

**Isolation, Purification and
Characterization of a Novel Glucose
Oxidase from *Penicillium canescens*
Tt42**

THESIS

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Abstract

A novel glucose oxidase from *Penicillium canescens* (Tt42) was isolated, purified and characterised. The *P. canescens* Tt42 was cultivated using an optimised growth medium from literature, and maximum glucose oxidase activities of 11.5 U/ml and 6.9 U/ml for the intra- and extracellular fractions were obtained. Maximum glucose oxidase production was achieved after 72 hours at 28°C which coincided with glucose depletion. A total of 1104 U (from 60ml) of glucose oxidase was produced with a biomass specific glucose oxidase activity of 1.08 U mg^{-1}

Four methods of cell disruption were evaluated for release of intracellular glucose oxidase from *P. canescens* Tt42 cells. These methods were; sonication, French press, Freeze-Thaw and a high pressure cell disrupter (Z-Plus Series) from Constant systems. All the methods were successful in releasing the intracellular glucose oxidase from *P. canescens* Tt42. The use of the Constant Systems high pressure cell disrupter was preferred, since it was the simplest and most rapid method.

Ammonium sulphate precipitation was shown to be effective as an initial purification step for extracellular glucose oxidase from *P. canescens* Tt42. Comparison of the intra- and extracellular glucose oxidase fractions using isoelectric focusing showed 2 isoenzymes in both fractions. The pI values of the isoenzymes were determined to be 4.30 and 4.67, with the former being dominant. Since both the intra- and extracellular fractions contained the same isoenzymes of glucose oxidase, further purification studies were performed using

the extracellular fraction. The glucose oxidase from *P. canescens* Tt42 was purified using 3 main techniques: ammonium sulphate precipitation (60% - 70% cut), anion exchange chromatography (Super Q 650M) and size exclusion chromatography (Sephadex S200HR). The glucose oxidase was determined to be $\pm 80\%$ pure by size exclusion chromatography. The final purified glucose oxidase was lyophilised, and an overall purification yield of 10.3% was achieved with an 8.6-fold purification. The purified glucose oxidase was confirmed to be catalase free.

Glucose oxidase from *P. canescens* Tt42 was determined to be a dimeric protein ($M_r \pm 148\text{kDa}$) likely consisting of 2 equal subunits ($M_r \pm 70\text{kDa}$). The temperature optimum range was shown to be 25-30°C. The optimum pH for the oxidation of β -D-glucose was pH 7. The enzyme was shown to be stable at 25°C for 10 hours, with a half life of approximately 30 minutes at 37°C. The lyophilised enzyme was stable at -20°C for 6 months. The properties of glucose oxidase from Tt42 were comparable to alternative glucose oxidase enzymes from *Aspergillus* and other *Penicillium* species.

Glucose oxidase from *P. canescens* Tt42 was shown to have distinct kinetic characteristics. The V_{\max} and K_m were shown to be 651 U mg^{-1} and 18.4 mM towards β -D-glucose. The catalytic k_{cat} and specificity k_{cat}/K_m constants for glucose oxidase from *P. canescens* Tt42 were shown to be 791 s^{-1} and $40 \text{ s}^{-1}\text{mM}^{-1}$ each respectively. The specificity constant (k_{cat}/K_m) of glucose oxidase from *P. canescens* Tt42 was determined to be 1.3-fold higher than that that of *A. niger*

(Sigma Type VII) and 8.7-fold lower than that of *P. amagasakiense* (ATCC 28686) from literature.

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Abbreviations

Tt42	<i>Penicillium canescens</i> from this study
GOX	Glucose Oxidase
FAD	Flavine Adenine Dinucleotide
CAT	Catalase
HRP	Horse Radish Peroxidase
H ₂ O ₂	Hydrogen Peroxide
ABTS	2, 2'-Azino-di-[3-ethylbenzthiazolin-sulfonat]
GOX-CAT	Glucose Oxidase – Catalase enzyme system
BSA	Bovine Serum Albumin
pI	Isoelectric Point
IEF	Isoelectric Focusing
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SDS	Sodium Dodecyl Sulphate
TEMED	N, N, N', N'tetramethylethylenediamine
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol

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Quote

After long and careful meditation, a conclusion reached I have - made me more intelligent, this thesis has, yes more intelligent, a master Jedi of biochemistry I now am!

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Chapter 1

Literature review

1 Literature review

1.1 Introduction

Soil, organic waste and plant cell material are a few of the diverse environments in which filamentous fungi are known to flourish. The success of fungi lies in their ability to produce a wide range of different enzymes enabling them to use many organic compounds as nutrient sources (Gouka *et al.*, 1997). Fungi have, for a long time, had an extremely important place in the realms of microbiology and biochemistry due to their ability to produce useful enzymes. Fungal enzymes have been employed for the rapid oxidation and decomposition of proteins, carbohydrates and fats (Kleppe, 1963). The peptides and proteins from fungi have been recently reviewed (Ng, 2004).

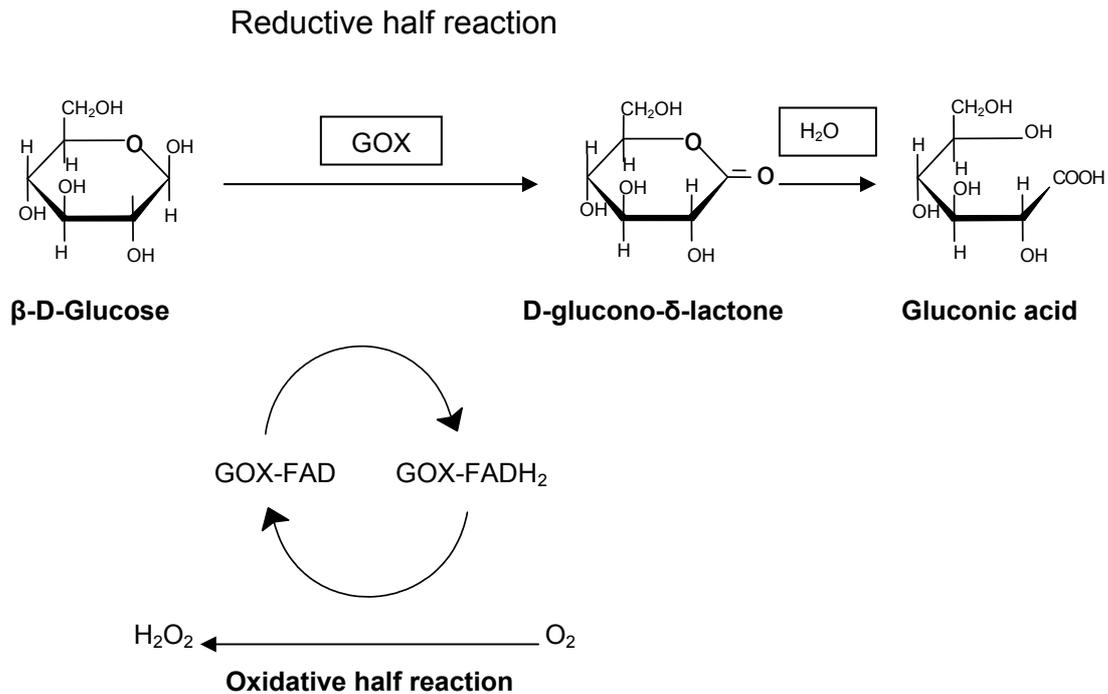
Filamentous fungi are capable of producing large amounts of specific enzymes although the levels are relatively too low for commercial exploitation in the naturally occurring wild type strains, thus the strains may be mutated (Punt *et al.*, 2002) or the genes coding for the enzymes of interest are cloned and over-expressed in suitable hosts. Mutagenesis of different fungi as a strategy for the improvement of glucose oxidase production has been successful in improving glucose oxidase activities up to 77% (Fiedurek and Gromada, 1997). Cloning and over-expression of glucose oxidase genes in *Saccharomyces cerevisiae* and *Escherichia coli* from *Aspergillus niger* and *Penicillium amagasakiense* respectively, have been successful (Kapat *et al.*, 2001 and Witt *et al.*, 1998). Fungi producing enzymes with attractive characteristics continues to be a field for exploitation.

Glucose oxidase (GOX) has been purified from a range of different fungal sources, mainly from the genus *Aspergillus* (Kleppe, 1963; Kalisz *et al.*, 1991 and Hatzinikolaou *et al.*, 1996) and *Penicillium* (Kusai *et al.*, 1960; Eryomin *et al.*, 2004; Sukhacheva *et al.*, 2004; Eriksson *et al.*, 1987 and Rando *et al.*, 1997). Despite the fact that GOX has been produced by a variety of filamentous fungi, *A. niger* is the most common fungus utilised for the production of GOX (Pluschkell *et*

al., 1996). The *Penicillium* species GOX has been shown to exhibit more advantageous kinetics for glucose oxidation than that of *A. niger* GOX (Kusai *et al.*, 1960 and Witt *et al.*, 1998). GOX has been used in large scale technological applications since the early 1950s (Fiedurek and Gromada, 1997). Major applications for GOX include: the enzymatic determination of glucose using biosensors (Vodopivec *et al.*, 2000 and Chudobova *et al.*, 1996), for the production of gluconic acid and as food preservative (Pluschkell *et al.*, 1996). Implantable glucose sensors have found application with diabetes patients (Gerritsen *et al.*, 2001). GOX in new forms with useful properties for applications in biotechnology continues to be of considerable interest despite the abundant availability of commercial GOX (Rando *et al.*, 1997).

1.2 GOX Reaction Mechanism

GOX (β -D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is a flavoprotein which catalyses the oxidation of β -D-glucose to D-glucono- δ -lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor (Pluschkell *et al.*, 1996; Hatzinikaou *et al.*, 1996; Witt *et al.*, 2000 and Beltrame *et al.*, 2004). The reaction can be divided into, a reductive and an oxidative step (Scheme 1.1). In the reductive half reaction, GOX catalyses the oxidation of β -D-glucose to D-glucono- δ -lactone which is non-enzymatically hydrolyzed to gluconic acid. Subsequently the flavine adenine dinucleotide (FAD) ring of GOX is reduced to FADH₂ (Witt *et al.*, 2000). In the oxidative half reaction the reduced GOX is re-oxidised by oxygen to yield hydrogen peroxide. The hydrogen peroxide is cleaved by catalase (EC 1.11.1.6) (CAT) to produce water and oxygen, (Beltrame *et al.*, 2004). Witteveen *et al.* (1992) stated that in *A. niger*, the enzyme lactonase (EC 3.1.1.17) was responsible for catalysing the hydrolysis of glucono- δ -lactone to gluconic acid, although the presence of lactonase was not necessary since the hydrolysis step does occur spontaneously but at a lower rate.



Scheme 1.1: Representation of the GOX reaction (This was adapted from Witt *et al.*, 2000).

1.3 The Composition of GOX

The GOX enzymes from ascomycetes are dimeric glycoproteins consisting of two identical polypeptide chain subunits that are covalently linked together via disulfide bonds (Rando *et al.*, 1997 and Kalisz *et al.*, 1997). Figure 1.1 below depicts the subunit structure of GOX from *P. amagasakiense* (Wohlfahrt *et al.*, 1999). Figure 1.2 depicts the FAD moiety and the key conserved active site residues of a GOX subunit from *P. amagasakiense* (Wohlfahrt *et al.*, 1999). The structure of the *P. amagasakiense* GOX (1GPE) <http://pd-beta.rcsb.org/pdb/explore.do?structureId=1gpe> was visualised on YASARA version 4.7.18 (www.yasara.org) for Figure 1.1 and 1.2 below. Each GOX subunit contains one mole of tightly bound, but not covalently linked, flavine adenine dinucleotide (FAD) as co-factor (Rando *et al.*, 1997 and Witt *et al.*, 2000). The GOX enzyme from *P. amagasakiense* is glycosylated, with a carbohydrate content of approximately 11-13%, which is of the high mannose type (Nakamura and Fujiki, 1968; Kusai *et al.*, 1960 and Kalisz *et al.*, 1997). GOX from *A. niger* and *P. amagasakiense* have been crystallized and intensively studied by Hecht *et al.* (1993) and Kusai, (1960), each respectively.

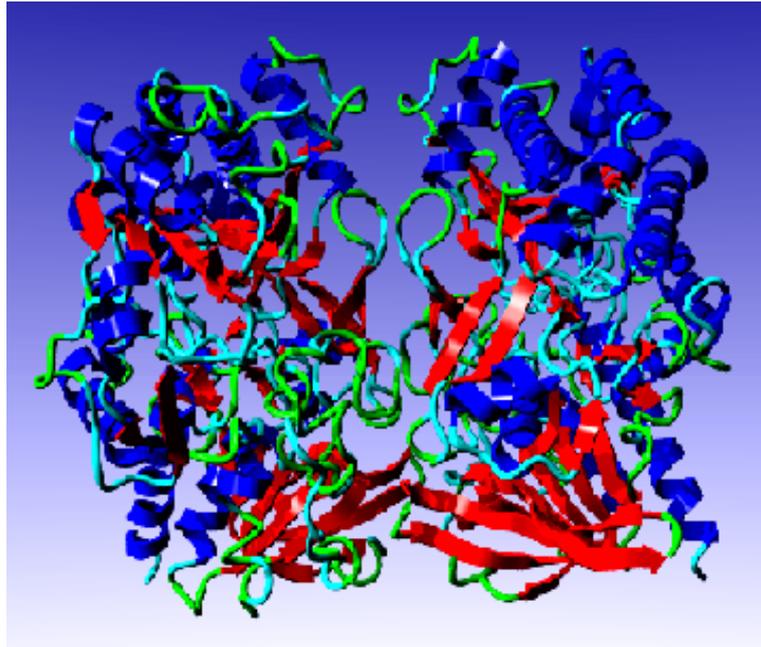


Figure 1.1: Overall structure of glucose oxidase from *P. amagasakiense* model based on 1GPE visualised using YASARA (Wohlfahrt *et al.*, 1999).

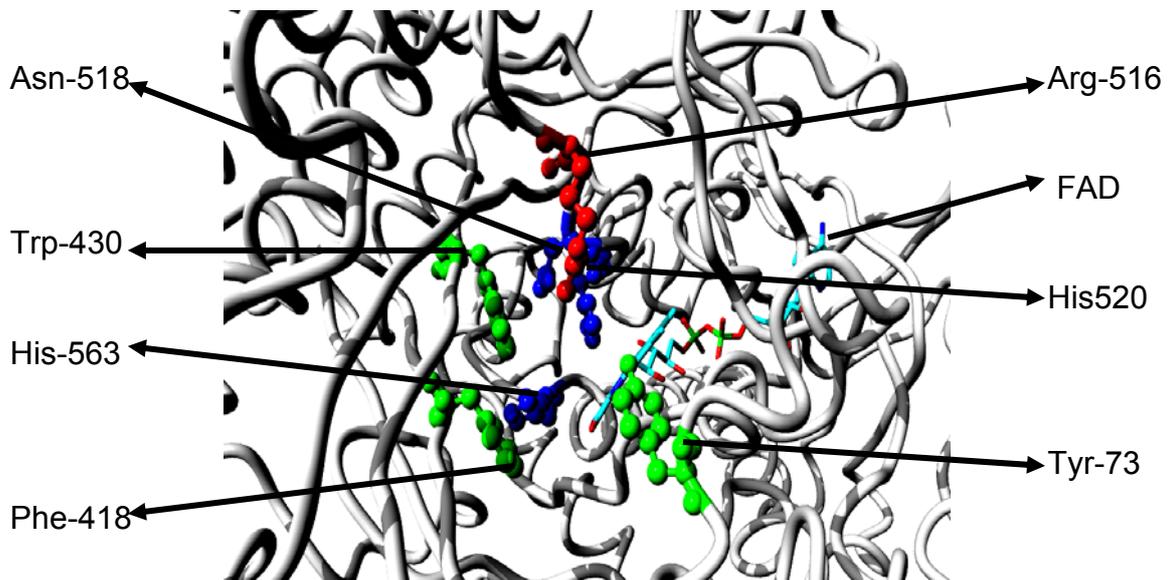


Figure 1.2: Glucose oxidase from *P. amagasakiense* showing the FAD moiety, indicating the key conserved active site residues (model visualised on 1GPE using YASARA (Wohlfahrt *et al.*, 1999)).

The key conserved active-site residues of GOX from *P. amagasakiense* are Tyr-73, Phe-418, Trp-430, Arg-516, Asn-518, His-520 and His-563 (Figure 1.2) (Witt *et al.*, 2000). Witt *et al.* (2000) determined which active site amino acid residues

were involved in the efficient binding of β -D-glucose for GOX. They concluded that Arg-516 was the most critical amino acid for the efficient binding of β -D-glucose by GOX, while Asn-518 contributed to a lesser extent. The aromatic residues - Tyr-73, Phe-418 and Trp-430 - were shown to be important for the correct orientation of the substrate as well as for the maximal velocity of glucose oxidation. The His 520 and His-563 form hydrogen bonds with the 1-OH of glucose during the reaction.

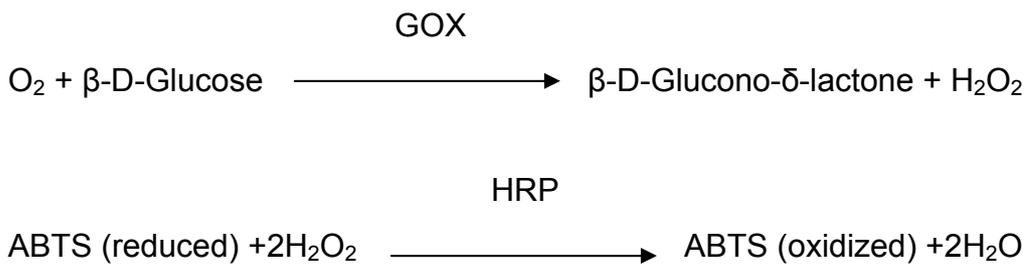
1.4 General Characteristics of GOX

The molecular weight of GOX ranges from approximately 130kDa (Kalisz *et al.*, 1997) to 175kDa (Eriksson *et al.*, 1987). The GOX enzyme is highly specific for the β -anomer of D-glucose, while the α -anomer does not appear to be a suitable substrate (Kusai, 1960). Low GOX activities are exhibited when utilizing 2-deoxy-D-glucose, D-mannose and D-galactose as substrates (Kusai, 1960). Inhibitors of GOX include p-chloromercuribenzoate, Ag^+ , Hg^{2+} , Cu^{2+} , hydroxylamine, hydrazine, phenylhydrazine, dimedone and sodium bisulphate (Kusai, 1960).

Nakamura and Fujiki (1968) performed comparative studies on the GOX enzymes of *A. niger* and *P. amagasakiense*. The molecular weights of the GOX enzymes from the 2 organisms were determined to be 150 kDa for the *P. amagasakiense* and 152 kDa for *A. niger*. The carbohydrate and amino acid compositions of the enzymes were also investigated and compared, which indicated that similar carbohydrates were contained in both enzymes which consisted mainly of glucose, mannose and hexosamine. The *A. niger* GOX contained more mannose and hexosamine than that of *P. amagasakiense*, but less glucose. The overall carbohydrate content was found to be 16% for *A. niger* and 11% for *P. amagasakiense*. The amino acid content of the 2 enzymes revealed that the *A. niger* GOX contained more histidine, arginine and tyrosine and less lysine and phenylalanine than the *P. amagasakiense* GOX (Nakamura and Fujiki, 1968). The optimum pH ranges for the *A. niger* GOX and *P. amagasakiense* GOX, were shown to be 3.5-6.5 and 4.0-5.5, respectively. *A. niger* GOX was found to have a broader pH range than the *P. amagasakiense* GOX (Nakamura and Fujiki, 1968).

1.5 Analysis of GOX

Various analytical methods for GOX determination have been described in literature (Ciucu and Pătroescua, 1984; Witteveen *et al.*, 1990; Fiedurek and Gromada, 1997 and Gerritsen *et al.*, 2001). GOX analysis is based on the principle that GOX oxidizes β -D-glucose in the presence of oxygen to β -D-glucono- δ -lactone and hydrogen peroxide. The hydrogen peroxide formed is used to oxidize a chromogenic substrate in a secondary reaction with horse-radish peroxidase (HRP) with a resultant colour change. This colour change is monitored spectrophotometrically (Scheme 1.2 and 1.3). Two of the chromogenic substrates used for the GOX reaction are; 2,2'-Azino-di-[3-ethylbenzthiazolin-sulfonat] (ABTS) (Witt *et al.*, 1998) and *o*-dianisidine (Bergmeyer, 1988 and Sukhacheva *et al.*, 2004). ABTS forms a greenish-blue oxidized product that is measured spectrophotometrically at 420nm. Below is a reaction scheme for the assay:



Scheme 1.2: Representation of the GOX reaction when using ABTS as a chromogenic substrate.

Oxidation of *o*-dianisidine forms a quinoneimine dye that is measured spectrophotometrically at 436nm.



Scheme 1.3: Representation of the GOX reaction when using *o*-dianisidine as a chromogenic substrate.

A new assay for GOX, using Fourier transform infrared spectroscopy, was developed by Karmali *et al.* (2004), who concluded that the method was useful to study the kinetic properties of GOX since the substrate and product of the reaction absorb at different frequencies. Distinct advantages over coupled assays were that it was faster, required only small amounts of substrate and enzyme, and the reaction could be followed by D-gluconolactone formation. The results obtained for the kinetic constants of GOX in purified form and as cell free extracts using the new method were comparable with the results obtained with the *o*-dianisidine-HRP linked spectrophotometric assay.

1.6 The influence of physical and chemical parameters on GOX production

1.6.1 Aeration and agitation

Aeration and agitation are both important factors for aerobic fermentation processes. Aeration of growing aerobic cultures is performed to fulfil the requirements of oxygen supply and to remove gaseous waste products. Oxygen for growth and production in fungal cultures can be ensured by the aeration and agitation of the mycelial culture (Zetelaki and Vas, 1968). Gas-liquid transfer is known to be a limiting factor in many aerobic fermentation processes. One of the main reasons for this occurrence is the low solubility of oxygen in fermentation media when compared to the solubility of the carbon and nitrogen sources and other nutrients. Oxygen supply is further hindered when conducting fermentations that are viscous that contain high concentrations of cells, such as found in fungal fermentations (Klein, *et al.*, 2002). Filamentous fungi grow in dense aggregated mycelial mats resulting in decreased accessibility of oxygen to the actively growing cells (Zetelaki and Vas, 1968). Solutions, to these problems, include agitation of broth cultures (Zetelaki and Vas, 1968; Zetelaki, 1970), the use of hydrogen peroxide as the sole oxygen source (Fiedurek and Gromada, 2000), and the use of pure oxygen over air (Zetelaki and Vas, 1968).

Agitation is used to increase the efficiency of aeration, by forcing the supplied air bubbles to disintegrate into smaller bubbles resulting in an increased interface between the gas and the liquid (Zetelaki and Vas, 1968). Increased agitator speed

corresponds to increases in the concentration of dissolved oxygen resulting in a faster growth rate and increased GOX production (Zetelaki and Vas, 1968; Zetelaki, 1970). Comparison of the GOX activities of 24 hour old cultures of *A. niger* agitated at 460, 700 and 900rpm, respectively showed that the activity of the culture agitated at 700rpm was approximately 20-24% higher than those agitated at 460rpm. Further increases in agitator speed did not result in improved growth rate or GOX production (Zetelaki and Vas, 1968).

Fiedurek and Gromada (2000) identified a novel method of increasing the dissolved oxygen in the growth media by adding hydrogen peroxide as the sole oxygen source. CAT produced by the organism catalysed the decomposition of the hydrogen peroxide to water and free oxygen, hydrogen peroxide acted as both the electron donor and acceptor in the reaction (Fiedurek and Gromada, 2000).

Pure oxygen was also shown to be beneficial to the growth rate of *A. niger*, demonstrated by submerged cultures supplied with pure oxygen. The growth rate increased from 61mg mycelial dry weight/100ml/h (aerated with air) to 95mg mycelial dry weight/100ml/h (aerated with pure oxygen). The oxygenated culture reached a higher maximum mycelial yield 8 hours earlier than in the aerated culture. It was however noted that autolysis was higher in the oxygenated culture medium. Also the viscosity of the oxygenated culture was found to be approximately 50% lower than that of the aerated culture in spite of the approximate 10-15% higher final cell concentration observed in the oxygenated culture. The cell walls of the aerated cultures were found to be thicker and more rigid than the cell walls of the oxygenated culture, thus causing the oxygenated cells to be less resistant to mechanical agitation which could have resulted in the lower viscosity observed in the oxygenated culture. At the same time the GOX activity of the oxygenated culture was determined to be double that of the aerated culture (Zetelaki and Vas, 1968). The disadvantage of the use of pure oxygen would be the large scale financial implications (Klein *et al.*, 2002).

