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EFFECT OF GAMMA IRRADIATION ON *ASPERGILLUS NIGER* FOR ENHANCED PRODUCTION OF GLUCOSE OXIDASE

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Abstract

Developing countries have a high prevalence of diabetes and their populations are suffering from associated adverse factors. Such a frequency requires more effective diagnosis, mostly achieved by glucose diagnostic kits. Although high priced kits are available in market but local production of such kits can be highly cost effective and may confer the decline in incidence of the disease. Glucose oxidase is the key enzyme for the determination of glucose in such analytical tools. Enhanced production of glucose oxidase was performed by mutagenesis of *Aspergillus niger* by gamma irradiation. A dose of 80krad was found as optimum for derivation of positive mutant strains. Following the screening by triton X-100 and 2-deoxy-D-glucose, the selected strains *A. niger* G-80-A, *A. niger* G-80-B and *A. niger* G-80-C showed 27.5, 23.20 and 20.55 UmL^{-1} glucose oxidase activity in enzyme diffusion zone test; which is much higher to parental strain (7.5 UmL^{-1}). *A. niger* G-80-A was subjected to submerged fermentation and obtained highest yields after 36 h, at CSL 2%, pH 6.5, 30°C, $KH_2PO_4$0.8% and urea 0.3%. Partial purification by ammonium sulfate resulted in 175UmL^{-1} of glucose oxidase activity after dialysis. Kinetic parameters like optimum pH, temperature, K_m and V_{max} were found to be 6.0 (180±2 UmL^{-1}), 30°C (185±0.5 UmL^{-1}), 5.26mM and 400U mL^{-1}, respectively. Active inhibition of the enzyme by increasing concentration of PLP in reaction mixture confirmed the presence of functional lysyl residue on the active site of enzyme.

Introduction

Among other south Asian countries, Pakistan has a high prevalence of diabetes mellitus. In associated risk factors, obesity ranks first. The Diabetic Association of Pakistan reported that out of 500 patients 43, 20 and 40 % were affected by chronic complications like retinopathy, nephropathy and neuropathy, respectively (Riaz, 2010). Rapid and reliable blood glucose determination is essential for diabetic patients. Enzymatic determination of glucose requires enzymes like glucose oxidase and peroxidase. In this coupled enzyme reaction mutarotase is sometimes also utilized for the fast conversion of glucose to beta anomer. In this method, glucose is oxidized to gluconic acid and hydrogen peroxide. The resultant H_2O_2 oxidizes the chromogen (ortho-dianisidine) into a dye in a catalyzed secondary reaction. Glucose oxidase is highly selective for the determination of glucose that reacts to β-D-glucose 150 times faster as compared to α-D-glucose. Such quick estimation makes the test more reliable and accurate (Raba & Mottola, 1995; Banauch *et al*., 1973).

Ionizing radiations like gamma irradiation have many advantages over chemical mutagens. Such ionizing radiations penetrate into tissues and generate the production of numerous compounds depending on the dose rate of radiation. By the reaction of gamma rays and water, some influential reducing and oxidizing species and molecular products like OH and H_2O_2 are produced respectively (Parker & Darby, 1995). Mutagenic effectiveness of gamma rays has been reported to be higher than the chemical like EMS (Dhulgande *et al*., 2011).

Due to selectivity for glucose, glucose oxidase has been most popular and selective agent for the quantitative glucose determination in biological fluids. The enzyme has been a clinical entity and of industrial interest for years. Considering its price per unit activity, the enzyme has been reported as one of the cost effective and suitable reagent for analytical purposes (Raba & Mottola, 1995).

Among the reported, *Aspergillus niger* is the potential source for the production of glucose oxidase and is preferred due to its high production ration of extracellular enzyme. The ability of *Aspergillus niger* to utilize a wide range of waste products as nutrition source makes it more economical source of the enzyme (Toscano *et al*., 2011). Other sources include *Penicillium* sp., *Escherichia coli* and *Botrytis cinerea* (Liu *et al*., 1998). Fermentation and optimization of glucose oxidase has been extensively performed formerly. In this regard, concentration of glucose, pH, temperature, fermentation period, nitrogen sources, KH_2PO_4 and $MgSO_4.7H_2O$ has been optimized for the enzyme from parental (wild) strain. Such an optimization for the potential mutant strain shall markedly improve the enzyme production (Khurshid *et al*., 2011).

