



**- REVIEW ARTICLE -**

## **Glucose Oxidase Applications and Comparison of the Activity Assays**

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### **Abstract**

Glucose Oxidase (GOD) oxidizes glucose to  $\delta$ -gluconolactone in presence of molecular oxygen by forming hydrogen peroxide. As a result of the catalysed reaction, GOD is widely used in cases where glucose or molecular oxygen should be removed to extend the shelf life of foods or used in the production of controlled hydrogen peroxide or gluconic acid. One of the most important application areas of GOD is the construction of the glucose biosensors. There are several studies about GOD purification, immobilization, industrial and analytical applications, so, fast and sensitive determination of GOD activity is essential for these studies. In this study, GOD activity determination methods were reviewed mainly four approaches: determination of decrease in glucose or oxygen concentration and determination of increase in hydrogen peroxide or gluconic acid levels.

### **Keywords:**

Glucose Oxidase, Activity assays, gluconic acid, hydrogen peroxide

### **Article history:**

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### **Introduction**

Glucose Oxidase (EC 1.1.3.4) catalyses the oxidation of  $\beta$ -D-glucose by molecular oxygen to  $\delta$ -glucono-1,5-lactone and hydrogen peroxide. Gluconolactone can be hydrolyzed simultaneously and non-enzymatically to gluconic acid in presence of water (Figure 1).

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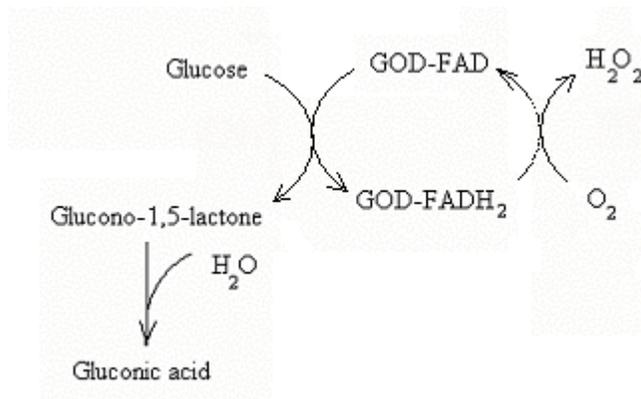


Figure 1. Reaction mechanism of GOD

As seen in Figure 1, glucose and O<sub>2</sub> concentrations decrease while products of reaction as H<sub>2</sub>O<sub>2</sub> and gluconic acid concentrations increase. The products cause two undesirable results: Decrease in pH owing to gluconic acid formation and inactivation of GOD because of oxidative properties of H<sub>2</sub>O<sub>2</sub> molecule (Kleppe, 1966). GOD is inactivated from H<sub>2</sub>O<sub>2</sub> produced in reaction medium, therefore, removing H<sub>2</sub>O<sub>2</sub> from reaction medium is important to hinder this inactivation. Catalase (CAT) which decomposes the H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O and O<sub>2</sub> is mostly used for this purpose (Figure 2). GOD is widely used with CAT at its free or co-immobilized form (Ozyilmaz 2007, Parpinello et al. 2002, Isaksen and Adler-Nissen 1997, Pickering et al. 1999, Harborn et al. 1997, Ramanathan et al. 2001, Vrbova et al. 1990, Blandino et al. 2002, Godjevargova et al. 2004a, Godjevargova et al. 2004b, Kang et al. 2003).

