Glucose oxidase — An overview

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A B S T R A C T

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase; EC 1.1.2.3.4) catalyzes the oxidation of β-D-glucose to gluconic acid, by utilizing molecular oxygen as an electron acceptor with simultaneous production of hydrogen peroxide. Microbial glucose oxidase is currently receiving much attention due to its wide applications in chemical, pharmaceutical, food, beverage, clinical chemistry, biotechnology and other industries. Novel applications of glucose oxidase in biosensors have increased the demand in recent years. Present review discusses the production, recovery, characterization, immobilization and applications of glucose oxidase. Production of glucose oxidase by fermentation is detailed, along with recombinant methods. Various purification techniques for higher recovery of glucose oxidase are described here. Issues of enzyme kinetics, stability studies and characterization are addressed. Immobilized preparations of glucose oxidase are also discussed. Applications of glucose oxidase in various industries and as analytical enzymes are having an increasing impact on bioprocessing.

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1. Introduction

Soil, organic waste and plant cell material are a few of the diverse environments in which filamentous fungi are known to flourish. Fungi produce a wide range of different enzymes which enables them to use many organic compounds as nutrient sources (Gouka et al., 1997). Among non-hydrolytic enzymes of fungal origin, glucose oxidase (EC 1.1.3.4) has seen large-scale technological applications since the early 1950’s (Fiedurek and Gromada, 1997). Glucose oxidase (GOD) has been purified from a range of different fungal sources, mainly from the genus Aspergillus (Kalisz et al., 1991; Hatzinikolaou and Macris 1995) and Penicillium (Eryomin et al., 2004; Sukhacheva et al., 2004), of which A. niger is the most commonly utilized for the production of GOD (Pluschkell et al., 1996). GOD from Penicillium species has been shown to exhibit more advantageous kinetics for glucose oxidation than that from A. niger (Kusai et al., 1996).

GOD (β-β-glucose:oxygen 1-oxidoreductase) catalyzes the oxidation of β-D-glucose to gluconic acid by utilizing molecular oxygen as an electron acceptor with simultaneous production of hydrogen peroxide (H2O2) (Hatzinikolaou and Macris 1995). GOD has found several commercial applications including glucose removal from dried egg; improvement of color, flavor, and shelf life of food materials; oxygen removal from fruit juices, canned beverages; and from mayonnaise to prevent rancidity. It has also been used in an automatic glucose assay kit in conjunction with catalase (Hatzinikolaou and Macris 1995). GOD has found many organic compounds as nutrient sources (Gouka et al., 1997). Produce a wide range of different enzymes which enables them to use many organic compounds as nutrient sources (Gouka et al., 1997).

1.1. Glucose oxidase reaction mechanism

GOD is a flavoprotein which catalyses the oxidation of β-D-glucose to D-glucono-δ-lactone and H2O2 using molecular oxygen as an electron acceptor (Pluschkell et al., 1996; Hatzinikolaou et al., 1996). This reaction can be divided into a reductive and an oxidative step (Fig. 1). In the reductive half reaction, GOD catalyzes 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 GOD is reduced to FADH2 (Witt et al., 2000). In the oxidative half reaction, the reduced GOD is reoxidized by oxygen to yield H2O2. The H2O2 is cleaved by catalase (CAT) to produce water and oxygen. Witteveen et al. (1992) found the enzyme lactonase (EC 3.1.1.17) in A. niger to be responsible for catalyzing the hydrolysis of glucono-δ-lactone to gluconic acid.

1.2. Composition of glucose oxidase

GOD from ascomycetes is a dimeric glycoprotein consisting of two identical polypeptide chain subunits that are covalently linked together via disulfide bonds (Rando et al., 1997; Kalisz et al., 1997). Fig. 2 depicts the FAD moiety and the key conserved active site residues of a GOD subunit from P. amagasakiense (Wohlfiath et al., 1999). The structure of the P. amagasakiense GOD shows each of its subunit to contain one mole of tightly bound, but not covalently linked FAD moiety as co-factor (Rando et al., 1997; Witt et al., 2000). GOD from P. amagasakiense is glycosylated with a mannose-rich carbohydrate content of approximately 11–13% (Nakamura and Fujiki, 1968; Kusai et al., 1960).

The key conserved active site residues of GOD from P. amagasakiense are Tyr-73, Phe-418, Trp-430, Arg-516, Asn-518, His-520 and His-563 (Fig. 2) (Witt et al., 2000). Witt et al. (2000) concluded that Arg-516 was the most critical amino acid for the efficient binding of β-D-glucose by GOD, while Asn-518 contributed to a lesser extent. The
acetic buffer containing 2% of the GOD. In this method, enzyme solution was added to sodium GOD. Tongbu et al. (1996) used titrimetric method for determination of GOD activities. It is evident that GOD from P. amagasakiense has a broader pH range compared to A. niger. GOD contained more histidine, arginine and tyrosine and less lysine and phenylalanine than the enzymes revealed that the GOD of A. niger and P. amagasakiense were shown to be 3.5–6.5 and 4.0–5.5, respectively. It is evident that GOD from A. niger has a broader pH range than that from P. amagasakiense GOD (Nakamura and Fujiki, 1968).

1.3. Characteristics of glucose oxidase

The molecular weight of GOD ranges from approximately 130 to 175 kDa (Kalisz et al., 1997). GOD is highly specific for the β-anomer of D-glucose, while α-anomer does not appear to be a suitable substrate. Low GOD activities were exhibited when utilizing 2-deoxy-D-glucose, D-mannose and D-galactose as substrates. Inhibitors of GOD include p-chloromercuribenzoate, Ag+, Hg2+, Cu2+ and hydroxylamine, phenylhydrazine, dimedone and sodium bisulphate (Kusai et al., 1960). Nakamura and Fujiki (1968) performed comparative studies on the GOD of A. niger and P. amagasakiense, and found their molecular weights to be 152 and 150 kDa, respectively. GOD produced by both the strains had similar carbohydrates, which consisted mainly of glucose, mannose and hexosamine. A. niger GOD 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 two enzymes revealed that the A. niger GOD contained more histidine, arginine and tyrosine and less lysine and phenylalanine than the P. amagasakiense GOD. The optimum pH ranges for GOD from A. niger and P. amagasakiense were shown to be 3.5–6.5 and 4.0–5.5, respectively. It is evident that GOD from A. niger has a broader pH range than that from P. amagasakiense GOD (Nakamura and Fujiki, 1968).

