**Journal of Enzyme Engineering (2022)**
**Clausius Scientific Press, Canada**

**DOI: 10.23977/enzyme.2022.010103**
**ISSN 2616-2962 Vol. 1 Num. 1**

# *Optimization and Production of Glucose Oxidase from a newly isolated strain of Aspergillus fumigatus*

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***Keywords:*** Isolation, *Aspergillus fumigatus* AFS4, 18S-rDNA sequence, Optimization, Glucose oxidase

***Abstract:*** Glucose oxidase (GOx) has several novel applications in chemical, pharmaceutical, textile, and other biotechnological industries. This study was aimed at optimizing the production of glucose oxidase from *Aspergillus fumigatus AFS4*. *Aspergillus fumigatus* was isolated from garden soil, obtained from staff quarters University of Nigeria, Nsukka and was screened for glucose oxidase production capability. Biochemical tests and 18S-rDNA sequencing were used to confirm the isolate as *Aspergillus fumigatus*. The isolate strain was tagged as *Aspergillus fumigatus AFS4*. GOx was produced from *Aspergillus fumigatus AFS4* under submerged fermentation system with an enzyme activity of 1591Uμmol/min and protein concentration of 3.89mg/ml. Different conditions for GOx production which include carbon sources, nitrogen sources, $CaCO_3$, pH and fermentation time were optimized. Glucose (80g/L) was found to be the best carbon source for GOx production with GOx activity of 1542μmol/min. Peptone (3g/L) was found suitable for GOx production with GOx activity of 1231μmol/min compared to other nitrogen sources tested. $CaCO_3$ enhanced GOx production when 30g/L of it was used to supplement the production medium. The optimum pH for GOx production was 6.5. The highest GOx production was obtained on the $7^{th}$ day with GOx activity of 1517μmol/min. The results proved that *Aspergillus fumigatus AFS4* has strong potential to produce glucose oxidase for various industrial applications.

## 1. Introduction

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase) catalyzes the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide ($H_2O_2$) using molecular oxygen as an electron acceptor. Glucose oxidase was first isolated from mycelia of *Aspergillus niger* and *Penicillium glaucum* by Muller (1928) and were detected in different sources (insects, honey, algae and micro fungi) and recently obtained from *Penicillum amagasakiense* (Todde *et al.*, 2014). Structurally, GOx is a dimeric glycoprotein consisting of two identical polypeptide subunits that are covalently linked together by disulfide bonds. Glucose oxidase (GOx) activity is dependent on Flavin-Adenine Dinucleotide (FAD), a cofactor which is transiently reduced along the reaction mechanism. This reaction can be divided into reductive and oxidative steps. Studies now focus on factors that regulate the production of GOx (Bankar *et al.*, 2008). Media constituents such as carbon, nitrogen,

$CaCO_3$ and environmental factors such as temperature, pH and agitation are required in the production of GOx. These factors have to be optimized for the effective production of GOx. There are few reports on optimization of these conditions necessary for GOx production from various fungi (Bankar *et al.,* 2008). Glucose oxidase is an important commercial enzyme due to its applications in diverse industries. It is widely used in chemical, energy, food industry, textile, pharmaceutical and medical fields (Sathiya, 2009). Among these applications, the most remarkable and novel application of GOx has been found to be in the biosensors and biofuels industries in recent years (Xu *et al.,* 2017). The objectives of this research were to isolate GOx producing fungi, molecular identification of the isolated strain and optimize conditions required for GOx productions using the isolated fungus.

## 2. Materials and Methods

### 2.1 Chemicals and Reagents

*O*-dianisidine dihydrochloride was purchased from Sigma, USA. Folin-Ciocalteau phenol used for protein determination was purchased from Sigma-Aldrich Chemicals Limited, USA. Horse radish peroxidase, D(+)-glucose, glycerol, peptone, ethanol was purchased from Sigma-Aldrich Chemicals Limited, USA. All equipment and apparatus for this study were assembled from the laboratory unit of Department of Biochemistry, University of Nigeria, Nsukka.

