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Optimization, Purification and Characterization of Glucose Oxidase from *Aspergillus oryzae* Strain MF13

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INTRODUCTION

Glucose Oxidase is a flavanoprotein which catayses the oxidation of β- D-glucose to β-D-glucono lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor. The H_2O_2 resulted from the glucose oxidase reaction promotes the disulphidic bonds forming between the glutenic proteins and oxidative gelation of water soluble pentosans[1]. Additional effect is that the H_2O_2 activates the flour catalase and peroxidase[2]. Most of the researchers state that the GOx addition to the dough leads to the occurrence of transversal bonds between wheat globulins and albumines, while the glutenins are less involved[3]. GOx research has been studied from various fungi due to its numerous application[4].

GOx has been used to produce gluconic acid, measurement of glucose in foodstuffs, and in diagnostics such as determination of glucose in blood, serum or plasma. GOx received numerous marketable applications in the improvement of color, flavor, texture and shelf life of food materials including the removal of oxygen and glucose from food and beverages[5], detection of enzymatic electrode for biofuel[6] and GOx is one of the most important enzyme in clinical analysis and the development of biosensor technology[7,8,9].

GOx is generally originated from fungus. Gox is normally synthesized by controlled fermentation process. GOx produced by many microbes especially, *Penicillium notatum, P. crysosporium, A. niger* and *Botrytis cinerea*. GOx from *A. niger* is a homodimeric secreted intracellular protein found in the mycelium of the organism[10]. The molecular weight of GOx about 155000 and consisting of two identical polypeptide chain the subunits connected with disulfide bonds. The enzyme holds optimum pH 5.5 with a broad range 4-7[11].

The current achievements of *Aspergillus* species for industrial scale and biotechnological goods is largely synthesize due to its metabolic versatility of the strain. Most of the commercially produced GOx is isolated from mycelium of the organism. Therefore, the improved production of GOx is certainly value for industrial application. Hence, the present work *A. oryzae* selected for synthesizes of intracellular GOx and its activities. *A. oryzae* which are Generally Recognized as Safe (GRAS) by the FDA[12]. The main goal of this report *A. oryzae* and its estimation of GOx activities under the optimized fermentation parameters and describes production, purification and characterization of GOx.

MATERIALS AND METHODS

Microorganism

The identified fungal culture of *A. oryzae* MF13 (Gen Bank ID: MG437005) used to examined the GOx production process. The fungal culture was maintained in Potato Dextrose Agar (PDA) medium and also the cultures were maintained at 4°C for further use.

Production of GOx

The GOx was synthesized by submerged fermentation of *A. oryzae* in 250 mL flask containing 100 mL medium. The fungi were grown on the medium containing (g/L); $(NH_4)_2$ HPO_4, 0.388; KH_2PO_4, 0.188; $MgSO_4$. $7H_2O$, 0.156; glucose, 80; peptone, 3.0[13]. The 5 mL of spore suspension was inoculated into the flask and incubated in a rotary shaker at 250 rpm (37° C) for 5 days.

Enzyme extraction

After shake flask fermentation, the cell biomass was harvested by centrifugation at 12,000 × g for 10 min at 4 °C. The cells were suspended in 50 ml distilled water and disrupted using ultrasonic disintegrator (Ultrasonic Homogenizer-Biologics, Inc-3000). The content was centrifuged at 10,000 × g for 10 min at 4 °C. The intracellular cell-free extract was collected and used for GOx activities.

GOx assay

The GOx enzyme activities were detected in standard assay experiments[14]. To make the reaction mixture O- dianisidine, 2.4 ml; Glucose, 0.50 ml; peroxidase, 0.01 ml the system was mixed and allowed to equilibrate water (2.5 ml) to this addition of 0.1 ml of crude enzyme. The reaction mixture was incubated at 30° C for 20 minutes. After incubation, the development of brownish red color then measured the intensity at 460 nm wavelength for assaying GOx. One unit of GOx catalyzes the conversion of 1 micromole of glucose to gluconic acid, consuming 1 micromole of oxygen and liberates 1 micromole of H_2O_2 as per assay condition.

Protein estimation

The protein content of the crude intracellular enzyme was determined by folin-phenol reagent according to the method[15] using bovine serum albumin as a standard.

