

## RESEARCH ARTICLE

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## Genetic and phenotypic diversity of naturally isolated wild strains of *Aspergillus niger* with hyper glucose oxidase production

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**ABSTRACT**

Glucose oxidase (GOx) is the basic stone for many of biological industry worldwide. The improvement of GOx production basically depends on selection of hyper producer strain of *Aspergillus niger*. Selective isolation and screening for natural hyper producer strains of *A. niger* and sequence analysis of the GOD gene, which is responsible for production of the enzyme, are very effective approaches to investigate the naturally modified strains of *A. niger* with hyper productive capacity of GOx enzyme. The aims of the current study were selective isolation of naturally hyper GOx producing strains of *A. niger* and evaluation of their GOx activities under optimized parameters in the laboratory. Five wild Egyptian isolates of *A. niger* were screened for GOx and catalase activity using two types of modified basal liquid media. The GOx activity was evaluated by high throughput liquid phase system. The isolates showed a variable activity for GOx production ranged from 0 to 28.7 U.ml<sup>-1</sup>. One isolate coded Strain 7 was negative GOx producer on Vogel's broth medium in comparison to other isolates, while its GOx activity on Cazpek Dox was considered as positive (7.28 U.ml<sup>-1</sup>). It was concluded that GOx production is affected by three controllable factors –the basal media components, time of incubation, and the strain with its adaption to the media components. Also, the catalase activity was tested and it was produced with a different degree of variability, which may be reflected on GOx stability. GOD genes of these wild variant of *A. niger* were cloned and sequenced to determine intraspecies diversity of GOD between the wild variants. The comparison of isolated wild variants to other reference hyper GOx producer strains of *A. niger* was performed to determine if the GOD sequence analysis of these strains can be distinguished based on their GOx activity. This is the first report for isolation and detection of naturally *A. niger* hyper GOx-producer strains with variable activity, which opening a way to improve GOx-production biotechnology.

**Key words:** *Aspergillus niger*, glucose oxidase, hyper production, GOD gene

**Introduction**

Genus *Aspergillus* was first recognized in 1729 by Micheli. It was found worldwide and consists of more than 180 recognized species. It comprises a particularly important group of filamentous ascomycete species (Bennett, 2010) and most of the members are used naturally in the degradation of

plant polysaccharides (de Vries *et al.*, 2000; 2003). Such industrial microorganisms are important for the large-scale production of both homologous and heterologous enzymes (Fawole & Odunfa, 2003; Wang *et al.*, 2003). Among these organisms, *A. oryzae* and *A. niger*, which are Generally Recognized as Safe (GRAS) by the FDA in the USA (Tailor & Richardson, 1979).

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Glucose oxidase (GOx) has been produced from a wide range of different fungal sources, mainly from the genus *Aspergillus* (Hatzinikolaou *et al.*, 1996) and *Penicillium* (Sukhacheva *et al.*, 2004). *A. niger* is considered the potential and the most commonly optimized producer of GOx (Pluschkel *et al.*, 1996). GOx is an enzyme, which catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (Hatzinikolaou & Macris, 1995). GOx has a broad range of applications including removal of oxygen and glucose for food and beverage preservation (Kleppe, 1966), biofuel (Soukharev *et al.*, 2004), production of gluconic acid (Klein *et al.*, 2002) and as glucose biosensor (Kim *et al.* 2001).

The catalase enzyme is necessary for detoxification of the hydrogen peroxide generated during the production of glucose oxidase. This is essential for the viability of the cells and the stability of the GOx (Miron *et al.*, 2002). Simultaneous production and synthesis of GOx and catalase by *A. niger* using a resting cell culture system was assayed by Liu *et al.* (1999).

Improvement of GOx production basically depends on selection of hyper producer mutant strain of *A. niger*. Several reports described the selection of *A. niger* mutants overproducing GOx (Kundu & Das, 1985; Fiedurek *et al.*, 1986; Markwell *et al.*, 1989). The main purpose of this report is selective isolation of wild naturally hyper GOx producer strains of *A. niger* and evaluation of its GOx activities under the optimized laboratory parameters. In order to maximize GOx production, it is necessary to optimize the culture condition prior production process beside the improvement of GOx producer strain. The most crucial factor in the optimization process is medium composition since it affects the productivity (Schmidt, 2005).

