

MUTANT DERIVATION AND PARASEXUALITY IN A CELLULOLYTIC STRAIN OF *Aspergillus niger*

Gustavo Henrique Goldman and João Lúcio de Azevedo

ABSTRACT

A genetic study was made of an industrial strain of *Aspergillus niger*. Growth on different culture media was tested and auxotrophic and morphological mutants were isolated. Heterokaryons were produced between pairs of auxotrophic mutants, and phototrophic colonies recovered from them. These were probably haploid, prototrophic recombinants derived from a parasexual process.

INTRODUCTION

Despite the great advances made in the genetics of filamentous fungi over the last few years, few studies are available on mutagenesis and recombination in filamentous fungi of industrial interest (Calam *et al.*, 1973; Bonatelli Jr., 1981; Bonatelli Jr. *et al.*, 1982; Ball and Hamlyn, 1982; Ball, 1984). In general, data obtained in studies on model organisms such as *Aspergillus nidulans* and *Neurospora crassa* are extrapolated to these microorganisms (Ball, 1984). *A. niger* is an industrially important species which produces several enzymes (amylase, glucoseoxidase, pectinase and cellulase), organic acids, antibiotics, etc. Genetical studies of industrial strains of *A. niger* have been carried out mainly in those which are citric acid producers. Following the discovery of the parasexual cycle in this species (Pontecorvo *et al.*, 1953a) studies were made into several aspects of the genetics of this fungus (Lhoas, 1961, 1967). In citric acid industrial strains, derived from high producing isolates (Bonatelli Jr. *et al.*, 1982), aspects of parasexuality and genetic instability were studied (Bonatelli

Jr., 1981; Bonatelli Jr. *et al.*, 1982, 1983; Masiero, 1988), resulting in the discovery of a variation of the parasexual cycle designated "parameiosis" (Bonatelli Jr. *et al.*, 1983); unstable diploids are produced inside the hyphae, followed by haploidization, and this results in the production of recombinant conidia direct from the heterokaryon. All these studies were carried out on a single industrial strain of *A. niger*. Despite its biotechnological importance, other strains of *A. niger* have not yet been studied extensively in terms of basic genetics (Bos, 1987).

In the present paper we describe genetic studies of the industrial strain, NRRL-337, of *A. niger*. It is currently used for amylase production, especially in the United States, and has been recently reported to be a good cellulase producer (Goldman, 1988). The aims of the present study were to determine fungal growth on different culture media, to isolate auxotrophic and morphological mutants, and to investigate the possible occurrence of parasexuality in this strain.

MATERIALS AND METHODS

Strain

A. niger NRRL-337 was kindly provided by Dr. Tobias Barreto de Menezes (ITAL, Campinas, Brazil).

Culture Media

Minimal medium (MM) and complete medium (CM) were those of Pontecorvo *et al.* (1953b) modified by Azevedo and Costa (1973); potato-glucose-agar (PDA). Czapek and corn-meal at respective concentrations of 3.9, 3.5 and 1.7% (Difco), and Sabouraud at a concentration of 6.5% (Oxoid Ltd) were also used.

Determination of growth media

The following growth media were used: Czapek, MM, PDA, corn-meal, Sabouraud and CM. Conidia were inoculated into the center of Petri dishes containing one of the above media and incubated at 28°C. Radial growth (mm) was measured 24, 48, 72, 96, 120 and 144 hours later.

Mutagenesis

A gamma-ray source (Co⁶⁰, CENA, USP) was used to obtain 5% survival. The dose was 800 Gray, given 2370 Gray per hour.

Number of nuclei per conidium

Nuclei were counted by the technique of Robinow and Caten (1969).

Genetic stability

Conidia from a single colony were inoculated into the center of Petri dishes containing CM. Plates were incubated for 96 hours, and sector production was determined at the end of this period.

Isolation of mutants

A suspension of 10^7 conidia/ml in tween-80 (0.1%, v/v) was treated with gamma-rays to obtain 5% survival. After treatment, conidia were plated onto Petri dishes containing CM at dilutions which gave isolated colonies, and were incubated for 3 to 4 days. Auxotrophic mutants were selected by the total isolation technique and classified by the auxonographic method of Pontecorvo *et al.* (1953b). Morphological mutants were isolated after visual inspection of CM plates. After conidial irradiation, mutants were also isolated by the filtration enrichment technique of Silveira and Azevedo (1984), with 12 hours being allowed between filtrations. Incubation was at 28°C in all experiments.

