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SOLID STATE FERMENTATION FOR THE PRODUCTION OF A-AMYLASE BY *PAENIBACILLUS AMYLOLYTICUS*

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Abstract

The potential of microorganisms as biotechnological sources of industrially pertinent enzymes has stimulated interest in the exploration of microbes having extracellular amylolytic activity. The present work is aimed to explore the potential of *Paenibacillus amylolyticus* for the production of thermostable α-amylase. In this context, different agricultural by-products (wheat bran, rice husk, rice bran and maize bran), potato peel and banana peel were tested as solid substrate for α-amylase production and wheat bran was found to be the best when moistened with diluent containing (%, w/v) nutrient broth 1.0, soluble starch 1.0, lactose 0.5, NaCl 0.5 and $CaCl_2$ 0.2. Considerable increase in enzyme activity was observed when initial pH of the optimized diluent and inoculum level were adjusted at 8.0 and 1.5 ml, respectively. The production of α-amylase was maximum (275.95±2.75 U/g/min) when 10 g of wheat bran was moistened with 10 ml of diluent i.e., substrate to diluent ratio was kept 1.1.

Introduction

Amylases are among the most important enzymes in present day biotechnology taking approximately 25% of the enzyme market. Alpha amylases (EC-3.2.1.1) degrade α-1,4-glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose and alpha limit dextrin (Dabai *et al.,* 2011). The use of amylases in starch hydrolysis goes back to the 9th century AD when malt was used to convert arrowroot starch to sweetener. Development and commercialization of α-amylases from fungal and bacterial sources started in the late 19th and early 20th centuries, respectively. By 1930s, these enzymes were used commercially in a variety of industrial applications including starch liquefaction, brewing, textile, pharmaceuticals, paper, detergents, drugs, toxic wastes removal and drilling for oil (Dabai *et al.,* 2011; Gangadharan *et al*., 2006; Haq *et al.*, 2003). These uses have placed greater stress on search for novel α-amylases for more efficient processes.

Bacterial species such as *Bacillus, Aeromonas, Lactobacillus, Streptococcus,* and *Micrococcus* have been tested for α-amylase production using submerged or solid state fermentations (Jeon *et al.,* 2010; Wu, 2010; Fan *et al*., 2009; Schallmey *et al*., 2004). Solid state fermentation (SSF) even though conventional but is still extensively employed due to less energy requirements, high product yield, less catabolic repression and end product inhibition, low capital investment and better product recovery (Regulapati *et al.,* 2007; Akpan and Adelaja, 2004; Pandey *et al*., 2000). A large number of by-products such as wheat bran, rice husk, cassava fibrous residues, rice starch, dried potato peel, banana peel, rapeseed cake, coconut oil cake and mustard oil cake have been used as substrates for SSF (Jeon *et al.,* 2010; Swain & Ray, 2007; Apar & Ozbek, 2004; Baysal *et al.,* 2003; Kokab *et al.,* 2003). Among these, agro residues are more economical substrates for SSF due to their nutritive compositions (Hmidet *et al*., 2010; Anto *et al*., 2006). However, wheat bran holds the key, and has most commonly been used substrate (Rajagopalan & Krishnan, 2010; Anto *et al.,* 2006).

When agro residues are used as primary substrates in SSF, there is a limitation in availability and accessibility of nutrients. Therefore, supplements of sugars and nitrogenous compounds in the form of diluents should be provided to enhance the microbial growth and enzyme productivity. Moreover, for the maximum production formation, microorganisms also need to facilitate with optimum cultural conditions such as incubation time, growth temperature, initial pH of diluents, moisture content and inoculum level.

In present research work, attempt has been made to optimize the cultural conditions for the α-amylase production by *Paenibacillus amylolyticus* as new potential source.

Materials and Methods

Organism and culture maintenance: The bacterial strain *Paenibacillus amylolyticus* NRRL B-14945 was taken from the available stock culture of Institute of Industrial Biotechnology, GC University, Lahore. The culture was maintained on slants containing nutrient broth (NB) agar medium and stored at 4°C in a cold cabinet (Model: MPR-1410, SANYO, Japan).