1.4. Analysis of glucose oxidase activity

Literature depicts various analytical methods for determination of GOD. Tongbu et al. (1996) used titrimetric method for determination of the GOD. In this method, enzyme solution was added to sodium acetate buffer containing 2%β-D-glucose and the reaction was stopped by adding sodium hydroxide solution. The resulting mixture was titrated with standard hydrochloric acid solution to calculate volume of added standard HCl and thereby to calculate GOD activity.

Most researchers use an analytical method for GOD that is based on the principle that GOD oxidizes β-D-glucose in the presence of oxygen to β-D-glucono-δ-lactone and H2O2. The H2O2 is then utilized to oxidize a chromogenic substrate in a secondary reaction with horseradish peroxidase (HRP) with a resultant color change that is monitored spectrophotometrically. Two of the chromogenic substrates used for the GOD reaction are: 2, 2′-Azino-di-[3-ethylbenzthiazolin-sulfonate] (ABTS) (Witt et al., 1998) and o-dianisidine (Bergmeyer et al., 1974). ABTS forms a greenish-blue oxidized product that is measured spectrophotometrically at 420 nm. The reactions involved are represented in Schemes 1 and 2.

O2 + β-D-Glucose → β-D-Glucono-δ-lactone + H2O2

ABTS(oxidized) + H2O → o-dianisidine(oxidized) + H2O

Scheme 1. GOD reaction with ABTS as a chromogenic dye (Witt et al., 1998).

2. Fermentative production of GOD

2.1. Microbial strains producing glucose oxidase

The most common microbial sources for fermentative production of GOD are Aspergillus, Penicillium, and Saccharomyces species. Most of the commercially produced GOD is isolated from mycelium of Aspergillus niger, grown principally for the production of gluconic acid or its salts such as sodium gluconate or calcium gluconate. Accordingly, the enzyme is obtained essentially as a by-product or co-product of gluconate production. Table 1 compiles detailed information on GOD production, production media and different assay methods used by various researchers.

2.2. Parameters affecting enzyme production

2.2.1. Carbon source

During microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but is also used in

O2 + β-D-Glucose + H2O → δ-Glucono-1,5-lactone + H2O2

Scheme 2. GOD reaction with o-dianisidine as a chromogenic dye (Bergmeyer et al., 1974).
the synthesis of polysaccharide and as energy source. The rate at which carbon source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites. Fast growth due to high concentration of rapidly metabolized sugars is often associated with high productivity of growth-associated products or primary metabolites (Stanbury et al., 1997).

Hatzinikolaou and Macris (1995) investigated the effects of different carbon sources on growth and total GOD activity for A. niger. Although A. niger grew on all the carbon sources that they tested, significant levels of GOD 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 GOD gene. Kona et al. (2001) used sucrose as carbon source when they used economical nutrient containing corn steep liquor as a fermenter. Petruccioli et al. (1993) studied GOD 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. variabile (3 strains), when cultivated on glucose as the carbon source produced GOD activity ranging from 0.61 U/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%).

A. niger glucose oxidase gene expressed in S. cerevisiae

Rogalski et al. (1988) and demonstrated the induction of GOD production by calcium carbonate (optimum 4%), which also maintained the pH of the cultivation media between 6.5 and 6.8. They showed that the activity of the glycolytic enzyme, glucose-6-phosphate isomerase, was higher in growth media without calcium carbonate, while that of GOD and catalase (CAT) were quite low. Inclusion of calcium carbonate to the growth media might cause a metabolic shift from glycolysis to the pentose phosphate pathway, thereby increasing GOD levels. Addition of calcium carbonate in the growth media prevented pH drop during cultivation, which is necessary for optimal GOD production.

2.2.2. Nitrogen source

Inorganic nitrogen is supplied as ammonia gas, ammonium salts or nitrates. Ammonia has been used for pH control. Ammonium salts such as ammonium sulphate usually produces acidic conditions as the ammonium ion gets utilized, and the free acid is liberated. On the other hand, nitrates will normally cause an alkaline drift as they get metabolized. Ammonium nitrate will first cause an acid drift as the ammonium ion is utilized, and nitrate assimilation is repressed. When the ammonium ion gets exhausted, there is an alkaline drift as the nitrate is then used as an alternative nitrogen source (Morton and MacMillan, 1954). Rogalski et al. (1988) showed that when cultivating A. niger mutant G-13 and supplementing the growth media with 3% peptone there was 36% and 42% increase in GOD activity and biomass production, respectively. Hatzinikolaou and Macris (1995) investigated different nitrogen sources on the growth and total GOD activity of A. niger cultivated on sucrose and molasses as sole carbon sources. They found that the peptone concentration had a marked effect on the total GOD production. With sucrose and molasses as carbon sources, maximum GOD activity was achieved at 1–2% and 0.2–0.3% peptone, respectively. Kona et al. (2001) investigated the effect of corn steep liquor as the sole nutrient source on the production of GOD from A. niger and found it to increase the GOD activity from 550 Uml⁻¹ to 640 Uml⁻¹, and that other nitrogen sources did not further improve the enzyme synthesis.

