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OPTIMIZATION OF CONDITIONS FOR THE PRODUCTION OF GLUCOAMYLASE FROM *ASPERGILLUS FUMIGATUS*: PURIFICATION AND KINETIC STUDIES OF GLUCOAMYLASE

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ABSTRACT

Filamentous fungi is the basic source of hydrolytic enzymes and widely used for industrial applications. *Aspergillus fumigatus* have been widely used for the production of hydrolytic enzymes. The present study was undertaken to demonstrate the production of glucoamylase from *Aspergillus fumigatus* using wheat bran as substrate. *A. fumigatus* was isolated from organic waste samples from river Ravi, Lahore, Punjab, Pakistan and the conditions were optimized for its growth. Various carbon and nitrogen sources were utilized for the supplementation of growth medium in order to produce higher level of glucoamylase. The produced enzyme was partially purified by fractional ammonium sulphate precipitation followed by dialysis for the removal of salt. The dialyzed sample was further purified by ion exchange and gel filtration chromatography. The purified protein was utilized for the kinetic studies. Purification studies resulted in 12 fold purification with 45% yield of the glucoamylase. SDS-PAGE analysis of the purified enzyme revealed an approximate size of 60 kDa. Under optimal conditions, we were able to produce 28 U/mL of glucoamylase. Highest enzyme activity was observed when *A. fumigatus* was grown on 3% wheat bran in the presence of 0.7% Tween-80 at 40^{o}C for 72 hours at pH 5.0. Kinetic studies demonstrated the K_m and V_{max} values 9.1 mg/mL and 40 µM min^{-1} mg^{-1} respectively when starch was utilized as substrate. Present study revealed a new source of glucoamylase, which have ability to utilize a variety of agriculture products as carbon sources that can be useful for enzyme industry. This study discovered a new strain, which is a unique machine that can act as an attractive alternative source for the production of industrial enzymes for starch hydrolysis. To our knowledge this is the highest glucoamylase activity from *Aspergillus fumigatus sp*. so far.

Key words: Glucoamylase, *Aspergillus fumigatus*, wheat bran, optimization.

INTRODUCTION

Glucoamylases (GAs) hydrolyze starch by catalyzing α 1-4 and α 1-6 linkage from non-reducing ends (Sauer *et al*., 2000). Several GAs have been characterized from a variety of bacteria (Zheng *et al*., 2010) and fungi (Puri *et al.,* 2013). Fungi have major contribution for the production of GAs (Chiquetto *et al.,* 2004). Microbial strains of genera *Aspergillus* and *Rhizopus* have been predominantly used for commercial production of GAs (Li *et al*., 2014; Pandey, 1995). Scientists used various substrates including rice bran, wheat bran and paddy husk for the economic bulk production of GAs (Puri *et al*., 2013; Singh and Soni, 2001) whereas, others utilize various sources for carbon, nitrogen and phosphorous for the optimal production of GAs (Bertolin *et al.,* 2001).

A. fumigatus is a fungus found in decaying organic matter and in soil i.e. compost heaps and has contributory role in nitrogen and carbon recycling in the environment (Klich and Pitt, 1988). Production of various enzymes from *A. fumigatus* has been reported previously which includes *β*-Glucosidase (Rudick and Elbein, 1975), xylolytic enzymes (Flannigan and Sellars, 1978), cellulolytic and proteolytic enzymes (Krikstaponis *et al*., 2001) and *β*-1,3-glucanases (Mouyna *et al*., 2013). In the present study, we reported the production of glucoamylase from *A. fumigatus* (GA_{AF}) and conditions were optimized for the production of $GA_{AF.}$

MATERIALS AND METHODS

Chemicals: All the chemicals utilized in the current study were purchased from Sigma Aldrich, Germany and were of purified grade.

Methods: *A. fumigatus* was isolated from organic waste soil samples from river Ravi located in Lahore, Punjab, Pakistan and was initially grown on Sabouraud Dextrose Agar (Dartora *et al.,* 2002). Hemocytometer was used for counting spores and 1x10^{6} spores per mL was utilized for inoculation (Strober *et al.,* 2001).