### 2.2 Isolation and screening of microorganism

Pure fungal cultures were isolated from garden soil collected from the University of Nigeria, Nsukka, Enugu State, according to the method described by Clark et al. (1958). The microorganism was identified as *Aspergillus sp*. according to Martin et al., (2004) using light microscope. The organism was identified as a glucose oxidase producer by screening using the method of Eun-Ha Park et al (2000). The isolated fungi were grown on a medium containing glucose (80g), peptone (3.0g), $(NH_4)_2$ $HPO_4$ (0.388g), $KH_2PO_4$ (0.188g), $MgSO_4$ $7H_2O$ (0.156g), Agar (20g), in one litre of sodium acetate buffer of pH 5.5. the broth was sterilized at 121$^oC$ for 15min; the broth was poured into petri dishes and were allowed to gel. A disc (2mm) of fungal culture was taken from the peripheral zone of pure culture colony and transferred into Petri plates containing the above medium. The plates were incubated at 35$^{\circ}C$ for 3 days and then treated with a solution containing glucose 5% (w/v), glycerol 2% (v/v), O-dianisidine 0.1% (w/v), Horse radish peroxidase 60IU/ml, agar 1% (w/v) in sodium acetate buffer (pH 5.5). The solution was used to overlay the 4 day old fungal culture and incubated for 1hr. A reddish brown colouration indicates the presence of GOx. The fungi with the highest GOx production were identified, selected and maintained on PDA slant at 4$^{\circ}C$.

### 2.3 Molecular identification and characterization

The fungi that showed GOx producing capability was identified using molecular characterization. Genomic DNA (gDNA) of the isolated fungus was extracted using AccuPrep® DNA extraction kits following the manufacturer's instructions. Primer pairs ITS 1(5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-GCTGCGTTCTTCTTCATGATGC-3') were used for PCR amplification of the fungal internal transcribed spacer (ITS) regions. The PCR conditions were set as follows: pre denaturation at 94oC for 3min, denaturation at 94oC for 40 sec, annealing at 54oC for 40 sec, extension at 72oC for 40 sec and final extension at 72oC for 10 sec. Agarose gel electrophoresis was carried out using 1.5% agarose gel (1.5g of agarose in 100ml of

Tris-acetate-EDTA (TAE) buffer). Agarose gel powder was dissolved by microwaving in 1 × TAE buffer. The mixture was cool to 55oC and 12µl of ethidium bromide was added and allowed to cool for 30min at 37oC. DNA ladder (6µl) and the amplicon (10µl) were loaded into the wells of agarose gel followed by electrophoresis at 100V for 1hr. the DNA bands on the gel was visualized using UV light box/gel imaging system. The DNA sequences obtained were subjected to BLAST (Basic Local Alignment Search Tools for Nucleotides) search algorithm and aligned using Multiple Sequence Alignment based on Fast Fourier transform (MAFFT) version 5. Phylogentic analysis of the ITS sequence data was conducted using molecular evolutionary genetic analysis (MEGA) version 7.

### 2.4 Optimization of medium components of GOx production

For the optimization of the submerged production of GOx as described by Simpson *et al.* (2006), the medium contains glucose 80% (w/v), peptone 0.3% w/v, $(NH_4)_2HPO_4$ 0.04% w/v, $KH_2PO_4$ 0.0188% w/v, $MgSO_4.7H_2O$ 0.0156% w/v, $CaCO_3$ 3.5% (w/v). The pH of the medium was adjusted to 6.0 with 1M NaOH prior to autoclaving at 121$^oC$ for 15 min. All the experiments were carried out in 250 ml Erlenmeyer flask containing 100 ml of the medium. The sterilized medium was inoculated with four disc each of pure culture of the microorganism and incubated in orbital shaker (150rpm.) at 30$^{\circ}C$. The fermented biomass in each case was harvested, filtered and centrifuged at 15000 rpm for 15 min. The supernatant constituted the crude enzyme.

#### 2.4.1 Effect of fermentation time on GOx production

Fourteen-day pilot study was also optimized to determine the day suitable for GOx production. The experiments were carried out in such a way that the parameter optimized in one experiment was maintained in the subsequent investigation (Silva et al. 2010).

#### 2.4.2 Effect of pH on GOx production

In this study, the pH of the medium was adjusted to various levels (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) to optimize GOx production.

#### 2.4.3 Effect of carbon sources on GOx production

Different carbon sources, glucose, fructose, maltose, sucrose and xylose at different concentration (i.e. 10 -120g/L) were used to optimize GOx production and *Aspergillus fumigatus* AFS4 growth.

