Characterization of glucose Oxidase from *Penicillium chrysogenum* MDC 8358: Prospects for application in food industry

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ABSTRACT

Backgrounds: Food enzymes play a crucial role in enhancing specific food attributes, such as improving texture, eliminating toxins and allergens, producing carbohydrates, and enhancing flavor and appearance characteristics. Glucose oxidase (GOX) catalyzes the oxidation of glucose to produce gluconic acid and hydrogen peroxide, making it applicable in various practical scenarios. This enzyme could be utilized to eliminate glucose, thereby facilitating the creation of functional food suitable for individuals with diabetes and glucose intolerance. Our main goal was to characterize GOX from a *Penicillium chrysogenum* strain isolated from Armenian ecosystems.

Methods: GOX of P. *chrysogenum* MDC 8358 was obtained by surface fermentation using glucose as a carbon source. The enzyme was purified 230-fold to homogeneity using a three-step purification scheme consisting of ion exchange, adsorption, and gel filtration chromatography.

Results: The specific activity of the purified enzyme reached 271.2 U/mg. GOX from *P. chrysogenum* MDC 8358 has shown to be dimeric with a molecular weight of 135.5 kDa, consisting of two equal subunits with a molecular weight of 70 kDa. Isoelectric focusing has revealed a pH of 4.6. The enzyme has displayed a temperature optimum of 40 °C, and a pH optimum of 6.8, being more thermostable at acidic pH. Characterized GOX was highly specific for β-D-glucose and has shown only minor activities to mannose, xylose, and galactose.

Keywords: *Penicillium chrysogenum* MDC 8358, surface fermentation, glucose oxidase, purification, characterization, functional food preservation

Characterization of GOX of *Penicillium chrysogenum* MDC 8358
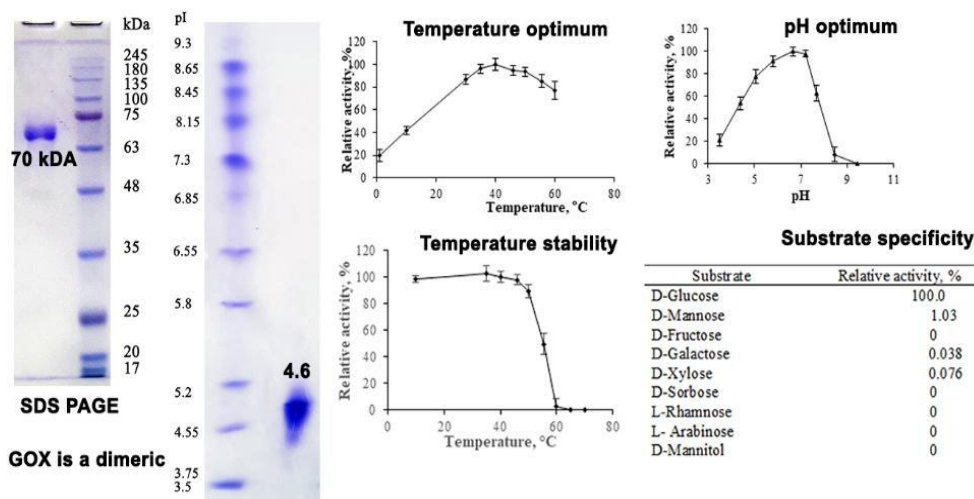
INTRODUCTION

The global enzyme market has been growing over the past few decades, as enzymes are preferable to chemicals because they are eco-friendly and sustainable alternatives. Glucose oxidase (GOX) is an industrially relevant enzyme which has promising applications in diverse fields of industries such as the food, medical, and pharmaceutical, textile and powder industries, etc. [1-3]. In contemporary applications, GOX is frequently employed in conjunction with other enzymes to achieve specific outcomes. For instance, the combination of GOX with tyrosinase is utilized for the analysis and differentiation of musts and wines [4]. The synergistic usage of GOX with α-amylases and xylanases enhances the quality of dough and bread products [5]. In medical contexts, GOX is paired with peroxidase for precise glucose level measurements in blood, saliva, and tears [5-6]. In recent years, glucose oxidase (GOX) has found novel applications in biosensors [7]. With a predicted annual growth rate of 7.6% per annum, the market value of the GOX increased to $6 billion by 2011 [3].

Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4.) catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide using molecular oxygen as electron acceptor. This enzyme was first obtained from *Aspergillus niger* mycelium by Muller in 1928, and from *Penicillium chrysogenum* cultural liquid by Coulthard and coauthors in 1945 [8]. In later case the enzyme was obtained by surface and submerged fermentation [9].

GOX is predominantly produced by fungi and insects. Among the identified species, a total of 50 distinct organisms were characterized to produce GOX, with 45 of these belonging to the fungi category and the remaining five originating from insects [10]. GOXs from different sources were isolated, purified and

characterized [11-16], whereas the GOX genes, that have been reported so far, were mainly from *Aspergillus* and *Penicillium* species, such as *Aspergillus niger* [17-19], *Aspergillus tubingensis* [20], *Penicillium amagasakiense* [21], *Penicillium notatum* [1, 22-23], *Penicillium variabile* P16 [24], *Penicillium funiculosum* [25-26], *Penicillium sp.* MX3343 [27], etc., while cold active glucose oxidase encoding gene (CngoxA) from *Cladosporium neopsychrotolerans* SL16 [28] and *Goffeauzyma gastrica* [29] have been cloned and the corresponding enzymes have been characterized recently.