Enzymes production: Sterilized fermentation medium supplemented with 2% wheat bran and nutrients [$ZnSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $FeSO_4$ $7H_2O$, $C_{12}H_{22}O_{11}$ and $MnSO_4$] (Arun *et al.,* 2008) was inoculated followed

by incubation at 37ºC in an orbital shaker at 130 rpm. The harvesting was done after 4 days and the biomass thus produced was centrifuge at 6500 rpm for 15 minutes (Gupta *et al.,* 2003). The supernatant was evaluated for enzyme activity.

Optimization of Conditions for the production of Glucoamylase: Production of GA_{AF} was analyzed by examining the *A. fumigatus* growth in wheat bran medium at various temperatures ranging from 30 to 50ºC and pH from 3 to 9 using 50 *mM* of each of sodium acetate buffer (3-5), sodium phosphate buffer (5-7) and Tris-HCl buffer (7-9) separately (Tayyab *et al.,* 2011). In order to examine the maximal glucoamylase production, the wheat bran medium was supplemented with additional carbon (sucrose & starch), nitrogen (urea & ammonium sulphate) and phosphate (potassium mono & dihydrogen phosphate) sources at various concentrations ranging from 0.5 to 1.5%. Fungal growth and GA_{AF} production was recorded in the above said medium after sterilization. Effect of incubation time for the maximal GA_{AF} production was recorded by examining the fungal growth up to 5 days. GA_{AF} production was also analyzed in the presence of Tween-80 a non-ionic detergent at a final concentration of 0.1 to 1% detergent.

Enzyme Assay: Enzyme activity was determined using starch as substrate at pH 5 in 50 *mM* sodium acetate buffer. Regarding the enzyme activity, 0.1 mL of enzyme was mixed with 1.9 mL substrate prepared in 50 *mM* sodium acetate buffer, followed by incubation at 40ºC for 40 mins. The production of glucose was measured by DNS method by recording the absorbance at 540 nm. The units for enzyme activity were calculated using standard curve for glucose. One unit of enzyme activity was the amount of enzyme required for the production of one micromole of reducing sugar in one minute at 40ºC (Miller, 1959).

Purification of enzyme: The soluble portion after centrifugation of fungal growth was utilized for 20, 40, 60 and 80% ammonium sulphate precipitation. The precipitation was done at 4ºC. Each level of ammonium sulphate precipitated sample (20, 40, 60 and 80%) was centrifuged at 6500 rpm for 15 min at 4ºC. The protein pellet obtained after each step was dissolved in 20 mL of 50 m*M* sodium acetate buffer (pH 5) and was utilized to examine the enzyme activity. The fraction with GA_{AF} activity was introduced into the dialysis tube and was dialyzed against 50 m*M* sodium acetate buffer (pH 5) for 48h by replacing the dialysis buffer after every 4h with fresh buffer. The dialyzed fraction with enzyme activity was utilized for further purification by column chromatography. The dialyzed sample (200 mg protein) was applied to pre-equilibrated DEAE Sephadex A-50 ion exchange column (12cm X 3cm). Unbound proteins were removed by washing the column with same buffer and elution of bound protein was done using NaCl gradient (0 to 1 *M*) in 50 m*M* sodium acetate buffer (pH 5) at a flow rate of 0.5 mL/min. The fractions were collected and utilized for enzyme activity analysis. The fractions with maximal enzyme activity were pooled and applied to pre-equilibrated Sephadex G-200 gel filtration column (12cm X 3cm) followed by elution with 50 m*M* sodium acetate buffer (pH 5) at a flow rate of 1mL/min. The purified protein was utilized for kinetic studies of GA_{AF}.

Kinetic Studies of Glucoamylase: Enzyme activity was examined by varying the concentration of starch from 1 to 10% under optimized conditions and the obtained data was utilized for developing the Line-weaver Burk Plot and for the estimation of kinetic parameters (Mansoor *et al.,* 2018).

RESULTS AND DISCUSSION

We have isolated and identified four strains of *A. fumigatus*. The strain with maximal production of glucoamylase (11 U/mL at 35ºC) was selected for the further studies. It was found that the initial increase in temperature from 30 to 40ºC resulted in the increased production of GA_{AF} from 9.33 U/mL to 17.4 U/mL. Further increase in temperature resulted in the decreased production of enzyme (Fig 1). The optimal production of GA_{AF} was observed at 40ºC that is much higher as compared to 30ºC for *Aspergillus oryzae* (Puri *et al.,* 2013) and 35ºC for GAs from *Aspergillus awamori* NRRL 3112 and *Aspergillus niger* (Aguero *et al.,* 1990).

