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Regular Article

Microbial Production of Amylase from Cassava Waste

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Bacterium mura **was isolated from cassava waste, (Tamil Nadu, India) for the production of extracellular amylase. On screening for amylase producing bacteria, 5 isolates showed positive results, of which *Bacterium mura* showed best amylase activity. The optimal conditions for the amylase activity were found at pH 6.0 (39 U/ml) and at temperature 37°C. Amylase activity was found to be higher when lactose (31 U/ml), casein, barley (42 U/ml) and SDS (32 U/ml) were used as the carbon source, nitrogen source, agro waste source and as additives respectively. The enzyme was partially purified by dialysis and the molecular mass was found to be 65kDa by SDS-PAGE. The partially purified and crude amylase was confirmed by zymogram. The partially purified amylase was used in bread making, which improved the softening of the bread and was used as a de-sizing agent.**

Keywords: *Bacterium mura*, zymography, agro waste, de-staining, Optimization.

Amylases are extracellular enzymes that catalyzes the hydrolysis of internal α-1,4-glycosidic linkages in starch to dextrin, and other small carbohydrate molecules constituted of glucose units (de Souza and de Oliveira Magalhaes, 2010). Based on the cleavage site, they are classified into three types: α- amylase, β- amylase, γ- amylase. The α- amylase acts at random locations along the starch chain, β- amylase hydrolysis second α-1,4 glycosidic bond, leading to cleaving off two glucose units whereas γ- amylase cleaves α(1-6) glycosidic linkages, in addition to cleaving the last α-(1-4)-glycosidic linkages at the non-reducing end of amylose and amylopectin, resulting in the formation of glucose (Shipra das *et al.*, 2011). These amylases can be obtained from natural sources like plants, animals, and microorganisms (Mageswari *et al.*, 2012). The use of efficient production strategies, the microorganism have established their

potential to contribute in industrial applications (Rajshree Saxena *et al.,* 2011). Amylases have attracted worldwide enzyme market because of their applications in textile, food, paper, detergent industries (Mageswari *et al.,* 2012). The use of amylase in pharmaceutical industries and clinical sectors requires complete purification (de Souza and de Oliveira Magalhaes, 2010). The use of agro-waste as substrate for the growth of the microorganism improves the production of amylase and is found to be cost effective (Rajshree Saxena *et al.,* 2011). The current study reports the production and partial purification of amylase from *Bacterium mura* isolated from cassava wastes.

MATERIALS AND METHODS

Sample collection:

Isolation of bacteria from cassava waste, collected from Rasipuram, Tamil Nadu, India. Bacterial isolation was carried out by serial dilution and spread plate method on nutrient agar plates. The bacterial isolates were further sub-cultured on to slants and maintained at -20^{o}C for preservation.

Screening and identification of amylase producer:

All the isolates were examined for the extracellular amylase production using starch agar media containing soluble starch as carbon source. After incubation the plates were flooded with 1% iodine solution for 1 min until the entire media perfectly colored in blue. Appearance of clear zone around the colonies in blue colored medium was taken for further studies. The positive strain that producing amylase enzyme was selected and was given for taxonomic identification in IBMS, University of Madras, Taramani.

Production of amylase:

The production medium for amylase consists of [g/ L]: Lactose-10g, yeast extract-20g, KH_2PO_4-0.05g, $MnCl_2.4H_2O$-0.0015g, $MgSO_4.7H_2O$-0.25g, $CaCl_2.2H_2O$- 0.05g, $FeSO_4.7H_2O$-0.01g. The enzyme production was carried out by shake flask fermentation [Hamilton *et al.,* 1999] at pH 7.0. The inoculated medium was incubated at 37^{o}C for 24 hours.

Amylase assay:

The amylase activity was determined by DNS method using starch as substrate. 1mL of culture filtrate was boiled in water bath for 20 min. Boiled samples were cooled suddenly and used as killed enzyme sample. Both killed and live samples were taken for the assay. The reaction mixture consists of 1mL of enzyme, 2.5mL of substrate; 2.5mL of sodium phosphate buffer and 1mL of sodium chloride, was incubated in a water bath at 37^{o}C for 15 min. Then, the reaction was terminated by adding 1mL of DNS, and immersing the tubes in boiling water bath. The absorbance was measured at 540nm. The OD value of killed enzyme was subtracted from the live enzyme value. One unit of enzyme activity was defined as the amount of enzyme releasing 1µmol of reducing sugars per minute under the standard assay conditions.

