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Full Length Research Paper

Hyper production of glucoamylase by *Aspergillus niger* through the process of chemical mutagensis

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The main objective of this study was to produce glucoamylase under optimum conditions and to study the effect of chemical mutagenesis on *Aspergillus niger* for the production of glucoamylase. The maximum activity of glucoamylase for mutant *A. niger* (3.185 ± 0.020 IU/ml/min) and *wild A. niger* (2.085 ± 0.021 IU/ml/min) was recorded in the culture filtration after 96 h of solid state fermentation of growth medium with 70% moisture level and in the presence of 0.3% yeast extract, 0.4% peptone and 4 ml Tween-80 at pH 4.8. The maximum fraction value after gel filtration for wild *A. niger* and mutant *A. niger* was 2.850 and 2.980 IU/ml/min, respectively. Purification through the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) revealed the indication of glucoamylase purification from *A. niger*. The high value of K_m shows that substrate had great affinity for glucoamylase. Glucoamylase enzyme had many useful applications in food processing industry and fermentation biotechnology.

Key words: *Aspergillus niger*, glucoamylase, ethidium bromide, fermentation, wheat bran.

INTRODUCTION

Glucoamylase (E.C. 3.2.1.3) is an enzyme that breaks the glucose units from the non reducing sides of amylose chain, glycogen and amylopectin involving in the hydrolysis of α (1 to 4) faster than α (1 to 6) and α (1 to 3) linkages and produce producing D glucose in successive manner (Fogarty, 1983).

Glucoamylase are produced through various microbial sources especially through bacteria, filamentous fungi and yeast. Glucoamylases of microbial sources have an advantage over the isolated, from other sources, because microorganisms have shorter life span, moreover, the enzyme of microbial origin can be isolated easily and their characteristics can be manipulated by genetic engineering and biotechnology techniques. This technique of enzyme biotechnology was extensively used in enzymes production (Selvakumar et al., 1994).

The extensive utilization of this glucoamylase enzyme is obtained by using a fungus, *Aspergillus niger* in enzyme production industry. Solid state fermentation (SSF) is a bright enzyme production practice, but the growth of many fungal species on solid substrates of wastes and organic minerals has been half-heartedly studied. Amylolytic enzymes production especially glucoamylase on solid support (substrate) is best studied in the fermentation technology (Wang et al., 2006; Ghildyal et al., 1985).

Glucoamylase enzyme having many applications in food processing industry, fermentation biotechnology, paper making and fabric industries, microbial origin of starch hydrolysing enzymes in the solid cultures of glucoamylase, uncover many applications in all types of industries. This enzyme is used in dextrose production, in the baking industry, in the brewing of low-calorie beer and in whole grain hydrolysis for the alcohol industry (Selvakumar et al., 1994).

MATERIALS AND METHODS

The wheat bran obtained from the local market of Rawalpindi is utilized as a substrate for glucoamylase production through solid state fermentation. Substrate was dried, ground to powder form (40 mm mesh) and stored in plastic jar. Wheat bran was subjected to proximate analysis by using standard method on dry matter basis

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Table 1. Compositions of growth medium of *Aspergillus niger*.

S/N	Ingredients	Quantity(g/100 ml)
1	Potato starch	2
2	Dextrose	2
3	Agar	2
4	Urea	0.3
5	$MgSO_4$ $7H_2O$	0.050
6	KCl	0.015
7	KH_2PO_4	0.008
8	$ZnSO_4.7H2O$	0.001
9	Distilled water	Up to 100 ml mark

(AACC, 2000).

All experiments were performed in triplicate flasks containing 70% moistened substrate. The growth medium (Table 1) was autoclaved for 15 min. After cooling the flasks, inoculum (5 ml) was added to each flask in the laminar air flow with the help of sterilized disposable syringe and flasks was incubated at 37°C for fermentation under still culture conditions. Glucoamylase was extracted from the fermented biomass by a simple contact method (Krishna and Chandrasekaran, 1996).

Optimization of conditions

Solid state fermentation process was optimized by studying the effects of varying fermentation period, moisture levels, inoculum size and varying concentration of peptone as additional nitrogen source. Yeast was extracted with Tween-80 (a surfactant) in triplicate flasks. The strategy was to maintain the previously optimized parameters and maintain them in the subsequent investigations (Krishna and Chandrasekaran, 1996).

Strain improvement techniques

Chemical mutagenesis was induced in wild type *A. niger* as mutagen by using ethidium bromide as mutagen by the following procedure.

Preparation of ethidium bromide stock solutions

Preparation of a stock solution was carried out by using 0.50 mg/ml of ethidium bromide and an addition of 1.0 ml of ethidium bromide (EB) stock solution into 9 ml of Vogel's media to make a volume of 10 ml containing spores of *A. niger* (1×10^8 spores/ml). After particular intervals of time such as 30.0, 60.0, 90.0, 120.0, 150.0 and 180.0 min of incubation, its centrifugation is carried out three times at about 10,000 revolutions per minute for 15 min. A dose (after 120 min) producing 76% kill, was said to be the best for further experiments.

Treatment of spores with ethidium bromide

After the treatment of spores with four concentrations of mutagens, 100 fold serials dilution of spores with mutation (each of mutant culture is treated) were arranged to give more or less 30 colonies per plate. In a dim room, the dilution of spores (0.10 ml) and spreading of spores on a Potato dextrose agar (PDA) medium with 1% ox-gall as a restrictor of colony. Spores having no mutation were also plated as a control. All treatments were performed with specific sterilized conditions in a laminar air flow (LFC). Then plates were enclosed with aluminium foil and placed in incubator at 37°C for 3 to 7 days or till colony formation. More than ten colonies were screened for the selection of desire mutant with high enzyme activity (Khattab and Bazaraa, 2005).

