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GLUCOAMYLASE PRODUCTION FROM *ASPERGILLUS NIGER* BY USING SOLID STATE FERMENTATION PROCESS

MUHAMMAD IMRAN[1], MUHAMMAD JAVAID ASAD[1], MUHAMMAD GULFRAZ[1], RAHMATULLAH QURESHI[2,*] HINA GUL[1], NAZISH MANZOOR[1] AND ARSHAD NAWAZ CHOUDHARY[3]

[1]*Department of Biochemistry, Pir Mehr Ali Shah Arid Agriculture University Murree Road Rawalpindi, Pakistan*
[2]*Department of Botany, Pir Mehr Ali Shah Arid Agriculture University Murree Road Rawalpindi, Pakistan*
[2]*Department of Soil Sciences, Pir Mehr Ali Shah Arid Agriculture University Murree Road Rawalpindi, Pakistan*
[*]*Corresponding author's e-mail: rahmatullahq@yahoo.com*

Abstract

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 (3.185±0.020 IU/mL/min.) by mutant *Aspergillus niger* and (2.085±0.021 IU/mL/min.) for *wild Aspergillus niger* was recorded in the culture filtration after 96 hours of Solid State Fermentation of growth medium with 70% moisture level and in 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 *Aspergillus niger* was 2.850 IU/mL/min and for mutant *Aspergillus niger* was 2.980 IU/mL/min. Purification through the SDS PAGE revealed the indication of glucoamylase purification from *Aspergillus niger*. The high value of Km shows that substrate had great affinity for glucoamylase. Glucoamylase enzyme has many useful applications in food processing industry and fermentation biotechnology.

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 (1-4) faster than α (1-6), α (1-3) linkages and produce producing D glucose in successive manner (Fogarty, 1983). This enzyme is 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 (Raza *et al*., 2011; Malik *et al*., 2011b).

The extensive utilization of this glucoamylase enzyme is obtained by using a fungus, *Aspergillus niger* in enzyme production industry (Malik *et al.,* 2011a; Malik *et al.,* 2011b). 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). Keeping its wider use, present study was designed to produce glucoamylase under optimum conditions and the effect of chemical mutagenesis on *Aspergillus niger* for the production of glucoamylase.

Materials and Methods

The wheat bran obtained from the local market of Rawalpindi 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 (Anon., 2000). All experiments were performed in triplicate flasks containing 70% moistened substrate. The growth medium was autoclaved for 15 minutes. 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 & Chandrasekaran, 1996).

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

Strain improvement techniques: Chemical mutagenesis was induced in wild type *Aspergillus 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 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 *Aspergillus niger* (1x 10^8 spores/mL). After

particular intervals of time such as 30.0, 60.0, 90.0, 120.0, 150.0, and 180.0 minutes of incubation, its centrifugation is carried out three times at about 10,000 revolution 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 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 aluminum foil and placed in incubator at 37ºC for 3-7 days or till colony formation. More than ten colonies were screened for the selection of desire mutant with high enzyme activity (Khattab & 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 & Bazaraa, 2005).

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

i) Ammonium Sulphate Precipitation.
ii) Gel Filtration
iii) 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 minutes. The sample was washed with 100 µL acetone and supernatant was removed and air dried the pellet and re-suspended 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 minutes 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:

i) Effect of pH on glucoamylase
ii) Effect of temperature
iii) Effect of substrate concentration, determination of Km and Vmax.

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 & Chandrasekaran (1996).

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

Statistical analysis: All the data were 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 Discussions

The proximate analysis (Table 1) 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%) analyzed by Anon., (2000).

Wheat bran's carbohydrate values determined after much identification tests for carbohydrates (Table 2, Fig. 1). Wheat bran gives positive test even in very small amount. Both tests used to identify carbohydrates (Molisch, Iodine and Barfoed's) are positive. Wheat bran has a large amount of starch which is a rich source of carbon for *Aspergillus niger* to grow on it for glucoamylase production. The proximate analysis highlighted the presence of nitrogen in the form of protein enhances the utilization of glucoamylase from *Aspergillus niger* (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 *Aspergillus niger* and produce many enzymes including glucoamylase (Pandey & Radhakrishnan, 2002).

