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Invertase Production on Solid-State Fermentation by *Aspergillus niger* Strains Improved by Parasexual Recombination

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Abstract

Invertase production by *Aspergillus niger* grown by solid-state fermentation was found to be higher than by conventional submerged fermentation. The haploid mutant strains Aw96-3 and Aw96-4 showed better productivity of various enzymes, as compared to wild-type parental strain *A. niger* C28B25. Here we use parasexual crosses of those mutants to increase further the productivity of invertase in solid-state fermentation. We isolated both a diploid (DAR2) and an autodiploid (AD96-4) strain, which were able to grow in minimal medium after mutation complementation of previously isolated haploid auxotrophic strains. Invertase production was measured in solid-state fermentation cultures, using polyurethane foam as an inert support for fungal growth. Water activity value (*Aw*) was adjusted to 0.96, since low *Aw* values are characteristic in some solid-state fermentation processes. Such diploid strains showed invertase productivity levels 5–18 times higher than levels achieved by the corresponding haploid strains. For instance, values for C28B25, Aw96-3, Aw96-4, DAR2, and AD96-4 were 441, 254, 62, 1324, and 2677 IU/(L·h), respectively. These results showed that genetic recombination, achieved through parasexual crosses in *A. niger*, results in improved strains with potential applications for solid-state fermentation processes.

Index Entries: *Aspergillus niger*; diploids; invertase; solid-state fermentation; parasexual recombination.

Introduction

Commonly found agricultural solid wastes have a potential as supports to produce high added-value products such as enzymes and other

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metabolites in solid-state fermentation cultures. However, it has been suggested that microbial strains require adaptation to those conditions generally found in solid-state fermentation *(1,2)*. Adaptation to solid-state fermentation by microorganisms includes genetic improvement of strains, which, in turn, results in better enzyme production *(2,3)*. Other advantages of solid-state fermentation over submerged fermentation cultures include a lower extent of catabolic repression *(4,5)* and higher enzyme productivity *(6–8)*. Parasexual crosses among *Aspergillus niger* strains have been used to improve enzyme titers *(9)* and citric acid production *(10,11)*. A previous study has shown a significant increase in enzyme production by a diploid construct of *A. niger* in submerged fermentation *(12)*. The present work compares, for the first time, invertase productivity in solid-state fermentation by both haploid mutants, previously characterized as enzyme-overproducing strains in solid-state fermentation *(2,12)*, and diploid constructs of *A. niger* originating from such haploid mutants. We also used polyurethane foam (PUF) as an inert support absorbed with culture medium to simulate those features typically present in solid-state fermentation *(8)*.

Materials and Methods

Microorganisms and Media Composition

Haploid mutant strains used (Aw96-3 and Aw96-4) were originated previously from the wild-type strain *A. niger* C28B25 *(2)* belonging to the IRD-UAM collection. Both mutant strains were selected to isolate auxotrophic strains in order to induce the parasexual crosses, as described below. This allowed isolation of two diploid strains (DAR2 and AD96-4) derived from the haploid mutants. All strains were maintained in minimal medium (MM) agar slants at 4°C and silica gel grains *(12)* for short- and long-term conservation, respectively.

MM composition was as follows: 12.6 g/L of $(NH_4)_2SO_4$, 11.8 g/L of K_2HPO_4, 3.0 g/L of urea, 0.2 g/L of $MgSO_4$, 0.29 g/L of $FeSO_4$, 0.001 g/L of $MnSO_4$, 0.001 g/L of $CuSO_4$, and 0.001 g/L of $ZnCl_2$ supplemented with sucrose at a final concentration of 100 g/L. Before adding agar (15 g/L) when required, the pH was adjusted to 5.0 and then the medium was sterilized for 10 min at 15 psi. Spore suspensions were obtained from cultures grown for 72 h at 30°C in 250-mL Erlenmeyer flasks containing 50 mL of MM with agar. For those cases in which amino acid supplement was required, each amino acid was added to a final recommended concentration *(13)*.

