Pectinase-hyperproducing mutants of *Aspergillus niger* C28B25 for solid-state fermentation of coffee pulp

Philip Antier*, Alfredo Minjares†, Sevastianos Roussos*, Maurice Raimbault* and Gustavo Viniegra-Gonzalez†

* *ORSTOM: French Research Institute for Development in Cooperation*
† *Department of Biotechnology, Universidad Autónoma Metropolitana-Iztapalapa*

The aim of this study was to improve mold strains for the production of pectinases by solid-state fermentation (SSF) of coffee pulp. A screening of 248 strains, isolated in Mexico's coffee-growing areas, permitted us to select a wild strain of Aspergillus niger *which in 72 h attains a peak production of 27.7 U ml^{-1} (138 U g^{-1} dry pulp) of pectinase measured by viscosimetry. Through the use of a selective culture medium with low water activity (a_w = 0.954) with 2-deoxy-glucose (2DG) it was possible to isolate pectinase-hyperproductive mutant strains for SSF (44.5 U ml^{-1}, 228 U g^{-1} dry pulp). Derepressed mutant strains that hyperproduced pectinase by submerged fermentation (SmF) were also obtained using a classic selective medium with high water activity (pectin + 2DG) with a_w = 0.999. A comparison between both classes of mutants, called AW96 and AW99, respectively, points out the need to design special selective media in order to obtain strains adapted either to SSF or SmF in which the a_w level would be a key selecting factor.*

Keywords: *Aspergillus niger;* improved strains; pectinases; water activity; solid-state fermentation; coffee pulp

Introduction

Coffee pulp consists of approximately 40% of several million tons of coffee cerises processed by the "wet method" of coffee milling used in Mexico, Central America, and Colombia.[1] Improving the value of coffee by-products has become a priority in coffee-producing countries, for economic and ecological reasons.[1–4] Various alternatives have been suggested for disposing of the pulp.[1,3–5] In particular, we have studied pectinase production by solid-state fermentation (SSF) using this by-product as a fermentable substrate.[5]

According to Pulgarin *et al.*,[2] coffee pulp contains around 6.5% pectin, and Zuluaga[6] has found 23–27% fermentable sugars (dry weight), mainly fructose (10–15%), sucrose (2.8–3.2%), and galactose (1.9–2.4%). Therefore, it constitutes an appropriate substrate for mold growth and pectinase production by the SSF[4,5] process. Trejo *et al.*[7] have shown that the pectinase productivity of the SSF process is much higher than the productivity obtained by submerged fermentation (SmF). Besides, the pectinase solution obtained may be utilized directly to speed up the coffee fermentation process used as part of the wet method to obtain coffee grains with a homogeneous quality.[5] Fogarty and Kelly[8] estimated a value of £165 million per annum in the pectinase market for various kinds of industrial processes. Pectinase-hyperproducing mutant strains of *A. niger*,[9–11] *P. occitanis*,[12,13] or *Verticillum albo-atrum*[14] have been obtained by different authors for SmF. For the great majority of these strains, the selection was made by looking for mutants not sensitive to catabolic repression (CR). Industrially, pectinases are produced using both SSF and SmF cultivation techniques with strains of *A. niger*, but SSF is generally considered more suitable for rendering higher yields of pectin esterase (E.C. 3.1.1.11) and polygalacturonase (E.C. 3.1.1.15).[15,16] According to some reports,[10,11] disruption of the mycelium decreases pectinase production in liquid fermentation. Shankaranand *et al.*[17] have indicated "the need for an extensive screening programme for the selection of a potent culture most suited to the SSF system." However, there is a lack of specific protocols for the selection of mold strains adapted to the SSF process, and such a lack of protocols limits the development of this fermentation technique.[18] The osmotic gradient due to heteroge-

Address reprint requests to Dr. G. Viniegra, Av. Purísima. esq. Michoacán, Apdo. 55-535. CP 09340 México, D. F., Mexico
Received 20 January 1992; revised July 1992

neous distribution of solutes, the matrix structure due to substrate porosity, and adsorption forces can be recognized as key factors in SSF.[19,20] More specifically, the low level of water activity (a_w) of the solid substrate has a significant effect on the physiological activity of microorganisms and enzyme production.[21] Oriol *et al.*[20] have calculated that SSF of cassava starch by *Aspergillus niger* reduced a_w from 0.95 to 0.85, indicating that this parameter may be indeed one of the most limiting factors of the SSF process.

