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REVERSION BY CALCIUM OF A YEAST-LIKE DEVELOPMENT TO THE ORIGINAL FILAMENTOUS FORM, OF THE 10V10 5-FLUOROCYTOSINE-SENSITIVE MUTANT OF *ASPERGILLUS NIGER*

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ABSTRACT

Some filamentous fungi present the phenomenon of dimorphism, their morphological structure alterations being capable of inducing metabolism changes. The *Aspergillus niger* strain 10v10, a producer of citric acid, was submitted to the mutagenic action of ultraviolet irradiation which respectively selects mutants sensitive or resistant to the antifungi agent 5 fluorocytosine (5-FC). 5-FC sensitive mutants presented a morphological alteration to a yeast-like form. The effects of pH changes, addition of salts (KH_2PO_4, NH_4NO_3, MgSO4 and $MgCl_2$), the presence of osmotic stabilizers, as well as of calcium chloride, on morphological reversal and acid production were studied. Morphological reversal to the filamentous form was observed only in the presence of $CaCl_2$ (500mM) for the mutants strains 1 and 2, while the acid production occurred in both, yeast-like and filamentous forms.

Key words: *Aspergillus niger*, 5-fluorocytosine, yeast-like, calcium, acid production, dimorphism

INTRODUCTION

Some fungi are dimorphic, presenting two morphological forms, filamentous or yeast-like depending on environmental factors, fungal physiological condition or the fungal genome. They include saprobic forms such as *Mycotypha* sp, *Aspergillus parasiticus* and *Mucor* sp, as well as several important mammalian pathogens such as *Blastomyces dermatidis*, *Histoplasma capsulatum* and *Sporothrix schenkii* (9,11).

Aspergillus niger is a filamentous fungus of high industrial value due to its use in the manufacture of citric acid; it is utilized among others, by the pharmaceutical, alimentary and cosmetic industries. The 10v10 strain of *Aspergillus niger* has been utilized as a producer of citric acid in surface fermentation processes (2).

Microorganisms deficient in protein synthesis, supply citric acid in higher yields. A frequently utilized technique to promote that deficiency is the employment of antibiotics or antimicotics to obtain protein synthesis resistant, or sensitive microorganism (20). In the presence of cytosine, the antimicotic drug 5-fluorocytosine (5-FC) is carried in to the cell's interior, altering protein and DNA synthesis (5,7).

During the process of selection of 5-FC sensitive mutants, the appearance of yeast-like characteristics among these mutants was noted. The objective of the present work was to evaluate the reversal to the filamentous form of such mutants, and the possible influence of this change on citric acid production.

MATERIALS AND METHODS

Microorganism

Aspergillus niger 10v10, a local citric acid producer used in a surface fermentation process by the industry in the past, kindly supplied by Dr. João Lucio de Azevedo - Escola Superior de Agricultura Luiz de Queiroz/USP, was used as the parental strain to obtain mutants, studied in the present work.

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Culture media

Dextrose Sabouraud Agar at 4% (SB 4%) was utilized for strain maintenance. FCL medium (20) containing (g/L): glucose-120, ammonium sulfate-3.0, monobasic potassium phosphate-1.0, dibasic potassium phosphate-1.0, magnesium sulfate-0.5, manganese sulfate-0.014, ferric chloride-0.01, was utilized. The pH was initially adjusted to 3.0, with hydrochloric acid. For the isolation of sensitive or resistant mutants to 5-FC, the FCL medium was supplemented with 5-FC (0.1 µg/mL). The 4% SB medium was also employed to verify the effects of salts and osmotic stabilizers (KCl, $MgSO_4$ and Sorbitol); to study the effect of calcium on the morphology, 500 mM $CaCl_2$ was added to the medium.