Optimization of cultural condition

Effect of incubation time

The optimum incubation time for the production of GOx was determined by marking the fermentation medium. The time course profile of maximum GOx production was set different incubation time at 24-120 h respectively. The sample were taken every 24 h of incubation and determined GOx activities.

Effect of pH

The optimum pH activity was determined by *A. oryzae*. The pH was adjusted in the range of 3-9 using 1N NaOH or 1N HCl and incubated at 250 rpm. After 3 days of incubation, the cell-free extract was assayed for GOx activities.

Effect of temperature

The effect of temperature for maximum GOx production was determined in the range of 20-50 °C by setting the fermentation medium with 250 rpm at pH (6.0). The cell-free extract was assayed for GOx activities.

Effect of carbon source

The effect of different carbon sources (8.5%, w/v of glucose, lactose, cellulose, starch, fructose and maltose) on GOx production was determined by incubated culture in a fermentation medium with 250 rpm at 35 °C pH (6.0). The cell free GOx activities were monitored by carbon source.

Effect of nitrogen Sources

To study the effect of supplementary sources, the fermentation medium containing glucose was supplemented with different concentration (0.5% peptone, yeast extract, beef extract, malt extract, and casein) with addition of glucose (0.5%) and incubated 250 rpm at 35 °C and pH 6.0 The GOx activities were carried out to determine the efficient nitrogen source.

Effect of NaCl Concentration

The effect of different concentration of NaCl on GOx production was determined in the range of (0.5-2.5% NaCl) supplemented with additional carbon source of glucose (8.5%), peptone (0.5%) in the production medium and incubated 35 °C for 250 rpm at pH (6.0). The GOx production was monitored up to different NaCl supplements.

Effect of metal ions

To study the mineral nutrients on GOx activities were determined with different minerals $MgCl_2$, $ZnCl_2$, K_2HPO_4, $FeSO_4$ and $CaCO_3$ (0.25%) supplemented in the production medium and incubated at 35 °C for 250 rpm at pH (6.0). After incubation the cell free extract of GOx activities were examined with mineral supplements.

Purification of GOx

The crude enzyme purification was subjected to the various distillation process. All purification process was maintained at 4 °C. (i) The precipitation of crude intracellular GOx enzyme preparation was examined by adding different concentration of ammonium sulphate 20-100% saturation. The enzyme solution was incubated overnight at 4°C until the complete precipitation. After complete saturation 12000 rpm for 10 minutes to remove the undissolved particles. The dissolved particles were tested for GOx activities. (ii) After ammonium sulphate precipitation the enzyme extract applied dialysis process. This purification process was carried out removes the traces of ammonium sulphate precipitates. The fraction was loaded inside the dialysis bag (cellulose bag) overnight against pure sucrose crystals[16]. The partially purified GOx preparation was diluted by citrate buffer at 4°C. (iv) The dialyzed fraction was

applied by a gel filtration chromatography column using Sephadex G200[17]. The column was packed with the sephadex gel matrix (5.0 * 75 cm). The column was equilibrated and eluted with 5 mM citrate buffer (pH 6.0). The flow rate was maintained for better resolution of the target protein. The flow rate was expressed in units of 25 ml/h. The finally eluted fractions were determined GOx and protein activity.

Protein expression

The target GOx protein was determined by gel electrophoresis. Crude cell-free extract containing soluble and purified enzyme were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli[18]. The GOx protein was confirmed by standard protein marker (Genei, Bangalore, India).

Characterization of purified GOx

Effect of pH and temperature

The characterization of purified GOx from *A. oryzae* was determined required optimum condition for activities. The effect of pH on the GOx activity was determined from the range of 4.5-7.5. The purified enzyme reaction mixture was prepared using various buffers for maintaining the pH, acetate buffer for pH 4 and 5.5, citrate buffer for pH 6.0-6.5, phosphate buffer for pH 7 and 7.5. The optimum pH was determined for GOx activity by *A. oryzae*.