Isolation of Mutants

Arginine and phenylalanine auxotrophs (Aw96-4arg$^-$ and Aw96-4phen$^-$) were isolated from strain Aw96-4, whereas an alanine auxotroph (Aw96-3ala$^-$) was isolated from strain Aw96-3. The auxotrophic mutants were isolated after an ultraviolet (UV) radiation process using low mutagen dose that resulted in a 50–60% survival rate *(14)*. Routine experiments consisted of 27 mL of a spore suspension, at a concentration of 8×10^6 spores/mL,

which were irradiated with UV light at 350 μW/cm^2. The low numbers of mutants induced in these conditions was compensated by a filtration enrichment method as described previously *(12,13)*.

Parasexual Crosses

Heterokaryon formation was induced by inoculating spores from two different strains (unable to grow in MM) in 5–10 mL of liquid amino acid–supplemented media for 24 h at 30°C. The resulting mycelia were harvested, rinsed with sterile distilled water, and then transferred to Petri dishes containing fresh MM. After an incubation period of 4–5 d at 30°C, vigorous growing sectors were detected, indicating heterokaryon formation. Heterokaryons were transferred twice to fresh MM until sporulation occurred. A spore suspension from sporulated heterokaryons was prepared in sterile water and 0.05% Tween-80, and then aliquots of this suspension were inoculated in MM sandwich plates. Diploid strains usually appeared on the surface of these plates as vigorously growing homogeneously sporulated colonies after 5–6 d. These colonies were then purified to obtain colonies from single spores on Petri dishes.

Solid-State Fermentation

PUF was cut into 0.5-cm cubes, washed three times with boiling water, and dried overnight in an oven at 70°C. Cultures were carried out in 250-mL Erlenmeyer flasks containing 1 g of dry PUF cubes. These cubes were sterilized at 15 psi for 15 min. Water activity (*Aw*) in MM was adjusted to 0.96 because this value has been commonly reported in solid-state fermentation trials *(2,12)*. Ethylene glycol at a final concentration of 8% was used as an inert *Aw* depressor *(7)*. MM was then sterilized separately as already described and then inoculated with corresponding spores. Every flask with PUF cubes was added to 25 mL of MM including spores from the corresponding strain at a concentration of 1×10^8 spores/g of carbon source. Incubation was carried out at 30°C for 48 h, and samples were collected periodically during time course cultures and then subjected to analysis.

Enzyme Assays

Crude extracts were obtained by gentle pressure on the PUF cubes within a 60-mL syringe (bringing the volume from 60 to 10 mL inside the syringe). The extracts were filtered through 0.45-μm Millipore membranes and then stored at 4°C for further analysis. Invertase activity was measured by the release of reducing sugars using 250 μL of 0.1 *M* sucrose as a substrate. The reaction mixture also comprised 200 μL of 0.1 *M* acetate buffer at pH 5.0 and 50 μL of properly diluted enzymatic extract. Incubation was set at 30°C for 30 min, and then the reaction was stopped by adding 1 mL of dinitrosalicylic acid reactive *(15)* and boiled afterward for 3 min to develop color. One invertase unit (IU) was defined as the amount of enzyme releasing 1 μmol of reducing sugars/min under assay conditions.

Protein determination in every sample was made according to Bradford *(16)* using a standard curve of bovine serum albumin.

Results and Discussion

Parasexual crosses between pairs of auxotrophic mutants allowed the isolation of two different diploid constructs: DAR2 (Aw96-3ala$^-$ × Aw96-4arg$^-$) and AD96-4 (Aw96-4arg$^-$ × Aw96-4phen$^-$). Putative diploids were streaked for single colony on MM and the spore size was determined under the microscope. The volume of diploid spores was found to be approximately twice that of haploid strains. Additionally, the diploid character was confirmed by the ability to generate auxotrophic sectors after haploidization on medium containing *p*-fluorophenylalanine at two different concentrations *(12)*: 60 and 90 mg/L. Auxotrophic sectors were recovered after 7 d from MM containing relevant amino acids plus *p*-fluorophenylalanine at either concentration analyzed. On the other hand, no signs of such sectors were detected after 10 d in the same MM without addition of *p*-fluorophenylalanine. The haploid strains (C28B25, Aw96-3, and Aw96-4) as well as the new diploid constructs were analyzed in solid-state fermentation at *Aw* 0.96 for volumetric and specific invertase production. As shown in Fig. 1, strain Aw96-4 showed an invertase activity peak of 2.2 IU/mL, which was even lower than the activity level observed for the wild-type strain C28B25 (10.5 IU/mL). Nevertheless, strain Aw96-4 was selected for the present work since this strain had been isolated originally as a pectinase-overproducing mutant in solid-state fermentation *(2,12)*. Recently, we have also shown that this strain, as well as another diploid, constructed between Aw96-4 and Aw99-iii (the latter strain is not part of the results described in this work), overproduce xylanases *(17)* and glucoamylases (unpublished results) in comparison with the wild-type strain C28B25.