The applicability of GOX primarily depends on its characteristics. Numerous research efforts have concentrated on discerning the fungal strains, which produce GOXs most suitable for specific application such as biosensor development, clinical investigations, and biochemical diagnostic tests, etc. [3]. An essential application of GOX lies in the realm of food preservation. It operates by oxidizing glucose in food products, generating gluconic acid and hydrogen peroxide. Notably, hydrogen peroxide has certain antibacterial and antimicrobial growth effects. It can inhibit the reproduction of microorganisms as well as mold, and thereby contributing to food preservation [30-32]. This enzyme may discover novel applications, as it has demonstrated the capability to stimulate the mobilization of long-term repopulating hematopoietic cells [33]. Recently, there has been a growing interest in functional foods [34], characterized by the inclusion of functional ingredients like biologically active compounds and probiotics. Many biologically active compounds are sensitive to heat (thermolabile), and the preservation of those compounds is critical in food production where traditional heat treatments such as pasteurization are commonly applied [35]. Therefore, in the production of functional foods, employing enzymes as a preservative presents an economical and environmentally friendly

alternative. This approach helps maintain the nutritional and sensory quality of the functional food. Moreover, with this enzyme it will be possible to selectively remove or regulate the amount of glucose in food. The widespread applications of glucose oxidase (GOX) have prompted extensive research aimed at improving its production and characterization to enhance stability, facilitating long-term usage. Currently, there are issues concerning production cost, purification difficulty, and low productivity, etc. To overcome these obstacles, molecular biology techniques as well as different heterologous expression systems have been developed. Moreover, a hybrid GOX with improved stability and catalytic efficiency has been created by combining elements from both *A. niger* and *P. amagasakiense* GOXes [36].

Production of GOX of *Penicillium* and *Aspergillus* using different expression systems, such as *E. coli*, *Saccharomyces cerevisiae, Pichia pastoris*, and *Yarrowia lipolytica* have been reported [17, 24, 27, 37-39]. *E. coli* expression system was not appropriate for GOX production as 60% of the recombinant protein was inactive [38]. The use of *Saccharomyces cerevisiae* and *Pichia pastoris* expression systems has proven advantageous in overcoming challenges associated with the E. coli expression system, including issues like the formation of inclusion bodies and non-glycosylation of recombinant GOX [24, 40-41]. However, these expression systems come with their own set of limitations, such as the potential for over-glycosylation of the recombinant protein and the absence of a cost-effective cultivation medium. Among available yeast expression systems, *Y. lipolytica* is most attractive for recombinant GOX production [39].

Despite the widespread use of heterologous expression systems for large-scale enzyme production, the extraction of glucose oxidase (GOX) from wild-type

strains remains a topic of great interest. Optimization of fermentation conditions as well as different purification strategies is being developed for obtaining an enzyme preparation with high purity and yield. Based on our knowledge glucose oxidases from *Penicillium chrysogenum* were purified only from cultural liquids [13, 42-44]. In this paper we report a simple method of obtaining of glucose oxidase of *Penicillium chrysogenum* MDC 8358 in nearly homogeneous state and characterize its some properties.

MATERIALS AND METHODS

Strains and maintenance: *Penicillium chrysogenum* MDC 8358 strains of Microbial Depository Center of Scientific and Production Center "Armbiotechnology" National Academy of Science of Republic of Armenia were used in this research. The strain was maintained on Czapek-Dox agar medium [8] and subcultured at 30-40-day intervals, single-spore isolations being carried out at intervals.

Preparation of seed material: Two different seed materials, namely, fungus mycelium cultivated on Czapek-Dox agar slants and aged spores grown on barley under 60% humidity conditions, were evaluated for their impact on glucose oxidase production. Both seed materials yielded comparable outcomes, and for the ease of procedure, aged *P. chrysogenum* spores were used as seed material at final concentration corresponding to 0.1 optical densities (OD$_{520}$). To obtain aged spores, a sterile substrate was inoculated with 7-8-day-old sterile mycelium of *P. chrysogenum* and placed in microbiological tubes sealed with cotton-wool plugs. The culture was incubated at a temperature of 28-30°C for 4-5 days and subsequently aged in a refrigerator at 4°C for 10-20 days. The formation of *P. chrysogenum* glucose oxidase was investigated under the conditions of flask fermentation of the fungus by the surface method, using as an inoculum an aqueous suspension of spores of the fungus grown on the above substrates.