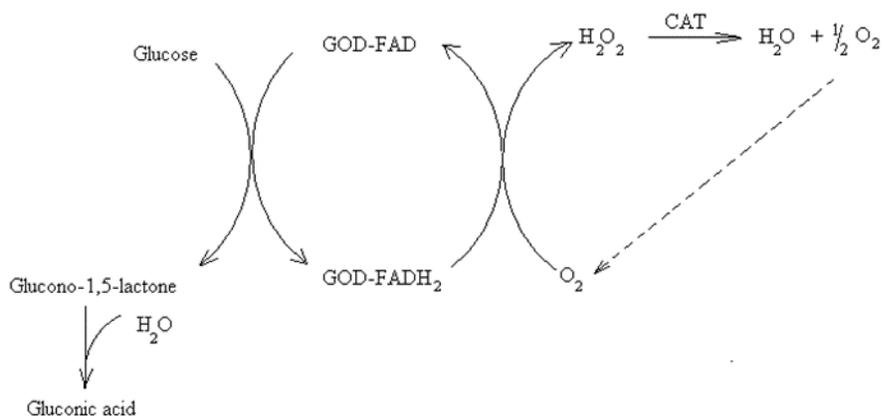


Figure 2. The co-use of GOD-CAT enzyme system

As seen in Figure 2, it is clearly seen that, part of the oxygen depleted by GOD is recovered by the CAT activity.

*Aspergillus niger* origin GOD is a dimeric protein with a molecular weight of 160 kDa, containing one, tightly but noncovalently bound, flavine adenine dinucleotide (FAD) per monomer as cofactor and glycosylated with a carbohydrate content of 16 % (w/w). GOD consists of two

identical polypeptide chain subunits covalently linked by disulfide bonds (O'Malley & Weaver 1972).

GOD has a number of industrial applications due to its ability to remove glucose and O<sub>2</sub> or production of gluconic acid and H<sub>2</sub>O<sub>2</sub>. There are several applications to use GOD for removing glucose from medium such as desugaring of egg-white (Sisak et al. 2006, Quan & Benjakul 2018), production of reduced alcohol wines (Pickering et al. 1999, Biyela et.al. 2009, Valencia et al. 2017, Ruiz et al.. 2018), removal of oxygen and browning control to increase storage time of fruit juices, purees and other stored foods (Parpinello 2002, Isaaksen and Adler-Nissen 1997, Xu et al. 2018, Cruz et al. 2013). GOD use is also an alternative way to production of H<sub>2</sub>O<sub>2</sub> for textile bleaching (Tzanov et al. 2002, Farooq et al. 2013, Reis et al. 2017, Qui et al. 2017, Madhu & Chakraborty 2019] or milk processing (Zhou &Lim 2009, Cruz et al 2013, Cruz et al 2012, Szweda et al 2013, Batista et al 2015). There are several studies about gluconic acid or gluconate salt production by GOD (Huang et al. 2019, Han et al. 2018, Ramezani et al. 2013, Mariam et al. 2010, Arimatsu et al. 2004, Bao et al. 2003, Shah & Kothari, 1993). GOD has analytical application to determine glucose levels of blood, serum, fruit juice *etc.*, and it is also the most used enzyme in preparation of glucose biosensors (Ozyilmaz et al. 2018, Khun et al. 2012, Guo et al. 2011, Salimi & Noorbahsh, 2011, Mazeiko et al. 2013, Ozyilmaz et al. 2007, Uang & Chou, 2003, Ekiz et al. 2011, Wang et al. 2013). Because of high degree of using areas, there are several studies about GOD.

When researchers study with any enzyme or its modified form such as immobilization, incubation or storage in the certain conditions, *etc*; they usually determine activity to reveal the effects of some parameters or modifications on the enzyme. So, at least one method should be used to determine activity for all enzymes. To determine GOD activity, there are several methods based on mainly four different change in the reaction medium: Consumption of dissolved O<sub>2</sub>, reducing the glucose concentration, production of gluconic acid and increase in concentration of H<sub>2</sub>O<sub>2</sub>.

#### ***Determination of decrease in dissolved O<sub>2</sub> concentration in the reaction medium***

Measuring the O<sub>2</sub> level in the reaction mixture was the earliest method for the determination the GOD activity (Kleppe 1966). Usually, O<sub>2</sub> depletion is measured with an amperometric oxygen electrode including Clark cell which was discovered by Dr. Clark in 1956.

GOD activity can be measured by oxygen meter basically. For this purpose, an oxygen meter probe is immersed in the stirred glucose solution at certain concentration. After measured O<sub>2</sub> concentration become steadily, GOD is added and decrease in O<sub>2</sub> concentration is measured for reaction duration. Thus, catalytic activity or specific activity can be calculated.