2.2.2.3. Calcium carbonate as an inducer

Petruccioli et al. (1995a) found that the addition of calcium carbonate to growth medium in shake flasks and in fermenters prevented pH drop during cultivation, which is necessary for optimal GOD production. Rogalski et al. (1988) showed that the synthesis of GOD was sensitively influenced by increasing concentrations of calcium carbonate (0–4.5%) with maximal GOD activity at approximately 3.5%. Hatzinikolaou and Macris (1995) reported calcium carbonate to be a strong inducer of GOD in A. niger and demonstrated it to be essential for increased levels of GOD production. Optimum calcium carbonate of 4 and 5% was observed for GOD production using sucrose and molasses respectively. Bankar et al. (2008) found 3% of CaCO₃ to be optimum for highest GOD production. Hatzinikolaou et al. (1996) cultivated A. niger using optimized cultivation media of Rogalski et al. (1988) and demonstrated the induction of GOD production by calcium carbonate (optimum 4%), which also maintained the pH of the cultivation media between 6.5 and 6.8. They showed that the activity of the glycolytic enzyme, glucose-6-phosphate isomerase, was higher in growth media without calcium carbonate, while that of GOD and catalase (CAT) were quite low. Inclusion of calcium carbonate increased the GOD and CAT activities with a simultaneous decrease in the glucose-6-phosphate isomerase activity. They suggested that the addition of calcium carbonate in the growth media might cause a metabolic shift from glycolysis to the pentose phosphate pathway, thereby increasing GOD levels. Addition of calcium carbonate in the growth medium caused changes in GOD, CAT, 6-phosphofructokinase (6-PFK) and glucose-6-phosphate dehydrogenase (G-6-PDH) activities. 6-PFK is a key regulatory enzyme of Embden–Meyerhof–Parnas

Table 1

Various microorganisms producing GOD.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Media composition (g/l)</th>
<th>Assay method</th>
<th>Yield (Unit)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillium variabile P16</td>
<td>Glucose,80; NaNO₃,5; KCl,0.5; KH₂PO₄,0.01; mycological peptone, 1; CaCO₃, 35</td>
<td>Reduction of benzoquinone by hydroquinone measured by the rate of absorbance increase at 290 nm</td>
<td>5.52 U ml⁻¹</td>
<td>Petruccioli et al. (1999), Petruccioli et al. (1995b)</td>
</tr>
<tr>
<td>A. niger glucose oxidase gene expressed in S. cerevisiae</td>
<td>Yeast peptone dextrose (YPD) medium: Yeast extract, 10; peptone,20; glucose,20.</td>
<td>Plate assay: with o-dianisidine Coupled o-dianisidine-peroxidase reaction.</td>
<td>125 U ml⁻¹</td>
<td>Hodgkins et al. (1993), Malherbe et al. (2003).</td>
</tr>
<tr>
<td>Aspergillus niger (RTL)</td>
<td>Sucrose,70; (NH₄)₂HPO₄,0.4; KH₂PO₄,0.2; MgSO₄·7H₂O,0.02; peptone,10; CaCO₃,35.</td>
<td>Coupled o-dianisidine-peroxidase reaction.</td>
<td>7.5 U ml⁻¹</td>
<td>Hatzinikolaou and Macris (1995), Kapat et al. (2001).</td>
</tr>
<tr>
<td>Recombinant Saccharomyces cerevisiae</td>
<td>Yeast extract,40; hycas, 5; glucose, 20; galactose,30.</td>
<td>Coupled ABTS reaction</td>
<td>3.43 U mg⁻¹ dry cell mass</td>
<td></td>
</tr>
<tr>
<td>Aspergillus niger AM1111</td>
<td>Glucose,80; peptone,30; NaNO₃,0.5; KH₂PO₄; CaCO₃,35.</td>
<td>Coupled reaction assay</td>
<td>2.5 U ml⁻¹</td>
<td>Fiedurek and Gromada (2000), Randi et al. (1997).</td>
</tr>
<tr>
<td>Penicillium pinophilum DSM 11428</td>
<td>Sucrose,40; Na₃PO₄·2H₂O,0.45; KH₂PO₄,15; NaNO₃, 19; MgSO₄·7H₂O,0.02; CaCl₂·2H₂O, 0.02; malt extract,10; yeast extract,5; Trace element,10; vitaminene,10.</td>
<td>Coupled ABTS -peroxidase reaction.</td>
<td>1.9 U ml⁻¹</td>
<td></td>
</tr>
<tr>
<td>Aspergillus niger ZBY-7</td>
<td>Glucose,150; inorganic salts,0.35; metal caronate,35;</td>
<td>Titrimetric</td>
<td>6 U ml⁻¹</td>
<td>Tonghu et al. (1996).</td>
</tr>
</tbody>
</table>
Metabolic pathway of glucose in the absence and presence of CaCO₃ by Aspergillus niger.

**2.2.7. Effect of growth temperature**

The internal temperature of the microorganism must be equal to that of its environment and, the microbial activity is well known to be sensitive to environmental temperatures. The influence of temperature on GOD production is related to the growth of the organism. Among the fungi, most GOD production studies have been done with mesophilic fungi within the temperature range of 25–37 °C. Optimum yields of GOD were achieved at 27–37 °C for *A. niger* (Caridis et al., 1991) studied the simultaneous production of GOD and CAT by *Alternaria alternate*, and revealed that GOD had its optimum temperature at 32 °C and CAT at 18.1 °C. Hatzinikolaou and Macris (1995) examined the effect of growth temperature on total GOD production at 22.5 to 32 °C, and found 27.5 °C as the optimum.
2.2.8. Fed-batch culture

The aim of fed-batch study is to determine the degree of improvement in enzyme production over that of batch culture, and to get an insight into the utilization of the carbon source. Sophisticated fed back controlled fed-batch systems are also available. Fed-batch cultivation for GOD production is reported by Kapat et al. (1998) who showed that the addition of part of the carbon source at a later stage could improve GOD formation. Out of four different feeding strategies tested by them for the production of extracellular recombinant GOD from Saccharomyces cerevisiae, constant feeding of galactose on exhaustion of initial glucose gave the highest yield of 154 U/ml, which was 62% above the yield achieved in batch operation (95 U/ml).

