INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
10–407/AWB/2010/12–6–964–966
http://www.fspublishers.org

Short Communication

Chemically Treated Strain Improvement of *Aspergillus niger* for Enhanced Production of Glucose Oxidase

M. ANJUM ZIA[1], KHALIL-UR-RAHMAN, MUNIR A. SHEIKH AND IFTIKHAR A. KHAN†
Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan
†*Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan*
[1]Corresponding author's e-mail: scientistuaf@yahoo.com

ABSTRACT

The objective of research was to enhance the production of glucose oxidase through mutagenesis of *Aspergillus niger*. Among colony restrictors, triton X-100 and ox-gall were used and it was found that 1% ox gall was the best. In order to produce depressed mutants for enzyme production, 2-deoxy-D-glucose was used at 1 mg mL^{-1} in PDA plates. A few colonies were selected based on large clearance zones than wild type microorganism. Glucose oxidase positive strain was identified on agar plate with o-dianisidine and peroxidase. The size and color of zone is an index of the formation of glucose oxidase, giving rise a brown color. The results indicated that *A. niger* mutant BCM-8 and BCE-6 produced 9 and 6 mm enzyme diffusion zone with 282 and 202% increased activity, respectively. © 2010 Friends Science Publishers

Key Words: Glucose oxidase; Strain improvement; Mutagenesis; *Aspergillus niger*

INTRODUCTION

The success of *Aspergillus niger* for industrial production is due to the metabolic versatility of this strain. *A. niger* is well known to produce a lot of organic acids, enzymes, plant growth regulators, mycotoxins and antibiotics. The industrial importance of *A. niger* is not only limited to its more than 35 native products but also on the development and commercialization of the new products, which are derived by modern molecular biology techniques. During the past few years numerous studies have been presented on *A. niger*, presumably the most important fungus for production and secretion of protein (Jeenes *et al.*, 1991; Yoon *et al.*, 2010).

Several microbial enzymes are known to have the capability of oxidizing glucose. Out of these, glucose oxidase also known as β-D-glucose: oxygen-oxidoreductase (EC 1.1.3.4), is of commercial interest, produces D-glucono-1,_5-lactone and a reduced acceptor. It removes hydrogen from glucose and reduces itself, which is then re-oxidized by molecular oxygen (Crueger & Crueger, 1990).

The importance of glucose oxidase comes from its wide applications in many fields as in food industries, is used to remove the glucose and oxygen from beverages and powdered eggs (Petruccioli *et al.*, 1999). It is used for the production of beer, wine and soft drinks. In pharmaceutical and analytical biochemistry, it is used for quantitative determination of glucose in biological fluids. Glucose "dip-sticks" became available for screening of blood/urine glucose (Worthington, 1988; Wang, 2008).

Strain improvement is a lengthy and laborious job, where we have to screen the better isolates among a mutagen-treated population. Various investigations have been conducted to improve glucose oxidase production by strain selection using classical screening and mutagenesis techniques. Chemical mutagens may induce mutations within a sequence originating mutagen-specific patterns of mutations. Still mutagenesis and selection are cost effective procedure for reliable short term strain improvement (Rowlands, 1984; Iftikhar *et al.*, 2010). The greatest advantage of screening methods is the simplicity that does not require any profound understanding of the molecular biology and physiology of the microorganisms being manipulated (Gromada & Fiedurek, 1997). The objective of the present study was strain improvement for the hyperproduction of glucose oxidase that may be used in glucose diagnostic kits or other purposes.

MATERIALS AND METHODS

The test organism used in this research work, *Aspergillus niger*, was procured from the National Fungal Culture Collection of Pakistan, Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan and maintained on potato-dextrose-agar.

Strain improvement techniques: The spores of *Aspergillus niger* were prepared in Vogel's media [g mL^{-1}: KH_2PO_4 0.5; NH_4NO_3 0.2; $(NH_4)_2SO_4$ 0.4; $MgSO_4.7H_2O$ 0.02; peptone 0.1; trisodium citrate 0.5; yeast extract 0.2; glucose 50; pH 5.5], using 250 mL Erlenmeyer flasks with working volume

To cite this paper: Zia, M.A., K.U. Rahman, M.A. Sheikh and I.A. Khan, 2010. Chemically treated strain improvement of *Aspergillus niger* for enhanced production of glucose oxidase. *Int. J. Agric. Biol.*, 12: 964–966

of 50 mL in rotary shaker operating at 220 rpm and temperature was adjusted at 30°C (Haq *et al.,* 2001).

