INCREASED INDUSTRIAL ENZYME PRODUCTION BY *PENICILLIUM LILACINUM* IBGE 04 USING SHAKEN FLASK TECHNIQUE OF SUBMERGED FERMENTATION

[*]Kashif Ahmed[1], Seharish munawar[2], Muhammad Ansar Khan[3]

[1]Department of Chemistry, N.E.D. University of Engineering & Technology, Karachi.

[*]kashif25473@yahoo.com

[2]Directorate of Fisheries Sindh, Research and Development, Karachi.

sehrish.rajput36@gmail.com

[3]Department of Chemical Engineering, N.E.D. University of Engineering & Technology, Karachi.

ansar_khan100@hotmail.com

ABSTRACT: *In this work optimization parameters for maximum production of alpha-amylase by Penicillium lilacinum IBGE 04 in submerged fermentation were studied. Various agricultural based by-products (sunflower waste, cotton stalk, rice husk, date syrup and molasses) as carbon sources and various nitrogen sources (corn steep, casein, potassium nitrate, albumin, ammonium sulphate, urea and yeast extract) were used for increased production. Optimal conditions for the production of α-amylase (7.68 U/mL) by Penicillium lilacinum IBGE 04 were observed when the strain was grown on culture medium (M1) containing yeast extract as a source of nitrogen, molasses as a source of carbon after 96 h of incubation at 40° C, initial pH 7.5, inoculum size of 6x10^6 conidia in 50 mL of culture medium and agitation rate of 200 rev/min. The strain was proved thermo (up to 60° C) and pH (up to 9.0) stable so it might be a potential strain for industrial utilization.*

Key words: *Penicillium lilacinum*, industrial enzyme, optimization, submerged fermentation

1. INTRODUCTION

Alpha-amylase (Enzyme Commission No. is 3.2.1.1) is an extracellular enzyme, which splits α-1, 4- glycosidic bonds of starch and produces glucose, maltose and alpha limit dextrin [1].The substrate of amylase is starch, which is a polysaccharide and composed of two types of polymers amylose and amylopectin. Starch is composed of 20-25 % amylose, which is a linear chain of glucose units joined by α-1, 4- glycosidic bonds and about 75-80 % amylopectin, which is branched macro molecule of glucose in which 1, 6-glycosidic bonds are also present [1].

Amylases are one of the most widely used commercial enzymes whose range of application has broadened in numerous areas such as food, medicinal, clinical and analytical chemistry. They catch uses in pharmaceutical, food and baking, brewing, paper, detergent and textile industries. These are essential enzymes used in starch treating activities for hydrolysis of polysaccharides such as starch into simple sugar components [1].

Agricultural based by-products in Pakistan are usually disposed of by environment non-friendly manner. So in the present study some of them were used as sources of carbon in order to reduce pollution related issues. In literature a number of nonconventional carbon sources such as starch, date syrup, sunflower waste, oilcakes, cassava starch, potato peel, fruit peel, corn and tapioca have been reported in submerged fermentation for various enzymes production [2, 3].

In this work the secretion of alpha-amylase by *Penicillium lilacinum* IBGE 04 in submerged fermentation with optimized parameters are being reported because no work is done on the strain to optimize all parameters for the production of alpha-

amylase by this strain.

2. MATERILS AND METHODS

2.1 Strain

P. lilacinum IBGE 04 was obtained from the Institute of Biotechnology & Genetic Engineering, University of Sindh and the culture was maintained as followed by Dahot [4].

2.2 Conidia count

Number of conidia of each fungus was counted by haemocytometer (BOE 13, Boeco Germany). Spore suspension was maintained about 4x10^6 conidia/mL and they were added to 50 mL of fermentation media in 250 mL flask.

2.3 Hydrolysis of agriculture waste

Each agricultural based by-product (cotton stalk, sunflower waste and rice husk) was treated as reported earlier [2, 3].

2.4 Alpha-amylase activity

Alpha-amylase activity was determined by Bernfeld method [5]. One unit of α-amylase is the amount of enzyme that will release 1 mg of reducing sugar in 3 min at 50° C and pH 7.0.

2.5 Optimization of Enzyme Production Parameters

All experiments were done in such a way that the parameter optimized in one experiment was fixed in the subsequent experiments for the maximum production of enzyme. Following were parameters:

2.5.1 Culture media

First of all the most suitable culture medium was determined. For optimization of α-Amylase production following culture media were used having composition (g/L).

