J Ind Microbiol Biotechnol (2005) 32: 289–294
DOI 10.1007/s10295-005-0249-7

ORIGINAL PAPER

A. A. Khattab · W. A. Bazaraa

Screening, mutagenesis and protoplast fusion of *Aspergillus niger* for the enhancement of extracellular glucose oxidase production

Received: 16 February 2005 / Accepted: 10 May 2005 / Published online: 11 June 2005

Abstract Various strains of *Aspergillus niger* were screened for extracellular glucose oxidase (GOD) activity. The most effective producer, strain FS-3 (15.9 U mL^{-1}), was mutagenized using UV-irradiation or ethyl methane sulfonate. Of the 400 mutants obtained, 32 were found to be resistant to 2-deoxy D-glucose, and 17 of these exhibited higher GOD activities (from 114.5 to 332.1%) than the original FS-3 strain. Following determination of antifungal resistance of the highest producing mutants, four mutants were selected and used in protoplast fusions in three different intraspecific crosses. All fusants showed higher activities (from 285.5 to 394.2%) than the original strain. Moreover, of the 30 fusants isolated, 19 showed higher GOD activity than their corresponding higher-producing parent strain.

Keywords Extracellular glucose oxidase · *Aspergillus niger* · Mutagenesis · 2-Deoxy D-glucose · Intraspecific protoplast fusion

Introduction

Glucose oxidase (GOD, β-D-glucose: O_2 1-oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone and H_2O_2 using oxygen as an electron acceptor. In a subsequent step, D-glucono-δ-lactone is hydrolyzed non-enzymatically to D-gluconic acid, and the reduced $FADH_2$-enzyme is reoxidized by O_2 [10].

A. A. Khattab
Genetic and Cytology Department,
National Research Center, Cairo, Egypt

W. A. Bazaraa (✉)
Faculty of Agriculture, Food Science Department,
Cairo University, Giza, Egypt
E-mail: wbazaraa@hotmail.com
Tel.: +20-2-5737362

GOD is of considerable commercial importance, as it is used extensively in food processing, production of gluconic acid, quantitative analysis of glucose, and in medical diagnostics [3].

Although several organisms have been reported to produce GOD [7, 14, 15], *Aspergillus niger* is the main organism used for industrial production [9]. Screening, genetic improvement, and evaluation of new GOD-overproducing strains is very important in improving the efficiency and economics of the industrial process. Several attempts have been made to improve GOD production in *A. niger* by strain selection using classical screening and mutagenesis techniques [4, 6, 16, 20], optimizing of cultivation conditions [7, 8, 12, 18], and genetic engineering [8, 19, 21].

Current literature did not reveal any use of protoplast fusion techniques to improve GOD production by *A. niger*. Therefore, this study was initiated to take advantage of mutagenesis in conjunction with the protoplast fusion technique in *A. niger* to obtain GOD-overproducing strains.

Materials and methods

Fungal strains and conidiospore suspensions

The code numbers and sources of *A. niger* strains used in this investigation are listed in Table 1. All strains were maintained on malt extract agar (Merck, Darmstadt, Germany) and transferred monthly. Conidiospores from 5-day-old malt extract agar slants were harvested, combined, counted microscopically (2.0×10^9 spores mL^{-1}), stored at 4°C and utilized as stock inoculum.

Media

Fermentation medium

The fermentation medium (FM) [4] used for GOD production contains (g L^{-1}): glucose, 80; peptone, 3;

Table 1 Code numbers and sources of the original strains of *Aspergillus niger*

Code number	Strain	Source
NR-99	599	Northern Regional
NR-26	326	Research Laboratories
NR-3	3	(NRRL),Peoria, IL
FS-1	1	Food Science Department,
FS-3	3	Faculty of Agriculture,
FS-5	5	Cairo University, Egypt

$(NH_4)_2$ PO_4, 0.388; KH_2 PO_4, 0.188; $MgSO_4 \cdot 7H_2O$, 0.156 and $CaCO_3$ (sterilized separately), 35.

Selective isolation medium

Selective isolation medium (SIM) [6] was utilized to isolate 2-deoxy-D-glucose (2-DG)-resistant mutants, and was prepared by the addition of 2-DG (1.5 g L^{-1}) to malt extract agar.

