Research Article

Cultural conditions for maximum alpha-amylase production by *Penicillium notatum* IBGE 03 using shaken flask technique of submerged fermentation

Kashif Ahmed^{1*}, Seharish Munawar² and Muhammad Ansar Khan³

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

2. Directorate of Fisheries Sindh, Research and Development, Karachi, Pakistan

3. Department of Chemical Engineering, N.E.D. University of Engineering and Technology, Karachi, Pakistan.

*Corresponding author's email: kashif25473@yahoo.com

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Abstract

Ever increasing biotechnological industries require an enlarged survey of microorganisms which might be useful for industries. In the present work optimization parameters for alpha amylase production by *Penicillium notatum* IBGE 03 in submerged fermentation were studied. Various agricultural based by-products (sunflower waste, cotton stalk, rice husk, date syrup and molasses) were used as sources of carbon. Optimal conditions for the production of α -amylase (6.58 U/mL) by *P. notatum* IBGE 03 were observed when the strain was grown on culture medium M1 containing corn steep liquor as a source of nitrogen, molasses as a source of carbon after 48 h of incubation at 30° C, initial pH 5.5, inoculum size of 5×10^6 conidia in 50 mL of culture medium and agitation rate of 150 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: Alpha-amylase; *Penicillium notatum* IBGE 03; Optimization.

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 are used in starch hydrolysis they also catch uses in pharmaceutical, food, 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]. The present study shows the optimized cultural conditions for the maximum production of alpha-amylase by *Penicillium notatum* IBGE 03 using different wastes as sources of carbon.

Materials and Methods

Strains

Strain of *Penicillium notatum* IBGE 03 was obtained from the Institute of Biotechnology & Genetic Engineering University of Sindh and the culture was maintained as followed by Dahot [4]. In the present study slants of 4 days old were used for inoculation.

Conidia count

Number of conidia of each fungus was counted by haemocytometer (BOE 13, Boeco Germany). Spore suspension was maintained about 4×10^6 conidia/mL and they were added to 50 mL of fermentation media in 250 mL flask.

Hydrolysis of agriculture waste

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

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.

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:

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, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.002, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 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, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 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: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.062, FeSO_4 0.068, copper sulphate pent hydrate 0.0001 and wheat bran 100 [9].

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.

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).

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.

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.

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.

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).

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).

Results and Discussion

Effect of culture media

Effects of various culture media on α -amylase production by *P. notatum* IBGE 03 after 24 h, at temperature 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min are presented (Fig. 1). The strain was grown on four different culture media i.e. M1, M2, M3 and M4. It was grown well on all types of culture media but production of α -amylase was maximum (1.42 U/mL) on culture medium M1, which was selected for the following study of α -amylase production.

Effect of incubation time period

The effects of incubation time periods on α -amylase production by *P. notatum* IBGE 03 in M1 at temperature 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation

rate 50 rev/min are plotted (Fig. 2). Activity of α -amylase was measured at regular interval of 24 h and it was found that the maximum activity (1.86 U/mL) was observed after 48 h of incubation. On prolonged incubation enzyme activity was decreased, which might be due to denaturing of enzyme or synthesis of inhibiting metabolite [10]. Khan and Yadav [8] also reported incubation time period of 48 h for α -amylase production by *Aspergillus niger*.

Effect of carbon sources

The effects of various carbon sources on α -amylase production by *P. notatum* IBGE 03 after 48 h in M1 at temperature 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min are presented (Fig. 3). It was observed that α -amylase activities were lower in case of 0.3 N sulphuric acid hydrolysed agriculture waste (0.86, 1.17 and 0.97 U/mL for cotton stalk, sunflower waste and rice husk respectively) and 0.5 % of molasses and date syrup (1.57 and 1.46 U/mL respectively). Activities of α -Amylase were closed to or higher than control, glucose (1.86 U/mL) when 0.6 N sulphuric acid hydrolysed agriculture waste (1.74, 2.13 and 1.84 U/mL for cotton stalk, sunflower waste and rice husk respectively) and 1 % of molasses (2.43 U/mL) and date syrup (2.24 U/mL) were used. Matthias [7] reported starch as the appropriate carbon source for α -amylase production by *Aspergillus*, *Mucor* and *Rhizopus* species.

Effect of nitrogen sources

The effects of various nitrogen sources on α -amylase production by *P. notatum* IBGE 03 after 48 h in M1 containing molasses as carbon source at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min are shown (Fig. 4). The strain showed the capability of utilizing well all types of nitrogen sources but corn steep liquor was found to be the best (2.18 U/mL in 0.25 % and 4.79 U/mL in 0.50 %).