## Materials and Methods

### *A. niger* macroscopic and microscopic identification

Samples were cultured on Malt Extract Agar (Merck, Darmstadt, Germany) and Czapek Yeast Extract Agar prepared in the laboratory according to Samson *et al.* (2000). The isolation and identification of *A. niger* was based on its morphological and microscopical characteristics according to the guidelines of Pitt & Hocking (1997) and Samson *et al.*, (2000).

### Screening for GOx enzyme activity of wild *A. niger* isolates by plate enzyme diffusion zone test

Screening of the GOx activity was performed according to El-Enshasy (1998) and Khattab & Bazaraa (2005). All isolates of *A. niger* were picked up and subcultured on agar plate containing 0.1 g/L *o*-dianisidine and 310 U/mg of purified horseradish peroxidase. The positive GOx strain was identified when a clear colored diffusion zone appeared after 4-5 days of incubation at 30°C. Depending upon the result of enzyme zone diffusion test, the top strains that showed wider clear zones were selected as the naturally mutant GOx-overproducing *A. niger* strains.

### High throughput screening and evaluating system for all the hyper GOx-producing *A. niger*

A stock culture of spore suspension ( $1 \times 10^7$  spores/ml) of positive GOx producer strains (five strains) on plate enzyme diffusion zone were prepared by obtaining a densely conidiating mature culture grown on Czapek Yeast Extract Agar medium for 48-72 h. The spores were harvested with a sterile physiological saline solution NaCl (0.9%, w/v), and counted using a counting slide.

### Modifications and optimization of the liquid growth media

The harvested spores from each culture have been cultivated on two different types of modified basal liquid media (Vogel's and Czapek Dox) at 30°C without agitation and limited aeration. The composition of both media was detailed in Table 1.

### Liquid phase screening with the ABTS assay in 96 well formats

The assay was applied according to Sun *et al.* (2002). Briefly, after injection of the selected ten *A. niger* strains into the previously mentioned two modified liquid media, the culture suspensions were taken on six days intervals starting from the third day post inoculation. These culture suspensions were centrifuged and only 50  $\mu$ l of the supernatant (containing GOx) were transferred into a 96 well flat-bottom microtiter plate and 250  $\mu$ l assay mix (333 mM  $\beta$ -D-glucose, 3.3 mM ABTS and 0.25 U HRP in 100 mM acetate buffer pH 5.5) was added into each well. The activity was measured by monitoring the absorbance at 405 nm at ambient temperature using a microtiter plate reader.

### Catalase activity assay

Catalase activity was quantified spectrophotometrically by following the decrease in absorbance at 240 nm during the decomposition of H<sub>2</sub>O<sub>2</sub> by the enzyme (Petruccioli *et al.*, 1993). The reaction mixture (3 ml) contained 10  $\mu$ l enzyme

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solution and 3 ml 30 mM H<sub>2</sub>O<sub>2</sub> in 50 mM sodium phosphate buffer (pH 7). One unit of catalase activity was defined as the amount of the culture liquid containing mycelia that reduced 1 mM substrate (H<sub>2</sub>O<sub>2</sub>) per one minute under the above-described conditions.

**Table 1.** Modified ingredients in the two types of selected basal media with different change in the components

	Modified Vogel's Broth Medium	Modified Czapek Dox Broth Medium
<b>Ingredients:</b>	Quantity (g/100 ml)	Quantity (g/100 ml)
<b>KH<sub>2</sub>PO<sub>4</sub></b>	0.5	0.1
<b>NH<sub>4</sub>NO<sub>3</sub></b>	----	0.3
<b>(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub></b>	----	----
<b>Peptone</b>	0.1	----
<b>Trisodium Citrate</b>	0.5	----
<b>Yeast extract</b>	0.2	----
<b>Sucrose</b>	50	3
<b>MgSO<sub>4</sub>·7H<sub>2</sub>O</b>	----	0.05
<b>KCl</b>	----	0.05
<b>FeSO<sub>4</sub>·7H<sub>2</sub>O</b>	----	0.001
<b>Urea</b>	0.2% (W/V)	----
<b>CaCO<sub>3</sub></b>	----	3.5% (W/V)

#### Total protein content in liquid broth:

Soluble protein content was measured by using the modified Lowry method (Peterson, 1977).

