Purification of Raw Starch Hydrolizing Alpha-amylase From *Mucor* spp.

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ABSTRACT

Three fungal species which produce raw starch degrading alpha-amylase were isolated from exposing to air and soil solution using raw cassava and potato substrates. These cultures were maintained in Potato Dextrose Agar (PDA) Slants. Identification of isolated fungal species was done by slide culture method. The culture conditions were optimized for α-amylase by measuring the activity, pH and protein content at 12 hours intervals. Among the fungal isolations, 48 hour *Mucor* spp culture supernatant that resulted the highest α-Amylase activity (1.25Absorbance) on raw starch was selected to purify α-amylase. Alpha-amylase was purified by 50% ammonium sulphate fractionation, dialysis and cation exchange column chromatography. Highest Raw Starch hydrolyzing amylase activity given fractions RSH I and RSH II were pooled. Different protein bands at each purification step were determined by Sodium Dodicyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE).

KEY WORDS: Fungal alpha-amylase, Ion Exchange Chromatography, Raw Starch, SDS-PAGE

INTRODUCTION

Microorganisms have been utilized to make a significant contribution to the production of food and beverages during last decades. Various industries such as food, brewing, textiles, pharmaceuticals and confectionaries largely depend specially on extra cellular enzymes produced by the microorganisms.

Amylases are enzymes, that are capable of digesting glycosidic linkages found in starch molecules to give diverse products including dextrin, and progressively smaller polymers composed of glucose units (Windish *et al.*, 1965). Amylases can be derived from a variety of sources which are present in all living organisms, but the enzymes vary in activity, specificity, and requirements from species to species and even from tissue to tissue in the same organism (Karkalas and John, 1995).

Alpha-amylases (alpha-1, 4-D-glucan glucanohydrolases, EC 3.2.1.1) are endoglucanases, which act upon large polymers of starch, at internal alpha 1,4-glucan bonds in polysaccharides containing 3 or more α-1,4-alpha linkages; results in a mixture of maltose and glucose (Karkalas and John,1995). Alpha-amylases are universally distributed throughout the animal, plant and microbial kingdoms. However, enzymes from fungal, bacterial and yeast sources have dominated applications in industrial sectors (Pandey *et al.*, 2000). Moreover many fungi had been found to be good source of amylolytic enzymes (Omemu *et al.*, 2005). An extra-cellular amylase, specifically raw starch digesting amylase has found important application in bioconversion of starches and starch-based substrates (Forgarty, 1983; Okolo *et al.*, 1995). Therefore only few micro-organisms have been reported to possess ability to produce raw starch degrading amylase (Abe *et al.*, 1988; Hayashida *et al.*, 1988; Okolo *et al.*, 1995).

Starch is a renewable resource; occur abundantly in most of the developing countries, despite their importance, a large portion of starch lost yearly due to inadequate and inefficient storage facilities (Jean-Claude *et al.*, 1993; Okolo *et al.*, 1995; Anthony *et al.*, 1996). These starches can be converted into reducing sugars by acid or enzymatic sacharification (Shambe *et al.*,1989). In terms of energy utilization and process simplicity, amylase conversion of raw starch is believed to be economically superior alternative to the conventional method that make use of pre-geletinized starch as substrate (Fogarty,1983; Achi and Njoku,1992; Okolo *et al.*, 1995; Adebiti and Akinyanju, 1998). The ethanol production from starch is now increasing as a solution for the energy demand. Therefore, in the present study, an attempt has been made to produce cassava derived raw starch digesting amylase, using an isolated fungal species.

MATERIALS AND METHODS

1. *Isolation of Alpha-amylase Producing Fungi*

Fungi species were isolated from raw cassava and potato by exposure to air and soil. They were purified and identified at the biotechnology laboratory, of the faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka.

All the glassware were washed in tap water, rinsed with distilled water and oven dried at 170°C for 1 h. Sterilization was done by autoclaving 120°C for 20 mins. Chemicals used were from Sigma, Analytical, and Spectrum chemical companies.

Four different culture media were used for the isolation; these include raw slices and macerated form of cassava and potato tubers. The basal medium for the isolation and selection of amylolytic fungi was PDA. Colonies with different morphological characters were isolated and the identification of isolated fungi was done by observing sporulating structures by slide culture technique as described by Riddell *et al.*, 1950. Periodically, fungal growth on plates was examined under low power of the microscope. When adequate sporulation was developed, the cover slip was removed, stained with Lactophenol Cotton Blue, and observed under light

microscope at low and high power. Photographs were taken from a camera attached to the microscope.