1.6.2 Calcium Carbonate

Petruccioli *et al.* (1995) stated that the addition of calcium carbonate to growth medium in shake flasks and fermenters, to prevent pH drop during cultivation, was

necessary for optimal GOX production. The effect of different buffering agents on the growth and GOX activity of *P. variable* (P16) was investigated in a 3 litre benchtop bioreactor (Petruccioli *et al.*, 1995). The buffering agents investigated were calcium carbonate, sodium hydroxide and calcium chloride. A combination of sodium hydroxide and calcium chloride was also investigated. Maximal enzyme activities were obtained with the addition of calcium carbonate to the medium, whereas the use of sodium hydroxide, even in conjunction with calcium chloride resulted in approximately 85% less GOX production. Maximal biomass yield was attained after 62 hours of fermentation using sodium hydroxide and calcium chloride, and 72 hours when using calcium carbonate. There was no apparent relation between GOX activity (U/ml) and mycelial growth (g L^{-1}) (Petruccioli *et al.*, 1995).

Rogalski *et al.* (1998) showed that the synthesis of GOX was sensitively influenced by increasing concentrations of calcium carbonate (0 – 4.5%), with maximal GOX activity reached at approximately 3.5%. The inclusion of the calcium carbonate to the growth medium caused an increase in the alkalinity to a pH of 5.7, at which point GOX production had reached an optimum. GOX production was minimal, below pH 4, thus Rogalski *et al.* (1988) concluded that calcium carbonate appeared to be important in preventing the acidification of the culture broth during cultivation.

Hatzinikolaou and Macris (1995) reported that calcium carbonate was a strong inducer of GOX in *A. niger*. They demonstrated that calcium carbonate was essential for increased levels of GOX production when *A. niger* was cultivated with sucrose or molasses as the carbon source. Optimum calcium carbonate concentrations of 4 and 5% were observed for GOX production using sucrose and molasses respectively. Hatzinikolaou *et al.* (1996) cultivated *A. niger* using the optimized cultivation media of Rogalski *et al.* (1988), and demonstrated the induction of GOX production by calcium carbonate (optimum 4%), which also contributed to maintaining the pH of the cultivation media between 6.5 and 6.8. Hatzinikolaou *et al.* (1996) showed that the activity of the glycolytic enzyme, glucose-6-phosphate isomerase, was higher in growth media without calcium carbonate, while the GOX and CAT activities were quite low. Inclusion of calcium

carbonate caused increases in the GOX and CAT activities with the simultaneous decrease in the glucose-6-phosphate isomerase activity. They suggested that the addition of calcium carbonate in the growth media may cause a metabolic shift from glycolysis to the pentose phosphate pathway, thereby increasing GOX levels.

1.6.3 Carbon and nitrogen source variations

Hatzinikolaou and Macris (1995) investigated the effects of different carbon sources on growth and total GOX activity for *A. niger*. *A. niger* was shown to grow on all the carbon sources tested but significant levels of GOX were only obtained using glucose, sucrose and molasses. Furthermore Hatzinikolaou and Macris (1995) stated that glucose (pure or as a product of sucrose hydrolysis by invertase) was the principal inducer for the transcription of the GOX gene. Petruccioli *et al.* (1993) studied GOX production by 84 strains of the genus *Penicillium* and reported that *P. expansum* (1 strain), *P. italicum* (1 strain), *P. chrysogenum* (3 strains) and *P. variable* (3 strains), when cultivated with glucose as the carbon source all produced GOX activity ranging from 0.61U/ml to 5.45 U/ml. The strains mentioned were investigated for their ability to oxidize glucose, fructose, mannose, galactose, arabinose and xylose. Only one of the *P. italicum* strains (NRRL 983) displayed enhanced oxidizing activity towards mannose, galactose, and xylose being 32.38%, 17.90% and 26.40% compared to glucose (100%), respectively (Petruccioli *et al.*, 1993). Petruccioli *et al.* (1997) investigated the effect of 10 different carbon sources on the growth and GOX production of *P. variable* mutant M80.10. Of the ten carbon sources investigated (glucose, fructose, galactose, xylose, arabinose, mannose, sucrose, maltose, mannitol and glycerol), only cultivation with glucose and mannose produced high levels of GOX, approximately 19 U/ml. Petruccioli *et al.* (1997) also determined that optimal production of GOX in *P. variable* (M-80.10) was obtained at a glucose concentration of 8% (m/v). Their findings were in agreement with Rogalski *et al.* (1988) who also reported a glucose concentration of 8% to be optimal for GOX in the *A. niger* G-13 mutant. Higher glucose concentrations caused undesired decreases in mycelial mass, culture pH and GOX concentrations. Kusai (1960) reported that sucrose was the best carbon source for the production of GOX by *P.*

amagasakiense, although if the pH of the growth medium was maintained during cultivation, glucose was the carbon source of choice.

Rogalski *et al.* (1988) showed that when cultivating *A. niger* mutant G-13 and supplementing the growth media with 3% peptone there was a 36% and 42% increase in GOX activity and biomass production, respectively. Hatzinikolaou and Macris (1995) performed investigations of different nitrogen sources on the growth and total GOX activity of *A. niger* cultivated with sucrose and molasses as sole carbon sources. They found that the peptone concentration had a marked effect on the total GOX production. With sucrose as the carbon source maximum GOX activity was achieved at peptone concentrations of 1-2%, and with molasses maximum activity was achieved at 0.2-0.3% peptone.

Kona *et al.* (2001) investigated the effect of corn steep liquor, as the sole nutrient source, on the production of GOX from *A. niger*. The use of corn steep liquor increased the GOX from 580 ± 30 U/ml, with sucrose as the sole carbon source, to 640 ± 36 U/ml.

1.6.4 Other medium components

GOX concentration in *A. niger* has been increased by the inclusion of various hydrocarbons during cultivation (Li and Chen, 1994). Increases in maximum intracellular GOX activity were 43%, 110% and 31% by the addition of *n*-dodecane, *n*-hexadecane and soybean oil, respectively. Increased yields of GOX were due to an increase in the efficiency of enzyme synthesis in the cells for *n*-dodecane and *n*-hexadecane, and an increase in cell concentration for the addition of soybean oil (Li and Chen, 1994).

Gromada and Fiedurek (1996), when investigating the effect of different medium components and metabolic inhibitors on GOX production in *A. niger*, found that substituting ammonium phosphate with sodium nitrate and lack of Mg^{2+} ions in their basal salt medium significantly increased GOX activity by 269.6%. Furthermore intracellular GOX activities were increased by 68.3% in the presence of sodium orthovanadate (1mM) and extracellular GOX activities were increased

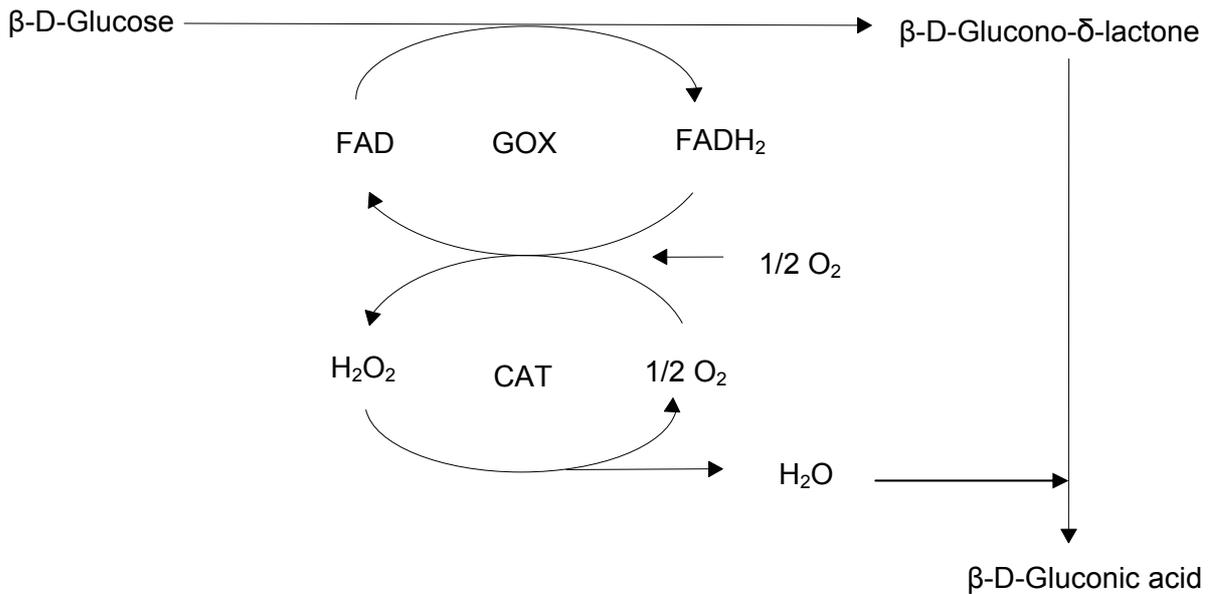
in the presence of hematin (1mM), choline (40mM) and Tween 80 (0.1%). The extracellular increase in GOX activity obtained was between 31.4 – 53.9%.

1.7 Applications of GOX

GOX is of considerable commercial importance due to its applications in food science, clinical chemistry and biotechnology. Raba and Mottola (1995) stated that GOX is the most widely used enzyme as an analytical reagent due to its application in the determination of glucose, and furthermore it's relatively low cost and good stability. Wilson and Turner (1992) also attribute the success of GOX as a diagnostic reagent to the enzyme's relative specificity. Raba and Mottola (1995) reviewed glucose oxidase as an analytical reagent and stated that the glucose/GOX system was a convenient model for method development especially in the area of biosensors.

GOX, usually in combination with CAT, is used to stabilize colour and flavour in beer, fish, tinned foods, and soft drinks, by the removal of oxygen (Crueger and Crueger, 1990). GOX is also used to remove glucose during the manufacture of egg powder, preventing browning during dehydration caused by the Maillard reaction (Crueger and Crueger, 1990). GOX has also found application in the baking industry, providing slight improvements to the crumb properties in bread and croissants (Rasiah *et al.*, 2005). GOX is also widely used to produce gluconic acid, which is used as a mild acidulant in the metal, leather and food industries (Crueger and Crueger, 1990; Nakao *et al.*, 1997 and Klein *et al.*, 2002). The most important application for GOX is for the diagnostic determination of glucose using biosensor technology (Wilson and Turner, 1992). Commercial diagnostic kits for the determination of glucose in blood, serum and plasma are supplied commercially in colorimetric diagnostic kits (Wilson and Turner, 1992). Various glucose biosensors are listed and elaborated on in Table 1.1.

GOX together with CAT or HRP has a range of applications in the food industry for glucose determination and as an antioxidant. Below is a reaction scheme for the GOX-CAT enzyme system:



Scheme 1.4: Representation of the glucose oxidase-catalase enzyme system (Adapted from Pickering, 2000)

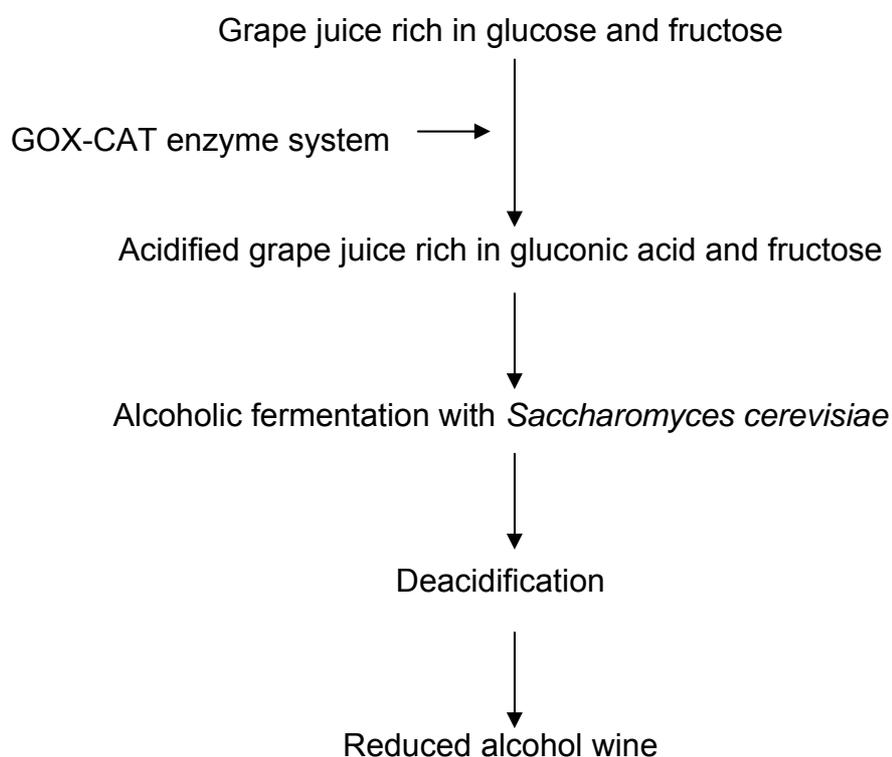
The GOX-CAT enzyme system was used by Isaksen and Adler-Nissen (1997) to scavenge oxygen in mayonnaises with different oxidative susceptibility. The investigation proved that the GOX-CAT enzyme system could be used to retard the lipid oxidation in mayonnaise stored at 5°C and 25°C, in mayonnaises containing pure soybean oil and where up to half the vegetable oil had been supplemented with fish oil. The enzyme system was responsible for scavenging the oxygen during glucose oxidation thereby decreasing the availability of the oxygen for lipid metabolism.

Parpinello *et al.* (2002) conducted preliminary studies into the use of the GOX-CAT enzyme system to control the browning of apple and pear purees by removing 99% of the oxygen content. Oxygen is known to be a key factor in the browning of fruit purees, and the enzyme system was shown to have the capability to control the non-enzymatic browning during fruit processing and purée storage. The scavenging of the oxygen by the enzyme system had a stabilising effect.

Table 1.1: Comparison of various GOX based biosensor applications

Application	Description	Response limit	Response time	Stability	Reference
On line glucose monitoring for fermentations	Macroporous connective interaction media (CIM) epoxy disks were used to immobilise GOX and were integrated in an enzyme reactor in a flow injection analysis system	90mM	-	10 days	Vodopivec <i>et al.</i> (2000)
Fibre optic biosensor for analysing glucose concentrations in soft drinks	GOX was covalently attached to a nylon net	10mM	10 seconds to 5 minutes	30 months	Chudobova <i>et al.</i> (1996)
Disposable strip-type biosensor for blood and serum monitoring	GOX was immobilised to a nitrocellulose strip (NC), sample volumes and amounts of reagent are precisely predefined by the dimension and pore size (8 µm) of the NC strip	27.7mM	30 seconds	-	Cui <i>et al.</i> (2001)
Strip-type biosensor for blood.	The GOX, peroxidase (HRP) and dye were physically adsorbed to the strip. The dye was 3-methyl-2-benzothiazdione and diethanolamine which reacted to form benzothiazolium dye.	20mM	15 seconds	300 days	Kim <i>et al.</i> (2001)
Miniaturised thermal biosensor for whole blood	Glucose was determined by measuring the heat evolved when samples containing glucose passed through a small column with immobilized GOX and catalase. Samples of whole blood (1 µl) can be measured directly, without any pre-treatment.	20mM	5 minutes	100 samples using the same column	Harborn <i>et al.</i> (1997)
Glucose sensor for whole blood	Co-immobilisation of GOX and catalase onto a gas permeable membrane. The hydrogen peroxide generated in the reaction had a high vapour tension and was able to cross the membrane. The hydrogen peroxide was oxidised at the platinum electrode by catalase. Interfering substances from blood were therefore blocked. (ascorbic acid, uric acid, urea and acetaminophen)	15mM	90-120 seconds	2-3 weeks per membrane	Santoni <i>et al.</i> (1997)
Glucose biosensor for serum from human blood.	Immobilisation of GOX by chitosan film on Prussian Blue layer. The chitosan film is selectively permeable for hydrogen peroxide, negating interference by ascorbic and uric acids in blood.	6mM	Less than 60 seconds	30 days	Zhu <i>et al.</i> (2002)

Another application of the GOX-CAT enzyme system is in the production of low alcohol wines investigated by Pickering *et al.* (1998). Reduction of the fermentation alcohol potential was achieved by pre-treating the grape juice with the GOX-CAT enzyme system to convert the available glucose to gluconic acid (Scheme 1.5). The low pH of the grape juice was determined to be a limiting factor, which was subsequently overcome by the use of calcium carbonate prior to the enzymatic treatment. A glucose conversion of 87% was achieved with this system.



Scheme 1.5: Procedure for producing low alcohol wine (reproduced from Pickering *et al.*, 1998)

The use of GOX has found application in the textile industry as a method for producing hydrogen peroxide for bleaching (Tzanov *et al.*, 2002). Tzanov *et al.* (2002) covalently immobilised GOX on alumina and glass supports, resulting in higher recoveries. Maximum hydrogen peroxide concentrations of 0.35 gL⁻¹ and 0.24 gL⁻¹ were reached after 450 minutes for GOX immobilised on the glass and alumina supports respectively (20g glucose in 50ml 0.1 M acetate buffer, pH 5, 35°C, and aerated at 5 L/min). The alumina support proved more stable at the

operational conditions and could be used for 3 consecutive runs. The hydrogen peroxide produced was tested for bleaching scoured woven cotton fabric and was found to be comparable to standard bleaching processes. No stabilisers were needed since the gluconic acid produced acted as a stabilising agent.

The applications for GOX and GOX-CAT enzyme systems are numerous and the need for microbial strains exhibiting enhanced GOX production will continue to be of interest.

An intensive screening programme of various environmental samples for the isolation of high GOX producing microorganisms was undertaken by the CSIR resulting in the isolation of *Penicillium canescens* Tt42. In view of the biotechnological and diagnostic importance of this enzyme it was deemed obligatory to investigate the properties of the enzyme.

1.8 Thesis hypothesis

The GOX from Tt42 was a novel enzyme that would have different properties to known GOX enzymes reported in relevant literature.

1.9 Objectives

- Cultivate Tt42 and determine the intra- and extracellular production levels of GOX,
- Purify the GOX from Tt42 for characterization and activity studies,
- Determine the kinetic parameters of GOX from Tt42,
- Compare the GOX of Tt42 with other GOX enzymes reported in relevant literature.

Chapter 2

Growth and GOX production by *Penicillium canescens* Tt42

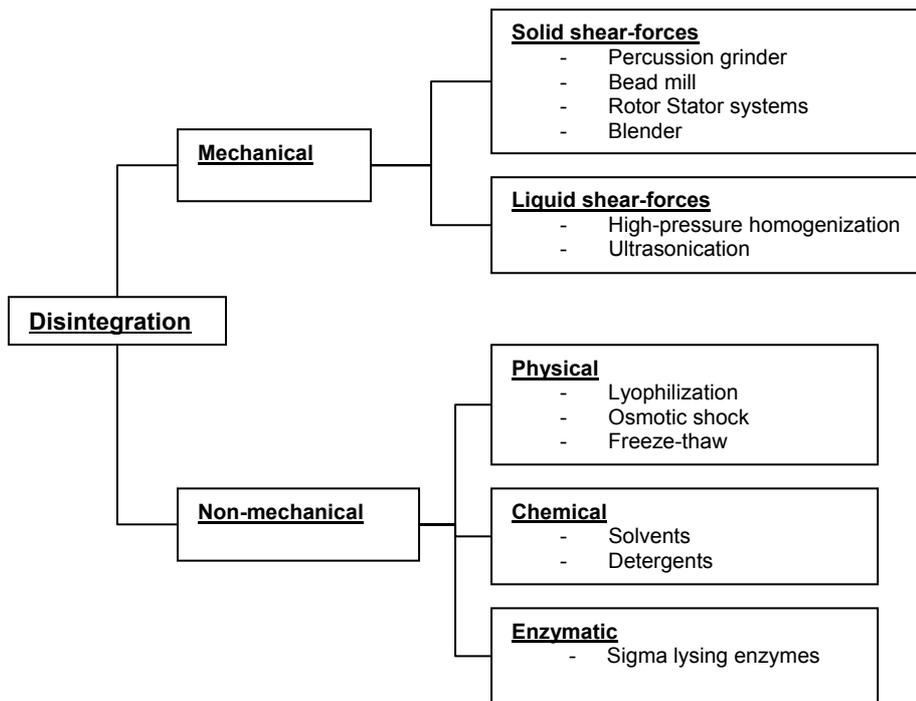
2 Growth and GOX production by *Penicillium canescens* Tt42

2.1 Introduction

Shake flask cultivation of Tt42 was performed using the growth medium described by Rogalski *et al.* (1988) for the culturing of *A. niger* G-13 mutant. Their conclusions were that concentrations of glucose (8% optimum), calcium carbonate (3.5% optimum) and peptone (3%) had a marked effect on the synthesis of GOX. The abovementioned medium constituents have also been used for GOX production by different *Penicillium* species (Petruccioli *et al.*, 1993; Fiedurek and Gromada, 1997 and Petruccioli *et al.*, 1997). Optimum cultivation time was determined to be 3 days and the optimum growth temperature was 28°C for both *A. niger* and *P. vitale* (Hatzinikolaou and Macris, 1995 and Petruccioli *et al.*, 1997).

Various methods of cell disruption have been used for filamentous fungi, including homogenisation (Hatzinikolaou *et al.*, 1996 and Gromada and Fiedurek, 1996), sonication (Lu *et al.*, 1996) and a combination of both (Hatzinikolaou and Macris, 1995). A comprehensive study of different methods for the disruption of two filamentous fungi, *Ganoderma applanatum* and *Pycnoporus cinnabarinus* was performed by Taubert *et al.* (2000). They concluded that fungal cells were particularly resistant to some disintegration methods commonly used for yeasts and bacteria and adapted the following schematic for cell disruption from Christi and Moo-Young (1986):

Optimal results were obtained with percussion grinding and, if necessary, in combination with a rotor-stator system – an Ultra-Turrax (Jahnke and Kunkel, Germany) – for complete disruption (Taubert *et al.*, 2000).



Scheme 2.1: Methods of cell disruption (reproduced from Taubert *et al.*, 2000; adapted from Christi and Moo-Young, 1986).

Due to the morphological differences between filamentous fungi, Taubert *et al.* (2000) found it difficult to recommend general operation parameters for fungal cell disruption and suggested that individual disruption studies on the particular fungus of interest should be carried out for optimal results.

2.2 Theory of techniques

Sonication relies on shear forces caused by amplified harmonic beats known to disrupt microbial cell walls. The mechanism involved is thought to be due to the cavitation principle associated with ultrasonication. Christi and Moo-Young (1986) explained the principle of cavitation as follows: at high acoustic power inputs micro bubbles are formed in the sample being disrupted. The bubbles increase in size during the rarefaction phase of the sound wave and collapses during the compression phase. The collapse of the bubble releases a violent shock wave that is transmitted through the medium. It is the formation, growth and collapse of these gas bubbles that is characterised as cavitation (Christi and Moo-Young, 1986). Disadvantages of sonication include heat generation, which may cause

denaturation of enzymes, and is limited to small scale applications (Wilson and Walker, 1995).

The Constant Systems (Z-Plus-series) cell disrupter utilises high pressure to force a sample through a small fixed orifice at high speed resulting in repeatable and effective cell disruption. During operation the sample is introduced into the high pressure cylinder and a high pressure piston forces the sample through the orifice, with a subsequent pressure drop, resulting in cell disruption. The disrupted cells are spread radially across a cooled heat exchange surface and collected. The cell disrupter can be used for large scale cell disruption applications. The French pressure cell is another cell disruption technique utilising a drastic drop in hydraulically generated high pressure. The pressure cell consists of a closed stainless steel chamber that is exposed to the environment by means of a needle valve. The chamber is closed off after the addition of the sample by a piston. Hydraulic pressure is applied to the piston (up to 108 Pa) and once the desired pressure is reached the needle valve is slowly opened, causing the drop in pressure. The decrease in pressure causes the cells to quickly expand and rupture. The technique is very useful for laboratory scale applications (Wilson and Walker, 1995).

Freeze-Thaw as a method of cell disruption is thought to result in the growth of ice crystals intracellularly during the freezing process. The forces exerted by the ice crystals on the cell walls are thought to bring about cell wall permeabilisation. The process is time consuming and inefficient for large scale applications where large volumes of cells are to be disrupted (Taubert *et al.*, 2000).

2.3 Aims

- Evaluate the growth and GOX production of Tt42 in Rogalski *et al.* (1988) medium,
- Validate the GOX assay (Bergmeyer, 1988) with purified GOX obtained from Seravac (Pty) Ltd, South Africa,
- Evaluate various methods of cell disruption for the release of GOX from Tt42 biomass,

- Compare volumetric titres of GOX from Tt42 with literature values for similar organisms.

2.4 Methods and Materials

2.4.1 Chemicals

GOX from *A. niger* and HRP were gifts from Seravac (Pty) Ltd South Africa. A total protein assay kit was purchased from Bio-Rad. Diammonium hydrogen phosphate and *o*-dianisidine were purchased from Sigma-Aldrich. All other chemicals were purchased from Merck. All chemicals were of reagent grade or better.