Solid state fermentation: Vegetative inoculum of *P. amylolyticus* was used for the production of α-amylase. Fifty milliliter of inoculum medium containing 0.8% (w/v) nutrient broth (pH 7.0) was transferred to the individual 250 ml Erlenmeyer flask, then cotton plugged and autoclaved them at 15 p.s.i. (121°C) for 15-20 min. After cooling to room temperature, flask was inoculated aseptically with a loop full of *P. amylolyticus* culture. Incubated the flasks in a shaking incubator (Model: 10X400.XX2.C, SANYO Gallenkamp, PLC, UK) at 200 rpm (37°C) for 24 h.

Ten gram of wheat bran was transferred in individual Erlenmeyer flasks then moistened with 10 ml of diluent and mixed well. The flasks were cotton plugged and sterilized in autoclave at 15 lbs/in^2 pressure and 121°C for 15 min. After cooling at room temperature each flask was inoculated with 24 h old bacterial inoculum (CFU 3.9×10^9) and incubated at 37°C for 48-96 h in an incubator with shaking twice a day. All the experiments were run parallel in duplicate.

Enzyme extraction: Phosphate buffer (0.02 M, pH 7.0) was used for the extraction of enzyme. Fermented broth was then centrifuged at 6,000 rpm for 10 min in a

centrifuge (Model: D-37520, Osterodeam-Harz, Germany). The supernatant was analyzed for α-amylase production. All the experiments were run parallel in duplicate.

Alpha amylase assay: Enzyme assay was performed according to Haq *et al*., (2010). Reducing sugars were quantified with Somogyi method (Nelson, 1944).

Enzyme unit: One unit of α-amylase is defined as the amount of enzyme that liberates 1.0 mg of reducing sugar as maltose per min under the standard assay conditions. It is expressed as U/g/min, where g is the grams of solid substrate (wheat bran) used.

Diluents: Following mineral salt solutions (g/l) were used as moistening agent:

M1: $(NH_4)_2HPO_4$ 5.0 g, soluble starch 40 g, yeast extract 5.0 g, sodium citrate 2.0 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2$ 0.08 g, lactose 20 g, pH 7.0 (Saito & Yamaoto, 1975).

M2: Nutrient broth 10.0 g, soluble starch 10.0 g, lactose 5.0 g, NaCl 5.0 g, $CaCl_2$ 2.0 g, pH 7.0 in 0.02 M phosphate buffer (Haq *et al*., 2003).

M3: KH_2PO_4 2.0 g, NH_4NO_3 10.0 g, NaCl 1.0 g, $MgSO_4.7H_2O$ 1.0 g, pH 7.0 **(**Gangadharan *et al.,* 2006).

M4: $(NH_4)_2SO_4$ 2.5 g, $MgSO_4.7H_2O$ 0.20 g, KH_2PO_4 3.0 g, $CaCl_2.H_2O$ 0.25 g, pH 7.0.

M5: Glucose 50.0 g, yeast extract 10.0 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4$ 1.0 g, KH_2PO_4 1.0 g, pH 7.0 (Abate *et al*., 1999).

M6: Soluble starch 5.0 g, yeast extract 10.0 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4$ 1.0 g, KH_2PO_4 1.0 g, pH 7.0 (Abate *et al*., 1999).

M7: Soluble starch 6 g, nutrient broth 1.6 g, $(NH_4)SO_4$ 0.45 g, $(NH_4)_2HPO_4$ 0.2 g, NaCl 2.0 g, $MgSO_4$ 0.10 g, $CaCl_2$ 0.02 g, pH 7.0**.**

M8: Glucose 6 g, nutrient broth 1.6 g, $(NH_4)SO_4$ 0.45 g, $(NH_4)_2HPO_4$ 0.2 g, NaCl 2.0 g, $MgSO_4$ 0.10 g, $CaCl_2$ 0.02 g, pH 7.0.

Preparation of potato peel and banana peel: Potato peel and banana peel were spread on trays and dried in oven for 24 h at 70°C. Then dried peels were ground and stored at room temperature (25±5°C) in polyethylene bags (Shukla & Rita, 2006)

Statistical analysis: Treatment effects were compared using computer software Costat. Significant differences among the replicates have been presented as Duncan's multiple ranges in form of probability ($p \leq 0.05$) values.