Selection of colony restrictor

In order to restrict fungal spores for the selection of mutants triton, X-100 (2% v/v) were used (Khattab and Bazaraa, 2005).

Enzyme purification

Glucoamylase was purified from crude enzyme extracts, by using the following comprehensive and important purification techniques (Khattab and Bazaraa, 2005).

1. Ammonium sulphate precipitation.
2. Gel filtration.
3. SDS polyacrylamide gel electrophoresis (SDS PAGE).

SDS poly acrylamide gel electrophoresis

For further purification, the sample obtained from dialysis and gel filtration was applied to poly acrylamide gel electrophoresis.

Sample preparation

Distilled water was added to sample containing 100 µg of protein added to bring volume up to 0.2 ml. TCA was added and incubated in ice for 10 min and centrifuged for 5 min. The sample was washed with 100 µl acetone and supernatant was removed and air dried the pellet and resuspended in 10 µl distilled water. Sample buffer was added to 10 µl of sample, 5 µl sample buffer was added and incubated in boiling water for 5 min and then spin quickly to collect the sample.

Characterization of glucoamylase

The purified glucoamylase was subjected to characterization through kinetic studies by studying the following:

1. Effect of pH on glucoamylase
2. Effect of temperature
3. Effect of substrate concentration, determination of K_m and V_{max}.

Protein estimation

The sample protein was estimated by Biuret method (Gornall et al., 1949).

Glucoamylase assay

Glucoamylase was extracted from the fermented biomass by a simple method proposed by Krishna and Chandrasekaran (1996).

$$\text{Enzyme activity} = \frac{\text{Absorbance of enzyme solution}}{\text{Time of incubation}} \times \text{Standard factor (IU/ml/min)}$$

Statistical analysis

All the data thus obtained were analyzed statistically by using analysis of variance (ANOVA) under the complete randomized design (CRD). The mean enzymatic activities under different treatments were compared by Duncan s Multiple Range Test (Steel et al., 1996).

RESULTS AND DISCUSSION

The proximate analysis (Table 5) highlighted that higher percentage (91.4 ± 0.41%) of dry weight of wheat bran and relatively low value of protein content (16.70 ± 0.04%), moisture content (8.6 ± 0.21%), ash content (18 ± 0.06%), oil content (6.9 ± 0.035%) and crude fibre (10.38 ± 0.05%) were analyzed (AACC, 2000).

Wheat bran's carbohydrate values were determined after much identification tests for carbohydrates. The proximate analysis highlighted the presence of nitrogen in the form of protein that enhances the utilization of glucoamylase from *A. niger* (Liu, 2001; Steel et al., 1996).

The proximate analysis of wheat bran also indicated its great potential for cultural growth of many fungi having well balanced ingredients including starch, nitrogen, fibre and some inorganic elements (Kulp et al., 1980). This makes wheat bran as one of the best substrate for fungal culture, especially *A. niger* and produce many enzymes including glucoamylase (Pandey and Radhakrishnan, 2002).

The *A. niger* provided the glucoamylase activity 1.345 ± 0.009 IU/ml/min for wild and 1.944 ± 0.009 IU/ml/min for mutant *A. niger* at 6 g substrate and the second at 10 g of substrate level. The results were highly significant with coefficient of variance 1.66% for wild and 2.62% for mutant *A. niger* glucoamylase activity (Pandey and Radhakrishnan, 2002). But the results of least significant difference (LSD) and Duncan's multiple range test (DMRT) showed that there was a significant difference among the treatments. This indicated that wheat bran has excellent ingredients for the growth of *A. niger*. Significant differences existed for the production of glucoamylase for both (Figure 2) wild and mutant *A. niger* at various substrate levels (Pandey and Radhakrishnan, 2002).

A. niger had high glucoamylase activity 1.147 ± 0.021 IU/ml/min for wild and 2.163 ± 0.053 IU/ml/min for mutant species at 96 h of incubation and the second at 120 h of incubation (Mehboob et al., 2011).

The results (Figure 3) were highly significant with coefficient of variance less than 10% for mutant *A. niger* glucoamylase activity (Biesebeke et al., 2005). This study showed that maximum activity for glucoamylase was achieved after the duration of four days (96 h). The *A. niger* in terms of glucoamylase activity was lower 1.333 ± 0.344 IU/ml/min for wild and relatively higher 2.135 ± 0.718 IU/ml/min for mutant fungus at 70% of moisture level (Figure 4). At 0 and 30%, *A. niger* had a low glucoamylase production as compared to other treatments as shown in Figure 4. Water serves as a good transport for various substrates and served as a best reactant, so it is confirmed that the level of water affects glucoamylase production during SSF (Pandey and Radhakrishnan, 2002).

Glucoamylase activity was shown by wild and mutant *A. niger* as 1.413 ± 0.005 IU/ml/min and 1.987 ± 0.007 IU/ml/min at 5 ml of inoculum level (Figure 5). The results were highly significant with coefficient of variance less than 5% for wild and mutant *A. niger* for glucoamylase activity (Pandey and Radhakrishnan, 2002). Glucoamylase optimum activity was greatly enhanced by *A. niger* at 5 ml inoculum level (Table 2) (Wang et al., 2006).

The *A. niger* glucoamylase activity 1.717 ± 0.015 IU/ml/min for wild was relatively lower than mutant fungus2.385 ± 0.006 IU/ml at 0.3% of peptone level and the second at 0.4% peptone level (Figure 6). At 0 and 0.1% of peptone level, *A. niger* had low glucoamylase production as shown in Figure 6. The glucoamylase production by *A. niger* was enormously increased with nitrogenous source like peptone (Pandey et al., 1994).