#### Molecular identification and detection of *A. niger* GOD gene

##### DNA extraction

DNA was extracted from *A. niger* strains according to Möller *et al.* (1992). Briefly, *A. niger* mycelia were cultured for 24 h at 28°C in PDA medium (20% potato extract and 2% sucrose). Only 30-60 mg lyophilized mycelium were ground with fine sand in a mortar. After then, the powdered mycelium was transferred into a microtube with 500 µl TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS) and 50-100 µg proteinase K were added and incubated for 30 min at 55-60°C with occasional gentle mixing. Salt concentration was adjusted to 1.4 M with 5 M NaCl. 10% CTAB was added and incubated for 10 min at 65°C. Gentle mixing was occurred and the mixture was incubated for 30 min at 0°C, and then spun for 10 min at 4°C, rpm<sub>max</sub>. The supernatant was transferred to a 1.5 ml tube, and 510 µl of isopropanol was added to precipitate DNA. Immediate centrifugation was done for 5 min, rpm<sub>max</sub>. After then, the pellet was washed twice with cold 70% ethanol and dissolved in 50µl TE.

#### GOD gene amplification from *A. niger*

The open reading frame (ORF) encoding the GOD gene was amplified by the polymerase chain reaction (PCR) technique using the following primers: GOD-F (5'-ATGCAGACTCTCCTTGTGAG-3') and GOD-R (5'-TCACTGCATGGAAGCATAAT-3'). The PCR procedure was performed through 30 cycles of: 50 sec denaturation at 94°C, 50 sec annealing at 50°C, 2 min extension at 72°C, followed by a final extension for 10 min at 72°C. The PCR product was purified from a 1.0% agarose gel using a DNA Gel Extraction kit (Jena Bioscience, Germany) according to the manufacturer's protocols, and then subcloned into pMD19-T vector. The resulting plasmid was used for sequence analysis.

#### GOD gene sequence analysis

Sequence analysis of strain 1, 3, 4, 5 and 7 GOD genes was done, representing high and negative GOx-producing isolates. Identification of homologies between nucleotide sequences of the selected *A. niger* strains (1, 3, 4, 5 and 7) and other strains, published in GenBank, was done using BLAST search programs (National Center for Biotechnology Information "NCBI" [http:// www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). The scores designated in the BLAST search have a well-defined statistical interpretation allow matches being easier to distinguish the tested sequences from the random background hits (Altschul *et al.*, 1997).

#### Computational and phylogenetic analysis

To allow appropriate phylogenetic analyses, sequences of *A. niger* GOD gene, that were included in the present study, were aligned to other references GOx hyper producer strains: GOD gene of *A. niger* J05242 (Frederick *et al.* 1990), GOD genes of *Penicillium amagasakiense* (Witt *et al.*, 1998), *Penicillium variabile* (Petruccioli *et al.*, 1999). The phylogenetic analysis, sequence divergence and percent of identity were calculated by MegAlign.

## Results

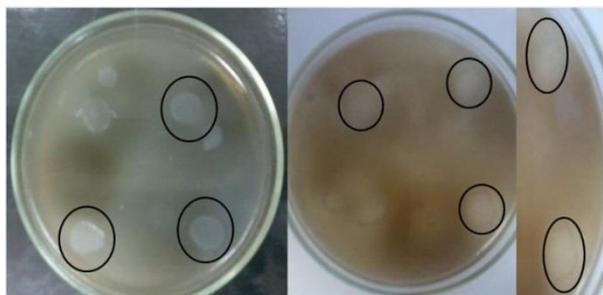
#### Macroscopic and microscopic identification of *A. niger*

According to the conventional mycological morphological and microscopic characteristics, five isolates were fully identified as *Aspergillus* section *Nigri*.

#### Plate enzyme diffusion zone test

The identified isolates of *A. niger* with GOx production activity exhibited growth showing a degradation clear zone (Figure 1 and Figure 2).

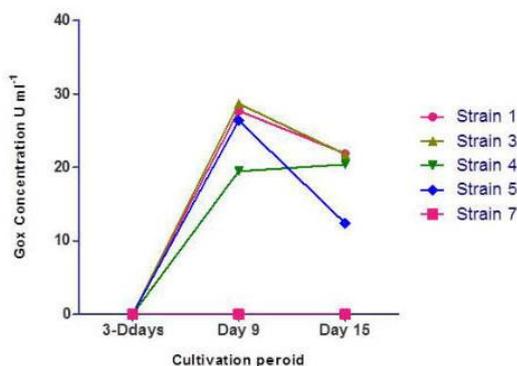
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**Figure 1.** Enzyme zone diffusion test of positive potent *A. niger* producing GOx enzyme. A clear zone of glucose oxidase activity.