#### 2.4.4 Effect of nitrogen sources on GOx production

The effect of nitrogen sources; protease peptone, yeast extract and bacteriological peptone were determined at different concentration, 1, 2, 3, 4, 5 and 6g/L.

#### 2.4.5 Effect of calcium carbonate on GOx production

The effect of $CaCO_3$ on GOx production was carried using different concentrations (10 – 60g/L) of $CaCO_3$.

## 2.5 Glucose oxidase assay

GOx activity was assayed according to the method described by Bergmeyer *et al*. (1974) using glucose as substrate, and *O*-dianisidine as coupling reagent. Reagents A, B, C, D and E were prepared for the assay. Reagent A is 0.05M sodium acetate buffer of pH 5.5; reagent B is *O*-dianisidine solution (0.21mM) dissolved in 100ml of reagent A; reagent C is β- D-glucose solution (10%w/v); reagent D is a cocktail of 24ml of reagent B and 5ml of reagent C; reagent E is freshly prepared solution of 60 purpurogallin units/ml of horseradish peroxidase type II. The reaction mixture contained 2.9ml of reagent D, 0.1ml of reagent E and 0.1ml of enzyme solution. Absorbance was determined every 15 sec for 5mins using UV-Visible spectrophotometer at 500nm.One unit of GOX activity is defined as the amount of enzyme that catalyses the conversion of 1µmole of β-D-glucose to D-gluconolactone and $H_2O_2$ per minute at 35°C and pH 5.5.

## 2.6 Determination of Protein

The protein content of crude GOx was determined as described by Lowry *et al*. (1951) using Bovine serum albumin (BSA) as standard.

## 2.7 GOx production

Submerged fermentation was carried out using the media composed of glucose 80% (w/v), peptone 0.3% w/v, $(NH_4)_2HPO_4$ 0.04% w/v, $KH_2PO_4$ 0.0188% w/v, $MgSO_4.7H_2O$ 0.0156% w/v, $CaCO_3$ 3.5% (w/v) in 50mM sodium acetate buffer pH 6.0 (Fiedurek and Gromada, 2000). 100ml of sterilized fermentation broth contained in 250ml conical flasks were inoculated with four disc each of pure culture of *Aspergillus fumigatus ASF4*. The flasks were incubated at 30°C on orbital shaker at 150rpm for seven days. The fermented biomass in each case was harvested, filtered and centrifuged at 15000 rpm for 15 min. The supernatant constituted the crude enzyme.

# 3. Results and Discussion

Chromosomal DNA of S4 isolate was extracted and the 18S rDNA was amplified by PCR. The agarose gel electrophoresis of the amplicons showed a clear band at approximately 500bp when compared to the DNA ladder (Figure 1). Multiple alignment of this sequence was done by comparing 15 other known sequences of *Aspergillus* species obtained from NCBI data bank. The results were expressed as phylogenetic tree which confirmed the isolate S4 as *Aspergillus fumigatus.* The fungus isolate of interest (**Query 136965**) showed 99% similarity with *Aspergillus fumigatus* strains *MG991595.1, MK719925.1* and *MH378448.1* (Figure 2).