2.3. Optimization by statistical methods

The conventional ‘one variable at-a-time’ approach is time consuming and often leads to confusion in the understanding of process parameters. Use of statistical methods enables easy selection of important parameters from a large number of factors, and also explains the interactions between important variables. A number of statistical experimental designs have been used for optimizing fermentation variables. Plackett–Burman design (Plackett and Burman, 1946) is well known and is a widely used statistical technique for screening and selection of most significant culture variables, while central composite design provides important information regarding the optimum level of each variable along with its interactions with other variables and their effects on product yield (Pardeep and Satyanarayana, 2006).

Placket–Burman saturated orthogonal designs work at two levels, and can be constructed on the basis of fractional replication of a full factorial design. This design allows reliable short listing of a small number of ingredients for further optimization and allows one to obtain unbiased estimates of linear effects of all the factors with maximum accuracy for a given number of observations, the accuracy being the same for all effects (Krishnan et al., 1998). Since this design is a preliminary optimization technique which tests only two levels of each medium component, it cannot provide the optimal quantity of each component required in the medium. This technique, however, provides indications of how each component tends to affect enzyme production (Yu et al., 1997). Bankar et al. (2008) highlighted the importance of Plackett–Burman experiments for optimizing culture variables in attaining higher GOD titers. Among the six variables which were expected to play a critical role in enhancing GOD production, three factors (calcium carbonate, proteose peptone, and magnesium sulphate) significantly affected enzyme production.

Response surface methodology (RSM) by central composite design (CCD) or by Box–behnken are the tools specifically used in present times in fermentation technology to find out the optimum concentration of the most effective variables for getting higher enzyme titers and to study their interactions. Various statistical software packages are available for statistical optimization of variables. Liu et al. (2003) applied RSM to optimize the speed of agitation and rate of aeration for maximum production of GOD by A. niger. They found aeration to have a more negative effect on GOD production than agitation. Significant negative interaction existed between agitation and aeration. RSM was also successfully employed for determination of optimum concentration of media components by Bankar et al. (2008). They found maximum GOD production at 3.08% calcium carbonate, 0.97% peptone and 0.1% magnesium sulphate.

2.4. Mathematical model for glucose oxidase kinetics

It is generally accepted that the GOD is a primary metabolite, but it is difficult for even Luedeking and Piret model (Luedeking and Piret, 1959; Crueger and Crueger, 1990) to explain the levels of GOD produced in the medium as alone. These levels depend on microbial production, as well on the processes that affect the enzyme once it has been released, amongst which the most noteworthy is its deactivation by the H2O2 produced by the enzymatic action itself. However, the microorganism also produces catalase, which breaks H2O2, thus opposing the previous effect and helping to preserve GOD (Miron et al., 2002).

Growth of the microorganism and the production of GOD both show the characteristics of a diauxic process. This diauxic nature, with logistic and linear phases, is also evident in the disappearance of glucose. The disappearance of glucose in the first phase of the culture, although partly due to microbial consumption, is mainly a consequence of its conversion to gluconic acid as a result of the action of GOD produced in the culture. Once the glucose is exhausted, the microorganism begins to use gluconic acid that accumulates during the first stage as a carbon source, with basically linear kinetics. GOD is considered as a semi-constitutive enzyme with a moderate rate of biosynthesis that remains constant in the absence of inducers, and increase in the presence of inducers (Miron et al., 2002).

Microbial processes do not necessarily follow the classical kinetic model of substrate-limited biomass growth and product formation as proposed by Monod in 1949. Therefore, the logistic equation, a substrate-independent model, is used as an alternative empirical function. In many fermentation systems, cell growth has been characterized by the logistic equation. The logistic equation (Eq. (1)) can be described as follows:

\[ \frac{dX}{dt} = \mu_m \left(1 - \frac{X}{X_m}\right)X \]  

where \( X \) is amount of biomass formed at time \( t \), \( X_m \) is maximum biomass formed; \( \mu_m \) is maximum specific growth rate and \( \frac{dX}{dt} \) is the rate of biomass formation (Wang et al., 2006).

Logistic equations are a set of equations that characterize growth in terms of carrying capacity (maximum cell mass; \( X_m \)). The usual approach is based on formulation in which specific growth rate is related to the amount of unused carrying capacity (Shuler and Kargi, 2002).

\[ \mu_s = k \left(1 - \frac{X}{X_m}\right) \]

where \( X_m \) is maximum cell mass-produced and \( k \) is carrying capacity coefficient.

Thus,

\[ \frac{dX}{dt} = k\left(1 - \frac{X}{X_m}\right) \]

Integrating Eq. (2) with boundary conditions \( X(0) = X_0 \) yields the logistic curve.

\[ X = \frac{X_m e^{k t}}{1 - \frac{X_m}{X_0} (1 - e^{k t})} \]

Eq. (2) can be rewritten as

\[ \frac{1}{X} \frac{dX}{dt} = k \left(1 - \frac{X}{X_m}\right) \]

or

\[ k = \frac{1}{X} \frac{dX}{dt} \left(1 - \frac{X}{X_m}\right) \]

The kinetics of product formation is based on the Luedeking–Piret equation (Luedeking and Piret, 1959). According to this model, the
product formation rate ($r_p$) depends on both the instantaneous biomass concentration and the growth rate in a linear manner.

$$r_p = \frac{dp}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (3)$$

where $\alpha$ and $\beta$ are the product formation constants and may differ under different fermentation conditions.