The mutagenesis of the parental strain was performed to seek the mutants having greater potential for production of glucose oxidase. In this work, we report the production and optimization of glucose oxidase from mutant derived strain of *Aspergillus niger,* which resulted in markedly high enzyme activity as compared to production using un-optimized conditions.

Materials and Methods

Sporulation medium: Vogel's broth of pH 5.5 was used for the preparation of spore suspension [glucose 2 % w/v, peptone 0.2g, trisodium citrate 0.5g, yeast extract 0.2g, KH_2PO_4 0.5g, NH_4NO_3 0.2g, $(NH_4)_2SO_4$ 0.4g, $MgSO_4.7H_2O$ 0.02g/100mL]. An amount of 50mL sporulation medium was sterilized and inoculated with fresh culture of *Aspergillus niger*. The medium was incubated in orbital shaker for 36h to achieve a spore suspension of 1×10^7spores mL^{-1} (Zia *et al*., 2010).

Strain improvement and selection: A 36h spore suspension of *Aspergillus niger* was transferred to sterilized McCartney vials, sealed with parafilm. These vials were exposed to gamma irradiation (source CS^{137}) (Gammacell 3000, Elan) at Nuclear Institute of Agriculture and Biology Faisalabad, Pakistan. The selected dose rate was as 20, 40, 60, 80, 100, 120 and 140 krad along with un-exposed control (Gromada & Fiedurek, 1997; Iftikhar *et al*., 2010a). Along with control, such irradiated samples of spore suspension were diluted to 100 folds and cultured on PDA supplemented with colony restrictor [g/100mL- glucose 2, agar 2, starch 2, KCl 0.015, KH_2PO_4 0.008, $MgSO_4.7H_2O$ 0.05, $ZnSO_4.7H_2O$ 0.001, urea, 0.3, triton X-100 1%]. A kill curve for the exposure of spores to gamma irradiation was prepared to select the significant colonies that resulted in selection of few mutant derived strains out of 1000 colonies on PDA. All the experiments were conducted in triplicate and under sterilized conditions.

Screening of mutants: Mutants were cultured on PDA supplemented with Triton X-100(1%) for 4-6 days for screening. The detergent triton X-100 restricted the colony size and limited the sporulation of the colonies, while potential colonies were further subjected to screening by using selective marker i.e., 2-deoxy-D-glucose at the level of 1mg mL^{-1}. Plates were incubated for 4-6 days at 30^0C and the selected colonies were tested for the production of glucose oxidase by enzyme diffusion zone test (Zia *et al*., 2010). For the test, the reaction mixture for glucose oxidase containing peroxidase and chromogen (coupling reaction enzyme 225U mL^{-1} and ortho-dianisidine 0.1g L^{-1} respectively) was applied on the fungal culture grown on PDA plates. Such reaction produced brown colored zone around the colonies as an indicator for the presence of glucose oxidase (El-Enshasy, 1998; Petruccioli *et al.,* 1995; Khattab & Bazaraa, 2005).

Production of glucose oxidase: The fungal strains were subjected to submerged fermentation in 250mL Erlenmeyer flasks, each containing 50mL fermentation medium [glucose 2%, urea 0.3g, KH_2PO_4 0.6g, CSL 2%]. A spore suspension of *Aspergillus niger* with 1×10^7 spores mL^{-1} was subjected to production of glucose oxidase using different substrates (corn steep liquor, rice polishing, molasses and sludge). Different concentrations of each substrate (0, 1, 2, 3, 4, and 5%), fermentation time (24, 36, 48, 60h), pH (4, 4.5, 5, 6, 6.5, and 7), temperature (20, 30, 37, 45 ^{0}C) and effect of different salts ($MgSO_4.7H_2O$, $CaCO_3$, KH_2PO_4 and Urea concentration) were optimized for the production of glucose oxidase (Zia *et al*., 2010; Kona *et al*., 2001; Hatzinikolaou & Macris, 1995).