In this work, the importance of a_w in SSF for pectinase production is stressed. A specific selective medium for obtaining pectinase-hyperproducing mutants is proposed. It is based on the use of selective media with low or high values of a_w together with the presence of deoxyglucose in order to obtain derepressed strains specifically adapted to SSF or SmF techniques, respectively.

Materials and methods

Microorganisms

The fungal strains used during the first stage of this work (screening of wild strains) belong to the ORSTOM-UAM fungal collection. These strains were previously isolated directly from coffee plants or from soil in coffee plantations.[22] The reference strain was *A. niger* CH4 from the Biomedical Research Institute, UNAM (Mexico).

Culture media

All microorganisms were maintained on potato dextrose agar (PDA) medium at 4°C. Pectin used was purchased from Sigma Chemical Co. (Cat. No. P-9135) having 85% galacturonic acid and 9.5% methoxyl content. Basal mineral medium (BMM) with 10 g l^{-1} pectin was used for assays in Petri dish and Erlenmeyer flasks, with the following additional nutrients (g l^{-1}): KH_2PO_4 1.3, Na_2HPO_4 0.60, $(NH_4)_2SO_4$ 0.75, urea 0.25, $MgSO_4$ 0.3, and $CaCl_2$ 0.3. For selecting colonies at high a_w level, after UV mutagenesis, Medium AW99 was used; it contained BMM, agar 15 g l^{-1}, pectin 10 g l^{-1}, and 2-deoxy-D-glucose (2DG) 0.01 g l^{-1}. For selecting colonies at low a_w, AW96 medium was used; it contained BMM, agar 15.0 g l^{-1}, pectin 10.0 g l^{-1}, ethylene glycol (EG) 15% (v/v), and 2DG 0.1 g l^{-1}. This medium helped to select very few fast-growing and sporulating colonies. For SSF, coffee pulp was obtained from a coffee processing plant near Jalapa City, Veracruz State (Mexico). The sample was sun-dried and ground (60 mesh). Coffee pulp composition has been reported by some authors[2,4,22] and has been commented on in the previous section. KH_2PO_4, $(NH_4)_2SO_4$, and urea were added to the dried pulp (DP) in proportions of 4.9, 8.0, and 4.3%, respectively. Coffee pulp was sterilized for 30 min at 10 psi. The initial pH for all media was adjusted to 5.5 by addition of H_3PO_4 0.1 M or NaOH 0.1 M.

Culture conditions

PDA medium was used for the production of spores, using an incubation temperature of 25°C. Spore suspensions for further experimentation were prepared following the technique described by Raimbault and Alazard.[24]

Erlenmeyer flask cultures. Fifty milliliters of BMM in 250-ml flasks containing 10 g l^{-1} pectin were inoculated with 5×10^4 spores per milliliter. All cultures were grown in a shaking incubator for 72 h at 25°C and 180 rev min^{-1} and then filtered through Whatman paper No. 4; the filtrate was collected and used for the enzyme assays.

SSF on coffee pulp. The SSF trials were carried out at laboratory scale, using glass cylinders (2.0 cm internal diameter) similar to those described by Raimbault and Alazard,[24] and maintained at 25°C within a thermostated water bath. Every cylinder was packed with coffee pulp (15 cm height) and aerated at the fixed rate of 60 ml min^{-1} with an air current saturated with water. Each fermenting column contained 20 g of coffee pulp (60 mesh) with a moisture of 60% (a_w = 0.960). It was found that after water saturation, the coffee pulp substrate reached a steady state a_w value of 0.960. The inoculum consisted of 2×10^7 spores g^{-1} DP. The a_w value remained within the range of 0.960 ± 0.005 throughout the fermentation time. Each experiment was repeated four times and the mean results are reported for each repetition.

