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Protoplast Fusion of β-Glucosidase-Producing *Aspergillus niger* Strains

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ABSTRACT

Protoplast fusion, induced by polyethylene glycol and Ca^{2+}, was carried out between two auxotrophic strains of *Aspergillus niger*. The fusion frequency ranged from 6.2×10^{-2}–9.1×10^{-2}. After induced haploidization of a diploid, various segregants showing combinations of the parental genetic markers were isolated. Unlike diploids, haploid segregants exhibited greater variations in their morphology and β-glucosidase activities. One segregant showed a 2.5-fold increase in β-glucosidase activity over those of the parents. Thus, this method appears promising for creating new recombinant strains of *A. niger* with improved β-glucosidase activities.

Index Entries: β-glucosidase; cellobiose hydrolysis; protoplast fusion; *Aspergillus niger*.

INTRODUCTION

Trichoderma spp. produce relatively low levels of extracellular β-glucosidase (cellobiase, EC 3.2.1.21) that result in limited hydrolysis of cellulose (*1*). The overall rate of saccharification, however, can be increased by culturing *Trichoderma* spp. with other fungi with higher β-glucosidase

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activities. In this respect, *Aspergillus* appears to be suitable because of its ability to produce high levels of β-glucosidase (*2*). There is increasing interest in *Aspergillus* β-glucosidase, with some of the research concentrating on the isolation of new and over producing strains in attempts to maximize saccharification (*3*). Protoplast fusion technique has been demonstrated to be an efficient tool for improving industrial micro-organisms (*4*). Thus, it is desirable to evaluate the feasibility of employing protoplast fusion technique to obtain strains of *Aspergillus niger* with improved β-glucosidase activities. In this study, haploid segregants obtained from protoplast fusion were screened for their ability to produce extracellular β-glucosidase, and the kinetic properties of the purified enzyme from high- and low-yielding haploid segregants were investigated.

METHODS

Strains and Culture Conditions

Protoplast fusion was carried out between *Aspergillus niger* USDB 0827 and USDB 0828. Both strains were UV-induced auxotrophic mutants from the Department of Botany Culture Collection, National University of Singapore. *A. niger* USDB 0827 has yellowish mycelium, black conidia, and requires Met (Met$^-$). *A. niger* USDB 0828 has white mycelium, white conidia, and requires Arg and His (Arg$^-$, His$^-$). Both strains were maintained on complete agar medium made up of minimal agar medium (*5*), supplemented with 0.5% (w/v) casamino acids, and 0.2% (w/v) yeast extract. In 2% (w/v) α-cellulose medium supplemented with Arg, His, and Met, β-glucosidase activity of *A. niger* USDB 0827 was twice that of *A. niger* USDB 0828 (*6*).

Isolation and Purification of Protoplasts

Protoplasts were isolated as in Hamlyn et al. (*7*) except that the osmotic stabilizer used was 0.4*M* $MgCl_2 \cdot 6H_2O$ in 0.05*M* maleate buffer, pH 5.8, and 20 mg wet wt mycelia were resuspended per mL osmotic stabilizer containing 5 mg each of Novozym 234 and Cellulase CP. Protoplasts were purified using 1*M* mannitol.

Protoplast regeneration was carried out as in Kirimura et al. (*8*) but with 0.6*M* sucrose as the osmotic stabilizer. The regeneration frequency was expressed as the ratio of the number of colonies formed on hypertonic complete medium to the number of protoplasts plated.

Protoplast fusion was carried out using polyethylene glycol (PEG) 6000, as described in Anne and Peberdy (*9*). Fusion frequency was expressed as the ratio of the number of colonies formed on hypertonic minimal medium to the number formed on hypertonic complete medium. Parental auxotrophic protoplasts were separately subjected to the same

PEG treatment and tested for reversion to prototrophy. Reversion frequency for each strain was expressed as the ratio of number of protoplasts of the strain regenerating on hypertonic minimal medium to the number on hypertonic complete medium.

Heterodiploid Formation from Heterokaryon

Heterodiploids were induced from a fusant (heterokaryon) by d-camphor treatment according to the modified procedure of Roper (*10*). Mycelial mats (5 mm×5 mm) from the fusant cultured on minimal medium were grown on minimal medium containing 0.1% (w/v) d-camphor at 30°C. After 7 d, this subculturing on d-camphor medium was repeated. Conidia from the culture were harvested, washed with sterile distilled water, and plated on minimal medium. Presumptive diploid colonies were obtained, and heterodiploids confirmed by their growth on complete medium with and without benlate (1.25 μg/mL), the greater diameter and DNA content of the conidia. Frequency of heterodiploid formation was expressed as the ratio of the number of colonies formed on minimal medium to the number of conidia plated.