Results and Discussion

Selection of substrate: Evaluation of different substrates i.e., wheat bran, rice husk, rice bran & maize bran, potato peel and banana peel for the production of α-amylase is shown in Fig 1. The maximum production of enzyme (80.5±2.12 U/g/min) was observed when 10 g of wheat bran was used as growth substrate for *P. amylolyticus*. It is because the wheat bran contains a higher proportion of readily metabolizing carbohydrates (80%) compared to other agro by-products, hence it serves as a better carbon source (Rajagopalan *et al*., 2010). Furthermore, it contains 17.1% proteins, 6.7% ash, 43.6 % insoluble dietary fibers, 2.5% soluble dietary fibers and various essential amino acids for the enzyme production and bacterial growth (Sievert *et al*., 1990). Potato peel with α-amylase activity 57 ± 1.41 U/g/min was found to be the second best substrate in the present study.

Time course study: Fig 2 depicts the rate of α-amylase fermentation (24-120 h) by *P. amylolyticus*. The production of α-amylase in the fermented wheat bran increased gradually and reached maximum after 72 h of inoculation. Further increase in incubation period decreased the enzyme production. Hence, incubation period of 72 h was optimized and used in the further experimental work. It might be due to the reason that *P. amylolyticus* entered the stationary phase after 72 h as maximum α-amylase accumulation takes place at the end of the logarithmic phase or during early stationary phase (Coleman, 1967). Rapid decrease in α-amylase production after 72 h may be due to the accumulation of toxic by-products, release of high levels of intracellular proteases activity concomitant with the spore formation (Abate *et al*., 1999; Anto *et al.,* 2006). Similar finding has been reported by Mishra & Behera (2008) for *Bacillus* sp.

Selection of diluents: Eight different diluents (M1, M2, M3, M4, M5, M6, M7 & M8) were tested for the production of α-amylase by *P. amylolyticus* (Fig. 3). The enzyme production was optimum (81.75±2.47 U/g/min) when M2 was used as moistening agent for wheat bran. All the other diluents gave relatively less enzyme production. The provision of additional carbon (soluble starch and lactose) and nitrogen sources (nutrient broth) by M2 for bacterial growth might be the reason for relatively high α-amylase production. Moreover, this diluent also contains NaCl that is reported to enhance the α-amylase production (Schwab *et al*., 2009; Kokab *et al*., 2003). Ca^{+2} ions, present in the medium in form of $CaCl_2$, are reported for activation and stability of α-amylase at higher temperature and prevent its thermal inactivation (Nielsen *et al*., 2003).

Effect of initial pH of diluents: Figure 4 shows the effect of initial pH (6.0-8.5) of diluent M2 on the production of α-amylase. The production of enzyme increased gradually with the increase in initial pH of optimized diluent. Maximum α-amylase activity was obtained when the initial pH was adjusted at 8.0. Further increase in the initial pH of the diluent decreased the enzyme production. Hence, initial pH 8.0 was selected for the subsequent parametric optimization.