The *A. niger* having glucoamylase activity 1.222 ± 0.009 IU/ml/min was slightly less than mutant fungus 2.221 ± 0.003 IU/ml/min at 0.3% of yeast extract level. At 0 and 0.1% of yeast extract level, glucoamylase production by *A. niger* was greatly reduced as shown in Figure 7 (Ellaiah et al., 2002). Glucoamylase activity optimum value for *A. niger* was observed at 0.3% of yeast extract level. The glucoamylase production was not enormously increased with nitrogenous source like yeast as compared to peptone (Mehboob et al., 2011; Joshi et al., 1999).

The glucoamylase activity was shown by *A. niger* 1.791 ± 0.007 IU/ml/min for wild and 2.236 ± 0.008 IU/ml/min for mutant species at 4 ml of Tween-80 level (Figure 8). The results were highly significant with coefficient of variance less than 5% for both wild and mutant *A. niger* for glucoamylase activity (Raimbault and Alazard, 1980).

The sample was also run in duplicate and the absorbance was recorded at 540 nm (Table 4).

Table 2. Composition of inoculum medium for *A. niger.*

S/N	Ingredients	Quantity(g/100 ml)
1	Dextrose	2
2	Urea	0.30
3	$MgSO_4$ $7H_2O$	0.05
4	KCl	0.015
5	KH_2 PO_4	0.008
6	$ZnSO_4.7H2O$	0.001
7	Distilled water	Up to 100 ml mark

Table 3. Biuret-protein assay table.

S/N	Distilled water (ml)	Volume of protein standard (2 mg/ml) in ml	Enzyme sample (ml)	Biuret reagent (ml)	Total volume (ml)	Protein concentration (mg/ml)	Absorbance at 540 nm
1*	0.5	-	-	1	1.5	0.0	0.000
2	0.4	0.1	-	1	1.5	0.4	0.018
3	0.3	0.2	-	1	1.5	0.8	0.040
4	0.2	0.3	-	1	1.5	1.2	0.075
5	0.1	0.4	-	1	1.5	1.6	0.093
6	-	0.5	-	1	1.5	2.0	0.128
7†	-	-	0.5	1	1.5	10.73	0.356
8†	-	-	0.5	1	1.5	10.83	0.353

*, Blank; †, our enzyme sample; -, zero.

Table 4. Absorbance values of known concentration of glucose at 550 nm.

S/N	Glucose concentration (µM/ml)	Absorbance	Standard factor
1	0.5	0.122	4.09
2	1	0.244	4.10
3	1.5	0.355	4.23
4	2	0.488	4.10
5	2.5	0.605	4.10
6	3	0.737	4.07
7	3.5	0.84	4.17
8	4	0.975	4.10
9	4.5	1.101	4.09
10	5	1.209	4.14

Table 5. Proximate analysis of wheat bran.

Protein content (%)	Moisture content (%)	Dry weight (%)	Ash content (%)	Oil content (%)	Crude fibre (%)
16.70 ± 0.04	8.6 ± 0.21	91.4 ± 0.41	4.2 ± 0.06	6.9 ± 0.035	10.4 ± 0.05

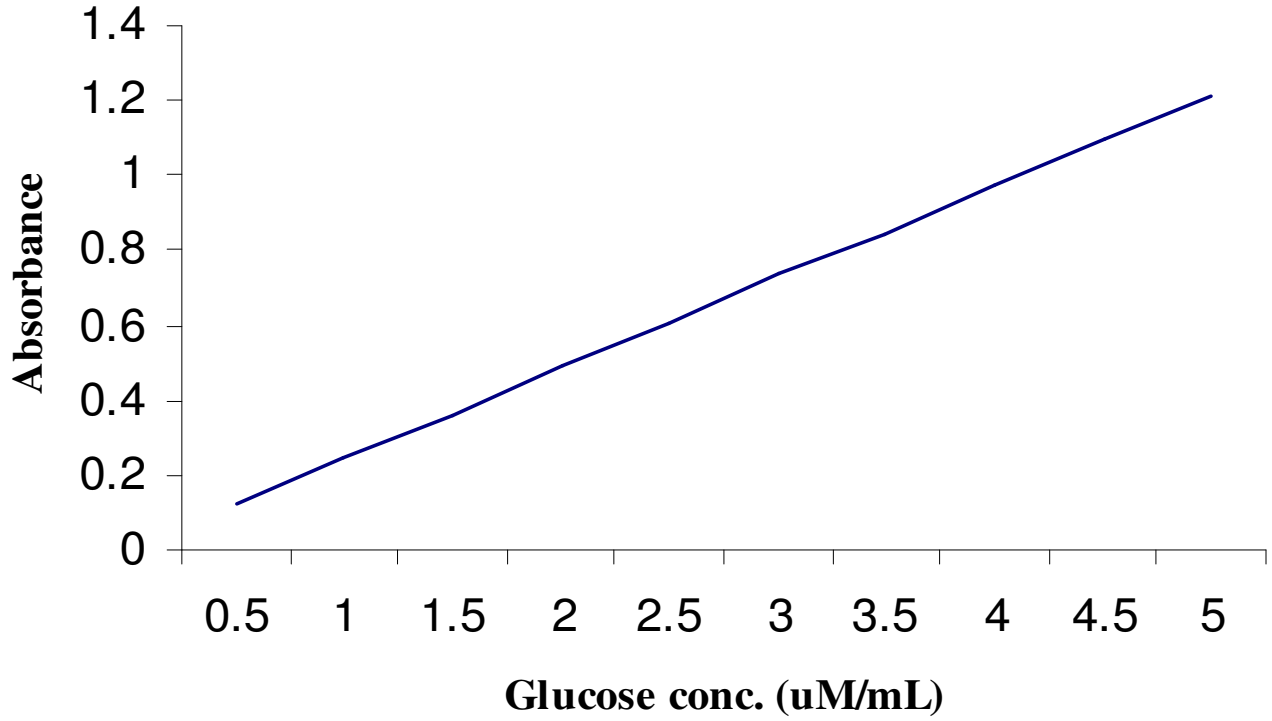

Figure 1. Standard curve for the known concentration of glucose at 550 nm.