**Figure 2.** Enzyme zone diffusion test of negative-oxidase mutant *A. niger* strains able to grow, but without a clear diffusion zone.

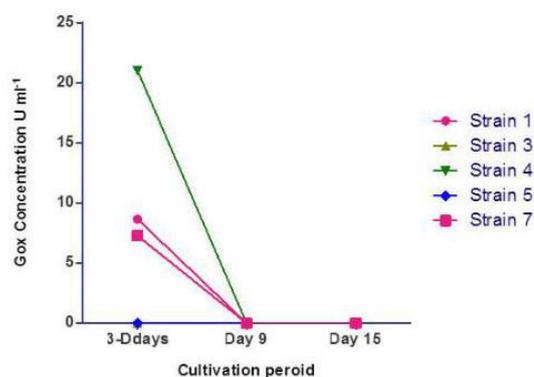


**Figure 3.** GOx enzymatic activity of 5 different *Aspergillus niger* strains on modified Vogel's medium on three intervals of screening 3,9 and 15-days post cultivation.

#### Screening for GOx in liquid phase with ABTS assay in 96 well formats

As shown in Table 2 and Table 3, the glucose oxidase activity of tested strains have been studied on two types of basal media. Results showed that the activity of GOx has been increased on modified Vogel's medium rather than Czapek Dox. The increasing activity of GOx was highly noticed in the strains. Results showed that GOx production

was affected by media components and cultivation time. In modified Vogel's medium, the production in all strains started at the 9<sup>th</sup> day post cultivation and the activity began to decline in the 15<sup>th</sup> days post cultivation except strain 7, which remained non-producer (Table 2 and Figure 3). In the other medium (Czapek Dox), the production of GOx from the five strains started much earlier (3 days post inoculation), but in lower levels if compared to the Vogel's medium (Table 3 and Figure 4).



**Figure 4.** GOx enzymatic activity of 5 different *Aspergillus niger* strains on modified Czapek Dox on three intervals of screening 3,9 and 15-days post cultivation.

#### Catalase activity assay

It is clear that the catalase activity begin from the 9<sup>th</sup> day of culturing and starts to decline till the 15<sup>th</sup> day post culturing on Vogel's medium. In case of Czapek Dox, the production of catalase begins in some strains at 9<sup>th</sup> day of spores inoculation (Figure 5).

As shown in Table 2, the highest yield of catalase enzyme was achieved into Vogel's broth media after 9 days post cultivation by Strain 7 (the negative mutant GOx strain) with average activity 29 U/mL. It seems that the production of catalase was parallel with GOx in the strains 1, 4 and 5.

#### GOD gene amplification of *A. niger*

GOD genes of tested strains were sequenced depending on the variable enzymatic activity of GOx according to the results of liquid phase screening. These strains were 1, 3, 4 and 5 representing hyper producing strains, in addition to strain 7 representing low GOx activity on Vogel's broth. As shown in Figure 6 and analysis of Gel-Pro Analyzer 3 software, the different strains produced variable sizes of amplicons for GOD gene (Table 5).

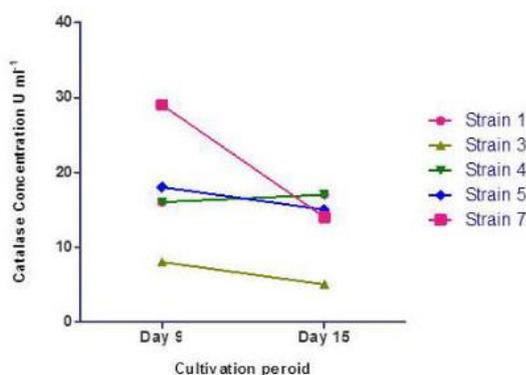
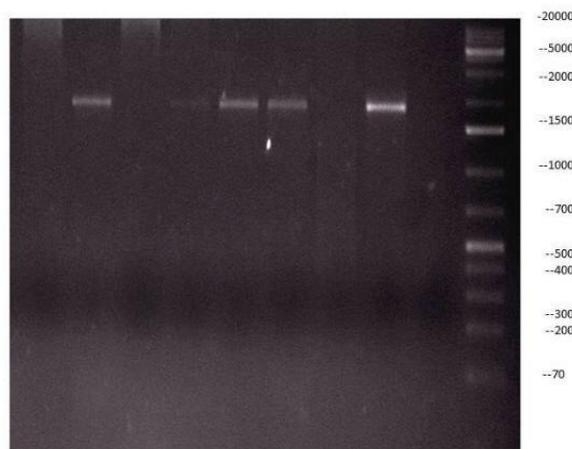
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**Table 2.** Mean values of three reading of *A. niger* wild variant extracellular GOx, catalase (CAT) and total protein (TP) activity on modified liquid phase Vogel's Broth

