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Production of α-Amylase by *Rhizopus microsporus* using Agricultural By-products in Solid State Fermentation

[1]P. Vijayaraghavan, [2]C.S. Remya and [3]S.G. Prakash Vincent

[1]Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam 629 502, Kanyakumari District, Tamilnadu, India

[2]Department of Enzyme Technology, Xpression Biotek, Marthandam, Kanyakumari District, Tamilnadu, India

[3]International Centre for Nanobiotechnology, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam 629 502, Kanyakumari District, Tamilnadu, India

Coressponding Author: P. Vijayaraghavan, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam 629 502, Kanyakumari District, Tamilnadu, India

ABSTRACT

α-Amylase is an important enzyme used in the industry and accounts for approximately 30% of the enzyme market. In the present study, Solid-State Fermentation (SSF) was carried out using banana peel, cabbage leaf, tapioca peel and wheat bran as substrates for α-amylase production by a fungus, *Rhizopus microsporus*. Among the four substrates used, the tapioca peel was found to yield the maximum (79 U g^{-1}) and wheat bran supported 64 U g^{-1} of amylase production. The present study proved that the tapioca peel was a superior medium for the production of α-amylase than wheat bran for *R. microsporus*. The maximum production of α-amylase was recorded after 96 h of fermentation, initial pH = 6.0 and moisture content 60% (v/w). Enzyme production was high in the solid substrate (tapioca peel) containing 30% fungal inoculum (v/w), 1% xylose and 1% urea. Enzyme activity was found to be high at the pH 6.0 and the temperature at 40°C with 2% starch (m/v) and 10 mM of calcium ion concentration. This enzyme was fractionated with solid ammonium sulphate and partially purified by sephadex G 75 gel filtration chromatography. The amylase activity was determined by native polyacrylamide gel electrophoresis. Results of the present study indicate that the tapioca peel is the potential medium for the production of α-amylase by *R. microsporus*. This study also suggests the use of banana peel and cabbage leaf for the production of α-amylase.

Key words: Agricultural waste, tapioca peel, banana peel, cabbage leaf, culture medium solid-substrate fermentation

INTRODUCTION

Amylases are a group of enzymes produced by several micro-organisms including fungi (Fadel, 2000; Wang *et al*., 2001). They are commercially used for processing starch to produce sugar syrups containing glucose, maltose and higher oligosaccharides (Hagihara *et al*., 2001). These enzymes account for about 30% of the world's enzyme production (Van der Maarel *et al*., 2002). *Rhizopus* sp. are considered good producers of amylolytic enzymes (Takahashi *et al*., 1994). Soccol *et al*. (1994) used *Rhizopus oryzae* MUCL 28168, MUCL 28627,

ATCC 34612 for the production of α and glucoamylase and applied raw and cooked cassava as the substrate; Peixoto-Nogueria *et al.* (2008) used *Rhizopus microsporus* var. rhizopodiformis for the production of α-amylase and glucoamylase using agricultural residues such as wheat bran, cassava flour, sugar cane bagasse, rice straw, corncob and crushed corncob as carbon sources Kunamneni *et al.* (2005) used *Thermomyces lanuginosus* to optimize the nutrional parameter for enhanced production of α-amylase by solid state fermentation.

The use of Solid State Fermentation (SSF) for enzyme production has many advantages over submerged fermentation (SmF) due to its simple technique, low capital investment, and better product recovery (Baysal *et al.*, 2003). During the last decade, an increased attention was paid to the use of various agro-industrial wastes for value addition using SSF by filamentous fungi (Pandey *et al.*, 1999). Utilization of agro-industrial wastes and by-products for productions of enzymes by microbial process has been developed in 1940's. Many researchers have exploited various agricultural wastes for amylase production for solid state fermentation. In majority of the cases wheat bran (WB) has been the preferred choice of substrate for the production of thermostable α-amylase in SSF. Usage of tapioca or mustard cake or rice husk (Baysal *et al.*, 2003) as a substitute to WB for the production of amylase.

Cassava fibrous residue constitutes high starch content (63%) and organic nature (rich in nutrient) and can serve as an important substrate for production of amylase enzyme (Ray, 2004). The industrial residues namely, oatmeal, cassava flour, pectin or sugar cane bagasse may serve as the alternative sources for the production of amylase (Peixoto *et al.*, 2003). In India, more than 1500 cottage and small industries crush over 5000 tonnes of cassava per day during harvest season (Jyothi *et al.*, 2005) for the manufacture of starch and sugar, thereby generating 1000 tonnes of dry residue equivalent per day. The residue, if effectively used, could lead to the valuable products such as α-amylase (Ray, 2004). The agro-industrial waste such as banana waste was used for amylase production (Pandey *et al.*, 1999) for amylase production. No report available in the use of cabbage leaf for the production of amylase. Wheat bran medium is widely implemented for glucoamylase production by using *Rhizopus* strains (Elegado and Fujio, 1993).