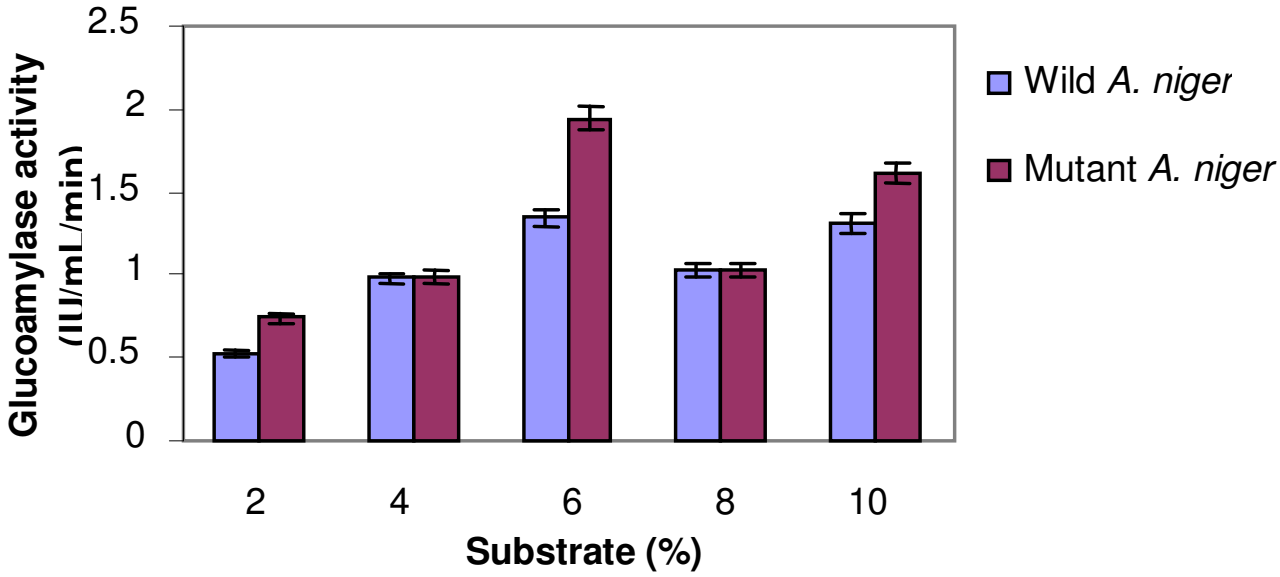

Figure 2. Activity of glucoamylase produced by wild and mutant *A. niger* with varying substrate level.

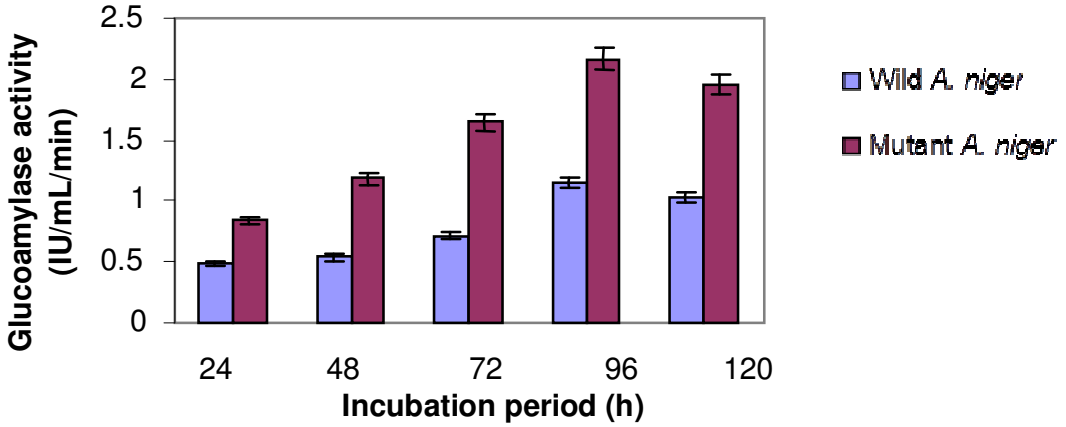

Figure 3. Activity of glucoamylase produced by wild and mutant *A. niger* with varying incubation periods.