Screening medium

Screening medium (SM) [4] was used for the preliminary selection of original strains as well as the superior GOD mutants on the basis of violet-blue enzymatic zones. SM contains(g L^{-1}):glucose,80;starch,10;KI,1.7;andsodium desoxycholate, 0.2 in Mcllvaine buffer (0.1 M, pH 5.6).

Protoplasting medium

Protoplasting medium (PM) [11], utilized for pre-growing strains for protoplasting, contains (g L^{-1}): glucose, 80; NH_4 NO_3, 2; KH_2 PO_4, 10; Mg $SO_4 \cdot 7H_2O$, 0.25; $FeCl_3 \cdot 6H_2O$, 0.02; $MnSO_4$, 0.014 at an initial pH of 4.25.

Mutagenesis

UV-Mutagenesis

About 5 mL of the stock inoculum of *A. niger* FS-3 was irradiated with a Phillips TUV-30-W-254 nm Lamp (Phillips, The Netherlands) for 4, 8 and 12 min at a distance of 20 cm. The treated spores were kept in the dark for 2 h to avoid photoreactivation repair. The suspension was then diluted and spread onto the surface of malt extract agar containing 0.1% (v/v) Triton X-100 to restrict radial colony growth. Plates were then incubated for 2 days at 28°C, and the growing colonies were transferred before sporulating on slants for further studies. At this stage, the drop in viability was about 90%. Mutants resistant to 2-DG were then selected on SIM plates (30°C, 3 days).

Ethyl methane sulfonate mutagenesis

The procedure for ethyl methane sulfonate (EMS) mutagenesis was similar to that for UV-mutagenesis except that phosphate buffer (0.1 M, pH 7.0) was used to prepare the spore suspensions, and EMS (Sigma, St. Louis, MO; final concentration 200 mM) was used as the mutagenic agent. Spores were treated with EMS for 20, 40 and 60 min. After exposure, spores were harvested by centrifugation (5,600 *g*, 4°C) and washed twice with the same buffer. In addition, the treated spores were not stored in the dark. After EMS treatment, a 90% drop in viability was observed.

Protoplast fusion

Isolation of antifungal-resistant mutants

Mutants with high levels of GOD activity were streaked on the surface of malt extract agar plates supplemented with specific antifungal agents (benomyl, cycloheximide, griseofulvin, miconazole or nystatin) and incubated for 5 days at 30°C. Mutants exhibiting resistance to specific antifungal agents were retested for stability of resistance.

Protoplast formation

For each antifungal-resistant mutant, 50 mL PM was inoculated with a stock spore suspension (2%, v/v) and incubated overnight (30°C) on a rotary shaker (180 rpm). The mycelium formed was recovered by centrifugation (5,600 *g*, 4°C), washed twice with sterile distilled water, resuspended in 5 mL citrate phosphate buffer (0.1 M, pH 5.8) containing 0.7 M NaCl, 0.2 M $CaCl_2$ and Novozyme 234 (10 mg mL^{-1}) and incubated (30°C) with gentle shaking for up to 3 h. The released protoplasts were photographed using a phase-contrast microscope.

Intraspecific protoplast fusion and fusant isolation

Equal volumes of crude protoplast suspension from two parents were mixed and centrifuged (1,000 *g*, 10 min, 4°C). The protoplast pellet formed was resuspended in 2 mL glycine-NaOH buffer (0.05 M, pH 7.5) containing 30% (w/v) polyethylene glycol 6000 (PEG), 50 mM $CaCl_2$ and 0.7 M NaCl, and incubated for 20 min at 30°C. Afterwards, PEG-treated protoplast suspensions were plated onto the surface of malt extract agar containing 0.7 M NaCl and supplemented with antifungal agents, and incubated for 5–7 days at 30°C. Colonies growing on the surface of the plates were considered as fusants.

Fermentation and GOD production

Fermentation flasks (250 mL) containing 50 mL FM were inoculated with 10% (v/v) stock spore suspension and incubated for 4 days (30°C) with shaking (225 rpm). The mycelia formed were harvested by centrifugation (5,600 *g*, 4°C) and GOD activity in the supernatant was determined.