Partial purification of amylase enzyme:

The total protein content from the sample was determined using Bradford method. The partial purification of amylase enzyme was performed by ammonium sulphate precipitation followed by dialysis. The sample was centrifuged and the cell free extract was saturated with ammonium sulphate up to 70%. The mixture was stored in cold room for 24 hours to precipitate all the proteins, and the precipitation was separated by centrifugation at 10,000rpm for 10min.

Then carefully the aqueous phase was discarded, and the remaining precipitate was dissolved with 5mL of 1M citrate phosphate buffer (pH 5).

The dialysis bag containing precipitated protein was freely suspended in a beaker containing 1M citrate phosphate buffer, and was stirred slowly using magnetic stirrer. The entire setup was positioned in cold room for 48 hours and the buffer was changed every 12 hours periodically. After the dialysis, the sample was shifted into a clean lyophilized flask.

Molecular weight determination by SDS PAGE:

The molecular weight of partially purified amylase enzyme was estimated by comparing with standard protein of known molecular weight. SDS PAGE was carried out with 15% polyacrylamide gel described by Smith (1984) and the proteins were stained with 0.2% coomassie brilliant blue.

Zymogram of amylase:

In Zymogram, the gel (7.5%) was incubated in a reaction solution containing 1% soluble starch, 20mM sodium acetate (pH 5.5) 1mM calcium chloride and then stained for starch-degrading activity with iodine solution (1% iodine, 10% potassium iodide and 50% ethanol). A clear hydrolytic zone in the gel shows the presence of amylase in the partially purified crude enzyme.

Optimization of fermentation conditions:

The fermentation conditions for the production of amylase were studied with various parameters. The parameters that intensify the production of amylase were studied by taking one factor at a time. The parameter such as temperature (28^{o}C to 52^{o}C), pH (4 to 9), carbon sources (xylose, lactose, galactose, fructose and sucrose), nitrogen sources (yeast extract, casein, gelatin, beef extract, urea, potassium nitrate and ammonium nitrate), natural and agro waste source (barely, corn, wheat, molasses, sweet potato peel and jack fruit seed) and additives (SDS, triton x- 100 and tween 20) were performed.

APPLICATION OF AMYLASE:

Amylase as de-sizing agent in textile industry:

The amylase enzyme produced from *Bacterium mura* was examined for de-sizing of cloth since it is an hydrolytic enzyme which on catalysis breaks down the starch. The experiment was carried out by boiling the cloth for 30 minutes along with the purified amylase enzyme. After the boiling process, it was treated with iodine solution to check the presence of starch (Etters and Annis, 1998).

Amylase in bread making

Wheat flour (500g) was mixed with dry yeast (5g), then 1.5% NaCl, 2% sucrose, 10ml sunflower oil and 2.5ml of partially purified amylase were blended with 60% water. Mixed thoroughly [mechanically] for 30min to produce dough. After 2 hours of fermentation it was baked at 275^{O}C for 30 minutes. Control was performed without amylase. Finally the breads were kept for shelf life and softness at different time intervals (Dogan, 2003).

RESULTS AND DISCUSSION

Isolation and screening of amylase producer:

The *Bacterium mura* used in this study was isolated by serial dilution method, and its 16s rRNA sequence was deposited in GenBank with accession number GU373971, which showed positive result for amylolytic activity (Fig.1) among 13 bacterial isolates from cassava waste. Hence it is used for the further studies.

Fig.1. Screening for amylase production in starch agar plate.

Effect of temperature on amylase production:

To optimize the optimum temperature for better production, various temperatures were monitored (Fig.2). The temperature requirement of the organism for its optimum growth is 37^{0}C and production of the enzyme was also found to be higher at 37^{0}C as well. Our result shows that the maximum amylase production was achieved with maximum growth of microbe as well as high protein content.

