African Journal of Biotechnology Vol. 10(65), pp. 14522-14533, 24 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1477
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Enhanced production and characterization of a novel β-D-glucose:oxygen-1-oxidoreductase by using *Aspergillus niger* UV-180-C mutant strain

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Accepted 18 August, 2011

Along with other emerging health consequences, diabetes mellitus is one of the critical threats spreading all over the world with continuity. The investigation of glucose level in diabetic patients requires the key enzyme β-D-glucose:oxygen-1-oxidoreductase/glucose oxidase, where its optimized production by mutant derived strain could affect the economic burden and accuracy of the test. Wild type *Aspergillus niger* was subjected to ultraviolet radiation for enhanced production of glucose oxidase. It was found that UV-180-C is a potential mutant derived strain, screened by using 2-deoxy-D-glucose. Optimum production of glucose oxidase from *A. niger* UV-180-C was carried out by using CSL (2%), fermentation period (36 h), pH (5.5 and 4.5 for wild and mutant respectively), temperature (30°C), $MgSO_4.7H_2O$ (0.0%), $CaCO_3$ (0.1%), KH_2PO_4 (0.8 and 1.0% for wild and mutant strains), Urea (0.3%). Crude enzyme was subjected to ammonium sulfate precipitation and resulted into 145.8 UmL^{-1} activity. Glucose oxidase from mutant derived *A. niger* exhibited optimum pH at 6, temperature 20°C, K_m 10mM and V_{max} 142 UmL^{-1}. The pyridoxal phosphate caused significant inhibition to the enzyme which indicates the presence of lysyl residues near or at the active site of the enzyme from both wild and mutant derived strains.

Key words: Enhanced production, UV radiations, β-D-glucose:oxygen-1-oxidoreductase, *A. niger*

INTRODUCTION

Diabetes mellitus is a general metabolic problem resulting in serious consequences which ultimately cause death. The diagnostic methods for diabetes have been compared to find the accurate method for glucose. The enzymatic method using glucose oxidase enzyme was most advantageous (Giampietro et al., 1982). Though, bed side glucose monitoring is 48.8% less costly and less time consuming but fails to surpass the enzymatic method (Baig et al., 2007).

Type 1 diabetes require 4 to 10 tests per day while for type 2 diabetes requires test less frequently. The total cost for the diabetes care has been investigated for the Pakistan in out patient clinics of Pakistan in 2007, that reported Rs. 515.8 are required per visit (Liaqat et al., 2007). The cost reduction at the diagnostics level could relief the economic pressure on the patients. So far, research has been focused on the enhanced production of enzymes by using mutant derived strains (Khattab and Bazaraa, 2005; Haq et al., 2001). The production optimization is an area of great interest for the maximal production of glucose oxidase using the mutant strains of *Aspergillus niger.*

β-D-Glucose:oxygen-1-oxidoreductase commonly known as glucose oxidase naturally produced by mycelia fungi and insects; which use hydrogen peroxide (the catalytic product of GOx) as anti-bacterial and antifungal agent. Glucose oxidase is used for the removal of oxygen for food preservation, for glucose estimation, for dry egg powder production, gluconinc acid and wine production

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and is an essential component of many therapeutic ointments. The predicted annual growth rate of glucose oxidase in enzyme market is 7.6% and by 2011, the market value shall increase from $4.1 to $6 billion (Wong et al., 2008). The augmentation in its consumption pleads for the search of worthy sources and superior methods for economic production of glucose oxidase.

For many years, the improvement of commercial applicability of microbial strains has been practice. Meanwhile, strain improvement of the enzyme producer offers the greatest opportunity for cost reduction without significant capital outlay (Stanbury et al., 1995). In recent years, new procedures such as balanced screening and genetic engineering have begun to make a significant contribution to this activity but random screening by mutagenesis and selection is still a cost-effective reliable and short term strain improvement. Moreover, the mutagenic procedures can be optimized in terms of type of mutagen and dose. Mutagen specificity effects can be taken into account and mutagenesis itself can be enhanced or directed in order to obtain the maximum frequency of desirable mutant types among the isolates to be screened (Semashko et al., 2004).

In this research, effort has been made to produce and purify the mutant strain of *A. niger* having a strong capability for glucose oxidase production under optimized and cheep medium components and could be used for the glucose oxidase at industrial scale.

MATERIALS AND METHODS

Preparation of inoculum

The wild strain of *A. niger* isolated, identified and purified from river soil was cultured and maintained on the potato dextrose agar (glucose 2, agar 2, starch 2, Urea 0.3, KH_2PO_4 0.008, $MgSO_4.7H_2O$ 0.05, $ZnSO_4.7H_2O$ 0.001, KCl 0.15%, pH 4 and temperature 30°C) (Haq et al., 2001). The spores on the PDA 4 days old culture were dislodged and transferred aseptically to Vogel's media [$(NH_4)_2SO_4$ 0.2, NH_4NO_3 0.4, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.02, peptone 0.1, trisodium citrate 0.5, yeast extract 0.2, glucose 2%, pH 5.5 and temperature 30°C] having a working volume of 50 mL and spore suspension of 1×10^7/mL served as inoculum (Zia, 2010).

Mutagenesis

A 36 h fresh inoculum was subjected to ultraviolet radiation (UV-Lamp: Type A-409, P.W. Allen and Co., 253-Liverpool, RD., London-N.1) for different exposure times that is, 30, 60, 90, 120, 150, 180, 210, 240 and 270 min. For each exposure time, a diluted volume of 100 µL was applied on the Petri plates containing PDA supplemented with triton X-100. The optimum exposure time of ultraviolet radiation was selected by formulating kill curve (Petruccioli et al., 1999).

Selection of positive mutants

The colonies present on the optimal dose rate were subjected to screening by the colony restrictor that is, 1% triton X-100. That 100 prevents the growth of colonies giving rise to a yellowish color around the colonies (Khattab and Bazaraa, 2005). Furthermore, the colonies tested on triton-X-100 were then subjected to the screening by glucose analogue 2-Deoxy-D-glucose at 1 mg/mL level for specific selection (Gromada and Fiedurek, 1997). 2-deoxy-D-glucose is a structural analogue of glucose (substrate for GOx) and is not metabolized by the organism due to its inadequacy to enter the glycolytic cycle. The potential mutant colonies were analyzed on agar plates for the glucose oxidase production using o-dianisidine (0.1 g/L) and peroxidase (225 U/mL). The formation of brown colored clearance zone of glucose oxidase around the colony was observed and measured (El-Enshasy, 1998). These colonies were scratched dissolved in phosphate buffer (0.1 M, pH 6) filtered and analyzed on spectrophotometer at 460 nm, for enzyme activity. The colonies producing larger zone size with higher activity were further studied for the enzyme production.