Syntrophism test

Conidia from the two different strains used in each cross were plated onto MM (10^5 conidia from each strain per plate) and incubated at 28°C for 7 days.

Genetic methods

Heterokaryons between mutant strains, and phototrophic colonies derived from these, were obtained by the technique of Roper (1952) by plating about 10^6 conidia from each heterokaryon on MM. Five colonies were isolated from each cross and classified for ploidy, relative to the parental mutants, by the following criteria: a) conidial diameter, measured with an ocular micrometer (Pontecorvo *et al.*, 1953b, Lhoas, 1967); b) segregation on plates containing CM plus 0.25, 0.5, 0.75, 1.0 or 2.0 microgrammes of benlate (Hastie, 1970) and c) polyacrylamide gel electrophoresis with specific detection of esterase activity (Laemmli, 1970; Paccola-Meirelles *et al.*, 1988).

RESULTS AND DISCUSSION

Table I shows the results of *A. niger* growth on different culture media. The Tukey test showed no significant difference at the 5% level between radial growth obtained on CM and on Sabouraud medium. Radial growth on Czapek, PDA and corn-meal also showed no significant differences. The CM was then used. Even though radial growth on Czapek medium was higher than that on the MM described by Pontecorvo *et al.* (1953), the MM was used because in this medium sporulation occurred 24 hours earlier, and a larger number of conidia were produced than on Czapek medium.

Table I - *A. niger* growth on different culture media.

Média*	Time (hours)**					
	24	48	72	96	120	144
MM	4.3	16.0	27.3	35.3	42.6	57.5 c***
Czapek	1.0	19.3	35.0	49.6	62.0	76.3 b
CM	7.7	22.7	54.0	78.0	89.0	89.0 a
PDA	9.7	34.0	54.0	63.3	68.6	76.3 b
Corn-meal	7.7	30.3	34.6	46.5	57.5	70.0 b
Sabouraud	9.0	37.5	62.5	75.5	80.5	85.0 a

* MM = minimum medium; CM = complete medium; PDA = potato-dextrose-agar.

** Colony diameter in mm.

*** Values followed by the same letter did not differ at the 5% level by the Tukey test.

Examination of 500 *A. niger* conidia showed that 21.4% were uninucleate, 77.2% binucleate and 1.4% trinucleate. No sectors were detected in 100 colonies tested on Petri dishes containing CM, showing that this strain has high vegetative stability and that the nuclei seem to have the same genetic constitution. According to Ball (1985), genetic stability is of fundamental importance in bioconversion processes at the industrial level.

Table II shows the seven morphological mutants obtained. Of these, four involved conidial color, two conidiophore arrangement, and one colony morphology. Table III shows the auxotrophic mutants with single and double deficiencies. Eleven auxotrophic mutants with a single deficiency and 15 with double deficiency were isolated. Most of these auxotrophic mutants showed a frequency of reversion to prototrophy of less than 1 in 10^6 to 10^7 conidia/ml. Mutants *leu1*, *leu2*, *pro1*, *phe2* and *lys2* showed a higher frequency of reversion, i.e., about 1 or slightly more than 1 in 10^6 to 10^7 conidia/ml.

Table II - Morphological mutants of *A. niger* obtained by gamma-ray irradiation.

Mutant	Phenotype
dbr	Dark brown conidia
fwn	Fawn conidia
lbr	Light brown conidia
whi	White conidia
con1	Sparse conidiation
con2	Sparse conidiation
pet	Reduced colony diameter

The yield of auxotrophic mutants from total isolation was 0.52%, and 2.0 to 4.8 times higher when filtration enrichment was used. The latter values agree with data reported for *Ophiostoma multiannulatum* (Fries, 1947), *Aspergillus awamori* (Fungaro, 1984), and *Metarhizium anisopliae* (Silveira and Azevedo, 1984; Bagalhi, 1987). However, these values are much higher than those obtained for the isolation of auxotrophic mutants in *A. niger* by Bonatelli Jr. *et al.* (1982) and by Masiero (1988). The filtration enrichment technique did not give a predominance of mutants with requirements for aminoacids as had been reported by Silveira and Azevedo (1984) and Bagalhi (1987). Silveira and Azevedo (1984) and Fungaro (1984) also reported that this technique was inefficient for the isolation of mutants with vitamin deficiencies. In the present study this was not the case, as mutants with biotin or nicotinic acid deficiency were obtained (Table III). These characteristics may possibly be specific to the biological material utilized rather than to the method employed.