Determination of glucose oxidase activity and protein contents: Glucose oxidase activity was determined by using o-dianisidine-buffer mixture and peroxidase enzyme (225 UmL^{-1}). An aliquot of 0.2mL glucose oxidase enzyme was subjected to spectrophotometric assay at 460nm. Protein contents were determined by using 0.5mL biuret reagent and 0.5mL enzyme extract. Optical density was observed at 540nm after incubation at 37 ^{0}C for 15 minutes (Worthington, 1988, Gornall *et al*., 1949).

Harvesting and Purification of the enzyme: Crude enzyme preparation was prepared by filtration from Whatman filter paper no.1. The preparation was centrifuged at 10,000 rpm for 15 min. at 4^oC to obtain crude extract. The extracellular preparation was subjected to partial purification by ammonium sulfate at 60-85% saturation (Zia *et al*., 2012). The sediments recovered after 85% saturation was subjected to dialysis in phosphate buffer pH 6.0 for 4h at 4^oC (Gromada & Fiedurek, 1997; Rasul *et al*., 2011).

Determination of amino acid residues and kinetic parameters: Specific modification of lysyl residues of active site of the enzyme was performed by pyridoxal phosphate (PLP). Different concentrations of pyridoxal phosphate (0.1, 0.2, 0.3 and 0.4mM) were prepared and investigated its effect on 0.03 mg of dialyzed glucose oxidase. The enzyme activity and activity of PLP-enzyme complex was analyzed spectrophotometrically at 460 and 432nm, respectively. Partially purified enzyme was subjected to kinetic studies by using substrate concentration 5-20% (w/v), keeping the enzyme concentration constant. To obtain V_{max} and K_m for glucose oxidase, we generated Lineweaver-burk plot by ploting $1/V_0$ as function of 1/[S] (Odebunmi & Owalude, 2007; Sukhacheva *et al*., 2004).

Results and Discussion

Aspergillus niger is a potent producer of many industrially important enzymes and may genotypically be improved by exposure to gamma rays. The mutants recovered after treatment by gamma rays were found to be effective producers of enzymes (Awan *et al*., 2011). Mutagenesis of *Aspergillus niger* by using chemicals has been reported earlier to improve many industrially important enzymes and other products. The cells of *Aspergillus niger* were subjected to mutagenesis by ultraviolet irradiation, resulting in 45.4% activity of cellulase (Junwei & Shuyun, 1987). Consistent death of cells occurs after their exposure to gamma irradiation for long time, as increase in exposure time results in an increase in dose of gamma rays. The total number of mutants and ratio of mutants to non-mutants increase in cellular population after treatment of gamma rays. The effect of increasing dose of gamma radiation is shown in Fig. 1.

Literature reported that physical mutagenesis is a cost effective method to generate potential mutant derived strains that may be used for commercial production of the enzymes. Kill curve formulation has been reported in literature for the selection of optimal dose of antibiotic against bacteria. Bapiraju *et al*., (2004) reported 99% killing and less than 1% survival for the spores of *Rhizopus* sp., for the enhanced production of *lipase*. A multistage mutagenization of *Aspergillus niger* resulted in 30% increase in glucose oxidase activity (Fiedurek *et al*., 2004). Selection from kill curve resulted in 80krad as best suited dose for the production of mutants (Fig. 2). All three colonies on the plate were named as *Aspergillus niger* G-80-A, G-80-B and G-80-C.

Triton X-100 (0.1-1.0%) has been reported to reduce the colony size of *Aspergillus niger* (Khattab & Bazaraa, 2005). Mutant colonies recovered after the selection from kill curve were subjected to 1% colony restrictor. Significant reduction of the colonies by parent strain was observed, while the mutant colonies showed good sporulation and colonies size. Javed *et al*., (2011) reported that, 0.5 gL^{-1} of triton X-100 for the selection of *Humicola insolens* mutant colonies; while Zia *et al*., (2010) used 10 gL^{-1} concentration of triton X-100 for the screening of *Aspergillus niger* mutant colonies. Further screening of the mutant colonies was performed by using 2-Deoxy-D-glucose which is an analogue of glucose molecule and has the 2-hydroxyl group replaced by hydrogen. The selective marker is uptaken by the glucose transporters of the cell but cannot undergo further glycolysis. The cell growth is inhibited because of slow glycolysis and loss of energy production (Pelicano *et al*., 2006).