2.4.2 Strain and Growth media

The composition of the medium used for cultivation of Tt42 was as follows (gL⁻¹): (NH₄)₂HPO₄, 0.388; KH₂PO₄, 0.188; MgSO₄·7H₂O, 0.156; peptone 30; glucose (autoclaved separately), 80 and CaCO₃ (sterilised separately), 35. Erlenmeyer flasks (300ml), each containing 60ml of the growth media were used for fungal growth. The flasks were each inoculated with 2ml of conidia suspension prepared from 3 day old Tt42 cultures grown on MEA plates at 28°C; at a concentration of $\pm 1.2 \times 10^8$ conidia/ml. Spore counts were determined with a haemocytometer. The spore suspension was prepared in a sterile Tween 80 solution (0.1% w/v). The flasks were cultivated for 120 hours on a rotary shaker (200rpm, 28°C). Whole flask harvesting was performed daily for the duration of the experiment, and analysed for the following: biomass concentration (dry weight in mg.ml⁻¹), pH, glucose concentration (gL⁻¹), and both intra- and extracellular GOX concentration (U/ml).

2.4.3 Biomass Measurement

Samples (10ml) were acidified to pH 2.5 using 4M HCl to convert the insoluble calcium carbonate to soluble calcium chloride and carbon dioxide. The samples were centrifuged at 2135 x g for 20 minutes in a Sorvall RT Du Pont bench top centrifuge, fitted with a swinging bucket rotor, and the supernatants were discarded. The mycelial pellet from the samples was washed with distilled water and dried (100°C, 24hours) to a constant mass. The biomass concentrations were

expressed in mg dry weight per ml of culture medium, and the measurement was done in triplicate.

2.4.4 Enzyme assays

The GOX assay as described by Bergmeyer (1988) was used for the determination of GOX activity. Protein determinations were performed using a Total Protein Assay Kit (Bio-Rad) following the manufacturer's protocol.

2.4.4.1 GOX Assay

GOX activity was measured using the coupled *o*-dianisidine – HRP reaction. The assay components and assay procedure are outlined in Appendix 1. The increase in absorbance (436nm) was followed using a Beckman Coulter DU800 spectrophotometer, and initial reaction rates determined. Enzyme and substrate blanks were also performed. All solutions were stored at 4°C and prepared fresh weekly. The glucose solution was allowed to stand for a minimum of 1 hour to allow for mutarotation before use. The phosphate buffer containing the *o*-dianisidine was sparged with oxygen for 5 minutes before use. One unit of GOX activity was defined as the amount of enzyme that catalyses the conversion of 1 μ mole β -D-glucose to gluconic acid and H₂O₂ per minute at 25°C and pH 7.

Commercial *A. niger* GOX, 133 U mg^{-1} , obtained from Seravac (Pty) Ltd (South Africa) was diluted to 0.15 U/ml and used for assay method validation. All the GOX assays were performed in triplicate.

2.4.4.2 Total Protein (Bio-Rad- Coomassie® Plus) assay

Where necessary the enzyme solutions were analysed for total protein content in mg/ml using the Total protein assay as per the manufacturer's protocol (Bio-Rad) (Appendix 2). Bovine Serum Albumin (BSA) was used as the protein standard. The absorbances of the BSA solutions were measured at 595nm on a Beckman Coulter DU800 spectrophotometer. The resultant absorbances were used to generate a standard curve and the linear equation of the curve was used to determine protein concentrations of the unknowns.

2.4.5 Cell disruption

Various methods of cell disruption were investigated for the release of GOX from Tt42. The 72 hour samples were centrifuged at 2135 x g in a Sorvall RT Du Pont benchtop centrifuge, fitted with a swinging bucket rotor, for 20 minutes, washed and resuspended to 20% wet weight/volume in 0.1M phosphate buffer at pH 7. All the experiments were performed in duplicate and represented as means \pm standard deviation.

2.4.5.1 Sonication

A Sonics and Materials Vibracell sonicator was used to investigate cell rupture by sonication. The sonication was conducted with a 10 ml sample for 40 minutes at 50% duty cycle using a micro tip pulsed for 10 second intervals. The jacketed glass sonication sample holder was connected to a water bath set at 4°C. Samples of 0.5 ml were removed every 5 minutes and centrifuged at 16060 x g for 5 minutes in a Biofuge Pico Heraeus benchtop centrifuge. The supernatants were assayed for GOX activity and total protein content.

2.4.5.2 Constant Systems Cell disrupter

A Constant Systems (Z-Plus series) cell disrupter was used to disrupt 20ml samples of Tt42 cells. The Tt42 cells were disrupted with 1 pass at 10Kpsi, 20Kpsi, 30Kpsi and 38Kpsi (maximum for the system) at 4°C. The samples were centrifuged at 2135 x g in a Sorvall RT Du Pont bench top centrifuge, fitted with a swinging bucket rotor for 20 minutes and the supernatants were analysed for GOX activity and total protein content.

Thereafter three separate 20ml samples were disrupted. The first sample was disrupted with 1 pass, the second with 2 passes and the third with 3 passes, all at 38Kpsi to determine the effect of consecutive passes on GOX and total protein release from Tt42.

2.4.5.3 French Pressure Cell

An Amicon French pressure cell was used to disrupt 20ml samples of Tt42 cells. A hydraulic press was used to generate maximum disruption pressures of 5Kpsi. A total of 9 consecutive passes were used, the samples were centrifuged at 2135 x

g in a Sorvall RT Du Pont bench top centrifuge, fitted with a swinging bucket rotor for 20 minutes and analysed for GOX activity.

2.4.5.4 Freeze – Thaw

Samples of Tt42 (20ml, 20% wet weight/volume) cells were subjected to 3 consecutive rounds of freezing (-20°C) and thawing (luke warm water). The samples were centrifuged at 16060 x g in a Biofuge Pico Heraeus bench top centrifuge for 5 minutes and analysed for GOX activity.

2.5 Results and Discussion

2.5.1 GOX Assay

For validation of the GOX assay (Bergmeyer, 1988), the assay was performed in triplicate using the commercial *A. niger* GOX. Results are depicted graphically in Figure 2.1.

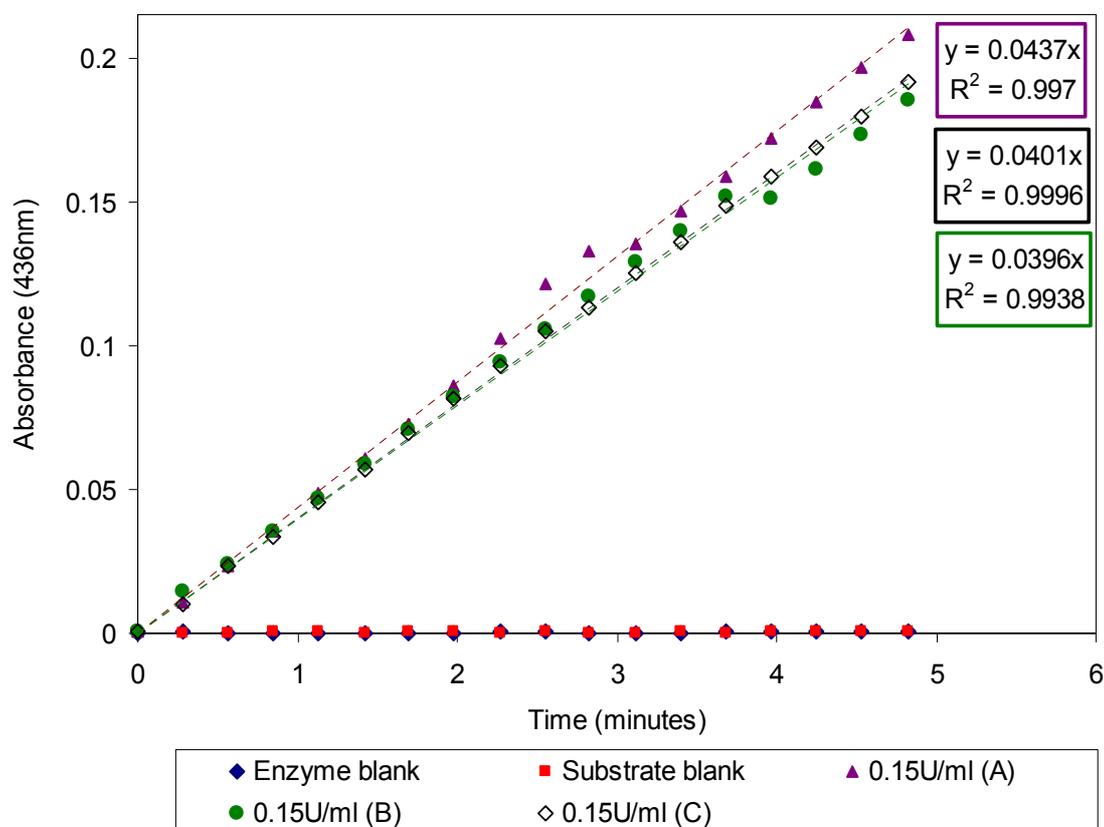


Figure 2.1: Validation of the GOX assay at 0.15U/ml GOX from *A. niger*.

The slope of the curve was used to calculate the results in U/ml GOX activity and were found to be 0.163 U/ml, 0.150 U/ml and 0.148 U/ml for each of the reactions. The average result was 0.153 ± 0.008 U/ml GOX (Appendix 1). The determination of GOX activities with the Bergmeyer (1988) method was thus found to be accurate and reproducible for the GOX from *A. niger*.

2.5.2 Cell Disruption

2.5.2.1 Sonication

The result for GOX and protein released by sonication from Tt42 cells is shown in Figure 2.2. The GOX activity of ± 2.8 U/ml observed before the onset of sonication was attributed to carry over from the cultivation medium during the formulation of the sample for sonication.

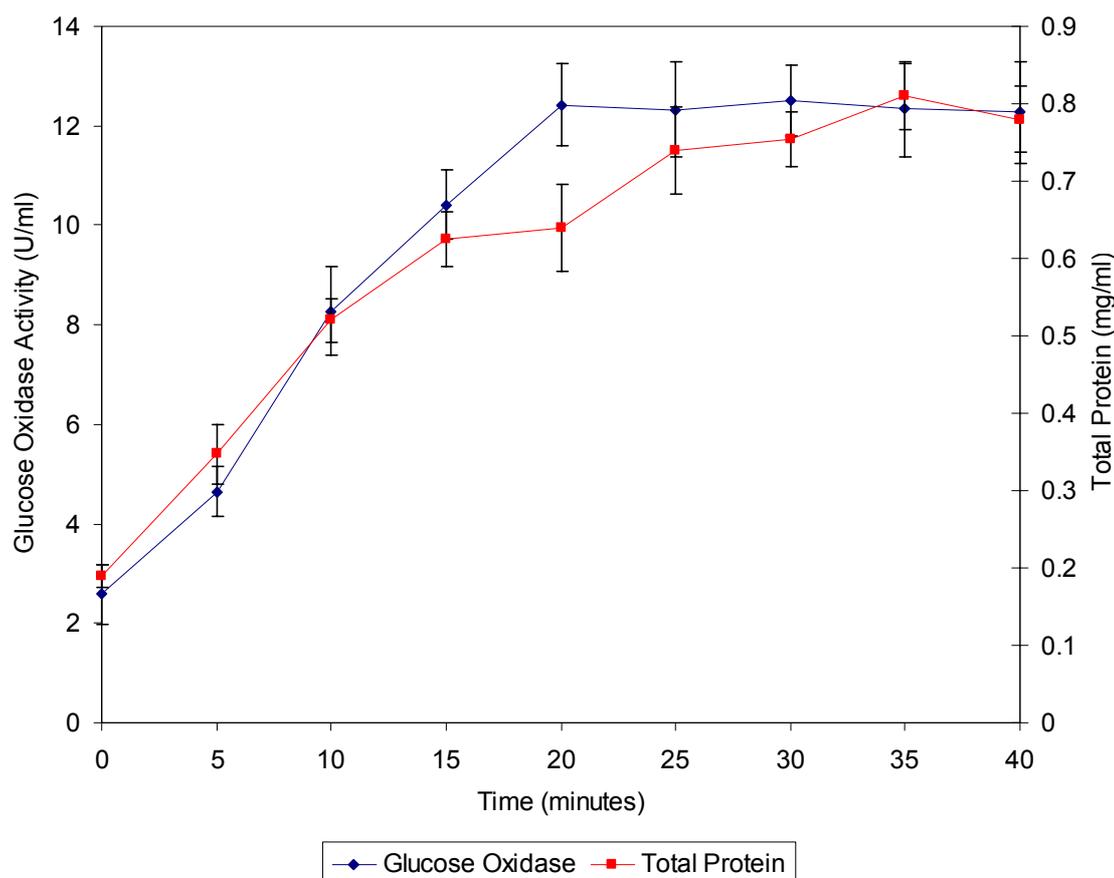


Figure 2.2: Time course of GOX activity and total protein release from 10ml of *P. canescens* Tt42 cells.

Maximum GOX release (12.5 U/ml) was reached after ± 20 minutes of sonication; the total protein released after 20 minutes of sonication was 0.64 mg/ml. The maximum total protein release (0.82 mg/ml) was only reached after 35 minutes of sonication; therefore total release of GOX was achieved before total protein release. A possible explanation for this would be that prolonged sonication resulted in the disruption of sub-cellular components, resulting in increased protein concentration.

Under the conditions of the experiment it was determined that 20 minutes of sonication was sufficient to obtain the intracellular concentration of GOX. No decrease in either GOX or total protein was observed thereafter, suggesting that the conditions of the experiment were gentle enough not to cause destruction of the intracellular enzymes and yet severe enough to disrupt the fungal cell walls. It is also possible that the experiment had reached a point of equilibrium, where the rate of intracellular GOX release was equivalent to the rate of GOX degradation under the experimental conditions.

Alternative methods of cell disruption were investigated to establish whether or not the GOX released was in fact the total amount that could be released.

2.5.2.2 Constant Systems (Z-Plus Series) cell disrupter

The results of different operating pressures on the release of the intracellular GOX and total protein, from a single pass, are tabulated in Table 2.1.

Table 2.1: The effect of a single pass at various pressures on the release of GOX and total protein from Tt42 cells using the cell disrupter.

Pressure (Kpsi)	GOX Release (U/ml)	Total protein (mg/ml)
10Kpsi	12.68 \pm 1.77	0.71 \pm 0.12
20Kpsi	12.53 \pm 2.23	0.73 \pm 0.10
30Kpsi	12.94 \pm 2.55	0.72 \pm 0.07
38Kpsi	12.91 \pm 2.50	0.74 \pm 0.15

The GOX and total protein released from the Tt42 cells were similar within the range of pressures tested, as between 12.53 and 12.94 U/ml GOX and 0.7mg/ml total protein were released. These results were similar to those obtained for 20

minutes of sonication as discussed above. A pressure of 38Kpsi for cell disruption was chosen and further investigated for improved release with further passes (Figure 2.3). This pressure was chosen for further investigation since it was possible that if Tt42 cells were cultured for longer than 72 hours, as in the growth curve, the cell walls may be more robust and therefore be more resistant to cell disruption at the lower pressures.

Consecutive passes of the Tt42 cells on the Z-plus series cell disrupter had an adverse effect on both the GOX and total protein released from the Tt42 cells (Figure 2.3).

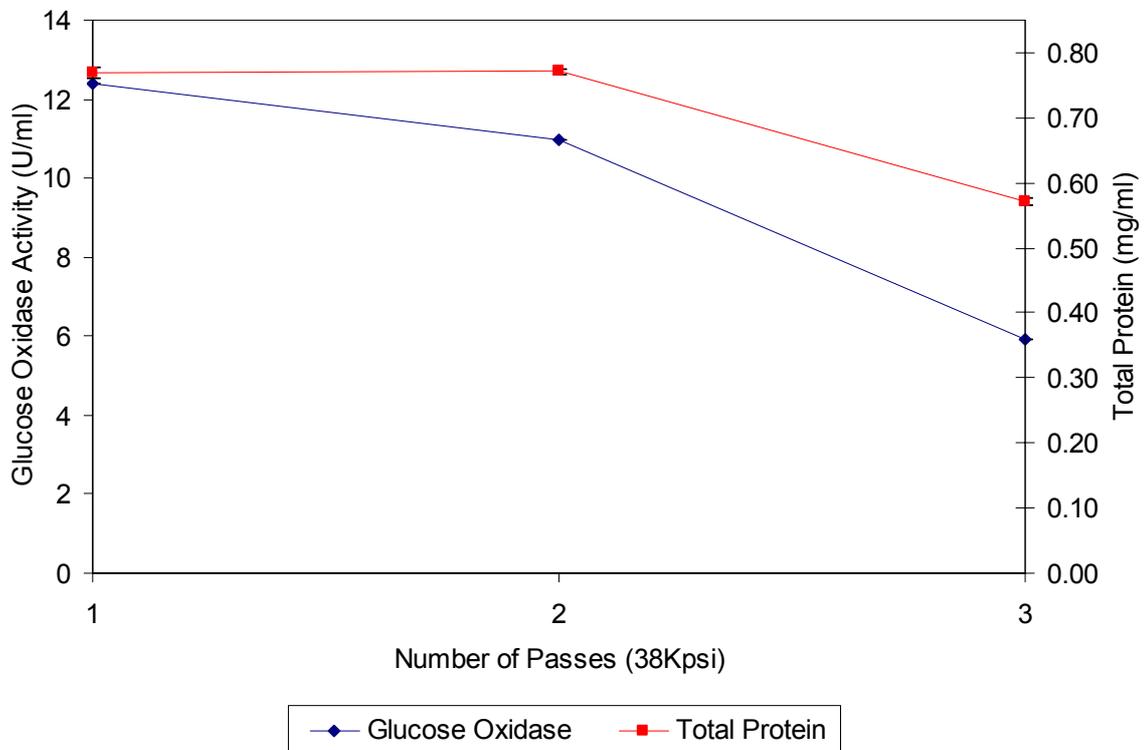


Figure 2.3: The effect of the 3 rounds of cell disruption on the intracellular release of GOX and total protein from Tt42 cells using the cell disrupter.

One pass resulted in the release of 12.39 U/ml GOX and 0.77mg of protein. The rate of GOX activity loss was more rapid than that of the total protein. Two passes caused a decrease of 11% in the GOX activity while the total protein released remained unchanged. Three passes caused a further decrease of 46% in the

GOX activity along with a 26% drop in the total protein. Total GOX release was achieved with a single pass on the cell disrupter; the decreases in GOX activity observed with further passes may be explained by enzyme denaturation. The decrease in total protein observed after 3 passes was surprising since denatured protein should still be detectable with the Bio-Rad protein assay, the loss may have been due to the protein sticking to the disrupted cells which were removed before analysis. The GOX and total protein released after 1 pass were similar to the results obtained, in the earlier studies, at the different disruption pressures (Table 2.1).

2.5.2.3 French Press and Freeze-Thaw

French press and freeze-thaw were evaluated as cell disruption techniques for the release of GOX from Tt42 cells. An Amicon French Pressure cell was applied at 5Kpsi and freeze-thaw cycles from -20°C to room temperature. A pressure of 5Kpsi was the only pressure tested with the French Pressure cell due to the constraints of the hydraulic system. Both methods were shown to release similar amounts of GOX from Tt42 as the previous methods tested (Table 2.2). A drawback of French press and freeze thaw as cell disruption options were that numerous passes were required for GOX release. The French press required 7 passes at 5Kpsi and freeze thaw required 3 cycles from -20°C to room temperature. Further passes or cycles with either of the methods had no significant effect on the release of GOX from Tt42. The optimum parameters for GOX release in U/ml of the 4 cell disruption techniques investigated are summarised in table 2.2 below:

Table 2.2: The effect of various methods of cells disruption on the GOX released from *P. canescens* Tt42 cultivated for ± 72 hours at 28°C.

Methods of Disruption	Description of Treatment	GOX Release (U/ml)
Sonication	20 minutes	12.42 ± 0.83
Cell Disrupter	38Kpsi (1 Pass)	12.91 ± 2.50
French Pressure Cell	5Kpsi (7 Passes)	12.34 ± 1.62
Freeze-Thaw	-20°C (3 Passes)	12.25 ± 2.42

All four methods of cell disruption investigated released similar amounts of glucose oxidase from Tt42. Cell disruption using the Constant System (Z-Plus Series) cell disrupter was considered to be the best disruption method as it was the most efficient. Added advantages of using the cell disrupter were ease of use and only 1 pass at 38Kpsi was required for optimal release.

2.5.3 Growth and Enzyme Production

Maximum GOX activity was achieved at 71 hours of cultivation, which coincided with complete glucose utilisation (Figure 2.4B). GOX was produced from the start of cultivation indicating constitutive production of this enzyme in Tt42. The biomass concentration increased exponentially for 48 hours, before glucose depletion was observed, with a linear increase thereafter. A possible explanation for the change in growth rate, observed before glucose depletion, may be oxygen limitation as oxygen mass transfer in shake flasks is poor.

The final biomass concentration achieved, after 120 hours, was ± 24 mg/ml (Figure 2.4A). During the growth of the Tt42 cells the pH decreased from 7 to ± 6.5 at ± 71 hours, after which the pH increased to a final value of ± 6.95 (Figure 2.4 A). The increase in pH after glucose depletion was probably due to the use of the organic nitrogen as a carbon source with the resultant release of ammonium ions. Petruccioli *et al.* (1995) when cultivating *P. variabile* mutant M-80.10, under similar conditions, recorded an increase in gluconic acid production to 78mg/ml (48 hours) followed by a steady linear decline in concentration to 40 mg/ml (120 hours). This increase in pH, and the increase in pH observed in the shake flasks for Tt42 could also have been due to the organism using the gluconic acid as a carbon source thereby lowering this acidic component in the medium.

Both the intra- and extracellular GOX concentrations increased until glucose depletion, at 71 hours, with maximum values of 11.5 and 6.9 U/ml, respectively (Figure 2.4B and Figure 2.5).

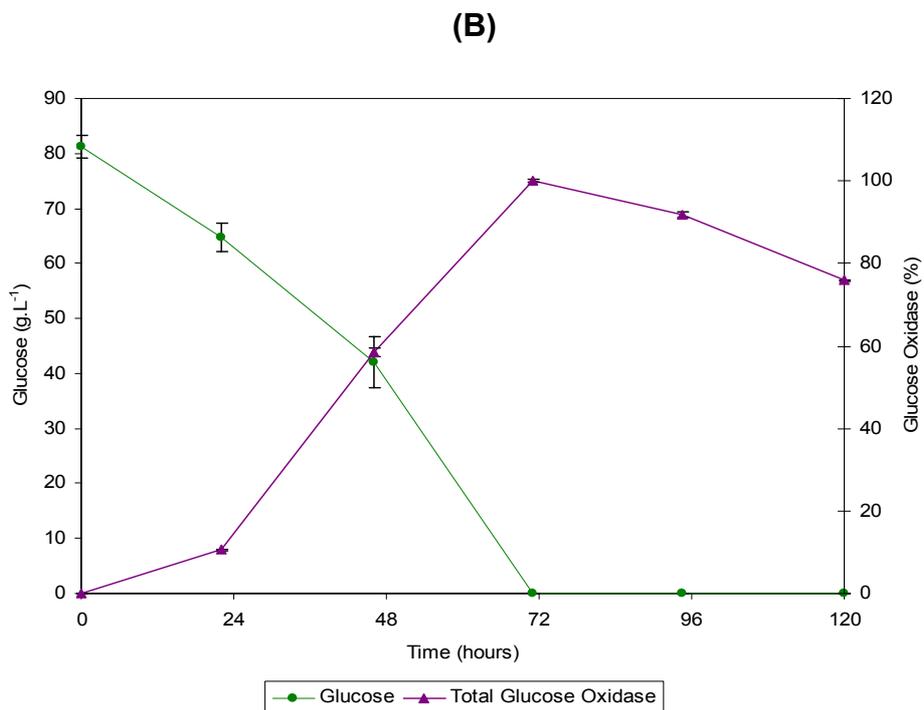
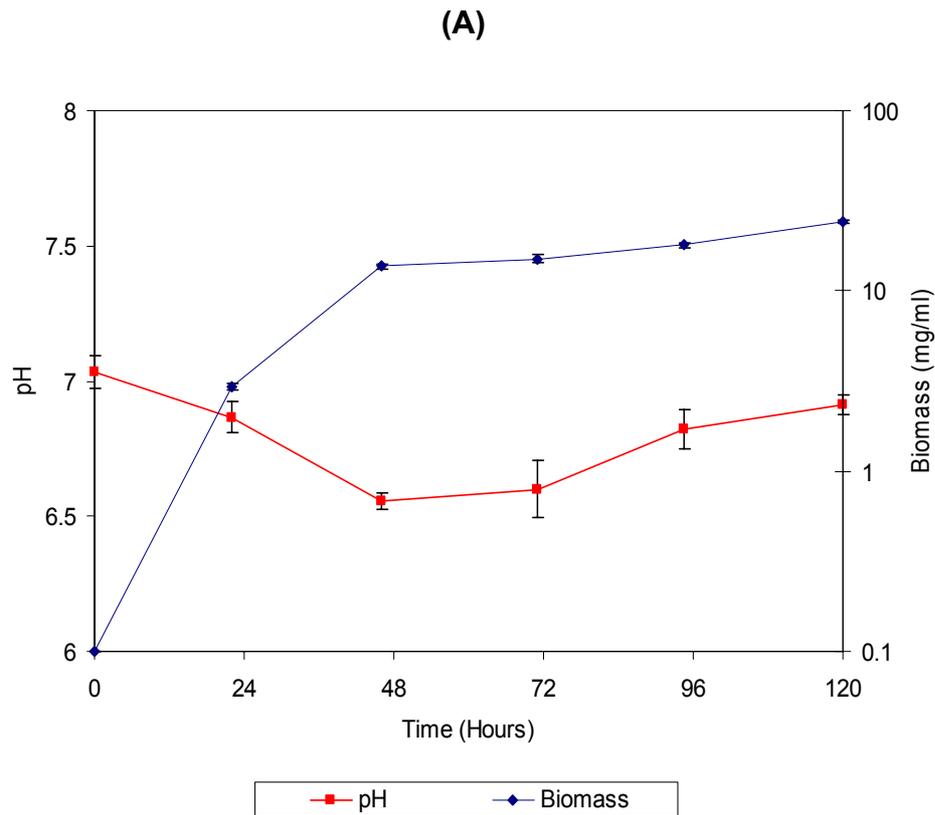


Figure 2.4: Shake flask cultivation of Tt42 using optimised media of Rogalski *et al.* (1988) over 120 hours (triplicate data). (A) – pH and Biomass; (B) – Glucose and Total Glucose Oxidase

On average, the intracellular GOX concentration was $\pm 70\%$ and the extracellular was $\pm 30\%$ of the total volumetric GOX produced (Figure 2.5). A total of 1104 U of GOX was achieved after 71 hours of cultivation from 60 ml of cultivation medium (based on the intra- and extracellular volumetric titres).