The effect of the different temperature on purified GOx activity was determined in the range of 30-50 °C. The optimum temperature was monitored by GOx assay in 5 mM citrate buffer (pH 6.0) at different temperature

Effect of pH and thermal stability

The pH stability of purified GOx was determined with the range of 5-7. The stability measurement of the enzyme was pre-incubated at various pH 5.0 (acetate buffer), pH 6.0 (citrate buffer) and 7.0 (phosphate buffer) for different time interval (20-60 min at 4 °C). The pH stability was determined periodically.

The temperature stability of GOx activity was determined by storing various temperature. The enzyme was pre-incubated in 45-75 °C (5 °C increment) at pH 7.5 for 10, 20 and 30 min interval. After pre incubation, the residual activity of purified GOx was monitored.

RESULTS

Optimized parameters

Optimization experiments of GOx production were conducted by flask scale fermentation. The culture condition was optimized for enhanced GOx production with desired parameters. The production and GOx activities were determined by desired incubation time (24-120 h). The time course profile was monitored every 24 h. During periodic checking of GOx production was observed logarithmic phase (72 h) the production was exhibited highest activity. The source *A. oryzae* synthesize of GOx activity was started 48-72 h, stretched maximum at 72 h. The time course of GOx production (Figure. 1a). The GOx production and activities was estimated in the range of pH 3-9. The optimum GOx activity was observed at pH 6.0, the value of 1.125U/mg protein and moderate activity observed in the pH 5.0 and 7.0 (Figure. 1b). Therefore, the GOx active on both acidic to the neutral condition.

The determination of temperature on GOx production was examined in the range of 20-50 °C incubation temperature. The favorable growth of *A. oryzae* was observed 30- 40 °C. The optimum GOx production was observed at 35 °C (1.175 U/mg protein). The result showed the temperature condition influencing the fermentation process (Figure. 1c).

The effect of carbon source on GOx production is given in (Figure. 2a). Different carbon sources were added to the production medium. The enzyme production of each carbon source was estimated. The GOx activity was highly influenced both glucose and fructose as a carbon source 1.225 and 1.351 U/mg of protein. The carbon source starch and maltose also provide moderate activity 0.926 and 0.631 U/mg of protein. The result gives glucose as a potential carbon for GOx production.

The effect of different nitrogen source on GOx production was estimated. The GOx production was highly observed in the substrate peptone (1.464 U/mg) and yeast extract (1.091 U/mg). The remaining nitrogen sources (Malt extract, Beef extract and Casein) also enhance the good GOx production (Figure. 2b). Therefore, the result indicated the nitrogen sources are very essential for GOx production.

The effect of various concentration of NaCl on GOx production (Figure. 2c). The NaCl concentration was applied in the range of 0.5 – 2.5 % respectively. The maximum production was observed in the percentage of 1.0% (1.08 U/mg) and 1.5% (1.486 U/mg) concentration used. The minimum production was exhibited 0.5-2.0 individually. The sodium chloride salt plays an essential role for GOx production because induce the activities of *A. oryzae* MF13.

The effect of metal ions on GOx production is given in (Figure. 2d). Generally, most of the fermentation process of microorganism metal ions occupied the vital production parameters. The very limited amount metals used for fermentation medium. In

this GOx fermentation the maximum activities were observed in K_2HPO_4 (1.538 U/mg). The favorable GOx production was monitored in the metal ions $MgSO_4$ (1.23 U/mg), $ZnCl_2$ (1.15 U/mg) individually. The calcium ions were reduced the GOx production.

Purification of GOx

The optimized crude GOx enzyme of *A. oryzae* was applied to few purification processes using ammonium sulphate precipitation, dialysis and gel filtration chromatography. The crude optimized GOx enzyme activity was observed at 1.538 U/mg protein. The crude enzyme was applied ammonium sulphate precipitation process; the GOx activity was exhibited at 1.868 U/mg protein. The protein fold 1.2 and the yield 75% observed. Furthermore, the precipitated GOx examined practical dialysis process the activity exhibited 2.213 U/mg protein. During dialysis the fold of purity 1.4 and the yield 64%. The intracellular GOx protein was purified by Sephadex G200 column chromatography. Throughout gel filtration chromatography, totally 45 fractions were collected. GOx activity was exhibited in the fraction numbers 14-35, the elements pooled concentrated for further use. The data of GOx purity is given in graphical representation (Figure. 3). The specific activity of GOx protein was exhibited 3.13 U/mg. The purity upgrading of GOx ~ 2.0 fold increased and overall protein yield 47.4 % (Table 1). The improved specific activity of GOx enzyme indicates the significant volume of purification was done by gel filtration chromatography.