The *Aspergillus niger* provided the glucoamylase activity 1.345±0.009 IU/mL/min for wild and 1.944±0.009 IU/mL/min for mutant *Aspergillus 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 *Aspergillus niger* glucoamylase activity (Pandey & Radhakrishnan, 2002). But the results of LSD and DMRT showed that there was a significant difference among the treatments. This indicated that wheat bran has excellent ingredients for the growth of *Aspergillus niger*. The significant differences existed for the production of glucoamylase for both (Fig. 2) in wild and mutant *Aspergillus niger* at various substrate levels (Pandey & Radhakrishnan, 2002).

Table 1. 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

Table 2. Absorbance values various concentration of glucose at 550 nm.

S. No.	Glucose conc. (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

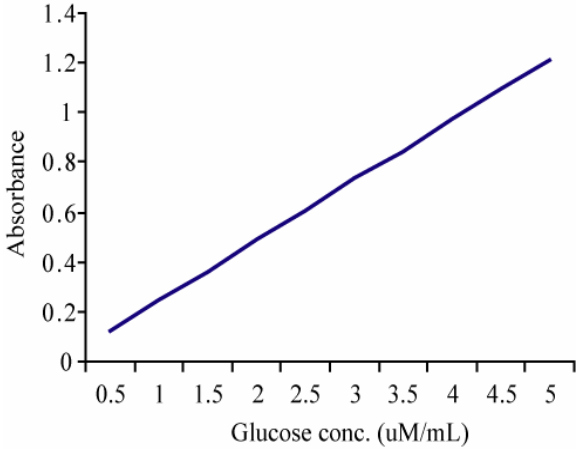

Fig. 1. Standard curve for the known concentration of glucose at 550 nm.

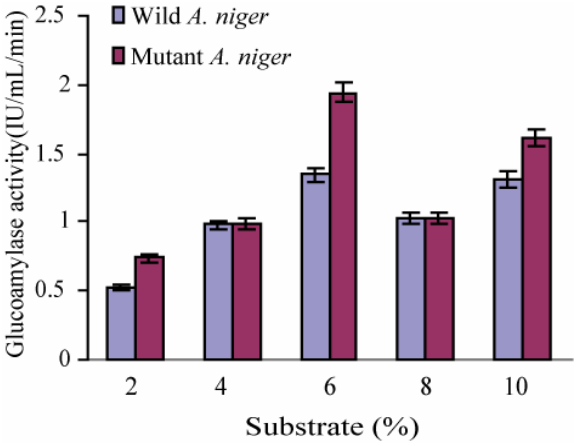

Fig. 2. Activity of Glucoamylase produced by wild & mutant *Aspergillus niger* with varying substrate level.

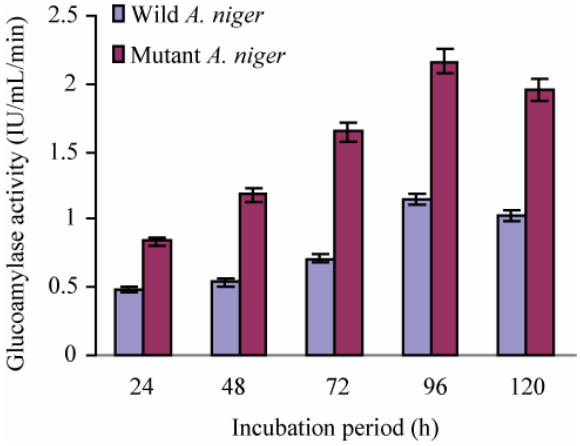

Fig. 3. Activity of Glucoamylase produced by wild & mutant *Aspergillus niger* with varying incubation periods.

The results (Fig. 3) were highly significant with coefficient of variance less than 10% for mutant *Aspergillus niger* glucoamylase activity (Biesebeke *et al.,* 2005). This study showed that maximum activity for glucoamylase was achieved after the duration of four days (96 hours). The *Aspergillus niger* in terms of glucoamylase activity which is 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 (Fig. 4). At 0 and 30%, *Aspergillus niger* had a low glucoamylase production as compared to other treatments as shown in the Figure 2. Water serves as a good transport for various substrates and served as a best reactant so it is confirmed that level of water effects glucoamylase production during SSF (Pandey & Radhakrishnan, 2002).

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

The *Aspergillus 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 hours of incubating and the second at 120 hours of incubation (Table 3).

