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BIOSYNTHESIS OF ALPHA AMYLASE FROM *ASPERGILLUS FUMIGATUS* (FRESENIUS 1863) IN SUBMERGED FERMENTATION

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ABSTRACT

In the present work optimization of some parameters for alpha amylase production by *Aspergillus fumigatus* (Fresenius 1863) 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 (7.01 U/mL) by *A. Fumigates* were observed when the strainwas grownonculture medium (M1) containing casein as a source of nitrogen, molasses as a source of carbon after 72 h of incubation at initial pH 5.5, temperature 35° C, inoculum size of $6x10^6$ 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.

KEYWORDS: Alpha-amylase, submerged fermentation, optimization parameters, *Aspergillus fumigatus*

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 (Ujjan*et al.,* 2004).It is widely distributed in nature(Abdullah *et al*, 2005). 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 % amylo-pectin, which is branched macro molecule of glucose in which 1, 6- glycosidic bonds are also present (Sundarram and Murthy, 2014).

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 poly-saccharides such as starch into simple sugar components (Sundarram and Murthy, 2014).

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 (Ahmed *et al.,* 2011; 2014).

The modern biotechnological setup due to increasing demand of enzymes has motivated the need for enlarged survey of microorganisms surviving and producing enzyme in extreme conditions. For the production of large quantities of enzymes filamentous fungi have biotechno-logical importance (Mamma *et al.,* 2008).

In this work optimization parameters in submerged fermentation were studied for the production of *α*-amylase from *Aspergillus fumigatus* (Fresenius 1863) because no comprehensive work of optimization for maximum enzyme production was done by this strain.

MATERILS AND METHODS

Strains: Strain of *Aspergillus fumigates* (Fresenius 1863) was obtained from the soil of NED University Karachi and culture were maintained as followed by Dahot (1986). 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 $4x10^6$ 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 (Ahmed *et al*., 2011).

α-Amylase Activity: *Amylase activity was checked by using Bernfeld, (1955) method.* Enzyme sample of 1.0 mL was added in1.0 mL of 1 % (w/v) soluble starch in 50 mM sodium phosphate buffer at pH 7.0 and then incubated for 3 min. at 50°C then added 1.0 mL DNS and boiled for 15 minutes and cooled at room temperature later on absorbance was noted at 540 nm (TOMOS).

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, $ZnSO_4.7H_2O$ 0.002, $MnSO_4.H_2O$ 0.001 and thiamine hydro-chloride 0.001 (Burrel *et al.,* 1966).

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 (Matthias, 2013).

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_4 Cl 2.0 (Khan and Yadav, 2011).

M4: $ZnSO_4.7H_2O$ 0.062, $FeSO_4$ 0.068, copper sulphate pent hydrate 0.0001 and wheat bran 100 (Hayashida and Teramoto, 1986).

1. **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 (which were observed the most suitable for all fungi under investigation) at various time periods from 24-240 h.
2. **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).
3. **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.
4. **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.
5. **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.
6. **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).
7. **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 Gallen-kamp, PLC, UK).

RESULTS AND DICUSSIONS

Effect of culture media: Effects of various culture media on *α*-amylase production by *A. fumigatus* after 24 h, at temperature 30°C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are presented (Figure 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 (0.72 U/mL) on culture medium M1, which was selected for the following study.

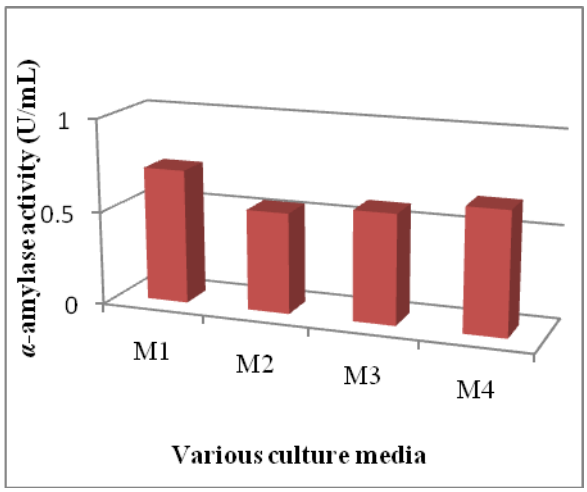

Figure 1: Effects of various culture media on α-amylase production by *A. fumigatus* after 24 h, at 30° C, initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min.

