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Evaluation of Antimicrobial Activity of Glucose Oxidase from *Aspergillus niger* EBL-A and *Penicillium notatum*

Muhammad Anjum Zia[1*], Ayesha Riaz[1], Samreen Rasul[1] and Rao Zahid Abbas[2]
[1]Enzyme Biotechnology Lab.; Department of Chemistry and Biochemistry; University of Agriculture; Faisalabad-Pakistan. [2]Department of Parasitology; University of Agriculture; Faisalabad- Pakistan

ABSTRACT

This work aimed to study the production and purification of glucose oxidase by Aspergillus niger *and* Penicillium notatum *using corn steep liquor as the substrate and evaluate its antimicrobial activity for use in pharmaceutical and food industries. The enzyme was purified by ammonium sulfate precipitation (60-85%), DEAE-cellulose ion exchange and Sephadex G-200 size exclusion chromatography. The crude enzyme extracts* of *A. niger* and *P. notatum showed 2.32 and 5.53 U mg^{-1} specific activities, respectively, which after desalting was 15.52 and 12.05 U mg^{-1}, and after ion exchange and gel filtration chromatography was 29.09 - 62 and 25.72 - 59.37 U mg^{-1} for A. niger and P. notatum, respectively. The antimicrobial activity was determined by disc diffusion method against selected microbial strains where glucose oxidase from A. niger showed anti-bacterial activity, while no fungicidal effects were shown by both* A. niger *and* P. notatum *glucose oxidases.*

Key words: Glucose oxidase, antimicrobial, *Aspergillus niger, Penicillium notatum*

INTRODUCTION

Glucose oxidase is a well-characterized glycosylated flavoprotein, consisting two identical subunits of 80 kDa. Enzymatic oxidation by glucose oxidase reduces FAD to $FADH_2$, releasing H_2O_2 in the presence of O_2. The enzyme has versatile ability to inhibit the growth of microbes through naturally produced hydrogen peroxide. The enzyme is produced by some fungi and insects. It acts as antifungal and antimicrobial agent. Glucose oxidase is generally recognized as safe (GRAS), having several industrial applications (Wong et al. 2008). Jeong et al. (1992) reported bactericidal effects of glucose oxidase in microbial growth of processed poultry meat. *Pseudomonas* and *Salmonella* growth was inhibited resulting into improvement in shelf-life of poultry meat. This property of glucose oxidase has economically strengthened many industries. Antimicrobial effects of glucose oxidase are utilized for food storage and packaging, thus improving the shelf-life of human and animal foods (Vartiainen et al. 2005). Glucose oxidase is extensively utilized in glucose estimation from clinical and biological fluids of human and animals. The enzyme is being used for de-sugaring of dry egg powder and in products of human and animal health-care systems (Kelemen and Lantz 2008).

There are many fungi that secrete a wide range of enzymes responsible of recycling, or lysis of biopolymers from both animals and plant tissues (Musser et al. 2004; Saleemi et al. 2012). Although most of these enzymes are hydrolytic and play important role in fungal nutrition, releasing carbon and nitrogen locked into

[*]Author for correspondence: scientistuaf@yahoo.com

insoluble macromolecules. Such characteristics make the fungi a better host for the production of enzymes and/or proteins (Jeens et al. 1991).

Aspergillus niger, as the most important fungus, is used for the production of food and diagnostics enzymes and many other products (Schuster et al. 2002). *Penicillium variabile* 16 has been characterized for its ability to produce high levels of glucose oxidase when grown on glucose rich media (Crognalea et al. 2008). Although several organisms have been reported for the production of glucose oxidase but *A. niger* and *P. notatum* are used for industrial scale fermentation (Khattab and Bazaraa 2005). Fermentation has been employed for the production of glucose oxidase for several years, providing a bulk of enzyme for biological applications and clinical trials. El-Sherbeny et al. (2005); Sabir et al. (2007) and Rasul et al. (2011) reported the production and purification of glucose oxidase from *A. niger* and *P. notatum*. Pathogenic and non-pathogenic bacteria have been reported as targets of antimicrobial activity of glucose oxidase. Tiina and Sandholm (1988) reported that *E. coli*, *S. aureus*, *S. infantis* and *B. cereus* etc were inhibited by this enzyme (Massa et al. 2001).

Thus, in this study, a comparison of strains *A. niger* and *P. notatum* was made for the production of glucose oxidase and to check the antimicrobial activity of glucose oxidase produced against clinically/industrially important microorganisms by using disc plate method (CLSI 2007).