The *Aspergillus niger* glucoamylase activity 1.717±0.015 IU/mL/min for wild which was relatively lower than mutant fungus 2.385±0.006 IU/mL at 0.3% of peptone level and the second at 0.4% peptone level (Fig. 6). At 0 and 0.1% of peptone level, *Aspergillus niger* had low glucoamylase production as shown in the Figure 6. The glucoamylase production by *Aspergillus niger* was enormously increased with nitrogenous source like peptone (Pandey *et al*., 1994).

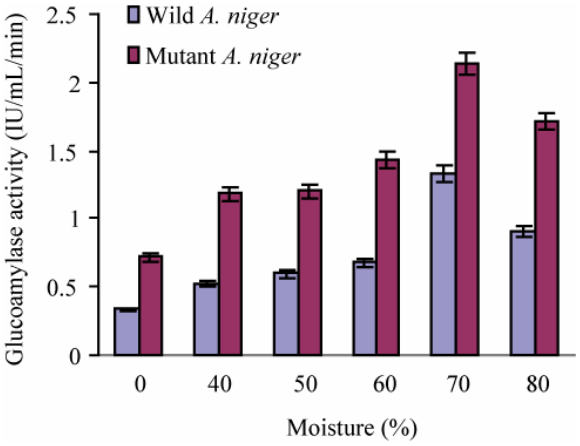

Fig. 4. Activity of Glucoamylase produced by wild & mutant *Aspergillus niger* with varying moisture levels.

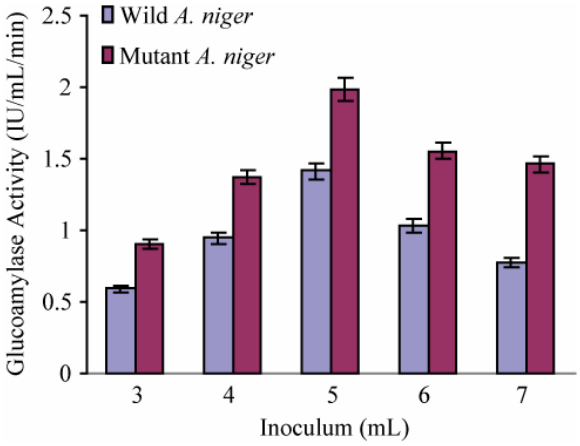

Fig. 5. Activity of Glucoamylase produced by wild & mutant *Aspergillus niger* with varying Inoculum's levels.

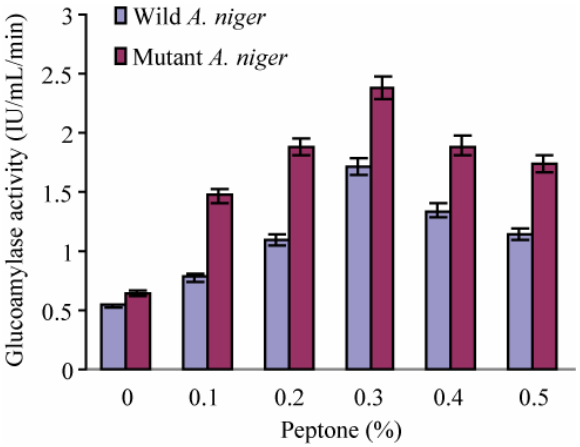

Fig. 6. Activity of Glucoamylase produced by wild and mutant *Aspergillus niger* with varying levels of peptone.

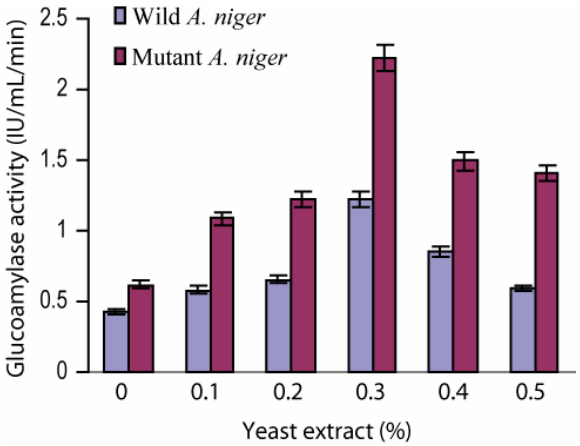

Fig. 7. Activity of Glucoamylase produced by wild & mutant *Aspergillus niger* with varying levels of yeast extract.

The *Aspergillus niger* had 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 *Aspergillus niger* was greatly reduced (Fig. 7) (Ellaiah *et al*., 2002). Glucoamylase activity optimum value for *Aspergillus 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 (Pandey *et al.,* 1994).