Determination of GOD activity

The violet-blue enzymatic zone method [4] was used for the preliminary screening of original strains as well as mutants with high GOD activity. Extracellular GOD activity in the cell-free culture medium was measured spectrophotometrically at 290 nm [2]. The reaction mixture consisted of: 2 mL D-glucose (1 M) in citrate phosphate buffer (0.1 M, pH 5.0), 1 mL benzoquinone (0.1%, w/v) and 100 μ L enzyme source. The mixture was incubated for 10 min at 35°C and the hydroquinone formed was determined at 290 nm. One unit of GOD activity was defined as the amount of enzyme producing 1 μmol of hydroquinone per milliliter per minute.

Determination of proteolytic activity

Proteolytic activity in the cell-free culture medium was determined by the lysis clear zone method [1], modified as follows: 50 μL clear cell-free culture medium was inoculated (6 mm well) into proteinase indicator medium (0.5% gelatin, 2% agar and 0.05 M Tris-HCl at pH 8.0). Plates were incubated at 35°C for 24 h and then flooded with proteinase indicator solution (15 g $HgCl_2$, 20 mL concentrated HCl and 60 mL distilled water). The clear zones formed were measured in millimeters.

Results and discussion

Screening of different *A. niger* genotypes for extracellular GOD production

Table 2 shows extracellular GOD production by the *A. niger* strains tested. Strains NR-99 and NR-26 exhibited no GOD activity, but had the highest proteolytic activities (12 and 17 mm proteolytic zones, respectively). Strain FS-3 exhibited the highest GOD activity (15.9 U mL^{-1} considered as 100%), followed by FS-5 (71.7%) and FS-1 (71.1%). It was of interest to note that strains FS-3 (the highest GOD producer), FS-1 and NR-3 show no proteolytic zones on plates. The data also indicated a very high correlation ($r = 0.98$) between GOD activities determined by the glucose oxidase zone method, where violet–blue zones are formed by iodine released from KI by GOD and combined with starch, and the spectrophotometric method, where benzoquinone was enzymatically reduced to hydroquinone. From the above results, strain FS-3 proved to be the highest GOD producer among the strains tested. Therefore, it was selected as the starting strain for all subsequent procedures aimed at improving GOD production.

Table 2 Extracellular glucose oxidase (GOD) and proteolytic activities of different genotypes of *A. niger*

Strain code	GOD		Proteolytic zone (mm)
	Violet–blue zone (mm)	U mL^{-1}	
NR-99	0	0.0	12
NR-26	0	0.0	17
NR-3	5	8.6	0
FS-1	6	11.3	0
FS-3	7	15.9	0
FS-5	6	11.4	8

Table 3 2-Deoxy D-glucose (2-DG) resistant mutants obtained after ultraviolet (UV) or ethyl methane sulfonate (EMS) mutagenesis

Exposure time (min)	Mutagen	Number of colonies tested	Number of resistant colonies[a]
4	UV	100	3
8	UV	50	6
12	UV	50	2
20	EMS	100	5
40	EMS	50	7
60	EMS	50	9

[a]Mutants were screened on malt extract agar containing 2-DG (1.5 g L^{-1})

Table 4 Extracellular GOD production of selected mutants obtained after UV or EMS mutagenesis

Mutant	GOD activities		Percent
	Violet–blue zone (mm)	U mL^{-1}	
FS-3 (wild type)	7	15.9	100.0
U-41	8	18.2	114.5
U-42	5	10.8	67.9
U-43	13	45.2	284.3
U-81	6	14.4	90.6
U-82	6	11.5	72.3
U-83	12	42.4	266.7
U-84	0	0.0	0.0
U-85	0	0.0	0.0
U-86	13	46.3	291.2
U-121	9	20.8	130.8
U-122	6	12.1	76.1
E-201	10	23.8	149.7
E-202	6	12.2	76.7
E-203	11	34.2	215.1
E-204	6	11.9	74.8
E-205	12	38.6	242.8
E-401	13	47.3	297.5
E-402	9	21.4	134.6
E-403	6	12.5	78.6
E-404	10	24.3	152.8
E-405	14	52.5	330.2
E-406	7	15.0	94.3
E-407	14	52.8	332.1
E-601	6	12.9	81.1
E-602	9	21.8	137.1
E-603	13	46.4	291.8
E-604	6	11.8	74.2
E-605	9	22.1	139.0
E-606	0	0.0	0.0
E-607	9	23.9	150.3
E-608	5	9.6	60.4
E-609	6	12.4	78.0