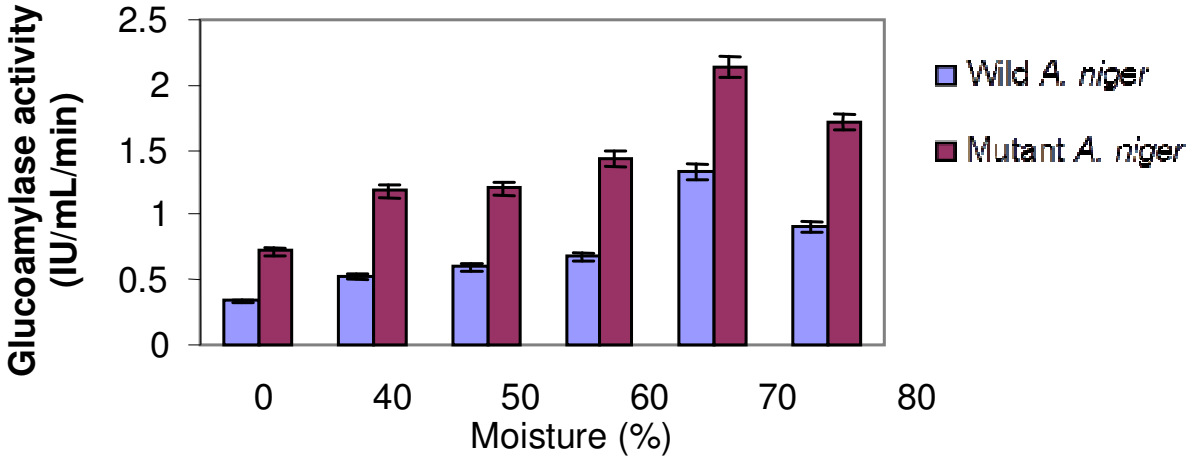

Figure 4. Activity of glucoamylase produced by wild and mutant A. *niger* with varying moisture levels.

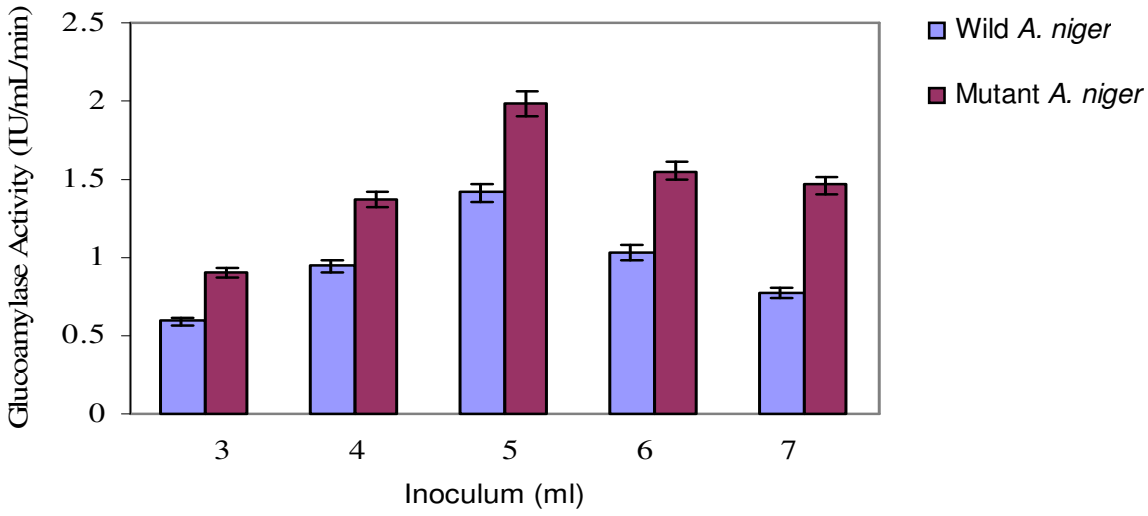

Figure 5. Activity of glucoamylase produced by wild and mutant *A. niger* with varying inoculums' levels.

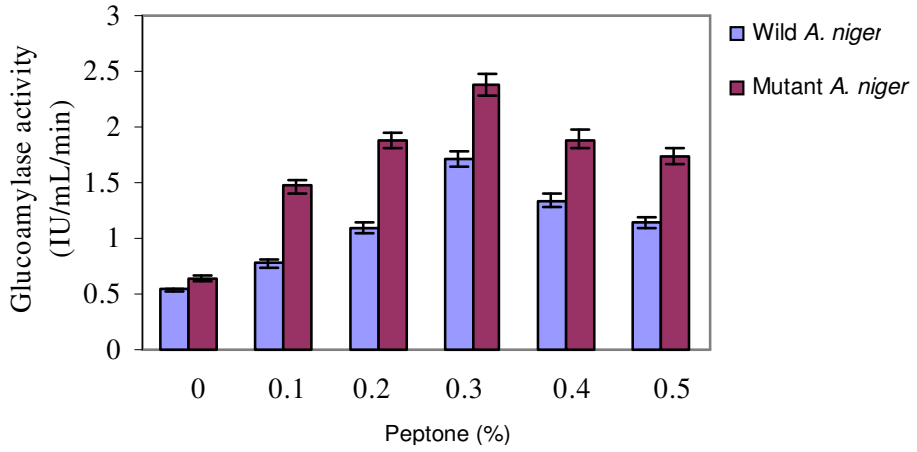

Figure 6. Activity of glucoamylase produced by wild and mutant *A. niger* with varying levels of peptone.