Mutagenesis of the parental strain of *A. niger* 10V10

Briefly, conidia from 5-7 day old colonies were collected in Tween 80 solution (0.1% v/v) and the suspension adjusted to 10^6/mL with saline (NaCl 0.85%w/v); 0.5 mL of the conidia suspension plus 9.5 mL of saline was put on a Petri dish and submitted to ultraviolet light treatment (Philips TUV,15w,254nm) for 15 min. under magnetic agitation as described before (12). Ten mL of the irradiated suspension were inoculated into 50 mL of FCL/ 5-FC medium, in 250 mL Erlenmeyer flasks, and incubated for 72 h in a water bath, at 30ºC, with a 125 rpm shaking movement, as described before (20). After each 24 h period, the FCL/5-FC was filtered through glass wool, so that the conidia that had germinated in the presence of 5-FC, would remain in the glass wool to be seeded into the SB/ 5-FC medium. Non-germinated conidia, considered sensitive, were collected from the filtrate; following centrifugation at 10,000 g for 2 min, they were washed with sterile distilled water, suspended in 0.5 mL of the FCL medium and re-inoculated into 50 mL of the FCL/5-FC medium. After completing the 72-h incubation period, the filtrate containing the sensitive conidia was seeded on Petri dishes containing the SB medium, to obtain isolated colonies.

Confirmation of sensitivity or resistance of selected *A. niger* mutants

Isolated mutants showing yeast-like morphology were inoculated into the 4% SB medium containing 5-FC (0.1 µg/mL) and incubated for 48 h at 30ºC. SB medium without 5-FC, was utilized to evaluate fungi growth. Mutants showing only growth in the 5-FC-free medium were considered to be sensitive to the drug; mutants presenting growth in both SB 4% 5-FC containing and 5-FC-free media were considered to be resistant to the drug. All mutants chosen were maintained on SB 4% plates at 4ºC, following the 48 h, 30ºC growth period.

Effect of pH alteration on chosen mutant strains

Selected yeast-like mutant strains were seeded in 4% SB and the medium pH adjusted with 0.5M HCl or 0.5M NaOH to evaluate growth at pH 4, 6 and 8, respectively. Yeast-like mutants were inoculated and incubated at 37ºC for 48 hours.

Effect of the addition of NH_4NO_3, $MgSO_4$, $MnCl_2$ and KH_2PO_4

Different salts were added at different concentrations to the 4% SB plated medium as follows: NH_4NO_3(0.4; 0.8; 1.2; 1.6; 2.0; 2.4 g/L); $MgSO_4$(0.01;0.02; 0.03; 0.04; 0.05; 0.06 g/L); $MnCl_2$(0.012; 0.018; 0.024; 0.030; 0.036; 0.048 mg/L) and KH_2O_4(0.2; 0.4; 0.8;1.0; 1.2; 1.4 g/L). The yeast-like strains were inoculated into dishes containing the various salt concentrations, incubated at 30ºC and their growth followed for 72 h.

Addition of osmotic stabilizers to the culture medium

The three osmotic stabilizers, KCl 0.6 M, $MgSO_4$ 1.2 M and Sorbitol 1.2 M, were added to the culture medium and the plates incubated with the yeast-like mutant strain for 72 h at 30ºC.

Effect of $CaCl_2$

The yeast-like mutant strains were inoculated into 100 mL of the FCL medium containing 500 mM $CaCl_2$ in 250 mL Erlenmeyer flasks, and incubated at 30ºC, with a 125 rpm shaking motion for nine days. The flasks with the cultures were then kept in the laboratory for further observation.

Citric fermentation, determination of titratable acidity and dry weight of the mycelia

Fermentation to citric acid was performed on a laboratory scale by the submerse procedure, using 250mL Erlenmeyer flasks, containing 100 mL of the FCL culture medium, at 30ºC, with constant shaking at 125 rpm, for nine days, in a Dubnoff bath. Following this period of fermentation, the biomass formed was separated from the liquid medium by filtration, washed with distilled water, and maintained in an oven at 98ºC for 24 h; following drying, it was weighed to obtain the weight of the mycelia (16), The determination of the total acidity produced was made by titration with 0.1 N NaOH and a phenolphthalein indicator (20).

RESULTS

Mutagenesis induced by ultraviolet irradiation of the conidia of the parental strain *A. niger* 10v10, and the inoculation into liquid FCL media containing 5-FC, permitted the isolation of eight mutants of strain 10v10, presenting wrinkled, brain-like colonies with a creamy-like consistency. After secondary subculture, the colonies presented growing bright, smooth, white mucoid aspect, denominated yeast-like. They were classified as 5-FC-sensitive. Nine 5-FC-resistant, filamentous mutants, of sporulated, dark brown to black colored colonies, showing morphological characteristics similar to those of the parental strain of *A. niger* 10v10, were also isolated, but not utilized in the present work.

The yeast-like mutant strains, were kept in 4% SB medium, and analyzed for the effects of pH changes, addition of salts, of osmotic stabilizers or $CaCl_2$, as shown on Table 1.