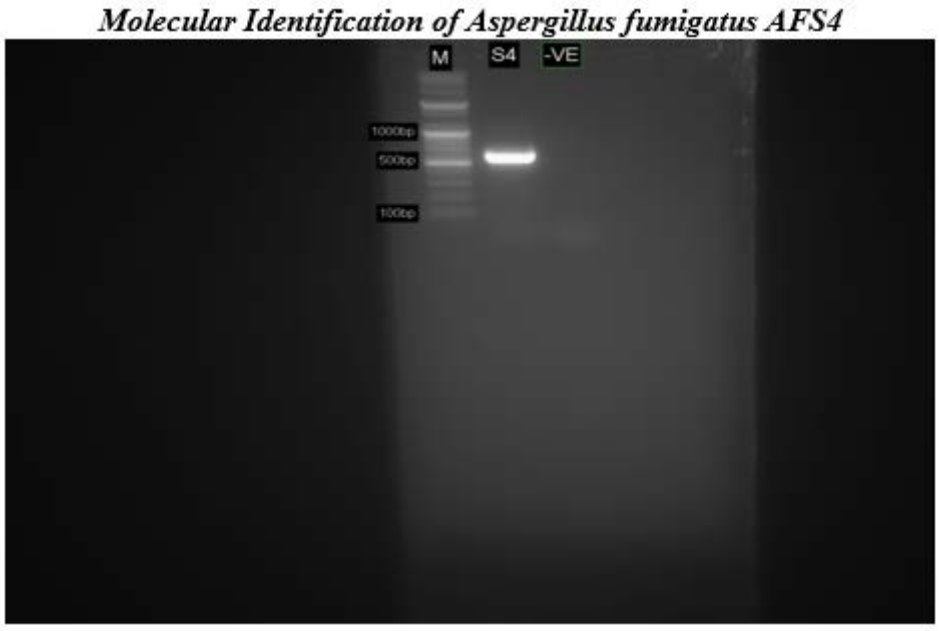


*Figure 1: Agarose gel electrophoresis of the amplicons.*

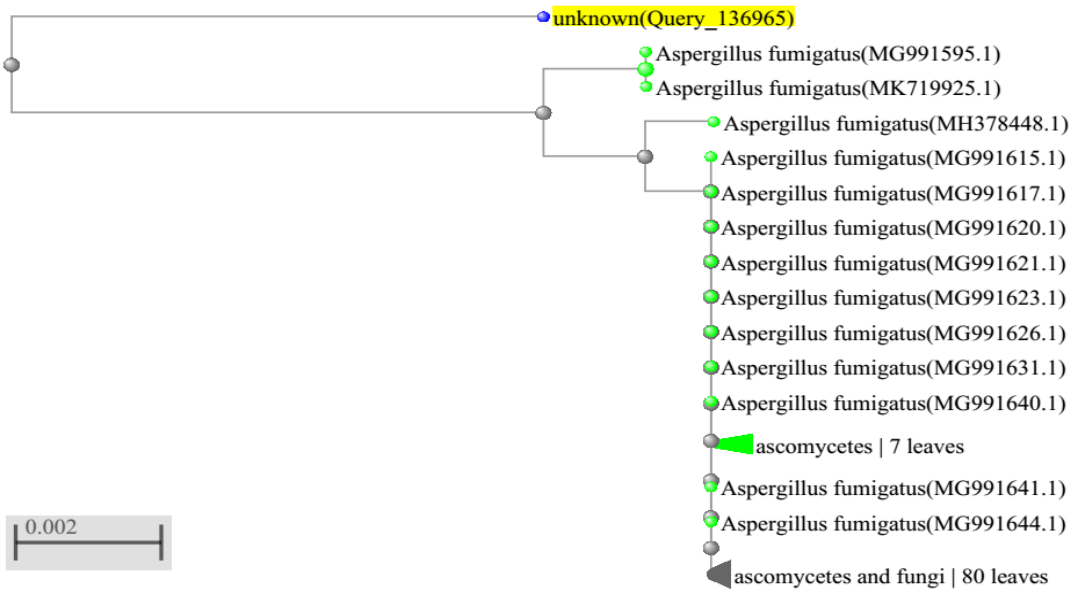


*Figure 2: Molecular phylogenetic tree of Aspergillus fumigatus AFS4 with other species of Aspergillus*

After a fourteen day pilot study, the highest GOx production (1517µmol/min) was obtained on the 7th day of fermentation after which, decline in enzyme production was observed, although Bankar *et al.* (2008) reported that the optimum fermentation time for GOx production was 4days. The variation might be as a result of fungal strains, size of inoculums used, type of inducer, or fermentation system, and environmental factors such as pH, temperature and available oxygen. Mukhtar *et al*. (2014) observed maximum production on day three (3). The decline in activity after the optimum fermentation could be as a result of growth or enzyme inhibition due to accumulation of $H_2O_2$ produced during GOx reaction with D-glucose (Bao *et al.*, 2003). The increase in GOx activity observed after the drop may probably be due to fungal adaptation to $H_2O_2$ or the fungus

might have been induced to produce catalase which decomposed the $H_2O_2$. This is essential for the viability of the cells and the stability of the GOx (Miron *et al.*, 2002). These indicate that catalase production was induced by decrease in GOx activity and high concentration of $H_2O_2$ and support the result obtained in this study.