Pure cultures were maintained throughout the research period on PDA slants in a refrigerator and sub cultured once a month.

2. *Alpha-amylase Production by the Isolated Fungi*

One liter fermentation medium comprised of soluble starch, 15g; yeast extract, 1.875g; $MgSO_4$, 0.1125g; $CaCl_2$, 0.1125g; KH_2PO_4, 2.625g, was sterilized in an autoclave for 15 mins at 121°C and pH was adjusted to 4.0 after cooling (Okolo *et al.*, 1995). Agar plugs of the each of the isolated three fungi species were inoculated into 50 ml of sterile fermentation medium in 100 ml Erlenmeyer flasks.

Cultures were incubated at room temperature (32°C) in a rotary shaker at 100rpm for 84 h. At 12 h intervals, samples were drawn from the supernatant and test for alpha amylase activity. Two replicate tests were carried out for each culture medium.

2.1 Alpha- amylase Assay.

Alpha-amylase activity on raw starch and soluble starch were assayed by the method described by Henry *et al.*, (1974), for the determination of α-amylase in serum, urine and duodenal fluids with some modifications. Soluble starch in buffer and raw starch flour which was prepared in the laboratory from cassava, modifying the method described by Jensz *et al.*, (1974) was used in the assay.

***2.2* Assay of Protein**

Protein content was determined by measuring the absorbance at 280nm as compared to a standared curve prepared using BSA protein dilution series.

2.3 Optimizing Culture Conditions for Alpha-amylase Secretion

Twenty four Erlenmeyer flasks containing 50ml of the fermentation medium were sterilized. Each seven out of eight were inoculated with 1cm^2 profuse agar plugs of the each of the three selected species {*Mucor* spp (Pma/2006), *Aspergillus* spp (Pmss/2006) and unidentified spp (Cmss/2006)}. Uninoculated medium was used as the blank. These were incubated at room temperature (32°C) for 84 h at 100rpm while measuring following parameters at 12h intervals,

- pH
- Supernatant amylase activity on raw starch
- Supernatant amylase activity on soluble starch

3. *Cultivation, Extraction and Purification*

Two hundred and fifty milliliters of the sterilized fermentation medium was inoculated with profuse agar plugs of the species selected *Mucor* sp (Psa/2006). This was incubated for the period of 48h at room temperature in a rotary shaker at 100rpm. The culture medium was filtered and the supernatant was taken for further purification of a-amylase. Total α-amylase activity on raw starch, total amylase activity on soluble starch and total protein content were measured at purification step.

3.1 Ammonuim Sulphate Fractionation of Alpha-amylase

Ammonium sulphate fractionation was done as described by Clowick and Kaplan, (1955). The supernatant of 172ml was brought up to 50% saturation by adding 62.8g of $(NH_4)_2SO_4$ with continuous stirring over a period of 30 mins. This was centrifuged at 3500rpm for 30mins. The supernatant was discarded and the pellet was dissolved in a minimum amount of 0.1M pH phosphate buffer.

3.2. Dialysis of Alpha-amylase

Redissolved precipitate was put into a cellulose membrane dialysis bag and dialyzed over a period of 24 h against 0.01N pH phosphate buffer. The buffer was changed at eight hour intervals. The dialyzed supernatant volume was measured and assayed for the α-activity on raw starch, soluble starch and protein content.

3.3. Ion Exchange Chromatography

Pre swollen, Dowex basic cation exchanger was used. Beads were treated with few drops of 0.1N NaOH and thoroughly mixed to denature any kind of adhered proteins. This was allowed to settle and the supernatant discarded and resuspended in starting 0.01M phosphate buffer pH 7.0. This gel was extensively washed with several volumes of the same buffer until the discharges reach its pH 7.0.The gel was then suspended in lesser amount of the same buffer to form thick slurry. This was filled in to glass column (13cm x 7.2cm) which was initially partially filled with the same buffer. The gel allowed settle while draining off the excess buffer from the valve.

3.3.1 Column Elution

Ammonium sulphate fractionated precipitate which was dialyzed against 0.01M phosphate buffer pH 7 was applied into the column at the rate of 1ml/ 1min.

The column was eluted with 20ml of the pH 7 phosphate buffers of different ionic strengths. Those are 0.01 M, 0.02M, 0.04M, 0.06M, 0.08M, 0.1 M, 0.2M, and 0.2M + 15% NaCl.

