.1
100%	1425	0.14

DISCUSSION

Microorganisms that produce amylase could be isolated from places such as soil, around mills, cassava farms and processing factories and flour markets [16]. During the study, amylase producing fungal strains were isolated from soil contaminated with decaying material, including kitchen waste from college canteen. Among the different fungal species Aspergillus carbonarius S-CSR-0002 has maximum amylase activity on raw cassava starch, and this species required lesser incubation (72 hr) showed a complete disappearance of the starch as compared to previous studies and a similar finding observed in G. candidum CMSS06 [3].

Most hydrolyzing enzymes have limited capacity against native starch granules but high energy is necessary for starch gelatinization stimulates the search for new amylases which act on raw starch [17].

This study showed that the susceptibility of raw starch to crude enzyme of Aspergillus carbonarius S-CSR-0002 was dependent on the starch source, this agrees with earlier reports [18]. Numerous fungi have shown the amylolytic activity but very few can hydrolyse raw starch. The important advantage of this enzyme compared to previous studies is that this enzyme can hydrolyze a wide variety of raw starches. Production of enzyme by solid state fermentation with this fungi is a production technique which is cost effective and low cost medium is used.

Optimization of medium composition is an important factor for overproduction of the enzymes to meet industrial demands [19]. Production of amylase by fungi depends on morphological state and metabolic state of the culture. Most production studies showed amylase production at a temperature range of 25-37 °C, our results also showed an optimum temperature of 40 °C with a range of 20-40 °C. A raw starch degrading amylase was produced at 30 °C by Aspergillus ficuum [20]. Results showed a pH of 5.0 for the optimal growth. Similar results were shown by earlier reports of Aspergillus species.

Aspergillus carbonarius S-CSR-0002 showed optimum amylase activity at pH 6.0 was 1130 U/ml. A Gradual decrease is observed after pH 6.0. A similar profile was observed in Streptomyces on raw corn starch as substrate [21]. Neutral p^H was found to be optimal p^H for amylase activity by B. thermooleovorans NP54 and also reported in B. coagulans [22], B. licheniformis [23] and B. Brevis [24].

Most of the raw starch digesting amylases are known to exhibit temperature optima between 40 °C and 60 °C and are stable at high temperature [25]. Surprisingly activity of enzyme in this study was at 30 °C. With increase in temperature enzyme activity increases until 30 °C and enzyme activity declined suddenly after 30 °C. This is due to the velocity of enzymatic reaction increases with the increase in temperature within limited range because of increase of kinetic energy of molecule until it reach to the degree of maximum reaction velocity, but the increase of temperature at ranged which lead to disruption of the three dimensional structure of the enzyme and then decreases its velocity [26]. How much active and stable an enzyme is during application and stronger condition is an important factor for its industrial application. This enzyme showed stability at 30- 35 °C and pH 6.5-7.5. Successful industrial use of amylases requires that they are sufficiently stable and active at application conditions such as temperature and pH.

Results suggest that Arrow root, rice and corn starch are more resistant to this enzyme action compared to raw tapioca starch. In previous study [27], one of the determining factor on adsorption is the ratio of surface area to volume. Larger granules have smaller ratio resulting in lower enzyme attachment. Rice have smaller granule size (<20 µm). Another factor determining the ratio is shape of granule, the more spherical the small is the adsorption ratio. Cassava starch is spherical in shape while rice is oval in shape. Raw starch adsorption efficiency is not correlated with amylase degradation on raw starches. Similar reports shown by adsorption of Rhizopus niveus, Aspergillus awamori var. F2035 and Aspergillus oryzae glucoamylases, which was inversely correlated with raw starch digestion [28, 29].

Based on the result, the crude enzyme shows very high enzyme activity at 20% ammonium sulphate saturation, indicated that the protein tend to aggregate and salt out of culture solution at low ammonium sulphate concentration. However the amylase enzyme activity decreases at 40-60% of ammonium sulphate concentration. This indicated that the enzyme couldn't precipitate at high ammonium sulphate concentration. The enzyme precipitated at 20% ammonium sulphate gave the highest enzyme activity of 1850 U/ml. When compared to crude enzyme the enzyme activity increased more than two times with 20% ammonium sulphate saturation. After each fractionation culture filtrate was centrifuged and the precipitate was dissolved in least volume of dialyzing buffer, which was then used for dialysis.

CONCLUSION

Soil is known to be repository of amylase producers. Conversion of raw starch by this enzyme means that some of them could be used as raw materials by starch industry for value added products. This will reduce wastage and improve economic gain. Of the different carbon sources used as sole carbon sources starch at 5% supported maximum enzyme secretion. In this study one fungal strain that belongs to the genus Aspergillus (coded as Aspergillus carbonarius S-CSR-0002), was isolated, culture conditions evaluated and its enzyme characterized. In this study the effect of carbon sources, temperature, pH of the medium at which maximum activity occurred and stability were evaluated. In conclusion, the crude amylase of A. carbonarius S-CSR-0002 selected for this study is capable of hydrolyzing tapioca, corn, arrow root, rice into glucose which can then be used directly in the production of ethanol and fructose. The raw tapioca starch was most susceptible to amylase action. Due to the importance of this finding further purification and commercial production of this enzyme is necessary.

CONFLICT OF INTEREST

The authors would like state that there is no conflict of interest in any manner regarding this research article.

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