MATERIALS AND METHODS

Strains culture conditions and inoculums development

Aspergillus niger EBL-A and *Penicillium notatum* strains were obtained from the Enzyme Biotechnology Laboratory, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan. Fungal strains were cultured on PDA (potato dextrose agar) slants at 30°C for 6 days. Cultures were stored at 4°C unless used (Ahmed et al. 2011).

Inocula for *A. niger* and *P. notatum* species were prepared in Vogel's broth as reported by Zia et al. (2010) and Iftikhar et al. (2010). Broth was sterilized at 121°C for 15 minutes and cooled at room temperature. A loopful culture of fungal spores was aseptically transferred to 250 mL Erlenmeyer flasks containing 50 mL broth medium and incubated at 120 rpm for 72 h in an orbital shaker at 30°C. The spores count was carried out using haemocytometer.

Production and analysis of glucose oxidase

Glucose oxidase production was carried out by liquid state fermentation using a medium containing glucose, urea, $CaCO_3$, KH_2PO_4 and CSL for *A. Niger*, for *P. notatum*, the medium contained KH_2PO_4, NH_2NO_3, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $Na_3C_6H_5O_7$, peptone, yeast extract and glucose (Sabir et al. 2007; Rasul et al. 2011). An inoculum size of 5% was added aseptically to Erlenmeyer flasks and incubated at orbital shaker (120 rpm) for 72 h at 30°C (Zia et al. 2010). The crude enzyme was obtained by filtering the fermented biomass and then subjected to centrifugation at 7826 x g at 0°C for 15 minutes. The activity of glucose oxidase was determined at each step of purification by the method of Worthington (1988) where an amount of 0.1 mL enzyme was added to reaction mixture of o-dianisidine. Along with 18% glucose solution, 0.1 mL of peroxidase having 225 U mL^{-1} activity from horseradish was added to reaction. One unit of enzyme activity was defined as micromoles of glucose converted into H_2O_2 per minute in one mL enzyme reaction mixture. Protein estimation was performed using biuret reagent by the method of Gornall et al. (1949).

Purification of the enzyme

Glucose oxidase from both the strains was subjected to ammonium sulfate at 60-85% saturation. It was stirred at 4°C for 4 h, centrifuged at 7826 x g and final pellets contained the glucose oxidase. Desalting of the enzyme was carried out against water at 4°C for 4 h and subjected to enzyme assay (Shin et al. 1993). DEAE-cellulose, an anion exchanger resin was used for the ion exchange chromatography of glucose oxidase. A 2 x 14 cm glass column was packed with DEAE-cellulose resin and equilibrated with potassium phosphate buffer pH 6.0 at 4°C (Zia et al. 2011). A total of 100 fractions were collected after application of 1.5 mL of desalted enzyme. Sephadex G-200 was used to elute the desired enzyme having highest specific activity (Sukhacheva et al. 2007). All the fractions collected were subjected to enzyme analysis and protein determination (Worthington 1988).

Analysis of antimicrobial activity

The antibacterial and antifungal effects of glucose

oxidase were analyzed by disc diffusion method. The test strains were *A. niger*, *A. flavus*, *Escherichia coli*, *Staphylococcus aureus* and *Pasteurella multoida*. *E. coli* and *S. aureus* were cultured on nutrient agar, while *A. niger* and *P. notatum* were grown on PDA. Round shaped filter paper discs of 1.5 cm diameter placed on the microbial cultures, were applied with 100 µL purified glucose oxidase. Incubation of loaded plates at 37°C for 24 h resulted in the formation of inhibition zones measured by zone reader (Hang et al. 2000; Choi et al. 2012).

RESULTS AND DISCUSSION

Production of glucose oxidase

Crude preparation of glucose oxidase from *A. niger* EBL-A and *P. notatum* showed 11.99 and 21.105 U mL^{-1} activity, respectively with 2.32 U mg^{-1} and 5.53 U mg^{-1} of specific activity. Bodade et al. (2010) investigated the production of glucose oxidase by *P. chrysogenum* SRT 19 using glucose as carbon source and reported 0.67 U mL^{-1} enzyme activity. Khursid et al. (2011) reported glucose oxidase activity of 2.96 U mL^{-1} after fermentation by *A. niger*. These activities were much lower than obtained in the present work. Kelly and Reddy (1986) reported glucose oxidase with 0.17 U mg^{-1} specific activity obtained from *P. chrysosporium*. Khattab and Bazaraa (2005) used *A. niger* and *P. notatum* for the production of glucose oxidase and utilized successfully as an antimicrobial agent against various disease causing agents.