Miron et al. (2002) found the kinetics of the GOD to be affected by substrate inhibition, competitive inhibition by gluconic acid, decrease of the reaction rate due to diffusional restrictions determined by the viscosity of the gluconic acid, and decrease in the reaction rate due to enzyme deactivation by H$_2$O$_2$. The last mentioned is a feature with some phenomenological resemblance to true substrate inhibition, and which disappears when catalase is present.

Liu et al. (2003) proposed a simple model using the Logistic equation for growth, the Luedeking–Piret equation for GOD production and Luedeking–Piret-like equation for glucose consumption. They showed the biosynthesis of GOD to be strongly linearly related to the growth, and that the correlation coefficient was very high. These results showed that the biosynthesis of GOD could be growth associated. The model provided a reasonable description for various kinetic model parameters during the growth phase.

3. Genetic expression for glucose oxidase production

As explained in section 1, the fungal GOD are homodimers of approximately 150–170 kDa containing two tightly, but non-cova-

lently bound FAD cofactors, and about 11–13% carbohydrate moiety of the high-mannose type. Typical problems that are usually encountered during their production are either low productivity or con-

comitant production of other enzymes such as CAT. To overcome these problems, use of genetically modified microorganisms rather than natural sources for the expression of this enzyme has been strongly suggested (Park et al., 2000).

Despite the abundant availability of commercial GOD, there is still considerable interest in its new forms with useful properties for special applications in biotechnology. With a view to improve the properties of enzyme by protein engineering, the GOD gene of A. niger was characterized (Frederick et al., 1990; Kriechbaum et al., 1989; Hatzinikolaou et al., 1996) and its crystal structure was elucidated (Hecht et al., 1993).

Malherbe et al. (2003) expressed the A. niger gene encoding GOD in S. cerevisiae and evaluated the transformants for lower alcohol production and inhibition of wine spoilage organisms such as acetate acid bacteria and lactic acid bacteria during fermentation. They devel-

oped tailored strains of S. cerevisiae for biopreserved wines with lower alcohol content. To test this novel concept, an antimicrobial yeast starter culture system, on selective agar plate and in liquid assay was done. The yeast transformants displayed antimicrobial activity in a plate assay due to production of H$_2$O$_2$, a final product of GOD enzymatic reaction and also a known antimicrobial agent. Production of α-glucono-1, 5-lactone and gluconic acid from glucose by GOD resulted in wines containing 1.8–2.0% less alcohol.

In the last few years, yeasts such as Hansenula polymorpha and S. cerevisiae have been investigated as promising high-yield production systems and suggested for heterologous GOD production; but further studies showed hyperglycosylation with yeast which may lead to serious limitations of usage (Romanos et al., 1992). Expression of recombinant GOD using E. coli (Witt et al., 1998) and S. cerevisiae (Frederick et al., 1990; Ko et al., 2002) has always shown limitations. In the case of E. coli, 60% of the recombinant protein was inactive, whereas the recombinant GOD expressed in S. cerevisiae was hypergly-

gosylated and thus characterized by reduced substrate binding capacity and catalytic activity (Kapat et al., 1998). Crognaile et al. (2006) used methylotrophic yeast Pichia pastoris as host for expression and secretion of recombinant GOD of the filamentous fungus P. variabile P16. They transformed the gene to P. pastoris X33, a strain largely used for selection on zeocin and large scale growth studies. They demonstrated P. pastoris to be an efficient host for expression of both secreted and intracellular heterologous proteins. Fermentation in 3 l fermenter lead to GOD production of up to 50 U/ml that represents approximately four times increase in the production as compared to P. variabile P16 cultivated under optimized conditions. Kriechbaum et al. (1989) described the cloning and sequencing of the GOD gene of A. niger NRRL-3 containing 5′ and 3′ flanking regions. They also represented the DNA-derived amino acid sequence of GOD and showed its identity with peptide sequences determined for parts of this protein.

The yeast has potential to perform many post-translational modi-

fications, typically associated with higher eukaryotes such as pro-

cessing of signal sequences, folding, formation of disulfide bridges, certain types of lipid addition, and O- and N-linked glycosylation. Since yeast requires minimal salt media, it contributes to cost effective industrial production. Recombinant yeast expression plasmids have been constructed by Frederick et al. (1990) containing a hybrid yeast alcohol dehydrogenase Il-glyceraldehyde-3-phosphate dehydrogen-

ase promoter, either the yeast α factor pheromone leader or the GOD presequence, and the mature GOD coding sequence. When trans-

formed into yeast, these plasmids direct the synthesis and secretion of between 75 and 400 μg/ml of active GOD. Analysis of the yeast-

derived enzymes showed that they are of comparable specific activity and have more extensive N-linked glycosylation than the A. niger protein.

Kriechbaum et al. (1989) constructed a library of the A. niger NRRL-3 genome in the phage A substitution vector EMBL3. They isolated one hybridizing clone which contained an insert of 15 kbp, from 12000 recombinant plaques with the nick-translated 800 bp cDNA fragment. The phage DNA was cleaved with Sall and the resulting fragments were subcloned into pBluescript SK (+). They used hybridization techniques and the shotgun sequencing method for identification of 1.8 kbp and 2.0 kbp Sall fragment containing the coding region of GOD as well as small 5′ and longer 3′ untranslated regions.

4. Downstream processing

Development of new and efficient separation processes is based on effectively exploiting differences in the actual physicochemical properties of the product such as surface charge / titration curve, surface hydrophobicity, molecular weight, biospecificity towards certain ligands (e.g. metal ions, dyes), isoelectric point (pI) and stability, compared to those of the contaminant components in the crude broth (Asenjo, 1993). The crucial step after completion of successful fermentation is recovery of GOD. Fig. 4 illustrates a general protocol for purification of GOD.