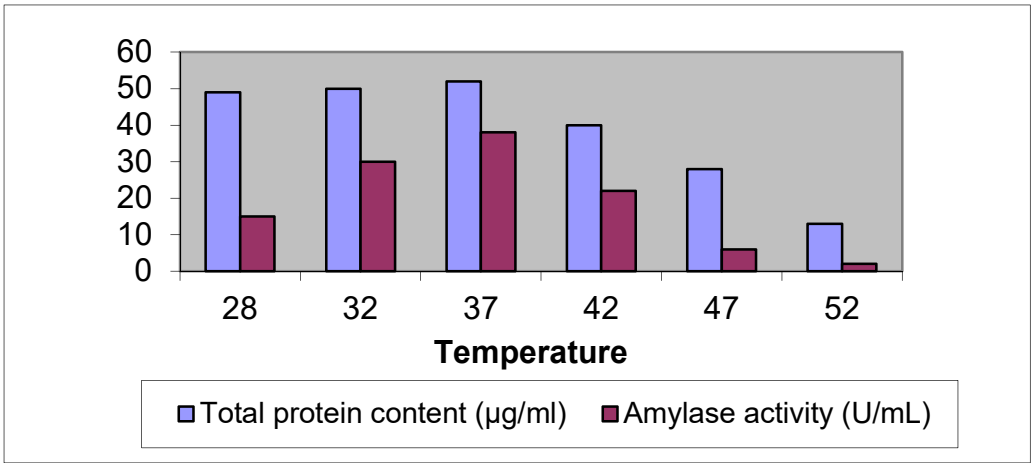

Fig.2. Effect of temperature on total protein production and enzyme production

Effect of pH:

The effect of pH was examined by varying the pH of the organism to improve the production of enzyme. Finally the amylase enzyme had its optical activity at pH 6.0 with the production of 39

U/ml (Fig.3), interestingly, amylase produced by *B. mura* was found to be acidic in nature. Notably, protein content in different pH range does not correlate with amylase activity. At acidic pH, enzyme activity was higher than at alkali. So, at alkaline conditions bacterium produces more proteins that may degrade or inactivate the enzyme. In that case, bacterium should be maintained at acidic conditions to have maximum amylase production.

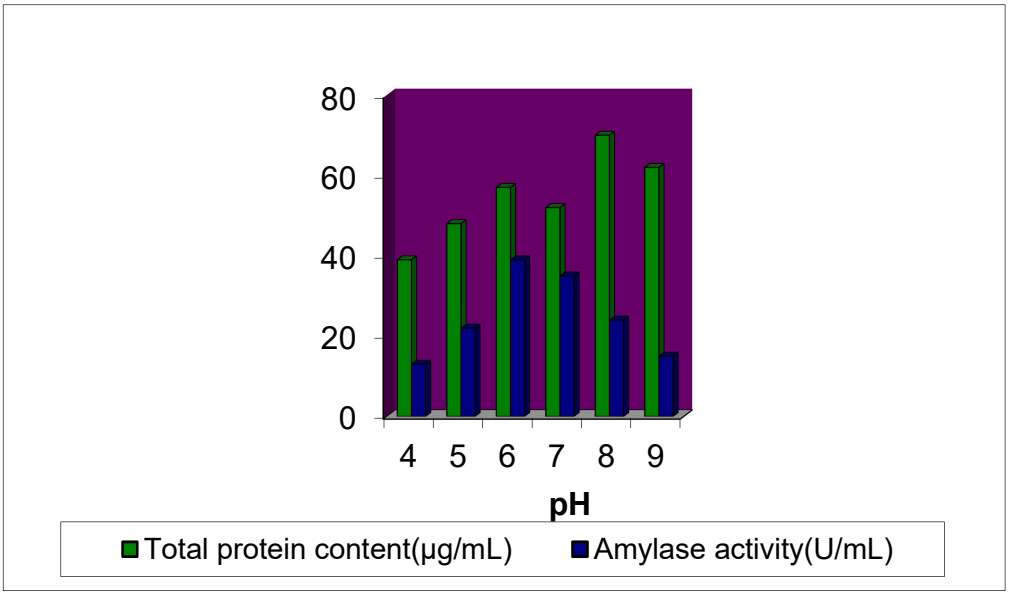

Fig.3. Effect of pH on total protein production and enzyme production.