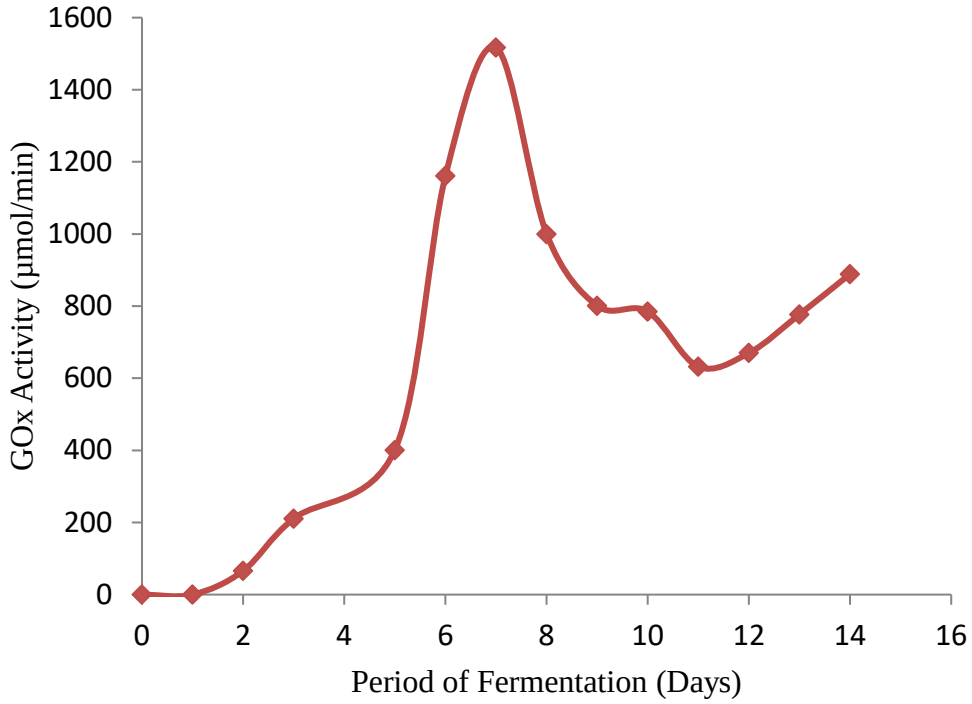


*Figure 3: Effect of fermentation time on GOx production*

Among the physiological parameters studied, the pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion. Maximum enzyme production was observed between the pH ranges of 5.0 – 6.5 with optimum at 6.5 (Figure 4). Optimum pH of 6.5 influenced the physiology of the microorganism by enhancing nutrient solubility and uptake, enzyme activity, cell membrane morphology, by-product formation and oxidative-reductive reactions (Bankar *et al.*, 2008). The pH change observed during the growth of organism affected product stability in the medium. Most of the strains used commercially for the productions of GOx have an optimum pH between 6.0 and 7.0 for growth and enzyme production (Singh and Verma, 2013), which correlates positively with our finding.