Molecular weight of GOx

The purity of GOx protein expression was determined by Sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE). The purified GOx protein expressed on single band on electrophoretic field. The relative molecular weight was estimated to be 66 Kda indicating the homogeneity of purified GOx (Figure. 4). The result showed the desired GOx protein relative molar mass compared to the standard protein ladder.

Characterization of purified GOx

Effect of pH and temperature

The effect of pH on purified GOx activity was determined in the range of pH 4.5-7.5. The relative activity was calculated in percentage vale of GOx activities. The optimum activity was observed only in pH 6.0 (97.2%) and the average activities examined pH 5.5 (81.1%) and 6.5 (85.7%) individually. The enzyme active at acidic pH 5.5-6.5 and activity decline in alkaline pH. The relative activity of pH value of purified GOx was plotted in the graph (Figure. 5a). The effect of temperature on purified GOx activity was determined in the range of 30 -50 °C. The optimum relative activity was exhibited at 40 °C (94.4%) and a considerable amount of activity observed at 35 °C (81.7%). The activity was decreased during high temperature above 45-50 °C, the relative values decline to 70.8-46.1% activity. Therefore, increasing the temperature 48.8% of activity was reduced. The result of optimum temperature of purified GOx was plotted in graphical presentation (Figure. 5b.)

Effect of stability on purified GOx

The stability of purified GOx activity was confirmed at various pH in the range of 5.0 -7.0. The purified GOx was pre-incubated at different incubation 20, 40 and 60 min. The stability of GOx at pH 6.0, the residual activity predicted the percentage of 98.3% at first stage (0-20 min); second stage, 95.9% (20-40 min); and 88.4% (40-60 min). Therefore, the storage of GOx enzyme at pH 6.0 (0-60 min), the half-life maintained at 60 min 89.9% activity retained only 8% activity reduced. The activity slightly reduced after 40 -60 min time interval. The moderate GOx stability exhibited pH 5.0 (97.1-75.1%) and 7.0 (97.01-80.1%) at 20-60 min, during this time ≥ 20% activity reduced. The GOx activity slowly reduced during the preincubation of pH (Figure. 5c). The temperature stability of purified GOx was determined in the range of 30-40 °C and pre-incubated with different time (0-60 min). The maximum stability was observed at 30 °C and 35 °C, the first stage of incubation (0-20 min; 97.0%), second stage (20-40 min; 81.0%), third stage (40-60 min; 50.1%). The half–life maintained at 35 °C for 40 min. Further, increasing the temperature of GOx assay reactions, the enzyme denatures slowly upto 45 °C (overall 0-60 min; 60.03- 19.1%). The enzyme denatures completely at 45 °C at 60 min, ≥ 70% reduction was observed (Figure. 5d). The purified GOx stable at pH 6.0 (0-60 min) and temperature up to 30-35 °C, therefore the result showed the purified GOx from *A. oryzae* can be applicable for industrial usage.