Table 5 Response of superior extracellular GOD mutants to various antifungal agents. *B* Benomyl, *C* cycloheximide, *G* griseofulvin, *M* miconazole, *N* nystatin

Mutant	GOD (U mL^{-1})	Antifungal agent (μg mL^{-1})				
		B (2.5)	C (100)	G (250)	M (5)	N (100)
U-43	45.2	+[a]	+	+	+	–
U-86	46.3	+	–	+	–	–
E-401	47.3	–	+	+	+	+
E-405	52.5	–	–	+	–	+
E-407	52.8	–	–	+	+	+
E-603	46.4	+	–	+	+	–

[a] + Resistant, – sensitive

Mutagenesis

After exposure to various UV or EMS treatments, mutants resistant to 2-DG were isolated (Table 3). After screening 200 UV-treated colonies, only 11 were characterized as 2-DG resistant, whereas 21 resistant colonies were obtained after EMS mutagenesis. The data also revealed an increase in the number of resistant colonies with increasing exposure to EMS mutagen (Table 3). In the case of UV-mutagenesis, the number of resistant colonies increased by increasing the exposure time to 8 min, then a sharp decline was noted.

The results presented in Table 4 show that of the 32 2-DG-resistant mutants obtained, only 3 (U-84, U-85 and E-606) did not produce GOD. Moreover, 12 mutants produced GOD in amounts less than their original parental strain. The remaining 17 mutants showed enhanced GOD production. The highest level of GOD was produced by mutant E-407, at 52.8 U mL^{-1} (332.1% production compared with the original untreated FS-3 strain). The GOD activities of the mutants selected were also determined by the violet–blue zone method (Table 4). The results demonstrated a very high correlation between the diameter of the zones formed on SM medium and GOD activities (U mL^{-1}) measured using the spectrophotometric assay, specially in the range of 0–10 mm (GOD zone) where the correlation was 0.98.

From the previous results, it could be concluded that increased resistance to 2-DG can be successfully used as a selection procedure to improve the enzymatic synthetic capacity of the original production strains. Resistance to the toxic glucose analogue (2-DG) has been used as a criterion to select glucoamylase [5] and cellulase [13] overproducer mutants.

Table 6 Design of crosses using different selective antifungal markers. *B* Benomyl, *N* nystatin, *M* miconazole

Cross	Parents	Selective marker
C_1	U-86 and E-405	B + N
C_2	E-405 and E-603	N + (B or M)
C_3	E-407 and E-603	B + N

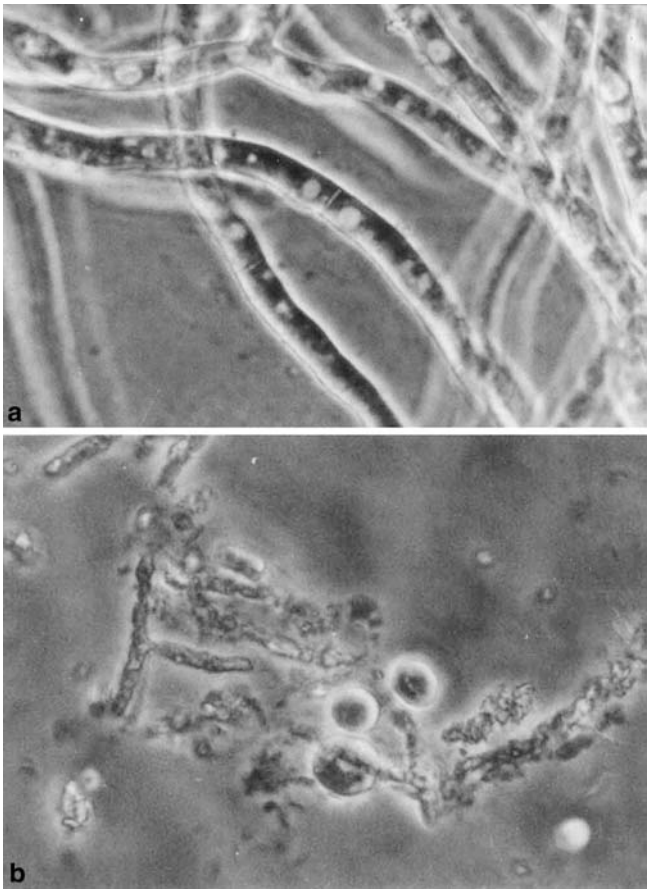

Fig. 1 Photomicrographs depicting protoplast formation. **a** Intact mycelia of *Aspergillus niger*. **b** Spherical protoplasts and cell wall debris after lysis