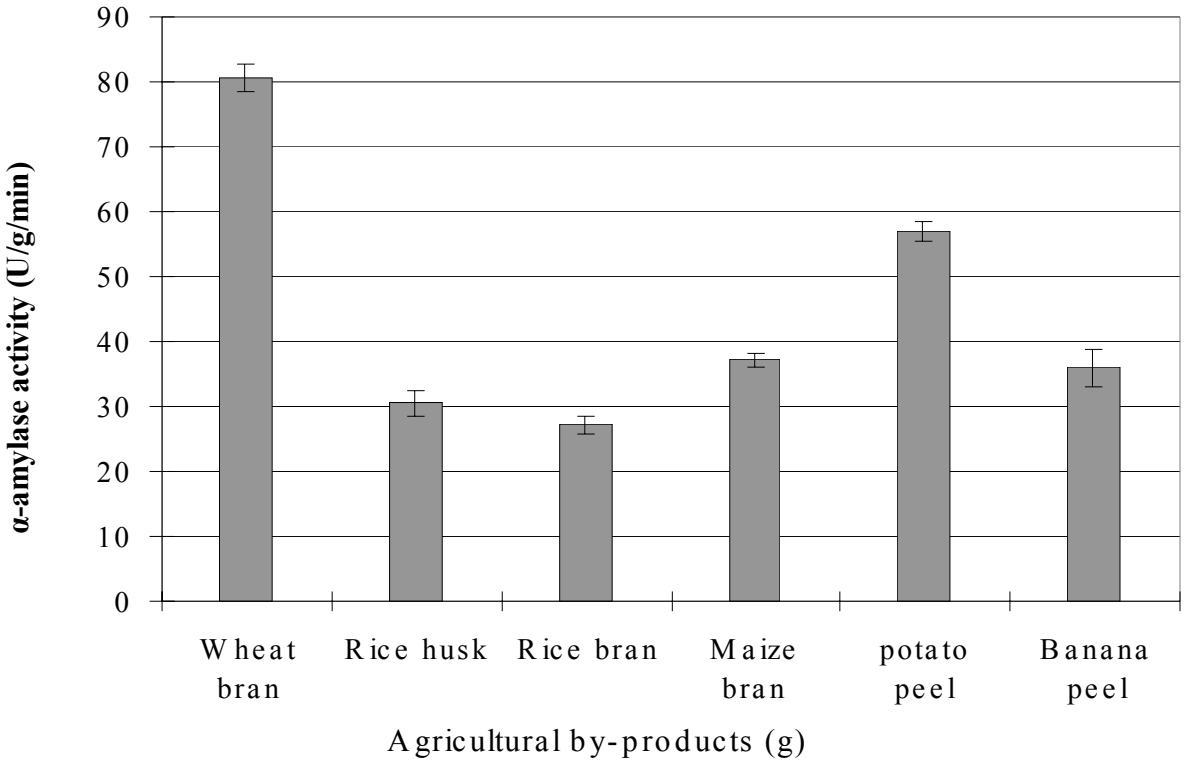

Fig. 1. Selection of substrate for the production of α-amylase by *P. amylolyticus* using SSF*
* Incubation temp 37°C, initial pH 7.0
Y-error bars indicate the standard deviation (±SD) among the parallel duplicates, which differ significantly at p≤0.05. LSD= 3.9