Effect of Carbon sources:

Amylase activity as observed on various carbon sources such as fructose, galactose, lactose, sucrose and xylose, of which, the amylase activity was higher in lactose amended medium with the production of about 31U/ml. Fructose supplement had a higher protein content but lesser amylase activity due to degrading nature of enzymes (Fig.4). Though total protein content had no much change against carbon sources, amylase activity does, and glucose was not tested individually.

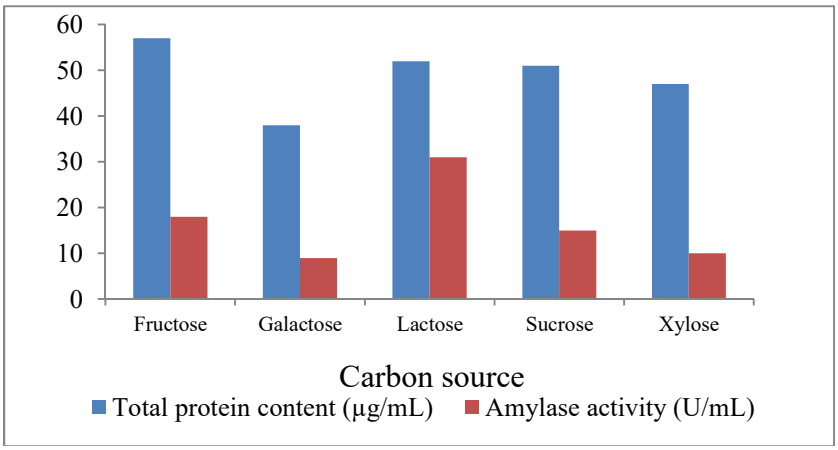

Fig.4. Enzyme production and total protein production on different carbon sources.

Effect of Nitrogen sources:

The nitrogen sources are of secondary energy sources for microorganisms, which play an important role in the growth of the organism as well as production of enzymes [Coronado *et al.*, 2000]. Here, the amylase activity was checked with different nitrogen sources, such as, yeast extract, casein, gelatin, beef extract, urea, potassium nitrate and ammonium nitrate. Among the nitrogen sources, the enzyme activity was higher in casein amended medium and is about 42U/ml. (Fig.5)

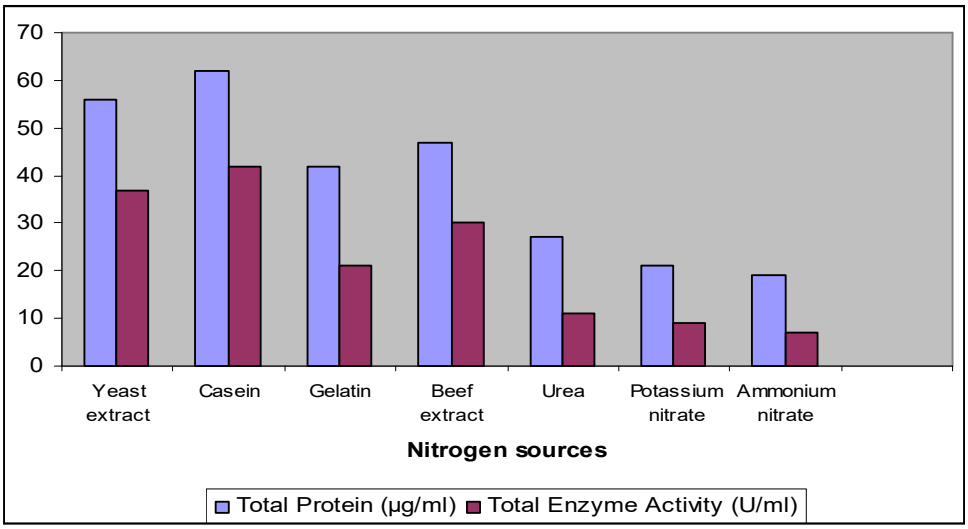

Fig.5. Effect of different nitrogen sources on enzyme production.