Intraspecific protoplast fusion

To investigate the effect of protoplast fusion on GOD production, six superior GOD-overproducing mutants (U-43, U-86, E-401, E-405, E-407 and E-603) were selected and tested for their resistance to the antifungal agents used as selective markers during detection of fusants after protoplast fusion of two strains. Table 5 shows that all mutants exhibited resistance to griseofulvin, but different responses to the other antifungal agents were noted. As a result, mutants U-86, E-405, E-407 and E-603 were selected for protoplast fusion. Due to the different responses of the selected mutants, three crosses were designed and executed (Table 6). The mycelium of each of the four mutants was induced to form protoplasts (Fig. 1), and the parental protoplast suspensions were used for fusion as designed. Fusants were isolated and their GOD activities determined (Table 7). The results revealed that all fusants obtained exhibited higher extracellular GOD activities than the original strain (FS-3). Moreover, seven fusants (obtained after cross 1) exhibited higher extracellular GOD activities than their higher-expressing parent strain (E-405). On the other hand, only six fusants with higher extracellular GOD activities than the parent strains

Table 7 Extracellular GOD production of fusants obtained after three different intraspecific protoplast fusion crosses

Parents and fusants	GOD (U mL^{-1})	Percent
FS-3 (wild type)	15.9	100.0
C1		
P_1 (U-86)	46.3	291.2
P_2 (E-405)	52.5	330.2
C-11	53.1	334.0
C-12	48.9	307.6
C-13	56.6	356.0
C-14	45.4	285.5
C-15	61.4	386.2
C-16	58.0	364.8
C-17	60.8	382.4
C-18	62.7	394.3
C-19	48.3	303.8
C-110	59.6	374.8
C2		
P_1 (E-405)	52.5	330.2
P_2 (E-603)	46.4	291.8
C-21	48.2	303.1
C-22	50.0	314.5
C-23	53.7	337.7
C-24	55.6	349.7
C-25	49.9	313.8
C-26	58.0	364.8
C-27	52.7	331.4
C-28	58.8	369.8
C-29	49.1	308.8
C-210	55.4	348.4
C3		
P_1 (E-407)	52.8	332.1
P_2 (E-603)	46.4	291.8
C-31	50.2	315.7
C-32	54.4	342.1
C-33	57.9	364.2
C-34	54.7	344.0
C-35	50.8	319.5
C-36	57.3	360.4
C-37	57.3	360.4
C-38	50.5	317.6
C-39	53.1	334.0
C-310	50.2	315.7

E-405 and E-407 were obtained after crosses 2 and 3, respectively. Fusant C-18 was the highest extracellular GOD producer, with an activity (62.7 U mL^{-1}) 3.94 and 1.19 times higher than that produced by the original strain (FS-3) and its higher-producing parent strain (E-405), respectively. It is of interest to report that GOD overproducer mutants U-86, E-405, E-407 and E-603, and fusants C-15, C-17 and C-18, exhibited stable GOD production for at least ten fermentation cycles (data not shown).

Mutagenesis of industrial microbial strains is widely used for the improvement of microbial synthesis of enzymes. Various mutagens have been used to obtain high-GOD-producing mutants [4, 6, 16, 17]. An increment of over 127% in GOD production was obtained after mutagenesis of *Penicillium variable* [17]. Similar results (>200–238.5% improvement) were noted after mutagenesis of *A. niger* [6, 16, 20]. In this respect, our results indicated a 393.8% improvement in GOD synthesis after applying mutagenesis followed by protoplast fusion in *A. niger*. Such an improvement counts amongst the highest reported in the literature.

In general, it can be concluded that mutagenesis (UV or EMS) proved to be an effective technique to enhance GOD production. In addition, intraspecific protoplast fusion between higher GOD producing mutants proved effective in achieving superior GOD-producing fusants.