M1: Dextrose 10, Peptone 5, Epsom salt 5, KH_2PO_4 5, Common salt 2.5, ferrous sulphate hepta hydrate 0.01, $ZnSO_4.7H_2O$ 0.002, $MnSO_4.H_2O$ 0.001 and thiamine hydrochloride 0.001 [6].

M2: Soluble starch 20, $NH_4NO_3$10, KH_2PO_4, 14, KCl, 0.5, Epsom salt 0.1, $FeSO_4.7H_2O$, 0.01 [7].

M3: NaCl 0.8, KCl 0.8, $CaCl_2$ 0.1, Na_2HPO_4 2.0, $MgSO_4$0.2, $FeSO_4$ 0.1, 8.0 Glucose, NH_4Cl 2.0 [8].

M4: Zn $SO_4.7H_2O$ 0.062, $FeSO_4$ 0.068, copper sulphate pent hydrate 0.0001 and wheat bran 100 [9].

2.5.2 Incubation time period

After the determination of the most suitable culture medium, optimum incubation time period was determined. It was done by growing the strain on M1 at various time periods from 24-240 h.

2.5.3 Carbon sources

After the optimization of incubation time the most suitable carbon source was determined. It was done by replacing the glucose (control) of culture medium (M1) by various wastes including sunflower waste, cotton stalk, rice husk, which were hydrolyzed by 0.3 *N* H_2SO_4 and 0.6 *N* H_2SO_4. Date syrup and molasses were used 0.5 % and 1 % in place of glucose (control).

2.5.4 Nitrogen sources

After the determination of the most suitable carbon source various nitrogen sources were checked for maximum production of enzymes. It was done by replacing peptone of culture medium (M1) by corn steep liquor, casein, potassium nitrate, albumin, ammonium sulphate, urea and yeast extract.

2.5.5 Incubation temperature

The most suitable culture medium M1 (with the most suitable carbon and nitrogen source) was tested on varying temperature from 20-70° C to determine the most suitable incubation temperature for the production of enzyme.

2.5.6 Initial pH of medium

The initial pH of a medium has an effect on growth and productivity of microorganism. A range of pH from 4.0-9.0 was checked for maximum enzyme production.

2.5.7 Inoculum size

Productivity was also checked in terms of number of conidia in 50 mL of optimized culture medium in order to obtain the optimized inoculum size of culture medium. The number of conidia was counted by haemocytometer (BOE 13, Boeco Germany).

2.5.8 Agitation rate

Effect of agitation rate was also checked for optimization at 50, 100, 150, 200, 250 and 300 rev/min in orbital shaking incubator (SANYO Gallenkamp, PLC, UK).

3. RESULTS AND DISCUSSIONS

3.1 Effect of culture media

Effects of various culture media on α-amylase production by *P. lilacinum* IBGE 04 after 24 h, at temperature 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are plotted (Fig 1). The strain was grown on four different culture media *i.e.* M1, M2, M3 and M4. It was capable of growing well on all types of culture medium but production of *α*-amylase was maximum (1.83 U/mL) on culture medium M1, which was selected for the subsequent study of *α*-amylase production.

3.2 Effect of incubation time period

The effects of incubation time periods on *α*-amylase production by *P. lilacinum* IBGE 04 in M1 at temperature 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are presented (Fig 2). Activity of *α*-amylase was measured at regular interval of 24 h and it was found that the maximum activity (2.76 U/mL) was observed after 96 h of incubation. On prolonged incubation enzyme activity was decreased, which might be due to denaturing of enzyme or synthesis of inhibiting metabolite [10]. The 96 h of incubation time period for maximum *α*-amylase production from *P. Fellutanum* was also reported [11]. Various incubation time periods reported for *α*-amylase production by various researchers [2, 3, 7, 13, 14].

3.3 Effect of carbon sources

The effects of various carbon sources on production *α*-amylase by *P. lilacinum* IBGE 04 after 96 h in M1 at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are plotted (Fig 3). It was observed that *α*-amylase activities were lower in case of 0.3*N* sulphuric acid hydrolysed agriculture waste (1.61, 1.42 and 1.35 U/mL for cotton stalk, sunflower waste and rice husk respectively) and 0.5 % of molasses and date syrup (2.49 and 2.31 U/mL respectively). Activities of *α*-amylase were higher than control, glucose (2.76 U/mL) when 0.6*N* sulphuric acid hydrolysed agriculture waste (2.77, 2.84 and 2.88 U/mL for cotton stalk, sunflower waste and rice husk respectively) and 1 % of molasses (3.52 U/mL) and date syrup (3.03 U/mL) were used. Various carbon sources were reported for *α*-amylase prodction by various researchers [2, 3, 7, 12, 13, 14].