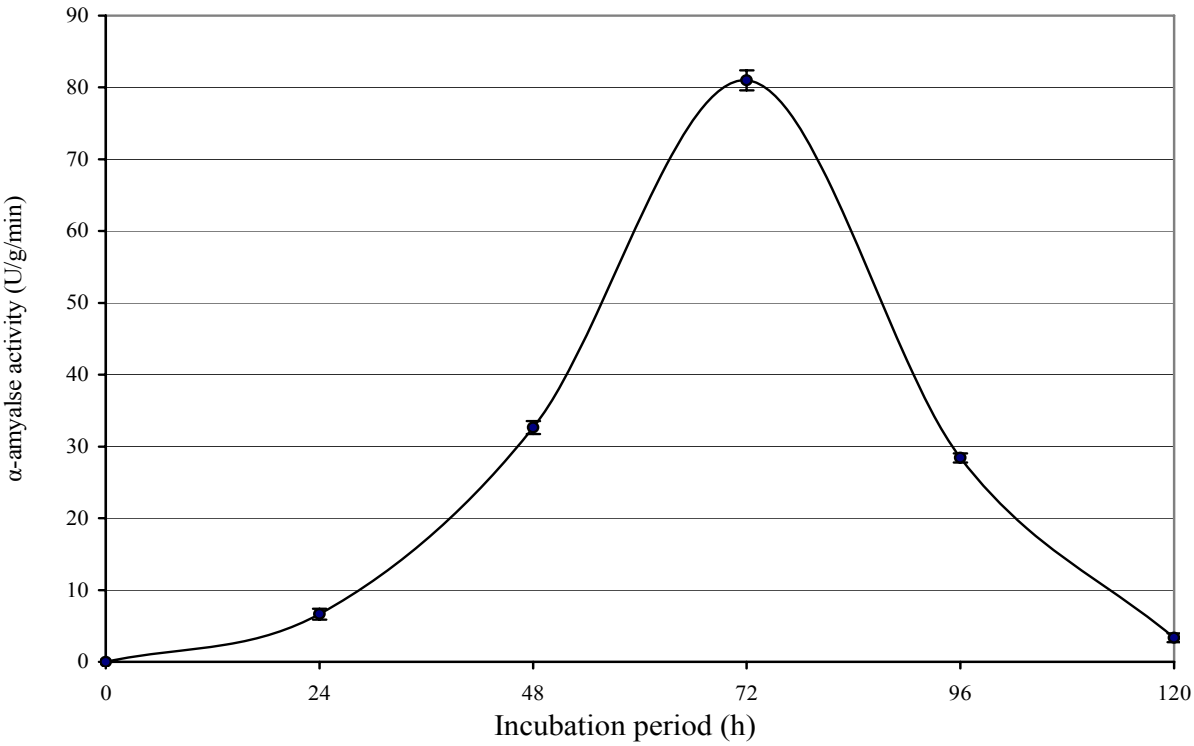

Fig. 2. Time course study for the production of α-amylase by *P. amylolyticus* using SSF*
*Incubation period range (24-120 h), Incubation temp 37°C, initial pH 7.0, wheat bran 10 g
Y-error bars indicate the standard deviation (±SD) among the duplicates, which differ significantly at p≤0.05. LSD= 2.35

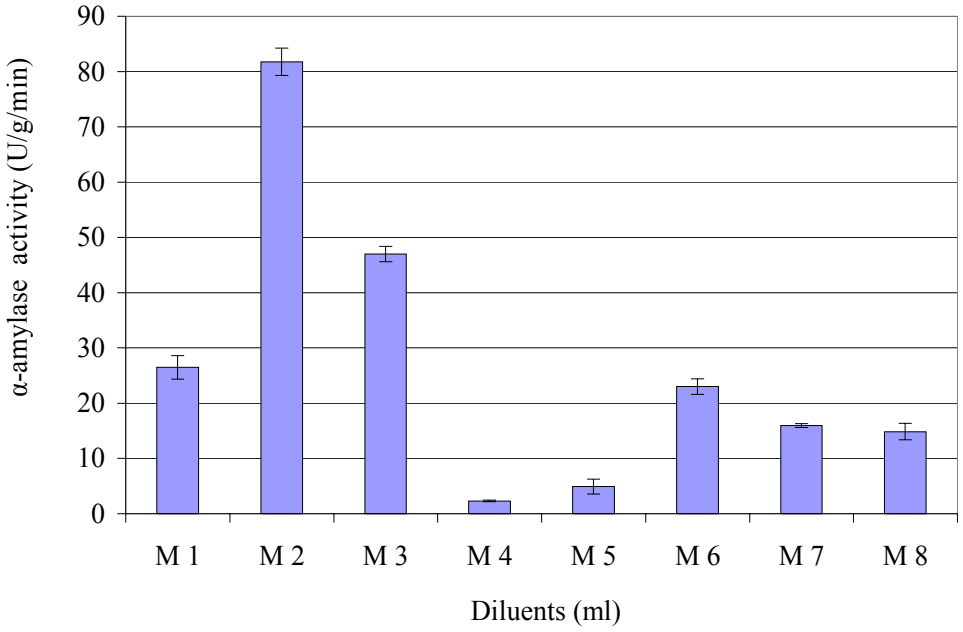

Fig. 3. Selection of diluent for the production of α-amylase by *P. amylolyticus* using SSF*
*Incubation period 72 h, Incubation temp 37°C, initial pH 7.0, wheat bran 10 g
Y-error bars indicate the standard deviation (±SD) among the duplicates, which differs significantly at p≤0.05. LSD = 3.68