A linear increase in the enzyme activity was recorded with the increase in pH. Maximum activity was recorded at pH 5 when 50 m*M* sodium acetate buffer was used for GA_{AF} activity (Fig 2). Our findings are comparable to previously reported results by Pandey and Radhakrishnan (1993). They examined 4.7 as an optimal pH for the production of glucoamylase by *A. niger* NCIM-1245.

The results demonstrated that 3% wheat bran was the optimal substrate concentration for the production of GA_{AF} (18 U/mL). Bhatti *et al.,* (2007) examined the maximum production of glucoamylase from *Fusarium solani* when 2% wheat bran was used under optimum growth condition. When the production of glucoamylase was examined, while growing the fungal strain on the medium supplemented with additional carbon source (0.5 to 1.5%) sucrose or starch; starch put a clear enhancing effect on GA_{AF} production. The presence of sucrose could produce a little effect on the production of enzyme. The presence of 2% wheat bran alone could produce the 11 U/mL of GA_{AF}. This was increased to 13.15 U/mL and 15.25 U/mL in the presence of 1.25% of sucrose and starch respectively (Fig 3). Our results are in agreement with studies on *A. awamori* which shows the

same pattern (Pavezzi *et al.,* 2008) where as these results are contradictory to the studies on *F. solani* (Bhatti *et al.,* 2007).

The supplementation of urea or ammonium sulphate (0.5 to 1.5%) as nitrogen source in the growth medium containing 2% wheat bran demonstrated that Urea enhanced GA_{AF} production from 11 U/mL to 16 U/mL when used at a final concentration of 1.25% (Fig 3). These results are in agreement with the studies of Ellaiah *et al.,* (2002), they examined the optimal glucoamylase production from *Aspergillus* sp. A3 when 1% urea was utilized as additional nitrogen source. The presence of ammonium sulphate put an inhibitory effect on the enzyme production. The presence of 1.5% ammonium sulphate decreased the production from 11 U/mL to 6.7 U/mL (Fig 3). This decrease in the activity is might be due to low fungal growth in the presence of ammonium sulphate. The presence of mono or di-potassium hydrogen phosphates didn't show a significant effect on the production of glucoamylase (data not shown).

The incubation time is very important parameter for the optimal production of enzyme. The incubation of *A. fumigatus* at 40 °C for a period of 2 days didn't put a significant effect on the production of GA_{AF} (11.51 U/mL). Whereas, incubation up to 3rd day resulted, increased production of GA_{AF} (21.02 U/mL), which clearly indicates, the fungal growth. Further incubation put an adverse effect on microbial growth. These results were contradictory to previously reported data, as maximum production of glucoamylase was recorded on 4th day from *F. solani* (Bhatti *et al.,* 2007) or 5th day from *A. oryzae* (Puri *et al.,* 2013) when, fungal strains were incubated at 30 °C.

The presence of Tween-80 showed enhancing effect on the glucoamylase production. The enzyme activity was found to be increased from 10.5 to 19.6 U/mL when the concentration of Tween-80 was increased from 0.1 to 0.7% respectively. Further increase in Tween-80 beyond 0.7% resulted in decreased activity.

GA_{AF} purification studies demonstrated 12 fold purification with 45% yield (Table 1) after ion exchange and gel filtration chromatography (Fig 4A). The purity analysis of the purified enzyme showed a clear band on SDS-PAGE having an approximate size of 60 kDa (Fig. 4B). Previous reports demonstrated the production of 74 kDa glucoamylase from *A. niger* (Deshmukha *et al*., 2011) while *A. awamori* has three glucoamylases having size 59.1, 87.1 and 109.6 kDa (Negi *et al*., 2011). The glucoamylase yield in present study was quite high as compared to 8% for glucosidase from the same strain (Rudick and Elbein, 1975). The Line-weaver Burk plot (Fig 5) demonstrated the K_m and V_{max} values of 9.1 mg/mL and 40 μM min^{-1} mg^{-1} respectively, when starch was used as a substrate.