Fermentation: Fermentation was done in cottonwool plugged 500 ml Erlenmeyer flasks filled with 100 ml liquid medium A of following composition (g/L): glucose – 100, NaNO$_3$ – 20, KH$_2$PO$_4$ – 5, MgSO$_4$ x 7H$_2$O – 0.5, KCl – 0.5, CaCO$_3$ – 5, FeSO$_4$ x 7H$_2$O – 0.01, pH 6.8 – 7.0, prepared on tap water. The fermentation process was done in static conditions, at temperature 28-30 °C. At the end of fermentation, the mycelium was wrung out, and the resulting liquid was combined with the culture liquid, filtered through multilayer gauze, and stored for future procedures.

Kept in a cool place, free from mycelium, and saturated with chloroform, culture filtrates retain glucose oxidase activity for periods of at least 3 months.

Enzymatic assay: Measurement of glucose oxidase activity was carried out based on the chromogenic peroxidase reaction using the modified Trinder method [45]. The reaction mixture (1.5 ml) contained of the final volume: 0.3 mM 4-aminoantipyrine; 5 mM phenol, 50 mM D-glucose, 1 unit horseradish peroxidase; 100 mM Na-acetate pH 5.8. The reaction was initiated by adding an aliquot of the sample of the enzyme solution studied. The amount of enzyme that catalyzes the formation of 1 μmol of hydrogen peroxide per 1 min was taken as a unit of enzymatic activity.

The protein concentration was measured by the method of Groves and Davis, by absorption difference at a wavelength of 224 and 236 nm [46].

Enzyme purification: Isolation and purification of GOX was carried out at a temperature of 4 °C. At the first stage of purification the cation exchange chromatography on SP-sephadex C-50 in hybrid mode (batch-column) was used. For this purpose, the pH of the filtrate containing

GOX activity was lowered to 3.3-3.5, dry powder of SP-sephadex C-50 was added (0.5 g for 10000 U of GOX) and left overnight with constant stirring in at 4 °C. On the next day the supernatant was decanted, and the resin was placed in a Whatman column (2.5 cm diameter) preloaded with 4 cm of same resin in 20 mM acetate buffer, pH 4. The column was washed with 20 mM Na-acetate buffer, pH 4, until the optical density of effluent was less than 0.05 at 280 nm. Then glucose oxidase was eluted from the column with 50 mM Na-phosphate buffer, pH 6.5. Active fractions were combined.

In the second step of purification the combined active fractions of GOX were applied to a hydroxyapatite column (2.5 x 7 cm). Absorbed proteins were eluted with a 0 - 0.3 M linear gradient of Na-phosphate, pH 6.7. The active fractions were combined and concentrated on MWCO 10000 centrifugal ultrafilters (Amicon Ultra Centrifugal Filters, EMD Millipore, USA) by centrifugation at 6000 rpm for 30 minutes on Bakman centrifuge and applied for gel filtration (third stage of purification) on Toyopearl 60 F (2.5 × 85 cm). 0.1 M NaCl containing 20 mM acetate buffer, pH 6.0 was used for column equilibration and protein elution. The active fractions were combined and concentrated by centrifugal ultrafilters in above mentioned condition and stored in 50 % glycerol solution at temperature -20 °C for future use.

Molecular weight determination: The molecular weight of the enzyme was determined by gel filtration and SDS electrophoresis methods. For gel filtration a Toyopearl 60F resin was used. The column was calibrated using the following proteins with known molecular weights: ferritin (450 kDa), catalase (250 kDa), bovine serum albumin (67 kDa), egg albumin (45 kDa), cytochrom C (12.3 kDa).

SDS PAGE electrophoresis was carried out in 12.5 % polyacrylamide gel in presence of 0.1 % SDS and at pH 8.9 by Pharmacia protocol [47] using Roti®Mark Tricolor prestained protein markers as standards.

Zymogram: Native disk-electrophoresis of GOX in 7.7% polyacrylamide gel (PAGE) with a Laemli buffer system, according to the instructions proposed by Pharmacia [47]. One part of the gel was stained for protein by Coomassie diamond blue R-250 [47], second part was stained by the activity to detect enzyme activity, gels were immersed in a solution containing 88 mM glucose, 0.5 mM diaminobenzidine, and 1 u/ml peroxidase, pH 5.8, for 30 min, and then incubated at 30 °C for 2-3 h until a brightly colored band appeared.

Isoelectric point determination: The isoelectric point of GOX was determined by isoelectric focusing in 5 % polyacrylamide gel plates using the Pharmacia protocol [47]. The 1.6 % of pharmalytes (Sigma-Aldrich, USA) in gel mass, with pH 3.0-10.0 was used for pH adjustment in the course of electrofocusing. Filter papers impregnated with 0.25 M NaOH and 0.25 M H_2SO_4 were placed under cathode and anode, respectively, in contact with the top and bottom parts of the gel plate. The wide range Pharmacia isoelectric point markers of following pI spectrum were used: trypsinogen (pI 9.30), lentil lectin basic group (pI 8.65), lentil lectin neutral group (pI 8.45), lentil lectin acidic group (pI 8.15), myoglobin basic group (pI 7.35), myoglobin acidic group (pI 6.85), human anhydrase (pI 6.55), bovine anhydrase (pI 5.85), β-lactoglobulin A (pI 5.20), soybean trypsin inhibitor (pI 4.55), amyloglucosidase (pI 3.50).