Production of glucose oxdiase

Mutant derived *A. niger* along with wild type was subjected to submerged fermentation for enhanced production of glucose oxidase. Basal fermentation media comprising (g/100mL) glucose 4, Urea 0.3, KH_2PO_4 0.6 and $CaCO_3$ 0.04, initial pH 5, temperature 30°C was inoculated by 5% inoculum and placed on an orbital shaking at 120 rpm for 36 h (Zia, 2007). Crude product was filtered using Whatman filter paper No. 1 and centrifuged for 15 min. at 10, 000 rpm and -10°C in order to remove the biomass (Gromada and Fiedurek, 1997). Furthermore, different growth conditions (carbon and nitrogen sources, different salts and other conditions) for the wild and mutant strains were inspected to meet the higher yield of glucose oxidase.

Enzyme analysis

An amount of 100 µL enzyme was analyzed using peroxidase 225 U/mL, orthodianisidine 1% solution and 18% of D-glucose (Substrate). The optical density was recorded at 460 nm on spectrophotometer for a maximum of 3 min. Worthington (1988) Biuret reagent was utilized for the determination of protein contents of the crude and other samples using bovine serum albumin as standard (Gornall et al., 1949).

Partial purification and determination of kinetic parameters

Crude enzyme was partially purified through ammonium sulfate precipitation at 60 to 85% saturation level (Simpson et al., 2007). The enzyme was examined for the optimum pH, temperature, substrate concentration and enzyme concentration. An aliquot of 0.1 mL purified enzyme was analyzed for activity at pH range of 4 to 8, temperature 20 to 45°C, substrate concentrations (5 to 20 mM) and enzyme concentrations (0.1 to 0.4 mL) to determine the kinetic parameters (Sun et al., 2008).

Determination of amino acid residues of the active site

Pyridoxal phosphate (PLP) is an active form of Vitamin B6. It has the capability of specifically modifying the lysyl residues of the enzymes by forming azolidine ring. The PLP was utilized for the determination of the active lysyl residues on the active site of the enzyme. Different concentrations of PLP (0.1, 0.2, 0.3 and 0.4 mM) were prepared and added to the enzyme. The enzyme activity and PLP-enzyme complex formed were observed on spectrophotometer at 460 and 432 nm, respectively (Janave et al., 1999).

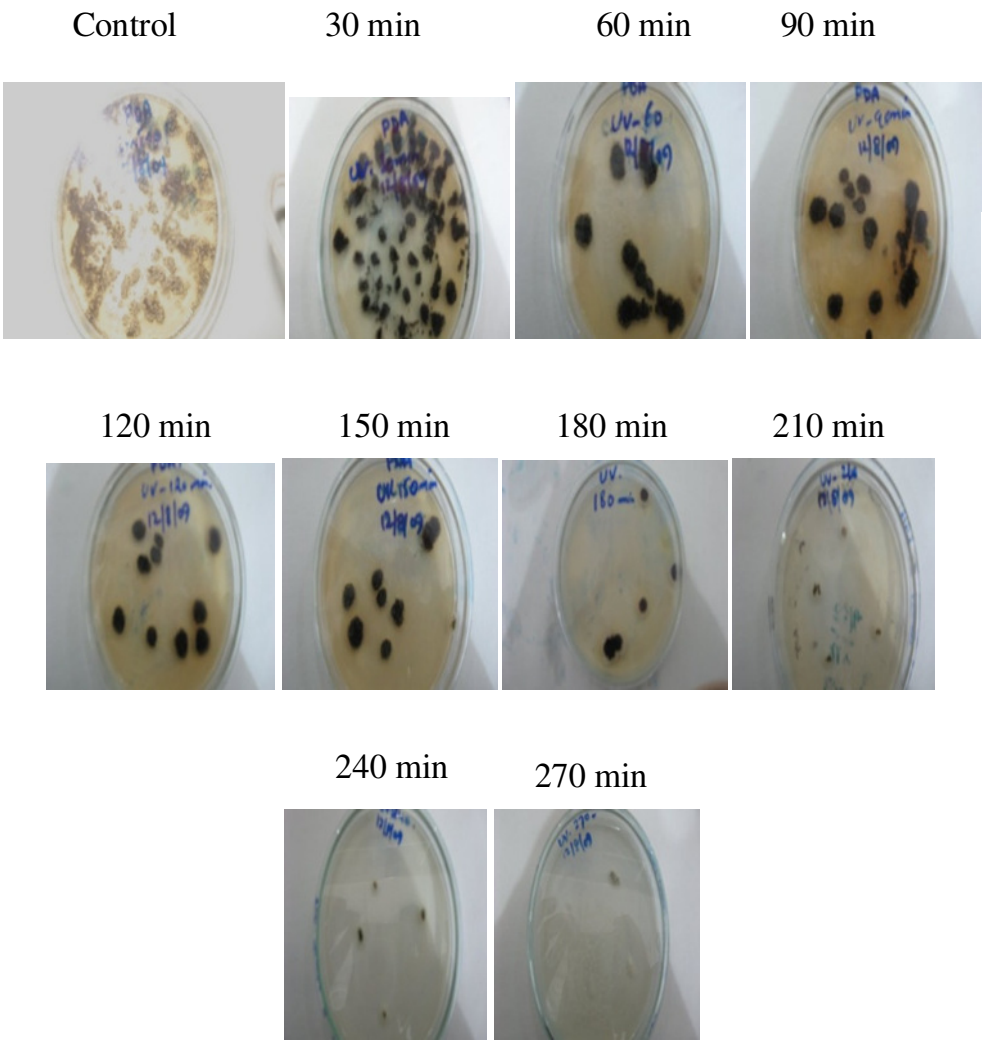

Figure 1. Treatment of *A. niger* with UV radiation at various exposure time intervals for the mutagenesis.

RESULTS AND DISCUSSION

The progress in fermentation productivity and a tremendous decrease in cost are achieved by implementation of strain improvement. It has become one of the most essential steps in the development of microbial production processes on industrial scale. Among the other available techniques, the established method of mutagenesis and selection is still a symbol of good probability for success. It is an evident verity that in recent period, mutagenesis has been successively used for the production of industrially important mutant strains (Haq et al., 2002). Ultraviolet radiations are potent inducers of strain mutations. The DNA pyrimidines are significantly affected by ultraviolet rays absorptions resulting in GC-AT transitions, these effects produce thymine dimmers and distortion in DNA structure pause the replication process. As a consequence vast range of genetic variants is produced; screening of the organisms under discriminatory conditions supply the desired organism (Shin et al., 1993).