Purification of glucose oxidase

The crude extracts of both enzymes were subjected to ammonium sulfate precipitation at 60-85% saturation. Enzyme fractions after 85% saturation showed 4.56 and 9.9 U mg^{-1} specific activity of the enzyme isolated from *A. niger* and *P. notatum*, respectively. Desalting of the enzyme resulted in an increase in specific activity to 15.52 and 12.05 U mg^{-1} respectively (Fig. 1). These results were in accordance with Khurshid (2008), who purified glucose oxidase by ammonium sulfate precipitation and reported an increase in enzyme activity by increasing ammonium sulfate concentration.

Results of specific activity of glucose oxidase after DEAE-cellulose treatment are presented in Figure 2. Glucose oxidase by *A. niger* and *P. notatum* showed specific activity of 29.09 and 25.72 U mg^{-1}, respectively. Singh and Verma (2010) reported the production of glucose oxidase by *A. niger* and obtained 3.3 U mg^{-1} specific activity of the enzyme after PEG based two-phase isolation system. After gel filtration chromatography, specific activity of enzyme was 62.0 and 59.37 U mg^{-1} by *A. niger* and *P. notatum*, respectively (Fig. 3). Khurshid (2008) showed an increase in enzyme activity from 11.9 - 37.42 U mL^{-1} after purification by gel filtration chromatography. El-Sherbeny et al. (2005) purified glucose oxidase by Sephadex G-200 treatment and obtained 65.203 U mg^{-1} specific activity. There was a small difference between the specific activity in these studies, which could be due to difference in strains and pH. However, specific activity of the enzyme increased after every step of purification as shown in Figures 1-3.

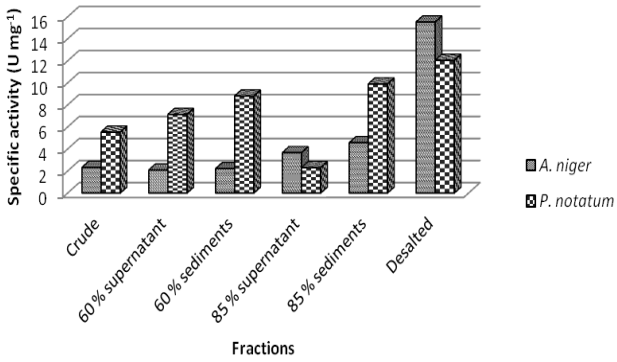

Figure 1 - Specific activity of glucose oxidase from *A. niger* and *P. notatum* after ammonium sulfate precipitation.

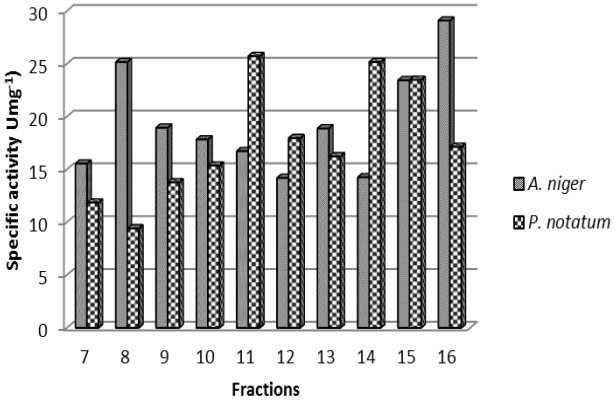

Figure 2 - Specific activity of glucose oxidase after ion exchange chromatography.

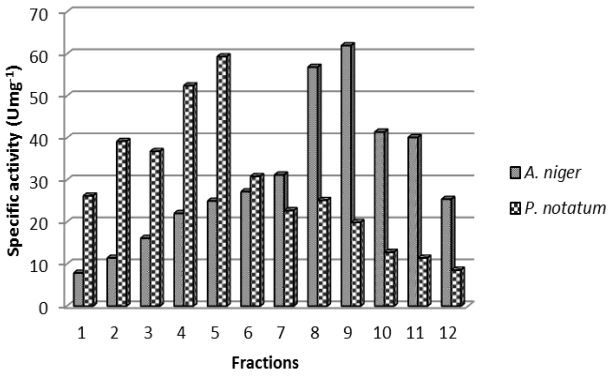

Figure 3 - Specific activity of glucose oxidase after gel filtration chromatography.