Substrate specificity: For substrate specificity determination the enzyme activity was determined in the presence of the following substrates (50 mM): D-glucose, D-manose, D-fructose, D-galactose, D-xylose, D-sorbose, I-rhamnose, I-arabinose, D-mannitol as described above method.

Optimal pH and temperature value: To determine pH optimum the above-mentioned reaction medium was used, changing only the initial buffer (50 mM citrate-, phosphate-, and tris-containing buffers were used, the pH of which was adjusted by adding concentrated solutions of HCl or NaOH, respectively). The pH range of 3.5-9.5 was examined. To determine the effect of temperature, the activity of the enzyme was measured in the temperature range of 2- 60 °C.

Thermal stability: To determine thermostability the enzyme was incubated at different temperatures in the range from 10 °C to 70 °C for 5 min, then the samples were quickly cooled in an ice bath, after which the residual activity was measured. To study the dependence of the thermostability of the enzyme on pH, the enzyme was incubated at 55 °C, at different pH values in the range of 3-11 for 5 min, after which it was cooled on an ice bath and the residual activity of the enzyme was measured.

RESULTS

The strain *P. chrysogenum* MDC 8358 was used in this study. Under surface fermentation conditions at 28-30 °C and pH 6.8-7.0, the strain exhibited significant enzyme production, with GOX activity ranging from 15 to 25 U/mL in the culture liquid within a fermentation period of 9-18 days. However, as the fermentation duration was prolonged, a decline in GOX activity was observed.

In this study, we have successfully developed a purification scheme for glucose oxidase (GOX) from the culture liquid of *P. chrysogenum* MDC 8358. The purification process involves sequential ion exchange, adsorption, and size exclusion chromatographies, resulting in a high-purity enzyme preparation with significantly enhanced specific activity. At the first stage of purification the hybrid method of ion exchange chromatography was applied. The batch method was used for the binding of GOX, followed by packing the solid phase with the bound enzyme onto a column. Afterwards washing and elution steps were done in the column. After elution all fractions have been analyzed based on the protein content and activity. Fractions containing GOX activity was combined and applied to adsorption and size exclusion chromatographies.

Table 1 presents the results of purification, demonstrating a remarkable improvement in the specific activity of GOX. In the result of purification, the specific activity of GOX increased by over 100 times, reaching 271.2 U/mg, with more than 45 % of total yield.

Table 1. Scheme of purification of GOX isolated of *P. chrysogenum* MDC 8358

Purification stage	Volume, ml	Protein, mg	Specific activity, U/mg	Yield, %
Filtrate	370	2035.0	2.6	100.0
SP-sepharose C-50	17	31.5	102.0	64.7
Hydroxyapatite	37	18.1	188.0	60.4
Toyoperl 60	1	8.8	271.2	45.1

Researchers have developed various purification schemes for isolating and purifying glucose oxidase (GOX) from different sources. The purification scheme proposed by Zia and coauthors include ammonium sulfate precipitation, DEAE-cellulose ion exchange chromatography and Sephadex G-200 size exclusion chromatography. This purification schema was successfully applied to purify GOX from *Aspergillus niger*

and *Penicillium notatum*. Following the purification steps, GOX from *A. niger* and *P. notatum* experienced significant purification with purification folds of 26 and 10, respectively. The specific activity of GOX from *A. niger* increased to 62 U/mg, while GOX from *P. notatum* reached a specific activity of 59.4 U/mg after purification [48]. Three-step purification scheme was applied to purify glucose oxidase (GOX) from *Penicillium notatum*, resulting in an enzyme with a specific activity of 62.4 U/mg [23]. Remarkably, the specific activity of the purified GOX obtained by us is significantly higher than that reported in the mentioned studies.

Fig. 1 demonstrates the gel filtration analysis, indicating that the molecular weight of GOX is approximately 135.5 kDa. In contrast, Fig. 2, which presents the SDS-PAGE results, shows a molecular weight of 70 kDa for GOX. By comparing these data, we conclude that GOX exists as a homodimer, comprising two identical subunits, each with a molecular weight of approximately 70 kDa. Furthermore, Fig. 2 provides additional information for the homogeneity of the GOX, suggesting a high level of purity in the enzyme preparation.
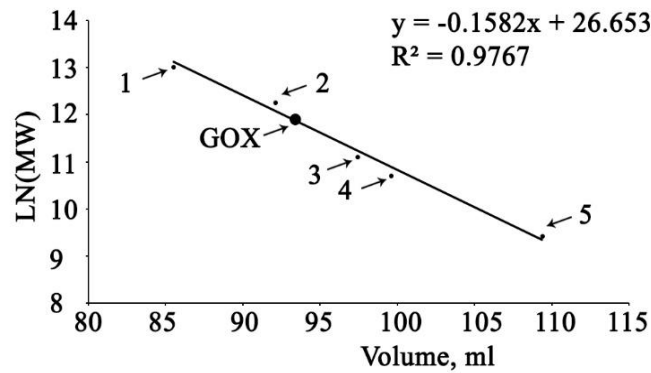