Table 1. Summary of Purification of Glucoamylase from *Aspergillus fumigatus.*

Purification Step	Total Protein (mg)	Total Enzyme Activity (U)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Crude Sample	790	6248	7.91	100	-
Ammonium Sulphate Precipitated	200	5500	27.5	88	3.48
Purified sample	30	2790	93	45	12

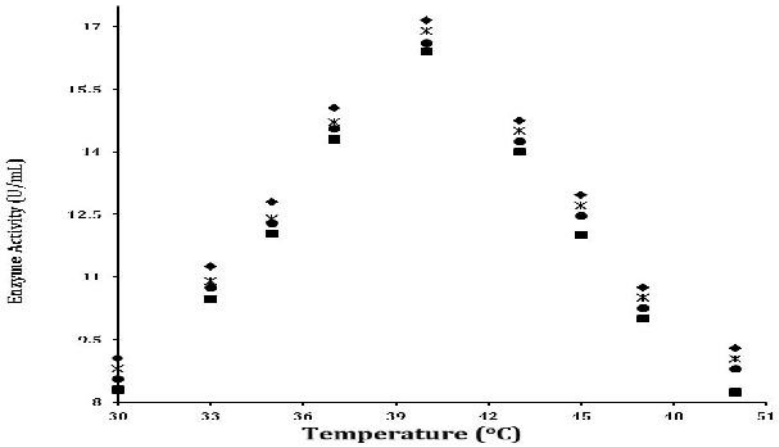

Fig. 1. Effect of Temperature on GA_{AF} production: The figure shows the effect of Temperature on the production of glucoamylase. X-axis presents temperature (°C) whereas Y-axis shows the glucoamylase activity (U/mL). In order to confirm the optimal temperature the medium was prepared by varying the concentration of wheat bran from 0.5%(■), 1%(●), 1.5%(×) and 2%(♦)and was utilized for examining the GA_{AF} production at various temperatures. Each point in the figure indicates the average of three independent experiments.

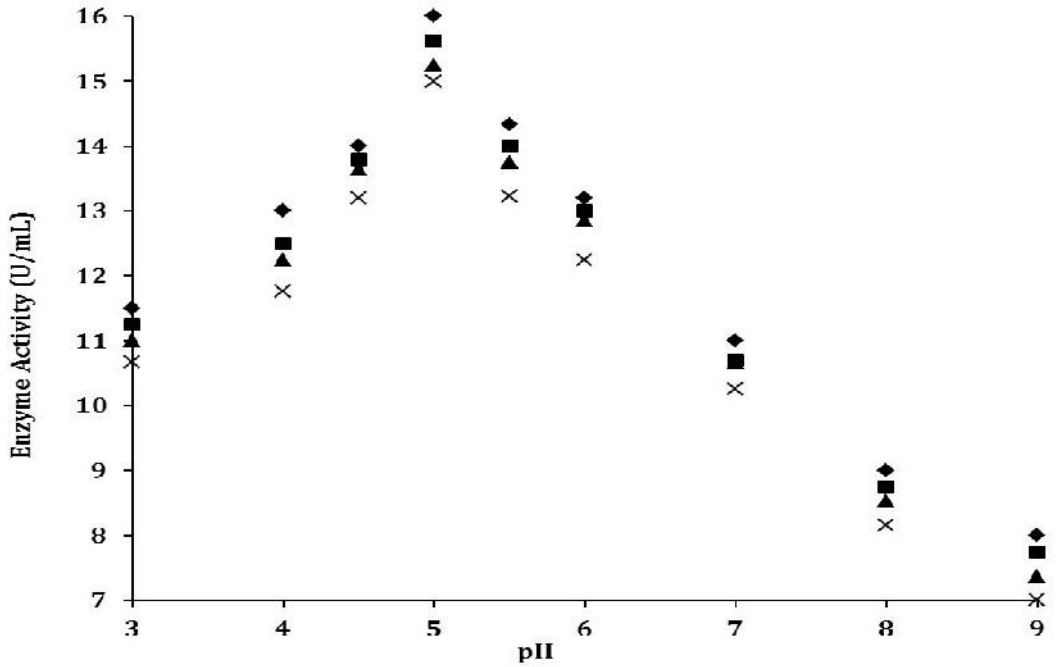

Fig. 2. Effect of pH on GA_{AF} production: The figure shows the effect of pH on the production of glucoamylase. X-axis presents pH ranging from 3 to 9. In order to check the effect of pH, the medium was prepared using 50 m*M* of each of Sodium Acetate buffer (3-5), Sodium Phosphate buffer (5-7) and Tris-HCl buffer (7-9) whereas Y-axis shows the glucoamylase activity (U/mL). In order to confirm the optimal pH the medium was prepared by varying the concentration of wheat bran 0.5%(×), 1%(▲), 1.5% (■)and 2%(◆)and was utilized for examining the GA_{AF} production at various pH using above said buffers. Each point in the figure indicates the average of three independent experiments.