Amylase was produced for various industrial applications from *Rhizopus* species including *Rhizopus stolonifer* (Pothiraj *et al.*, 2006); *Rhizopus* sp. W-08 (Wang *et al.*, 2007) and *Rhizopus* sp. (Nahar *et al.*, 2008). In the present study, amylase production was carried out using four different agriculture by-products, namely banana peel, cabbage leaf, tapioca peel and wheat bran by *Rhizopus microsporus*. Among the four substrates, the superior substrate was utilized for the production of α-amylase by *Rhizopus microsporus* and the process parameters such as initial moisture content, inoculum, fermentation period and pH were optimized. The crude enzyme was partially purified by ammonium sulphate fractionation, sephadex G-75 gel filtration chromatography and the enzyme properties were studied. The main objective of the present study is to identify suitable agro-industrial residue for the production of α-amylase from *R. microsporus*.

MATERIALS AND METHODS

Isolation and screening of *Rhizopus microsporus:* The fungus, *Rhizopus microsporus* was isolated in March-2009, from the soil sediments obtained from the coir retting pond, near to Centre for Marine Science and Technology, Rajakkamangalam, Tamilnadu, India by serial dilution technique and plated on potato dextrose agar (PDA) plates. The plates were incubated at 37°C for 4-7 days. Fungal colonies were examined under direct microscopy and *R. microsporus* was identified according to standard morphological criteria as stated by Jennessen *et al.* (2008). *R. microsporus* appeared as fluffy, broad, readily twisted, ribbon-like hyphae, the presence of sporangium size of

less than 100 μm, sporangiophore length of less than 1000 μm and the absence of azygospores as reported by Jennessen *et al.* (2008). *R. microsporus* was screened for amylase activity on starch agar plate method as described by Hols *et al.* (1997).

Organism and inoculum preparation: *R. microsporus* was maintained on PDA slants and grown for 6-8 days at 37°C for sporulation. Ten milliter sterile double distilled water was transferred to a sporulated PDA slant culture. The spores were dislodged using an inoculation needle under aseptic conditions and the suspension was used as inoculum. One milliter of the spore suspension contained about 5×10^6 spores.

Substrates: The agro-industrial by-products (banana peel, cabbage leaf, tapioca peel and wheat bran) were obtained from the local market. They were washed, first with tap water, followed by double distilled water to remove dust particles. They were then air dried followed by sun drying for 8-10 days and ground into a powder.

Solid State Fermentation (SSF): Five grams each of various substrates (banana peel, cabbage leaf, tapioca peel and wheat bran) were placed in 250 mL Erlenmeyer flasks. To adjust the moisture levels, 50 mM of citrate phosphate buffer (pH = 6.0) was sprayed onto the solid substrates. The contents of the flasks were mixed thoroughly and autoclaved at 121°C for 20 min. After sterilization, each flask was inoculated with 1 mL of the spore suspension and incubated at 37°C for 4 days. The incubating flask was rotated gently every 12 h to facilitate to mix the contents.

Enzyme extraction: The fermented substrates were mixed thoroughly with 50 mL of sterile distilled water. The mixture was shaken for 30 min at room temperature in an orbital shaker (155 revolutions min^{-1}) and the slurry was squeezed using a muslin cloth. The extract was filtered through a filter paper (Whatman No. 1) and the filtrate was used as the crude enzyme.

Enzyme assay: The amylase enzyme was assayed accordingly to the method described by Miller (1972) using the UV-visible spectrophotometer (Eltek, India). One unit of α-amylase activity was defined as the amount of enzyme that releases 1 mg of reducing sugar as glucose per mL per min under the assay conditions.