Pure culture of *A. niger* was exposed to UV radiation at the time range of 30 to 270 min. The first selection was based on the kill curve which was formulated to determine the optimum exposure time for killing the fungal spores at 37°C. The curve presented 81.41% killing rate while the survival rate was 18.58% (Figures 1 and 2). The conidial killing of 43% and survival of 57% has been reported after ultraviolet treatment of *A. niger* (Singh, 2006).

Triton X-100; a synthetic surfactant is capable to reduce the fungal and bacterial growth. It causes a delay in logarithmic growth phase of microbes. Triton X-100 (100 mg/mL) caused the maximum degradation of 42.2% of substrate by *Flauobacterium* sp. Q14 (Sun et al., 2008). More than 10^{-6} cells/mL were subjected to

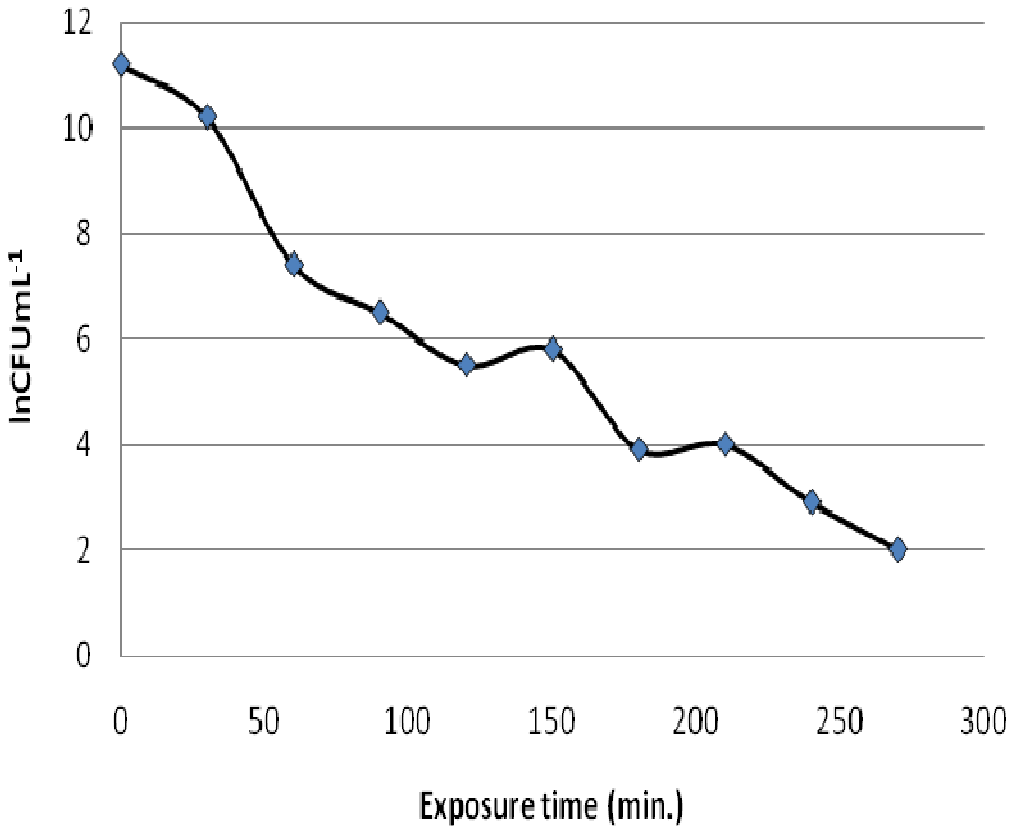

Figure 2. Effect of UV-radiation on *A. niger* to formulate the kill curve.

mutagenesis which resulted in approximately 100 colonies after diluted culturing on PDA (supplemented with Triton-X-100). The selected exposure time of 180 min after kill curve formulation contained five colonies, which were subjected to potato dextrose agar media supplemented with Triton-X-100 (1%). Out of many colonies, five were selected and named as UV-180-A, UV-180-B, UV-180-C, UV-180-D and UV-180-E. All the colonies showed approximately equal resistance against the colony restrictor except UV-180-C which had more resistance comparatively.

The use of 2-deoxy-D-glucose, a toxic analogue of glucose has been a routine practice for the selection of positive mutants (Azin and Noroozi, 2001). Five selected strains after UV mutagenesis were subjected to PDA supplemented with 2-deoxy-D-glucose (1 mg/mL) as selective marker. The mutant strains checked after 6 days of growth period exhibited difference in growth potential on selection media. UV-180-A, UV-180-B and UV-180-C showed a comparatively stronger growth potential, while the UV-180-D and UV-180-E presented more spore formation than wild but lesser than other three mutants.

Khattab and Bazaraa (2005) used 2-deoxy-D-glucose to examine the resistance of mutants produced after UV and EMS for the enhanced production of glucose oxidase. 2-Deoxy-D-glucose has been also used as a criterion for the selection of positive mutants for the enhanced production of amylase, glucose-6-phosphate dehydrogenase, catalase and β-fructofuranosidase (Maresma et al., 2010). The mutants having the greater resistance against 2-deoxy-D-glucose were subjected to enzyme diffusion zone test for visual analysis and quantitative analysis using spectrophotometer.

The mutant colonies grown on the agar plates containing 2-deoxy-D-glucose as a constituent were analyzed by enzyme diffusion zone test; which is a simple and reliable test for the estimation of enzyme production from the specific strain. The reaction mixture 0.1 mL was applied on the mutant colony which produced glucose oxidase as a metabolic product. The brown colored clearance zone around the colony indicates the enzyme production. The mutant colonies were dislodged from the agar plate and were analyzed for optical density on spectrophotometer after filtration (Figure 3). The mutant colonies produced the zone size of 9.7, 9.9 and 10 mm for UV-180-A, UV-180-B and UV-180-C, respectively while the zone size for wild strain was 9 mm. The activity was recorded as 8.5, 8.9 and 9 U/mL for three mutants (Table 1); wild strain contained 8.0 U/mL.