3.4 Effect of nitrogen sources

The effects of various nitrogen sources on *α*-amylase production by *P. lilacinum* IBGE 04 after 96 h in M1 containing molasses as carbon source at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are exhibited (Fig 4). The strain showed the capability of utilizing well all types of nitrogen sources but yeast extract was found the best (3.48 U/mL in 0.25 % and 4.35 U/mL in 0.50 %). Various other nitrogen sources have been reported for *α*-amylase prodction by various researchers [2, 3, 7, 12, 13, 14].

3.5 Effect of temperature

The effects of incubation temperatures on *α*-amylase production by *P. lilacinum* IBGE 04 after 96 h in M1 containing molasses as carbon source, yeast extract as nitrogen source, at initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are presented (Fig 5). The fermentation medium was incubated at a range of temperatures 20-70° C. Activity of *α*-Amylase was the highest (5.29 U/mL) about 40° C. The strain showed thermo stability up to 60° C (1.18 U/mL). Various incubation temperatures were reported for *α*-amylase prodction by various researchers [2, 3, 7, 12, 13, 14].

3.6 Effect of initial pH

The effects of initial pH of fermentation medium on *α*-amylase production by *P. lilacinum* IBGE 04 after 96 h in M1 containing molasses as carbon source, yeast extract as nitrogen source, temperature 40^o C, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are presented (Fig 6). The range of pH (4.0 to 9.0) was studied and found that initial pH of 7.5 was the best for maximum enzyme production (6.21 U/mL). Various initial pH of fermentation medium was reported for *α*-amylase prodction by various researchers [2, 3, 7, 12, 13, 14].

3.7 Effect of inoculum size

The effects of inoculum sizes on *α*-amylase production by *P. lilacinum* IBGE 04 after 96 h in M1 containing molasses as carbon source, yeast extract as nitrogen source, at 40° C, initial pH 7.5 and agitation rate 50 rev/min are plotted (Fig 7). Flasks were added with $4x10^6$-$8x10^6$ conidia and

maximum α-amylase activity (6.98 U/mL) was observed when $6x10^6$ conidia were added to the medium. Literature survey revealed that researchers used varying inoculum sizes [2, 3, 7, 12, 13, 14]. Large inoculum size caused overgrowth and nutritional imbalanced resulting less production of enzyme [4, 10, 14].

3.8 Effect of agitation rate

The effects of agitation rates on α-amylase production by *P. lilacinum* IBGE 04 after 96 h in M1 containing molasses as carbon source, yeast extract as nitrogen source, at 40° C, initial pH 7.5 and inoculum size $6x10^6$ conidia are exhibited (Fig 8). The fermentation medium was agitated at 50, 100, 150, 200, 250 and 300 rev/min. Activity of α-Amylase was maximum (7.68 U/mL) at 200 rev/min. Literature survey revealed that researchers reported various agitation rates (100-200 rev/min) for enzymes production by different microorganisms [4, 8, 10, 14].

4. CONCLUSION

Optimal conditions for the production of α-amylase (7.68 U/mL) by *Penicillium lilacinum* IBGE 04 were observed when the strain was grown on culture medium M1 containing yeast extract as a source of nitrogen, molasses as a source of carbon after 96 h of incubation at 40° C, initial pH 7.5, inoculum size of 6x106 conidia in 50 mL of culture medium and agitation rate of 200 rev/min. The strain showed enzyme activity up to pH 9.0 (2.76 U/mL) and temperature 60° C (1.18 U/mL) *i.e.* it was thermo and pH stable which is basic requirement of a microorganism for its industrial use.