Table 1. Evaluation of the morphological effect of different agents which promote metabolic alterations in the morphology of the yeast-like mutants (1 to 8) from the 10v10 *A. niger* parental strain.

Agents		Morphological alteration		
		Growth		Reversal
		+	-	
pH	4.0	1,2,3,4,5,6,7,8	-	-
	6.0	1,2,3,4,5,6,7,8	-	-
	8.0	1,2,3,4,5,6,7,8	-	-
NH_4NO_3	0.4	2,4,5,7	1,3,6,8	-
(g/L)	0.8	2,4,5,7	1,3,6,8	-
	1.2	2,4,5,7	1,3,6,8	-
	1.6	2,4,5,7	1,3,6,8	-
	2.0	2,4,5,7	1,3,6,8	-
	2.4	1,2,3,4,5,6,7,8	-	-
$MgSO_4$	0.01	2,5,7	1,3,4,6,8	-
(g/L)	0.02	2,5,7	1,3,4,6,8	-
	0.03	2,5,7	1,3,4,6,8	-
	0.04	2,5,7	1,3,4,6,8	-
	0.05	2,5,7	1,3,4,6,8	-
	0.06	2,5,7	1,3,4,6,8	-
KH_2PO_4	0.2	2,5	1,3,4,6,7,8	-
(g/L)	0.4	2,5	1,3,4,6,7,8	-
	0.8	2,5	1,3,4,6,7,8	-
	1.0	2,5	1,3,4,6,7,8	-
	1.2	2,5	1,3,4,6,7,8	-
	1.4	2,5	1,3,4,6,7,8	-
$MnCl_2$	0.012	1,2,3,4,5,6,7,8	-	-
(g/L)	0.018	1,2,3,4,5,6,7,8	-	-
	0.024	1,2,3,4,5,6,7,8	-	-
	0.030	1,2,3,4,5,6,7,8	-	-
	0.036	1,2,3,4,5,6,7,8	-	-
	0.048	1,2,3,4,5,6,7,8	-	-
KCL	0.6 M	1,2,3,4,5,6	7,8	-
$MgSO_4$	1.2 M	1,5	2,3,4,6,7,8	-
Sorbitol	1.2 M	1,2,5,6,7	3,4,8	-
$CaCl_2$	500 mM	3,4,5,6,7,8	-	1,2

Mutants strains 1, 2, 5 and 6 were selected to be photographed under the optical microscope during the four following months. Added at a 500-mM concentration, $CaCl_2$ promoted morphological reversion of the yeast-like mutant strains 1 and 2, to the filamentous condition. As it can be observed in Fig. 1- a, b, c and d both strains initially showed globular cells, subsequent budding characteristic of the yeast-like state, and finally cell grouping and formation of hyphae, characterizing the filamentous original fungus.

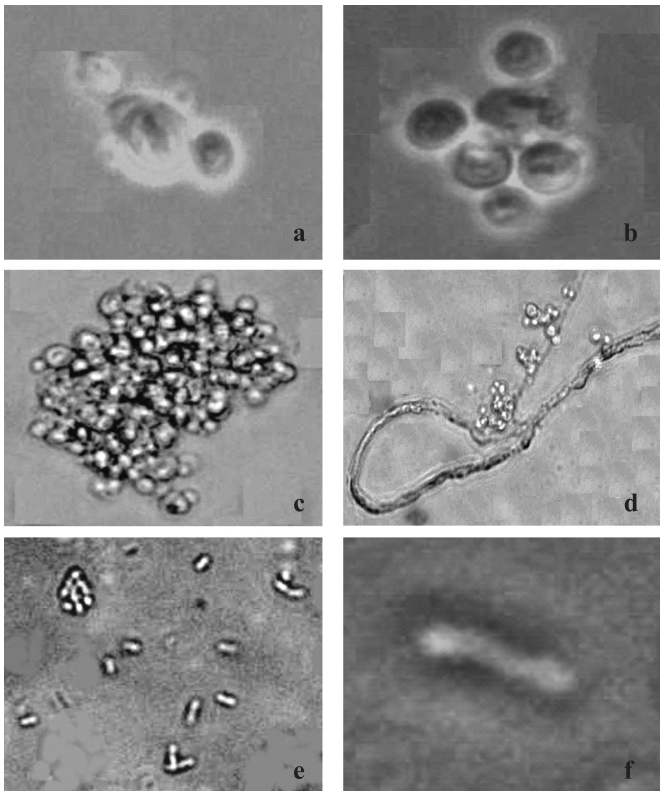

Figure 1. Visualization under the optic microscope: a) globose, yeast-like cell (1000x); b) cell grouping (1000x); c) cell grouping (40x); d) hyphae formation (400x); e) morphological aspect of strain 5 (400x); f) morphological aspect of strain 5 (1000x).