Optimization of culture conditions: Various process parameters that affect α-amylase production in *R. microsporus* by solid-state fermentation were optimized. The best fermentation medium from four different agricultural by-products (banana peel, cabbage leaf, tapioca peel and wheat bran) was initially determined and used as the substrate for subsequent experiments. The strategy was to optimize each parameter independently of the others and subsequently, optimal conditions were employed in all experiments. The process parameters tested were the fermentation period, pH, the moisture content, the inoculum concentration, the carbon and nitrogen sources. To determine the optimum fermentation period for enzyme production, *R. microsporus* was grown on tapioca peel medium, incubated at 37°C and the enzyme was extracted at every 24 h starting from 1day of incubation till 7 day. The effect of pH on enzyme production was studied by adjusting the substrate pH (pH = 3.0-7.0), which was achieved by the addition of buffer prior to sterilization. The effect of moisture content was determined by adjusting the substrate moisture (40-90%) by using citrate phosphate buffer (50 mM, pH = 6.0). The effect

of inoculum concentrations on the production of enzymes was found by inoculating the spore suspension at various concentrations (10-80%) in the Erlenmeyer flask containing the solid substrate. The effect of carbon source on enzyme production was studied by supplementing 1% carbon source (cellobiose (CE), glucose (GL), lactose (LA), maltose (MA), starch (AT) and xylose (XY)) in 2.5 g of solid substrate before sterilization. The effect of nitrogen source on enzyme production was determined by supplementing 1% nitrogen source (ammonium chloride (AC), ammonium sulphate (AS), peptone (PE), urea (UR) and yeast extract (YE)) in 2.5 g of solid substrate before sterilization. One milliter of *R. microsporus* spore suspension was used along with the substrate and incubated for 96 h at 37°C. After the fermentation period, the enzyme was extracted and the amylase activity was assayed.

Protein estimation: The total protein content of the sample was determined as described by Lowry *et al.* (1951) using bovine serum albumin as standard.

Effect of pH on enzyme activity and stability: The effect of pH on enzyme activity was determined by reacting 1 mL of enzyme solution with 1 mL of starch substrate (1%, w/v) at various pH values (3.0-8.0). It was incubated for 30 min at 37°C. The effect of pH on enzyme stability was determined by holding the enzyme solution at various buffers (3.0-8.0) for 30 min. The remaining enzyme activity was assayed at 37°C.

Effect of temperature on enzyme activity and stability: The influence of different temperatures on amylase activity was determined by incubating 1.0 mL of enzyme solution with a 1.0 mL of 1% soluble starch at various temperatures (20-70°C) for 30 min during the standard enzyme assay. Thermal stability was determined by holding the enzyme solution without the addition of substrate at various temperatures (20-70°C) for 1 h. The remaining activity was measured in standard assay condition.

Effect of substrate concentration and ions on enzyme activity: To study the effect of the substrate, the sample was incubated with the substrate at various concentrations (0.5-2.5%, m/v) and the enzyme activity was assayed. To study the effect of ions on enzyme activity, the sample was incubated with 10 mM of calcium chloride, magnesium chloride, mercuric chloride, potassium chloride, EDTA and incubated for 30 min at 37°C. The enzyme activity was assayed at 37°C and pH of 6.0.

Partial purification of amylase from *R. microsporus*: The crude amylase enzyme was precipitated with solid ammonium sulphate and loaded on a Sephadex G-75 (Amersham Biosciences, Sweden) column. The column was equilibrated and the sample was eluted with sodium phosphate buffer (50 mM, pH = 7.0). The flow rate was adjusted to 1.0 mL^{-1} min and 25 fractions were collected (5.0 mL each). The extinction was measured at 280 nm and enzyme activity was assayed.

Enzyme activity staining: The amylase produced by *R. microsporus* in SSF was purified by the chromatography step and loaded into the 8.5% native polyacrylamide gel electrophoresis. To determine amylase activity, the gel was incubated and equilibrated in sodium phosphate buffer for 30 min. It was incubated with 1% soluble starch for 1 h and flooded with 1% iodine solution.