Table IV shows the crosses performed between different mutants. From 10^6 conidia plated onto MM a variable number of phototrophic colonies were recovered in each cross ranging from few to almost full growth on the plate. In some, but not all crosses where colour mutants were present some non-black conidia colonies were produced.

Five colonies were selected on MM from the heterokaryon obtained in each cross, and haploidization was attempted through the use of benlate. These colonies, considered to be possible diploids, did not produce sectors on benlate. The conidial diameter of the parental mutants and of these putative diploids was measured, and no difference in size was detected. According to Rosim *et al.* (1978), there is some evidence that this criterion of conidial size measurement for the differentiation of haploids is not quite valid in *A. niger*. These investigators determined the differences in conidial size in haploids, diploids and heterokarya of three *A. niger* strains. The

Table III - Auxotrophic mutants of *A. niger* obtained by gamma-ray irradiation.

Mutant	Deficiency*
<i>pro1</i>	proline
<i>ths1</i>	sodium thiosulphate
<i>ade1</i>	adenine
<i>arg1</i>	arginine
<i>nic1</i>	nicotinic acid
<i>bio1</i>	biotin
<i>cyt1</i>	cytosine
<i>leu1</i>	leucine
<i>pro1 lbr</i>	proline
<i>pro1 dbr</i>	proline
<i>leu2 whi</i>	leucine
<i>bio1 arg1</i>	biotin and arginine
<i>bio1 lys1</i>	biotin and lysine
<i>bio1 yex1</i>	biotin and yeast extract
<i>leu1 yex2</i>	leucine and yeast extract
<i>leu1 ths1</i>	leucine and sodium thiosulphate
<i>leu1 bio2</i>	leucine and biotin
<i>leu1 arg2</i>	leucine and arginine
<i>leu1 lys2</i>	leucine and lysine
<i>leu1 nic1</i>	leucine and nicotinic acid
<i>pro1 met1</i>	proline and methionine
<i>pro1 phe1</i>	proline and phenylalanine
<i>pro1 arg3</i>	proline and arginine
<i>pro1 bio3</i>	proline and biotin
<i>pro1 lbr lys3</i>	proline and lysine
<i>pro1 lbr met2</i>	proline and methionine

* The symbols *lbr*, *dbr* and *whi* respectively represent morphological markers for light brown, dark brown and white conidial color.

results obtained in their study showed that the classification of haploid and diploid strains of *A. niger* on the basis of conidial size is not reliable. The high frequency of binucleate conidia in the present strain also made the distinction of haploid and diploid on the basis of conidial size unreliable.

Table IV - Crosses performed between different *A. niger* mutants.

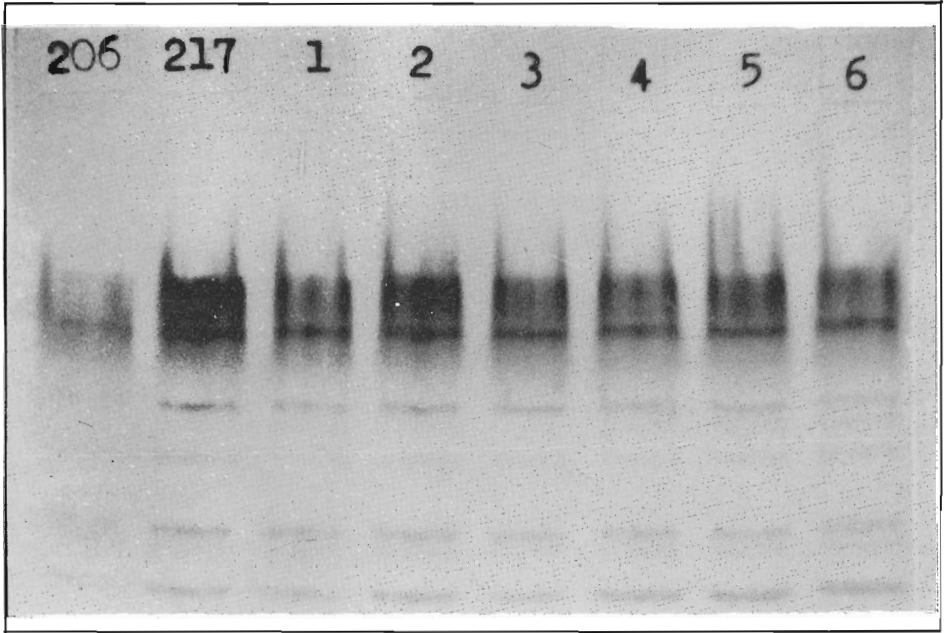
Crosses	
leu1 ths1	x pro1 lbr lys3
leu1 ths1	x pro1 phe1
bio1 lys1	x pro1 phe1
pro1 phe1	x leu2 whi
leu1 lys2	x pro1 lbr met2
leu1 lys2	x pro1 met1
leu2 whi	x pro1 lbr lys3