N-methyl-N-nitro-N-nitrosoguanidine (MNNG; 0.15 mg mL^{-1}) and ethidium bromide (0.5 mg mL^{-1}) were used to induce mutagenesis in *A. niger* for enhance production of glucose oxidase (Gromada & Fiedurek, 1997; Khattab & Bazaraa, 2005; Iftikhar *et al.,* 2010). One mL of MNNG/ethidium bromide solution and 9 mL of Vogel's media containing spores of *A. niger* (1×10^7 spores mL^{-1}) were added in flask and kept in water bath (37°C). After intervals of 30, 60, 90, 120, 150 and 180 min., 1 mL sample was drawn and washed thrice for 15 min. at 10,000 rpm. A dose (after 120 min.) producing 82% kill in case of MNNG and 76% kill by ethidium bromide, was found to be the best.

Selection of mutant: After chemical mutagenesis, 100 fold serial dilutions were prepared and 0.1 mL was spread onto PDA media containing 1% ox gall. It was placed in an incubator at 30°C for till colony formation. Non-treated spores were plated as control. Out of about 1000 colonies, a few mutant derived were isolated on PDA plates to determine glucose oxidase activity. Such, mutant derived colonies; showing bigger zone in comparison to wild type, were further sub-cultured (Petruccioli *et al.,* 1999).

2-Deoxy-D-glucose was used at 1 mg mL^{-1} for selection of mutant derived strains having the potential of enhance production of glucose oxidase. The mutant spores were allowed to grow in PDA at 30°C up to 8 days. The colonies grown were subjected to the glucose oxidase identification (Khattab & Bazaraa, 2005).Mutant derived strains were screened on agar plate containing chromogen and coupling enzyme. The occurrence of reddish/brown color will indicated the presence of glucose oxidase (El-Enshasy, 1998; Khattab & Bazaraa, 2005). Mutant strains having maximum diffusion areas (mm) were scratched and the reaction for enzyme activity was analyzed on spectrophotometer (Hittachi U-1100, Japan) at 460 nm.

RESULTS AND DISCUSSION

In this study, the purpose of mutagenesis was to select the colonies of *Aspergillus niger* which could hyperproduce glucose oxidase enzyme. Mutagenic procedures can be optimized in terms of the type of mutagen and dose.

Kill curve determination: It has been well documented that N-methyl-N-nitro-N-nitrosoguanidine (MNNG) is one of the strong and multi-potential carcinogen that has been frequently reported (Zhu *et al.,* 2000). Chemical mutagenesis of *Cellulomonas* was carried out with ethyl methane sulfonate (EMS) and MNNG as mutagens to obtain a hyper-xylanoytic mutant with 2.5 time higher enzyme production than parent strain when grown on sugarcane bagasse as a carbon source (Lino & Teresa, 1998).

To obtain an instant *A. niger* mutant, 0.15 mg mL^{-1} MNNG for 120 min. dose rate, produced 82% killing. Ethidium bromide (0.5 mg mL^{-1}) produced 76.13% killing/23.87% survival after the exposure of 120 min. These results are in agreement with Witteveen *et al.* (1990) who isolated glucose oxidase overproducing mutants of *A. niger* when conidial survival ranged between 33 and 78%. Chemical mutagenesis of *A. niger* was carried out with ethyl methane sulfonate by Khattab and Bazaraa (2005) and found 52.8 U mL^{-1} glucose oxidase activity (with 14 mm of enzyme zone size).

Selection and evaluation of mutant: The use of ox gall (1%) was found to be optimal for colony restriction and clearance as the colony size was small and showed good ones around colonies. Ox gall is being used as colony restrictor due to the presence of bile salts (Khattab & Bazaraa, 2005). So, all further studies were based on this concentration for the selection of colonies. A few colonies were selected based on large clearance zones than wild type microorganism by using selective marker. It is reported that 2-deoxy-D-glucose is one of the best agent to screen and isolate the mutants with increased glucose oxidase activity (Gromada & Fiedurek, 1997), glucoamylase (Fiedurek *et al.,* 1987) and cellulase (Labudova & Farkas, 1983).