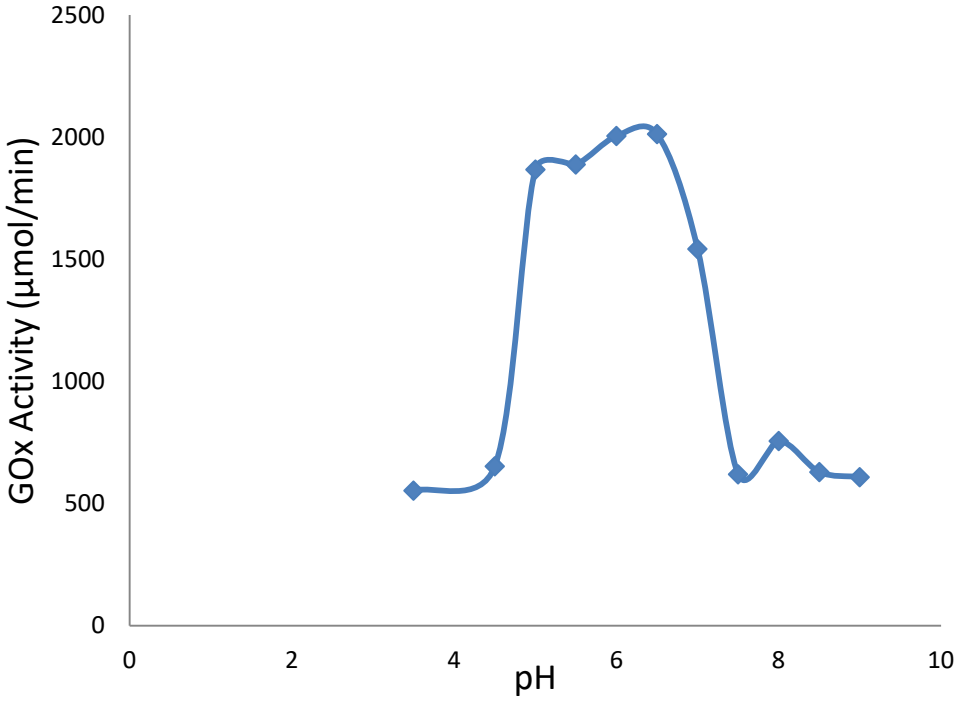


*Figure 4: Effect of pH on GOx production*

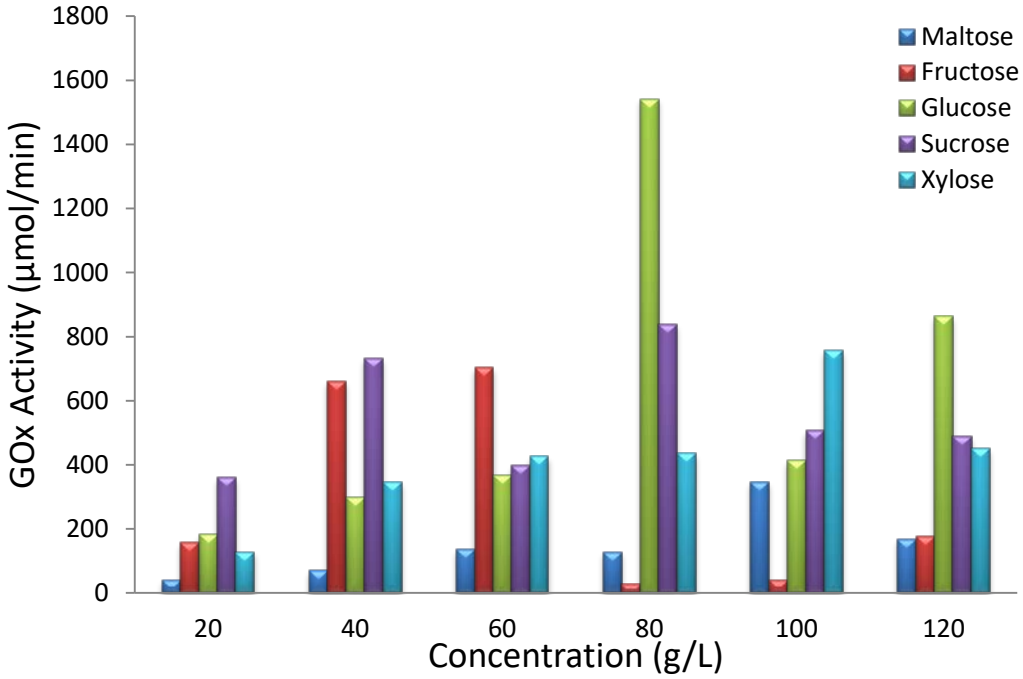


*Figure 5: Effect of carbon sources on GOx production*

Different concentrations of carbon sources (20 - 120g/L) were used to supplement the basal medium. Although *Aspergillus fumigatus AFS4* grew on all carbon sources that were tested, significant levels of GOx activities were obtained when glucose and sucrose were used as carbon sources. Bankar *et al.*, (2009) reported that significant levels of GOx were obtained using glucose, sucrose and molasses. In this study, GOx production was enhanced as concentration of glucose was

increased from 20 to 80g/L, after which there was no significant increase in GOx production. The optimum concentration of glucose for GOx production was found to be 80g/L with an enzyme activity of 1542µmol/min. The highest GOx activity was obtained in glucose supplemented medium followed by sucrose when compared to other carbon sources, although Singh and Verma, (2013) reported sucrose to have cause significant increase in the levels of GOx activity. Glucose serves as the principal inducer for the transcription of the GOx gene (Sathiya, 2009). GOx has a high specificity for glucose when compared to other carbohydrates. During microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but it is also used in the synthesis of polysaccharide and as energy source (Bankar *et al.* 2008). The rate at which carbon source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites. This study correlates with Bankar *et al.* (2008) who reported that glucose and sucrose were more effective in the production of GOx in *Aspergillus Niger*.