3.3.2 Fraction Collection

Two milliliter fractions were collected manually and the following parameters were taken for each fraction.

1. Absorbance at 280 nm.
2. Total alpha-amylase activity on raw starch
3. Total alpha-amylase activity on soluble starch

The fractions having high enzyme activity were pooled and total α-amylase activity on raw starch, soluble starch and the total protein content for each of the pooled samples were determined.

4 *Sodium Dodicyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE)*

The purified proteins at each step were determined by SDS-PAGE with the standard proteins. After electrophoresis the gel was stained for 30minutes in a Coomessie Brilliant Blue solution.

RESULTS AND DISCUSSION

Six fungal species with different morphological characters were isolated from the culture media exposed to air and soil solution. Out of six, three species were selected for further studies. They were designated as Psa/2006 (Potato sliced air exposed, Figure 2a), Pmss/2006 (Potato macerated soil solution exposed, Figure 2b), and Cmss/2006 (Cassava macerated soil solution exposed, Figure 2c).

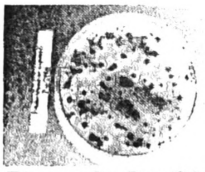

Figure 2a.Psa/2006

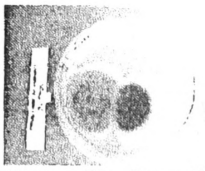

Figure 2b. Pmss/2006

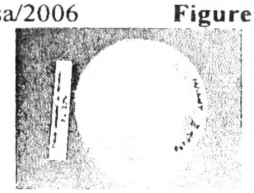

Figure 2c. Cmss/2006

Figure 2 - Morphological characters of isolated fungal colonies:

Structures of the isolated species were observed under light microscope. Psa/2006 and Pmss/2006 had filamentous, branched and septate hyphae and long delicated sphorongiophores. They showed sporulating structures. Based on the sporulating structure and color of the spores, Psa/2006 was identified as Mucor spp, Pmss/2006 as *Aspergillus* spp (Figures 3a & 3b) and Cmss/2006 was unidentified (Figure 3c).

Fig3a.Psa/2006

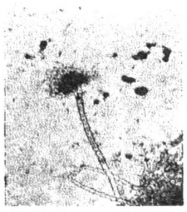

Fig3b.Pmss/2006

Fig3c.Cmss/2006

Figure 3 - Structural features of isolated fungal species under light microscope:

1 *Determination of Optimum Culture Conditions for Extra Eelluler Alpha-amylase Production*

Mucor spp (Psa/2006) culture supernatant showed maximum absorbance 1.25 at 36 h for raw starch. A maximum of 0.9495 absorbance was obtained at 48 h for soluble starch.The protein content at 280nm showed a maximum absorbance of 0.689 at 48 h and pH of the culture supernatant 4.58 was the maximum at 12 h (figure 4).

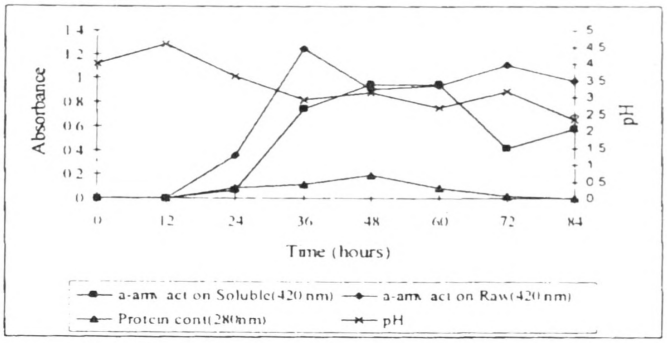

Figure 4 - Alpha-amylase Production by *Mucor* spp (Psa/2006):

Aspergillus spp (Pmss/2006) showed maximum absorbance of the culture supernatant of 0.456 for soluble starch at 84 h, and for raw starch, the maximum absorbance 0.876 was observed after 72 h. The maximum protein content of 0.823 absorbance was obtained at 24 h culture supernatant and the pH 4.88 was the maximum at 24 h (figure 5).