When GOD activity is measured using oxygen meter, reaction medium must be stirred, that cause O<sub>2</sub> diffusion through interface between air and reaction solution. So, while O<sub>2</sub> concentration decreases due to reaction, O<sub>2</sub> concentration increases slowly at the same time causing O<sub>2</sub> diffusion.

There are several studies about GOD at which enzyme activity was determined by measuring O<sub>2</sub> concentration (Gulla et al, 2004, Solomon, 1977; Weibel & Brights, 1971; Okuda & Miva, 1970; Okuda & Miva, 1971; Rogers & Brandt, 1971; Haouz et al., 2001, Valentova et al. 1981). As can be seen, GOD activity was determined by using oxygen meter formerly and GOD activity was calculated by using Equation 1.

$$GOD \text{ Activity} = \frac{(C_0 - C) \cdot V}{t} \quad (1)$$

Where

$C_0$ : Initial  $O_2$  concentration (mM)

$C$ : Last  $O_2$  concentration (mM)

$V$ : Reaction volume (mL)

$t$ : Reaction time (min)

According to Equation 1, GOD activity can be calculated as  $\mu\text{mol } O_2 \cdot \text{min}^{-1}$

### Determination of reduced glucose concentration

There are several chemical methods to determine concentrations of reducing sugars including glucose such as Folin –Wu, Somogyi- Nelson, o-Toluidine, Dinitrosalicylic acid method, *etc.* In GOD system, glucose concentration is reduced continuously and GOD activity can be determined by measuring of this decrease by a proper method. In the literature, to measure glucose concentration for determining the GOD activity, usually DNSA reagent is used (Blandino 2001, Ozyilmaz et al. 2005). DNSA reagent was developed by Sumner and co-worker (Sumner & Noback 1924) for the determination of reducing sugar. The determination of the reducing sugars is based on the reduction of 3,5-dinitrosalicylic acid in alkaline medium at 80 °C, forming gluconic acid and 3,5-diaminosalicylic acid which is measured with a spectrophotometer. The stoichiometric ratio between the DNSA and glucose is 1 to 6 and involves 12 interchanged electrons, where 2 nitro groups are reduced to amines due to the exchange of 6 electrons per each nitro group (Canizares-Macias et al. 2001). Reaction of DNSA with glucose was given in Fig.3

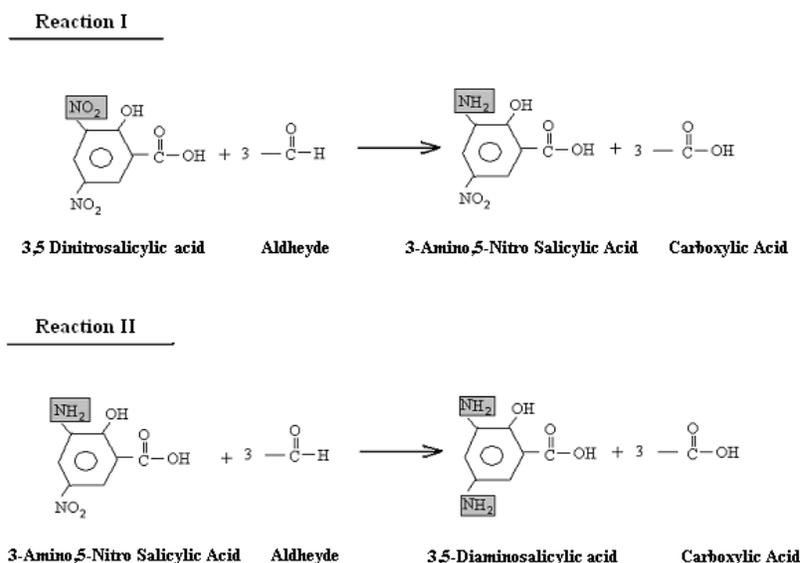


Figure 3. Reaction of DNSA with aldehyde group of glucose

According to Sumner and Noback, the reagents phenol, Rochelle salt (potassium sodium tartrate) and sodium bisulfite are needed to carry out the reaction. The roles of these reagents were analyzed in subsequent works. It was found that, phenol has no reducing effect on the DNSA but