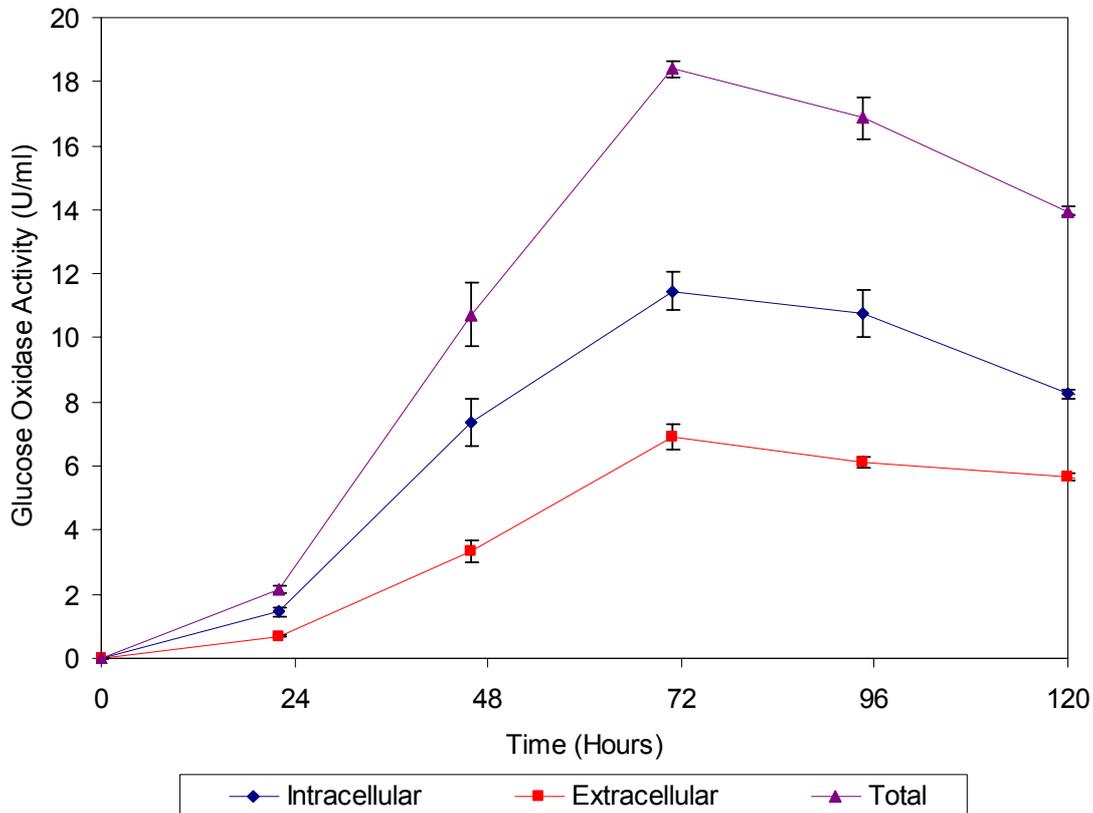


Figure 2.5: Volumetric GOX production by *P. canescens* Tt42 over 120 hours of cultivation.

The determination of specific GOX activity in Umg^{-1} protein was not investigated since the cultivation medium used in this study contained an organic nitrogen source. Therefore biomass specific GOX activity was investigated (Figure 2.6). The maximum biomass specific GOX activity (Umg^{-1} biomass) was achieved approximately 3 days after inoculation (71 hours) and coincided with glucose depletion, with a maximum activity value of 1.08 Umg^{-1} (Figure 2.6).

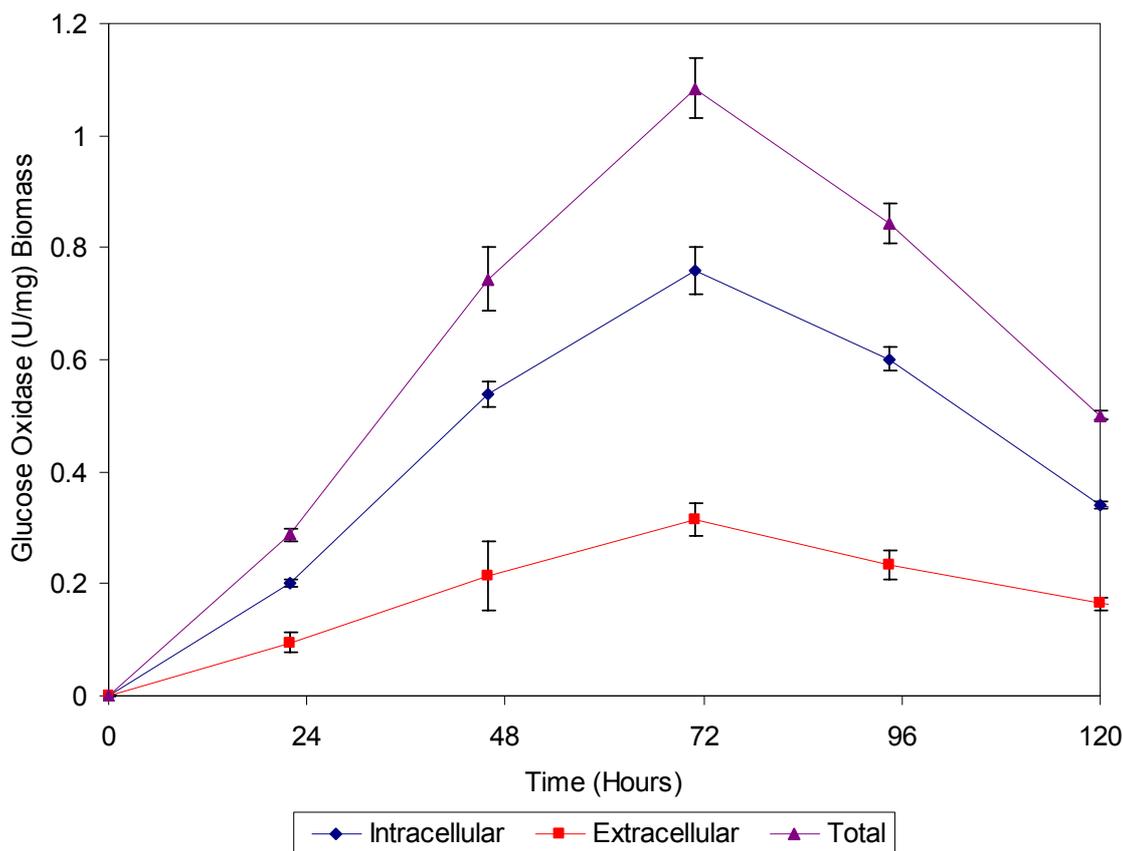


Figure 2.6: Biomass specific GOX activity for the shake flask cultivation of Tt42 over 120 hours.

Thereafter the biomass specific activity decreased, probably due to a lack of available glucose for maintenance of cellular metabolism. The optimum cultivation time for GOX production by Tt42 was thus found to be approximately 3 days when cultivated on the medium optimised by Rogalski *et al.* (1988). Maximum growth coincided with glucose depletion, suggesting that increased GOX levels could be achieved with a glucose feed after initial glucose depletion. This, however, is not the goal of this study and was not investigated further.

A comparison of the volumetric titres of GOX activity obtained with Tt42 was performed with those compiled from literature (Table 2.3). Accurate comparison of total GOX produced proved difficult since published values are scarce and only *A. niger* M-6 (Fiedurek and Gromada, 1997) and *A. niger* BTL (Hatzinikolaou *et al.*, 1996) have been reported for intra- and extracellular GOX activity. These were $\pm 20\%$ and $\pm 70\%$, respectively, lower than those found in this study. *Aspergillus*

niger strains G-IV-10 and AM-11 and *P. variable* P16 produced similar extracellular enzyme concentrations as that obtained with Tt42. *Penicillium variable* M-80.10 produced 2.76-fold more GOX extracellularly than Tt42 and was the only organism that performed significantly better. When compared to other wild type organisms, only *P. variable* P16 produced 7.67 U/ml extracellular GOX, performed at a similar level to Tt42.

Table 2.3: Comparison of GOX production from different fungal strains in growth media containing 8% glucose, 3,5% calcium carbonate and 3% peptone.

Organism	GOX (U/ml)			Reference
	Intracellular	Extracellular	Total	
<i>A. niger</i> M-6 (mutant)	5.8	9	14.8	Fiedurek and Gromada (1997)
<i>A. niger</i> G-13 (mutant)	ND	2.8	N/A	Rogalski <i>et al.</i> (1988)
<i>A. niger</i> G-IV-10(mutant)	ND	6.75	N/A	Gromada and Fiedurek (1996)
<i>A. niger</i> BTL (wild type)	3.53	1.9	5.43	Hatzinikolaou <i>et al.</i> (1996)
<i>A. niger</i> NRRL-3 (wild type)	0.177	ND	N/A	Sharif <i>et al.</i> (1992)
<i>A. niger</i> AM-11 (mutant)	ND	7	N/A	Fiedurek (1998)
<i>P. chrysogenum</i> DBVPG 8552 (wild type)	ND	1.54	N/A	Petruccioli <i>et al.</i> (1993)
<i>P. expansum</i> UCD 2-53-2 (wild type)	ND	0.78	N/A	Petruccioli <i>et al.</i> (1993)
<i>P. italicum</i> NRRL 983 (wild type)	ND	1.05	N/A	Petruccioli <i>et al.</i> (1993)
<i>P. variable</i> P16 (wild type)	ND	7.67	N/A	Petruccioli <i>et al.</i> (1999)
<i>P. variable</i> M-80.10 (mutant)	ND	19.09	N/A	Petruccioli <i>et al.</i> (1995)
<i>P. canescens</i> Tt42 (wild type)	11.47	6.92	18.39	Current work

N/A – not applicable since either the intra- or extracellular data was not available

ND – not determined by authors

2.6 Conclusions

The Bergmeyer (1988) GOX assay method was shown to be reliable and reproducible for the measurement of GOX activity using the instrumentation and chemicals available in this study.

The disruption of Tt42 cells cultivated for 72 hours, and formulated to 20% wet weight/volume in 0.1M phosphate buffer at pH 7 was investigated using four different methods which were: a sonicator, a Constant Systems (Z-plus series) cell disrupter, an Amicon French pressure cell and freeze-thaw at -20°C. Optimal GOX release was achieved with each of the methods tested, although Constant Systems (Z-Pus Series) cell disrupter was shown to be the simplest and most rapid method as only 1 pass was required. Sonication required 20 minutes for optimal enzyme release, French press required up to 6 consecutive passes at 5Kpsi and freeze thaw required 3 rounds. The Constant Systems (Z-plus series) cell disrupter required only 1 pass for optimal GOX release independent of the 4 pressures investigated i.e. 10, 20, 30 and 38Kpsi. Further passes were shown to cause a decrease in the GOX concentration, likely as a result of enzyme denaturation. This method was chosen for all subsequent studies.

Shake flask cultivation of Tt42 cells was successfully performed in 300ml Erlenmeyer flasks containing 60ml of optimised Rogalski *et al.* (1988) medium at 28°C and 200rpm. Optimum GOX production coincided with glucose depletion. Of the total GOX activity, 70% was found to be intracellular and 30% extracellular. The maximum volumetric GOX activity was 18.4 U/ml and the maximum specific GOX activity was shown to be 1.08 Umg⁻¹ biomass.

Unfortunately only 2 organisms reported in literature showed data for both intra- and extracellular GOX activity, namely *A. niger* M-6 (mutant) and *A. niger* BTL (wild type) which produced 20% and 70%, lower GOX yields than Tt42 each respectively. The mutated strain *P. variable* M-80.10 produced 2.76-fold more glucose oxidase extracellularly than that from Tt42.

To accurately determine the catalytic efficiency of the GOX from Tt42 a sufficiently pure preparation of the enzyme was needed. Due to the relatively high GOX yield from Tt42, as well as the various industrial applications for GOX, it was decided to purify and characterise the enzyme from shake flask cultivation.

Chapter 3
Purification of GOX from *Penicillium*
***canescens* Tt42**

3 Purification of GOX from *Penicillium canescens* Tt42

3.1 Introduction

CAT is known to be produced by *Penicillium* species, which catalyses the decomposition of hydrogen peroxide to water and free oxygen, where hydrogen peroxide acts as both the electron acceptor and donor (Fiedurek and Gromada, 2000). Since the hydrogen peroxide formed in the GOX reaction (Appendix 1) is essential to the accurate determination of GOX activity, it was imperative to remove CAT to accurately characterise the enzyme.

GOX has been purified for commercial application from different fungi including *A. niger* (Hatzinikolaou *et al.*, 1996; Kalisz *et al.*, 1990 and Swoboda and Massey, 1965) and *Penicillium* species; *P. pinophilum* (Rando *et al.*, 1997), *P. amagasakiense* (Kusai *et al.*, 1960 and Kalisz *et al.*, 1997); *P. chrysogenum* (Eriksson *et al.*, 1987); *P. notatum* (Gorniak and Kaczkowski, 1974) and *P. funiculosum* (Eryomin *et al.*, 2004).

On average, the isoelectric point of GOX has been shown to fall between pH 4 and pH 5 (Eriksson *et al.*, 1987; Kalisz *et al.*, 1997 and Kusai, 1960) hence the use of anion exchange chromatography for its purification has been commonly used (Kalisz *et al.*, 1997; Swoboda and Massey, 1964; Dai *et al.*, 2002 and Hatzinikolaou *et al.*, 1996). GOX is mainly eluted with salt gradients using NaCl (Hatzinikolaou *et al.*, 1996; Rando *et al.*, 1997 and Dai *et al.*, 2002), although mixed pH and salt gradients have previously been used (Kalisz *et al.*, 1997). Various precipitation techniques have also been used before and after ion exchange chromatography to purify GOX including: ammonium sulphate (Kalisz *et al.*, 1997; Kusai *et al.*, 1960 and Swoboda and Massey, 1964), uranyl acetate (Gorniak and Kaczkowski, 1974), potassium hexacyanoferrate (II) and copper sulphate (Eriksson *et al.*, 1987). The pI of CAT from *P. chrysogenum* was reported to be 6.5 (Eriksson *et al.*, 1987). The differences in pI values between GOX and CAT could therefore be exploited to assist in the purification of GOX to ensure it is CAT free by using anion exchange chromatography since separation with this

method is by pI differences. GOX from *P. amagasakiense* has been reported to contain 4 different isoenzymes of GOX with pI values of 4.37; 4.42; 4.46 and 4.51 (Kalisz *et al.*, 1997).

3.2 Theory of techniques

Ammonium sulphate precipitation (salting out) is a technique used to precipitate proteins from solution by increasing the ionic strength of the solution. The technique is reliant on the hydrophobic nature of proteins, since they contain hydrophilic and hydrophobic groups. When the proteins are dissolved, water is forced into contact with the proteins' hydrophobic groups and in the process becomes ordered around the proteins. Increasing the concentration of salt ions by the addition of ammonium sulphate causes the water to be removed from around the protein exposing the hydrophobic portions of the proteins. Precipitation of the proteins will then occur due to the aggregation of proteins via the exposed hydrophobic portions. The technique is used to fractionate the proteins from solution since proteins with larger or more hydrophobic portions will aggregate and precipitate before those with smaller or fewer portions of hydrophobic groups. Ammonium sulphate is an ideal salt for the process because it is sufficiently soluble and relatively inexpensive (Harris and Angal, 1994)

The isoelectric point (pI) for an enzyme is defined as the pH at which the enzyme has no net charge, it's zwitterionic form, in aqueous solution. Isoelectric focusing (IEF) can be used to separate enzymes based on their pI values. The separation is obtained by applying a potential difference across horizontal gels on glass or plastic sheets. The gels contain a pH gradient formed by the addition of ampholytes into the gels. Ampholytes are complex mixtures of synthetic polyamino-polycarboxylic acids. IEF is able to separate enzymes which differ in their pIs by as little as 0.01 of a pH unit, and is thus very effective. The IEF may also be used to determine the pI of a particular enzyme by staining the gel with a mixture of enzyme assay reagents (substrates and buffer) or by running a set of standards of enzymes of known pI values (Wilson and Walker, 1995).

The principle of ion exchange chromatography as a protein purification technique relies on the fact that proteins are ionisable, carrying net negative or net positive charges. Thus they can be separated based on the attraction between oppositely charged particles. There are 2 types of ion exchangers, cation and anion exchangers. The cation exchangers have negatively charged groups and thus attract and exchange positively charged groups, and anion exchangers contain positively charged groups and thus exchange negatively charged groups. There are five steps involved in the ion exchange mechanism (Wilson and Walker, 1995):

- Diffusion of the ions to be exchanged to the exchanger surface,
- Diffusion of the ions through the exchanger matrix to the site of ion exchange,
- Exchange of the relevant ions at the exchange site in the matrix,
- Diffusion of the exchanged ions back out through the exchange matrix to its surface,
- The final step involves the selective desorption by the elution buffer and diffusion of the molecule into the external elution buffer.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a standard technique used for the qualitative analysis of protein mixtures. Since the method involves the separation of proteins according to size, it is useful in determining the relative molecular weight of proteins and monitoring enzyme purification. SDS is an anionic detergent and under defined experimental conditions the relative electrophoretic mobilities of proteins in the presence of SDS are related to their relative molecular masses. Samples to be run on SDS-PAGE are boiled for 5 minutes in the presence of β -mercaptoethanol and SDS. The β -mercaptoethanol results in the reduction of any tertiary structure disulphide bonds and the SDS strongly binds the proteins and denatures them. Each protein in the mixture is fully denatured into a rod-shaped structure containing a series of negatively charged SDS molecules along the polypeptide chain. A current is passed through the gel once the samples are loaded. The SDS-protein complexes migrate through the gel and separate due to the molecular sieving properties of

the gel. The smaller the protein the less hindered it is from migration and thus the proteins are separated based on their relative molecular masses. The sample buffer usually contains an ionisable tracking dye that allows the run to be monitored. Once the run has reached completion it is stained with a staining solution like Coomassie Brilliant Blue for a few hours and washed in an appropriate destain solution overnight. The proteins are visible as blue bands on a clear background. Solutions containing a mixture of proteins with known molecular masses are run with the unknown samples in order to determine the relative molecular masses of the proteins of interest (Wilson and Walker, 1995)

Size exclusion chromatography is a technique used to separate molecules based on their size and shape. A column is packed with an appropriate porous gel material spanning a relatively narrow size range. Examples of gels used for size exclusion chromatography are: cross-linked dextrans, polyacrylamide, agarose and polystyrene. When passing a solution containing molecules of various sizes through columns containing these materials, the larger molecules that are excluded from the pores will pass through the interstitial spaces and will appear in the eluent first. The smaller molecules get caught up in the mobile phase, both inside and outside the molecular sieve and thus pass through at various rates. The molecular weight of the smallest of the large molecules that are unable to enter into the pores of a given gel is known as the exclusion limit of that gel. Molecules that have molecular weights below the exclusion limit of the gel will elute from the gel based on their molecular weight, from largest to smallest. There is an approximate linear relationship between the relative elution volume of a substance and the logarithm of its molecular mass, used for determination of the approximate molecular mass of proteins (Wilson and Walker, 1995 and Voet and Voet, 1995).

3.3 Aims

- To purify the Tt42 GOX for characterisation of its kinetic properties,
- Determine the ammonium sulphate concentrations at which the GOX from Tt42 would precipitate, both intra- and extracellularly,

- Determine the number of isoenzymes of the Tt42 GOX, both intra- and extracellularly,
- Employ ammonium sulphate precipitation, ion exchange and size exclusion chromatography as methods for the purification of the GOX from Tt42,
- Confirmation of GOX purity with gel electrophoresis and size exclusion chromatography.

3.4 Materials and Methods

3.4.1 Chemicals

Toyopearl[®] Super Q 650 M quaternary ammonium anion exchange resin was purchased from TosohBiosep. A Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad) was used for SDS-PAGE. Fermentas PageRuler[™] Protein ladder molecular weight standards were purchased from Inqaba Biotechnical Industries. Immobiline Dry Strips pH 4-7 (13 cm, linear), IPG buffer, pH 3-10, N,N,N',N'-tetramethylethylenediamine (TEMED) and bromophenol blue were obtained from Amersham Biosciences. Acrylamide/bis-Acrylamide solution (29:1), sodium dodecyl sulfate (SDS) and ammonium persulphate were obtained from Sigma-Aldrich. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), Perhydrol (30% hydrogen peroxide) solution and ammonium sulphate were obtained from Merck. Bio-Rad Gel Filtration Standards used for size exclusion chromatography were purchased from Bio-Rad. CAT from beef liver was obtained as a gift from Seravac Pty Ltd. Omega[™] 30kDa low protein binding polyethersulphone ultrafiltration membranes were obtained from Pall Filtration. All chemicals were reagent grade or better.

3.4.2 Growth conditions and cell disruption

Five Erlenmeyer flasks, each containing 200ml of Rogalski *et al.* (1988) growth medium were used to cultivate Tt42. The media was prepared as described in Chapter 2. Each of the flasks was inoculated with 6ml conidia suspension at a concentration of $\pm 1.2 \times 10^8$ conidia/ml, grown on MEA plates (3 days, 28°C). The flasks were cultivated for 72 hours on a rotary shaker at 200rpm and 28°C. After 72 hours the flasks were pooled and harvested. The culture broth was centrifuged at 2135 x g in a Sorvall RT Du Pont benchtop centrifuge, fitted with a swinging

bucket rotor, for 20 minutes, washed and resuspended to 20% wet weight/volume in 0.1M phosphate buffer at pH 7 (GOX assay buffer – Appendix 1). The supernatant was kept aside and constituted the extracellular GOX fraction. The formulated pellet fraction was passed once through the Constant Systems (Z-Plus) series cell disrupter at 38Kpsi, and centrifuged as before. The disrupted pellet was discarded while the supernatant constituted the intracellular GOX fraction.

3.4.3 Enzyme assays

GOX and total protein were determined as described in Appendices 1 and 2. CAT was determined in the final purified preparation of GOX from Tt42 according to Bergmeyer (1988) (Appendix 3). The decomposition of hydrogen peroxide by catalase was measured spectrophotometrically at 240nm and 25°C using a Beckman Coulter DU800 spectrophotometer. Initial decreases in absorbance at 240nm were measured and the linear portion of the curve was used to determine the catalase activity. One unit of catalase activity was defined as the amount of enzyme catalysing the decomposition of 1µmole of hydrogen peroxide per minute under the assay conditions. All assays were performed in triplicate.

3.4.4 Preliminary GOX purification investigations

3.4.4.1 Ammonium sulphate precipitation

Initial ammonium sulphate precipitation experiments were done on both the intra- and extracellular fractions of GOX from Tt42. The experiment was performed on a 20ml sample. The extracellular fraction was diluted 1:1 with 50mM potassium phosphate buffer (pH 7) since ammonium sulphate is known to slightly acidify the extract. The precipitation was performed with 20ml cell free fractions for each of the respective ammonium sulphate concentrations (0 to 100% saturation) in 100ml beakers at 4°C while stirring gently. The ammonium sulphate was pre-weighed and added slowly to each of the respective samples and allowed to stir gently for 1 hour. The samples were centrifuged at 3000 x g for 1 hour in a Beckman J2-21 centrifuge using a JA20.1 rotor, and the supernatants were separated from the precipitated pellets. The pellets were resuspended to 20ml with GOX assay buffer. All fractions were analyzed for GOX activity and total

protein in triplicate. The 100% (intracellular) and 70% (extracellular) fractions were freeze dried using a LABCONCO LYPH.LOCK[®] 12 freeze dryer.