On the basis of the present results the following possibilities were proposed:

a) there was no heterokaryon formation but, rather, syntrophism between the two strains used for each cross; b) diploid colonies of high genetic stability were obtained; or c) the selection process on MM used in the present study may have selected haploid, phototrophic recombinants formed by a parameiotic process. In fact, no syntrophic interactions occurred between the various pairs of mutant strains used. Thus, heterokaryon formation did occur, and additional evidence for this was the recovery of prototrophic recombinants.

In haploid strains of fungi the diploid state is unstable, and haploid sectors are produced early in the presence of haploidizing agents. Although other haploidizing agents, such as p-fluorophenylalanine and chloroneb (Lhoas, 1961, and Azevedo *et al.*, 1977) were not tested, in the presence of benlate, a powerful haploidizing agent, no instability was detected in the prototrophic recombinants colonies recovered from the heterokaryons. Also, all strains crossed, and recombinants derived from them, were submitted to polyacrylamide gel electrophoresis for specific detection of esterase activity; this was in an attempt to detect possible pattern distinctions between the original strains and their recombinants. Figure 1 shows one of these electrophoretic runs involving the *pro1 x leu2 whi* cross. There was no difference in the pattern of alpha- and beta-esterase distribution between the possibly diploid colonies and the respective parental mutants. All the electrophoretic runs had the same patterns of band distribution as that shown in Figure 1. In addition, no bands with greater density than those of the parental types were observed. Thus, this method did not permit the detection of differences between parental strains and phototrophic recombinants. The high stability of the latter are, however, a strong indication that they are not diploid.

The hypothesis that haploid prototrophic recombinants were selected, would



206 = *pro1 lbr*;

217 = *leu2 whi*;

1 to 5 = prototrophic colonies derived from the heterokaryon;

6 = heterokaryon

Figure 1 - Polyacrylamide gel electrophoresis, with specific detection of esterase activity, of the cross *pro1 lbr lys3* x *ley 2 whi*.

require a highly unstable diploid nucleus. In such a process, termed "parameiosis" by Bonatelli Jr. *et al.* (1983), these heterozygous unstable diploid nuclei may show a high haploidization and recombination rate in heterokaryotic hyphae because of their instability. This phenomenon has been reported already for *A. niger* (Bonatelli Jr. *et al.*, 1983) and *M. anisopliae* (Bagalhi, 1987; Silveira and Azevedo, 1987) and may occur also in unstable diploids such as has been observed in *Penicillium patulum* and *Cephalosporium acremonium* (Calam *et al.*, 1973; Ball and Hamlyn, 1982). Although further genetic studies on the NRRL-337 strain of *A. niger* must be carried out, mainly using different strategies of recombinant selection, as was done for an industrial strain producer of citric acid (Bonatelli Jr. *et al.*, 1983), the results reported here are an indication that parameiosis is not a particularity of that strain but may occur also in other strains of *A. niger*.

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RESUMO

Um estudo genético foi realizado em uma linhagem industrial de *Aspergillus niger*. O crescimento em diferentes meios de cultura foi testado e mutantes morfológicos e auxotróficos foram isolados. Foram também produzidos hererocários entre pares de mutantes auxotróficos, sendo que colônias prototróficas foram recuperadas. Provavelmente, estas colônias eram recombinantes haplóides prototróficas derivados de um processo parameiótico.

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