GOD has been purified for commercial application from different fungi including A. niger (Hatzinikolaou et al., 1996; Kalisz et al., 1991; Svoboda and Massey, 1965) and Penicillium species such as P. pinophilum (Rando et al., 1997), P. amagasakiense (Kusi et al., 1960; Kalisz et al., 1997), P. chrysogenum (Eriksson et al., 1987), P. notatum (Gorniak and Kączkowski, 1974), and P. funiculosum (Eryomin et al., 2004).

GOD is known to be produced intracellularly or extracellularly or sometimes as mycelia-associated enzyme. Hence cells have to be disrupted for complete release of GOD into the broth. The intra- or extracellular location of the enzyme of A. niger and Penicillium species has been the subject of numerous discussions. In the meantime, the periplasmatic location of the A. niger GOD was clearly demonstrated (Witteveen et al., 1992), which is in agreement with the presence of a signal sequence preceding the A. niger GOD gene (Kriechbaum et al., 1989; Frederick et al., 1990). As a consequence of peripheral location, the release of the enzyme from mycelium may be facilitated by mechanical and physical forces, e.g. agitation and/or sonication.
GOD has a negative charge in double distilled water due to its pI being 4–5. It is therefore adsorbed at the beginning of the column. On eluting with the elution buffer (pH 3.6), GOD becomes positively charged and gets desorbed from the anionic exchange resin. The different eluted fractions of GOD carries different amounts of negative charges and this separates them from each other. The first separated part, GOD A has less charge than GOD B in the purification process; therefore influence of charge impulse to the conformation of GOD A is small and has a higher enzyme activity. GOD B carries more negative charge than GOD A, and therefore it can only be eluted after GOD A and also destroys the native conformation and decreases the enzyme activity (Dai et al., 2002). Rando et al. (1997) used a very efficient and elaborate procedure for the purification of GOD from \textit{P. pinophilum}. They purified GOD to apparent homogeneity with a yield of 74% by including an efficient extraction step of the mycelium mass at pH 3.0 and ion-exchange chromatography followed by gel filtration.

5. Immobilization of glucose oxidase

Enzyme immobilization has attracted a wide range of interest from fundamental academic research to many different industrial applications. To date, several immobilized enzyme-based processes have proved to be economical. They have been implemented on a larger scale, mainly in the food industry, where they replace free enzyme-catalyzed processes, and in the manufacture of fine specialty chemicals and pharmaceuticals, particularly where asymmetric synthesis or resolution of enantiomers to produce optically pure products are involved (Krajewska, 2004).

Varieties of supports have been used for immobilization of enzymes such as cellulose, solid glass particles, porous glass particles, and nickel screen. Because of the advantages in catalytic activity offered by materials having relatively high surface areas, porous glass and cellulose have been the most popular supports. For example, the preparation and to improve the storage stability of GOD on porous glass by $\gamma$-amino-propyltriethoxysilane (APTES) was used by various researchers for silanization of the glass; prior to immobilization of GOD. Immobilized GOD on both solid and porous glass was also performed by some researchers (Bouin et al., 1976; Herring et al., 1972; Sreedivya et al., 1998; Wasilewska et al., 1987; Wassman and Hultin, 1980). In addition, nickel oxide screen can be silanized and GOD can be coupled by thiophosgene method, but the screen offers little surface area (Herring et al., 1972). GOD is difficult to crosslink with an agent like glutaraldehyde, but does crosslink rather easily in the presence of certain proteins that have high number of reactive amino groups such as polyethyleneimine (Bouin et al., 1976).

Co-immobilization of GOD and CAT were studied by several investigators (Tarhan and Telefoncu, 1990; Blandino et al., 2002; Tarhan and Telefoncu 1992; Godjevargova et al., 2004; Podual et al., 2000). Ozyilmaz and Tukel (2007) used inorganic and porous magnesium silicate (Florisil) as a support for simultaneous co-immobilization of GOD and CAT. Basic property of carrier may play a special role in partial neutralization of gluconic acid produced by bound GOD in the pores of the carrier. This prevents a dramatic decrease in pH of microenvironment of bound enzymes, which may hinder their denaturation and thereby enhance the stability of co-immobilized GOD/CAT.

Enzymes are covalently linked to the support through functional groups in the enzymes, which are not essential for catalytic activity. But, it is known that immobilization decreases enzyme activity due to blocking of the active site or due to changes in the enzyme geometry at the end of the coupling procedure. Addition of a substrate or a competitive inhibitor to the coupling mixture protects the active site on the enzyme against loss of activity (Sree et al., 1976). Several studies are available in literature on immobilization of enzymes in the presence of their substrates.

Various methods of cell disruption have been used for filamentous fungi, including homogenization (Hatzinikolaou et al., 1996; Fiedurek and Gromada, 1997), 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, \textit{Candida\textit{}aplanatum} and \textit{Pycnoporus cinnabarinus} was performed by Taubert et al. (2000). They concluded that fungal cells were particularly resistant to some of the disintegration methods commonly used for yeasts and bacteria as well as the mechanical and non-mechanical cell disruption methods described by Christi and Moo-Young (1986). For release of intracellular as well as cell-bound GOD into the liquid broth, various methods like sonication, bead mill, homogenizer and freeze-thawing were applied. After the disruption of the cells, GOD is released in the fermentation broth which may be separated from the cells either by differential centrifugation or by filtration.

Figure 4. General protocol for purification of glucose oxidase.
To protect the active sites of GOD and CAT, co-immobilization was carried out in the presence of the glucose which is a substrate of GOD. During the coupling period, GOD oxidizes glucose to produce H$_2$O$_2$ which is a substrate of CAT. Thus, co-immobilization is carried out in the presence of both glucose and H$_2$O$_2$, which are substrates of GOD and CAT, respectively (Ozyilmaz and Tukel, 2007). Ozyilmaz and Tukel (2007) further reported the maximum activities of co-immobilized GOD and CAT in the presence of 15 and 20 mM glucose, respectively. Co-immobilization of GOD and CAT in the presence of their substrates significantly improves the activity and reusability of both enzymes. GOD is inactivated by H$_2$O$_2$, the concentration of which increases during the catalytic turnover (Kleppe, 1966). H$_2$O$_2$ inactivation can be reduced with CAT which reduces H$_2$O$_2$ and eventually removes it from the system. By this reaction, some oxygen is recovered and made available for the oxidation of glucose (Ramchandran and Perlmutter, 1976).