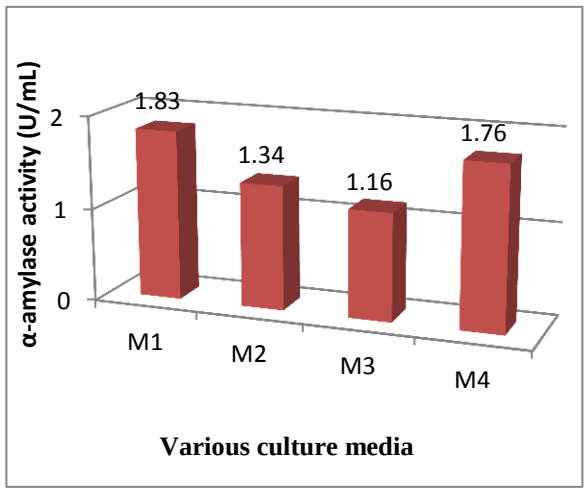

Fig 3.1: Effects of various culture media on α-amylase production by *P. lilacinum* after 24 h, at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

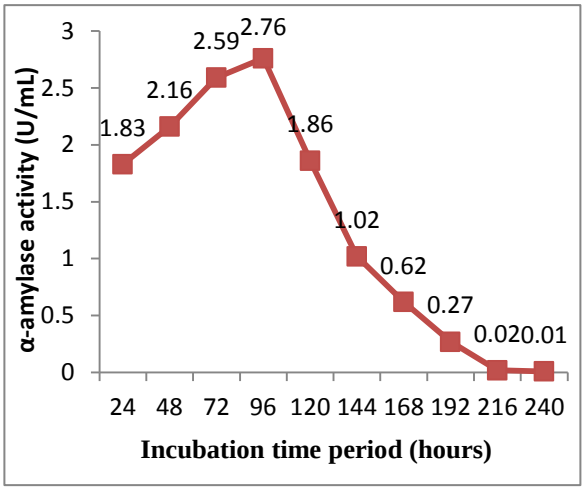

Fig 3.2: Effects of incubation time periods on α-amylase production by *P. lilacinum* in M1 at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50

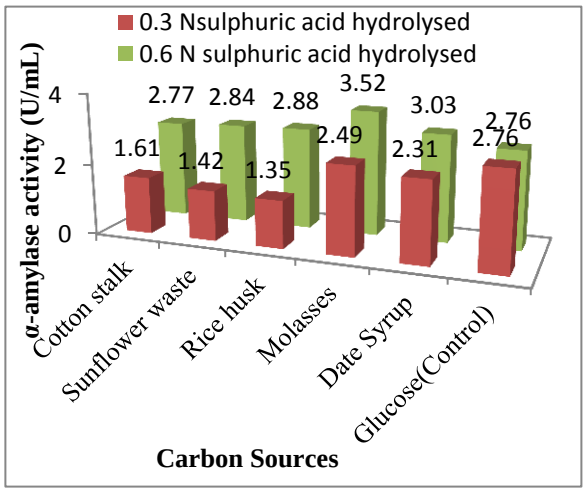

Fig 3.3: Effects of various carbon sources on production α-amylase by *P. lilacinum* after 96 h in M1 at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50

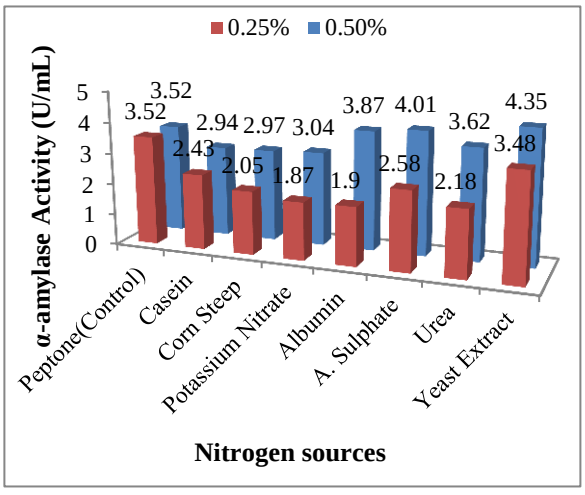

Fig 3.4: Effects of various nitrogen sources on α-amylase production by *P. lilacinum* after 96 h in M1 containing molasses as carbon source at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate

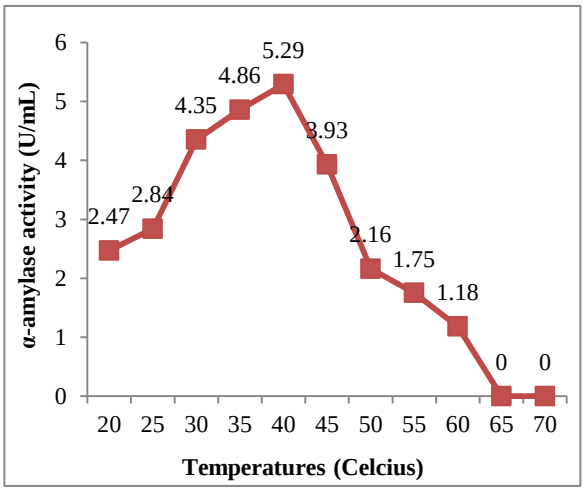

Fig 3.5: Effects of incubation temperatures on *α*-amylase production by *P. lilacinum* after 96 h in M1 containing molasses as carbon source, yeast extract as nitrogen source, at initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min.

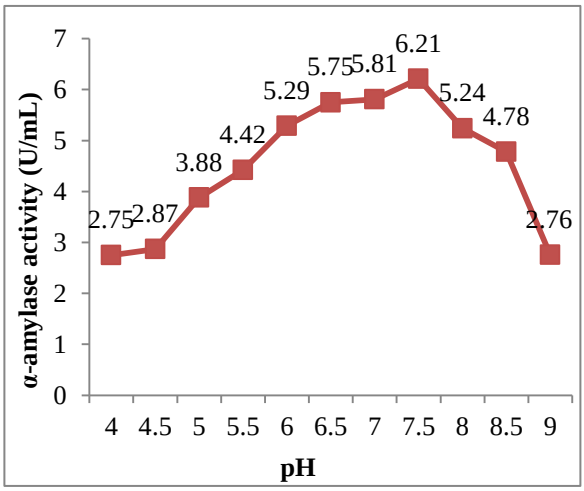

Fig 3.6: Effects of initial pH of fermentation medium on *α*-amylase production by *P. lilacinum* after 96 h in M1 containing molasses as carbon source, yeast extract as nitrogen source, at 40° C, inoculum size 4x10^6 conidia and agitation rate 50 rev/min.

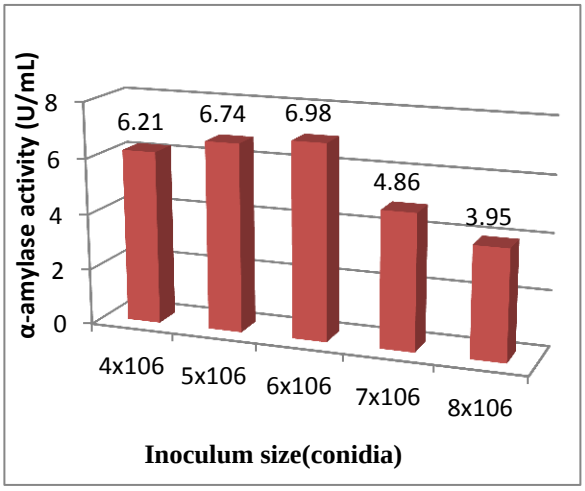

Fig 3.7: Effects of inoculum sizes on *α*-amylase production by *P. lilacinum* after 96 h in M1 containing molasses as carbon source, yeast extract as nitrogen source, at 40° C, initial pH 7.5 and agitation rate 50

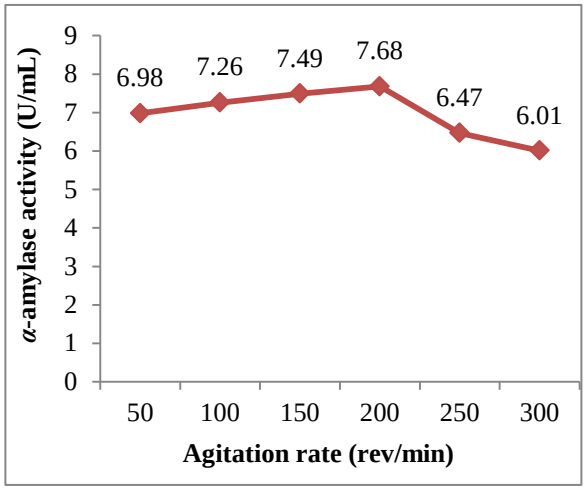

Fig 3.8: Effects of agitation rates on *α*-amylase production by *P. lilacinum* after 96 h in M1 containing molasses as carbon source, yeast extract as nitrogen source, at 40° C, initial pH 7.5 and inoculum size 6x10^6

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