increases the color produced by the glucose (apprx 300 %). Sodium bisulfite is added to prevent instability of the product after heating. The Rochelle salt reduces the oxygen concentration before heating, avoiding degradation of the sugar by the action of the oxygen in alkaline medium; therefore, the intensity of color increases due to higher DNSA reduction. Miller (Miller 1959) adjusted each of these compounds and mainly optimized the Rochelle salt concentration. Components and their compositions of DNSA reagent are 1% of dinitrosalicylic acid, 30 % Rochelle salt (potassium sodium tartrate), 0.5 % phenol, 0.5 % sodium bisulfite and 1 % sodium hydroxide.

Spectrophotometric measurements were carried out at various wave lengths such as 480 nm (Canizares-Macias et al. 2001), 575 nm (Miller 1959, Marsden et al. 1982, Breuil & Saddler 1985), 546 nm (Yu et al 1998), 540 nm (van Staden & Mulaudzi, 2000, Huang et al. 2019), 550 nm (Blandino et al.,2001).

There are several literature in which GOD activity was determined by measuring decrease in glucose concentration using standard curve prepared by glucose (Ozyilmaz et al. 2005, Huang et al. 2019, Blandino et al.,2001). In DNSA assay, initial and also final glucose concentration are determined by DNSA reagent. Sample is added to the DNSA reagent and left in a boiling water bath for 10 minutes. After cooling in ice-water bath, samples are diluted by deionized water. After 20 minutes for colour stability, absorbance values are read and concentrations are calculated with the help of the standard curve prepared with glucose. GOD activity was calculated as  $\mu\text{mol glucose} \cdot \text{min}^{-1}$  by using Equation 2 which is given below:

$$\text{GOD Activity} = \frac{(C_0 - C) \cdot V}{t} \quad (2)$$

Where

$C_0$ : Initial glucose concentration (mM)

$C$ : Last glucose concentration (mM)

$V$ : Reaction volume (mL)

$t$ : Reaction time (min)

### *Following the gluconic acid production*

Gluconic acid is produced from nonenzymatically hydrolysis of D-glucono-1,5-lactone produced in reaction mixture from glucose by GOD (Figure 4).

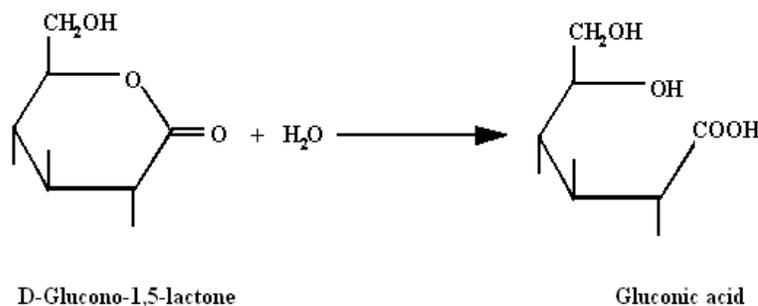


Figure 4. Non-enzymatic hydrolysis of D-glucono-1,5-lactone to gluconic acid

In this method, during or after stopping GOD activity, produced gluconic acid is neutralized by NaOH. As the volume of NaOH spent is proportional to the gluconic acid concentration, GOD activity is calculated as  $\mu\text{mol gluconic acid}\cdot\text{min}^{-1}$ . There are several studies in which GOD activity was determined by monitoring gluconic acid concentration (Casolaro & Barbuccu, 1991, Bhat et al. 2013, Iturbe et al. 1989, Ramezani et al. 2013, Yang et al. 2004, Cui et al.2019, Zhao et al. 2002).