Clear distinction between GOD and pyranose oxidase, which is a structural features and the regioselectivity of the reaction allows a differentiation of molecular oxygen at C-1 position to β-D-glucose and show only marginal activity determinations. The initial rates begin to decrease which lead to inconsistent activity determinations.

6.1. Substrate specificity

GOD is highly specific for β-D-glucose and show only marginal activities with other sugars. β-D-glucose gets oxidized in the presence of molecular oxygen at C-1 position to δ-glucono-1, 5-lactone, which is, in turn is spontaneously hydrolyzed to p-gluconic acid. These structural features and the regioselectivity of the reaction allows a clear distinction between GOD and pyranose oxidase, which is a homotetramer, and oxidizes β-glucose at the C-2 position (Danneel et al., 1993; Hatzinikolaou et al., 1996; Pluschkell et al., 1996).

6.2. pH optima and stability

Since enzyme activity is dependent on the ionization state of the amino acids in the active site, pH plays an important role in maintaining the proper conformation of an enzyme. Most proteins are only active within a narrow pH range, usually in the range of 5–9 (Wilson and Walker, 1995; Voet and Voet, 1995). The pH optima of GOD vary from 5.0 to 7.0. GOD from most fungi and yeast have pH optima in the acidic to neutral range such as A. niger and P. chrysogenum shows pH optima of 5.0 to 6.0. (Kalisz et al., 1991; Eriksson et al., 1987). In contrast, the GOD obtained from P. funiculosum 433 and P. canescens show slightly alkaline pH optima of 6 to 8.6 (Sukhacheva et al., 2004).

6.3. Optimum temperature and stability

Enzymes are known to be sensitive to changes in temperature. 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 doubles. At temperature range between 40 °C and 70 °C most enzymes get denaturated and lose their activity. Enzymes are known to display maximal activity at a temperature known as the optimum temperature of the enzyme (Wilson and Walker, 1995). The lowest optimum temperature for GOD is reported to be 25–30 °C from P. funiculosum 433 (Sukhacheva et al., 2004) and the highest of 40–60 °C from A. niger and P. amagasakiense ATCC 28686 (Kalisz et al., 1991, 1997).

6.4. Variation of the initial rate with enzyme concentration

Michaelis and Menten determined that the initial rate or velocity of catalysis of an enzyme varied hyperbolically with substrate concentration (Voet and Voet, 1995). The initial rate increases with an increase in substrate concentration to a point where it would reach maximum velocity ($V_{max}$). At low substrate concentrations, initial rate is proportional to the substrate concentration, referred to as first order kinetics. At high substrate concentrations the initial rate is independent of substrate concentration, referred to as saturation or zero order kinetics. GOD at 0.2 U/ml could be accurately determined using the GOD dye binding assay (Bergmeyer et al., 1974). At higher GOD activities, the initial rates begin to decrease which lead to inconsistent activity determinations.

6.5. Kinetic parameters variability

The Michaelis constant ($K_m$) and the maximal limiting rate velocity ($V_{max}$) for GOD were calculated by various researchers and they found slight differences in their values. The $K_m$ value of the GOD from T. favus is 10.9 mM (Kim et al., 1990) and is 33 mM from A. niger (Swoboda and Massey 1965). P. amagasakiense ATCC 28686 and P. funiculosum 433 shows lower $K_m$ value of 5.7 mM and 3.3 mM respectively (Witt et al., 1998; Sukhacheva et al., 2004). It should also be noted that reestimations of the $K_m$ values of glycosylated and deglycosylated P. amagasakiense GOD gave values of 3.4 mM and 2.7 mM respectively (Kim and Schmid 1991). $V_{max}$ value of GOD ranges between 450 to 1000 U/mg. A. niger shows $V_{max}$ value of 458 U/mg while P. amagasakiense ATCC 28686 exhibited 925 U/mg (Witt et al., 1998; Kalisz et al., 1991).

6.6. Storage stability

GOD has half-life of approximately 30 min at 37 °C. Immobilized GOD would be more effective for applications at 37 °C. Polyhydric alcohols including ethylene glycol, glycerol, erythritol, xylitol, sorbitol...
and polyethylene glycol have shown stabilizing effect on GOD from A. niger (Ye et al., 1988). The lyophilized GOD preparation remains stable for a minimum of 6 months at −20 °C.

7. Applications of glucose oxidase

GOD has gained considerable commercial importance in the last few years due to its multitude of applications in the chemical, pharmaceutical, food, beverage, clinical chemistry, biotechnology and other industries. GOD is the most widely used enzyme as an analytical reagent for determination of glucose due to its relatively low cost and good stability. Its uses range from a glucose biosensor for the control of diabetes, to a food preservative and color stabilizer. With co-immobilized enzymes, improved stability, reusability, continuous operation, possibility of better control of reactions, high purity and product yields and economical process can be expected (Kleppe, 1966). Some of its current applications in industry are described here.

7.1. Glucose biosensor for diabetes monitoring

People with diabetes mellitus need to constantly monitor their blood glucose levels in order to detect fluctuations in glucose levels that could lead to hyperglycemia (high blood glucose levels) and hypoglycemia (low blood glucose levels) so as to control the disease. Currently, such monitoring is done using finger-prick blood samples and a portable meter several times a day. Biosensors are being developed to measure blood glucose levels. GOD is one of the possible enzymes that can be used in biosensor. Biosensors work by keeping track of the number of electrons that pass through an enzyme by connecting it to an electrode and measuring the resultant charge. Alternatively, some biosensors use sensitive fluorescence measurements, monitoring changes in the intrinsic FAD fluorescence of GOD (Wilson and Turner, 1992).