		Concentration	Strain 1	Strain 3	Strain 4	Strain 5	Strain 7
9 <sup>th</sup> day post culture	GOx	U ml <sup>-1</sup>	27.7	28.7	19.5	26.4	0
	CAT	U ml <sup>-1</sup>	16	8	16	18	29
	TP	mg ml <sup>-1</sup>	40.1	32	38.9	41.1	55.9
15 <sup>th</sup> day post culture	GOx	U ml <sup>-1</sup>	21.9	21.8	20.4	12.4	0
	CAT	U ml <sup>-1</sup>	17	5	8	15	14
	TP	mg ml <sup>-1</sup>	43.5	40.4	40.1	42.7	53.6

**Table 3.** Mean values of three reading of liquid phase screening with the ABTS assay for different 10 *A. niger* strains on modified Czapek Dox Broth

		Strain 1	Strain 3	Strain 4	Strain 5	Strain 7
Day 3	Conc. U ml <sup>-1</sup>	8.66	0.00	21.0	0.00	7.28
Day 9	Conc. U ml <sup>-1</sup>	0.00	0.00	0.00	0.00	0.00
Day 15	Conc. U ml <sup>-1</sup>	0.00	0.00	0.00	0.00	0.00

**Figure 5.** Catalase enzymatic activity of 5 different *Aspergillus niger* strains on modified Vogel's Broth after 9 and 15-days post cultivation.**Figure 6.** Electrophoretic profile of GOD gene of the potent *A. niger* GOx producer and low GOx.**Table 4.** Maximum GOx yield of different wild variant of *A. niger* to different reference hyper GOx producer strains

Microorganism	Accessions No.	Assay method	Maximum Yield	References
<i>A. niger</i> Str. 1 (EGY-GOD1)	KF758449	Coupled ABTS -peroxidase reaction	27.7 U ml <sup>-1</sup>	This study
<i>A. niger</i> Str. 3 (EGY-GOD3)	KF758450	Coupled ABTS -peroxidase reaction	28.7 U ml <sup>-1</sup>	This study
<i>A. niger</i> Str. 4 (EGY-GOD4)	KF758451	Coupled ABTS -peroxidase reaction	19.5 U ml <sup>-1</sup>	This study
<i>A. niger</i> Str. 5 (EGY-GOD5)	KF758452	Coupled ABTS -peroxidase reaction	26.4 U ml <sup>-1</sup>	This study
<i>A. niger</i> Str. 7 (EGY-GOD7)	KF758453	Coupled ABTS -peroxidase reaction	0.00	This study
<i>A. niger</i> NRLGOx gene expressed in <i>S. cerevisiae</i>	J05242	Coupled reaction assay	300-400 µg ml <sup>-1</sup>	Fiedurek et al. (1990)
<i>Penicillium amagasakiense</i>	AF012277	Coupled ABTS reaction	6.6-9.26U mg <sup>-1</sup>	Witt et al. (2000)
<i>Penicillium variable</i>	AJ583233	Reduction of benzoquinone by hydroquinone	5.52 U ml <sup>-1</sup>	Petruccioli et al. (1999).

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**Table 5.** Molecular weight of amplified GOD gene of potent five strains of *A. niger*. by analysis of Gel-Pro Analyzer 3

Lane No.	Lane 2	Lane 4	Lane 5	Lane 6	Lane 8
	Strain 1	Strain 3	Strain 4	Strain 5	Strain 7
GenBank accession No.	KF758449	KF758450	KF758451	KF758452	KF758453
(MW)	1939	1976	2000	2022	2065

**Table 6.** Sequence distance showing percent identity and divergence values. Percent identity of GOD gene of selected five strains of *A. niger*. and reference strains