Enzyme diffusion zone analysis is the specific procedure to screen and identify specific mutant based on enzymatic reaction on plate media. The size and intensity of zone color is an index of the formation of glucose oxidase. These results indicated that mutant BCM-8 (MNNG treated for 120 min) 9 mm and BCE-6 (ethidium bromide treated for 120 min) 6 mm with 282 and 202 increased activities (Fig. 1; Table I). However, another test was also employed on the colonies obtained by zone analysis. Petruccioli *et al.* (1995) found the distribution of the irradiated colonies according to the size of halos of glucose oxidase diffusion into the agar plates and 54 colonies showed diffusion halo larger than 7 mm in diameter as compared to parent of 3-5 mm. Malherbe *et al.* (2003) screened for the secretion of biologically active *A. niger* glucose oxidase by selecting the colonies surrounded by a brown halo in the glucose oxidase agar plate assay and were identified as positive.

The glucose oxidase activities of the mutants selected were determined by the violet-blue zone method (Khattab & Bazaraa, 2005). The results demonstrated a very high index between the diameter of the zones formed on screening medium and glucose oxidase activities measured using the spectrophotometric assay, especially in the range of 0-10 mm (glucose oxidase zone). Park *et al.* (2000) also adapted the enzymatic diffusion zone method to select the active mutant for glucose oxidase activity.

The larger and darker zone producing strains were scratched, dissolved in 0.1 M phosphate buffer pH 6, filtered, homogenized and then the reaction for glucose oxidase activity was done spectrophotometerically (Table I).

CONCLUSION

Depending upon these trials, it was suggested that *A.*

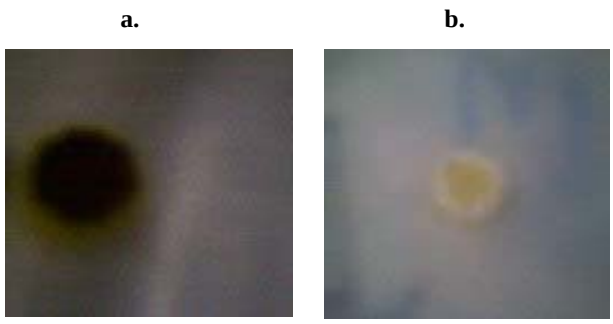
Table I: Analysis of glucose oxidase activity shown by mutant derived strains

A. niger mutant strains	Zone size (mm)	Activity (U mL^{-1})	%age increased activity
Wild type/control	2	3.89	100
BCM-1 (MNNG: 120 min.)	3	2.53	65
BCM-2 (MNNG: 120 min.)	5	5.66	146
BCM-3 (MNNG: 120 min.)	4	4.02	103
BCM-4 (MNNG: 120 min.)	6	5.78	149
BCM-5 (MNNG: 120 min.)	8	10.00	257
BCM-6 (MNNG: 120 min.)	6	8.97	231
BCM-7 (MNNG: 120 min.)	3	4.75	122
BCM-8 (MNNG: 120 min.)	**9**	**10.96**	**282**
BCE-1 (EB: 120 min.)	5	4.33	111
BCE-2 (EB: 120 min.)	5	5.14	132
BCE-3 (EB: 120 min.)	3	1.96	51
BCE-4 (EB: 120 min.)	4	3.52	91
BCE-5 (EB: 120 min.)	2	2.32	86
BCE-6 (EB: 120 min.)	**6**	**7.87**	**202**
BCE-7 (EB: 120 min.)	4	5.01	129
BCE-8 (EB: 120 min.)	5	4.45	115

Key: BCM: MNNG treated BCE: Ethidium bromide treated

Fig. 1: Selection of glucose oxidase hyperproducing mutants by enzyme diffusion zone

a. Enzyme diffusion zone of *A. niger* BCM-8 obtained at 0.15 mg mL^{-1} MNNG for 120 min. **b.** Enzyme diffusion zone of *A. niger* BCE-6 obtained at 0.5 mg mL^{-1} ethidium bromide for 120 min



niger BCM-8 and BCE-6 would be potential mutants for the maximum production of glucose oxidase. So, this enzyme can be used in as an analytical reagent in biochemical, clinical, food or pharmaceutical laboratories/industries.

Acknowledgement: This research paper is a part of Ph.D. thesis work of M.A. Zia. The authors are thankful to Directors of CABB, NIAB and NIBGE for providing lab facilities and technical help.