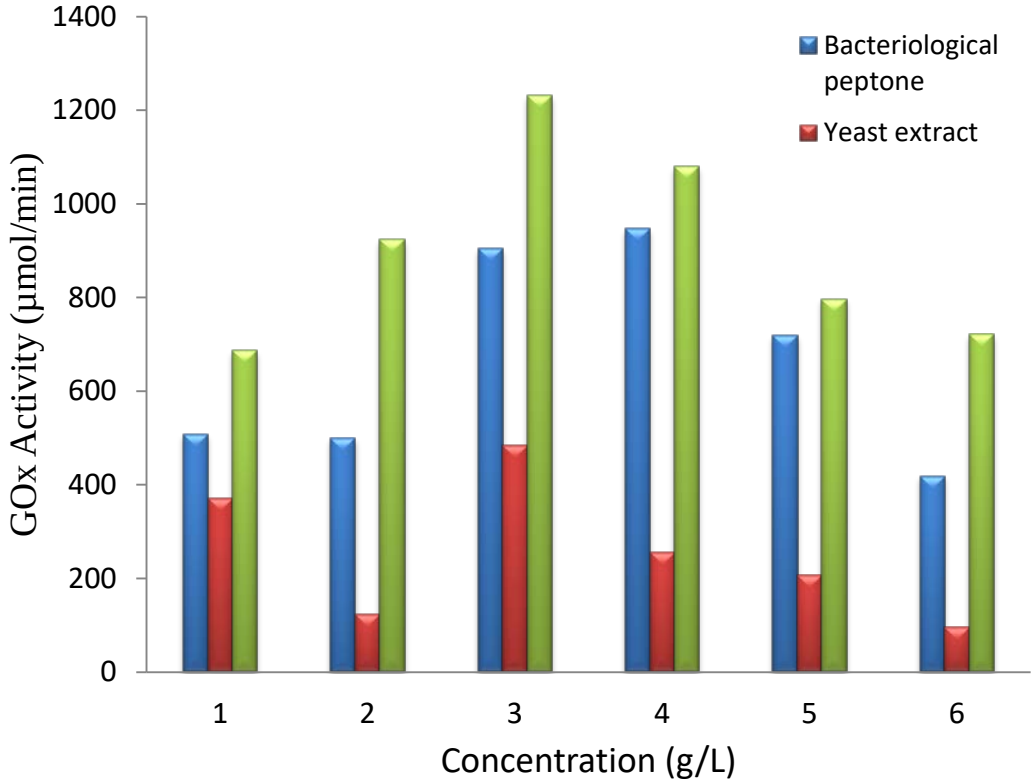


*Figure 6: Effect of different nitrogen sources on GOx production*

Different concentrations (1- 6g/L) of protease peptone, yeast extract and bacteriological peptone were used to optimize for maximum GOx production. Protease peptone was found to be an effective nitrogen source for GOx production. There was increase in GOx production with increased concentration of protease peptone from 1-3g/L, having the maximum activity (1231 µmol/min) at 3g/L concentration. This concentration was in line with the report of Fiedurek and Gromada (2000). However, Singh and Verma (2013) reported 1.5g/L protease as the optimum for GOx productionfrom *Aspergillus niger*.Increasing concentration of nitrogen source produced higher biomass, but reduces the GOx production. This study correlates with similar result obtained by Bankar *et al.* (2008).