Mutant strains 5 and 6 presented a microscopic appearance, which remained unchanged during the four months of the photographic follow- up. The cylindrical cell aspect can be seen on Fig. 1 (e and f).

The parental strain of A. *niger* 10v10 was developed to be utilized in processes of surface fermentation (2). In the present work, the submerged fermentation process was utilized, the parental strain serving as control. After nine days of fermentation, the dry weight of the mycelium of each mutant yeast–like strain and the accumulation of the titratable acidity produced, were measured (Table 2). Only the four selected mutants (1,2,5,6) were evaluated.

The yield of acid produced by the strains which underwent morphological reversal from the yeast-like to the filamentous form, was greater in the filamentous form for mutant 1 than in the yeast-like form and smaller in the filamentous form for mutant 2 than in the yeast-like form (Table 2). The dry weight of strain 1 presented only a slight variation according to their morphological state however, strain 2 when in the filamentous form, showed a significant increase in dry weight (Table 2).

Table 2. Determination of mycelial dry weight and acid production by yeast-like mutant strains which underwent reversion from the yeast-like to the filamentous state in the presence of 500 mM $CaCl_2$, and of the parent strain of *A .niger* 10v10.

Strain	Morphological state of the culture					
	Yeast-like			Filamentous		
	Dry weight g/L	Acid production mmol H^+/ L	mmol H^+/ g dry weight	Dry weight g/L	Acid mmol production H^+/ L	mmol H^+/g dry weight
1	1.301	59	45.38	1.349	80	59.70
2	1.170	32	27.35	11.682	97	8.30
3	1.043	48	-	-	-	-
4	1.090	34	-	-	-	-
5	1.398	33	23.74	-	-	-
6	0.960	28	29.16	-	-	-
7	0.411	30	-	-	-	-
8*	-	-	-	-	-	-
10v10	-	-	-	7.209	155	21.52

* Mutant strain 8 did not show enough growth.

DISCUSSION

Studies of great importance, involving pathogenic fungi have been centered on the investigation of dimorphism, employing the technique of inducing hyphae growth and examination of one or more of their specific properties (1,3). Factors inducing hyphae development of *C. albicans* are quite varied, and include pH, minerals, vitamins, amino acids and carbohydrates (11).The pH and temperature activate genetic elements related to fungi morphology, that regulate the formation of either yeast-like, or filamentous forms. Yeast-like cells of *C. albicans* grow at pH 4.5 and 25ºC, while filamentous growth is induced by pH 6.8 at 37ºC. *Penicillium marnefei* grows in culture at 25ºC on Sabouraud agar as a mycelial fungus, typical of the genus; however, it shows yeast-like cells when growing at 37ºC *in vitro* on appropriate media, or *in vivo* in infected animals and humans (19).Generally, *in vitro* conditions used to induce hyphae formation require a pH close to neutrality (3,19).

Change of pH to 4, 6 or 8, and of temperature to 37ºC, tested in this work, did not yield filamentous development by mutant yeast-like strains of *A. niger* 10v10; this does not agree with the results obtained by others (3,19), that verified the expression of morphology changes associated to pH.

No morphological reversal was observed by us in the presence of various concentrations of $MgSO_4$, $MnCl_2$ or KH_2PO_4, that permitted yeast-like development of the strains studied. The salts tested are related to the yield of citric acid production and some morphological changes of the fungi, observed in their presence or absence. Several authors (4,13,21,22) have verified such morphological alterations.

Osmotic stabilizers are widely used to maintain protoplasm integrity following the removal of the cell wall. Among the most common ones utilized, are inorganic salts, sugars and sugar alcohols. In general, inorganic salts have been proven to be most efficient for yeasts (24). In the presently studied mutant strains, KCl, Sorbitol and $MgSO_4$, useful as osmotic stabilizers, did not lead to morphological reversion.