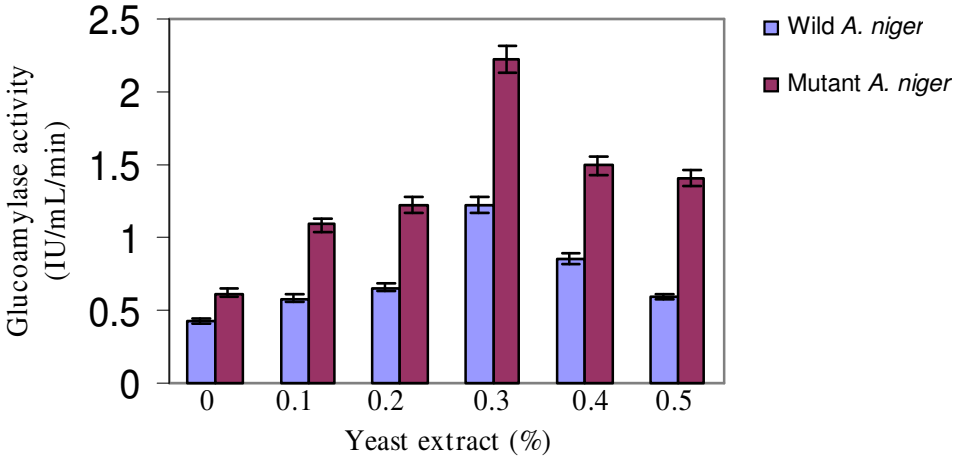

Figure 7. Activity of glucoamylase produced by wild and mutant *A. niger* with varying levels of yeast extract.

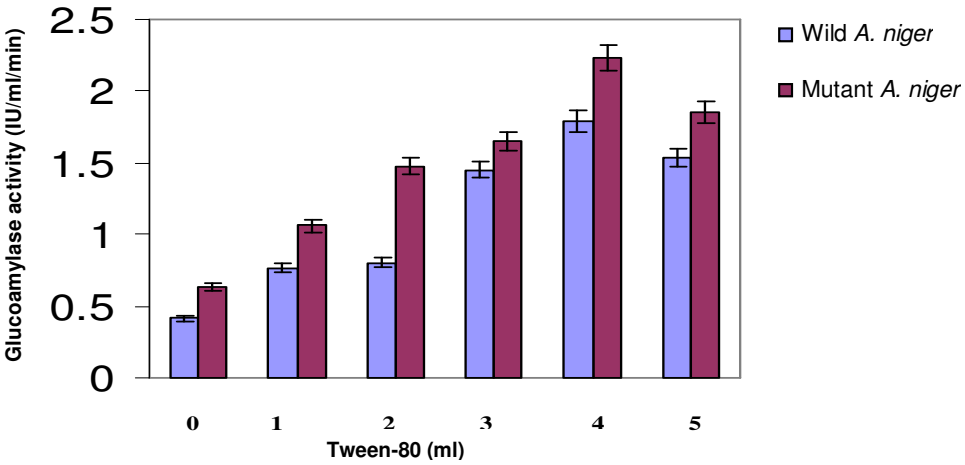

Figure 8. Activity of glucoamylase produced by wild and mutant *A. niger* with varying levels of Tween-80.

Table 6. Gel filtration fraction.

Gel filtration fractions	Glucoamylase activity (IU/ml/min) Wild *A. niger*	Glucoamylase activity (IU/ml/min) Mutant *A. niger*
I	0.132	2.632
II	2.06	2.06
III	1.48	2.48
IV	0.640	2.14
V	2.236	0.236
VI	2.850	2.980
VII	1.632	0.432
VIII	0.160	2.16
IX	0.48	2.48

Table 7. Activity of glucoamylase production by wild and mutant *A. niger* for all optimized conditions.

Glucoamylase activity (IU/ml/min) (wild *A. niger*)	Glucoamylase activity (IU/ml/min) (mutant *A. niger*)
2.085 ± 0.021	3.185 ± 0.020

Table 8. Purification.

	Volume (ml)	Volumetric activity (U/ml/min)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor
Crude enzyme	100	3.185	318.5	894.66	0.356	-
AS purified enzyme	100	2.067	206.7	58.33	1.52	4.88
Gel filtration	50	1.867	93.35	10.70	8.72	5.73

AS: Ammonium sulphate.

Glucoamylase activity increased tremendously for both wild *A. niger* (2.085 ± 0.021 IU/ml/min) and mutant *A. niger* (3.185 ± 0.020 IU/ml/min) at all preoptimized conditions (Table 7). The comparison of both wild and mutant fungus showed that mutant *A. niger* had a greater potential for glucoamylase production than wild species (Pandey and Radhakrishnan, 2002).

Ammonium sulphate is used to find out preferentially, because it is soluble in water and high ionic strength is attained. In this study, maximum glucoamylase activity was obtained with 60% of ammonium sulphate $(NH_4)_2SO_4$ concentration and the enzyme activity was found to be 1.52 U/mg, which were 4.88 folds more activity than the activity obtained without ammonium sulphate purification (0.356 U/mg). The specific activity of the crude also increased from 0.356 U/mg to 1.52 U/mg (Table 8) when purified (Aquino et al., 1990).

After ammonium sulphate purification, gel filtration is carried out to purify more glucoamylase. In this study, maximum glucoamylase activity was obtained with 0.1 M sodium concentration buffer and the enzyme activity was found to be 8.72 U/mg, which were 5.73 folds more active than the activity obtained without gel filtration (1.52 U/mg). The specific activity of the crude also increased from 1.52 to 8.72 U/mg (Table 8) when purified. Filtration by method gave good results (Table 8) for enzyme purification (Nahas and Waldermarin, 2002).

Protein estimation

Protein was estimated using protein-biuret assay. For this purpose, different concentrations of bovine serum albumin (BSA) were prepared and were run on the spectrophotometer to take the absorbance (Table 3). A standard curve of the protein was prepared from the absorbance shown by standard solutions of BSA (Figure 1).

Regression equation was used to calculate the protein of the unknown sample and their mean was taken (Figure 9). Protein of the crude sample as well as ammonium sulphate partially purified glucoamylase was also determined (Aguero et al., 1990).