3.4.4.2 Isoelectric focusing

The freeze dried intra- and extracellular fractions, were reconstituted in distilled water to a concentration of 1mg/ml. These samples were diafiltered with distilled water in an Amicon (8200) 200ml stirred tank ultrafiltration unit, fitted with a 30kDa cut-off polyethersulphone membrane. The pIs of the intra- and extracellular GOX from Tt42 were determined using an Amersham IPGPhor[™] isoelectric focusing unit on 13cm pH 4-7 linear Immobiline Strip (Amersham Biosciences). Rehydration buffer was modified from the manufacturer's protocol and consisted of glycerol (40%) and CHAPS (4%) and 1µl of IPG buffer (Amersham Pharmacia). Samples (200µl diluted 1:1 with rehydration buffer) were loaded onto the Immobiline strip using the rehydration loading protocol from the manufacturer's handbook. The loaded samples were rehydrated at room temperature ($\pm 25^{\circ}\text{C}$) for 12 hours. The samples were focused stepwise: 500V for 2 hours, 1000V for 2 hours, and 5000V for 5 hours.

3.4.4.3 GOX zymogram

After isoelectric focusing the strips for the intra- and extracellular GOX fractions were stained with a GOX assay stock. The GOX assay stock was made up to 30ml containing 24ml GOX assay phosphate buffer (0.1M, pH 7) solution containing *o*-dianisidine.2HCl (0.006% w/v), 5ml of glucose aqueous solution (10%w/v), and 1ml HRP (60 U/ml). The focused strips were placed into the solution and allowed 30 minutes to react.

3.4.5 Final GOX purification

Three main methods of protein purification were used for the final purification of GOX from Tt42; they were ammonium sulphate precipitation, ion exchange chromatography and size exclusion chromatography.

The extracellular fraction from cultured Tt42 (600ml) was used to purify the GOX. The sample was mixed with 150ml of GOX buffer (Appendix 1) and centrifuged at 9632 x g for 10 minutes in a Beckman J2-21 centrifuge using a JA14 rotor to

remove the resultant calcium phosphate precipitate. The sample was precipitated with ammonium sulphate (60-70% saturation) and centrifuged at 3000 x g for 1 hour in a Beckman J2-21 centrifuge using a JA14 rotor. The precipitate was resuspended in 200ml distilled water and diafiltered with distilled water in an Amicon (2800) ultrafiltration stirred cell reactor using a 30kDa cut-off polyethersulphone ultrafiltration membrane. The diafiltered sample was diluted to 50ml with 20mM Tris buffer containing 50mM NaCl (pH7.2) and was applied to a Super Q 650M (TosohBiosep) anion exchange resin column (40cm x 2.6cm) pre-equilibrated with 20mM TRIS buffer (pH7.2). Bound protein was eluted with a 500 ml linear salt gradient from 50mM to 500mM NaCl in 20mM Tris buffer (pH 7.2) at a flow rate of 4 ml/min, collecting 7 to 10 ml fractions. Fractions were assayed for protein by monitoring ultraviolet absorption at 280 nm, and GOX activity was determined for each fraction using the GOX assay (Appendix 1). Fractions containing high GOX activity were pooled and diafiltered with distilled water. The dialyzed eluate was then lyophilized and stored at -20°C. Size exclusion chromatography was used as the final purification step; furthermore the experiment was used to determine the approximate molecular weight of the native protein as well as providing an indication of the purity of the final purified GOX. The lyophilized sample was dissolved in a minimum amount of distilled water and subjected to size exclusion chromatography using a XK16 Pharmacia Biotech column (550mm X 16mm) packed with Sephacryl S200HR (Amersham) resin. The sample was eluted from the column at a flow rate of 0.7ml/minute with 20mM potassium phosphate buffer at pH 7.4. The peak displaying high GOX activity was reapplied to the size exclusion chromatography column to confirm purity and approximate molecular weight. The approximate molecular weight of the GOX was determined by calibration against Bio-Rad gel filtration standards of known molecular weight which were: thyroglobulin (670kDa), bovine gamma globulin (158kDa), chicken ovalbumin (44kDa), equine myoglobin (17kDa) and vitamin B12 (1.35kDa). The final lyophilized purified preparation was analyzed for CAT activity.

3.4.6 Confirmation of purity

Two standard biochemical techniques were employed to confirm the purity of the GOX from the extracellular fraction of Tt42 namely, SDS-PAGE and size exclusion chromatography. Size exclusion chromatography fulfilled a dual role in

being the final purification step and allowing for the determination of the GOX approximate molecular weight. Final visual confirmation of GOX purity and molecular mass was done using SDS-PAGE on 12% acrylamide vertical slab gels (10 x 8cm). The final purified sample, after size exclusion chromatography was examined at a protein load of 1.3 µg. SDS-PAGE sample buffer (0.0625 M Tris, pH 6.8, 10% glycerol, 2 % SDS, 5 % β-mercaptoethanol, 0.05 % bromophenol blue) was added to the sample (1:1), after which sample was boiled at 95°C for 5 minutes. The 12 % acrylamide separating gel (0.375 M Tris, pH 8.8, 0.1 % SDS, 12 % acrylamide) and 4 % stacking gel (0.125 M Tris, pH 6.8, 0.1 % SDS, 4 % acrylamide) was prepared. Polymerisation of both the separating and stacking gels was induced by the addition of 0.05 % ammonium persulphate (APS) and 0.005 % N,N,N'N'-tetramethylethylenediamine (TEMED). The SDS-PAGE running buffer consisted of 25 mM Tris, 192 mM glycine and 1 % SDS. The gel was resolved for at least one hour at 200 V. The staining of the polyacrylamide gels was performed using Coomassie stain (40 % methanol, 0.7 % acetic acid, 0.075 % Coomassie dye) and destained using Coomassie destain (40 % methanol, 0.7 % acetic acid). The approximate subunit molecular mass of GOX from Tt42 was determined by calibration against a set of protein standards. The PageRuler™ Protein ladder contains 14 protein bands of 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 40kDa, 50kDa, 60kDa, 70kDa, 85kDa, 100kDa, 120kDa, 150kDa and 200kDa.

3.5 Results and Discussion

3.5.1 Initial GOX purification investigations

3.5.1.1 Ammonium sulphate precipitation

Ammonium sulphate protein precipitation was used to fractionate the intra- and extracellular protein fractions. Profiles of GOX activity against ammonium sulphate saturation are shown in Figures 3.1 and 3.2.

The GOX from the extracellular fraction was effectively precipitated with a 60% - 70% ammonium sulphate cut (Figure 3.1). At 70% ammonium sulphate saturation there was 60% recovery of total GOX (U) and a 14% recovery of total protein (mg). Therefore for the extracellular GOX fraction, the 60%-70% ammonium

sulphate precipitation was found to be an effective initial purification step increasing the specific GOX activity from 28 U mg^{-1} to 122 U mg^{-1}

The intracellular GOX was precipitated using ammonium sulphate with a 50% - 100% cut (Figure 3.2). At 100% ammonium sulphate saturation an approximate recovery of 80% for total GOX (U) and total protein (mg) was observed. Ammonium sulphate precipitation was determined to be ineffective as a purification step for the intracellular GOX from Tt42 since no significant change in the specific GOX activity was found, remaining ± 20 U mg^{-1} total protein.

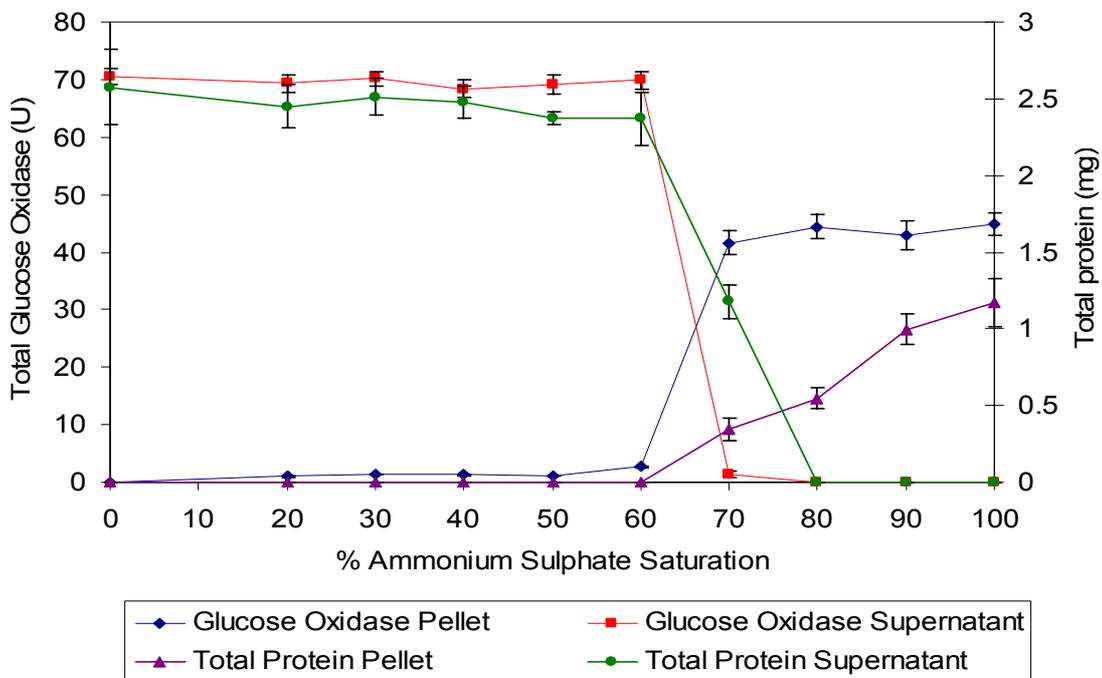


Figure 3.1: Ammonium sulphate precipitation of the extracellular fraction of GOX from Tt42.

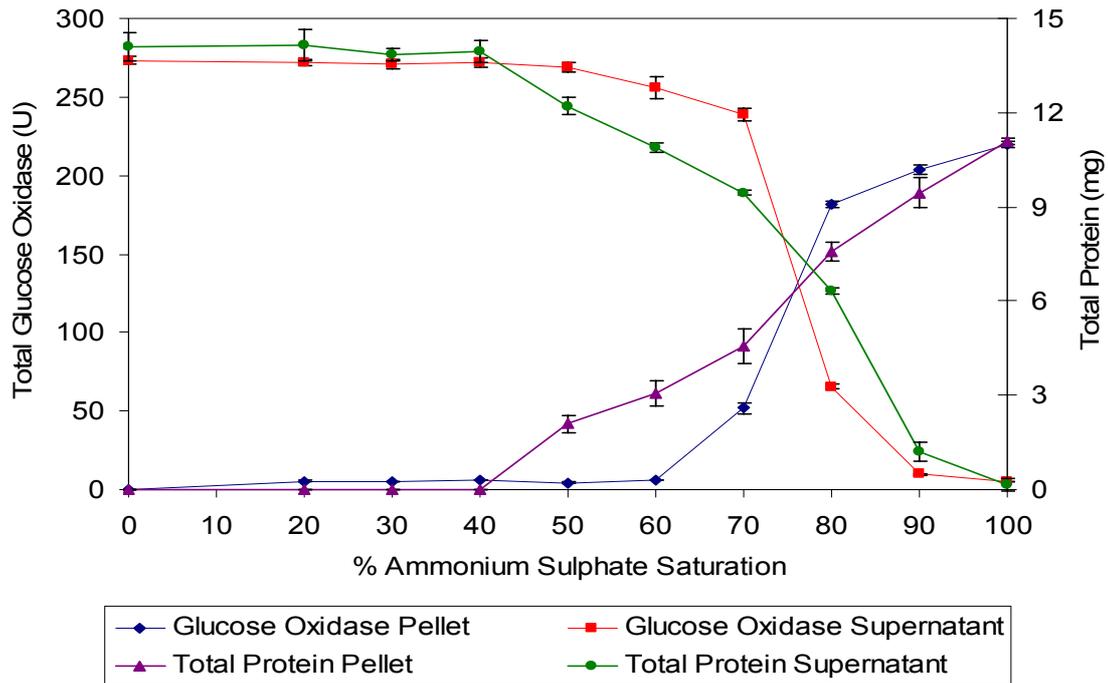


Figure 3.2: Ammonium sulphate precipitation of the intracellular fraction of GOX from Tt42.

The differences in ammonium sulphate precipitation characteristics for the intra and extracellular GOX from Tt42 may be attributed to the fact that GOX from *Penicillium* species are known to be glycosylated. The GOX from *P. amagasakiense* is a glycoprotein which contains 11 – 13% carbohydrate described as the high-mannose type (Kusai *et al.*, 1960; Eriksson *et al.*, 1987 and Nakamura and Fujiki, 1968).

The GOX in the extracellular fraction would, probably, have all been glycosylated and therefore precipitated more efficiently and over a narrower ammonium sulphate saturation concentration. The losses in total GOX for the intra- and extracellular GOX were probably due to denaturation caused by the high ammonium sulphate concentrations. As mentioned, during ammonium sulphate precipitation as the ionic strength of the solution increases the hydrophobic groups become exposed and the proteins aggregate causing the proteins to precipitate. When the precipitated proteins are resuspended it is also possible that some of the aggregates do not completely dissociate and would also cause the decrease in GOX activity.

3.5.1.2 Isoelectric focusing and GOX zymogram

The intra- and extracellular GOX from ammonium sulphate precipitation were subjected to isoelectric focusing and stained with the GOX zymogram solution (Figure 3.3).



Figure 3.3: Zymogram of GOX isoenzymes of the intra- and extracellular fraction from Tt42 (extracellular – top; intracellular – bottom). A and B represent the 2 isoenzymes of GOX.

The experiment was done to determine how many isoenzymes of GOX were produced by Tt42 and their approximate pI. The results revealed 2 isoenzymes for GOX in both the intra- and extracellular fractions. The isoenzymes were determined to have approximate pI values of 4.30 and 4.67, as indicated by A and B respectively in Figure 3.3.

Using the information obtained for the initial purification investigations it was decided to purify the GOX of Tt42 from the extracellular fraction (600ml) only. The reasons for the decision were:

- The ammonium sulphate precipitation was more effective for the extracellular GOX.
- The intra- and extracellular GOX fractions were confirmed to both contain the same isoenzymes at the same pI values.

3.5.2 GOX purification

3.5.2.1 Ammonium sulphate precipitation

An extracellular fraction of GOX from Tt42 (600ml) was purified as outlined in table 3.1. The extracellular GOX was precipitated using a 60%-70% ammonium

sulphate cut, after adding 150ml of 0.1M potassium phosphate buffer (pH 7) buffer, to minimise pH effects. The addition of the buffer caused the sample to go murky and form a precipitate. The sample was centrifuged and the pellet was assayed for GOX activity and was found to contain no GOX activity. The precipitate may have been calcium phosphate formed from the addition of the phosphate buffer since the growth media (Rogalski *et al.*, 1988) contained a high concentration of calcium carbonate (35 gL⁻¹). Ammonium sulphate precipitation resulted in the elimination of 87.5% of the total protein and a 4.9-fold purification of the GOX.

Table 3.1: Purification table of extracellular GOX from Tt42

Sample description	Protein (mg)	GOX (U)	Specific Activity (Umg ⁻¹)	Yield (%)	Fold Purification
Initial Sample	153.9	4308.0	28.0	100.0	1.0
Calcium Precipitation	146.0	4082.0	28.0	94.7	1.0
Ammonium Sulphate Precipitation	19.3	2748.0	137.7	63.8	4.9
Diafiltration (Salt Removal)	12.7	1947.0	153.0	45.2	5.5
Anion Exchange Chromatography	4.1	1096.0	268.1	25.4	9.6
Diafiltration (Salt Removal)	2.9	880.0	300.0	20.4	10.7
Lyophilisation	2.4	677.0	288.0	15.7	10.3
Size Exclusion	2.0	609.0	312.0	14.1	11.1
Lyophilisation	1.9	445.0	240.5	10.3	8.6

3.5.2.2 Anion Exchange Chromatography

Following ammonium sulphate precipitation the sample was subjected to anion exchange chromatography (Figure 3.4). The samples collected between ±190ml and ± 250ml contained high concentrations of GOX, and were pooled. Three main protein peaks other than GOX were observed at ±10ml, 110ml and 330ml. The

anion exchange chromatography resulted in a 32% decrease in total protein with a 56% recovery of GOX in relation to the sample applied to the chromatography step (Table 3.1). Anion exchange chromatography resulted in an overall 9.6-fold purification of the GOX (table 3.1).

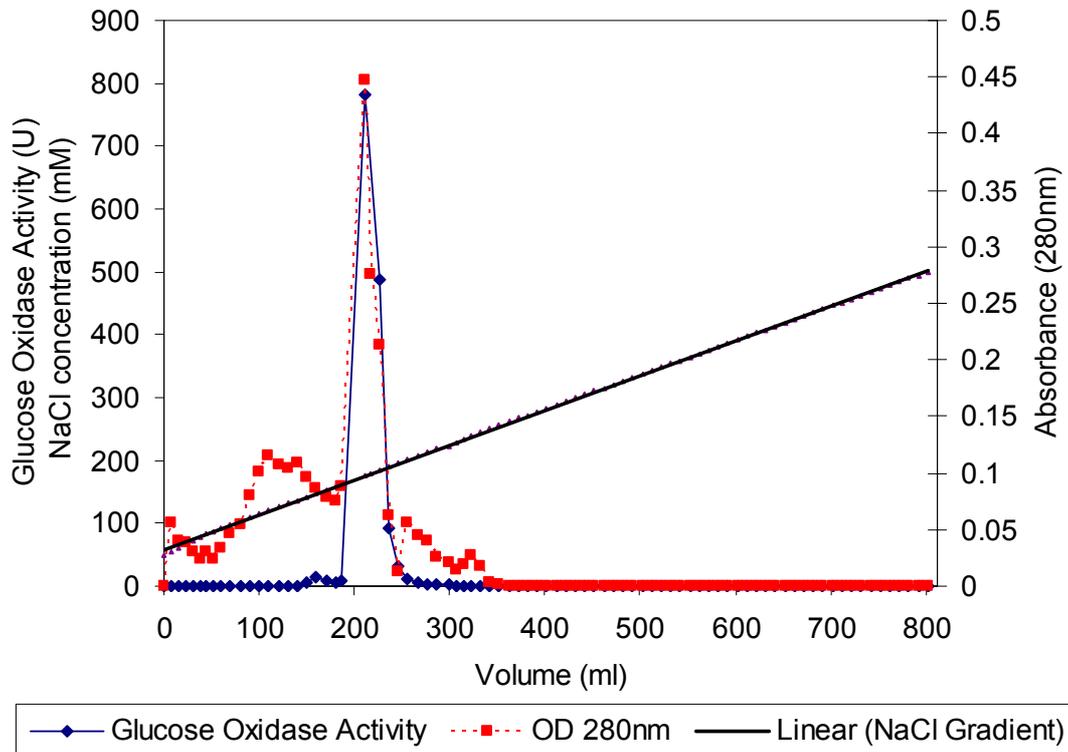


Figure 3.4: Anion exchange chromatography elution profile of extracellular GOX and total protein from Tt42.

After the ammonium sulphate precipitation and the anion exchange chromatography steps the samples were diafiltrated (30kDa) to remove the salts from the sample. For each of the ultrafiltration steps the filtrate was shown to be GOX free. The ultrafiltration after ammonium sulphate precipitation caused a 30% loss in total GOX activity and after anion exchange chromatography a 20% loss was observed. These losses in activity may possibly be attributed to shear in the ultrafiltration stir cell reactor and binding of protein to the 30kDa polyethersulphone ultrafiltration membranes.

Purification, with ammonium sulphate precipitation and anion exchange chromatography, resulted in a GOX yield of 15.7% with 10.3 fold purification. The partially purified GOX was determined to have a specific activity of 288 U mg^{-1} total protein (Table 3.1).

3.5.2.3 Size exclusion chromatography

The lyophilised GOX from Tt42 was subjected to size exclusion chromatography as a final purification step.

Three peaks were obtained from the size exclusion chromatography profile for the partially purified GOX from Tt42 (Appendix 5 B). The standard curve for size exclusion chromatography was determined to have a linear correlation of 0.99986 for bovine gamma globulin (158kDa), chicken ovalbumin (44kDa), equine myoglobin (17kDa) and vitamin B12 (1.35kDa) (Appendix 5A). The thyroglobulin (670kDa) was larger than the exclusion limit of the resin since it was eluted in the void volume which was determined to be 43ml. The three peaks were determined to correspond to approximate molecular weights of 259kDa, 176kDa and 14kDa in order of elution calculated from the molecular weight standard curve (Appendix 5A and 5B). The peak displaying high GOX activity, correlated to an approximate molecular weight of 176kDa, was applied to the size exclusion column again (Appendix 5C). The size exclusion profile produced 4 peaks with 1 peak more prominent than the rest (Appendix 5C). The 4 peaks corresponded to approximate molecular weights of 148kDa, 103kDa, 39kDa and 16kDa in order of elution calculated from the molecular weight standard curve (Appendix 5A). Based on the area under the peaks the GOX from Tt42 was calculated to be approximately 80% pure. Size exclusion chromatography resulted in an 11.1-fold purification of the GOX from Tt42 (Table 3.1).

The final purified GOX from Tt42 sample was lyophilised and shown to have a specific GOX activity of 240.5U mg^{-1} (Table 3.1). The overall yield of GOX from Tt42 was calculated to be 10.3% with an 8.6-fold purification. The drops in the fold purification observed for each of the lyophilisation steps in Table 3.1 were thought to be due to the absence of GOX stabilisers during the freeze drying process.

The final lyophilised GOX preparation was analysed for CAT activity and found to be catalase free. The result was an important one since the Bergmeyer (1988) assay for GOX determination uses the hydrogen peroxide generated in the first reaction as a substrate in the second reaction for the formation of the quinoneimine dye.

3.5.2.4 SDS-PAGE

SDS-PAGE was performed on the final purified GOX sample and the results are shown in Figure 3.6. The experiment was performed to confirm GOX purity and approximate the subunit molecular weight. The sample was loaded at 1.3 µg total protein. The SDS-PAGE showed a subunit molecular weight for GOX from Tt42 of approximately 70kDa calculated from the standard curve of the molecular weight standards (Appendix 4). The results obtained for the size exclusion and SDS-PAGE, together, indicated that the GOX from Tt42 was a dimeric enzyme probably consisting of 2 equal subunits (M_r 70kDa each) with an overall molecular weight of approximately 148kDa.

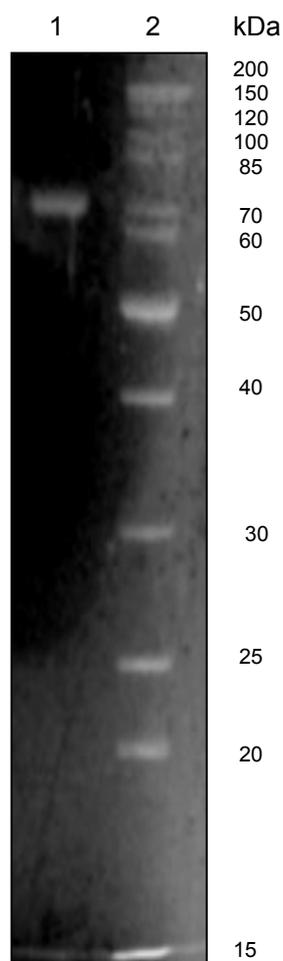


Figure 3.5: SDS-PAGE (12%) of purified GOX from Tt42 after size exclusion chromatography. Lane 1, final purified GOX from Tt42; lane 2, molecular weight standards.

3.6 Conclusions

It was shown that the GOX from Tt42 consisted of 2 isoenzymes where one was dominant over the other, with the dominant having a pI of 4.3 and the less dominant having a pI of 4.67. Due to the dominance of the one GOX isoenzyme, it was concluded that activity characterisation investigations would be performed for the dominant GOX isoenzyme. Both the intra- and extracellular fractions of GOX were shown to contain the same two isoenzymes.

Three main purification techniques were employed to successfully achieve the aims of the chapter, namely: ammonium sulphate precipitation, ion exchange chromatography and size exclusion chromatography. The three techniques were

successful in purifying the GOX from Tt42 with an overall yield of 10.3% with a 8.6-fold purification. The final purified GOX preparation was shown to be approximately 80% pure (by size exclusion chromatography) and free of CAT.

Size exclusion chromatography and SDS-PAGE were effectively used to determine that the GOX from Tt42 was a dimeric protein probably consisting of 2 identical subunits with a molecular weight of 70kDa.

To accurately compare the GOX from Tt42 with those described in literature the enzyme would need to be compared based on its characteristics. Ultimately the catalytic efficiency of the GOX from Tt42 would need to be compared with other GOX enzymes in order to determine if this novel enzyme displayed enhanced or different characteristics. Therefore, a kinetic characterisation of purified GOX from Tt42 was undertaken.