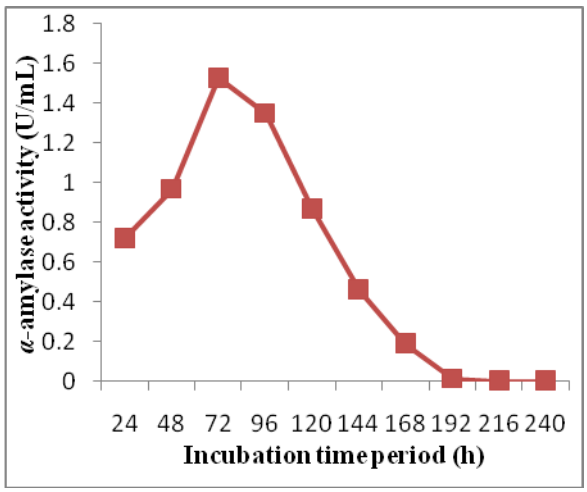

Figure2: Effects of incubation time periods on α-amylase production by *A. fumigatus*in M1 at 30ºC, initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min.

Selection of the most suitable culture media has the deep effect on enzyme production. Many investigators all over the world have reported different culture media for maximum α-amylase production (Hayashida and Teramoto, 1986; Khan and Yadav, 2011; Matthias, 2013; Ahmed *et al*., 2014).

Effect of incubation time period: The effects of incubation time periods on α-amylase production by *A. fumigatus* in M1 at temperature 30°C, initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min are plotted (Figure 2).Activity of α-amylase was measured at regular interval of 24 h and it was found that the maximum activity (1.53 U/mL) was observed after 72 h of incubation. On prolonged incubation enzyme activity was decreased, which may be due to denaturing of enzyme or synthesis of inhibiting metabolite (Mamma *et al*., 2008). Khan and Yadav (2011) reported incubation time period of 48 h for α-amylase production by *Aspergillus niger* while Archana and Satyanarayana (2011) reported 36 h and 72 h in batch and fed batch fermentations by *Bacillus acidicola.*

Effect of carbon sources: Effects of various carbon sources on α-amylase production by *A. fumigatus* after 72 h in M1 at temperature 30º C, initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min are presented (Figure 3).It was observed that α-amylase activities were lower in case of 0.3*N*sulphuric acid hydrolysed agriculture waste (1.44, 1.48 and 1.35 U/mL for cotton stalk, sunflower waste and rice husk respectively) and 0.5 % of molasses and date syrup (1.01 and 1.47U/mL respectively). Activities of α-amylase were higher than control, glucose (1.53 U/mL) when 0.6 *N* sulphuric acid hydrolysed agriculture waste (1.86, 2.13 and 1.86 U/mL for cotton stalk, sunflower waste and rice husk respectively) and 1 % of molasses (2.01 U/mL) and date syrup (1.91 U/mL) were used. Singh *et al*. (2014) have reported pomegranate peel as appropriate carbon source by *Aspergillus fumigates* for α-amylase production.

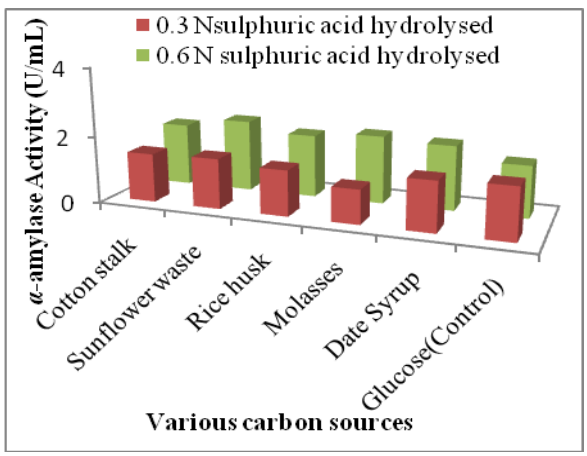

Figure 3: Effects of various carbon source on α-amylase production by *A. fumigatus* after 72 h in M1 at initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min.