### Measuring the $\text{H}_2\text{O}_2$ produced

Nowadays, measurement of the amount of  $\text{H}_2\text{O}_2$  produced in reaction medium is the most used method to determine the GOD activity. In general, peroxidase based methods were reported in the literature. A proper reagent is used to react with  $\text{H}_2\text{O}_2$  produced by GOD and spectrophotometric measurement is used to calculate glucose oxidase activity. The most used chemicals are *o*-dianisidine and 4-amino antipyrine for this purpose. Oxidation and colour change of *o*-dianisidine and 4-amino antipyrine by peroxidase activity in presence of  $\text{H}_2\text{O}_2$  are given in Figure 5 and 6, respectively.

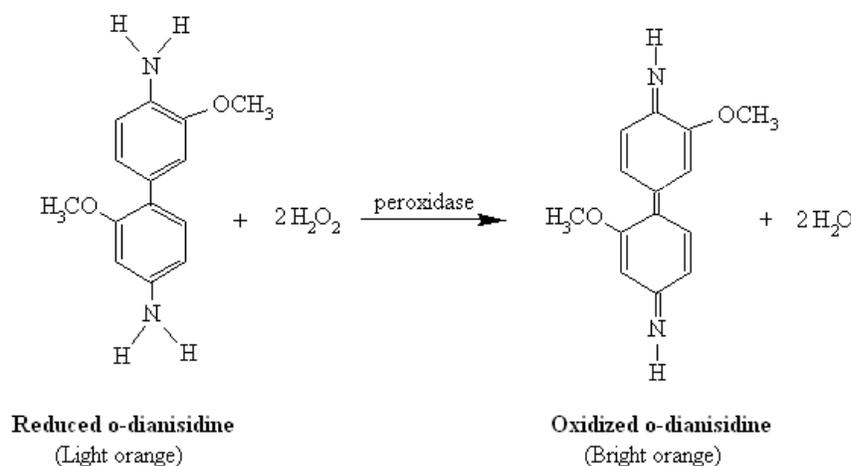


Figure 5. Reaction of *o*-dianisidine and hydrogen peroxide with peroxidase

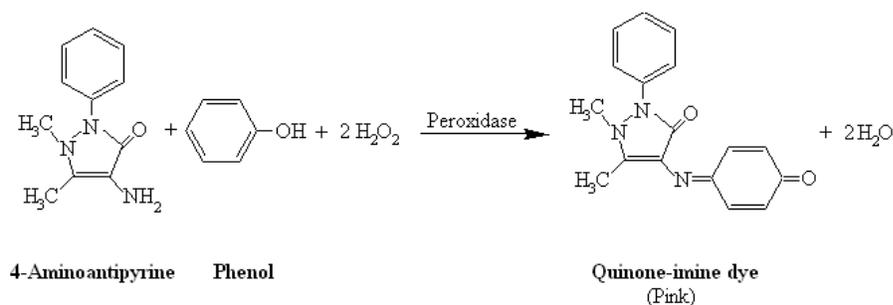


Figure 6. Reaction of 4-aminoantipyrine, phenol and hydrogen peroxide with peroxidase

Table 1 gives the literatures about determination of GOD activity by using peroxidase with different chromogenic compounds. In this assay, peroxidase and chromogenic compound are added into GOD medium. Produced  $H_2O_2$  is used in peroxidase activity after by GOD activity, so, the color of oxidized chromogenic compound increases during reaction time.

Table 1. Chromogenic compounds are used to determine GOD activity in peroxidase assay

Used substrates of peroxidase for the determination of the GOD activity	References
o-dianisidine	Ruiz et.al 2018, Atia and Batal, 2005; Godjevargova et al. 2004; Gouda et al., 2003; Ferreira et al., 2005; Godjevargova et al., 2005; Aken et al., 2000; Ying et al., 2002; Arica and Bayramoğlu, 2004; Kang and Bae, 2003; Elçin et al., 1993; Godjevargova et al., 2000; Bulmuş et al., 1997; Yavuz et al., 2002; Chen et al, 2002; Frederick et al. 1990; Sukhacheva et al., 2004
4-Aminoantipyrine	Miron et al., 2004; Brahim et al., 2002, Suye et al., 1998; Appleton et al., 1997; Mugo et al. 2019, Zhao et al. 2018
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	Mugo et al. 2019; Betancor et al., 2005; Portaccio et al., 2002; Betancor et al., 2004; Ukeda et al., 1998; Witt et al., 2000; Szweda et al. 2013, Mahdizadeh et al. 2014
Dimethylaminobenzoic acid	Brahim et al, 2002