The glucoamylase activity was shown by *Aspergillus 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 (Fig. 8). The results were highly significant with coefficient of variance less than 5% for both wild & mutant *Aspergillus niger* for glucoamylase activity (Raimbault & Alazard, 1980).

The sample was also run in duplicate and the absorbance was recorded at 540 nm. Glucoamylase activity was increased tremendously for both wild *Aspergillus niger* (2.085±0.021 IU/mL/min) and mutant *Aspergillus niger* (3.185±0.020 IU/mL/min) at all preoptimized conditions. The comparison of both wild and mutant fungus showed that mutant *Aspergillus niger* had a greater potential for glucoamylase production than wild species (Pandey & 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 1.52U/mg when (Table 4) purified (Aquino *et al*., 2001).

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 activity than the activity obtained without gel filtration (1.52 U/mg). The specific activity of the crude also increased from 1.52 U/mg to 8.72 U/mg (Fig. 8) when purified. Filtration by method gave good results for enzyme purification (Nahas & Waldermarin, 2002).

Table 3. Activity of Glucoamylase production by wild & mutant *Aspergillus niger* for all optimized conditions.

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

Table 4. Purification table.

	Vol. (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	----
A.S. 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

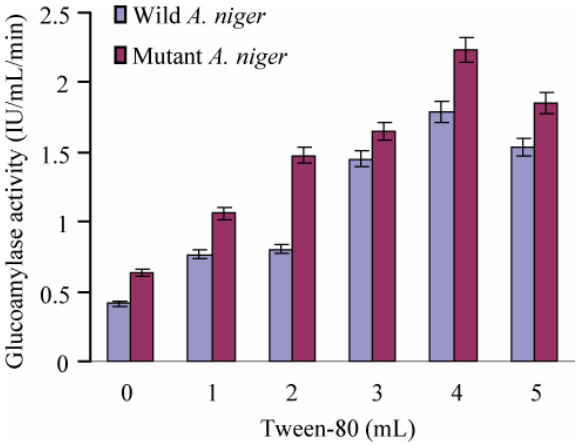

Fig. 8. Activity of Glucoamylase produced by wild & mutant *Aspergillus niger* with varying levels of tween-80.

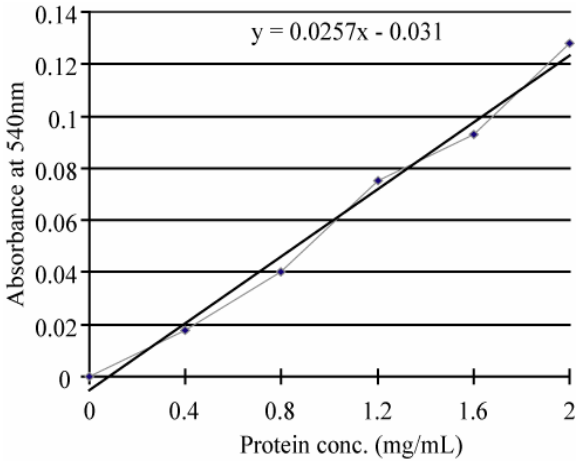

Fig. 9. Protein concentration determination at 540nm.

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 5), a standard curve of the protein was prepared from the absorbance shown by standard solutions of BSA (Fig. 9).

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

The average protein of the crude protein became to be 9.185 mg/mL and that of the ammonium sulphate partially purified glucoamylase became to be 8.567 mg/mL and after gel filtration its value is 5.867 mg/mL per mL of protein **(**Table 8) in the purified sample (Nahas & Waldermarin, 2002).

SDS poly acrylamide gel electrophoresis: For further purification, the sample obtained from dialyzing tube and gel filtration was applied to Poly Acrylamide Gel Electrophoresis. Figure 10 showed that all fractions (II, V, VI and VII) had a clear band of glucoamylase enzyme having weight 60 k D for wild *Aspergillus niger*. The band comparison with marker of glucoamylase (60 kD) (novozyme) indicated glucoamylase which was the confirmation of its purification (Aquino *et al*., 2001).

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

Characterization of glucoamylase incubation temperatures: The comparison of wild and mutant *Aspergillus niger* for the glucoamylase production and characterization of glucoamylase activity under different levels of incubation period (Fig. 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 *Aspergillus niger* after 40°C of incubation. At 60 and 70°C of incubation, *Aspergillus niger* activity was decreased considerably (Fig. 12). The significant differences existed for the production of glucoamylase for both (Fig. 12) in wild and mutant *Aspergillus niger* (Wang *et al*., 2006).