Various GOD based biosensors are as listed below:

1. On line glucose monitoring for fermentations (Vodopivec et al., 2000).
2. Fibre optic biosensor for analyzing glucose concentrations in soft drinks (Chudobova et al., 1996).
3. Disposable strip-type biosensor for blood and serum monitoring (Cui et al., 2001).
5. Miniaturized thermal biosensor for whole blood (Harborn et al., 1997).
6. Glucose sensor for whole blood (Santoni et al., 1997).
7. Glucose biosensor for serum from human blood (Zhu et al., 2002).

7.2. Biofuel cells

Bio-electronic devices are energy demanding, requiring small power sources to sustain operations. Biofuel cells convert biochemical energy into electrical energy using a biocatalyst. One type of biofuel cell uses enzymes as a biocatalyst. For example, GOD and microperoxidase-8 can be used on the cathode, where the H₂O₂ produced by GOD oxidizes microperoxidase-8 to directly accept electrons from the carbon rod electrode. Biofuel cells consist of a two electrode set of any stable and electrically conducting material modified by biocatalytic enzymes to specifically oxidize/reduce substrates. One approach towards the design of an implantable, membraneless and biocompatible biofuel cell consists of catalyzing the oxidation of glucose at the anode using either GOD or glucose dehydrogenase enzymes. These enzymes are coupled to the reduction of dioxygen at the cathode by a dioxygen-reducing enzyme such as laccase, bilirubin oxidase or cytochrome oxidase (Chen et al., 2001; Mano et al., 2002; Soukharev et al., 2004). Electron transfer to/from the biocatalytic active sites can be mediated by polymer bound or entrapped redox complexes (Barton et al., 2004). Both water-soluble fuel molecules (glucose and O₂) are found in body fluids and in blood at 10 and 0.1 mM, respectively. Besides, these molecules are converted at the electrodes into naturally occurring degradation molecules in low concentration (gluconolactone and water). The maximum theoretical electromotive force (emf) allowed by the thermodynamics of glucose oxidation and dioxygen reduction at physiological pH is approximately 1 V.

7.3. Food and beverage additive

GOD has been used successfully to remove residual glucose and oxygen in foods and beverages in order to prolong their shelf life. The H₂O₂ produced by the enzyme acts as a good bactericide, and can later be removed using a second enzyme, CAT that converts H₂O₂ to oxygen and water. GOD/CAT is used to remove glucose during the manufacture of egg powder, preventing browning during dehydration caused by the Maillard reaction for use in baking industry, providing slight improvements to the crumb properties in bread and croissants (Rasiah et al., 2005; Crueger and Crueger, 1990).

GOD can also be used to remove oxygen from the top of bottled beverages before they are sealed. GOD/CAT system is shown to control non-enzymatic browning during fruit processing and puree storage. The scavenging of the oxygen by the enzyme system had a stabilizing effect. In addition, GOD is used to prevent color and flavor loss as well as to stabilize color and flavor in beer, fish, tinned foods and soft drinks by removing oxygen from foods and beverages (Crueger and Crueger, 1990). For example, they are used to reduce the discoloration occurring in wines and mayonnaises. GOD/CAT enzyme system can 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 (Isaksen and Adler-Nissen, 1997). Bonet et al. (2006) studied the effect of GOD on dough rheology and bread quality and showed the strengthening of wheat dough and an improvement in bread quality on addition of GOD. However, the enzyme level must be very carefully added, since adverse effects were obtained on addition of excessive enzyme.

7.4. Low alcohol wine production

GOD has potential for use in the wine industry, where it can lower the alcohol content of wine through the removal of some of the glucose (by converting it to δ-glucono-1, 5-lactone), which would otherwise be converted to alcohol. Tests showed that the GOD treatment of wine-must could reduce the potential alcohol content of wine by about 2%. In addition, GOD is able to inhibit wine spoilage through its bactericidal effect on acetic acid bacteria and lactic acid.

![Fig. 6. Preparation of low alcohol wine from GOD/CAT treated grape juice](Pickering et al., 1998).
bacteria during the fermentation process. The bactericidal effect of an enzyme means fewer preservatives need to be added to the wine (Malherbe et al., 2003). Pickering et al. (1998) reduced the fermentative alcohol potency by pre-treating the grape juice with the GOD/CAT enzyme system to convert the available glucose to gluconic acid (Fig. 6). They achieved 87% of glucose conversion with this system. 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.

7.5. Oral hygiene

GOD as well as lactoperoxidase can be used as anti-microbial agents in oral care products (Asfeth and Rolla, 1983). The oral cavity houses several species of Streptococci such as Streptococcus mutans, which is a significant contributor to tooth decay and is carried by virtually everyone. The H$_2$O$_2$ produced by GOD acts as a useful bactericide. The ability of GOD to kill S. mutans appears to be enhanced by the fusion of the enzyme with heavy chain antibodies (Etemadzadeh et al., 1985).

7.6. Gluconic acid

GOD is also used as a commercial source of gluconic acid, which can be produced by the hydrolysis of $\alpha$-glucono-1,5-lactone, the end-product of GOD catalysis. Gluconic acid has been used as a food additive to act as an acidity regulator, in sterilization solution or bleaching in food manufacturing, and as a salt in chemical components for medication. Gluconic acid is also used as a mild acidulant in additives to act as an acidity regulator, in sterilization solution or product of GOD catalysis. Gluconic acid has been used as a food additive to act as an acidity regulator, in sterilization solution or product of GOD catalysis. Gluconic acid has been used as a food additive to act as an acidity regulator, in sterilization solution or product of GOD catalysis. Gluconic acid has been used as a food additive to act as an acidity regulator, in sterilization solution or product of GOD catalysis. 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