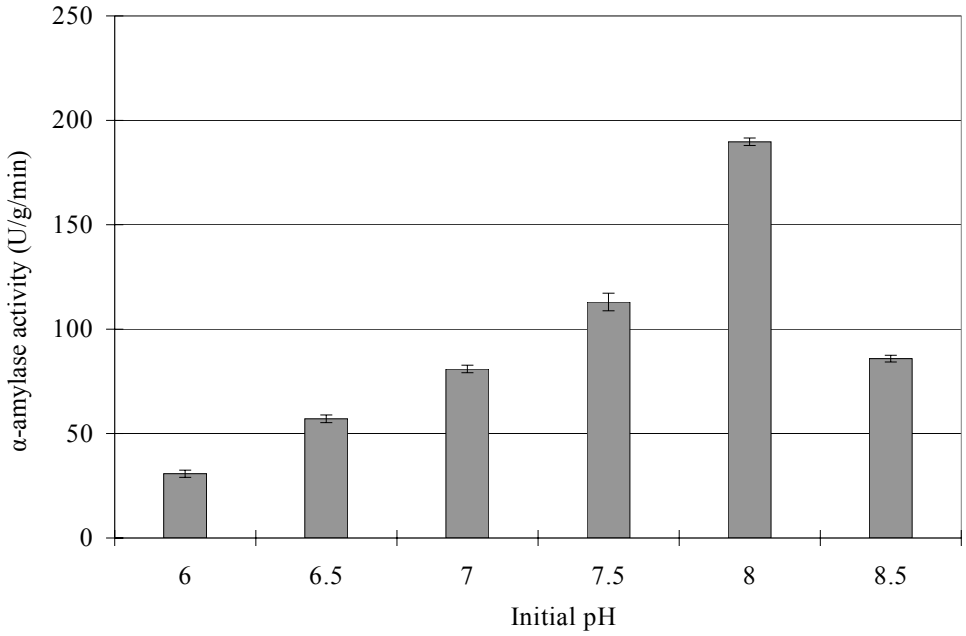

Fig. 4. Effect of initial pH of diluent on the production of α-amylase by *P. amylolyticus* using SSF*
*Incubation period 72 h, Incubation temp 37°C, wheat bran 10 g
Y-error bars indicate the standard deviation (±SD) among the duplicates, which differs significantly at p≤0.05. LSD = 5.79

Effect of inoculum size: The effect of size of inoculum (0.5, 1.0, 1.5, 2.0 & 2.5 ml) was also investigated on α-amylase production by *P. amylolyticus* (Fig. 5). Maximum amount of enzyme (276.65±2.61 U/g/min) was obtained when fermentation flask containing 10 g of wheat bran was inoculated with 1.5 ml of 24 h old vegetative inoculum. The enzyme production with all the other inoculum levels was rather low. The less enzyme production at lower inoculum level (<1.5 ml) might be because less number of viable cells in the production medium require more time to grow to an optimum number to utilize the nutrients in substrate and for enzyme production (Kashyap *et al.*, 2002). However, less enzyme production at higher inoculum level (>1.5 ml) may be due to decreased nutrient availability for the large number of viable cells, or rapid accumulation of toxic metabolites (Zhang *et al*., 2002).