RESULTS AND DISCUSSION

Solid state fermentation : Solid Substrate Cultivation (SSC) or Solid State Fermentation (SSF) is envisioned as a prominent bioconversion technique to transform natural raw materials into a wide variety of chemical as well as bio-chemical products. Various agro-industrial wastes have been tested for amylase production which included raw cassava (Soccol *et al.*, 1994) tapioca, mustard cake, rice husk (Baysal *et al.*, 2003), wheat bran (WB), corncob leaf, wheat straw and rye straw (Balkan and Ertan, 2006). In the present study, the agro-industrial residues namely banana peel, cabbage leaf, tapioca peel and wheat bran were used for amylase production by *R. microsporus*. Among the all four substrates, *R. microsporus* produced more quantities of amylolytic enzyme on tapioca peel substrate (79 U g^{-1}) (Fig. 1) and was superior in the production of α-amylase by this particular fungus than wheat bran substrate (64U g^{-1}). Soccol *et al.* (1994) grew the *Rhizopus* species such as *R. oryzae* 28168, 28627, 34612 on cassava powder in solid state fermentation and produced 42.2, 76.0 and 40.4 U g^{-1} of α-amylase activity which was similar to the present study. It is also evident in *R. microsporus* var *rhizopodiformis,* amylase activity was high in the production medium containing cassava flour as the carbon sources (6.5 U mg^{-1} protein), which was 5.21, 3.40, 2.20 U mg^{-1} protein for oat meal, sugar cane bagasse and raw wheat (Peixoto *et al.*, 2003). Babu and Satyanarayana (1995) found that, thermostable α-amylase production using maize bran was similar to that with WB, but the extraction is reported to be much more difficult with maize bran. Ramesh and Lonsane (1990) supported our view, and stated that the production of thermostable α-amylase may vary from one micro-organism to another depending on the organism's physio-chemical requirements. Based on the fact mentioned above, it is concluded that tapioca peel is the best substrate for the production of amylase for *R. microsporus* than wheat bran.

Effect of fermentation period on enzyme production in SSF: Enzyme production was maximum (38 U g^{-1}) at 96 h of fermentation at 37°C (Fig. 2). It declined to 23, 13, 12 U g^{-1} substrate for 120, 144 and 168 h respectively, Soccol *et al.* (1994) stated that amylase enzyme production was high at 48 h incubation at 35°C for *Rhizopus* sp. Peixoto-Nogueria *et al.* (2008) found that the optimum fermentation period for amylase production was 6 days, at 45° in *Rhizopus microsporus* var. *rhizopodiformis*.

Effect of pH on enzyme production: In *R. microsporus* amylase production was found to be high (49 U g^{-1} t pH 6.0 (96 h, 37°). Enzyme activity was 6.0, 9.8, 23.6, 31 and 17 U g^{-1} or the pH = 3.0, 4.0, 5.0, 7.0 and 8.0, respectively. Similar results were obtained in *Rhizopus microsporus* var. *rhizopodiformis* (Peixoto-Nogueria *et al.*, 2008).

Moisture: In *R. microsporus,* enzyme production was found to be high (29 U g^{-1} at 60% (v/w) of moisture content. At 40 and 50% of moisture, it secreted 16, 20 U g^{-1} substrate. Enzyme activity was found to be 24, 18 and 17 U g^{1} for 70, 80, and 90% of moisture content at 96 h of incubation at 37°. Peixoto-Nogueria *et al.* (2008) found that 76% of moisture content was optimum for the production medium containing wheat bran and corncob for *Rhizopus microsporus* var. *rhizopodiformis*.

Inoculum: Enzyme production was found to be 29 and 36 U g^{-1} for 10 and 20% (v/w) inoculum after 96 h of fermentation period at 37°C. Amylase production was high (68 U g^{-1} substrate) at 30%

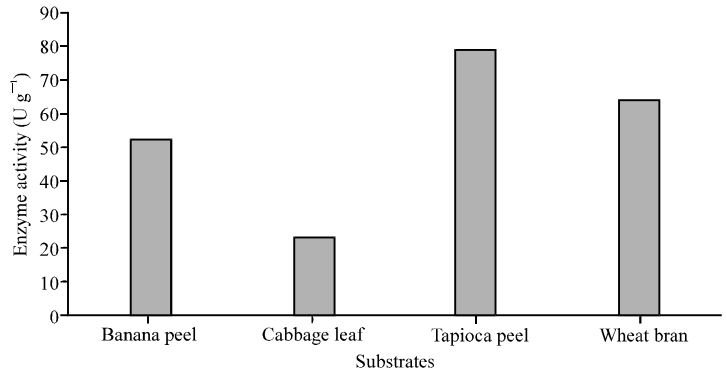

Fig. 1: Effect of agro industrial wastes on amylase production. R. microsporus produced more amylolytic enzyme in tapioca peel substrate (79 U g^{-1}) and 23, 52 and 64 U g^{-1} for cabbage leaf, banana peel and wheat bran respectively