Table 5. Biuret-protein assay.

S. No.	Dist. water (mL)	Volume of protein standard (2mg/mL) in mL	Enzyme sample (mL)	Biuret reagent (mL)	Total volume (mL)	Protein conc. (mg/mL)	Absorbance At 540nm
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

Key: *Blank, † Our enzyme sample, - Zero mL

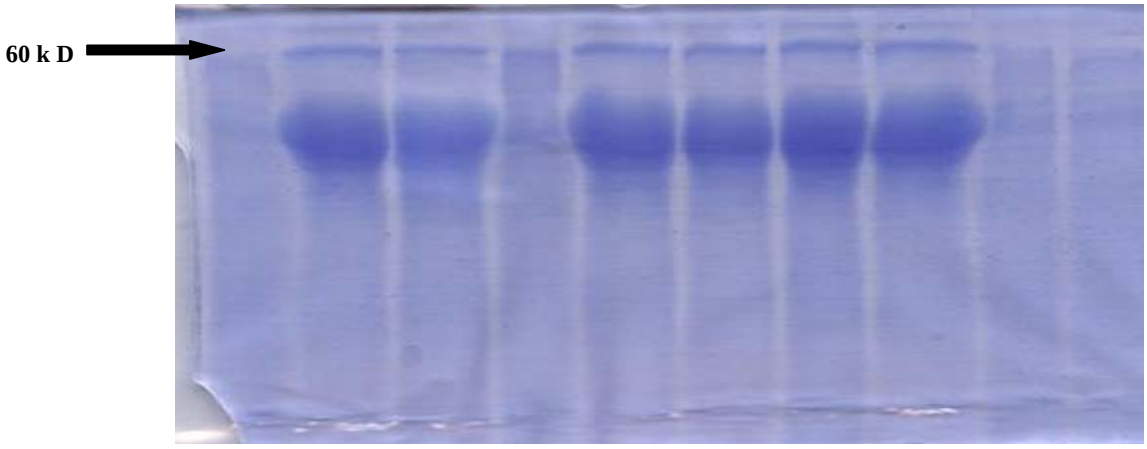

Fig. 10. SDS PAGE Characterization of Glucoamylase from wild *Aspergillus niger*
M: Marker for Glucoamylase (Novozyme 60 k D)
Lane II, V, VI and VII: Glucoamylase band present; **Lane I, IV, XIII and IX:** Glucoamylase band is absent

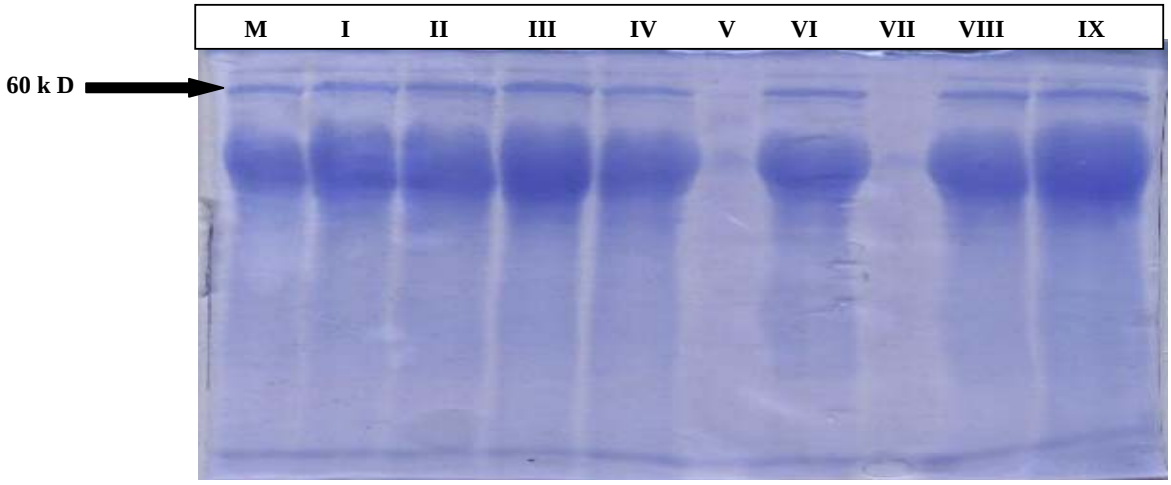

Fig. 11. SDS PAGE Characterization of Glucoamylase from mutant *Aspergillus niger*
M: Glucoamylase marker (Novozyme 60 k D)
Lane: I, II, III, IV, VI. VIII. IX: Glucoamylase band is present; **Lane:** V and VII: Glucoamylase band is absent