Chapter 4
Characterisation of GOX from
***Penicillium canescens* Tt42**

4 Characterisation of GOX from *Penicillium canescens* Tt42

4.1 Introduction

Determination of the characteristics and catalytic constants of the purified ($\pm 80\%$) GOX from Tt42 was performed to better understand the enzyme and compare it with other GOX enzymes from literature.

GOX from various fungal species have been kinetically characterised including *A. niger* (Kalisz *et al.*, 1990 and Hatzinikolaou *et al.*, 1996) and various *Penicillium* species, including *P. funiculosum* (Sukhacheva *et al.*, 2004), *P. chrysogenum* (Eriksson *et al.*, 1987), *P. pinophilum* (Rando *et al.*, 1997) and *P. amagasakiense* (Kalisz *et al.*, 1997).

The kinetic constants for free GOX from *A. niger* (Sigma Type VII) and *P. amagasakiense* (ATCC 28686) were determined by Witt *et al.* (1998) and Kalisz *et al.* (1991) respectively. The literature reporting kinetic constants of free GOX was found to be scarce since most kinetic parameter investigations have been performed on immobilised GOX.

4.2 Theory of the techniques

Enzymes are known to be sensitive to changes in temperature since the relationship between reaction rate of an enzyme and temperature is exponential. For every 10°C rise in temperature the rate of an enzyme reaction will double. At temperature ranges of between 40°C and 70°C most enzymes are denatured and lose their activity. Enzymes are known to display maximal activity at a temperature known as the temperature optimum of the enzyme (Wilson and Walker, 1995).

Enzyme activity is pH dependent since the activity is dependent on the ionisation state of the amino acids in the active site (Wilson and Walker, 1995 and Voet and Voet, 1995). Most proteins are only active within a narrow pH range, usually in the range of 5-9 (Wilson and Walker, 1995 and Voet and Voet, 1995).

Leonor Michaelis and Maude Menten (1913) determined that the initial rate or velocity of catalysis of an enzyme varied hyperbolically with substrate concentration (Voet and Voet, 1995). The initial rate increased with an increase in substrate concentration to a point where it would reach maximum velocity (V_{max}). At low substrate concentrations the initial rate was determined to be proportional to the substrate concentration, referred to as first order kinetics. At high substrate concentrations the initial rate was found to be independent of the substrate concentration, referred to as saturation or zero order kinetics. Michaelis and Menten derived a mathematical equation to express the relationship between the initial rate and substrate concentration:

$$v_o = \frac{V_{max}[S]}{K_m + [S]}$$

Where v_o is the initial rate, V_{max} is the maximal velocity of the reaction and K_m is the Michaelis constant. The K_m value was found to be equal to the substrate concentration at half the maximal velocity of the reaction, and was shown to be independent of the enzyme concentration and a characteristic of the system (Wilson and Waller, 1995).

The K_m value is a measure of the enzymes substrate affinity or the binding strength of the enzyme and its substrate. V_{max} is the limiting value of the initial velocity. The turnover number is an expression of the maximum number of moles of substrate that 1 mole of enzyme can convert to product in 1 second; it is the rate of the catalytic process or a measure of catalytic activity. The specificity constant (k_{cat}/K_m) is a measure of the relative reaction rate at low substrate concentrations; it is a second order rate constant that is a direct measure of the efficiency of the enzyme (Wilson and Walker, 1995).

4.3 Aims

- Determine the accurate range of the GOX assay,
- Determine the temperature and pH activity profiles for GOX,
- Determine of the stability of the GOX at 25°C and 37°C,

- Determine the storage stability of GOX,
- Determine the kinetic constants of the GOX,
- Compare the kinetic constants of GOX from Tt42 with those reported in relevant literature.

4.4 Materials and Methods

4.4.1 Chemicals

GOX from Tt42, purified according to Chapter 3, was used to perform all the characterisation investigations. All the reagents for the GOX assay were purchased from Merck, with the exception of *o*-dianisidine which was purchased from Sigma-Aldrich. All chemicals were of reagent grade or better.

4.4.2 GOX characterisation studies

All the characterisation investigations were performed using a Beckman Coulter DU800 spectrophotometer fitted with a Peltier cooling system. The GOX assay (Bergmeyer, 1988) was performed as outlined in Appendix 1, unless otherwise stated. All assays were done in triplicate.

4.4.2.1 Variation of the initial rate with enzyme concentration

The relationship between GOX concentration (U) and the initial rate was investigated. Different concentrations of GOX, ranging from 0U/ml to 1U/ml, were made up in distilled water and the initial rates were determined.

4.4.2.2 Variation of the initial rate with temperature

The GOX assay reagents were added to plastic cuvettes and equilibrated at the respective temperatures (ranging from 10°C to 65°C) for 10 minutes before initiating the reactions with the addition of the GOX at a concentration of 0.15U/ml. The assays were performed at the respective temperatures.

4.4.2.3 Variation of the initial rate with pH

The pH profile for GOX from Tt42 was performed in universal buffer containing 50 mM phosphate (disodium hydrogen orthophosphate), 33 mM citrate (citric acid) and 50.7 mM borate (boric acid) (Perrin and Dempsey, 1974). The universal buffer

was pH adjusted to the respective pH values using 1M potassium hydroxide solution. GOX diluted to 0.4 U/ml was used in the investigation, the concentration was chosen as only 50% of the GOX activity was apparent in universal buffer.

4.4.2.4 Stability at 25°C and 37°C

The stability of GOX from Tt42 was determined at 25°C and 37°C, due to the two major applications for GOX, that of implantable glucose biosensors (37°C) and glucose determination test strips ($\pm 25^\circ\text{C}$).

GOX from Tt42 at a concentration of 0.15 U/ml was made up in distilled water and filter sterilised through a 0.2 μm cellulose acetate filter before being placed into pre-equilibrated water baths at 25°C and 37°C. Samples were removed periodically and analysed for activity at 25°C. The GOX activity of the samples was compared to an initial sample taken at the onset of the experiment.

4.4.2.5 Stability of freeze dried GOX

The freeze dried GOX preparation obtained from the purification described in Chapter 3 was stored at -20°C and periodically analysed for enzyme activity over a period of 6 months. The investigation was performed to give an indication of the shelf-life of the enzyme.

4.4.2.6 Variation of the initial rate with substrate concentration

In order to determine if the GOX from Tt42 displayed Michaelis – Menten kinetics, various concentrations of glucose ranging from 5mM to 650mM were used to determine the initial rates of the GOX reaction. GOX from Tt42 was diluted to a concentration of 0.15U/ml and used in the study.

4.4.2.7 Determination of the kinetic constants

The kinetic constants for GOX from Tt42 were determined from the Hanes – Woolf linear plot. The Michaelis constant (K_m), the maximal limiting rate velocity (V_{max}), the turnover number (k_{cat}) and the specificity constant (ratio of k_{cat}/K_m) were all calculated (Appendices 6 and 7).

4.5 Results and Discussion

4.5.1 Variation of the initial rate with enzyme concentration

The relationship between the concentration of GOX (U) from Tt42 in the assay and the initial rate was shown to be linear up to approximately 0.2 U/ml (Figure 4.1).

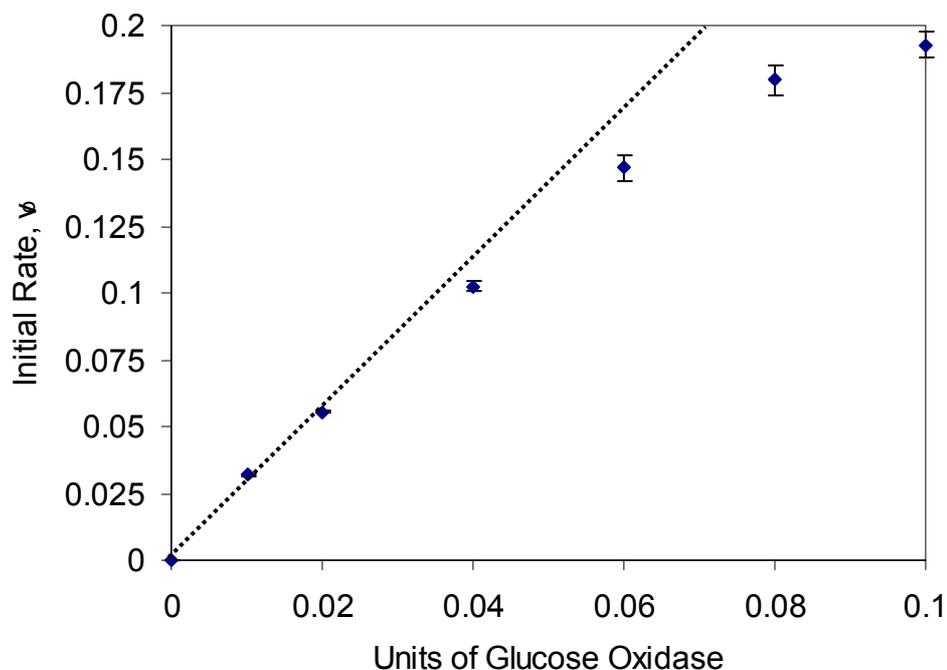


Figure 4.1: The correlation between concentration of GOX from Tt42 and the initial rate v_0 (Units of GOX in the assay).

The GOX from Tt42 to a concentration of 0.2 U/ml (0.02 U in the actual assay) could be accurately determined using the (Bergmeyer, 1988) GOX assay. At higher GOX concentrations the initial rates began decreasing which lead to inconsistent activity determinations. Therefore the maximum initial rate of β -D-glucose hydrolysis was shown to be $\pm 0.055 \mu\text{mol}/\text{min}/\text{ml}$.

4.5.2 Variation of the initial rate with temperature

The GOX from Tt42 was optimally active at 25°C and exhibited more than 70% of the maximum activity between 10°C and 45°C. Above 45°C the activity decreased rapidly (Figure 4.2).

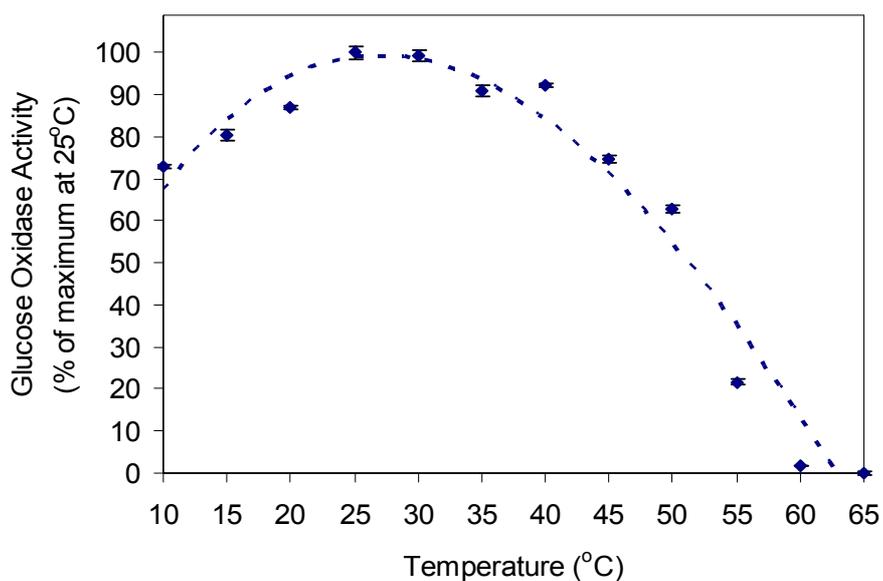


Figure 4.2: Temperature activity profile of GOX from Tt42

4.5.3 Variation of the initial rate with pH

GOX from Tt42 displays highest activity at neutral to alkaline pH. The GOX was optimally active at pH 7 and exhibited more than 70% of the maximum activity between pH 4.9 and pH 8.9. Below pH 4.9 the activity decreased to a minimum activity of $\pm 25\%$ at pH 3.4 (Figure 4.3).

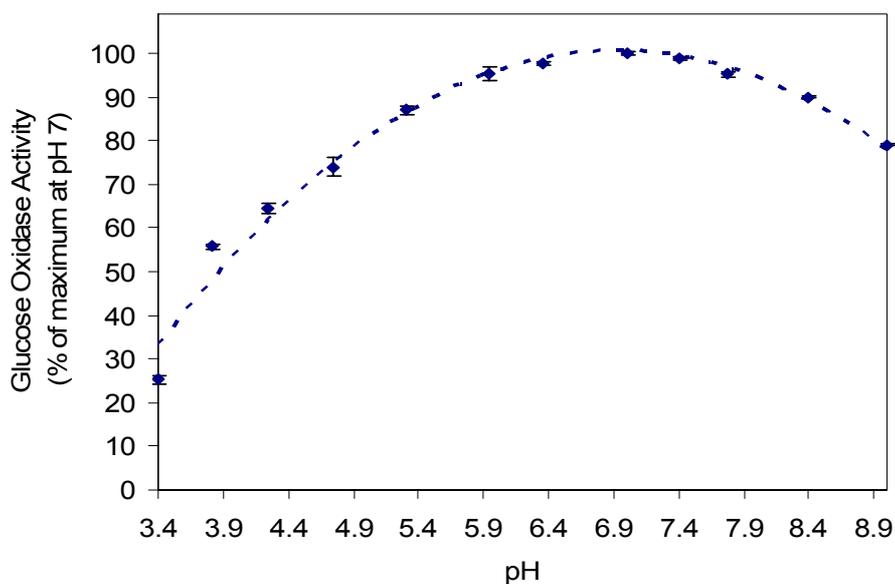


Figure 4.3: pH activity profile GOX from Tt42

4.5.4 Stability at 25°C and 37°C

The residual activity of GOX from Tt42 remained relatively unchanged for 10 hours at 25°C (Figure 4.4). Applications of the enzyme at room temperature would thus be possible based on the stability of GOX from Tt42 at 25°C.

The GOX from Tt42 was shown to have a half life of approximately 30 minutes at 37°C (Figure 4.5). The GOX from Tt42 would not be effective for applications at 37°C without stabilisation (e.g. immobilisation). Polyhydric alcohols including, ethylene glycol, glycerol, erythritol, xylitol and sorbitol and polyethylene glycol have been shown to have a stabilising effect on GOX from *A. niger* (Ye *et al.*, 1988).

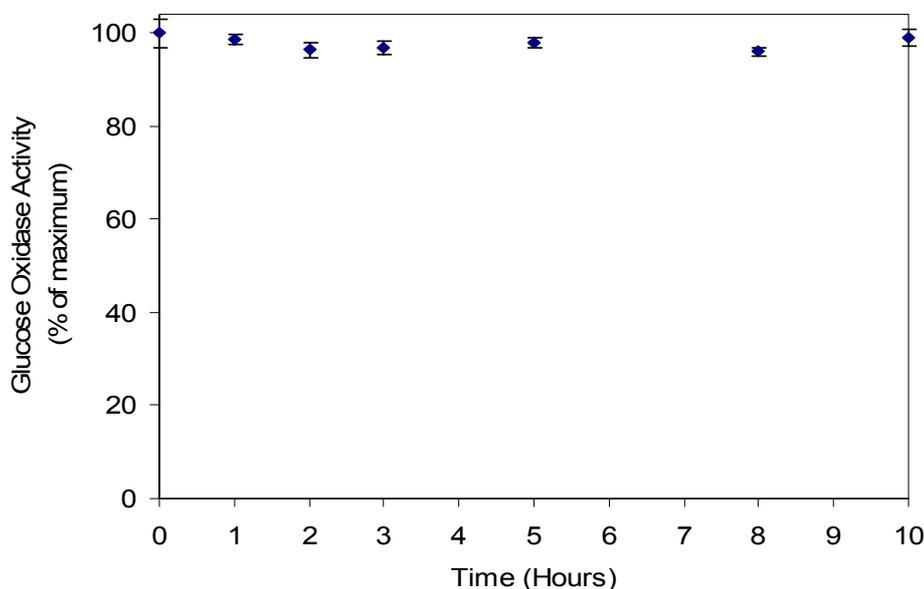


Figure 4.4: Stability profile of GOX from Tt42 at 25°C.

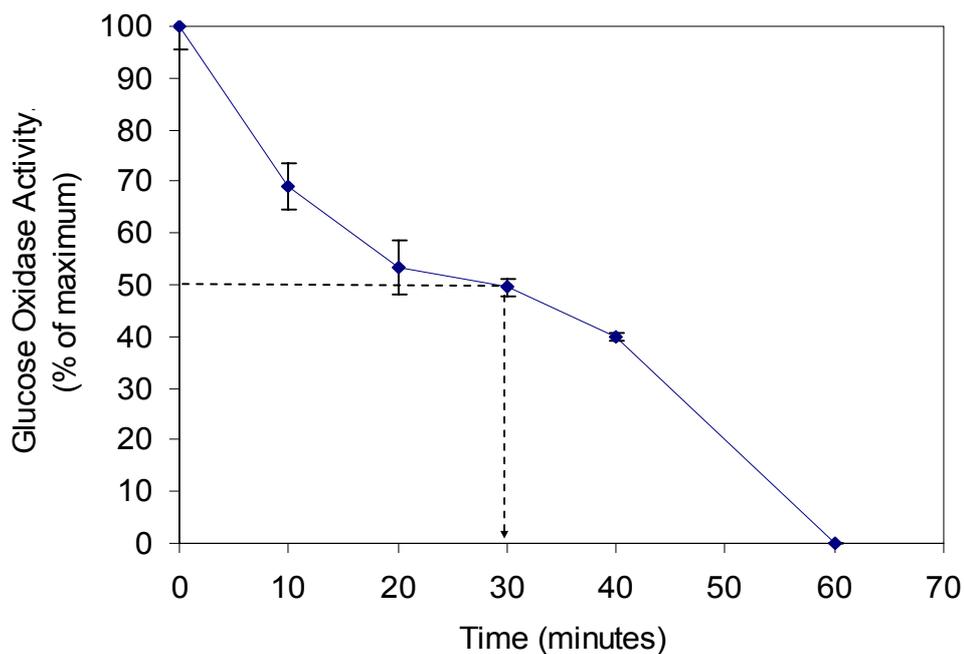


Figure 4.5: Stability profile of GOX from Tt42 at 37°C.

4.5.5 Storage Stability

The lyophilised GOX preparation from Tt42 remained stable for a minimum of 6 months at -20°C (Figure 4.6). The shelf life of the purified GOX without the addition of any stabilisers is thus an attractive feature of this enzyme for commercial applications.

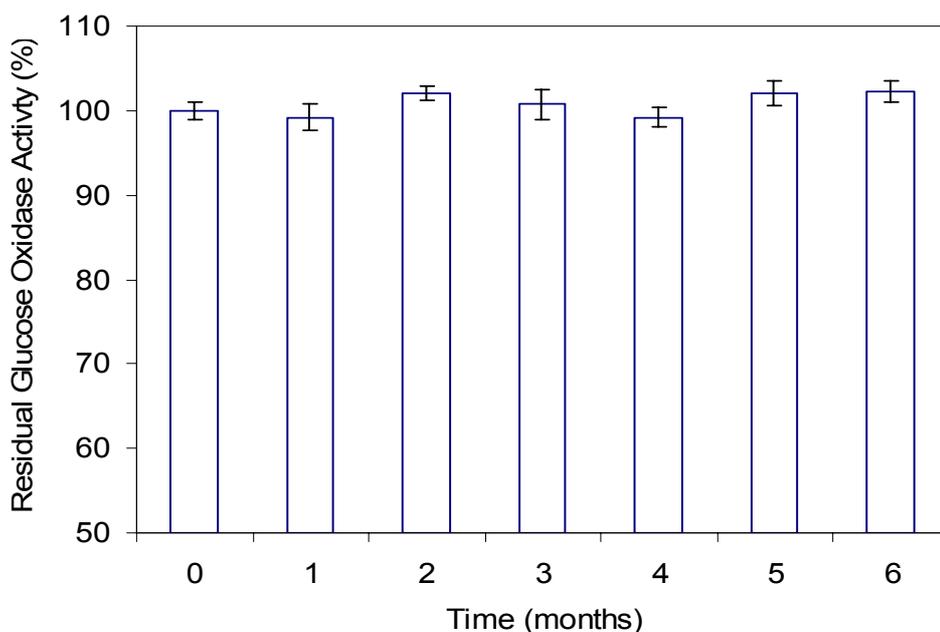


Figure 4.6: Storage stability of lyophilised GOX from Tt42 at -20°C

4.5.6 Variation of the initial rate with substrate concentration

Upon examination of the initial rate of the GOX reaction at different substrate concentrations it was determined that the reaction was subject to standard Michaelis – Menten kinetics (Figure 4.7). The hyperbolic curve in Figure 4.7 maintained the Michaelis – Menten deductions mentioned earlier. Based on the Michaelis – Menten equation it would appear that the values for V_{\max} and K_m were $4.5 \times 10^{-2} \mu\text{mol/minute}$ and $\pm 14 \text{ mM}$ glucose respectively (Figure 4.7). It should be noted that the glucose concentration depicted in figures 4.7 and 4.8 are the concentrations as calculated in the actual enzymatic reactions. The accurate determination of the initial rate at high substrate concentrations is known to be difficult and therefore the calculations of the kinetic values mentioned are subject to error. Therefore linear transformations of the Michaelis – Menten equation are preferred (Wilson and Walker, 1995). The Hanes – Woolf plot is based on one such transformation equation and was used to accurately determine the V_{\max} , and K_m of GOX from Tt42.

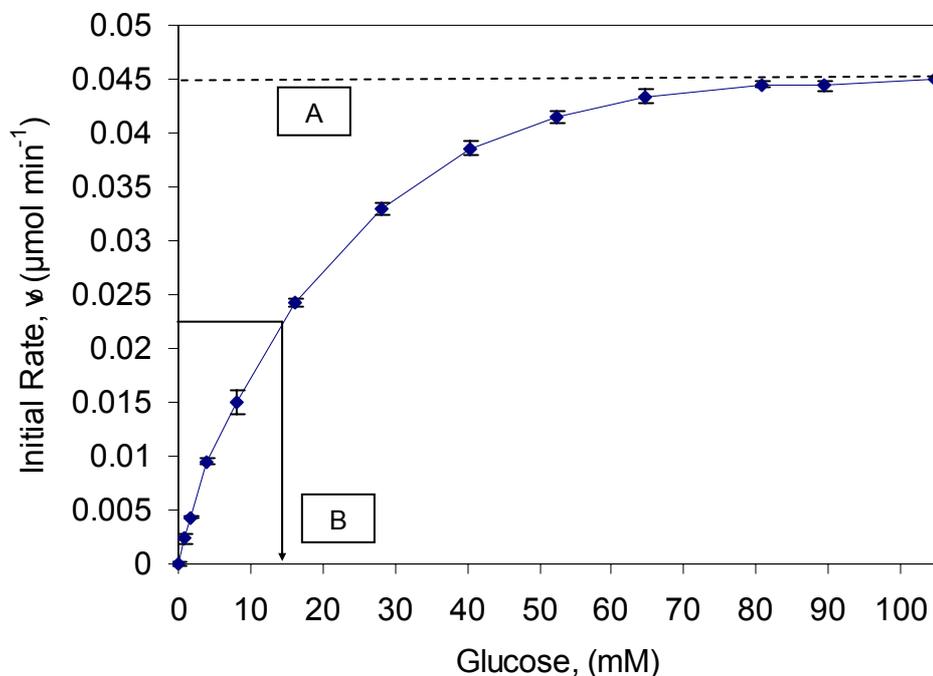


Figure 4.7: The effect of glucose concentration on the initial rate of the GOX reaction where: A – V_{\max} , B – K_m .

4.5.7 Determination of the kinetic constants

The Hanes – Woolf plot was used to determine the kinetic constants of the GOX from Tt42. The V_{\max} and K_m values of the GOX reaction were determined to be $5.41 \times 10^{-2} \mu\text{mol/minute}$ and $18.43 \text{ mM } \beta\text{-D-glucose}$ respectively (Appendix 6). The turnover number (k_{cat}) of GOX from Tt42 was determined to be 741 s^{-1} (Appendix 7). Therefore 1 mole of native GOX from Tt42 can convert 741 moles of glucose to product in 1 second. The specificity constant (k_{cat}/K_m) for GOX was determined to be $40 \text{ s}^{-1}\text{mM}^{-1}$ (Appendix 7).