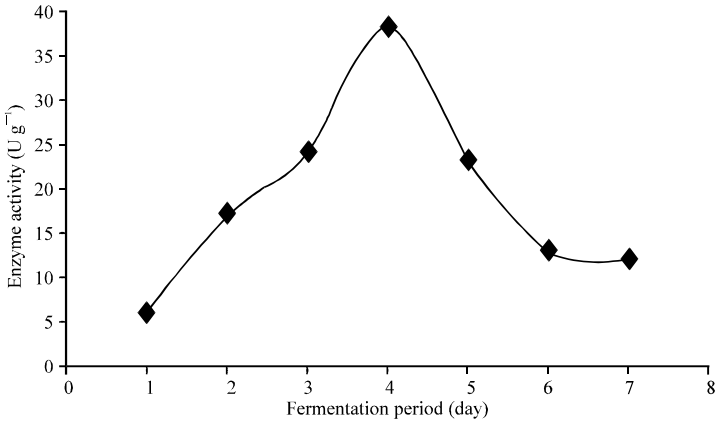

Fig. 2: Effect of fermentation period on amylase production in solid state culture. Enzyme production was found to be high (38 U g^{-1}) at 96 h of incubation at 37°C

of inoculum for *R. microsporus* using tapioca peel as a fermenting medium. Its production reduced over 30% of inoculum and the enzyme activity was 48, 37, 35, 29 and 26 U g^{-1} substrate for 40, 50, 60, 70 and 80% of inoculum, respectively.

Effect of carbon and nitrogen sources: In this study, among all the supplementary carbon (cellobiose, glucose, lactose, maltose, starch and xylose) and nitrogen sources (1%, w/w) (ammonium chloride, ammonium sulphate, peptone, urea and yeast extract), xylose and urea have been found to be the best carbon and nitrogen sources for amylase production by *R. microsporus*. Xylose enhanced the production (57 U g^{-1}) of amylase and enzyme activity was 52 U g^{-1} for urea (Fig. 3). Peixoto *et al.* (2003) found that 2% carbon sources such as glucose, maltose, trehalose, arabinose and sucrose stimulated the production of amylase and the enzyme activity was 2.45, 0.47, 8.44, 9.29 and 6.65 U mg^{-1} protein, respectively for *R. microsporus* var. *rhizopodiformis*.

Effect of pH on enzyme activity and stability: The effect of pH on enzyme activity was studied by incubating the enzyme at different pH values (3.0-8.0). Enzyme activity was found to be 10, 26,

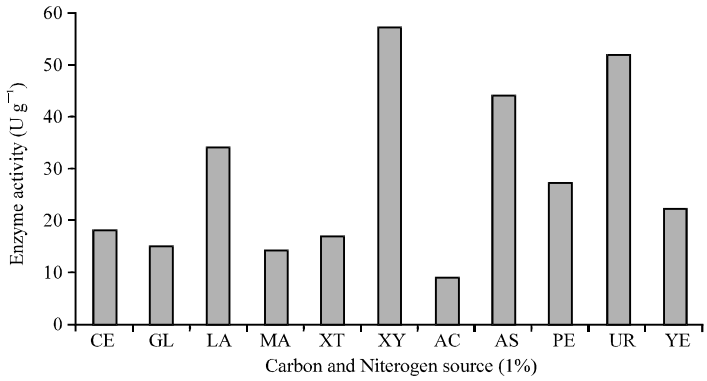

Fig. 3: Effect of carbon and nitrogen sources on amylase production. Xylose enhanced the production of amylase (57 U g^{-1}) and enzyme activity was 52 U g^{-1} for urea

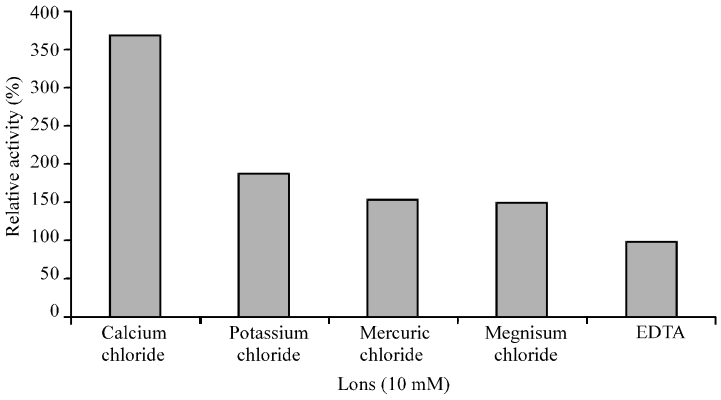

Fig. 4: Effect of ions on enzyme activity. The relative activity was 368, 189, 154, 149 and 99% for calcium chloride, potassium chloride, mercuric chloride, magnesium chloride and EDTA

44, 52, 50 and 16 U g^{-1} for pH = 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0, respectively. Similar result was obtained in *R. microsporus* var *rhizopodiformis* (Peixoto-Nogueria *et al*., 2008). The enzyme was highly stable (100%) at pH = 6.0 for 30 min and the remaining enzyme activity was 8, 30, 72, 85 and 52% at pH = 3.0, 4.0, 5.0, 7.0 and 8.0, respectively. Similar results were obtaine d from *Rhizopus oryzae* mutant where the glucoamylase was stable over a pH range of 4.0-6.0 (Suntornsuk and Hang, 1997).