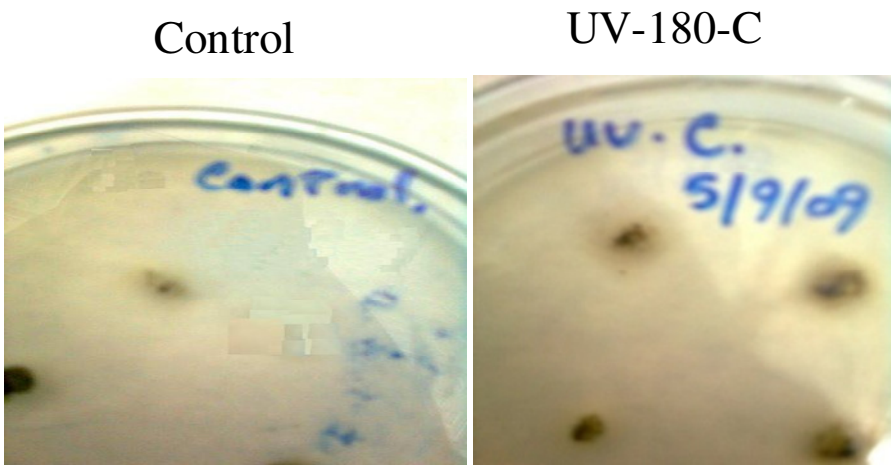

Figure 3. The enzyme diffusion zone test for the wild strain and the UV-180-C mutant derived strain.

Table 1. The zone size, activity and percentage increase in activity of mutant derived strains from *Aspergillus niger*.

S/N	*A. niger* mutant strains	Zone size (mm)	Enzyme activity (U mL^{-1})	% increase in the activity
1	Wild type/Control	9	8.0	100
2	UV-180-A	9.7	8.5	106.3
3	UV-180-B	9.9	8.9	111.3
4	UV-180-C	10	9.0	112.5

The percentage rise in activity of mutants was 106.3, 111.3 and 112.5% for UV-180-A, UV-180-B and UV-180-C mutant strains respectively. It was clear from the analysis that UV-180-C was the more significant mutant as compared to the other strains. Fiedurek et al. (1986) reported the UV mutant of *A. niger* having 1.5 to 18% increased activity than wild strain after diffusion zone analysis.

Production of the enzyme

Corn steep liquor was used as an economical substitute for glucose which is a high cost substrate for *A. niger* production. Fermentation media economics are driven by the profitability of the market product and manufacturing cost of the product per unit. Fermentation was carried out in duplicate using a concentration of 2% corn steep liquor for 36 h at 30°C and 120 rpm. Harvested enzyme from *A. niger* wild and UV-180-C contained 23.7 and 37 U/mL activity (Figure 4). A noticeable increase in activity of mutant strain was observed on using corn steep liquor (2%). Singh, (2006) observed the glucose oxidase activity of 2.62 U/mL by using molasses as a carbon source for UV mutant strain of *A. niger*. Upon using sucrose as carbon source, Semashko et al. (2004) obtained 8.23 U/mL glucose oxidase activities from mutant strain of *P. funiculosum*.

Medium components were further optimized for glucose oxidase over-production. Experiments were carried out at 24, 36, 48 and 60 h fermentation period using *A. niger* UV-180-C mutant. The successive culture of mutant strain was achieved after 36 h of fermentation possessed an increase in enzyme activity (59.2 U/mL); while a deflation in enzyme production was observed at higher fermentation time (Figure 5). Ragini et al. (2010) found 72 h optimum time period for the production of glucose oxidase using *A. niger* and *P. chrysogenum* mutant strain, with *Penicillium sp.* mutant resultant activity of 1.56 U/mL. Semashko et al. (2004) found the maximum enzyme production between 48 to 52 h. The production of the enzyme at shorter duration is a considerable quality of the mutant strain to minimize time consumption in production process at industrial scale.

The medium pH induces changes in morphology and enzyme secretion by the microbial strain. The optimal pH for the production of glucose oxidase for *A. niger* UV-180-C mutant strain was 4.5 (Figure 6). Nakamatsu et al. (1975) and Simpson et al. (2007) reported optimum pH at 6 and 7, respectively, for the glucose oxidase production with specific activity of 28 U/mg for later. It was observed that 30°C is the best temperature for the production of

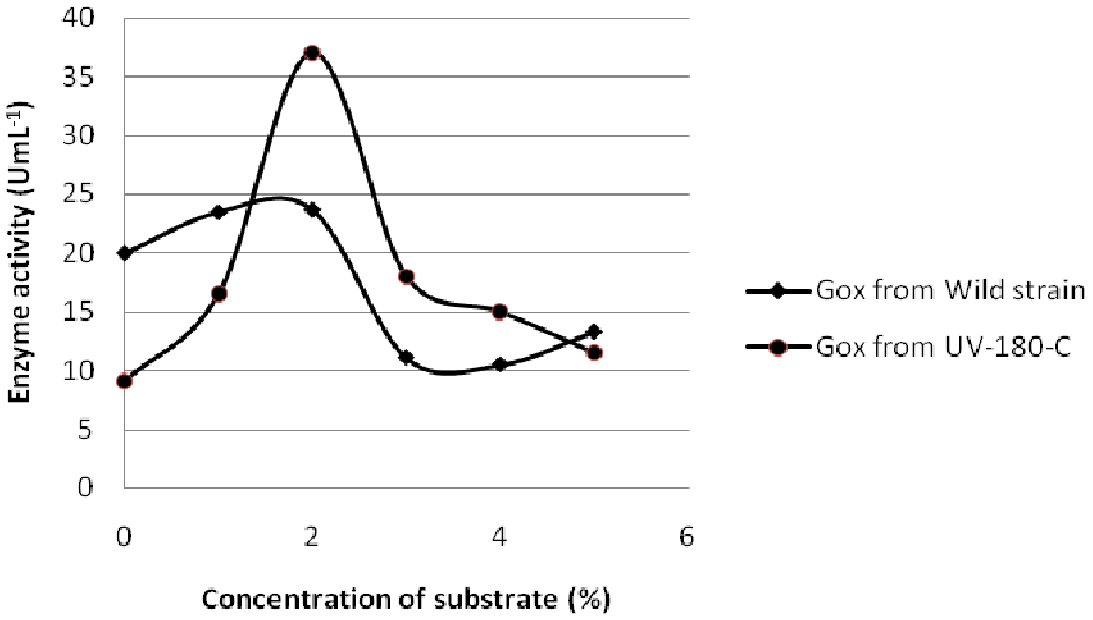

Figure 4. Effect of substrate concentration of glucose oxidase production from wild and mutant derived strains of *Aspergillus niger.*