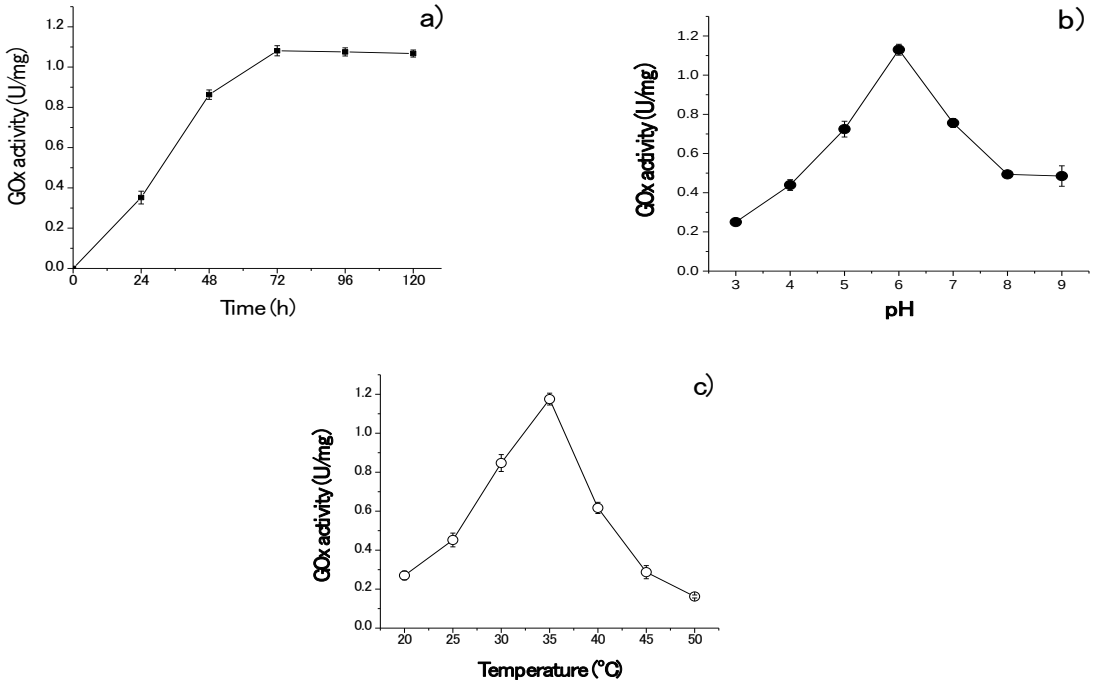


Figure 1. Effects of incubation time, pH and temperature on glucose oxidase production: a) Effects of incubation time on GOx production; b) Effects of pH on GOx production was determined in the range of pH 6.0-9.0; c) Effects of temperature on GOx production.

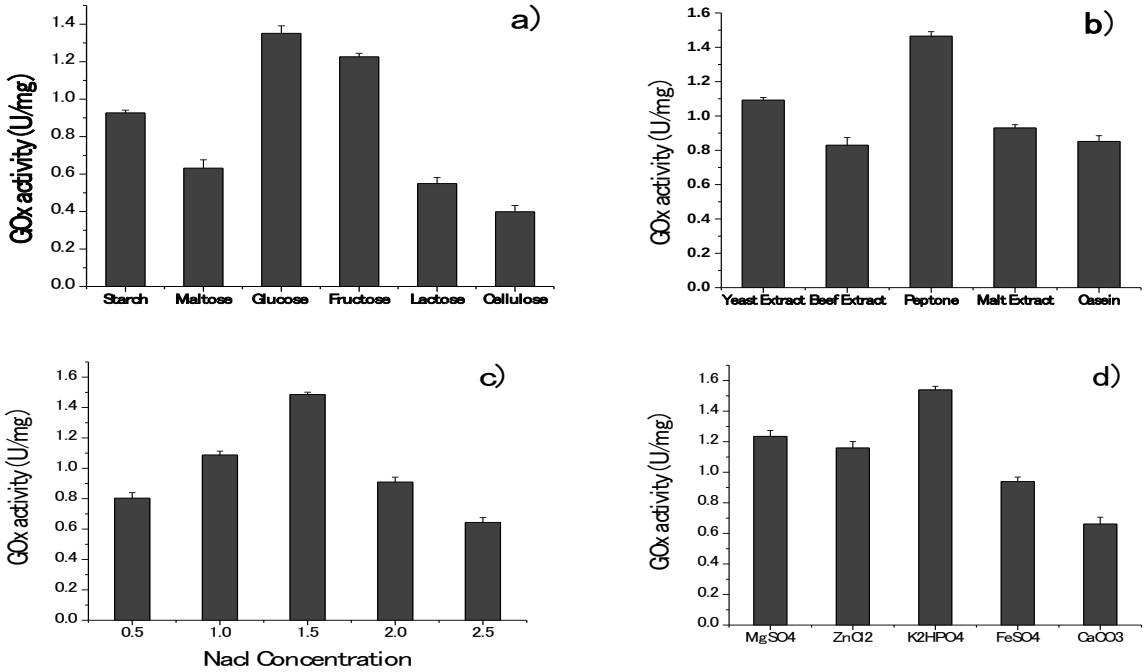


Figure 2. Effects of carbon, nitrogen sources, NaCl, and metal ions on glucose oxidase production: a) Effects of carbon sources on GOx production, b) Effects of nitrogen sources on GOx production, c) Effects of different metal ions on GOx production.