$$y = -0.1582x + 26.653$$
$$R^2 = 0.9767$$

Figure 1. Determination of molecular weights of GOX of *P. chrysogenum* MDC 8358 by gel filtration. 1) ferritin (450 kDa), 2) catalase (240 kDa), 3) bovine serum albumin (67 kDa); 4) egg albumin (45 kDa); 5) cytochrom C (12.4 kDa).
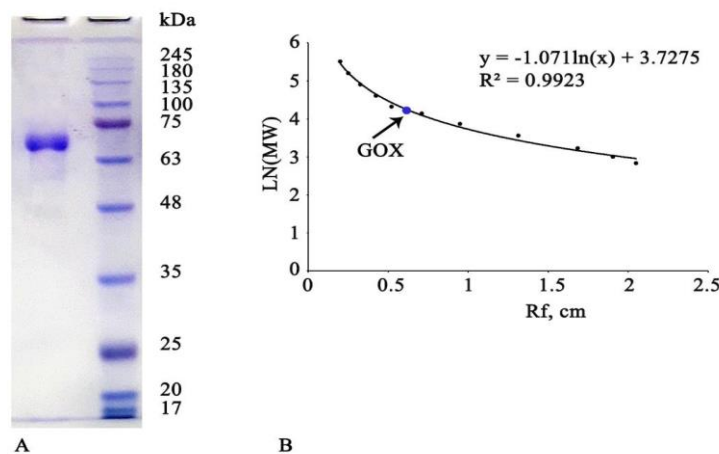

$$y = -1.071\ln(x) + 3.7275$$
$$R^2 = 0.9923$$

Figure 2. Determination of molecular weight of subunits of GOX *P. chrysogenum* MDC 8358 SDS-PAGE in 12.5 % gel. A) The gel stained by Coomassie diamond blue R250, B) calibration curves of known molecular weight proteins.

The results of native PAG electrophoresis and zymogram analysis are presented in Fig 3. As follows from Fig. 3A there are three protein bands in native PAGE plate, while in the SDS PAGE plate (Figure 2A) only one protein band is visible. Moreover, in Fig. 3B where the gel was stained for enzymatic activity with diaminobenzidine again three bands are presented. The results of the work allow us to conclude that in the native state, protein molecules can form dimers, tetramers, etc. Similar results have been obtained Ahmadifar and coauthors identifying more than one protein and activity bands in native PAGE for GOX enzyme [49]. The results of determination of isoelectric point are presented in Fig. 4. The isoelectric points of enzyme appear to be 4.6.
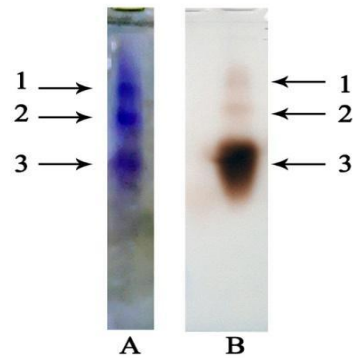

Figure 3. Native disk-electrophoresis and zymography of GOX *P. chrysogenum* MDC 8358 in 7.5% polyacrylamide gel (PAGE). A) the gel was stained for protein by Coomassie diamond blue R250, B) the gel was stained for enzymatic activity with diaminobenzidine.
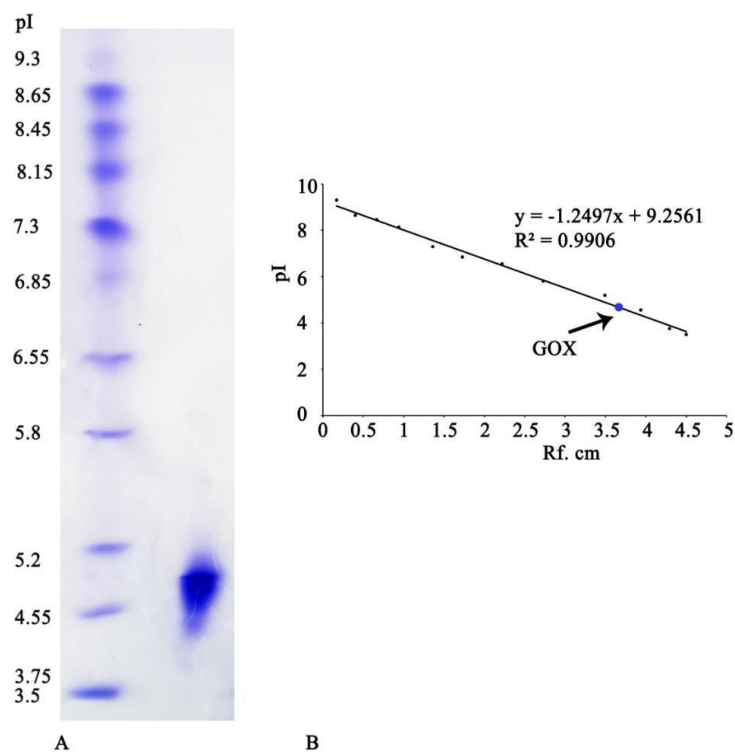

Figure. 4. Determination of isoelectric points of GOX of *P. chryzogenum A*) The gel stained by Coomassie diamond blue R250, B) calibration curve for determination of isoelectric points.