The wild-type strain, however, also presented a rapid loss of activity as invertase detection decreased after the maximal peak detected (Fig. 1). By contrast, strain Aw96-3 exhibited constant growth in enzymatic production, reaching a peak of 12.2 IU/mL at the end of the experiment. Invertase production was higher for diploid strains; for instance, diploid DAR2 surpassed after 12 h of cultivation (15.9 IU/mL) those levels achieved by its parental haploid strains. Interestingly, autodiploid strain AD96-4 presented an unexpected increase in invertase production since maximal levels obtained with the corresponding parental strain Aw96-4 were considerably lower (48.18 and 2.2 IU/mL, respectively). Similarly, once protein levels had been estimated, specific activity revealed a significant increase for diploid strains. Crude extracts from DAR2 and AD96-4 strains obtained a specific activity of 2990 and 8050 IU/mg in only 12 and 18 h after inoculation, respectively. Haploid strains C28B25, Aw96-3, and Aw96-4 achieved a specific activity of 1540 IU/mg after 24 h, 1650 IU/mg after 48 h, and 410 IU/mg after 18 h of cultivation, respectively. This is an indication that

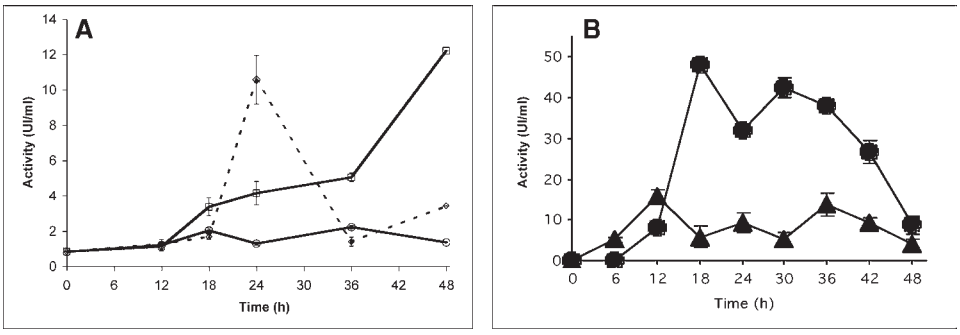

Fig. 1. Invertase production by *A. niger* strains in solid-state fermentation. **(A)** Haploid strains: C28B25 (– – ◇ – –), Aw96-3 (— □ —), and Aw96-4 (—○—); **(B)** diploid strains: DAR2 (—▲—) and AD96-4 (—●—). Every point is the mean of two independent replicates and SEs are also shown.

Table 1
Kinetic Parameters from Solid-State Fermentation Cultures at $Aw = 0.96$ Using Haploid and Diploid Strains of *A. niger*[a]

Strain	μ (h^{-1})	X_{max} (g/L)	E_{max} (IU/L)	$Y_{E/X}$ (IU/g X)	Productivity (IU/[L·h])
C28B25	0.114	40 ± 2.2	10,577 ± 1124	679	441
Aw96-3	0.055	36 ± 2.6	12,214 ± 203	342	254
Aw96-4	0.081	33 ± 3.1	2217 ± 180	99.4	61.6
DAR2	0.10	50 ± 4.2	15,893 ± 1070	26,225	1324
AD96-4	0.15	44 ± 3.3	48,185 ± 712	7967	2677

[a]Every point is the average of two independent replicates and SEs are shown. Specific growth rate (μ), maximal biomass production (X_{max}), maximal invertase production (E_{max}), and enzymatic yield ($Y_{E/X}$) are given. Estimation of μ was done using the best fit to logistic equation *(8)*.

these diploid strains not only increased the production of invertase in solid-state fermentation cultures, but also were capable of producing enriched extracts with good implications in terms of recovery costs in enzyme purification procedures.