REFERENCES

Crueger, A. and W. Crueger, 1990. Glucose transforming enzymes. *In*: Forgarty, W.M. and C.T. Kelly (eds.), *Microbial Enzymes and Biotechnology*, 2nd edition, pp: 177–227. Elsevier, New York

El-Enshasy, A.H., 1998. Optimization of glucose oxidase production and excretion by recombinant *Aspergillus niger*. *Ph.D. Thesis*, Biochemical Engineering Department, Gesellschaft für Biotechnologische Forschung mbH (GBF), Braunschweig, Germany

Fiedurek, J., A. Paszczynski, G. Ginalska and Z. Ilczuk, 1987. Selection of amylolytically active *Aspergillus niger* mutants to 2-deoxy-D-glucose. *Zentralbl. Mikrobiol.*, 142: 407–412

Gromada, A. and J. Fiedurek, 1997. Selective isolation of *Aspergillus niger* mutants with enhanced glucose oxidase ptoduction. *J. Appl. Microbiol.*, 82: 648–652

Haq, I., S. Khurshid, S. Ali, H. Ashraf, M.A. Qadeer and M.I. Rajoka, 2001. Mutation of *Asperigllus niger* for hyper-production of citric acid from black strap molasses. *World J. Microbiol. Biotechnol.*, 17: 35–37

Iftikhar, T., M. Niaz, S.Q. Abbas, M.A. Zia, I. Ashraf, K.J. Lee and I.U. Haq, 2010. Mutation induced enhanced biosynthesis of lipases by *Rhizopus oligosporus* var. *Microsporus. Pakistan J. Bot.*, 42: 1235–1249

Jeenes, D.J., D.A. MacKenzie, D.A. Roberts and D.B. Archer, 1991. Heterologous protein production by filamentous fungi. *Biotechnol. Gen. Eng. Rev.*, 9: 327–367

Khattab, A.A. and W.A. Bazaraa, 2005. Screening, mutagenesis and protoplast fusion of *Aspergillus niger* for the enhancement of extracellular glucose oxidase production. *J. Indian Microbiol. Biotechnol.*, 32: 289–294

Labudova, J. and V. Farkas, 1983. Enrichment techniques for the selection of catabolite repression-resistant mutants of *Trichoderma* as producer of cellulase. *FEMS Microbiol. Lett.*, 20: 211–215

Lino, M.R. and P.N. Teresa, 1998. Isolation of a hyperxylanolytic *Cellulomonas flavigena* mutant growing in continuous culture. *Biotechnol. Lett.*, 20: 443–446

Malherbe, D.F., M. Du-Toit, R.R.C. Otero, P. Van-Rensburg and I.S. Pretorius, 2003. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production. *Appl. Microbiol. Biotechnol.*, 61: 502–511

Park, E.H., Y.M. Shin, Y.Y. Lim, T.H. Kwon, D.H. Kim and M.S. Yang, 2000. Expression of glucose oxidase by using recombinant yeast. *J. Biotechnol.*, 81: 35–44

Petruccioli, M., P. Piccioni, F. Federici and M. Polsinelli, 1995. Glucose oxidase overproducing mutants of *Penicillium variabile* (P16). *FEMS Microbiol. Lett.*, 128: 107–111

Petruccioli, M., U.F. Federici, U.C. Bucke and T. Keshavarz, 1999. Enhancement of glucose oxidase production by *Penicillium variabile* P16. *Enzyme Microbial. Technol.*, 24: 397–401

Rowlands, R.T., 1984. Industrial strain development: mutagenesis and random screening procedures. *Enzyme Microbial Technol.*, 6: 3–10

Wang, J., 2008. Electrochemical glucose biosensors-A review. *Chem. Rev.*, 108: 814–825

Witteveen, F.B.C., P. Van De Vondervoort, K. Swart and J. Visser, 1990. Glucose oxidase overproducing and negative mutants of *Aspergillus niger*. *Appl. Microbiol. Biotechnol.*, 33: 683–686

Worthington, C.C., 1988. *Worthington Enzyme Manual: Enzymes and Related Biochemicals*, pp: 155–158. Worthington Biochem. Corporation, USA

Yoon, J., T. Aishan, J. Maruyama and K. Kitamoto, 2010. Enhanced production and secretion of heterologous proteins by the filamentous fungus *Aspergillus oryzae* via disruption of vacuolar protein sorting receptor gene *Aovps10*. *Appl. Environ. Microbiol.*, 76: 5718–5727

Zhu, J.S., Q. Su, J.G. Zhou, P.L. Hu and J.H. Xu, 2000. Study of primary leiomyosarcoma induced by MNNG in BALB/C nude mice. *World J. Gastroenterol.*, 6: 128–130

(Received 03 August 2010; Accepted 13 September 2010)