Fig. 1. Effects of various culture media on α -amylase production by *Penicillium notatum* after 24 h, at temperature 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 2. Effects of incubation time periods on α -amylase production by *P. notatum* in M1 at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 3. Effects of various carbon sources on α -amylase production by *P. notatum* after 48 h in M1 at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 4. Effects of various nitrogen sources on α -amylase production by *P. notatum* after 48 h in M1 containing molasses as carbon source at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 5. Effects of incubation temperatures on α -amylase production by *P. notatum* after 48 h in M1 containing molasses as carbon source, corn steep liquor as nitrogen source, at initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 6. Effects of initial pH of fermentation medium on α -amylase production by *P. notatum* after 48 h in M1 containing molasses as carbon source, corn steep liquor as nitrogen source, at 30° C, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 7. Effects of inoculum sizes on α -amylase production by *P. notatum* after 48 h in M1 containing molasses as carbon source, corn steep liquor as nitrogen source, at 30° C, initial pH 5.5

Fig. 8. Effects of agitation rates on α -amylase production by *P. notatum* after 48 h in M1 containing molasses as carbon source, corn steep liquor as nitrogen source, at 30° C, initial pH 5.5 and inoculum size 5×10^6 conidia.

Various nitrogen sources have been reported in literature for α -amylase production for example Singh *et al.* [11] reported beef extract by *Aspergillus fumigatus* while Matthias [7] reported ammonium nitrate for *Aspergillus*, *Mucor* and *Rhizopus* species.

Effect of temperature

The effects of incubation temperatures on α -amylase production by *P. notatum* IBGE 03 after 48 h in M1 containing molasses as carbon source, corn steep liquor as nitrogen source, at initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min are plotted (Fig. 5). The fermentation medium was incubated at a range of temperatures 20-70° C. Activity of α -amylase was the highest (4.79 U/mL) about 30° C. Saleem & Ebrahim [12] reported similar incubation temperature for α -amylase production by *Aspergillus niger* and *Rhizopus stolonifer*. The strain showed thermo stability up to 60° C (0.03 U/mL).

Effect of initial pH

The effects of initial pH of fermentation medium on α -amylase production by *P. notatum* IBGE 03 after 48 h in M1 containing molasses as carbon source, corn steep liquor as nitrogen source, temperature 30° C, inoculum size 4×10^6 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 5.5 would be the optimum for maximum enzyme production (5.24 U/mL). After and before the pH the decrease in enzyme activity was observed. Saleem & Ebrahim [12] have reported pH 6.0 as the appropriate for α -amylase production by *Aspergillus niger* and *Rhizopus stolonifer*.

Effect of inoculum size

The effects of inoculum sizes on α -amylase production by *P. notatum* IBGE 03 after 48 h in M1 containing molasses as carbon source, corn steep liquor as nitrogen source, at 30° C, initial pH 5.5 and agitation rate 50 rev/min are presented (Fig. 7). Flasks were

added with 4×10^6 - 8×10^6 conidia and maximum α -amylase activity (5.73 U/mL) was observed when 5×10^6 conidia were added to the medium. Literature survey revealed that researchers used varying inoculum sizes [3, 13]. Large inoculum size caused overgrowth and nutritional imbalance resulting less production of enzyme [4, 10, 13].

Effect of agitation rate

The effects of agitation rates on α -amylase production by *P. notatum* IBGE 03 after 48 h in M1 containing molasses as carbon source, corn steep liquor as nitrogen source, at 30° C, initial pH 5.5 and inoculum size 5×10^6 conidia are presented (Fig. 8). The fermentation medium was agitated at 50, 100, 150, 200, 250 and 300 rev/min. α -amylase activity was maximum (6.58 U/mL) at 150 rev/min. Literature survey revealed that researchers reported various agitation rates (100-200 rev/min) for enzymes production by different microorganisms [4, 10, 13].

Conclusion

Optimal conditions for the production of α -amylase (6.58 U/mL) by *Penicillium notatum* IBGE 03 were observed when the strain was grown on culture medium M1 containing corn steep liquor as a source of nitrogen, molasses as a source of carbon after 48 h of incubation at 30° C, initial pH 5.5, inoculum size of 5×10^6 conidia in 50 mL of culture medium and agitation rate of 150 rev/min. The strain was proved pH (up to 9) and thermo stable (up to 60° C) therefore can be used in industries for alpha-amylase production.

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