Isolation of mutants

Five milliliters of conidia suspension in a Petri dish were exposed to ultraviolet (UV) radiation at a distance of 11.5 cm. The UV-treated conidial suspension was cultured on the appropriate selective medium. Mutants isolated on AW99 with 10 mg l^{-1} of 2DG were labeled as *dgrAW99* (deoxyglucose-resistant at high a_w) and mutants isolated on AW96 (15% EG and 100 mg l^{-1} 2DG) were labeled as *dgrAW96* (deoxyglucose-resistant at low a_w). The later mutants were selected because of their faster rate of colonial expansion and earlier sporulation, which distinguished them from very slowly expanding colonies.

Analytical methods

Growth in liquid fermentation was determined by drying biomass (DW) at 80°C for 24 h. Water activity was measured at equilibrium by means of a Water Activity System-Decagon device. For the analysis of samples obtained by SSF, 10 g of fermented coffee pulp was mixed with 10 ml of water and pressed to 1000 psi with a hydraulic press. The extract obtained was then centrifuged at 6000*g* for 5 min. Supernatants were used for enzyme assays. The pectinase activity (presumably to be mainly of the type, poly-1, α-D-galacturonide glycanohydrolase, E.C. 3.2.1.15[25]) was assayed through the measurement of the reduction of viscosity of a pectin solution (20 g l^{-1}, buffer citrate-phosphate, 0.1 M, pH 5.5) using a Brookfield Viscosimeter. Thus 18 ml of the pectin solution and 1 ml of the enzyme

Table 1 Screening of 248 wild strains according to their growth on SC medium in Petri dishes and the corresponding diameter of the visible hydrolyzed zone

			$H_i/C_i > 1$			
	$C_i = 0$	$H_i/C_i = 1$	$A' < 0$	$A' = 0$	$A' > 0$	Total
Tested strains	12	117	97	7*	15*	248
(%)	(5)	(47)	(39)	(3)	(6)	(100)

C_i, Diameter of colony i
H_i, Diameter of hydrolyzed zone around colony i
C_{ref} and H_{ref}, Reference values (*A. niger* CH4)
* Strains selected to be cultivated by SSF

$$A' = \frac{(H_i/C_i - H_{ref}/C_{ref})}{(H_{ref}/C_{ref})}$$

solution were incubated at 45°C for 10 min. One unit (U) of pectinase activity was defined as the amount of enzyme which reduced the initial viscosity by 50% in 10 min.

In Petri dishes the diameter of the hydrolyzed zone around a colony was considered to be an indicator of the pectinase activity. To visualize the pectinase clearance zones, medium SC was used. It contained BMM, 15.0 g l^{-1} agar, and 2.0 g l^{-1} pectin, and was developed by flooding the plates with a 1% w/v aqueous solution of hexadecyltrimethylammonium bromide (HTAB; Sigma Chemical Co. Cat. No. H-5882).[23] This activity was expressed through a coefficient A', which represents the percentage of surplus activity with respect to the reference strain:

$$A' = \frac{(H_i/C_i - H_{ref}/C_{ref})}{(H_{ref}/C_{ref})}$$

where

H_i = diameter of hydrolyzed zone
C_i = diameter of the colony
ref = reference *A. niger* CH4
A' value of the reference = 0

Results and discussion

Screening of wild strains

Our first objective was to select, from the UAM-ORSTOM mold collection, the pectinase-producing wild strains best suited to SSF of coffee pulp.

Table 1 shows the results obtained from the analysis of 248 strains cultured in Petri dishes. Nearly 5% of those did not produce colonies on the selective medium SC ($C_i = 0$). This might be due to the absence of the enzyme system needed to metabolize pectin. However, 47% of the strains grew on this medium, but they did not produce a visible pectin hydrolysis zone ($H_i/C_i = 1$). The remaining 48% grew and produced a clear zone. From this latter group, we selected 22 strains (9%) to be cultivated by SSF because their A' coefficients