Fractions with high specific activities were pooled and applied for the determination of antimicrobial effect of glucose oxidase on bacterial and fungal strains. Specific activity of the purified enzyme was appreciably higher than crude fractions from both the sources while the fold purification of glucose oxidase from *A. niger* was 2.5 times higher than *P. notatum*. The results are summarized in Table 1. The antimicrobial activity was determined using the purified glucose oxidase, as the impurities in the crude extract could interfere with the antimicrobial effects of the enzyme. In addition, some non-enzymatic agents might be present in the crude enzyme suspensions that could mimic the enzymatic antimicrobial activity, thus end up in false positive results (Ahmadi 2012; Ahmad et al. 2012).

Table 1 - Stepwise increase in the purity of glucose oxidase from *A. niger* and *P. notatum.*

Purification step	Specific activity (U mg^{-1})		Fold purification	
	A. Niger	***P. notatum***	***A. niger***	***P. notatum***
Crude extract	2.32	5.53	1	1
Desalted enzyme	15.52	12.05	6.69	2.17
Ion exchange	29.09	25.72	12.53	4.65
Gel filtration	62	59.37	26.36	10.73

Antimicrobial activity of glucose oxidase

Incidence of various microbial infections has consistently increased in human and animal populations. However, the resistance against the conventional antibiotics has also increased due to prolonged antimicrobial therapies. Proteins such as glucose oxidase with potential antimicrobial activity are ubiquitously present in a variety of microorganisms. *A. niger* and *P. notatum* are potent producers of glucose oxidase, having momentous bactericidal and fungicidal effects (Leiter 2004).

P. multocida and *S. aureus* are pathogenic bacteria causing avian cholera and human infections such as pimples, bacteraemia and sepsis. The purified glucose oxidase by *A. niger* had bactericidal action on both of these pathogenic microbes; while no such effect was noticed for *E. coli.* Glucose oxidase obtained after purification by *P. notatum* was effective against *S. aureus*, showing no effectiveness against *E. coli* and *P. multocida*. The investigations on the antimicrobial effects of glucose oxidase revealed its property to produce H_2O_2 in the presence of oxygen. Acidity of the medium is due to gluconic acid produced non-enzymatically from δ-D-gluconolactone and the decrease in pH ultimately results in fungicidal effects (Bradshaw 2011). Mundo et al. (2004) and Leiter et al. (2004) reported growth inhibition of *P. chrysogenum* and *A. niger* by glucose oxidase.

As reported earlier, glucose oxidase has a strong capacity to work as antifungal and antibacterial agent. An antibiotic talaron produced by fungus *Talaromyces flavus* contained 40% of its preparation as glucose oxidase (Kim et al. 1990). *In vitro* antimicrobial activity of the culture was examined against clinical bacterial isolates namely, *Bacillus subtilis* and *E. coli*. It was observed that glucose oxidase inhibited the growth of *B. subtilis* and *E. coli* (Onyegeme-Okerenta et al. 2009). Vartiainen et al. (2005) reported the inhibition of *B. subtilis* and *E. coli* strains by glucose oxidase. Table 2 shows the effect of glucose oxidase from two different sources on bacterial and fungal strains; while Table 3 represents the mathematical signs and grading of the zone size for the antimicrobial activity of glucose oxidase. Glucose oxidase from both the sources was examined for antifungal and antibacterial effects by using 100 µL glucose oxidase.

Table 2 – Effect of glucose oxidase as an antibacterial and antifungal agent.

Source of glucose oxidase	Bacterial strains			Fungal strains	
	E. coli	*P. multicotida*	*S. aureus*	*A. niger*	*A. flavus*
A. niger	-	+	+	-	-
P. notatum	-	-	+	-	-

Table 3 - Grading of results for antimicrobial activity of glucose oxidase by disc diffusion.

Serial no.	Mathematical sign	Zone size	Interpretation
1.	-	0.0-5.0	No or poor activity
2.	+	1-11	Activity present
3.	++	12-20	Moderate activity
4.	+++	21-30	Strong activity
5.	++++	31-40	Highly strong activity

Pure enzyme was introduced on the discs placed on the fungal and bacterial cultures. A major effect of glucose oxidase from *A. niger* were observed against *P. multicida* and *S. aureus*; while no antifungal affects has been displayed by the enzyme. In case of glucose oxidase by *P. notatum*, inhibition of *S. aureus* was observed only.

CONCLUSIONS

It was concluded that glucose oxidase from *A. niger* was more effective against bacterial strains as compared to *P. notatum*. These findings showed that glucose oxidase from different sources could have difference in their effects against different bacteria, or fungi and further studies should be carried out towards the development of new enzyme -drugs.

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