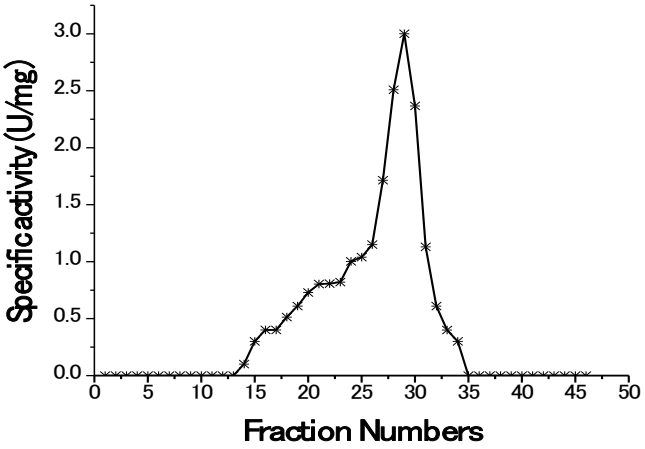


Figure 3. Elution profile of glucose oxidase from a Sephadex G200 gel filtration chromatography: The eluted 48 fractions were analyzed for protein and GOx activity.

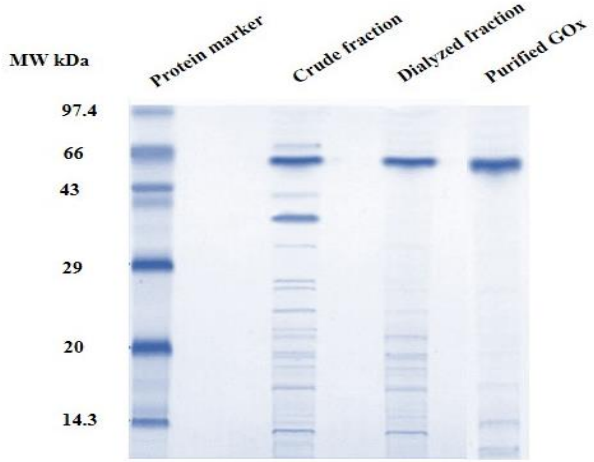
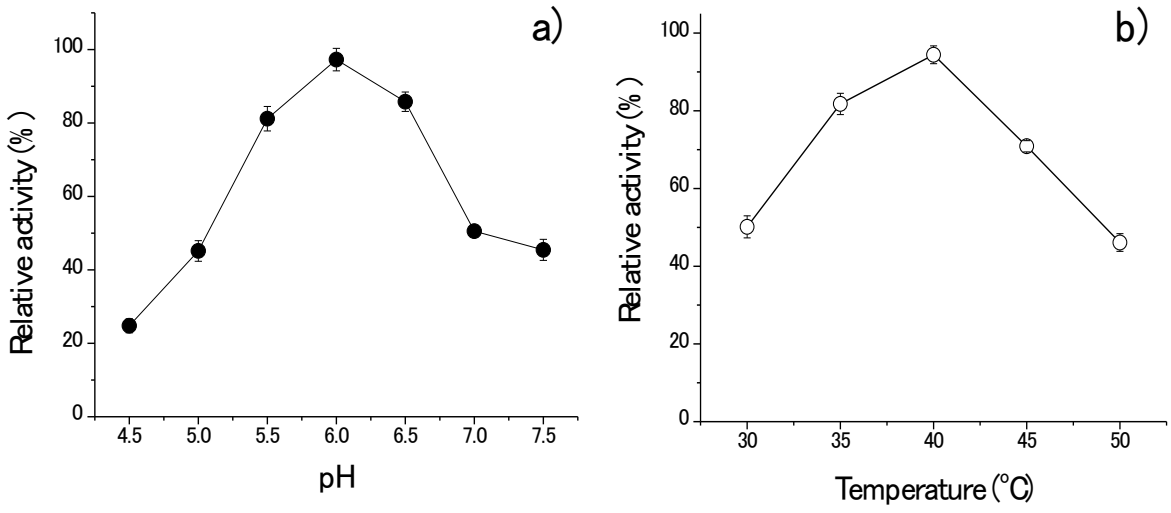


Figure 4. SDS Page Gel Electrophoresis separation of GOx protein. A different fraction of GOx protein were electrophoresed on a 12% gel under denaturing condition. The predicted molar mass 66 kDa presumed to be a GOx enzyme.