Final confirmation of potential of the colonies for the production of glucose oxidase was determined by glucose oxidase agar plate test. The reaction on agar plate resulted in the formation of zones around the colonies. A comparative zone formulation, for the glucose oxidase production by the mutant strains was observed as compared to the parent strain. The zone sizes for the mutant derived strains G-80-A, G-80-B and G-80-C and parent strain of *Aspergillus niger* were 24, 20, 19 and 7mm respectively. The colonies were further tested spectrophotometrically by scratching and filtering reaction mixture on the colonies and noting the absorbance at 460nm. Glucose oxidase activity by mutant strains, i.e. G-80-A, G-80-B and G-80-C was 366.6, 309.3 and 274%, respectively. Iftikhar *et al.,* (2010b) reported 311% increase in lipase activity by *Aspergillus niger* after treatment with gamma irradiation. As mutant strain G-80-A showed maximum production of glucose oxidase, it was further studied for the optimization of conditions.

A number of production parameters have been studied for the optimized production of glucose oxidase from *Penicillium* and *Aspergillus* sp. Such parameters include carbon sources, nitrogen sources, fermentation time, pH of the medium, incubation temperature and various salts concentrations. Optimization of fermentation concluded CSL (2%), fermentation time (36h), pH (6.5), temperature (30°C), $CaCO_3$ (0.1%), KH_2PO_4 (0.8%), and Urea (0.3%) as optimum concentrations/levels, as the production of the enzyme was noted to increase significantly for these concentrations. A considerable decrease in glucose oxidase production was observed with increasing concentration of $MgSO_4$.7H2O, so it was taken as 0% in production medium. Gunny *et al*., (2011) performed screening of fermentation medium for the production of glucose oxidase. CSL has been reported by many researchers as cost effective substrate/carbon source for the industrial and laboratory scale enzyme production (Nascimento *et al*., 2008). The production of glucose oxidase was increased by using CSL as substrate, while rice polishing, molasses and sludge gave relatively less enzyme production, respectively. Results for optimization of conditions are presented in Fig. 3. Khurshid *et al*., (2011) reported the decreased activity of glucose oxidase after the increase in the $MgSO_4$ concentration, while a pH 5.5 and 30°C temperature were found optimum.