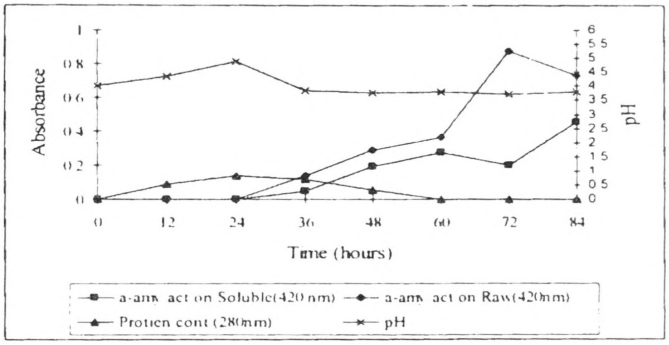

Figure 5 - Alpha-amylase Production by *Aspergillus* spp (Pmss/2006):

And unidentified spp (Cmss/2006) showed maximum absorbance of 0.816 for soluble starch at 48 h and for raw starch, the maximum absorbance 0.956was observed after 48 h. Protein content of the culture supernatant was very low and the pH did not show wide variation from 4.0 (figure 6).

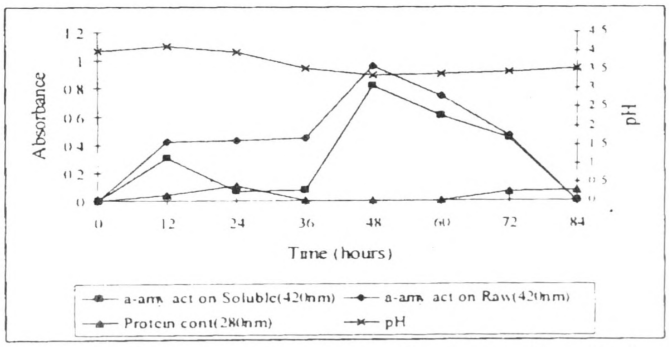

Figure 6 - Alpha-amylase Production by Unidentified spp (Cmss/2006):

It was considered the absorbance is proportional to the activity of amylase.In this study we have tested α-amylase activity of the supernatnt only as the α-amylase activity of the pellet was less as previously reported by De Silva S.N.T., 1997.

According to the results, *Mucor* spp (Psa/2006) at pH of 3.15 was selected for the further studies.

2 *Ammoniumsulphate Fractionation*

Preliminary studies of the ammonium sulphate fractionation showed that the alpha-amylase activity was higher in the 0-50% saturated precipitate.

Therefore, 172 ml of culture supernatant of the 48 hours cultivated culture medium was immediately brought up to 50% saturation. This precipitate was dissolved in minimum amount of 0.01M phosphate buffer and dialysis was done against with the same buffer to remove the salts from the protein purified. After dialysis the final volume of the sample was 48 ml.

3 *Ion Exchange Chromatography*

According to the absorbance value measured, highest alpha amylase activity was given by the fractions from 40 to 45 at 0.06M strength buffer and fractions from 66 to 70 at 0.2M strength (Figure 7) which were labeled as RSH I and RSH II respectively. The highest activity on soluble starch was given by the fractions from 62 to 65 were pooled and labeled as SS I.

According to the absorbance value observed at 280 nm, most of the proteins were eluted at low ionic strength but the raw and soluble starch α-amylase activity was low in these fractions (Figure 7 and 8).

4 *SDS-PAGE*

Unresolved bands were obtained for culture supernatant, 50 % ammonium sulphate fractionated samples and its dialyzed fraction, and for the standard markers. However, bands were not observed for RSH I and RSH II. But the absorbance measured at 280 nm for protein indicates the presents of protein in low concentration which was not possible to detect by Coomassie staining.

CONCLUSION

A *Mucor* spp (Psa/2006) containing α-amylase activity was isolated. Thus this species can be used as an enzyme source to digest raw starch together with enzyme glucoamylase to produce ethanol.

Enzyme purification procedure for column exchange chromatography was established to separate the raw starch hydrolyzing α-amylase activity containing fractions from the isolated species.