Effect of nitrogen sources: The effects of various nitrogen sources on α-amylase production by *A. fumigatus* after 72h in M1 containing sunflower waste as carbon source at temperature 30°C, initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min are exhibited (Figure 4). The strain showed the capability of utilizing well all types of nitrogen sources but yeast extract was found to be the best (2.08 U/mL in 0.25 % and 3.89 U/mL in 0.50 %) for α-amylase production. Ahmed *et al*. (2014) reported yeast extract while Khan and Yadav (2011) reported peptone as the most appropriate nitrogen source by *Aspergillus niger.*

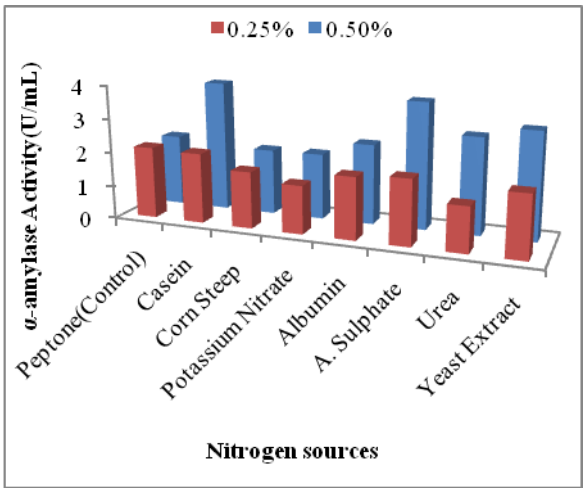

Figure 4: Effects of various nitrgen sources on α-amylase production by *A. fumigatus* after 72 h in M1 containing sunflower waste as carbon source at initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min.

Effect of temperature: The effects of incubation temperatures on α-amylase production by *A. fumigatus* after 72 h in M1 containing sunflower waste as carbon source, casein as nitrogen source, at initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min are presented (Figure 5).The fermentation medium was incubated at a range of temperatures 20-70° C.Activity of α-amylase was the highest (4.53 U/mL) about 35° C. Interestingly the strain showed thermo stability up to 65° C (0.86 U/mL), which is a requirement for industrial use of a microorganism (Mamma *et al.,* 2008). Singh *et al*. (2014) also reported 35° C for α-amylase production by *Aspergillus fumigatus.*

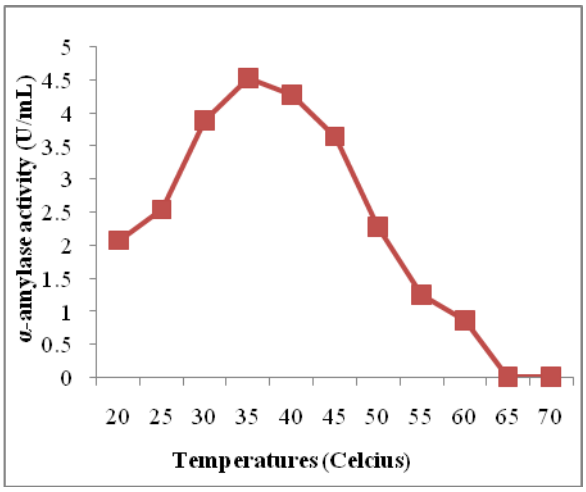

Figure 5: Effects of incubation temperatures on α-amylase production by *A. fumigatus* after 72 h in M1 containing sunflower waste as carbon source, casein as nitrogen source, at initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rev/min.

Effect of initial pH: The effects of initial pH of fermentation medium on α-amylase production by *A. fumigatus* after 72 h in M1 containing sunflower waste as carbon source, casein as nitrogen source, temperature 35°C, inoculum size 4x10^6 conidia and agitation rate 50 rev/min are shown (Figure 6). The range of pH (4.0 to 9.0) was studied and found that initial pH of 5.5 would be optimum for maximum enzyme production (5.16 U/mL). Saleem and Ebrahim (2014) reported pH 6.0 as the optimum for α-amylase production by *Aspergillus niger* and *Rhizopus stolonifer.*

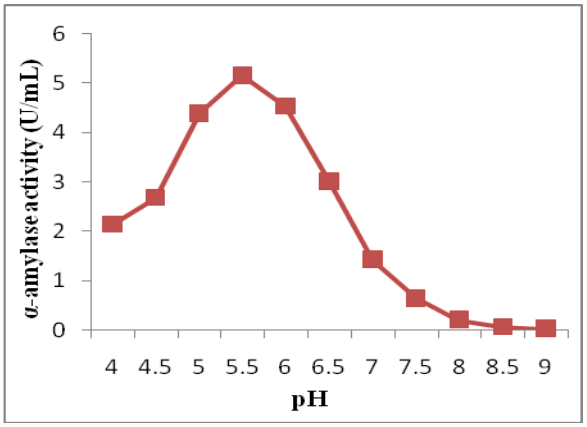

Figure 6: Effects of initial pH of fermentation medium on α-amylase production by *A. fumigatus* after 72 h in M1 containing sunflower waste as carbon source, casein as nitrogen source, temperature 35° C, inoculum size 4x10^6 conidia and agitation rate 50 rev/min.