		Percent Identity									
		1	2	3	4	5	6	7	8		
Divergence	1	■	82.2	90.8	71.6	82.2	83.1	54.2	55.0	1	EGY-GOD1
	2	20.3	■	84.8	86.5	99.1	82.5	53.9	55.0	2	EGY-GOD3
	3	9.8	17.0	■	72.2	85.4	88.0	56.7	57.9	3	EGY-GOD4
	4	35.6	14.9	34.7	■	86.8	70.2	45.0	45.8	4	EGY-GOD5
	5	20.3	0.9	16.3	14.5	■	83.1	53.9	55.0	5	EGY-GOD7
	6	19.2	20.0	13.2	37.9	19.2	■	63.3	64.2	6	J05242
	7	69.4	70.1	63.5	94.3	70.1	50.0	■	90.3	7	AF012277
	8	67.4	67.4	61.0	91.7	67.4	48.4	10.5	■	8	AJ583233
		1	2	3	4	5	6	7	8		

**GOD gene sequence analysis**

According to PSI-BLAST search score, GOD genes of the selected strains (1, 3, 4, 5 and 7) were sequenced. The degree of similarity between different five strains and reference GenBank strains was analyzed by MegAlign software (Table 6).

**Discussion**

Glucose oxidase is a main basic stone for many of commercially interesting applications in the industry. Different approaches were applied for production of GOx depending on improvement of GOx producer strain in combination to optimizing GOx fermentation production condition. The most common microbial sources for fermentative production of GOx are *Aspergillus*, *Penicillium*, and *Saccharomyces* species. *A. niger* is the most used species for commercial production of GOx (Bankar et al., 2009). In this study, five isolates were identified as *Aspergillus* of section *Nigri*, according to the conventional mycological morphological and microscopic characteristics (Pitt & Hocking, 1997; Samson et al., 2000). Plate screening assay is the most common used technique for detection of mutant hyper GOx producer strains based on acidification of the medium around a colony (Kundu & Das, 1985; Fiedurek et al., 1986; Markwell et al., 1989). Depending from the formation of a clear zone on the plate enzyme diffusion test, these isolates varied in level of GOx production (Figures 1

and 2). This was clear from the different diameters of zones on plate enzyme diffusion test agar (Witteveen et al., 1990). The detection of natural GOx variability among wild *A. niger* isolates is a scientific fact of spontaneous variability between GOx producing fungus. This was encountered in *Penicillium* spp. by Mikhailova et al. (2007). For detection and screening of extra cellular GOx production, as shown in Tables 2 and 3, the high throughout liquid phase assay was applied on the selected five strains by plate enzyme diffusion test. The strains GOx production have been studied on two types of liquid broth basal media. The cultivation of variant wild strains of *A. niger* on the two types of liquid broth media revealed that the optimum GOx activity was achieved in the modified Vogel's medium in comparison to modified Czapek Dox liquid medium. The increase in activity of GOx was highly noticed in strains 1, 3, 4, and 5, GOx production varied between potent GOx producer strains (four isolates) and was affected by media components and cultivation time.

In modified Vogel's medium, the production in all tested strains was started at the 9<sup>th</sup> day post cultivation and the activity start to decline in the 15<sup>th</sup> days post cultivation except strain 7, which remained inactive (Table 2 and Figure 3). In the other medium (Czapek Dox), the production of GOx from the five strains differed completely as it begins much more earlier (3 days post inoculation) in strain 1, 3 and 7, but in lower levels compared to the Vogel's medium (Table 3 and Figure 4), while the other two strains remains inactive. The pronounced difference between two basal media Vogel's and Czapek Dox components is sugar content, which strongly affects the GOx activity (Petruccioli et al., 1997). In Vogel's medium, sucrose was not less than 50 %, in addition to urea (nitrogen source) and KH<sub>2</sub>PO<sub>4</sub>. On the other hand, the components of modified Czapek Dox liquid medium lack the sugar source (only 3% of sucrose) and so, it did not give the high production and initiation of an obvious levels of GOx activity even with the presence of 3.5% of CaCO<sub>3</sub> (Hatzinikolaou DG & Macris, 1995; de Vries et al., 2000). Moreover, the level of FeSO<sub>4</sub> 7H<sub>2</sub>O at a concentration of 0.001 g 100 ml<sup>-1</sup> in Czapek Dox (Petruccioli et al., 1997) also could not support GOx production. Interestingly, GOx

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produced from strains 4 and 7, which cultured in Czapek Dox, showed high levels in the 3<sup>rd</sup> day post inoculation and then dramatically regressed (Table 3 and Figure 4). The results revealed that the GOx activity is not only dependent on the media components, but also is controlled by the time of growth and strain itself via GOD gene, which is responsible for expression of GOx enzyme. The optimum production was achieved in the 9<sup>th</sup> day post inoculation, which disagrees with the common concept that reports the maximum production activity in the 3<sup>rd</sup> day of cultivation process (Petruccioli *et al.*, 1997; 1999).