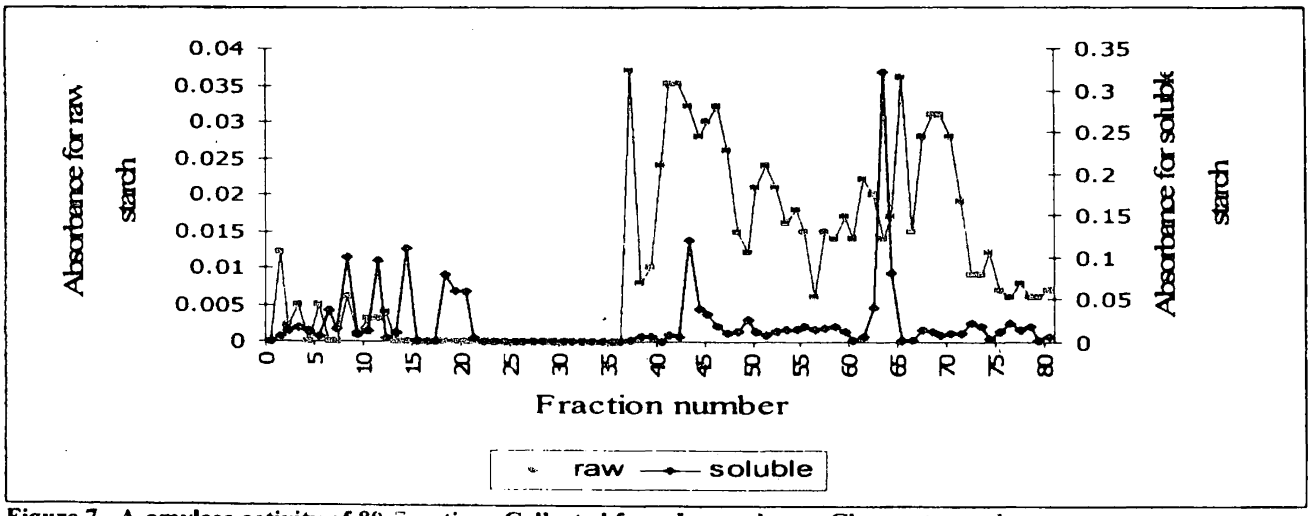

Figure 7 - A-amylase activity of 80 Fractions Collected from Ion exchange Chromatography:

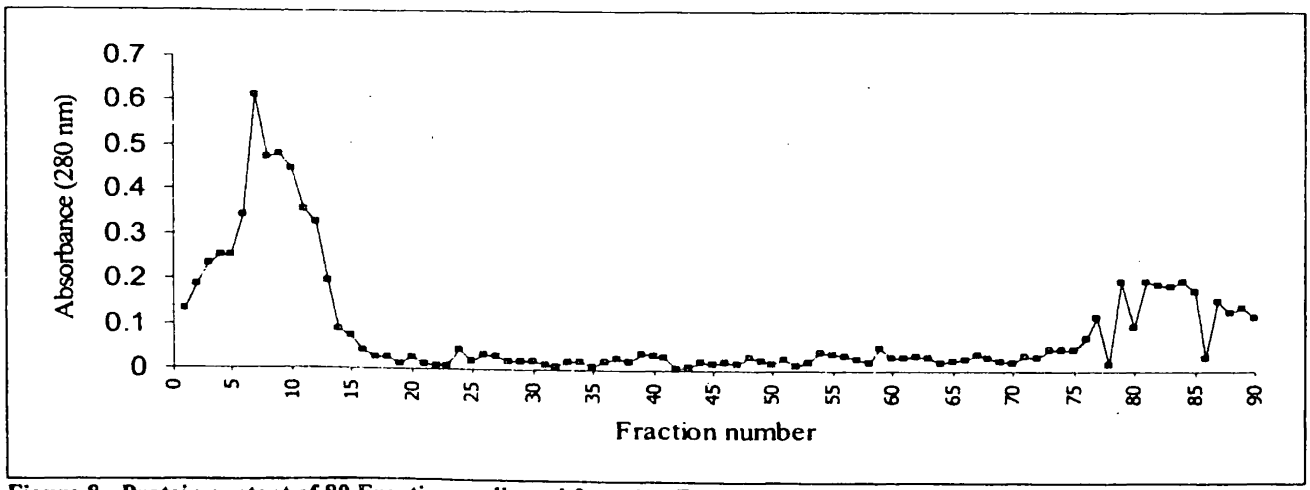

Figure 8 - Protein content of 80 Fractions collected from ion Exchange Chromatography:

AKNOWLEDGEMENT

Authors wish to thank, laboratory staff of the dept of biotechnology, WUSL for their great support. Dr. D.B.Kelaniyangoda senior lecturer, Dept of Horticulture and Landscape Gardening, and Dr. K.D.R.R.Silva, Head of the Dept of Applied Nutrition, WUSL for their cooperation given us to conduct this study. Ms.Subashini, Plant Pathology Division, National Plant Quarantine Service, Katunayake, and the staff of the Computer service unit, Makandura premises of WUSL for their noble assistance.

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