The substrate specificity test indicates that GOX *P. chryzogenum* in the presence of natural sugars exhibits almost exceptional specificity in relation to D-glucose (Table 2). Of all investigated hydrocarbons, only mannose, xylose, and galactose reliably react with GOX and the GOX activity in their presence is ~ 1%, as compared to D-glucose. Characterised GOXs are highly specific for D-glucose and show only marginal activities with other sugars.

Table 2. Substrate specificity of GOX of *P. chrysogenum* MDC 8358

Substrate	Relative activity %
D-Glucose	100.0
D-Mannose	1.03
D-Fructose	0
D-Galactose	0.038
D-Xylose	0.076
D-Sorbose	0
L-Rhamnose	0
L- Arabinose	0
D-Mannitol	0

In Fig. 5A shows the temperature dependence of GOX activity. The enzyme activity increases linearly to a maximum at 40 °C, after which it slowly decreases due to thermal inactivation of the enzyme. In Fig. 5B shows that the enzyme exhibits maximum activity in the pH range 5-7.5, with a peak at pH 6.8. Based on the results obtained (Fig. 5C), it can be concluded that after 5 min of incubation in the temperature range up to 50 °C, the enzyme is sufficiently thermostable at 55 °C, 50% of the initial activity is lost.

During the incubation of the enzyme at a temperature of 55 °C (Fig. 5D), more than 70% of the activity remains in the pH range 3.6 - 6. In the alkaline pH range, the enzyme is rapidly inactivated. When GOX was stored in a phosphate buffer pH 6.5 at 4 °C for a year, its activity did not change.
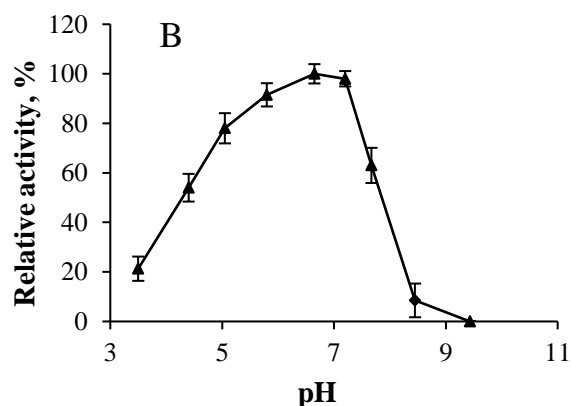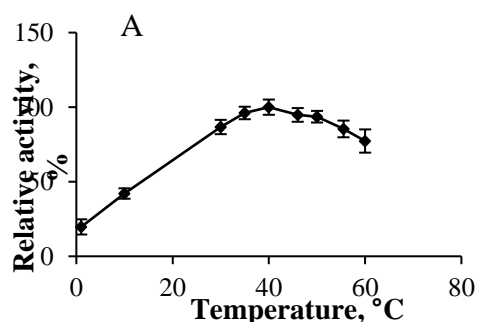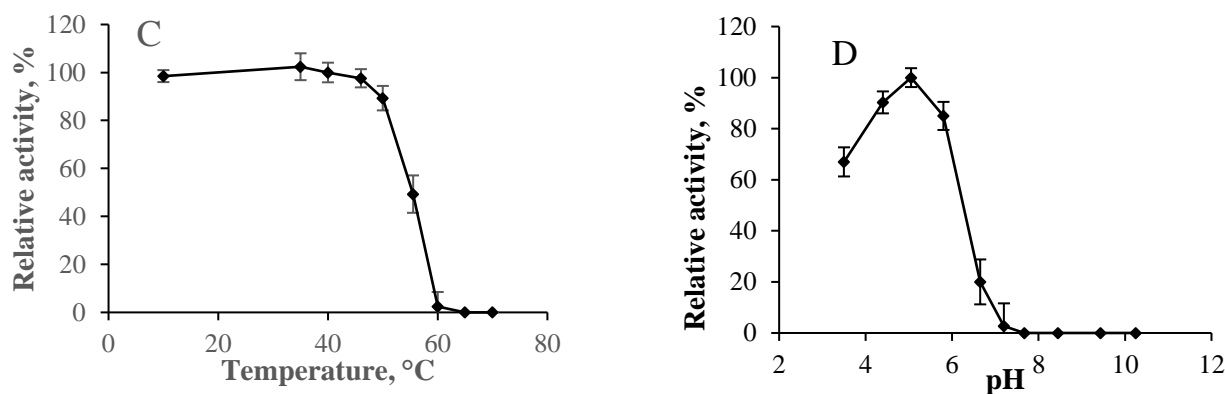
Figure 5 A, B) The temperature and the pH dependence of GOX *P. chrysogenum* MDC 8358 activity; C, D) the thermal stability and the thermal stability on the pH dependence of GOX *P. chrysogenum* activity.

Storing the GOX enzyme from *P. chrysogenum* MDC 8358 in a phosphate buffer at a temperature of 4 °C and pH 6.5 for a year did not result in any alteration in its activity. Nevertheless, a more accurate approach for storage would involve placing the enzyme in either an acetate buffer at pH 5 or in a lyophilized state.