### *Comparison of GOD activity methods*

GOD as green catalyst is widely used for various reasons such as removing glucose or molecular oxygen or production of hydrogen peroxide and gluconic acid salts. Therefore, the activity of the GOD enzyme, accuracy and sensitivity of the determined activity value are of great importance. There are several methods are used to determine the GOD activity. At the beginning, the first method for determination of the GOD activity was to measure the decrease of dissolved  $O_2$  concentration in the reaction medium. But, nowadays, there have been many literatures about GOD in which activities have been determined by using peroxidase and a chromogen system. Activity methods are also used which are based on decrease in glucose concentration or increase in the amount of the produced gluconic acid. GOD activity determination methods have advantages and disadvantages compared to each other in terms of usage area, consumption expenses, convenience and sensitivity. Table 2 summarizes the GOD activity methods with their advantages and disadvantages.

Table 2 is summarized the advantages and disadvantages of the methods used for determination of GOD activity, also suitability for GOD-CAT system.

Table 2. Comparison of activity assays of GOD for 10 min of reaction time.

	<b>Determination of depleted O<sub>2</sub></b>	<b>Glucose determination with DNSA</b>	<b>Titration of the produced gluconic acid</b>	<b>Peroxidase-chromogen system</b>
<b>Required equipment</b>	Oxygenmeter	Spectrophotometer	Burette	Spectrophotometer
<b>Time required</b>	10 min	40 min	15-20 min	10 min
<b>Cost of reagents</b>	Reagentless	Cheap	Very cheap	Expensive due to using of peroxidase enzyme
<b>Easiness</b>	Required Oxygenmeter stability	Laborious	Medium	Easy
<b>Repeatability</b>	Low	High	Moderate	High
<b>Suitable for use in GOD+CAT system</b>	Unsuitable	Suitable	Suitable	Unsuitable

As seen in Table 2, all methods have some advantages and also disadvantages in terms of required equipment and time, easiness of method and repeatability and also its suitability on GOD-CAT system. Titration method not only the cheapest method but also it can be easily applicable in all laboratories as no device required such as spectrophotometer or oxygenmeter, and also it is suitable for GOD-CAT system. However, in this method, GOD activity must be sufficient to produce enough gluconic acid to be determined by titration. Peroxidase-chromogen system is the most preferred method recently due to its simplicity and repeatability. However it is the most expensive method due to requiring another enzyme system, and also it is unsuitable for GOD-CAT system owing to depletion of H<sub>2</sub>O<sub>2</sub> by CAT activity. Nevertheless, even very low GOD activity can be measured with high sensitivity by this method. The method depends on oxygen concentration measurement is preferable because it does not require any reagents and no additional processing. In this method, ensuring the stability of the oxygen meter during the measurement of oxygen concentration is very important for the reliability of the results. However, long-term stabilization of the oxygen meter and the fact that oxygen in the air diffuses into the reaction medium during the reaction time significantly affect the sensitivity of this method. Also, The DNSA method is one of the most commonly used methods to determine GOD activity because of its high reproducibility and relatively low cost. It also suitable for GOD-CAT system. The main drawback of this method is that it is time consuming and laborious.

## Conclusion

GOD is widely used for different purposes such as glucose or oxygen removal from the medium or production of controlled peroxide or gluconic acid. It is also widely used in the design of glucose biosensors. For this reason, the determination of GOD activity in many studies varies according to the purpose of the study. In this study, four different GOD activity methods are compared and their usage areas have been reviewed. It is concluded that peroxidase system is widely used in cases where GOD is used alone, whereas DNSA method is frequently used in systems including CAT.

As a result, it can be said that the choice of GOD activity method will be determined according to the presence of CAT enzyme in the environment, the catalytic efficiency of the GOD, and the sensitivity requirement of the results.

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