Effect of temperature on enzyme activity and stability: The effect of temperature on enzyme activity was determined by incubating the reaction mixture at various temperatures (20-70°C). The enzyme activity was found to be high (66 U g^{-1}) at 40°C and was 41, 51, 50, 49 and 45 U g^{-1} at temperatures 20, 30, 50, 60 and 70°C. Temperature optima and thermal stability for amylase activity varied among *Rhizopus* species. Khoo *et al.* (1994) found that the optimum temperature for purified α-amylase was 55°C, which was 65°C for *R. microsporus* var *rhizopodiformis* crude amylase enzyme (Peixoto-Nogueria *et al*., 2008), and the temperature optimum was reported as 35°C in *Rhizopus* species by Soccol *et al.* (1994). Effect of temperature on enzyme stability was studied by thermally denaturing the enzyme sample at various temperatures (20-70°C) for 1 h. Enzyme activity was found to be 57, 65 U g^{-1} at the denaturizing temperature 20 and 30°C. The enzyme retained 100% activity at 40°C and which decreased to 97, 94, 86 U g^{-1} for the

Fig. 5: Amylase activity staining. Starch hydrolysis pattern of polyacrylamide gel (8.5%) with purified enzyme. The colourless zone (indicated in arrow) represents area of enzymatically hydrolyzed starch against the dark background of the starch-iodine complex

temperatures 50, 60 and 70°C. This enzyme was highly active over a broad temperature range of 30-60°C. Similar results were reported in *Rhizopus* sp. (Takahashi *et al*., 1994) and also in *Rhizopus nodosus* (Mutbukumaran and Dhar, 1983).

Effect of substrate concentration and ions on enzyme activity: Enzyme activity was 13, 28 and 32 U g^{-1} for 0.5, 1.0 and 1.5% substrate concentration. Enzyme activity was high (38.4 U g^{-1}) at 2% of substrate in the reaction mixture and decreased to 25 U g^{-1} at 2.5% of substrate.

To study the effect of ions on enzyme activity, the sample was incubated with 10 mM calcium chloride, potassium chloride, mercuric chloride, magnesium chloride and EDTA and the percentage relative activity was high (368%) at 10 mM of calcium ion concentration. EDTA had little effect on amylase activity (99.46%). Potassium chloride, mercuric chloride and magnesium chloride showed 189, 154 and 149% of relative activity (Fig. 4). Similar result was obtained from *R. oryzae,* where EDTA had no inhibitory effect on the enzyme (Yu and Hang, 1991) and inhibitory effect was found in the presence of Hg^{2+}.

Partial purification of amylase from *Rhizopus microsporus*: In the present study, the amylase enzyme was precipitated by ammonium sulphate. The fractionated amylase was purified by gel filtration chromatography and the amylase activity was detected in 8.5% native polyacrylamide gel electrophoresis (Fig. 5). Similar salting out and chromatography procedures were employed for *R. oryzae* (Yu and Hang, 1991) and *R. oryzae* mutant 4U2 (Suntornsuk and Hang, 1997) for amylase purification.

CONCLUSION

The present study clearly reveals that compared to wheat bran, tapioca peel was a better medium for the production of α-amylase by *R. microsporus*. Although many authors suggested the

α-amylase produced by fungal isolates utilizing wheat bran medium were found to be best, the present study found that tapioca peel medium is the better substrate for the production of α-amylase by the fungus, *R. microsporus*. Banana peels can be a better substrate for the production of α-amylase, as it supported 80% of amylase production when compared with wheat bran that was used elsewhere. Based on the facts provided, we suggest that tapioca and banana peel are the best substrates for the production of α-amylase for *R. microsporus*. Cabbage leaf was also useful substrate for the production of á amylase which was not used as a fermenting medium so far. However, further research could be benefit for the production of α-amylase by other fungal species using the substrates namely, tapioca peel, banana peel and cabbage leaf could be the alternate source to wheat bran.

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