The average protein of the crude protein became 9.185 mg/ml and that of the ammonium sulphate partially purified

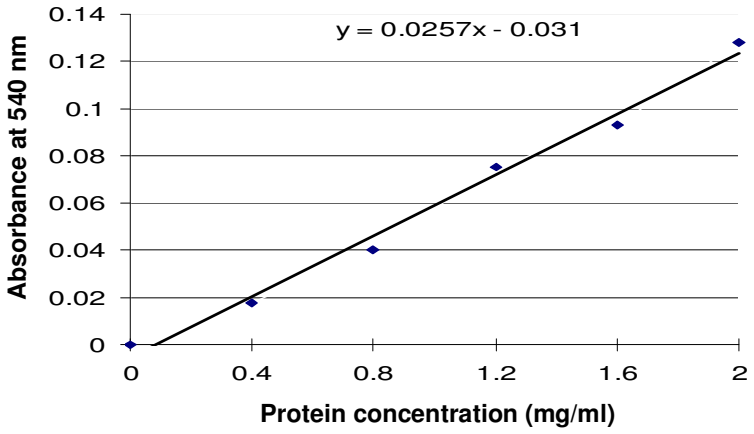

Figure 9. Protein concentration determination at 540 nm.

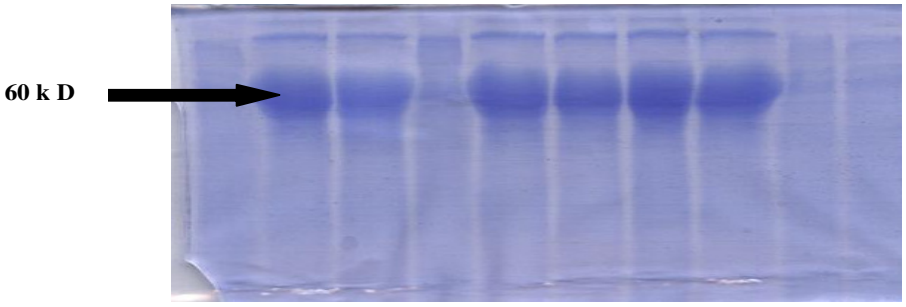

Figure 10. SDS PAGE characterization of glucoamylase from wild *A. niger*. M: Marker for glucoamylase (Novozyme 60 kD); Lane II, V, VI and VII: Glucoamylase band present; Lane I, IV, XIII and IX: Glucoamylase band is absent.

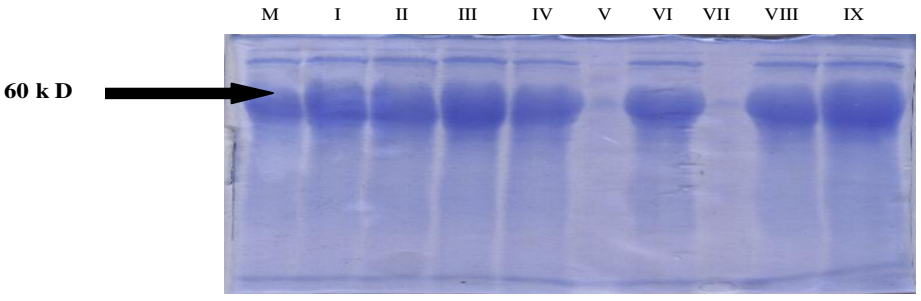

Figure 11. SDS PAGE characterization of glucoamylase from mutant *A. niger*. M: Glucoamylase marker (Novozyme 60 kD); Lane I, II, III, IV, VI, VIII and IX: Glucoamylase band is present; Lane V and VII: Glucoamylase band is absent.

glucoamylase became 8.567 mg/ml and after gel filtration, its value was 5.867 mg/ml of protein (Table 8) in the purified sample (Nahas and Waldermarin, 2002).

SDS poly acrylamide gel electrophoresis

For further purification, the sample obtained from dialysing tube and gel filtration was applied to poly acrylamide gel electrophoresis. Figure 11 showed that all fractions (II, V, VI and VII) had a clear band of glucoamylase enzyme having weight 60 kD for wild *A. niger*. The band comparison with marker of glucoamylase (60 kD) (novozyme) indicated glucoamylase which was the confirmation of its purification (Liu, 2001) (Figure 10).

The fractions (I, IV, XIII and IX) did not contain any band

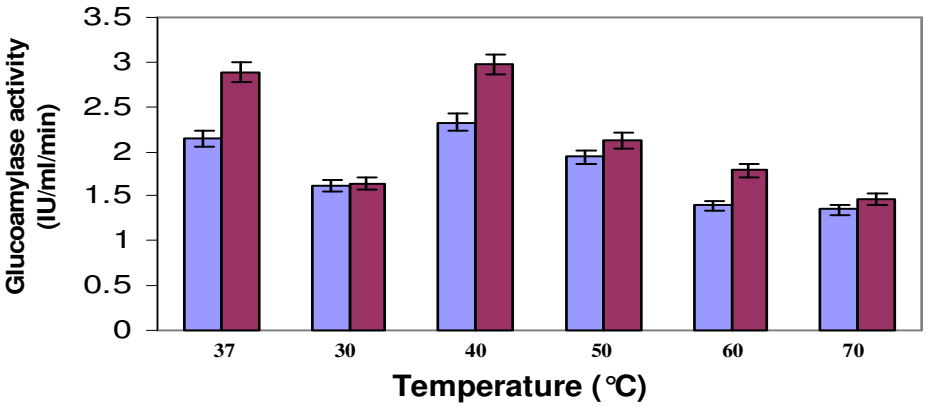

Figure 12. Characterization of glucoamylase produced by wild and mutant *A. niger* with varying incubation temperatures.