Effect of Additives:

The addition of additives sometimes either increases or decreases the enzyme production. The addition of the detergent SDS has shown highest enzyme production of 32U/ml, in contrast other additives like Triton –X 100 and Tween - 20 shows almost same amount of effect on the enzyme production (Fig.6).

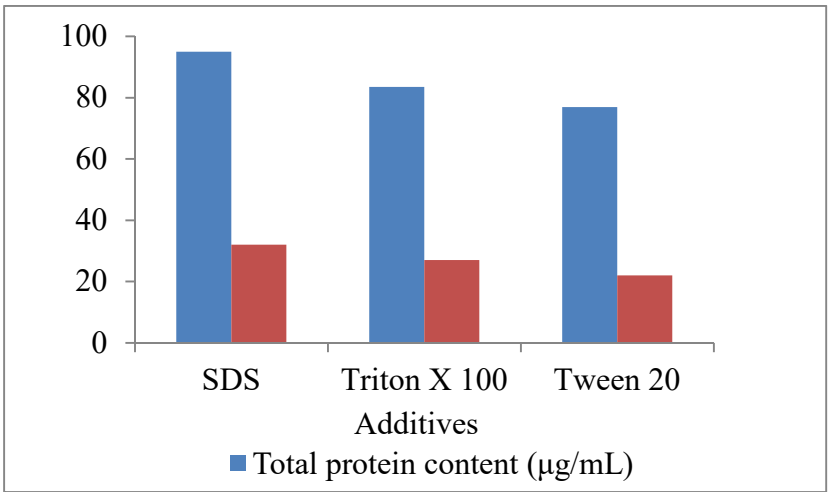

Fig.6. Enzyme and total protein production on different additives

Production of amylase from agro products:

The most economical and valuable enzymes are produced from the natural sources and industrial wastes. In this study, several natural and agro products have been used as substrates namely barely, corn, wheat, molasses, sweet potato and jack fruit seed. The results showed that, maximum production of 27U/ml was observed in barley because it has the higher content of starch in addition, the strain was isolated from the cassava waste which is rich in starch (Fig.7)

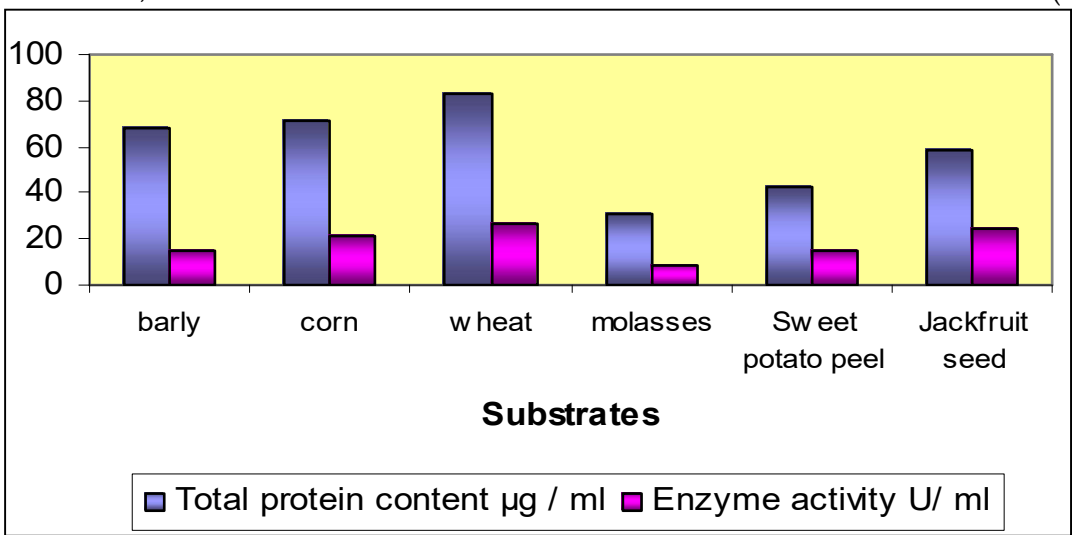

Fig.7. Enzyme production and total protein production on different agro substrates.