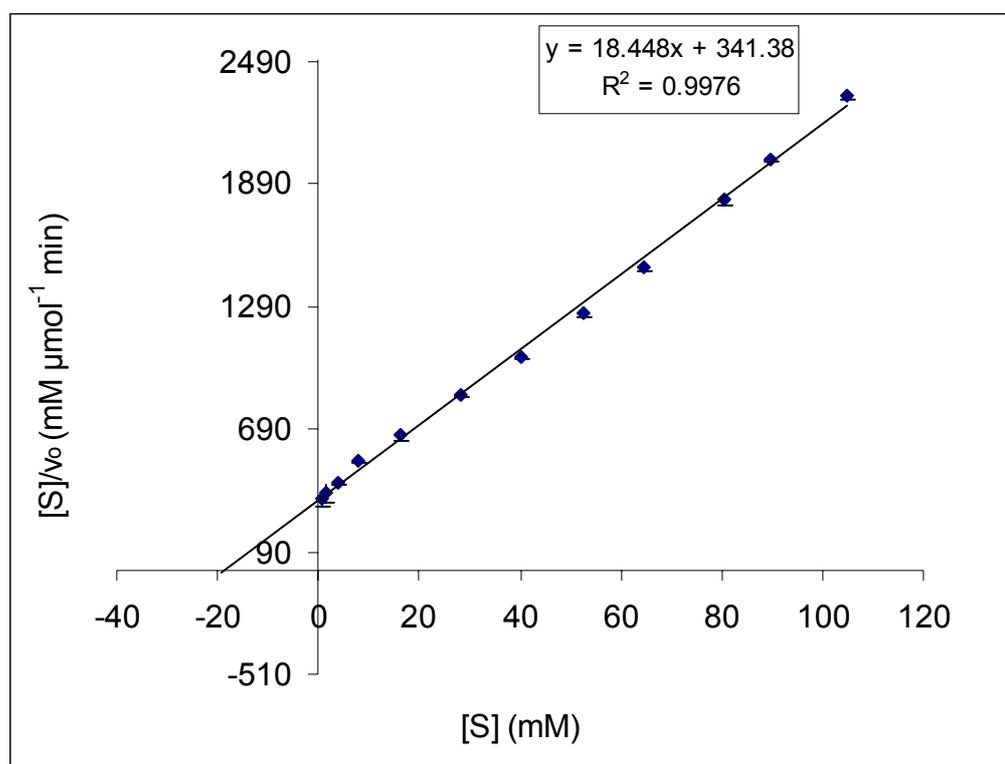


Figure 4.8: Hanes - Woolf linear plot for the determination of V_{\max} and K_m of GOX from Tt42.

The kinetic parameters calculated for GOX from Tt42 were compared with those from literature (Table 4.1). The comparisons outlined in table 4.1 are for free enzyme and the k_{cat} values were calculated per mole of native GOX. With reference to Table 4.1 the V_{\max} of GOX from Tt42 was 1.42-fold higher than that for *A. niger* (Sigma type VII) and 1.42-fold lower than that of *P. amagasakiense* (ATCC 28686). The GOX from Tt42 was calculated to have 1.63-fold higher affinity for $\beta\text{-D-glucose}$ than that of *A. niger* (Sigma type VII), and *P.*

amagasakiense (ATCC 28686) GOX was calculated to have an affinity for β -D-glucose 3.23-fold higher than that of Tt42 GOX. The turnover number of GOX from Tt42 was calculated to be 2.7-fold and 1.2-fold lower than that of *P. amagasakiense* (ATCC 28686) and *A. niger* (Sigma type VII) GOX, each respectively. The relative rate of reaction at low substrate concentration or specificity constant of GOX from Tt42 was 1.3-fold higher than that of *A. niger* (Sigma type VII) but calculated to be 8.7 fold lower than that of *P. amagasakiense* (ATCC 28686). Overall the GOX from Tt42 displayed more advantageous kinetic properties compared to GOX from *A. niger* (Sigma type VII) due to the enzyme having a higher affinity for β -D glucose and a higher specificity constant despite the lower turnover number.

Table 4.1: Kinetic parameters for β -D-glucose of GOX from different sources

GOX	Kinetic Constants				Reference
	V_{max} (Umg ⁻¹)	K_m (mM)	K_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	
<i>P. amagasakiense</i> ATCC 28686	925	5.7	2001	351	Witt <i>et al.</i> (1998)
<i>A. niger</i> (Sigma Type VII)	458	30.0	920	31	Kalisz <i>et al.</i> (1991)
<i>P. canescens</i> Tt42	651*	18.4	741	40	Present work

* Calculated V_{max} /mg of GOX in the reaction at V_{max} .

The GOX from Tt42 was found to contain 2 subunits of approximate molecular weight of 70kDa each (Refer Table 4.2). The approximate native molecular weight was determined to be 148kDa. Both molecular weight and subunit molecular weight of GOX from Tt42 were comparable to those for various *Aspergillus* and *Penicillium* species which ranged from 130kDa – 175kDa (native) and 70kDa – 80kDa (subunit). The pI values for the different GOX enzymes from the *Aspergillus* and *Penicillium* species were shown to range between 3.7 and 4.51. Only the less dominant isoenzyme from Tt42 was determined to be approximately 4.67 only slightly higher than those it was compared to in table 4.2. Two other strains were found to contain more than one isoenzyme of GOX, these being *A. niger* (Sigma Type VII) and *P. amagasakiense* (ATCC 28686) containing 5 and 4 isoenzymes of GOX, respectively. The optimum pH of the GOX from Tt42 (6 to 8) was higher than those of the other GOX enzymes reported in literature, except for

the GOX enzyme from *P. funiculosum* 433 with an optimum pH range of 6 to 8.6. The other GOX enzymes all displayed optimum pH values in the range 4 to 6. The temperature optimum of GOX from Tt42 was comparable to that of *P. funiculosum* 433 both displaying optimal activity in the region of 25°C to 30°C. The optimum temperatures for the other GOX enzymes in table 4.2 were relatively higher and reported to be in the range of 40 to 60°C. The substrate affinity (K_m) values for GOX towards β -D-glucose from Tt42 was 1.29 and 1.63 fold lower than those obtained for *A. niger* BTL and *A. niger* (Sigma Type VII), respectively (Table 4.2). The substrate affinities of the *Penicillium* strains reported in Table 4.2 were all higher than those for Tt42. The K_m values for *P. funiculosum* 433, *P. pinophilum* (DSM 11428) and *P. amagasakiense* (ATCC 28686) were 5.6; 2.96 and 3.5 fold lower for D-glucose than the GOX from Tt42, respectively.

Table 4.2: Comparison of characteristics of GOX from different sources

Parameter	GOX Source						
	<i>A. niger</i> (Sigma Type VII)	<i>A. niger</i> BTL	<i>P. funiculosum</i> 433	<i>P. Chrysogenum</i>	<i>P. pinophilum</i> (DSM 11428)	<i>P. amagasakiense</i> ATCC 28686	<i>P. canescens</i> Tt42
Molecular weight (kDa)	157	ND	140	175	155	130	148
Subunit Molecular weight (kDa)	80	75	70	72	78	77	70
Isoelectric Point	5 bands between 3.97 and 4.16	3.7	ND	4.2	ND	4 bands of 4.37, 4.42, 4.46 and 4.51	2 bands of 4.3 and 4.67
Optimum pH range	5.5 to 6	ND	6 to 8.6	5 to 6	4 to 6	4.5 to 6.5	6 to 8
Optimum Temperature (°C)	40 to 60	ND	30	ND	ND	40 to 50	25 to 30
K _m for β-D- glucose (mM)	30	23.7	3.3	ND	6.2	5.2	18.4
Reference	Kalisz <i>et al.</i> (1990)	Hatzinikolaou <i>et al.</i> (1996)	Sukhacheva <i>et al.</i> (2004)	Eriksson <i>et al.</i> (1987)	Rando <i>et al.</i> (1997)	Kalisz <i>et al.</i> (1997)	Present work

4.6 Conclusions

The kinetic constants and some of the main characteristics for GOX from Tt42 were successfully determined from the purified GOX preparation and compared to those reported in literature.

GOX concentrations up to 0.2 U/ml were shown to be accurately measurable using the Bergmeyer (1988) assay.

The temperature optimum for GOX from Tt42 was determined to be in the region of 25°C to 30°C. The GOX retained activity at 25°C for at least 10 hours. The results were helpful in concluding that the GOX from Tt42 could potentially find application in glucose determination kits and biosensors used at room temperature. The GOX from Tt42 was determined to be less effective at 37°C, exhibiting a half life of approximately 30 minutes. Thus the application to implantable biosensors operating at body temperature may not be possible without stabilisation investigations. Furthermore the GOX in purified and lyophilised form retained its activity when stored at -20°C for 6 months, indicating a good shelf life.

Optimum Tt42 GOX activity was displayed at a more neutral pH range than most of those cited in the literature. Only one other organism *P. funiculosum* 433 displayed optimal GOX activity with a similar range. Most of the GOX enzymes cited displayed optimal activity in more acidic environments.

Effective comparison of the catalytic constants of GOX from Tt42 with those cited in literature proved difficult due to the paucity of relevant literature. The reason is likely that the catalytic constants for GOX are generally determined for immobilized enzymes based on their relevant application. The specificity constant of GOX from Tt42 was 1.3-fold higher than that of *A. niger* (Sigma Type VII) and 8.7-fold lower than that of *P. amagasakiense* (ATCC 28686).

The necessity of comparing enzymes based on their catalytic and kinetic constants proved invaluable in this study since further process and application

research of GOX from Tt42 would only be relevant if the enzyme was characteristically favorable for current applications.

Chapter 5

Final Conclusions

5 Final Conclusions

5.1 Introduction

A novel GOX from *P. canescens* (Tt42) was identified and shown to be produced at comparatively high levels. A study was undertaken to isolate, purify and characterize the enzyme. Due to the various current applications of GOX in biotechnology and diagnostics, the search for new GOX enzymes with superior properties is of value.

5.2 Isolation and GOX production from *Penicillium canescens* Tt42

A practical and reproducible method for determination of GOX activity was achieved with the Bergmeyer (1988) GOX assay method. The method was validated using GOX from *A. niger* obtained from Seravac Pty Ltd. Accurate determination of the GOX activity was imperative to this study.

Different methods of cell disruption were investigated for their ability to release the intracellular GOX of Tt42. All the methods investigated were successful, and the use of the Constant Systems (Z-Pus series) high pressure cell disrupter was determined to be the simplest and most efficient method.

GOX was successfully produced with high titres in the GOX production medium of Rogalski *et al.* (1988) in shake flask culture. The GOX from Tt42 was shown to be produced both intra- and extracellularly, reaching maximum volumetric titres of 11.5 U/ml and 6.9 U/ml, respectively, after 3 days of growth. Accurate comparison of the total GOX produced by Tt42 with the GOX production by similar organisms was difficult due to the scarcity of both intra- and extracellular GOX titres reported in literature. The total GOX produced by Tt42 was determined to be higher than any of the titres reported for *A. niger* organisms, and found to be comparable to the *P. variable* P16 extracellular titres reported.

5.3 Purification and characterisation of GOX from Tt42

Both the intra- and extracellular GOX from Tt42 was shown to contain 2 isoenzymes with pI values of 4.3 and 4.67, the former being dominant. For ease of purification only the extracellular GOX was purified.

Purification of GOX for activity characterisation from the extracellular fraction of Tt42 was achieved with the aid of three principal purification techniques. Ammonium sulphate precipitation, anion exchange chromatography and size exclusion chromatography were all used successfully, to purify the GOX from Tt42. The GOX was shown to be 80% pure by size exclusion chromatography. The purified GOX was used for enzyme characterisation and some of the main characteristics were determined along with the kinetic and catalytic constants.

The GOX from Tt42 was determined to be a dimeric protein ($M_r \pm 148\text{kDa}$) most likely consisting of 2 equal subunits ($M_r \pm 70\text{kDa}$). The temperature optimum for the GOX from Tt42 was shown to be 25-30°C. The pH optimum for the oxidation of β -D-glucose was found to be pH 7. The enzyme was shown to be stable at 25°C for a minimum of 10 hours, while displaying a half life of 30 minutes at 37°C. The lyophilised enzyme was stable at -20°C for a minimum of 6 months. These properties of GOX from Tt42 were comparable to those of other GOX enzymes reported in literature.

The specificity constant (k_{cat}/K_m) for GOX from Tt42 was calculated to be $40 \text{ s}^{-1} \text{ mM}^{-1}$, which was 1.3-fold higher than that of *A. niger* (Sigma Type VII) and 8.7-fold lower than that of *P. amagasakiense* (ATCC 28686). Interestingly, despite the fact that the GOX from Tt42 was shown to have a lower turnover number than that of *A. niger* (Sigma Type VII) its specificity constant was higher, since its affinity for β -D-glucose is higher.

5.4 Future work

Further investigations of the GOX from Tt42 could include:

- Fed-batch fermentation, with glucose as a carbon source, of the *P. canescens* Tt42 to maximise the production of glucose oxidase. Alternate carbon sources such as sucrose and molasses could also be investigated.
- Alternate more efficient and cost effective purification techniques could be investigated to attempt to recover higher yields of glucose oxidase. Anion exchange chromatography as a purification step could be optimised using a shallow salt gradient than was used in this study to better purify the GOX with this technique.
- The kinetic constants of immobilised GOX could be determined and compared with relevant values reported in literature.
- Additional characteristics of the GOX from Tt42 could be investigated including specificities for other substrates, the effect of different activators and inhibitors on the enzyme activity and the effect of different stabilisers to improve thermostability may be of interest.

5.5 Conclusion

The author has successfully isolated, purified and partially characterised the GOX from *P. canescens* Tt42 and shown that the enzyme is in fact novel based on its distinct biochemical and kinetic characteristics. The characteristics of the GOX were shown to be comparable to similar GOX enzymes reported in literature. The kinetic constants of GOX from Tt42 were more advantageous than those reported for *A. niger* (Sigma Type VII), and less advantageous than those cited for *P. amagasakiense* (ATCC 28686).

Another conclusion based on the investigations conducted in this thesis could be the necessity to compare enzymes based on their kinetic properties, and that high enzyme titres are not of primary importance when isolating novel enzymes.

6 Appendices

Appendix 1: The Glucose Oxidase assay (Bergmeyer, 1988)

Appendix 2: The Bio-Rad Total protein assay

Appendix 3: The Catalase assay (Bergmeyer, 1988)

Appendix 4: Standard curve of the Fermentas PageRuler™ Protein ladder molecular weight standards used for SDS-PAGE.

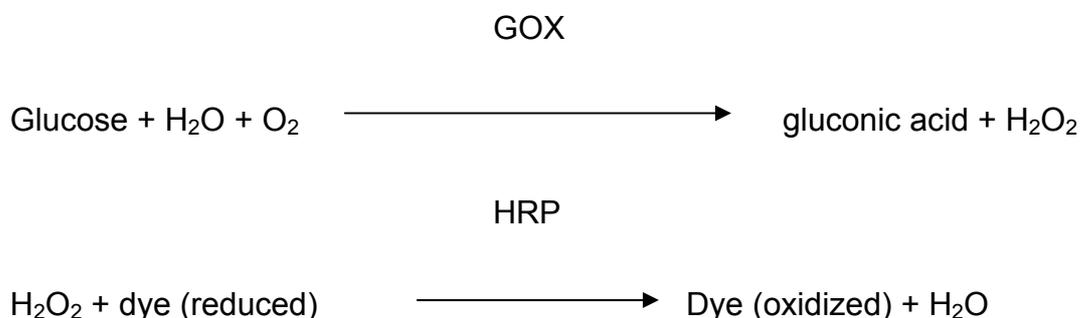
Appendix 5: Size exclusion chromatography of GOX from Tt42. **(A)** standard curve, **(B)** size exclusion profile after anion exchange chromatography and lyophilisation, **(C)** final size exclusion profile of GOX (lyophilised).

Appendix 6: Hanes – Woolf Equations

Appendix 7: Kinetic constant equations

Appendix 1: The GOX assay method (Bergmeyer, 1988)

GOX reaction



Reagents

- *o*-dianisidine:2HCl: Prepare 6mg *o*-dianisidine:2HCl / ml distilled water. Thereafter dilute 1ml to 100ml with enzyme diluent buffer below. Oxygenate this solution with pure oxygen for at least 5 minutes before use.
- HRP: Dilute the HRP to 60U/ml in distilled water for the assay. Store on ice.
- Glucose substrate: prepare a 10% (m/v) glucose solution in 100ml-distilled water. Use anhydrous glucose and allow the solution to stand for 1 hour to mutarotate before use.
- Enzyme diluent (0.1M potassium phosphate buffer pH 7): Dissolve 5.3g KH_2PO_4 and 10.6g K_2HPO_4 and 0.1mg/ml BSA (100mg) in distilled water, adjust the pH to 7 and then dilute to 1000ml. Store on ice

Sample

Dissolve 10mg/ml glucose oxidase enzyme/ml distilled water. Dilute the stock solution to 0.15U/ml in enzyme diluent immediately before the assay.

Procedure

- Into a 4.5ml quartz cuvette pipette the following:
- 2.4ml *o*-dianisidine solution
- 0.1ml of the HRP solution
- 0.5ml of the glucose substrate

- Mix and equilibrate to 25°C
- Thereafter add 0.1ml of the glucose oxidase enzyme
- Mix and record the increase in absorbance at 436nm for no more than 5 minutes
- For each new glucose oxidase assay 2 blanks must be run, an enzyme blank and a substrate blank
- Enzyme blank contains glucose oxidase enzyme and no glucose; the glucose is compensated for using potassium phosphate buffer.
- Substrate blank contains glucose and no glucose oxidase and the enzyme is compensated for using potassium phosphate buffer.

To calculate the enzyme activity the rate is multiplied with a factor of 3.73 which is obtained from the following calculation:

$$U/ml = \frac{\Delta A/min \times 3.1}{8.3 \times 0.1}$$

Total volume	3.1ml
Extinction coefficient	8.3 mM ⁻¹ cm ⁻¹
Volume of enzyme	0.1ml

Appendix 2: Bio-Rad Total protein assay

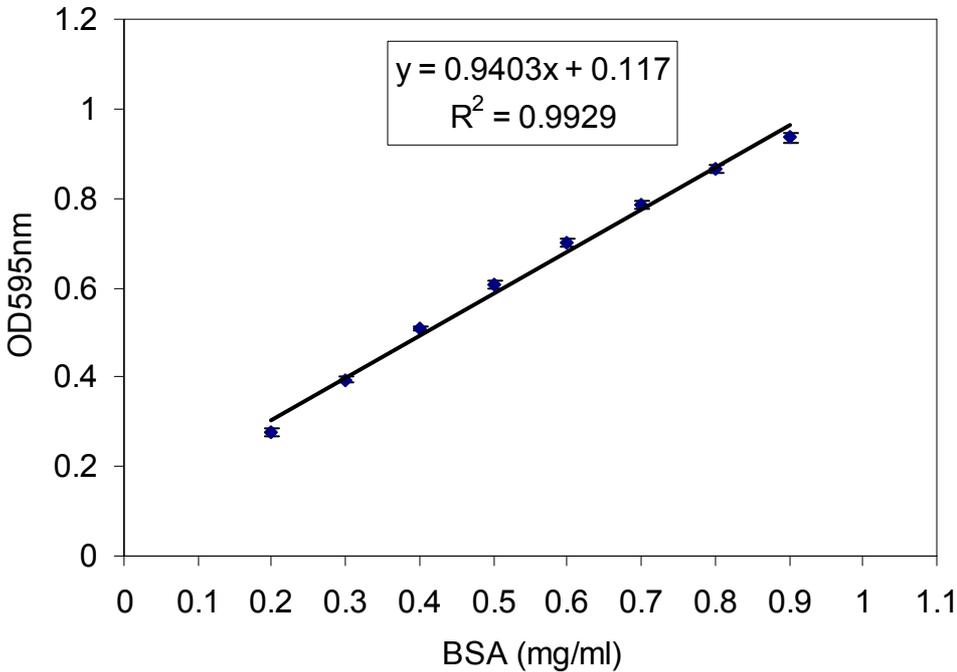
The Coomassie®-Protein reaction scheme

Protein + Coomassie® G250 in acidic medium \longrightarrow Protein-Dye complex (blue colour measured at 595nm)

Procedure

- Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionised water. Filter through Whatman #1 filter to remove particulates. This diluted reagent may be used for approximately 2 weeks when kept at room temperature.
- Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of the assay for BSA is 0.2 to 0.9 mg/ml.
- Pipette 50 μ l of each standard and sample solution into a clean, dry plastic or glass cuvettes. Protein solutions are normally assayed in duplicate or triplicate.
- Add 2.5 ml of diluted dye reagent to each cuvette and mix by inversion.
- Incubate at room temperature for at least 5 minutes. Absorbance will increase over time, samples should incubate at room temperature for no more than 1 hour.
- Measure absorbance at 595 nm. Generate a standard curve of A_{595nm} against the protein concentration standards. The linear portion of the curve is used to calculate the protein concentration of unknown test samples.

Typical standard curve for the Bio-Rad Protein Assay, bovine serum albumin (Fraction V).



Appendix 3: The Catalase assay (Bergmeyer, 1988)

CAT reaction



Reagents

- 0.1M Potassium Phosphate buffer, pH 7: Dissolve 13.6 g KH_2PO_4 in 800ml distilled water, check pH to 7 with 1M KOH and dilute to 1 L. Store on ice. Solution is stable for 1 month at 2-8°C.
- Hydrogen peroxide: Dilute hydrogen peroxide (30% Perhydrol) with 50ml buffer (above) and adjust to an absorbance at 240nm of 0.50 ± 0.01 , measured against a buffer blank. Store at ambient temperature. Prepare fresh daily. 69.12mg hydrogen peroxide made up to 50ml with buffer – this must be checked each time. If the OD is below 0.50 add more hydrogen peroxide and if it is above 0.50 then add more buffer.

Sample

Sample preparation: Dilute enzyme to yield 14.9 – 37.2 units/ml with ice-cold buffer immediately before the assay is carried out.

Procedure

- Pipette the following into a 4.5ml quartz cuvette:
- 3 ml of the substrate and equilibrate at 25°C for 2 minutes reading absorbance at 240nm
- Add 0.05ml of the enzyme at time zero.
- Record the rate of decrease in absorbance at 240nm over the linear portion of the reaction for approximately 5 minutes.

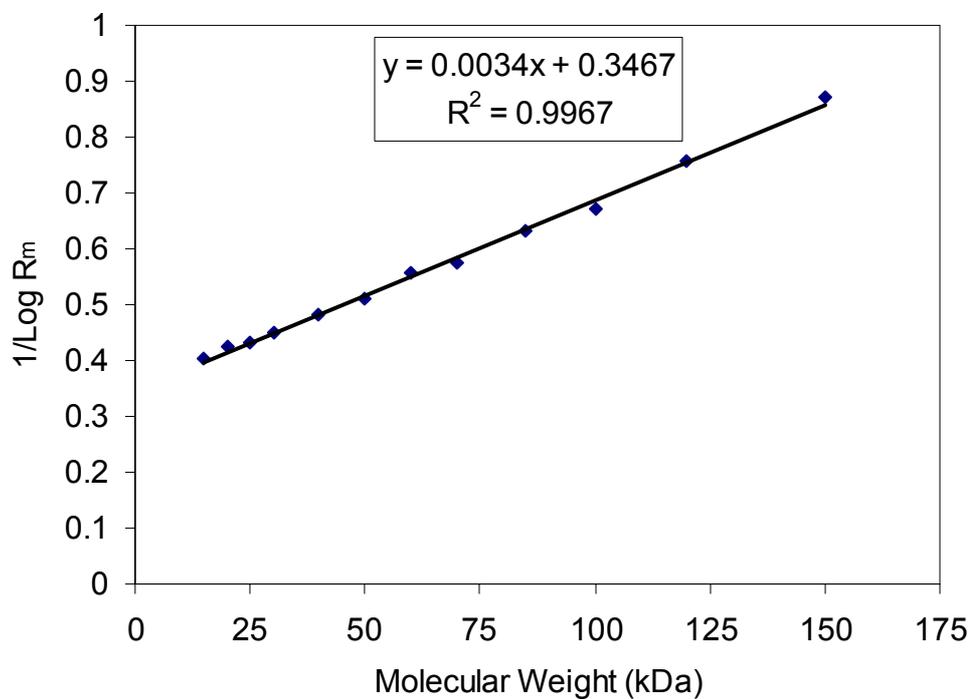
- In between each $A_{240\text{nm}}$ reading the contents of the cuvette should be mixed using a 1ml pipette to avoid bubbles from interfering with the readings.
- For each assay an enzyme and substrate blank should be read and recorded. The values should be subtracted from the enzyme rates.
- The enzyme blank contains 3ml buffer with 0.05ml of the enzyme
- The substrate blank contains 3ml substrate and 0.05ml buffer.
- When assaying the glucose oxidase samples for catalase then the sample is substituted in the place of the catalase enzyme.

To calculate the enzyme activity the rate is multiplied with a factor of 1487.8 which is obtained from the following calculation:

$$\text{U/ml solution} = \frac{\Delta\text{Abs}/\text{min} \times 3.05}{0.041 \times 0.05}$$

Total volume	3.05ml
Extinction coefficient	0.041 $\text{mM}^{-1} \text{cm}^{-1}$
Volume of enzyme	0.05ml

Appendix 4: Standard curve of the Fermentas PageRuler™ Protein ladder molecular weight standards used for SDS-PAGE.