Isolation of Haploid Segregants

Diploids were induced to haploidize by treatment with benlate. Mycelial mats (5 mm×5 mm) of the diploid strain were inoculated on complete medium containing benlate (1.25 μg/mL) as a haploidizing agent (*11*). After 7 d, isolates from haploid sectors were grown on complete medium containing benlate for another 7 d. The ploidy of segregants was verified by their growth on complete medium containing benlate, and the diameter and DNA content of the conidia. Haploid segregants were subcultured on minimal medium supplemented with Arg, His, and/or Met for phenotypic characterization.

Diameter, DNA Content, and Nuclear Staining of Conidia

The diameter of 200 conidia of each strain was measured with an eyepiece micrometer of a light microscope. DNA of conidia (1×10^9 conidia were used for each determination) was extracted with 0.5*M* perchloric acid according to Herbert et al. (*12*). DNA content was assayed according to Burton (*13*), with calf thymus DNA as standard. Nuclei of conidia were stained with Hoechst 33258 (*14*) and detected under a fluorescence microscope.

Measurement of Purification, and Characterization of β-Glucosidase

Mycelia for enzyme induction were cultured in complete medium broth, harvested and grown in α-cellulose medium (6 g wet wt/25 mL) as

described in Hoh et al. (*6*). β-Glucosidase activity and mycelial growth of cultures were monitored daily for 12 d. β-Glucosidase activity was determined as described in Hoh et al. (*6*) and was expressed as nkat/mL of culture broth.

β-Glucosidase (cellobiase) was purified and the kinetic properties investigated as described in Hoh et al. (*6*). The molecular size of β-glucosidase was determined by electrophoresis in different polyacrylamide gel concentrations (*15*). Protein content was determined according to Lowry et al. (*16*), with bovine serum albumin as standard.

Mycelia were recovered by filtration, washed with distilled water and dried at 100°C overnight. The total nitrogen content of the mycelia was determined by micro-Kjeldhal method (*17*).

RESULTS AND DISCUSSION

Protoplast yields from *A. niger* USDB 0827 and *A. niger* USDB 0828 were 2.8×10^5 and 3.0×10^5 per mg wet wt mycelium respectively. These yields are comparable to those obtained for other *A. niger* strains (*7,8*) but are 18-fold more than that obtained for *A. niger* strain 097 using cellulase, snail digestase, and lysozyme (*18*). The enhanced protoplast yields were probably caused by the higher chitinase and α-1,3-glucanase activities of Novozym 234 used compared to those in the snail enzyme preparation (*19*). The cell wall of *A. niger* is composed mainly of chitin and α-glucans (*20*). Protoplasts of *A. niger* USDB 0827 and *A. niger* USDB 0828 regenerated on hypertonic complete medium containing 0.6*M* sucrose, with frequencies of 23 and 26%, respectively.

The frequency of fusion between protoplasts of *A. niger* USDB 0827 and *A. niger* USDB 0828 ranged from 6.2×10^{-2}–9.1×10^{-2}, more than 10,000 times the reversion frequencies of the parental auxotrophs (Table 1). This fusion frequency is comparable to those obtained for other *A. niger* strains (*8*). The fusants grew slowly compared to the parental auxotrophic mutants, had thin mycelial mats, and irregular colony morphology. They had sparse conidiophores and produced conidia of the parental auxotrophic mutants. The morphology of the fusants remained stable through ten generations of subculturing on minimal medium. When transferred to complete medium, they produced sectors and segregated into the parental auxotrophic mutants. These observations showed that the fusants were heterokaryons.

Heterozygous diploids were obtained from conidia that germinated on minimal medium. The frequency of heterodiploid formation was 1.7×10^{-2}. The diploids grew faster than the heterokaryons, and showed compact and regular colony morphology. Diploids were generally characterized by white, dense mycelia and hyaline conidia lacking the black pigment. A similar phenomenon was observed in fusions involving *A. niger* strains with

Table 1
Protoplast Fusion Frequency of *A. niger* USDB 0827 and *A. niger* USDB 0828

Protoplast pair	Reversion frequency, less than	Protoplasts regenerated on hypertonic complete medium	Fusants formed on hypertonic minimal medium	Fusion frequency
A. niger USDB 0827	2.5×10^{-6}	(i) 2.5×10^{5}	(i) 3.2×10^{4}	6.2×10^{-2}
		(ii) 2.5×10^{5}	(ii) 3.7×10^{4}	7.1×10^{-2}
		(iii) 2.4×10^{5}	(iii) 4.1×10^{4}	8.3×10^{-2}
		(iv) 2.4×10^{5}	(iv) 4.5×10^{4}	9.1×10^{-2}
A. niger USDB 0828	2.1×10^{-6}	(i) 2.7×10^{5}		
		(ii) 2.7×10^{5}		
		(iii) 2.6×10^{5}		
		(iv) 2.5×10^{5}		

complementary auxotrophic and spore color mutations (*21*). The diploid conidia showed twice the DNA content (7.1×10^{-8} μg per conidium) and 1.3-fold the conidial diameter (5.8 ± 0.3 μm) of the parental mutants (*A. niger* USDB 0827: 3.5×10^{-8} μg DNA per conidium, diameter of conidia = 4.3 ± 0.2 μm; *A. niger* USDB 0828: 3.6×10^{-8} μg DNA per conidium, diameter of conidia = 4.1 ± 0.2 μm). On complete medium, the diploid colonies were uniform and segregation did not occur. However, on complete medium containing 1.25 μg/mL benlate, the diploid colonies grew slowly and formed sectors of the parental auxotrophic mutants or recombinants.