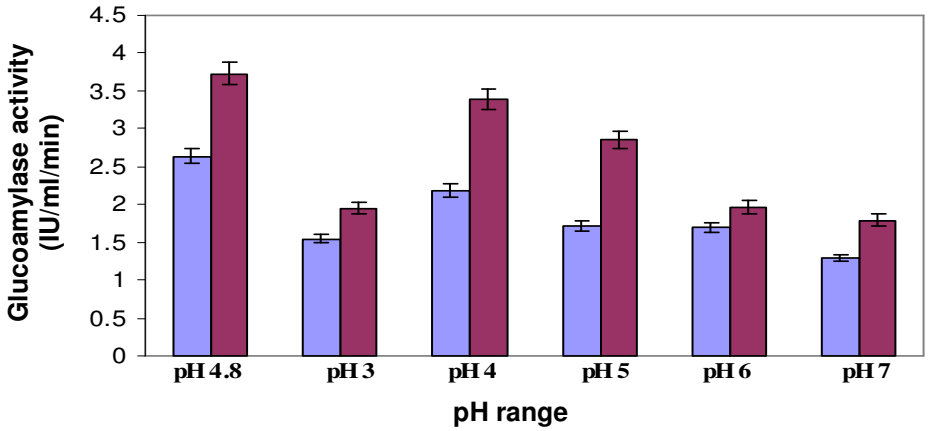

Figure 13. Characterization of glucoamylase produced by mutant *A. niger* with varying pH level.

band which indicated the absence of glucoamylase. This purification was carried out by wild type *A. niger* which showed less glucoamylase activity as compared to the mutant type of *A. niger*. The fractions after gel filtration were very sucessful (Table 6). The results from Figure 11 show that all fractions (I, II, III, IV, VI, VIII and IX) contained a clear band of glucoamylase enzyme having weight 60 kD for mutant *A. niger*. The fractions (V and VII) did not contain any band which indicated the absence of glucoamylase. The comparison of both fungi indicates that mutant *A. niger* had more purification as compared to wild type (Liu, 2001).

Characterization of glucoamylase

Incubation temperatures

There was comparison of wild and mutant *A. niger* for the glucoamylase production and characterization of glucoamylase activity under different levels of incubation period (Figure 12). The glucoamylase activity was increased to 2.326 ± 0.008 IU/ml/min for wild and 2.975 ± 0.005 IU/ml/min for mutant *A. niger* after 40°C of incubation. At 60 and 70°C of incubation, *A. niger* activity was decreased considerably (Figure 12). Significant differences existed for the production of glucoamylase for both (Figure 12) wild and mutant *A. niger* (Joshi, et al., 1999; Wang et al., 2006).

There was also comparison of wild and mutant *A. niger* for the characterization of glucoamylase activity under different levels of incubation period (Figure 13). The glucoamylase had its high activity 2.185 ± 0.021 IU/ml/min for wild and 3.385 ± 0.020 IU/ml/min for mutant at pH 4. The results were highly significant with coefficient of variance less than 5% for mutant *A. niger* glucoamylase activity (Ellaiah et al., 2002). Results from DMRT showed that there was a significant difference

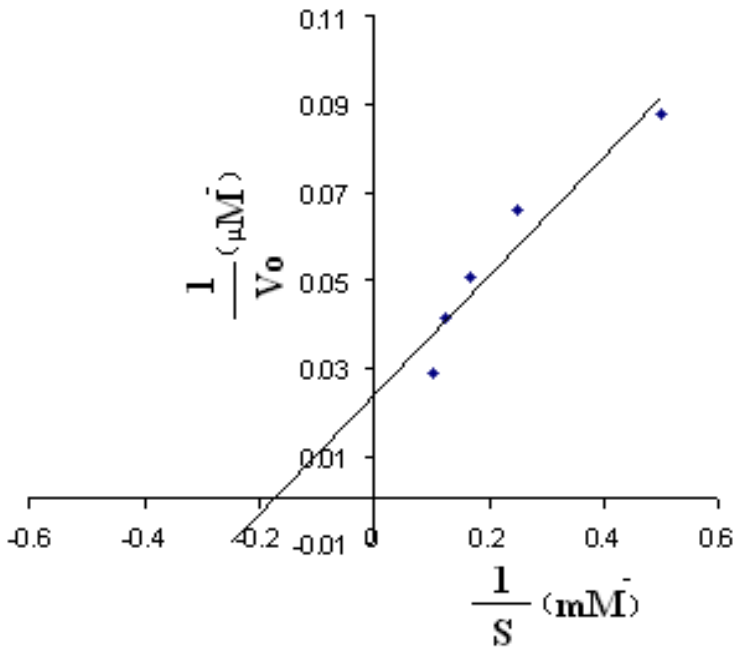

Figure 14. V_{max} and K_m for glucoamylase.

among the treatments. The drastic decrease of glucoamylase activity at pH shows that enzyme was denatured at this pH (Nahas and Waldermarin, 2002).

Substrate concentration plays an important role in the enzyme activity. Lower concentration may cause a lower activity. Therefore, the determination of K_m and Maximum velocity (V_{max}) is an important step while characterizing an enzyme. An initial increase in the enzyme activity was observed with slight increase in the substrate concentration and then, the increase in the glucoamylase activity (Figure 14). Maximum velocity (V_{max}) observed from the graph was 40.12 µM and K_m was 4.31 mM. Maximum velocity (V_{max}) can be measured, but cannot be achieved in reality and K_m is an opposite to measure the attraction or power of binding between the enzyme and its substrate. So the lower concentration of substrate indicated that glucoamylase had a strong affinity for substrate (Wang et al., 2006).

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