DISCUSSION

Glucose oxidase is an enzyme that has widespread applications in industry and biotechnology. GOX is primarily produced by fungi. Despite the widespread and diverse use of this enzyme it has been reported that only 27 sequences are available in databases, which have been divided into three main groups: The two sequences belongs to *Aspergillus* species, which are sharing 85% identity, next are the sequences of GOX belonging to *Penicillium* and *Talaromyces* species with 63–67% identity to *A. niger* (with one exception), and last ones are all other sequences belonging to other species, which have only 27–35% identity (ref). Thus, the GOX isolated and characterized in this study belongs to the second group. In the present work, GOX from *P. chrysogenum* MDC 8358 was isolated and characterized. The experimental work thus indicates an economically feasible process for GOX production using surface fermentation. It was found that the maximum formation of GOX was noted when spore inoculum obtained from oat and wheat grains was used. Despite it was possible to obtain about 25 U/mL enzyme activity in culture liquid, we tend to think, that the production rate has a potential to be increased by optimization of media composition and culture conditions. Industrially, the most important producers of GOX are species belonging to the genera *Aspergillus* and *Penicillium.* GOXs are being produced by solid state fermentation and submerged fermentation. For compression it was reported that *Penicillium* strains accumulate GOX activity in culture liquid [44, 50] while GOX of *Aspergillus* is intracellular [51-52]. *P. chrysogenum* SRT19 strain accumulated 1.702 U/mL of GOX activity in the submerged fermentation conditions after 72 h of incubation period (at 20° C and pH 6) [44].

P. chrysogenum MDC 8358 GOX was successfully purified from the culture supernatant using an approach aimed at simplifying the process by minimizing purification steps.

Purified GOX has a molecular weight of 135.5 kDa consisting of two identical subunits. The molecular weight of characterised GOXs ranges from approximately 130 to 175 kDa. All characterised GOXs from ascomycetes are a dimeric glycoprotein consisting of two identical subunits [18] Enzymes of *A. niger* and *P. amagasakiense*

have been 152 kDa and 150 kDa, respectively determinate by sedimentation equilibrium method [53]. Similar size has been obtained for GOX of *Penicillium chrysogenum* according to the gel filtration data [42]. It needs to be pointed out that the molecular weight of recombinant GOXs strongly depends on the glucalization level. Thus, recombinant GOX produced in yeast expression system has higher molecular weights than the native enzyme, which is probably due to the hyperglycosylation [54]. Furthermore, when the glycosylation was removed using Endoglycosidase H, the molecular weight of GOX of *Cladosporium neopsychrotolerans* SL16 was significantly changed reaching to approximately 68 kDa from 120 kDa [55]. This is a good agreement with the results obtained for GOX of *Penicillium variabile* P16 conforming that GOXs are highly glycosylated proteins [24].

We have shown that GOX of *P. chrysogenum* MDC 8358 consists of two identical subunits with 70 kDa molecular weight. The pI of GOX was 4.6. The results obtained in this study exhibit both disparities and parallels when compared to the findings outlined in the existing literature. Thus, the GOX characterized from *P. chryzogenum* has similar pI (4.2) and subunit molecular weight (72 kDa), but there is noticeable deference between gel filtration results (MW= 175 kDa) [13]. Concerning the GOX of *Penicillium chrysogenum* NCAIM 00237 the published enzymological characteristics (MW=155 kDa, subunit MW=76 kDa, pI=5.4) were quite different from those shown in this work (MW =135.5 kDa, subunit MW=70 kDa, pI=4.6) [42]. Additionally, our findings demonstrate that even though the enzyme possesses a dimeric structure, an "adsorption" effect is typical for this enzyme. Enzyme molecules have the potential to aggregate, leading to the emergence of multiple active protein bands in native PAGE (Fig 3).

Substrate specificity results have shown that GOX of *P. chrysogenum* MDC 8358 has higher specificity to D-glucose but also has relatively small amount of activity against other sugars such as D-galactose (0.038 %), D-mannose (1.03) and D-xylose (0.076 %) (Table 2.). Thus, for compression the native enzyme of *P. variabile* P16 shows 7.2 % and 3% relative activity to D-mannose and D-xylose, respectively [24], native GOX of *Penicillium funiculosum* shows 0.4 %, 18 %, 8.6 % and 0.3% relative activity to arabinose, galactose, xylose and mannitol, respectively, [25], recombinant GOX of *Penicillium sp.* shows 10.3 %, 7.67 %, 3.54 % and 7.37 % relative activity to D-galactose, D-mannose, D-sorbitol and D-xylose, respectively [27]. The obtained substrate specificity results indicate the higher selectivity and specificity of GOX of *P. chrysogenum* MDC 8358 compared to characterized enzymes of other *Penicillium* species.