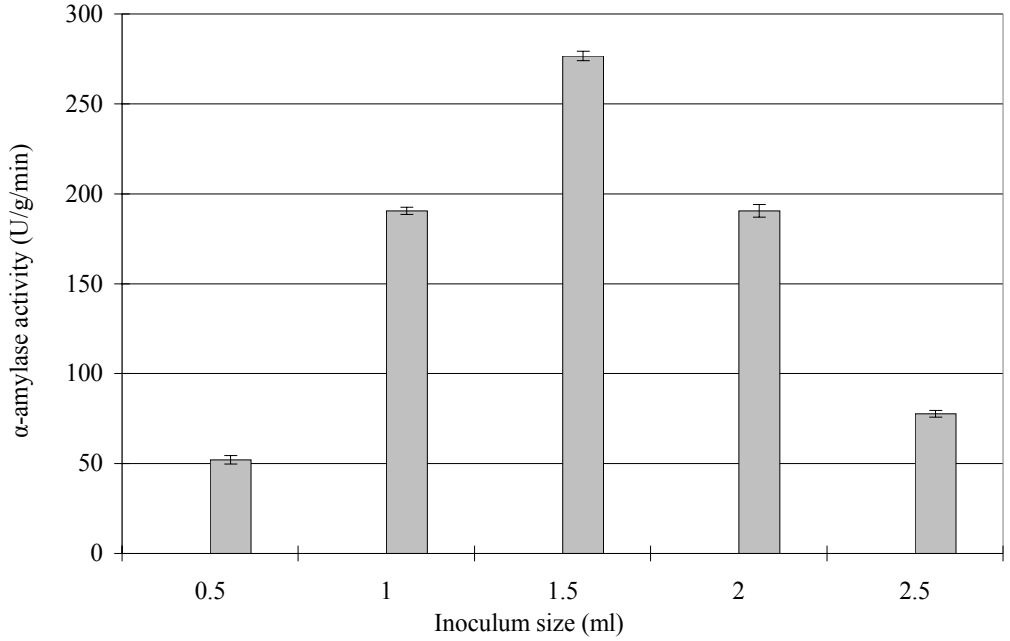

Fig. 5. Effect of inoculum size on the production of α-amylase by *P. amylolyticus* using SSF*
*Incubation period 72 h, Incubation temp 37°C, wheat bran 10 g, initial pH 8.0
Y-error bars indicate the standard deviation (±SD) among the duplicates, which differs significantly at p≤0.05. LSD = 6.53

Effect of diluent to substrate ratio: The effect of diluent to substrate ratio on the α-amylase production by *P. amylolyticus* is shown in Fig 6. The maximum enzyme production was obtained when 10 g of wheat bran was moistened with 10 ml of M2 diluent i.e., 1:1 substrate to diluent ratio. Any other variation from this optimal value (10 ml) decreased the production of α-amylase. It may be because high moisture contents decrease the porosity of substrate, promotes development of stickiness altering the particle structure and resulting in low oxygen transfer rate. Similar finding have been reported by other researcher (Lonsane *et al*., 1985; Zadrazil & Brunnert, 1981).

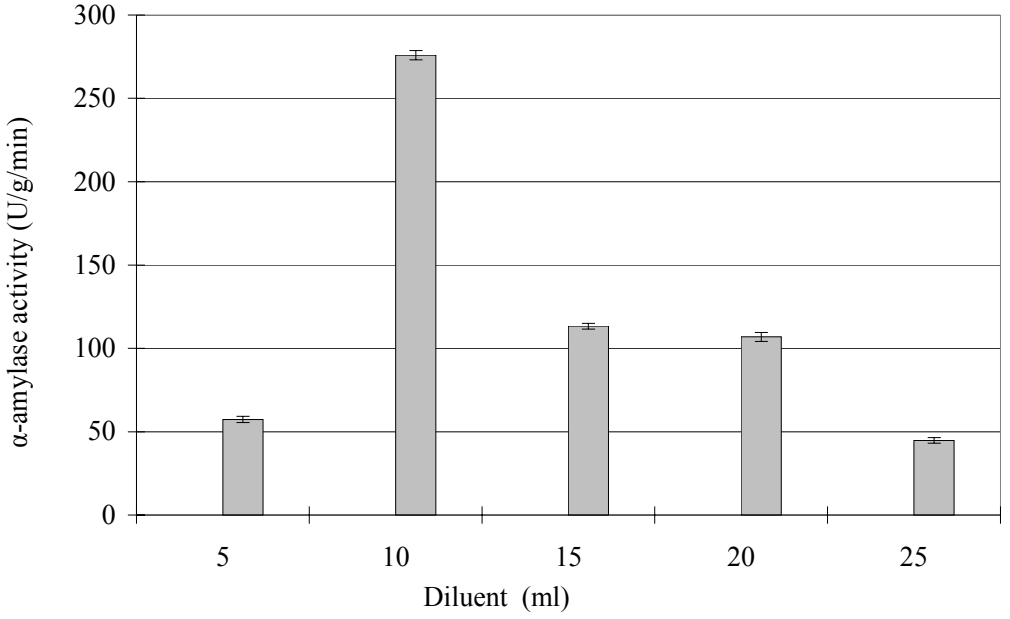

Fig. 6. Effect of diluent to substrate ratio on the production of α-amylase by *P. amylolyticus* using SSF*
*Incubation period 72 h, Incubation temp 37°C, wheat bran 10 g, initial pH 8.0
Y-error bars indicate the standard deviation (±SD) among the duplicates, which differs significantly at p≤0.05. LSD = 5.65

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