Detroy and Ciegler (4), verified hyphae - yeast-like dimorphism in *Aspergillus parasiticus* in respectively, the presence or absence of manganese ions in the culture medium, observing also that the addition of amino acids, vitamins, and traces of other metals did not induce significant effects on morphogenetic development. The cultures were mostly yeast-like in the presence of $7.3x10^{-4}$mM manganese; however, high manganese concentrations resulted in hyphae formation.

Calcium is an important element for growth, metabolism and differentiation of several fungi and yeasts (6,23). It participates in multiple regulatory functions including sporulation, ramification, tip growth and hyphae reorientation, direction of localized stimuli, bud germination, regulation of dimorphism, sexual reproduction and control of the cell cycle has been described (23). At high concentration, calcium is toxic; for this reason, cells utilize a complex homeostatic system to maintain low levels of calcium concentration (6).

Pera and Callieri (18), verified that the addition of 0.5 g/L of $CaCl_2$ to the culture medium, promoted an increase in mycelium ramification in the filamentous strain of *A. niger.* The presence of short and tall ramifications of the hyphae, stimulated pellet formation, as it was also observed by others (10,15).

Madi *et al*. (14) studied the effect of the addition of calcium to cultures of *Aureobasidium pullulans*, In the presence of low

Ca^{2+} concentrations, increased formation of yeast-like cells and reduced morphology transition were observed. When the concentration of Ca^{2+} was raised, the development of a 3-4 mm diameter pellet was observed, with a predominance of filamentous development. In the present study, 500-mM $CaCl_2$ induced the formation of hyphae in yeast–like mutant strains of *A. niger* 10v10. It must be observed that the induction of yeast-like mutants was found only for the 10v10 strain; other strains like N402 (8) did not show this effect after the same treatment, even if repeated several times.

A long time interval, of approximately 120 days, was required to obtain the yeast-like reversion to filamentous form, as also observed by Paula *et al*.(17) who, in their experiments, obtained morphological reversion of *Scopulariosis brevicaulis* after a two-month period of cultivation. This indicated that the strains remained for a long time in a transition state, of partial reversal. In the present study, strains 5 and 6 did not show reversal after four months, indicating that their morphological stability remained in a transition phase between filament and yeast, as also previously verified (17).

5-FC employed in chemotherapy of systemic candidiasis, interferes with nucleic acid synthesis. It is taken into fungal cells by a cytosine permease, deaminated to 5-fluorouracil, converted to the nucleosideo triphosphate and incorporated into RNA, where it causes miscoding. In addition, 5-FC is converted to deoxynucleoside, which inhibits thymidylate synthase and thereby DNA biosynthesis (7).However, there still exist doubts about its side effects.

Acid production, a characteristic of the *A. niger* 10v10 strain, was found both in the yeast-like mutants, and in the revertant filamentous form (Table 2).

In summary, this study shows the induction of the yeast-like development in *A. niger* strain 10v10 and its reversion to filamentous morphology in some mutants, in the presence of 500mM calcium chloride.

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RESUMO

Reversão pelo cálcio de um desenvolvimento leveduriforme para a forma filamentosa original em um mutante sensível a 5-fluorocitosina na linhagem 10V10 de *Aspergillus niger*

Alguns fungos filamentosos apresentam o fenômeno de dimorfismo, sendo que as alterações da estrutura morfológica podem induzir alterações metabólicas. A linhagem de *Aspergillus niger* 10v10, produtora de ácido cítrico foi submetida à ação mutagênica da radiação ultravioleta selecionando mutantes sensíveis ou resistentes ao antifúngico 5-fluorocitosina (5-FC). Os mutantes selecionados como sensíveis a 5-FC apresentaram uma alteração morfológica com desenvolvimento leveduriforme. Nestes mutantes foram avaliados o efeito da alteração de pH, a adição de sais (KH_2PO_4, NH_4NO_3, $MgSO_4$e $MnCl_2$), a presença de estabilizadores osmóticos, cloreto de cálcio, e o seu efeito sobre a reversão morfológica e a produção de ácido. A reversão morfológica para a forma filamentosa ocorreu apenas na presença de $CaCl_2$ (500mM) para as linhagens mutantes 1 e 2, enquanto que a produção de ácido ocorreu nas duas formas, leveduriforme e filamentosa.

Palavras-chave: *Aspergillus niger*, 5-fluorocitosina, leveduriforme, cálcio, produção de ácido, dimorfismo

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