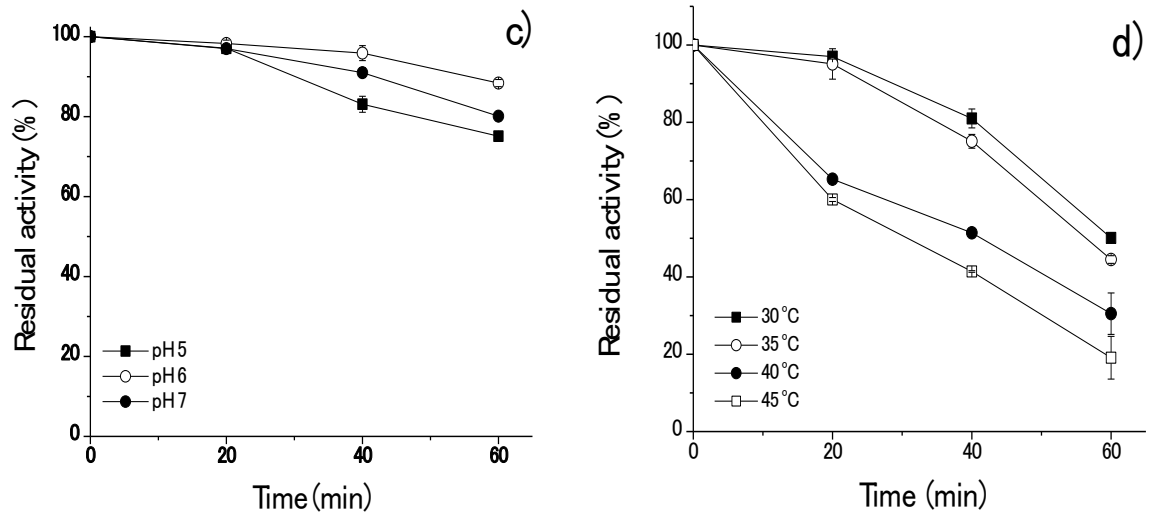


Figure 5. Effects of pH and temperature on purified GOx activity: a) optimum pH on purified GOx activity; b) optimum temperature on purified GOx activity; c) pH stability of purified GOx; d) temperature stability of purified GOx activity.

Enzyme	Total activity (µmol/pro/ml/min)	Protein (mg/ml)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude Enzyme	2.511 ± 0.023	1.631 ± 0.015	1.538 ± 0.004	-	100
$(NH_4)_2SO_4$ precipitation	2.303 ± 0.041	1.232 ± 0.059	1.868 ± 0.002	1.2	75.53
Dialysis	2.344 ± 0.005	1.059 ± 0.004	2.213 ± 0.010	1.43	64.91
Sephadex G200	2.432 ± 0.029	0.775 ± 0.021	3.138 ± 0.005	2.03	47.49

GOx assay experiments were performed triplicates and the mean values along with standard error mean (n=3) are presented.

Table 1. Glucose oxidase activities in crude and purified extracts

DISCUSSION

The goal of the present study aimed to improving the productivity of GOx from *A. oryzae* strain MF13 to accelerate the optimization of fermentation process also and purification and characterization of GOx. The most crucial fermentation parameters in the optimization process, which standardize the medium composition subsequently it marks synthesize of desired enzyme[19]. The cell growth and GOx activity of *A. oryzae* was monitored during the period of 0-120 h and the optimum incubation was observed at 72 h obviously enhancing GOx activity. The report was similar incubation time 72 h was previously reported and GOx production was studied at 96 h[20,4] whereas, during 36 h of incubation GOx production from *A. niger* was studied[21].