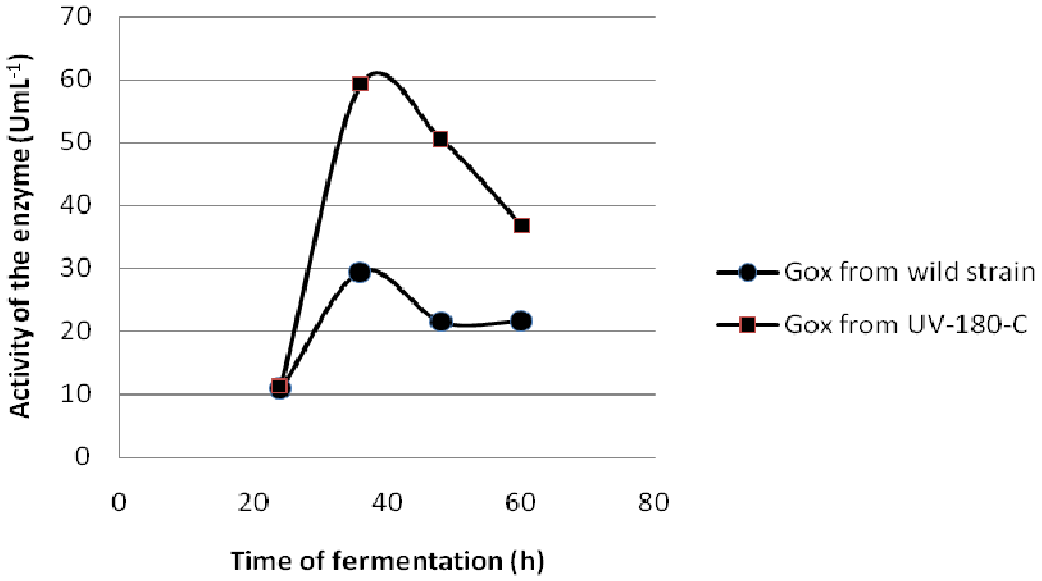

Figure 5. Production of glucose oxidase from parental and mutant derived strain for different fermentation periods.

glucose oxidase (89 U/mL), however, an increase in temperature reduced the enzyme production (Figure 7). El-Sherbeny et al. (2005) showed optimum production of glucose oxidase from *A. niger* at 30°C and studies by Ragini et al. (2010) and Fiedurek (1998) exhibited the glucose oxidase activity of 1.702 and 4.15 UmL^{-1} at 20 and 50°C, respectively from mutant derived strain.

Salt concentrations play an important role on the enzyme activity. Increasing concentrations of $MgSO_4.7H_2O$ caused a sharp decrease in the activity of the enzyme and the highest activity was found without the addition of $MgSO_4.7H_2O$. Lu et al. (1996) found that increasing concentrations of the salts including $MgCl_2$ cause the decrease in activity. Higher salt concentrations

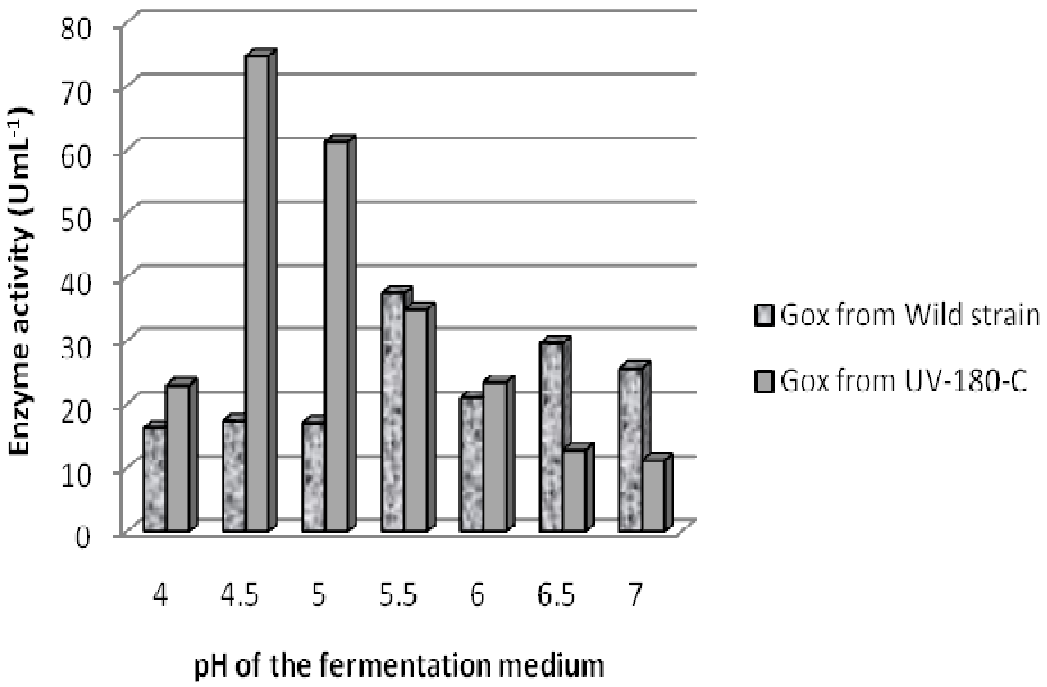

Figure 6. Effect of varying medium pH on the production of glucose oxidase from wild strain and UV-180-C mutant derived strain.