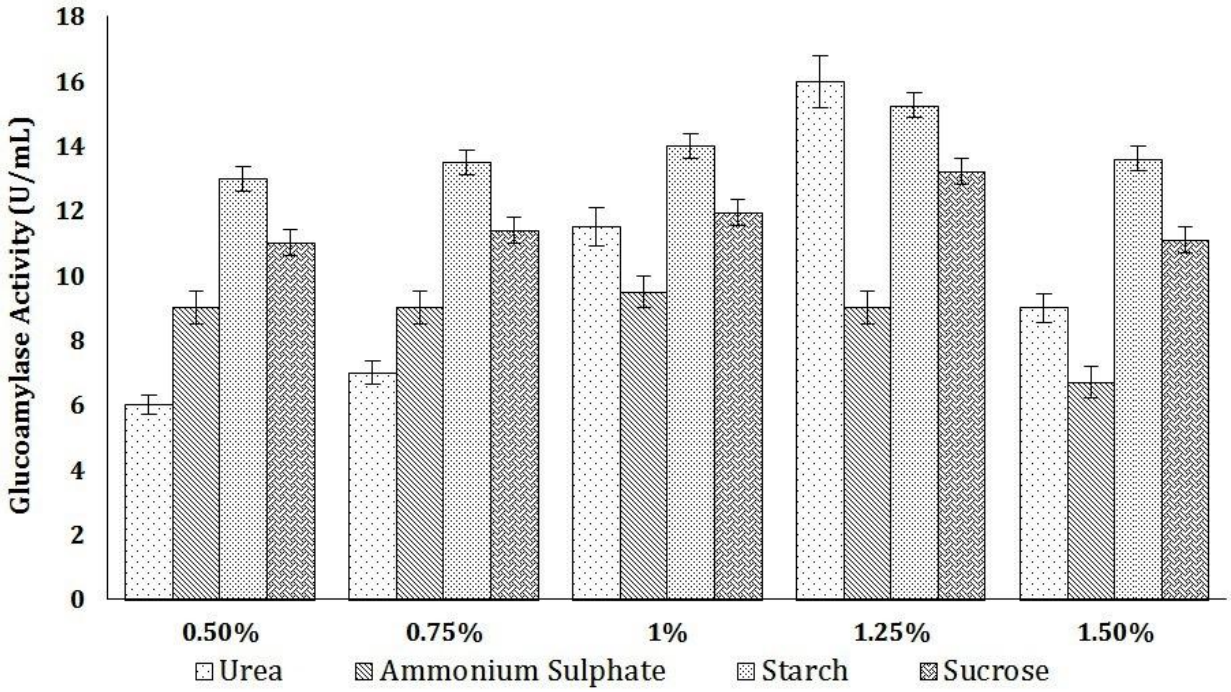

Fig. 3. Effect of supplementation of additional carbon and nitrogen sources on the glucoamylase production. Glucoamylase production was examined in the growth medium supplemented with wheat bran (2%) in sodium acetate buffer (pH 5).