In addition to direct effect of media components on GOx production level and variability, we can attribute the clear difference between different tested strain to nature of strain itself. Visser *et al.* (1994) classified *A. niger* according its GOx activity into three main mutational groups: *goxC* (negative mutant) or *goxB* and *goxE* (hyper producer strains). They described *A. niger* strains, which are less dependent on the oxygen level for glucose oxidase expression as *goxB* mutant strains. In our fermentation procedure, we limited aeration and agitation on two types of basal media. In the description of the effect of sugar content on GOx production, Visser *et al.* (1994), considered the *goxE* mutants is that strain able to produce glucose oxidase on several carbon sources and thus seem to have lost the dependency on the presence of glucose for glucose oxidase induction and that fact clarify, the main reason for strain 4, which was cultured on Czapek Dox and showed high levels in the 3<sup>rd</sup> day post inoculation even with low level of sugar in the media (Table 3 and Figure 4). All over the GOx activity, we can classify our wild variant strains into the three mutational classes according to Visser *et al.* (1994): strains 7 as *goxC-type*, negative mutant on Vogel's broth, while strains 1, 3 & 5 as *goxB-type* overproducer mutant non-oxygen dependent strains and finally strain 4 as *goxB-type* overproducer mutant non-sugar dependent strain. This classification is not restricted classes as simply can changed according type of media, which approve that fact the GOx production is a multifactorial complex intertwined. Our results indicate the GOx production is completely depended on the strain adaptively to media composition. This means that there are not enough sufficient elements for inductions and production of GOx, but also, the adaption and compatibility of this strain is not sufficient to the ingredients of the media. Catalase activity was detected simultaneously and parallel with GOx in case of strains 1 and 5, whereas this activity was very high (29 U ml<sup>-1</sup>) in strain 7, the negative GOx producer. In

addition, the low and imbalanced CAT activity in the case of strains 3 and 4, suggests that the production of GOx and CAT is not necessary to be simultaneous.

On molecular basis, detection of GOD gene in variant potent as well as non GOx-producing strains is a very important finding, which needs further investigations to compare the sequence analysis between potential hyper GOx-producers and the others negative-GOx mutant strains. The strains were 1, 3 and 5 representing hyper producing strains (*goxB-type*), strain 4 (*goxE-type*) in addition to strain 7 representing negative mutant (*goxC-type*). By analysis of Gel-Pro Analyzer 3 software, the different strains produced variable sizes of amplicons for GOD gene (Figure 5 and Table 4). According PSI-BLAST search score top related hits, GOD genes of the selected strains (1, 3, 4, 5 and 7) were identified. The degree of similarity between different five strains and reference GenBank strains were analyzed by MegAlign software (Table 5). Analysis of GOD gene sequences of the selected five wild strains showed variable degrees of homology and identity between each other's. This means there is no typical identity between such strains and they are genetically different. This information could be useful in a further study investigating production of recombinant strains to obtain GOx hyper producers. It is clear that the highest degree of similarity was achieved between two strains EGY-GOD3 and EGY-GOD7 by 99.1% identity. On the other hand, when our local *A. niger* isolates have been compared with other standard genetically engineered strains, the maximum degree identity scored between strain EGY-GOD4 (19.5 U ml<sup>-1</sup>) and J05242 *A. niger* NRRL (300-400µg ml<sup>-1</sup>) with 88%. In spite of EGY-GOD7 is considered a negative mutant GOx on Vogel's broth and producer on Czapek DOx broth. It is known that *A. niger* NRRL is a recombinant strain produces up to four times extracellular GOx under laboratory condition. Such interesting finding can lead us to catch a genetically modified GOx hyper producing strain in the laboratory. Since several attempts have been made to improve GOx production in *A. niger* by strain selection using classical screening and mutagenesis techniques (Fiedurek *et al.* 1986; Petruccioli *et al.*, 1997; Sukhacheva *et al.*, 2004) we conclusively recommend the selective isolation and screening for new naturally hyper GOx-producing wild strains of *A. niger* could be easily achieved by further investigations in optimizing laboratory factors (media, time/temperature, cloning and genetic engineering).

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