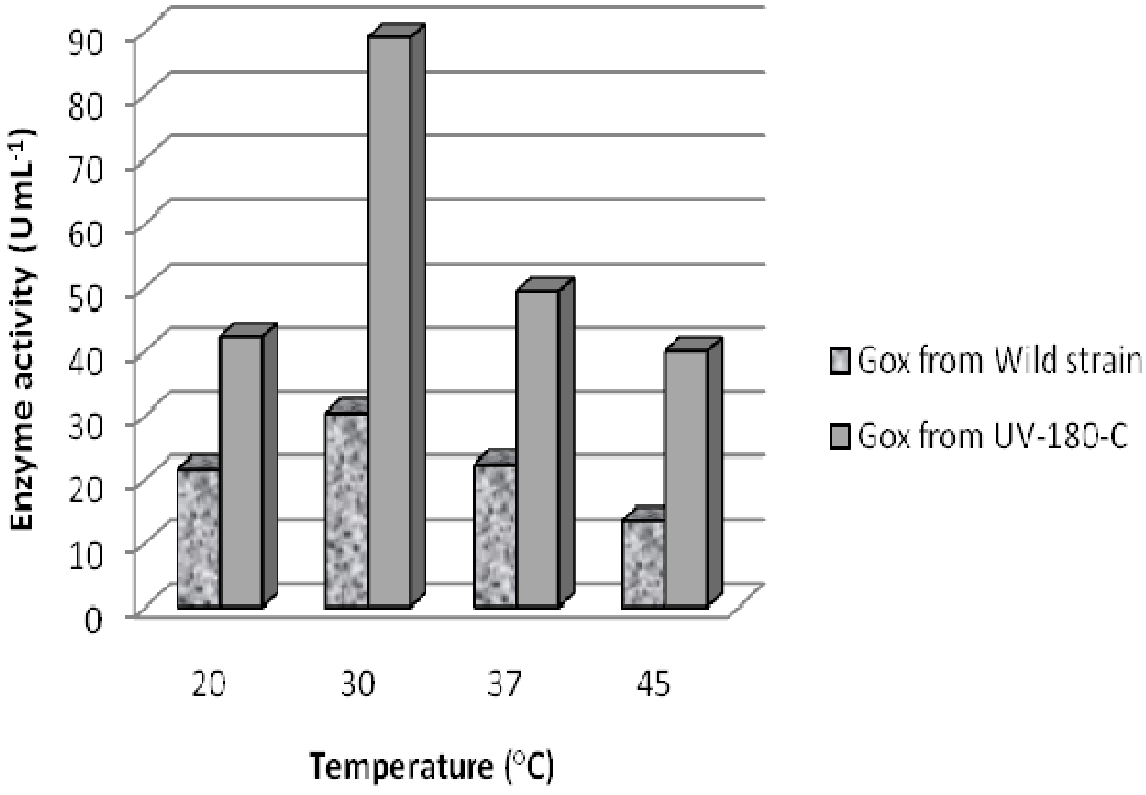

Figure 7. Optimization of temperature for the production of glucose oxidase using wild strain and UV-180-C mutant strain.

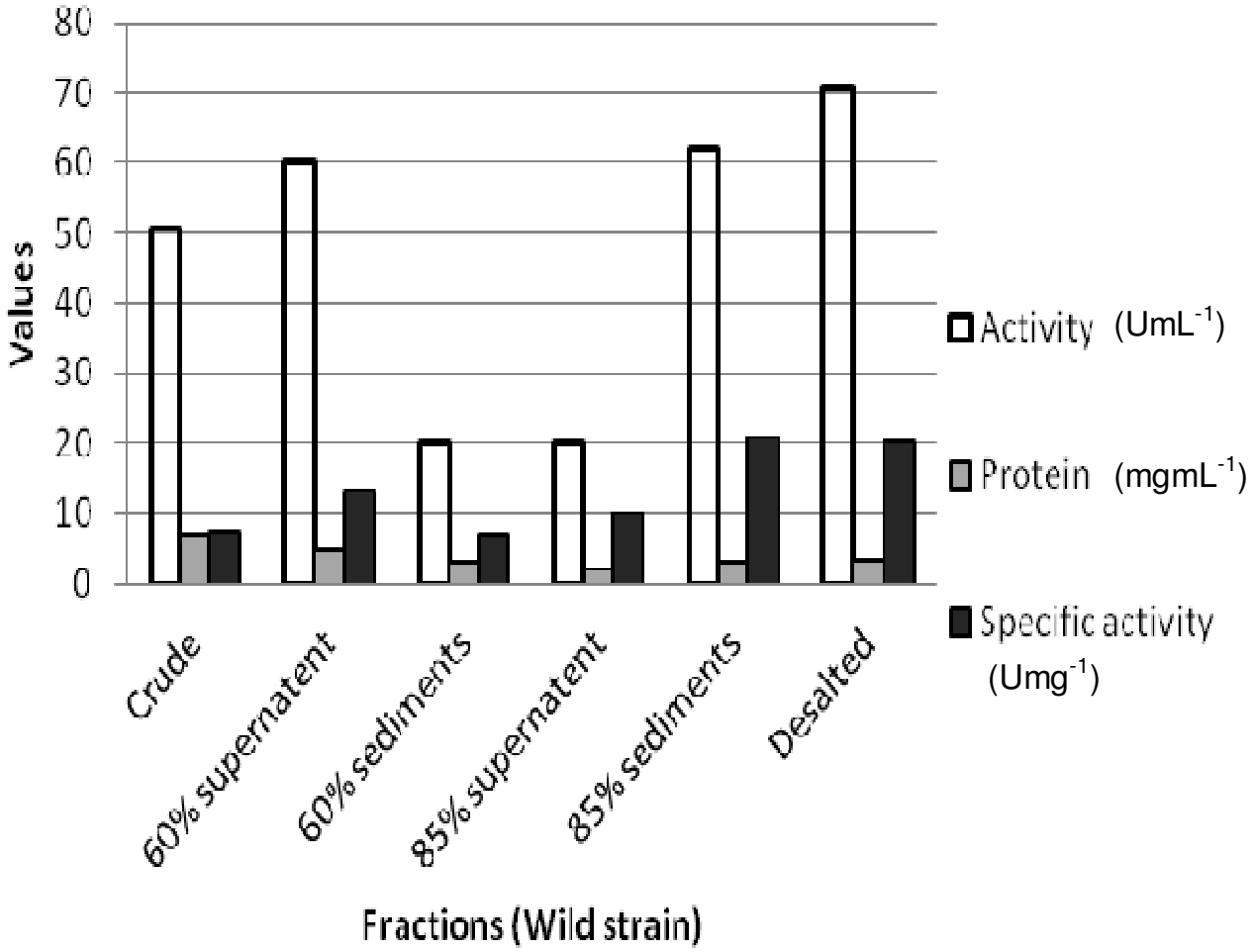

Figure 8. Ammonium sulfate precipitation of GOx produced from wild strain.

produce metal ions which compete with substrate for binding sites on the enzyme, so glucose oxidase activity is partly inhibited. Enzyme production decreased by the addition of $MgSO_4.7H_2O$ even at small concentrations, the production however increased without $MgSO_4.7H_2O$, resulting in an increment in enzyme activity of 96.2 U/mL.

It has been reported that $CaCO_3$ is frequently used in gluconinc acid and glucose oxidase production and suggested as the mechanical support for the fungal growth (Kundu and Dass, 1985). Moreover, it has been employed as buffering agent against the pH drop during fermentation (Petruccioli et al., 1999). A remarkable increase in glucose oxidase biosynthesis was observed by adding $CaCO_3$ and maximum activity 101.02 U/mL was observed on adding upto 0.1% $CaCO_3$. KH_2PO_4 addition upto 0.8% effected the enzyme production and activity reached upto 101.2 U/mL. The overall production of glucose oxidase from *A. niger* UV-180-C was increased by 3.53 fold after optimization of fermentation conditions. Singh (2006) reported the 3.43 fold increase in glucose oxidase production using UV mutant of *A. niger*.

The production was carried out using optimized medium conditions and crude enzyme activity from wild and mutant strain was observed to be 50.7 and 130.7 UmL^{-1}. Guo et al. (2010) reported the enzyme activity of 40 U/mL at optimized culture conditions. The activity increased after ammonium sulfate precipitation to 70.78 and 145.8 U/mL, while the specific activity was 20.22 and 50.27 U/mg for wild and UV-180-C mutant strain (Figures 8 and 9). El-sherbenny et al. (2005) reported glucose oxidase activity of 1.02 U/mL in crude extract and 0.995 U/mL after 80% ammonium sulfate precipitation, respectively.