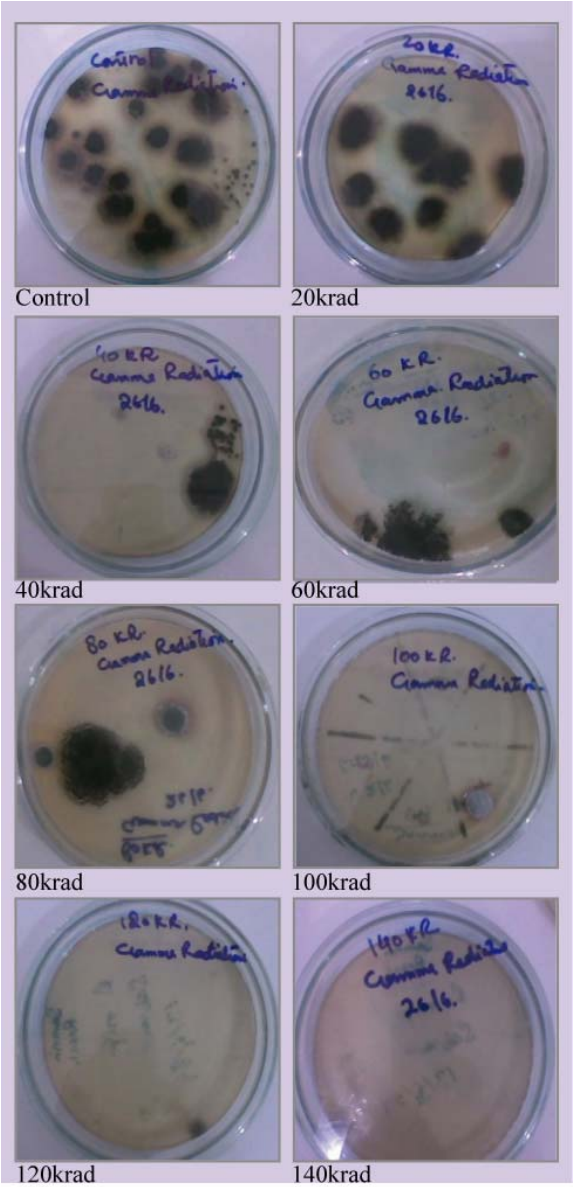

Fig. 1. Exposure of *Aspergillus niger* parent strain to different doses of gamma radiation.

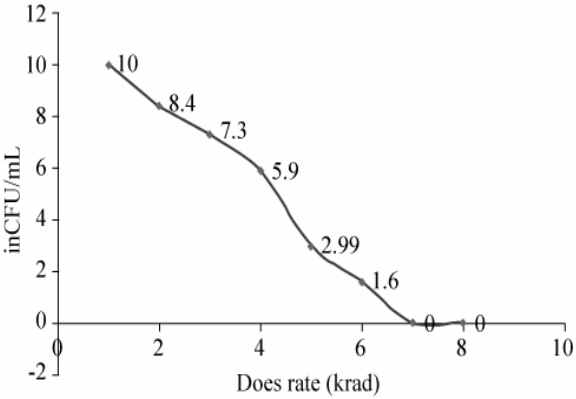

Fig. 2. Formulation of kill curve for the mutagenesis of *Aspergillus niger* by gamma radiation.

They also reported that KH_2PO_4 0.4%, urea 0.2%, temperature 30°C, pH 5.5 were best optimal conditions for the enzyme production. Shaheen *et al*., (2008) reported a decrease in relative activity of protease production using 20mM $MgSO_4$ in fermentation medium, concluding glucose, Urea and $CaCO_3$ as important parameters affecting positively on the enzyme production; while $MgSO_4$ resulted in a fall of glucose oxidase production. Riboflavin production from the *Aspergillus niger* is affected by the addition of Mg^+ ions, that affect the glucose oxidase activity (Gunny *et al*., 2011).

The precipitation of proteins by ammonium sulfate is an ideal method due to high solubility of salt, cheapness and lack of toxic effects. Increase in ionic strength reduces the repulsive forces among the protein molecules and cause precipitation. Ammonium sulfate precipitation of glucose oxidase produced by *Aspergillus niger* G-80-A mutant derived strain of *Aspergillus niger* under optimum conditions was performed at 60-85% saturation level. Removal of ammonium sulfate by dialysis resulted in the 175 UmL^{-1} and 86.2Umg^{-1} specific activity which is 5 times higher than crude enzyme (Fig. 4). El-Sherbeny *et al*., (2005) reported 35.54 Umg^{-1} glucose oxidase specific activity at 80% saturation level. Simpson *et al*., (2007) precipitated the protein at 60-70% saturation and obtained 137Umg^{-1} specific activity.

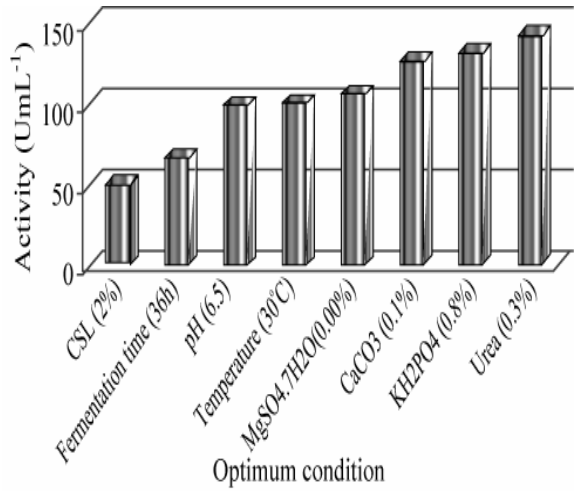

Fig. 3. Production of glucose oxidase using mutant strain *Aspergillus niger* G-80-A