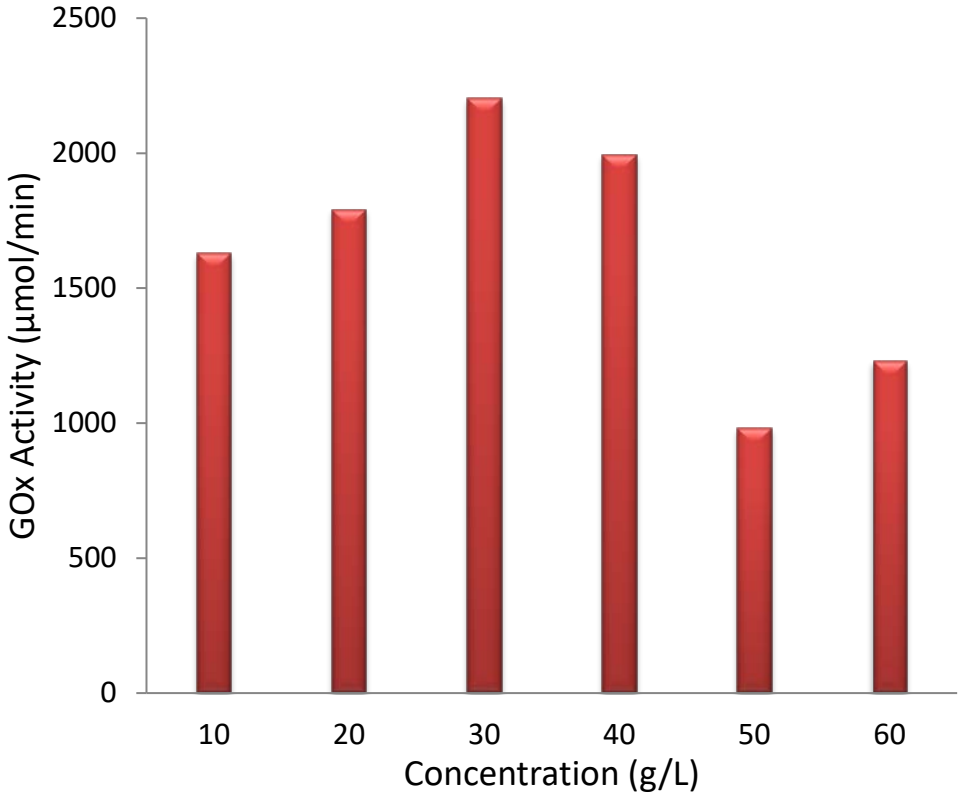


*Figure 7: Effect calcium carbonate on GOx production*

Increase in the concentration of $CaCO_3$ from 10 – 30g/L was accompanied by increase in GOx production, beyond which the enzyme production decreased making, 30g/L the optimum concentration of $CaCO_3$ for GOx production from *Aspergillus fumigatus AFS4*. The addition of calcium carbonate in the growth media might cause a metabolic shift from glycolysis to the pentose phosphate pathway, thereby increasing GOx levels. Bankar *et al.*, (2008) reported that calcium carbonate might be a strong inducer of GOx in *Aspergillus Niger* and demonstrated it to be essential for increased levels of GOx production. Bankar *et al.* (2008) found 30g/L of $CaCO_3$ to be optimum for highest GOx production. However, Singh and Verma, (2013) reported that $CaCO_3$ enhanced GOx production at a concentration of 20g/L. Addition of calcium carbonate in the growth medium causes changes in GOx, catalase (CAT), 6-phosphofructokinase (6-PFK) and glucose-6-phosphate dehydrogenase (G-6-PDH) activities. Lui *et al.* (2001) reports that the addition of $CaCO_3$ into the growth medium made an alteration in the production of GOx by 6- phosphofructokinase and glucose-6-phosphate dehydrogenase. The 6-PFK is a key regulatory enzyme of Embden–Meyerhof–Parnas (EMP) pathway in most living cells (Liu *et al.*, 2001). Liu *et al.* (2001) further showed that cells grown in media without calcium carbonate produced high levels of 6-PFK and low amounts of G-6-PDH, GOx and CAT. Addition of calcium carbonate to the growth medium increased the production of GOx and CAT, and decreased the synthesis of 6-PFK. Therefore, this observation which correlates with our study indicates that the inclusion of calcium carbonate is accompanied by a metabolic shift from the glycolytic pathway (EMP) to direct oxidation of glucose by GOx.

Based on the optimized conditions (glucose 80g/L, peptone 3g/L, $CaCO_3$ 30g/L and pH 6.5 with a fermentation time of 7days), GOx was produced from *Aspergillus fumigatus AFS4* under submerged fermentation system with an activity of 1591Uµmol/min and protein concentration of 3.89mg/ml.

## 4. Conclusion

These studies provide optimal conditions for large scale production of GOx needed for industrial and biotechnological applications. These add to the wealth of information available on GOx producing fungi. Also, the results suggest that *Aspergillus fumigatus AFS4* can be a potential source for glucose oxidase.

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