The results of temperature and pH effect on the enzyme activity show that GOX of *P. chrysogenum* MDC 8358 is quite active in the wide range of temperature (0-60 °C) and pH (3.5-8). The optimum temperature range reported for most GOXs characterized from *Penicilium* spaces as from 25 to 65 °C (Table 3). In comparison, *Aspergillus niger* GOX exhibited optimum activity at 40-55°C [14, 17, 19, 40] while more thermostable GOX from *Aspergillus tubingensis* has been reported as well [20]. With the interest of being applied in food preservation cold adapted GOXs have been characterizes from actinomycetes which represent about 70 % of its activity at 0-4°C [27, 55]. The GOX enzyme from *P. chrysogenum* MDC 8358 exhibits approximately 20% of its optimal activity at 1 °C and around 60 % at 20 °C. Furthermore, its activity demonstrates a rising trend with temperature elevation, peaking at 40 °C. Although Sukhacheva et al discovered that GOX from *Penicillium* could maintain about 80 % at 20 °C and the enzyme activity reduced rapidly with decreasing of temperature [26]. The pH

optima of GOXs varies from 5.0 to 7.0. GOX from most fungi have pH optima in the acidic to neutral pH range [18]. In contrast, the GOX obtained from *P. funiculosum 433* and *Cladosporium neopsychrotolerans* SL16 have shown optimal activity at neutral and slightly alkaline pH conditions (Table 3) [26, 55]. Table 3 outlines the characteristics of GOX of *Penicillium chrysogenum* MDC 8358 in comparison to the GOXs isolated from diverse *Penicillium* species.

Table 3. Comparison of the properties of GOX of *Penicillium chrysogenum* MDC 8358 with different GOXs of *Penicillium* spaces

GOX sources	Molecular weight kDa, subunit molecular weight, Kda isoelectric point	Temperature optimum, °C	pH optimum	Thermostability, retained activity, %	Thermostability depending on pH, retained activity, %	Reference
P. chrysogenum MDC 8358	135.5, 70, 4.6	40	6.8	50 (after 5 min incubation at 55 °C)	70 (after 5 min incubation at 55 °C, pH 3.6-6.0)	This study
P. chrysogenum	175, 72, 4.5	ND*	5-6	ND	100 (after 3 h incubation at room temperature, pH 3.5-7.0)	[13]
P. variabile P16	175, 82, ND	55	6.0	ND	ND	[24]
P. amagasakiense	120, 60, 4.5	28-40	5.2-6.2	ND	90 (after 72 h incubation at room temperature, pH 5-7)	[38]
P.notatum	ND, 65, ND	35	6.2	83 (after 30 min incubation at 50 °C)	65 (after 1 hour incubation at 35 °C, pH 3.0-7.0)	[1]
P. notatum	87, 78, ND	45	5.5	48 (after 15 min incubation at 40 °C)	72 (after 15 min incubation at room temperature, pH 4.5)	[23]
P. notatum	ND, ND, ND	45	5.4	50 (after 99 min incubation at 45 °C)	ND	[22]
P. funiculosum 46.1	ND, ND, ND	65	6	ND	ND	[25]
P. funiculosum 433	140, 70, ND	25-30	6-8.6	40 (after 1 h incubation at 70 °C)	100 (after 2 h incubation at 30-45 °C, at pH 4.5-8)	[26]
Penicillium sp. MX3343	ND, 70, 5	35	5.5	80 (after 10 min incubation at 10-45 °C)	70 (after 2 h incubation at 25 °C, pH 2.0-5.0	[27]

*ND represents the non-determined data.

CONCLUSION

An efficient and easy-to-implement method for the purification of GOXs from the culture liquid of *P. chrysogenum* MDC 8358 is proposed, which makes it possible to obtain the enzyme with a high yield and purity.

The purified GOX exhibits remarkable stability, high catalytic activity, and strong substrate affinity, which makes this enzyme a promising candidate for practical applications.

Hence, GOX derived from *Penicillium chrysogenum* MDC 8358 is a non-toxic and no side effect green food additive. The exceptional characteristics of the GOX from *Penicillium chrysogenum* MDC 8358 open up promising perspectives for commercial use in various food processing and preservation industries. Thus, a patented method has been developed to enhance the durability of food items by employing GOX. This method is suitable for extending the shelf life of various packaged and prepared foods, including meat and poultry patties, smoked fish, vegetable salads with mayonnaise, sausages, bread, and egg-based spreads. This enzymatic preservation method operates by diminishing oxygen levels, thereby establishing an environment that is both microbicidal and microbistatic [56]. Due to its broad pH profile of activity, this enzyme holds promise for application within the winemaking industry. In the process of the GOX reaction, oxygen is utilized, effectively positioning GOX as a capable oxygen scavenger. This process contributes to establishing anaerobic conditions. Simultaneously, the presence of GOX reaction leads to a reduction in glucose content within the wine which terminates anaerobic fermentation. In short, GOX of *Penicillium chrysogenum* MDC 8358 is a good candidate for commercial use as indicate number of safety evaluation results [43]. Further studies are being carried out to elucidate the other characteristics to reveal broader applications of GOX of *P. chrysogenum* MDC 8358.

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Investigation: Protein purification, electrophoresis– AP; biochemistry, characterization – KD, AH

Resources – AP, AH

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