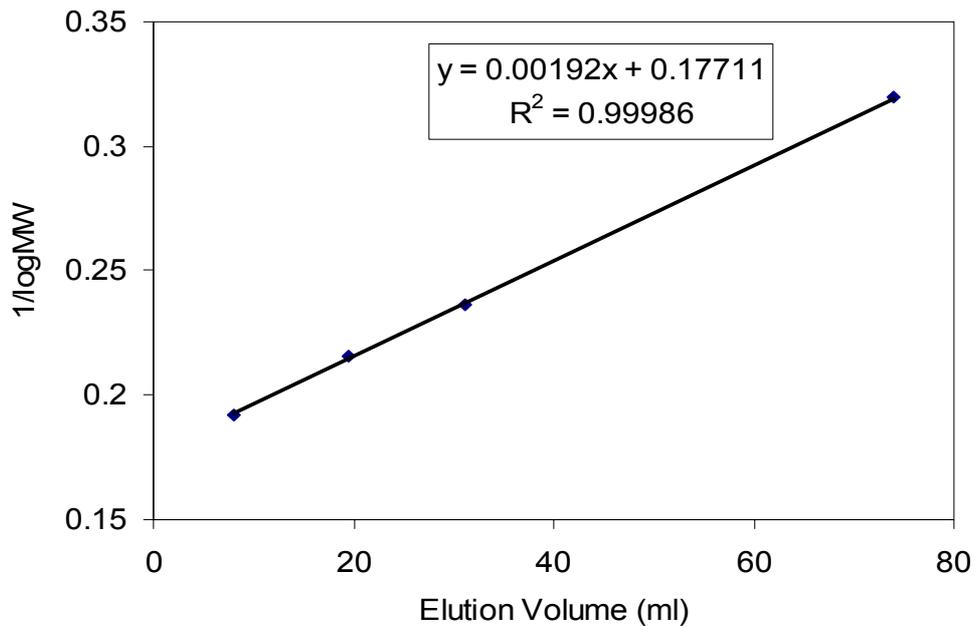


Note: The standard curve above does not include the $1/\log R_m$ values for 200kDa and 10kDa since it was not possible to distinguish the 200kDa and 150kDa bands from each other, as well as it was not possible to distinguish the 14kDa and 10kDa bands from one another (figure 3.5). Therefore these values were not included in the standard curve.

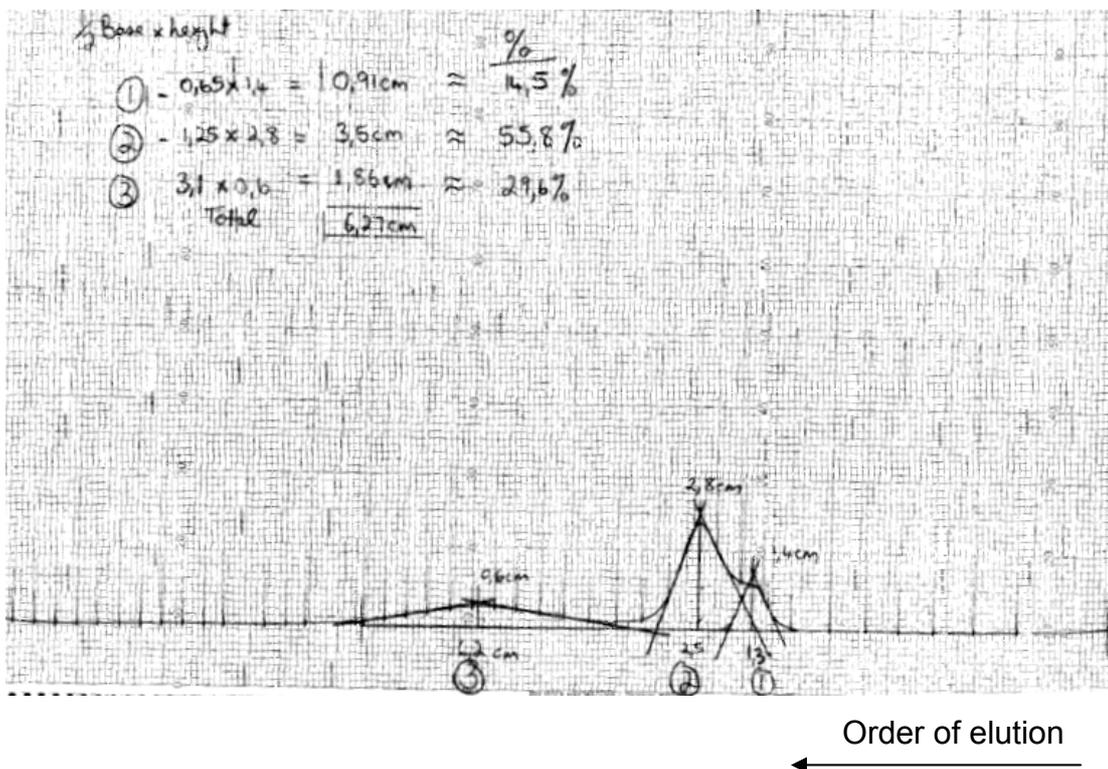
Appendix 5: Size exclusion chromatography of GOX from Tt42.

(A) Standard curve, (B) size exclusion profile after anion exchange chromatography and lyophilisation, (C) final size exclusion profile of GOX (lyophilised).

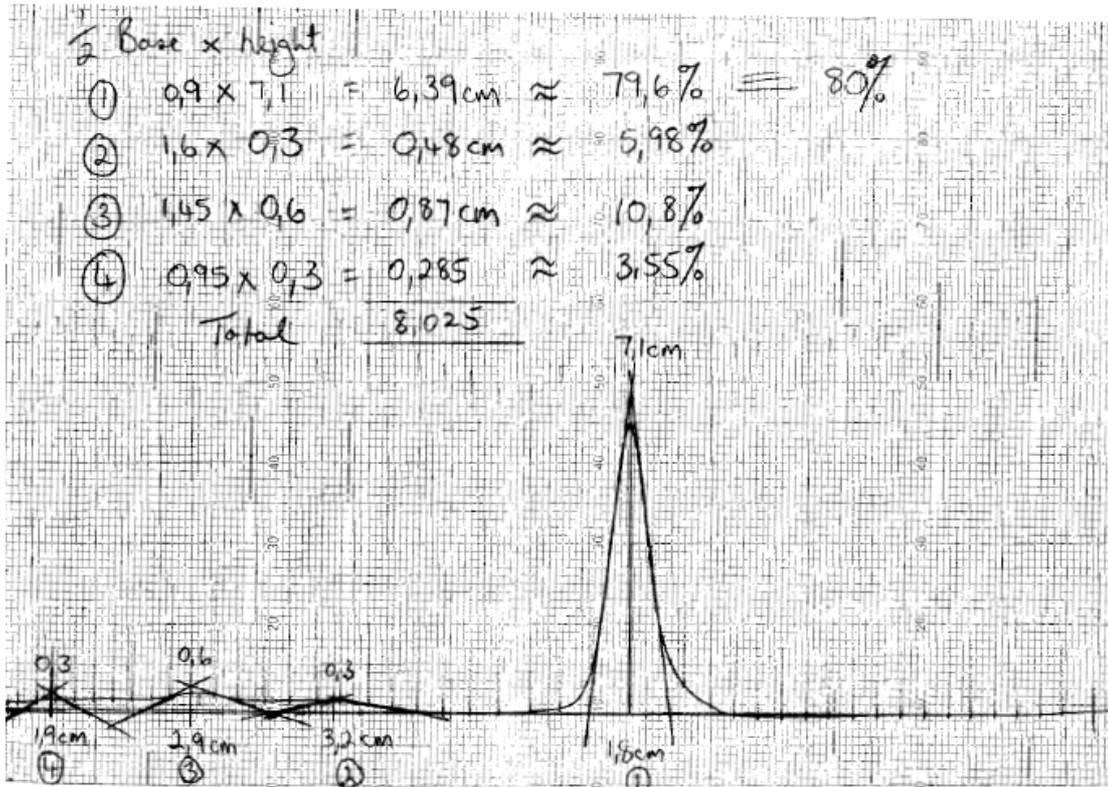
(A)



(B)



(C)



Order of elution
←

Appendix 6: Hanes – Woolf Equation

$$[S]/v_o = K_m/V_{\max} + [S]/V_{\max}$$

The equation of the straight line for GOX from Tt42 is $Y = 18.461 X + 340.26$
where:

- X-intercept = $-K_m$
- Y-intercept = K_m / V_{\max}
- Slope = $1/V_{\max}$

Appendix 7: Kinetic constant equations

The equation for the determination of k_{cat} is:

$$k_{cat} = V_{max} / [E_t]$$

Where $[E_t]$ is calculated from the following equation:

$$[E_t] = \frac{\mu\text{g of enzyme/ml}}{\text{Molecular weight } (\mu\text{g}/\mu\text{mol})}$$

The equation for enzyme efficiency is:

$$k_{cat}/K_m$$

7 References

Beltrame, P, M. Comotti, C. Della Pina, and M. Rossi. 2004. Aerobic oxidation of glucose I. Enzymatic catalysis. *J. Catalysis*. **228**:282-287.

Bergmeyer, H.U. 1988. *Methods of Enzymatic Analysis*. 3rd Ed. Vol.II: Samples, Reagents, Assessment of results.

Christi, Y, and M. Moo-Young. 1986. Disruption of microbial cells for intracellular products. *Enzyme Microb. Technol.* **8**:194-204.

Chudobova, I, E. Vrbova, M. Kodicek, J. Janovcova, and J. Kas. 1996. Fibre optic biosensor for the determination of d-glucose based on absorption changes of immobilized glucose oxidase. *Analytica. Chimica. Acta.* **319(1)**: 103-110.

Ciucu, A, and C. Pătroescua. 1984. Fast spectrometric method of determining the activity of glucose oxidase. *Analytical Lett.* **17 (B12)**:1417-1427.

Crueger, A, and W. Crueger. 1990. Glucose transforming Enzymes In: *Microbial Enzymes and Biotechnology* (W.M. Fogarty, and C.T. Kelly, Eds). Elsevier Applied Science, London, New York. 177-226.

Cui, G, J.H. Yoo, B.W. Woo, S.S. Kim, G.S. Cha, and H. Nam. 2001. Disposable amperometric glucose sensor electrode with enzyme-immobilized nitrocellulose strip. *Talanta*. **54**:1105-1111.

Dai, G, J. Li, and L. Jiang. 2002. Difference in enzyme activity and conformation of glucose oxidase before and after purification. *Colloids and Surfaces B: Biointerfaces.* **24**:171-176.

Eriksson, K-O, I. Kourteva, K. Yao, J-L. Liao, F. Kilár and S. Hjertén. 1987. Application of high-performance chromatographic and electrophoretic methods to

the purification and characterization of glucose oxidase and catalase from *Penicillium chrysogenum*. J. Chromatography. **397**:239-249.

Eryomin, A.N, A.P. Droshdenyuk, G.K. Zhavnerko, T.V. Semashko, and R.V. Mikhailova. 2004. Quartz Sand as an Adsorbent for Purification of Extracellular Glucose Oxidase from *Penicillium funiculosum* 46.1. Appl. Biochem. Microbiol. **40(2)**:178-185.

Fiedurek, J. 1998. Effect of osmotic stress on glucose oxidase production and secretion by *Aspergillus niger*. J. Basic Microbiol. **38**:107-112.

Fiedurek, J, and A. Gromada. 1997. Screening of mutagenesis of moulds for improvement of the simultaneous production of catalase and glucose oxidase. Enzyme Microb. Technol. **20**:344-347.

Fiedurek, J, and A. Gromada. 2000. Production of catalase and glucose oxidase by *Aspergillus niger* using unconventional oxygenation of culture. J. Appl. Microbiol. **89(1)**:85-89.

Gang, C, J.H. Yoo, B.W. Woo, S.S. Kim, G.S. Cha, and H. Nam. 2001. Disposable amperometric glucose sensor electrode with enzyme-immobilized nitrocellulose strip. Talanta. **54**:1105-111.

Gerritsen, M, A. Kros, J.A. Lutterman, R.J.M. Nolte, and J.A. Jansen. 2001. A percutaneous device as model to study the in vivo performance of implantable amperometric glucose sensors. J. Materials Science: Materials in Medicine. **12(2)**:129-134.

Gibson, Q.H, B.E.P. Swoboda, and V. Massey. 1964. Kinetics and Mechanism of Action of Glucose Oxidase. J. Biological Chem. **.239(11)**:3927-3934.

Gouka, R. J, P. J. Punt, and C. A. M. J. J. van den Hondel. 1997. Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. Appl Microbiol. Biotechnol. **47**:1-11.

Gromada, A, and J. Fiedurek. 1996. Influence of Medium Components and Metabolic Inhibitors on Glucose Oxidase Production by *Aspergillus niger* Mycelium. *Acta Microbiologica Polonica*. **45(1)**:37-43.

Gromada, A, and J. Fiedurek. 1997. Optimization of catalase biosynthesis in submerged cultures of *Aspergillus niger* mutant. *J. Basic Microbiol.* **37**:2, 85-91.

Gōrniak, H, and J. Kączkowski. 1974. Isolation and Characterization of Glucose Oxidase from Mycelium and Nutrient of *Penicillium notatum* Cultures. *Biochem.* **Vol XXII**:351-355.

Harborn, U, B. Xie, R. Venkatesh, and B. Danielsson. 1997. Evaluation of a miniaturized thermal biosensor for the determination of glucose in whole blood. *Clinica. Chimica. Acta.* **267**:225-237.

Harris, E.L.V, and S. Angal. 1994. Protein purification and methods: A practical approach. Oxford University Press Inc, New York.

Hatzinikolaou, D.G, O.C. Hansen, B.J. Macris, A. Tingey, D. Kekos, P. Goodenough, and P. Stougaard. 1996. A new glucose oxidase from *Aspergillus niger* characterization and regulation studies of enzyme and gene. *Appl Microbiol Biotechnol.* **46**:371-381.

Hatzinikolaou, D.G, and B.J. Macris. 1995. Factors regulating production of glucose oxidase by *Aspergillus niger*. *Enzyme. Microb. Technol.* **17**:530-534.

Hecht, H.J, H.M. Kalisz, J. Hendle, R.D. Schmid, and D. Schomburg. 1993. Crystal Structure of Glucose Oxidase from *Aspergillus niger* Refined at 2.3 Å Resolution. *J. Mol. Biol.* **229**:153-172.

Isaksen, A, and J. Adler-Nissen. 1997. Antioxidative Effect of Glucose Oxidase and Catalase in Mayonnaises of Different Oxidative Susceptibility. I. Product Trials. *Lebensm.-Wiss. u.-Technol.* **30**:841-846.

Kalisz, H.M, H.J. Hecht, D. Schomburg, and R.D. Schmid. 1991. Effects of carbohydrate depletion on the structure, stability and activity of glucose oxidase from *Aspergillus niger*. *Biochim. Biophys. Acta.* **1080(2)**:138-142.

Kalisz, H.M, J. Hendle and R.D. Schmid. 1990. Purification of the glycoprotein glucose oxidase from *Penicillium amagasakiense* by high-performance liquid chromatography. *J. Chromatography.* **521**:245-250.

Kalisz, H.M, J. Hendle, and R.D. Schmid. 1997. Structural and biochemical properties of glycosylated and deglycosylated glucose oxidase from *Penicillium amagasakiense*. *Appl Microbiol Biotechnol.* **47**:502-507.

Kapat, A, J.-K. Jung, and Y.-H. Park. 2001. Enhancement of glucose oxidase production in batch cultivation of recombinant *Saccharomyces cerevisiae*: optimization of oxygen transfer condition. *J. Appl. Microbiol.* **90**:216-222.

Karmali, K, A. Karmali, A. Teixeira, and M.J. Marcelo Curto. 2004. Assay for glucose oxidase from *Aspergillus niger* and *Penicillium amagasakiense* by Fourier transform infrared spectroscopy. *Analytical Biochem.* **333**:320-327.

Kim, S-H, S-M. Lee, D-U. Kim, J-Z. Cui, and S-W. Kang. 2001. Enzyme-based glucose biosensor using a dye couple system. *Dyes and Pigments.* **49**:103-108.

Klein, J, M. Rosenberg, J. Markoš, O. Dolgoš, M. Krošlák, and L. Krištofiková. 2002. Biotransformation of glucose to gluconic acid by *Aspergillus niger* – study of mass transfer in an airlift bioreactor. *Biochemical Engineering Journal.* **3568**:1-9.

Kleppe, K. 1963. Purification and properties of a Glucose Oxidase from *Aspergillus niger*. Nebraska Univ. Phd thesis.

Kona, R.P, N. Qureshi, and J.S. Pai. 2001. Production of glucose oxidase using *Aspergillus niger* and corn steep liquor. *Bioresource Technol.* **78**:123-126.

Kusai, K. 1960. Crystalline Glucose Oxidase. Ann.Rep.Sci.Works, Fac. Sci. Osaka Univ. **8**:43-74.

Kusai, K, I. Sekuzu, B. Hagihara, K. Okunuki, S. Yamauchi, and M. Nakai. 1960. Crystallization of glucose oxidase from *Penicillium amagasakiense*. Biochim. Biophys. Acta. **40**:555-557.

Li, T.-H, and T.-L. Chen. 1994. Enhancement of Glucose Oxidase Fermentation by Addition of Hydrocarbons. J. Ferm. Bioeng. **78(4)**:298-303.

Lu, T, X. Peng, H. Yang, and L. Ji. 1996. The production of glucose oxidase using the waste myceliums of *Aspergillus niger* and the effects of metal ions on the activity of glucose oxidase. Enzyme Microb. Technol. **19**:339-342.

Nakamura, S, and S. Fujiki. 1968. Comparative Studies on the Glucose Oxidases of *Aspergillus niger* and *Penicillium amagasakiense*. J. Biochem. **63(1)**:51-58.

Nakao, K, A. Kiefner, K. Furumoto, and T. Harada. 1997. Production of gluconic acid with immobilized glucose oxidase in airlift reactors. Pergamon: Chemical Engineering Science. **52**:4127-4133.

Ng, T.B. 2004. Peptides and proteins from fungi. Peptides. **25**:1055-1073.

Parpinello, G.P, F. Chinnici, A. Versari, and C. Riponi. 2002. Preliminary Study on Glucose Oxidase-Catalase Enzyme System to Control the Browning of Apple and Pear Purées. Lebensm.-Wiss. u.-Technol. **35**:239-243.

Perrin, D. D, and B. Dempsey. 1974. Buffers for pH and Metal Ion Control. Halsted Press, John Wiley and Sons Inc, New York, p. 38.

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

Petruccioli, M, M. Fenice, and P. Piccioni. 1993. Distribution and typology of glucose oxidase activity in the genus *Penicillium*. *Lett in Appl. Microbiol.* **17**:-285-288.

Petruccioli, M, P. Piccioni, and F. Federici. 1997. Glucose oxidase overproduction by the mutant strain M-80.10 of *Penicillium variable* in a benchtop fermenter. *Enzyme & Microb. Technol.* **21**: 458-462.

Petruccioli, M, P. Piccioni, F. Federici, and M. Polsinelli. 1995. Glucose oxidase overproducing mutants of *Penicillium variable* (P16). *FEMS Microbiol. Lett.* **128**:107-112.

Pickering, G.J. 2000. The use of Glucose Oxidase in Winemaking. Adapted from: Pickering, G.J. (2000). The use of Glucose Oxidase in Winemaking. *Proceedings of the 1st EIT Oenology and Viticulture Seminar Series.* Eastern Institute of Technology, New Zealand. G.J. Pickering (ed.), Campus Press, New Zealand. pp 11-21.

Pickering, G.J, D.A. Heatherbell, and M.F. Barnes. 1998. Optimising glucose conversion in the production of reduced alcohol wine using glucose oxidase. *Food Research International.* **31(10)**:685-692.

Pluschkell, S, K. Hellmuth, and U. Rinas. 1996. Kinetics of Glucose Oxidase Excretion by Recombinant *Aspergillus niger*. *Biotechnol. Bioeng.* **51**:215-220.

Punt, P.J, N. van Biezen, A. Conesa, A. Albers, J. Mangnus, and C. van den Hondel. 2002. Filamentous fungi as cell factories for heterologous protein production. *Trends in Biotechnol.* **20(5)**:200-206.

Raba, J. and H.A. Mottola. 1995. Glucose Oxidase as an Analytical Reagent. *Critical Reviews in Analytical Chemistry.* **25(1)**:1-42.

Rando, D, G.-W. Kohring, and F. Giffhorn. 1997. Production, purification and characterization of glucose oxidase from a newly isolated strain of *Penicillium pinophilum*. *Appl Microbiol Biotechnol.* **48**:34-40.

Rasiah, I.A, K.H. Sutton, F.L. Low, H.-M. Lin, and J.A. Gerrard. 2005. Crosslinking of wheat dough proteins by glucose oxidase and the resulting effects on bread and croissants. *Food Chem.* **89**:325-332.

Rogalski, J, J. Fiedurek, J. Szczordrak, K. Kapusta, and A. Leonowicz. 1988. Optimization of glucose oxidase synthesis in submerged cultures of *Aspergillus niger* G-13 mutant. *Enzyme Microb. Technol.* **10**:508-511.

Santoni, T, D. Santianni, A. Manzoni, S. Zanardi, and M. Mascini. 1997. Enzyme electrode for glucose determination in whole blood. *Talanta.* **44**:1573-1580.

Sharif, F.A, and N. G. Alaeddinoglu. 1992. Expression and overproduction of glucose oxidase in *Aspergillus niger*. *Appl Microbiol Biotechnol.* **38**:115-116.

Sukhacheva, M.V, M.E. Davydova, and A.I. Netrusov. 2004. Production of *Penicillium funiculosum* 433 Glucose Oxidase and Its Properties. *Appl. Biochem. Microbiol.* **40 (1)**:25-29.

Swoboda, B. E. P, and V. Massey. 1965. Purification and properties of the glucose oxidase from *Aspergillus niger*. *J. Biol. Chem.* **240**:2209-2215.

Taubert, J, U. Krings, and R.G. Berger. 2000. A comparative study on the disintegration of filamentous fungi. *J. Microbiological Methods.* **42**:225-232.

Tzanov, T, A. Silgia, G.M. Gübitz, and A. Cavaco-Paulo. 2002. Hydrogen peroxide generation with immobilized glucose oxidase for textile bleaching. *J. Biotechnol.* **93**:87-94.

Vodopivec, M, M. Berovic, J. Jancar, A. HRPgornik, and A. Strancar. 2000. Application of Convective Interaction Media disks with immobilized glucose oxidase for on-line glucose measurements. *Analytica. Chimica. Acta.* **407**:105-110.

Voet, D, and J.G. Voet. 1995. *Biochemistry.* 2nd Ed. John Wiley & Sons, Inc.

Wilson, K, and J. Walker. 1995. *Practical Biochemistry: Principles and Techniques.* 4th Ed. Cambridge University Press.

Wilson, R, and A.P.F. Turner. 1992. Glucose oxidase: and ideal enzyme. *Biosensors & Bioelectronics.* **7**:165-185.

Witt, S, G. Wohlfahrt, D. Schomburg, H-J. Hecht, and H.M. Kalisz. 2000. Conserved arginine-516 of *Penicillium amagasakiense* glucose oxidase is essential for the efficient binding of *b*-D-glucose. *J. Biochem.* **347**:553-559.

Witt, S, M. Singh, and H.M. Kalisz. 1998. Structural and Kinetic Properties of Nonglycosylated Recombinant *Penicillium amagasakiense* Glucose Oxidase Expressed in *Escherichia coli*. *Appl. Enviro. Microbiol.* **64(4)**:1405-1411.

Witteveen, Cor F.B, M. Veenhuis, and J. Visser. 1992. Localization of Glucose Oxidase and Catalase Activities in *Aspergillus niger*. *Appl. Enviro. Microbiol.* **58(4)**:1190-1194.

Witteveen, Cor F.B, 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.

Wohlfahrt, G, S. Witt, J. Hendle, D. Schomburg, H.M. Kalisz, and H.J. Hecht. 1999. 1.8 and 1.9 Å resolution structures of the *Penicillium amagasakiense* and *Aspergillus niger* glucose oxidases as a basis for modelling substrate complexes. *Acta Crystallogr, Sect.D.* **55**:969-977.

Ye, W. N, D. Combes and P. Monsan. 1988. Influence of additives on the thermostability of glucose oxidase. *Enzyme Microb. Technol.* **10**:498-502.

<http://www.yasara.org/>

Zetelaki, K, and K. Vas. 1968. The role of Aeration and Agitation in the Production of Glucose Oxidase in Submerged Culture. *Biotechnol. Bioeng.* **Vol.X**:45-59.

Zetelaki, K.Z. 1970. The role of Aeration and Agitation in the Production of Glucose Oxidase in Submerged Culture. II. *Biotechnol. Bioeng.* **Vol.XII**: 379-397.

Zhu, J, Z. Zhu, Z. Lai, R. Wang, X. Guo, X. Wu, G. Zhang, Z. Zhang, Y. Wang, and Z. Chen. 2002. Planar Amperometric Glucose Sensor Based on Glucose Oxidase Immobilized by Chitosan Film on Prussian Blue Layer. *Sensors.* **2**:127-136.