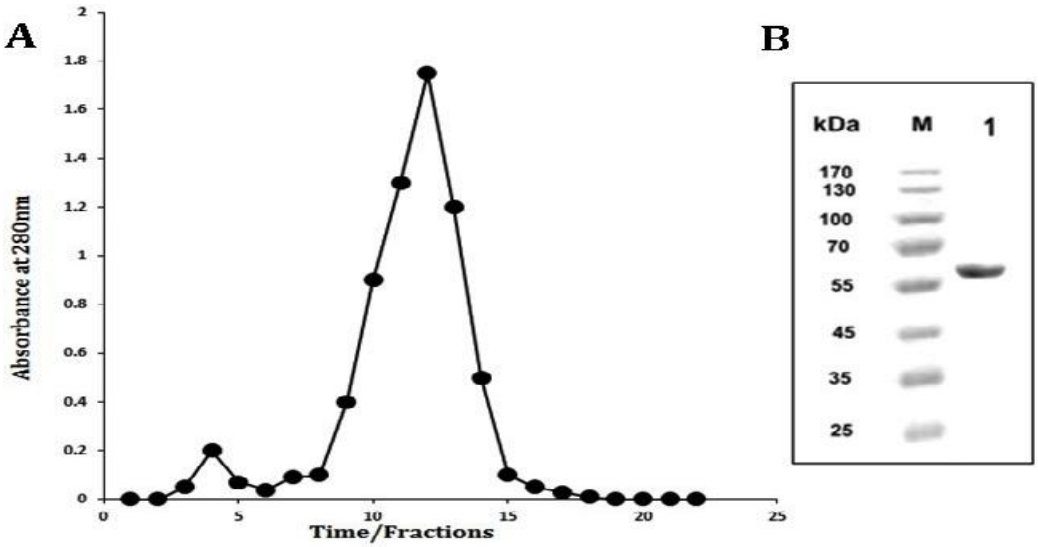

Fig. 4. Purification profile of Glucoamylase: A: Chromatogram of purified Glucoamylase, The figure shows the elution pattern of glucoamylase from gel filtration column. The fraction from ion exchange chromatography with enzyme activity was applied to the pre-equilibrated Sephadex G-200 gel filtration column. X-axis presents elution Time or fraction number whereas Y-axis presents the absorbance at 280 nm. **B: SDS-PAGE analysis of purified glucoamylase**, Lane M shows the protein molecular weight marker while Lane 1 shows the purified protein (Fraction no.12 from the gel filtration column)

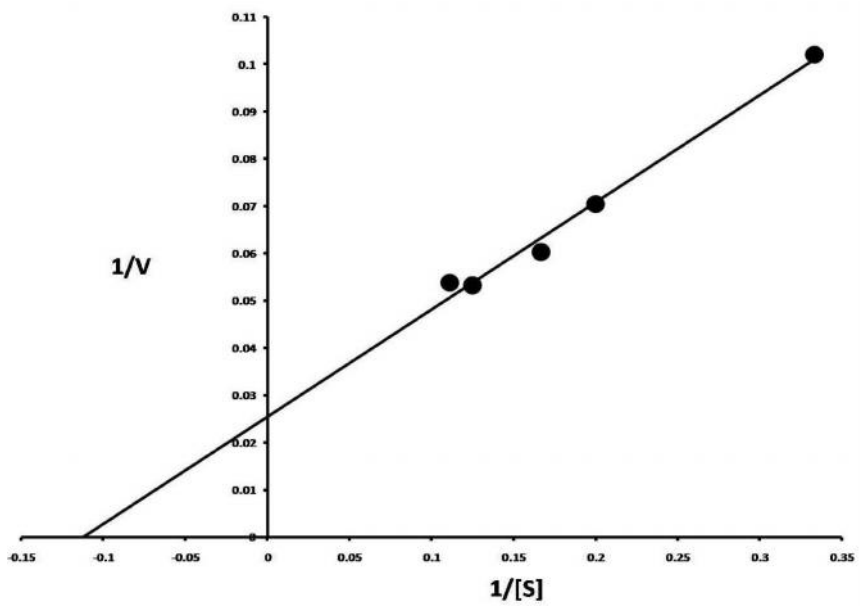

Fig.5. Kinetic studies of glucoamylase: Activity of glucoamylase was determined using various concentrations of starch as substrate in sodium acetate buffer pH 5. X-axis shows the concentration of 1/substrates and Y-axis shows the 1/velocity of glucoamylase.

Conclusion: This study demonstrated that *Aspergillus fumigatus* has strong potential for the glucoamylase production using agricultural wastes as carbon source. Optimization studies revealed the highest glucoamylase activity in shake flasks containing 3% wheat bran supplemented with 0.7% Tween-80 (23 U/mL) followed by 1.5% of urea (28 U/mL) at a temperature of 40^{o}C in the presence of 50 m*M* sodium acetate buffer (pH 5). The ability of the fungal strain to utilize the agricultural waste as carbon source for the production of glucoamylase makes it unique machine for the production of enzymes. To our knowledge this is the highest glucoamylase activity produced by *Aspergillus fumigatus* sp. reported so far.

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