Yeasts are traditionally used to produce invertase by submerged fermentation and considerable improvements have been reported *(18,19)*. However, invertase levels achieved by diploid AD96-4 (48.18 U/mL) in our study were similar to those reported using a *Saccharomyces cerevisiae* strain (45 U/mL) in a submerged fermentation *(20)*. This achievement is also a substantial improvement (up to 10-fold increase) in invertase production by different *A. niger* strains on solid-state fermentation *(8)*.

Table 1 summarizes kinetic data from solid-state fermentation cultures using different strains, including specific growth rate (μ), maximal biomass production (X_{max}), enzymatic yield ($Y_{E/X}$), and productivity measured at the maximal invertase production time for each strain. Diploid strains presented higher invertase levels in the crude extracts, as shown in

Fig 1. Additionally, these strains resulted in an up to sixfold increase in enzyme productivity (Table 1). Interestingly, yields ($Y_{E/X}$) were substantially improved in diploid strains, which were rebounded in better enzyme titers and productivities in solid-state fermentation.

Parasexual crosses between *A. niger* strains have been used to isolate diploid strains with improved enzyme production in submerged fermentation processes *(9,12)*. In fact, some of the parental strains were able to overproduce enzyme other than pectinases and invertase, such as xylanases, but the corresponding diploids have shown even better production patterns for those enzymes analyzed *(12,17)*. There have been successful attempts to increase citric acid production using strains isolated after diploid constructs between outstanding strains *(10,11)*.

The present work describes, for the first time, the improvement in invertase production using diploid strains isolated from parasexual crosses between mutants previously characterized as pectinase-overproducing strains specially adapted for solid-state fermentation *(2)*. The reason for these results is not yet clear, and further studies are required to elucidate the events behind an improved diploid strain. The strategy originally used to isolate those overproducing mutants led to the recovery of strains with an altered metabolism showing better performance in solid-state fermentation cultures *(2,12,21)*. The random mutagenesis could have targeted different regulatory genes whose alteration resulted in better adaptation for solid-state fermentation. Speculation for mutation in regulatory genes, rather than structural genes in particular, stems from the fact that in our laboratory we have detected overproduction patterns for several enzymes in these mutant strains and in the first diploid isolated as mentioned *(12,17)*. The isolation of auxotrophic mutant strains, even at low mutagen levels, might have introduced secondary mutations influencing protein production. However, when the arginine auxotrophic strain, derived from Aw96-4, was tested originally for pectinase production either on solid-state fermentation or submerged fermentation, it did not perform any better than the original mutant, even when media were supplemented with arginine *(22)*. This result can be explained since developmental defects resulting from arginine auxotrophy have been reported for *A. nidulans*, which include a poor induction of permeases involved in arginine utilization *(23)*. Additionally, the strain Aw96-4 was isolated also as a 2-desoxyglucose (2DG)-resistant mutant, and the corresponding arginine auxotrophic mutant did not change this 2DG resistance profile *(12)*.

Once some pairs of mutants with both similar genetic mutations and overproducing enzymatic patterns in solid-state fermentation have been selected, parasexual crosses may be induced between these preferred pairs. Resulting diploids would be expected to have an increase in enzyme gene doses with at least one noncomplemented mutation originally responsible for creating better performance in solid-state fermentation, especially for those cases in which more than one mutation favored production in solid-state fermentation in every haploid strain. Diploid strains resulting from

those crosses may behave quite differently, as was described with strain DAR2 and AD96-4 in our work. In fact, diploid construction has not always been useful in terms of enzyme production since in some studies activity levels have decreased *(24)*. However, as suggested earlier *(12)*, for diploids DAR2 and AD96-4, we used pairs of mutants previously known to overproduce enzymes in solid-state fermentation, thus increasing the possibilities of isolating further improved strains, as was demonstrated here.

To take advantage of all the conveniences of solid-state fermentation over submerged fermentation, it is important to use strains adapted to those conditions encountered in solid-state fermentation *(1,2)*. Using these improved strains will make more profitable those processes aimed at exploring and optimizing production of other commercially important enzymes in solid-state fermentation cultures.

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