Determination of kinetic parameters

An enzymes 3D structure is disturbed by minor changes in temperature and pH, thereby resulting in negative changes in activity. Optimum temperature was determined by carrying out the standard assay procedure

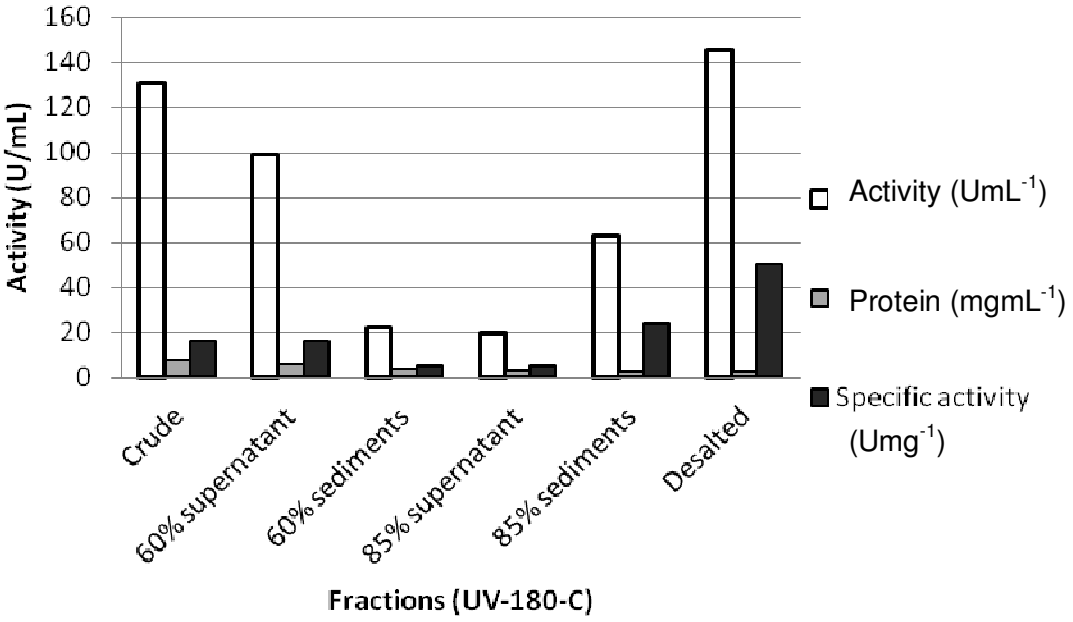

Figure 9. Ammonium sulfate precipitatuin of GOx produced from UV-180-C mutant strain.

Table 2. Activity of glucose oxidase at optimum temperature and pH from wild and mutant UV-180-C mutant strains.

Parameter at optimum activity and activity range of enzyme	Activity (UmL^{-1})	
	Wild strain	Mutant Strain UV-180-C
Optimum pH (6)	73.0	150.7
Optimum Temperature (20°C)	75.2	152
pH range (6 to 8)	68±5	145±5
Temperature range (20 to 40°C)	67.7±7	151±1

at 20, 30, 40 and 50°C. The results show that enzyme is highly active at lower temperature that is, activity of 75.2 and 152 U/mL for wild and mutant strain at 20°C. The activity remained nearly constant upto 40°C while deviated at 50°C. Wong et al. (2008) reported several authors who presented the maximum enzyme activity at temperature range of 25 to 30°C. The results obtained are in agreement with Zia (2007) who reported 40°C as optimum temperature, with a significant decrease in enzyme activity above 50°C. The purified enzyme showed optimum activity of 73 and 150.7 U/mL at pH 6, while activity of 68.5±5 and 145±5 UmL^{-1} for wild and UV-180-C at pH ranges of 6 to 8. At 20°C the optimal activity was 75.2 and 152 U/mL, while the enzyme was still active within a range of 20 to 40°C with an activity of 67.7±7 and 151±1 U/mL for wild and UV-180-C, respectively (Table 2). Ko et al. (2002) reported that glucose oxidase from mutant strain had maximum activity at 6 pH. Simpson et al. (2007) observed maximum glucose oxidase activity in range of 6 to 8.

The rate of enzyme catalyzed reaction depends on the enzyme concentration, substrate concentration and other factors including temperature and pH of the reaction mixture. In this regard, the V_{max} and K_m for the enzyme recovered from wild and mutant strain showed an apparent K_m of 2.3 and 10 mM, respectively where the V_{max} for wild and mutant was 10 and 142 U/mL (Figures 10 and 11). K_m for the glucose oxidase from wild strain is significant while from UV-180-C mutant strain the higher K_m was observed, while the V_{max} for the UV-180-C glucose oxidase is significantly higher than the wild strain glucose oxidase. The increased K_m and V_{max} for the glucose oxidase from UV-180-C might be due to some structural changes in the enzyme active site. Odebunmi and Owalude (2007) reported the K_m of 0.117 M and 0.143 /min V_{max}. The apparent Km 57.0 µg/mL has been reported by Xiong et al. (2005). Kinetic parameters for glucose oxidase have also been reported by Leiter et al. (2004) and recorded the K_m 9.5 mM.

The active site lysyl residues were found to be most important for the active site performance of the enzyme both in wild strain and the mutant obtained after ultraviolet irradiation. Previously, it has been confirmed that the lysyl and histidine residues were the most

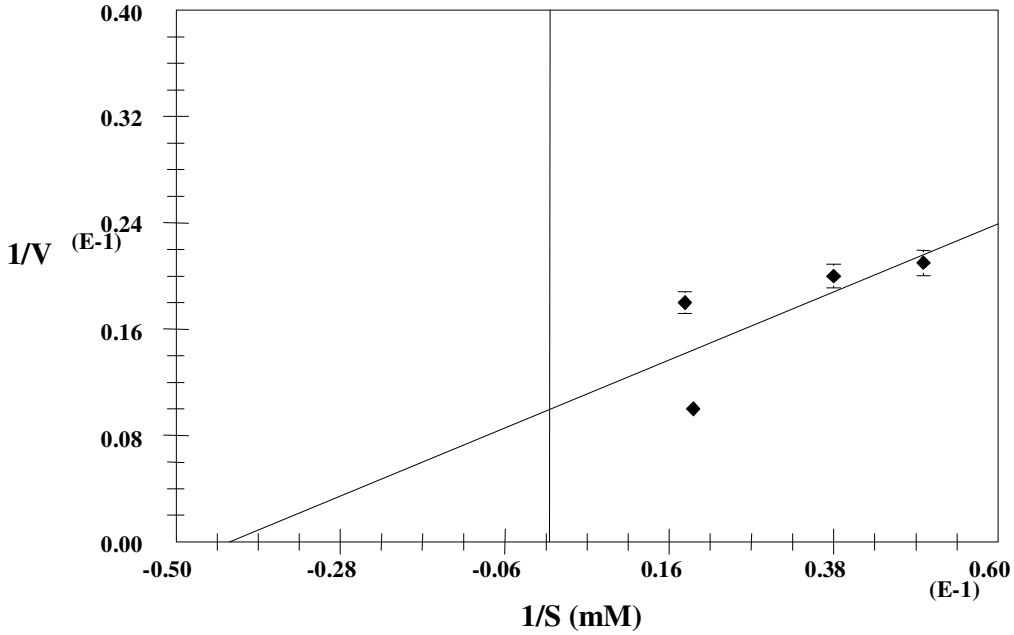

Figure 10. Lineweaver-burk plot for the determination of kinetic parameters for GOx from wild strain.