Effect of inoculum size: The effects of inoculum sizes on α-amylase production by *A. fumigatus*

after 72 h in M1 containing sunflower waste as carbon source, casein as nitrogen source, at 35° C, initial pH 5.5 and agitation rate 50 rev/min are presented (Figure 7). Flasks were added with $4x10^6$-$8x10^6$ conidia and maximum α-amylase activity (6.21 U/mL) was observed when $6x10^6$ conidia were added to the medium. Researchers used varying inoculum sizes (Dahot, 1986; Archana and Satyanarayana, 2011; Ahmed *et al*., 2014). Large inoculum size caused overgrowth and nutritional imbalanced resulting less production of enzyme (Mamma *et al.,* 2008; Archana and Satyanarayana, 2011).

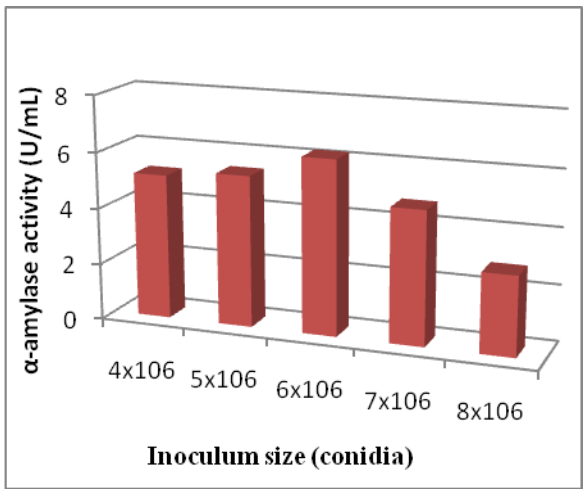

Figure 7: Effects of inoculum sizes on α-amylase production by *A. fumigatus* after 72 h in M1 containing sunflower waste as carbon source, casein as nitrogen source, at 35º C, initial pH 5.5 and agitation rate 50 rev/min.

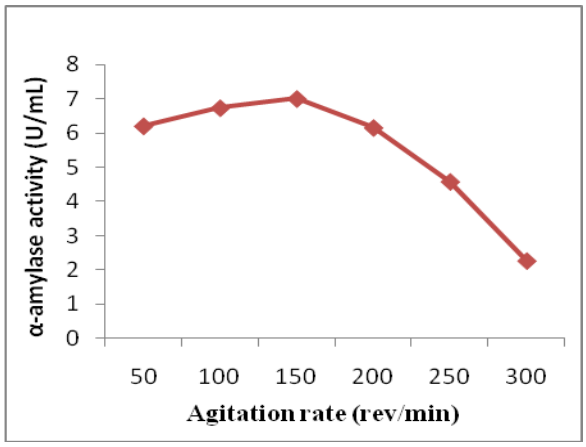

Figure-8: Effects of agitation rates on α-amylase production by *A. fumigatus* after 72 h in M1 containing sunflower waste as carbon source, casein as nitrogen source, at 35ºC, at initial pH 5.5 and inoculum size $6x10^6$ conidia.

Effect of agitation rate: The effects of agitation rates on α-amylase production by *A. fumigatus* after 72 h in M1 containing sunflower waste as carbon source, casein as nitrogen source, at 35°C, initial pH 5.5 and inoculum size $6x10^6$ conidia are presented (Figure 8).The fermentation medium was agitated at 50, 100, 150, 200, 250 and 300 rev/min. Activity ofα-amylase was maximum (7.01 U/mL) at 150 rev/min. Researchers have reported various agitation rates (100-200 rev/min) for enzymes production by different microorganisms (Dahot, 1986; Mamma *et al.,* 2008; Archana and Satyanarayana, 2011).

CONCLUSION

Optimal conditions for the production of α-amylase (7.01 U/mL) by *Aspergillus fumigates* (Fresenius 1863) were observed when the strainwas grownonculture medium M1 containing casein as a source of nitrogen, molasses as a source of carbon after 72 h of incubation at initial pH 5.5, temperature 35° C, inoculum size of $6x10^6$ conidia in 50 mL of culture medium and agitation rate of 150 rev/min. The strain showed enzyme activity up to pH 9.0 (0.6 U/mL) and temperature 60° C (0.86 U/mL) which is basic requirement of a microorganism for its industrial use.

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