The effect of optimum pH for GOx activity was observed at pH 6.0. The GOx of *A. oryzae* was active on both 5.5, 6.0 and 7.0. Our findings were similar to the reports of GOx production by *A. niger* in the range of pH 5.5-6.5[22]. The earlier researcher noticed GOx stability always depends with the pH of the medium[23]. The effect of optimum temperature for GOx production by *A. oryzae* was determined in the range of 25-50 °C. The optimum production was observed at 30 and 35 °C which is comparable to the growth temperature of earlier GOx production[24,25]. Increasing high temperature GOx has lost the activity at 39 °C[26].

A number of carbon source used for the GOx production by *A. oryzae*. In this result both glucose and fructose was act as an optimum carbon source for enhancing GOx production. The results coincide with the earlier reports on GOx production used glucose as an essential nutrient. The percentage of glucose 8%,[27,28] and 15%[29] were studied. The high rate of GOx production was observed on both yeast extract and peptone. The result coincides with the earlier research on GOx production was achieved by peptone as an essential nutrient [30]. Therefore, peptone was selected a good nutrient source for GOx production.

In this study, optimize the effect of various concentration of NaCl used fermentation process.

Generally, marine microorganism growth depends on the salt concentration. Hence, to determine the effect of NaCl for GOx production. The high level of GOx production noticed in the range of 1.0-1.5%. The optimization of different metal ions used to determine GOx production. The optimum activity of GOx production achieved by K_2HPO_4. The similar mode of GOx production also examined both $MgSO_4$ and $ZnCl_2$. Addition of K_2HPO_4 in the production medium increasing the enzyme activity. The result coincides with the reports on GOx optimization by *A. niger* [31].

A. oryzae producing crude GOx was applied to initial purification of ammonium sulphate precipitation. The fractional precipitation provides partial purification of GOx. Numerous reports have been used ammonium sulphate for the precipitation of intracellular GOx [32,33]. This work closely related to previous findings of GOx purification [34]. The *A. oryzae* was producing GOx purified by gel filtration chromatography [35]. The purified GOx predicted to be homo- dimeric form with a molar mass of 148 kDa, consisting of two identical subunits. SDS page showed a single band indicated a monomeric form of GOx subunit approximately similar to 70 kDa[36].

The purified GOx temperature stable at ≥ 30-35°C. The enzyme was stable at 35°C, the half-life maintained above 40 minutes. The results were coinciding with the previous studies[37,38] whereas, GOx from *A. niger* active at 30 °C[31]. The purified GOx enzyme was stable at pH 5.0-7.0 for 40 minutes. The enzyme half-life maintained 60 minutes. Therefore, the GOx stable in the range of 5.0-7.0. The desired characteristics of GOx highly applicable for industrial practice. This work related to the previous findings of GOx, pH stability comparable to fungal GOx[39,40]. The purified GOx optimum activity was determined in the range of pH 4.5- 7.5. The GOx activity was observed in the pH 6.0 also and the desired activity at pH 5.5 and 7.0. This work comparable with the reports on GOx production by *Penicillium* sp. CBS 120262 optimum at broad pH 4.9-8.0[36].

The optimum temperature for GOx activity was reported by various fungal sources, notably *A. niger* at 40-60 °C[41], and *P. funiculosum* 433 at 25-30°C[42]. GOx has more recently been used biosensor and nano-technological applications[43]. In this work, GOx producing *A. oryzae* demonstrate great importance for biotechnological applications.

CONCLUSION

The present study successfully demonstrated GOx optimization and production by *A. oryzae* strain MF13. The optimized parameters of GOx producing *A. oryzae* active at pH 6.0, temperature 35 °C, glucose (carbon source), fructose (nitrogen source), 1.5% NaCl and K_2HPO_4, incubation 72hrs, agitation speed 250 rpm. Therefore, the easily available and low-priced substances highly influencing the GOx production. The purified GOx was characterized regarding the optimum pH and temperature activity and stability. The purified GOx active at optimum pH in the range of 5.5-6.5 and temperature 35 °C. The maximum residual activity of purified GOx stable at pH 5.0-7.0 (98%) and temperature ≥ 30-35 °C (97.01%). Hence, the present study describes the purified GOx from *A. oryzae* strain MF13 holds vital properties for the industrial application. In the future, desired GOx gene will be used for scale-up process, and utilization of agricultural substrates should be examined to mark the higher production for increasing economic value of the GOx.

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