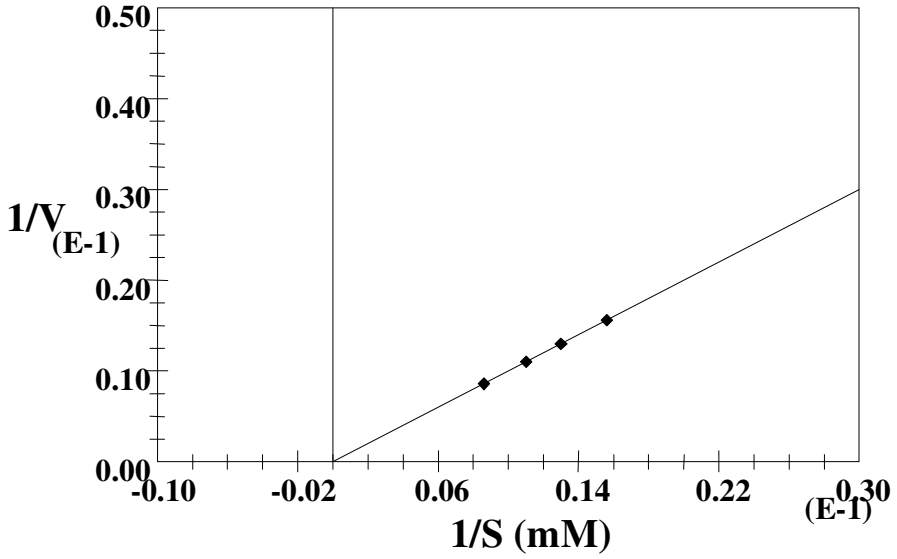

Figure 11. Lineweaver-burk plot for the determination of kinetic parameters for GOx from *A.niger* UV-180-C mutant strain.

important for the production of H_2O_2, while other amino acids like aspartic acid and arginine were not important for the enzymatic activity (Buschle-Diller et al., 2005). Janave et al. (1999) studied the effect of pyridoxal phosphate (PLP) on enzyme activity. The enzyme activity was observed at the various concentrations of PLP, the effective binding of PLP by the enzyme lysyl residues was demonstrated by the sharp decrease in the activity of glucose oxidase after 10 min treatment. The reaction results in the formation of PLP-enzyme complex by the ε-amino group. The specific binding results in the formation of azolidine ring system X-CH(R)-NH, where R is the phosphopyridoxal residue. The presence of the complex was confirmed by spectrophotometeric analysis at 432 nm, decreased presence of the product and a significantly increased presence of PLP-enzyme complex

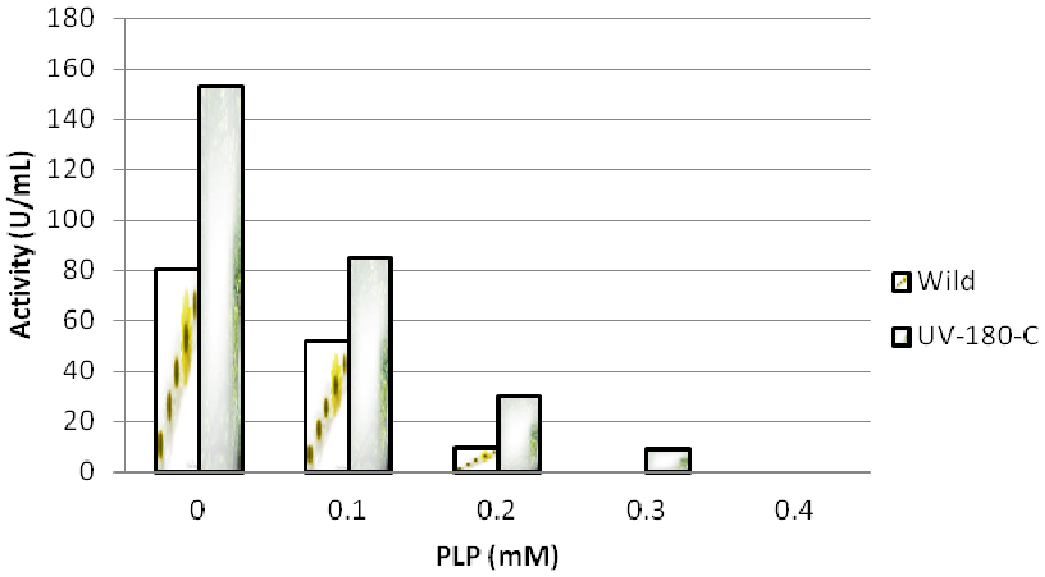

Figure 12. Effect of lysine modifying reagent on enzyme activity from *A. niger* wild type and mutant strains.

indicated the presence of lysine residues and it can be predicted that the lysine residues are at or near the active site of the enzyme from both wild and mutant derived strains of *A. niger* (Figure 12). The purified retinal oxidase was used for the identification of substrate binding sites using some chemical modification reagents. It was observed that lysyl-specific pyridoxal 5′-phosphate (PLP) and cysteinyl-specific *p*-chloromercuribenzoate (PCMB) competitively inhibited the activity of the retinal oxidase (Huang and Ichikaw, 1995).

Conclusion

Depending on the mentioned results, it is concluded that *A. niger* UV-180-C has a significant potential for the β-D-glucose:oxygen-1-oxidoreductase/ glucose oxidase production using optimized fermentation medium and could be applied for further research and commercial production.

ACKNOWLEGEMENTS

This research work is a part of M.Phil. thesis of S.R. and funded by Higher Education Commission, Pakistan through grant No. 1119 to M.A.Z. The author is grateful to HEC for financial support and T.I. and M.A.S. for providing laboratory facilities.

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