SDS-PAGE analysis:

The protein was analyzed using SDS-PAGE; the total protein content of the isolate is moderately high and showed multiple bands (Fig.8).The molecular weight of the amylase enzyme was observed around 65-80 KDa.

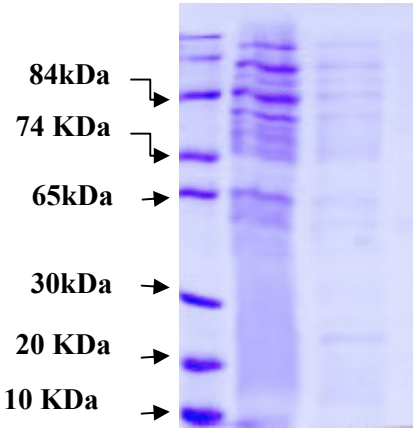

Fig.8. SDS-PAGE results for crude and dialyzed amylase. Lane 1: Protein marker, Lane 2: Crude enzyme, Lane 3: Purified enzyme.

In SDS- PAGE zymogram, the gel (7.5%) were incubated in a reaction solution containing 1% soluble starch, 20mM sodium acetate (pH 5.5) 1mM calcium chloride and then stained for starch-degrading activity with iodine solution (1% iodine, 10% potassium iodide and 50%

ethanol). A clear hydrolytic zone in the gel shows the presence of amylase in the partially purified crude enzyme (Fig.9).

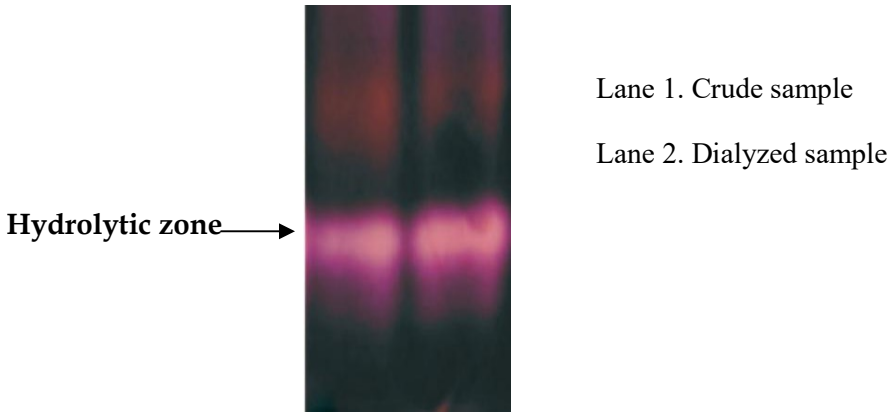

Fig.9. Zymogram of amylase. Clear zone around the dark background showed the amylolytic activity.

APPLICATION OF AMYLASE:

Amylase as de-sizing agent in textile industry

The sized cloth was de-sized by means of the amylase enzyme; the hydrolysis of starch was confirmed by treating the cloth with iodine. Appearance of blue color indicates the presence of starch in the cloth. In this study, the blue color did not appear after amylase treatment, which indicates the starch content was removed by the action of amylase.

Amylase in bread making

The effect of partially purified amylase from *Bacterium mura* strain on dough rheological properties was studied. Increasing the enzyme addition level to the wheat flour decreased dough stability and increased softening. Dough stability is very important for bread production. Amylase on the physical and rheological properties makes the dough soft and alters the handling properties. Softening effects on the dough as a result of amylase activity during mixing reduces the baking absorption and ultimately the dough yield**.**

Conclusion

The optimization studies like pH and temperature revealed that *Bacterium mura*, an efficient amylase producer. The amylase enzyme was partially purified by ammonium precipitation followed by dialysis. The amylase presence was confirmed by zymogram. SDS-PAGE results revealed the molecular mass of the partially purified to be 65 kDa. The partially purified enzyme was found to have applications towards bread making and as a de-sizing agent. Hence, we conclude that *Bacterium mura* as a potential source for amylase production for industrial purpose.

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