The comparison of wild and mutant *Aspergillus niger* for the characterization of glucoamylase activity under different levels of incubation period (Fig. 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 *Aspergillus niger* glucoamylase activity (Ellaiah *et al*., 2002). Results from DMRT showed that there was a significant difference among the treatments. The drastic decrease of glucoamylase activity at pH showed that enzyme was denatured at this pH (Nahas & Waldermarin, 2002).

Table 6. Gel filtration fraction's Table.

Gel filtration fractions	Glucoamylase activity (IU/mL/min.) wild *Aspergillus niger*	Glucoamylase activity (IU/mL/min.) mutant *Aspergillus 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

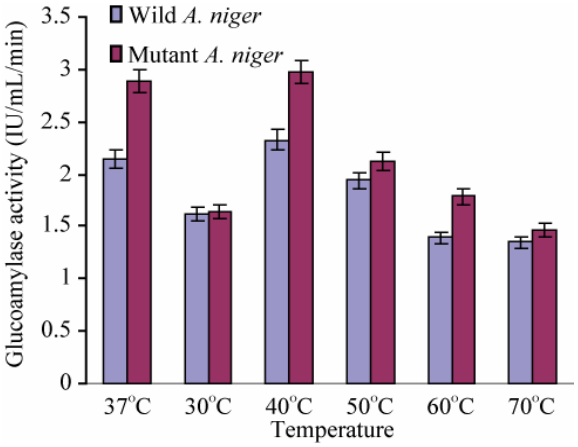

Fig. 12. Characterization of Glucoamylase produced by wild & mutant *Aspergillus niger* with varying Incubation temperatures.

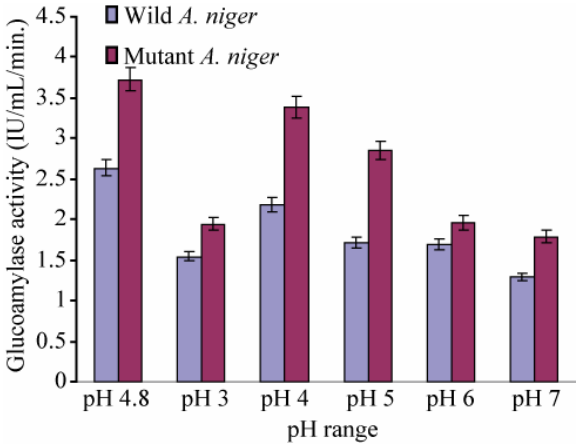

Fig. 13. Characterization of Glucoamylase produced by mutant *Aspergillus niger* with varying pH level.

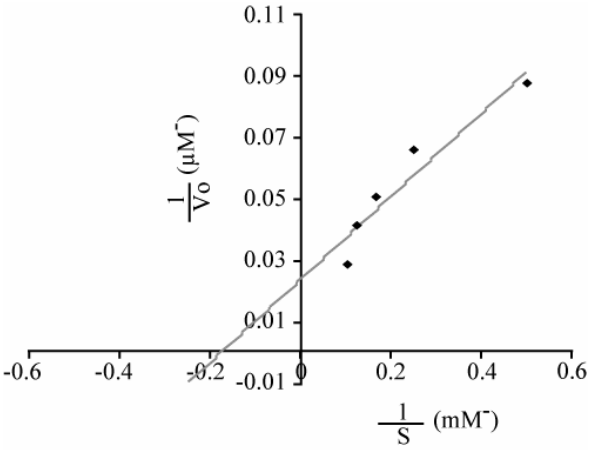

Fig. 14. Vmax., and Km for Glucoamylase.

Substrate concentration plays an important role in the enzyme activity. Lower concentration may cause a lower activity. Therefore, the determination of Km and Vmax is an important step while characterizing an enzyme. An initial increase in the enzyme activity was observed with the slight increase in the substrate concentration and then the increase in the glucoamylase activity (Fig. 14). Vmax observed from the graph was 40.12 µM and Km was 4.31 mM. V_{max} can measure but can not be achieved in reality and Km is an opposite to measure of 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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