References

1. Badr UM (1995) Gene transfer in *Bacillus megaterium* in relation to proteinases. PhD Thesis, Faculty Agriculture of Ain Shams University, Egypt
2. Ciucu A, Patroescu C (1984) Fast spectrometric method of determining the activity of glucose oxidase. Anal Lett 17:1417–1427
3. Crueger A, Crueger W (1990) Glucose-transforming enzymes. In: Fogarty WM, Kelly CE (eds) Microbial enzymes and biotechnology, 2nd edn. Elsevier, London, pp 177–227
4. Fiedurek J, Rogalski J, Ilczuk Z, Leonowicz A (1986) Screening and mutagenesis of moulds for the improvement of glucose oxidase production. Enzyme Microb Technol 8:734–736
5. Fiedurek J, Paszczynski A, Ginalska G, Ilczuk Z (1987) Selection of amylolytically active *Aspergillus niger* mutants to 2-deoxy-D-glucose. Zentralbl Mikrobiol 142:407–412
6. Gromoda A, Fiedurek J (1997) Selective isolation of *Aspergillus niger* mutants with enhanced glucose oxidase production. J Appl Microbiol 82:648–652
7. Hatzinikolaou DG, Macris BJ (1995) Factors regulating production of glucose oxidase by *Aspergillus niger*. Enzyme Microbiol Technol 17:530–534
8. Hellmuth K, Pluschkill S, Jung J, Ruttkowski E, Rinas U (1995) Optimization of glucose oxidase production by *Aspergillus niger* using genetic- and process-engineering techniques. Appl Microbiol Biotechnol 43:978–984
9. Kapat A, Jung J, Park YH (1997) Enhancement of extracellular glucose oxidase production in pH-stat feed-back controlled fed-batch culture of recombinant *Saccharomyces cerevisiae*. Biotechnol Lett 20:683–686
10. Kapat A, Jung J, Park YH (1998) Improvement of extracellular recombinant glucose oxidase production in fed-batch culture of *Saccharomyces cerevisiae*: effect of different feeding strategies. Biotechnol Lett 20:319–323
11. Kirimura K, Yaguchi T, Usami S (1986) Intraspecific protoplast fusion of citric acid-producing strains of *Aspergillus niger*. J Ferment Technol 64:473–479
12. Kona RP, Qureshi N, Pai JS (2001) Production of glucose oxidase using *Aspergillus niger* and corn steep liquor. Bioresour Technol 78:123–126
13. Labudova J, Farkas V (1983) Enrichment technique for the selection of catabolite repression-resistant mutants of *Trichoderma* as producers of cellulase. FEMS Microbiol Lett 20:211–215
14. Liu JZ, Yang HY, Weng LP, Ji LN (1999) Synthesis of glucose oxidase and catalase by *Aspergillus niger* in resting cell culture system. Lett Appl Microbiol 29:337–341
15. Lu T, Peng X, Yang H, Ji L (1996) The production of glucose oxidase using the waste myceliums of *Aspergillus niger* and the effects of metal ions on the activity of glucose oxidase. Enzyme Microb Technol 19:339–342
16. Markwell J, Farkes L, Brott E, Osterman J, Wanger F (1989) *Aspergillus niger* mutants with increased glucose oxidase production. Appl Microbiol Biotechnol 30:166–169
17. Petruccioli M, Piccioni P, Federic F, Polsinelli M (1995) Glucose oxidase over producing mutants of *Penicillium variable* (P16). FEMS Microbiol Lett 128:107–112
18. Rogalski J, Fiedurek J, Szczordrak J, Kapusta K, Leonowicz A (1988) Optimization of glucose oxidase synthesis in submerged cultures of *Aspergillus niger* G-13 mutant. Enzyme Microb Technol 10:508–511

19. Sharif FA, Alaeddinoglu NG (1992) Expression and over-production of glucose oxidase in *Aspergillus niger*. Appl Microbiol Biotechnol 38:115–116
20. Witteven CFB, Vondervoort PJI, Swart K, Visser J (1990) Glucose oxidase over-producing and negative mutants of *Aspergillus niger*. Appl Microbiol Biotechnol 33:683–686
21. Witteven CFB, Vondervoort PJI, Broeck HC, Engelenburg FAC, Graaff LH, Hillebrand MHBC, Schaap PJ, Visser J (1993) Induction of glucose oxidase, catalase and lactonase in *Aspergillus niger*. Curr Genet 24:408–416