Five diploid strains were assayed for β-glucosidase activity which ranged from 19.0–20.8 nkat/mL. The strain that showed the highest activity (20.8 nkat/mL), designated as *A. niger* USDB 0900, was selected for induced haploidization with benlate to obtain segregants. Seventy segregants were isolated (Table 2). Of these, nine had phenotypes similar to those of the parental auxotrophs while the other sixty-one showed wide phenotypic variations from the parental auxotrophs. These data indicated that whole genomes from both parents had been successfully integrated into the heterozygous diploids and that genetic recombination had occurred through haploidization of these diploids.

The segregants were morphologically stable and showed no sign of sectoring on complete medium with benlate. The conidia of all the segregants were similar in diameter (4.0 ± 0.2–4.2 ± 0.2 μm) and DNA content (3.5×10^{-8}–3.7×10^{-8} μg per conidium) to those of the parental strains. The conidia of all the strains, regardless of ploidy, were uninucleate as seen with Hoechst 33258 staining. These results indicated that the segregants were haploids.

All seventy haploid segregants showed positive β-glucosidase activities ranging from 9.3–44.6 nkat/mL. While most of the segregants had activities

Table 2
Segregants Obtained by Benlate Treatment of Diploid Strain, *A. niger* USDB 0900

Color of conidia	Phenotypes: Nutritional requirements			Number of segregants isolated
	Arg	His	Met	
black*	+	+	+	11
black*	–	+	+	10
black**	+	+	–	5
white*	+	+	+	9
white*	+	+	–	24
white*	+	–	+	7
white**	–	–	+	4

+ Nonrequiring.
– Requiring.
*Recombinant phenotypes.
**Phenotypes similar to parental auxotrophic mutants.

Table 3
Distribution of β-Glucosidase Activities of *A. niger* Haploid Segregants

β-glucosidase activities, nkat/mL	Number of segregants
<9.8	1
9.8*–10.0	3
10.1–12.0	17
12.1–14.0	12
14.1–16.0	13
16.1–17.7**	19
17.8–19.1	4
44.6	1

*β-glucosidase activity of *A. niger* USDB 0828.
**β-glucosidase activity of *A. niger* USDB 0827.

distributed between those of the two parents (*A. niger* USDB 0828, 9.8 nkat/mL; *A. niger* USDB 0827, 17.7 nkat/mL), four strains had activities that were slightly higher than that of *A. niger* USDB 0827. One haploid segregant, designated as *A. niger* USDB 0837 showed an activity of 44.6 nkat/mL, a 2.5-fold increase over that of *A. niger* USDB 0827 (Table 3).

In order to explain the different levels of enzyme activity among the haploid segregants, three segregants with vastly different levels of enzyme activity were selected for further investigation. These were *A. niger* USDB 0829, *A. niger* USDB 0847, and *A. niger* USDB 0837 with activities of

Table 4
Biomass and Kinetic Parameters
of β-Glucosidase of *A. niger* Haploid Segregants

	A. niger USDB 0829	*A. niger* USDB 0847	*A. niger* USDB 0837
Biomass (mg N/25 mL)			
Day 0	4.4	4.5	4.3
Day 9	17.7	18.4	18.9
K_m, cellobiose (m*M*)	1.71±0.13	1.67±0.10	1.64±0.13
V_{max}, cellobiose (nkat/mg protein)	3375±26	3310±49	3452±38

10.5, 19.6, and 44.6 nkat/mL respectively. As shown in Table 4, the increase in biomass for the three strains over a period of nine days was comparable and hence, the different levels of enzyme activity were unlikely to be caused by differences in growth rate. β-Glucosidases purified from the three strains also showed comparable K_m (1.64–1.71 m*M*) and V_{max} (3310–3452 nkat/mg protein) values (Table 4). Thus, the different levels of enzyme activity could not be attributed to differences in affinity of the enzyme for the substrate. β-Glucosidases isolated from the three strains also showed the same molecular size as those of the parental auxotrophic strains (230 kDa). It is possible, therefore, that the three haploid segregants produced different levels of β-glucosidase and that *A. niger* USDB 0837 is a hyperproducing strain. These observations also suggest that induced haploidization by benlate might have affected genes that control the synthesis and/or secretion of the enzyme. While previous studies employed UV and/or chemical mutation for strain improvement (*3,22–24*), this study shows that protoplast fusion technique could be an alternative strategy for improving β-glucosidase activity in *A. niger*.

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