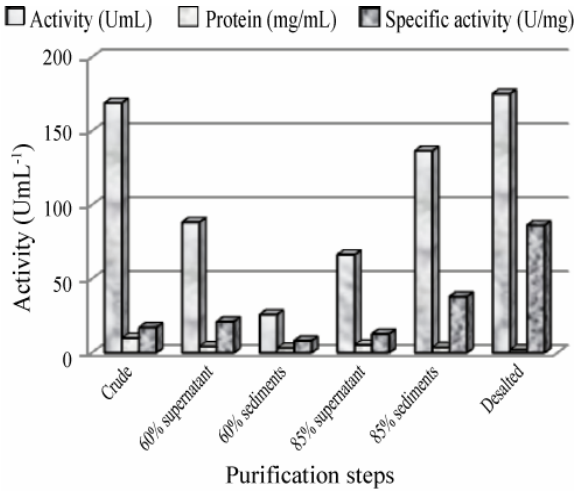

Fig. 4. Activity of glucose oxidase by mutant strain *Aspergillus niger* G-80-A at different steps of partial purification.

The superficial K_m and V_{max} for partially purified glucose oxidase from *Aspergillus niger* G-80-A was determined by studying the enzymatic reaction over the varying glucose concentration. Apparent K_m and V_{max} values were observed to be 5.26mM and 400 UmL^{-1}, respectively (Fig. 5). Kelley & Reddy, (1986) reported K_m of 38mM for glucose oxidase from *P. crysosporium* using glucose as substrate. Odebunmi & Owalude, (2007) reported K_m value of 0.117M and V_{max} for glucose oxidase was 0.143 min^{-1}. Ragini *et al*., (2010) also reported the K_m and V_{max} of 3.4 $\times 10^{-1}$mM and 8.3×10^{-3} per minute, respectively for glucose oxidase from *Penicillum crysogenum* SRT19 strain.

For many enzymes, pyridoxal phosphate is a lysine binding coenzyme and is essentially active amino acid in catalysis on active site. Vermeersch *et al*., (2004) demonstrated that PLP is an inhibitor that binds to the active site lysine and competitively inhibits topoisomerase I. Schiff-base is formed as a result of reaction of aldehyde group of PLP with ε-group of lysine. Such a reaction yields enzyme-PLP complex having azolidine ring system X-CH(R)-NH. The complex was determined by obtaining a decreased OD at 432nm with increasing concentration of PLP (Table 1). Buschle-Diller *et al*., (2005) reported the lysine and histidine residues on the active site are critical for enzyme activity and production of H_2O_2. The enzyme activity was observed at the various concentrations of PLP, the effective binding of PLP by the enzyme was demonstrated by the sharp decrease in the activity of glucose oxidase after 10 minutes treatment. The enzyme activity was observed to sharply decrease with increasing concentration of PLP in reaction mixture.

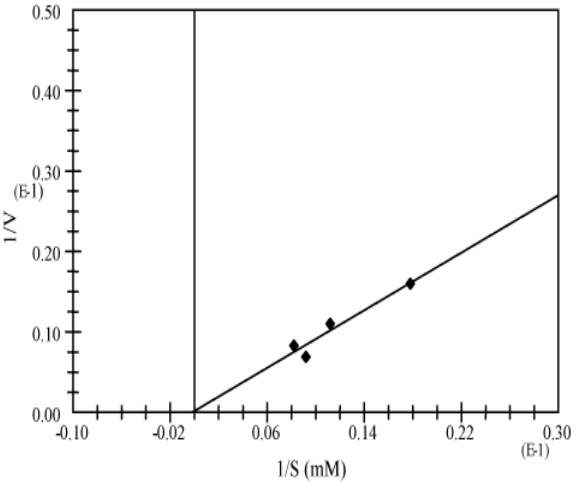

Fig. 5. Lineweaver-burk plot for the determination of K_m and V_{max} for partially purified glucose oxidase.

Table 1. Trend of glucose oxidase activity and % inhibition of the enzyme by increasing concentration of PLP.

Concentration of PLP (mM)	*A. niger* G-80-A	
	Activity (UmL^{-1})	% inhibition
0	185.6	0
0.1	60.02	58.74
0.2	12	91.75
0.3	0.3	99.7
0.4	0	100

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