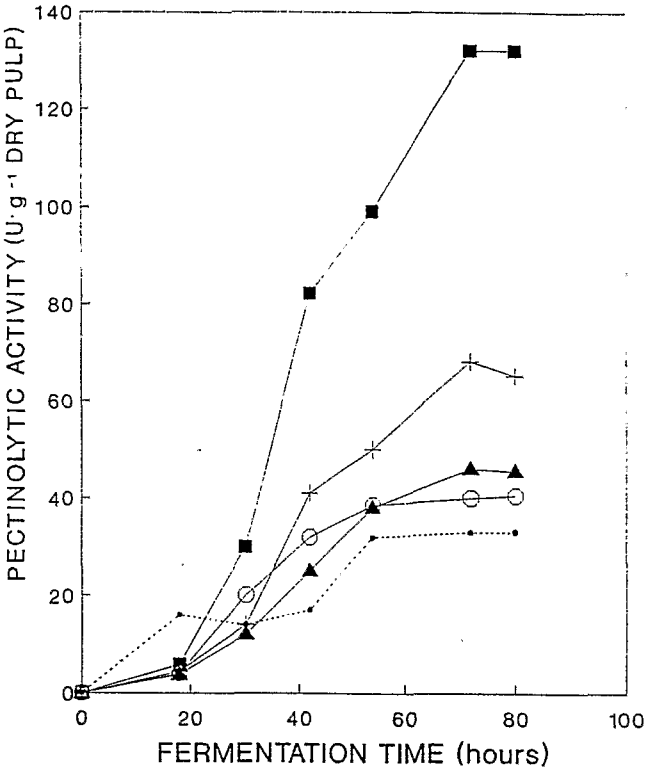


Figure 1 Pectinase production during SSF on coffee pulp by the wild strains of molds with higher enzyme activity than the reference strain. *A. niger* CH4 (ref) (● ··· ●); *A. niger* C28B25 (■–■); *A. niger* C16C25 (O–O); *A. niger* C17B25 (+–+); *Penicillium* sp C15B25 (▲–▲)

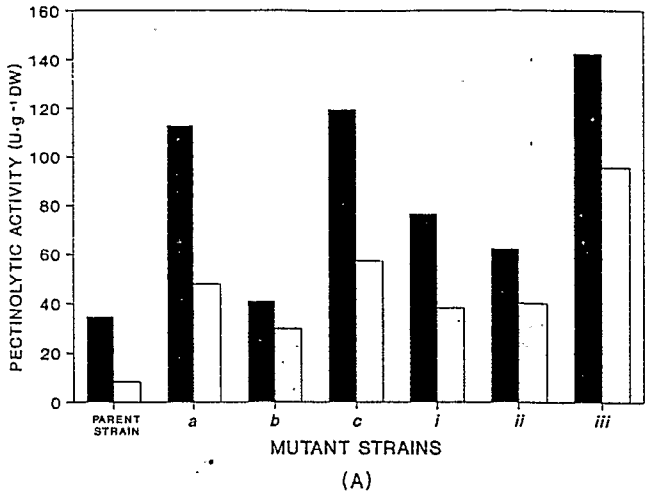
corresponded to "pectinolytic activity" levels equal to or above that of the reference strain.

When the latter strains were grown on coffee pulp by the SSF technique, four of them were found with pectinase activities above the reference level of *A. niger* CH4 strain (*Figure 1*). Three of these strains were found to be *A. niger* (C16C25, C17B25 and C28B25), and the other one was *Penicillium* sp. (C15B25). The highest activity levels were produced by these strains after 3 days of coffee pulp fermentation and remained constant for 5 days. The highest activity value was 135 U g^{-1} DP for the *A. niger* C28B25 and well above the reference level (35 U g^{-1} DP).The extract obtained by pressing fermented coffee pulp contained 27.7 U ml^{-1} of pectinase activity. This strain was, therefore, chosen to be mutagenized in the next step of this work.

The results of SmF experiments with *A. niger* C28B25 incubated with pectin alone or pectin plus glucose or sucrose are shown in *Table 2*. There it can be seen that the pectinase system was sensitive to CR if the sugar concentration was between 5 and 10 g l^{-1}, because the total and specific activities decreased below the basal level (solely pectin). However, the addition of smaller levels of sugar (1 g l^{-1}) produced an increase of pectinase production (*Table 2*). This latter effect has also been found by other workers,[26,27] but it has not been understood as yet.

Table 2 *A. niger* C28B25 pectinase production during SmF in pectin alone or in pectin plus glucose or sucrose (72 h, 25°C). Note the catabolic repression effect at 5 and 10 g l^{-1} of glucose or sucrose

Culture media (g l^{-1})		Biomass (g l^{-1})	Pectinolytic activity (U ml^{-1})	Pectinolytic activity (U g DW^{-1})
Pectin (P)	10	6.30	0.22	34.92
P + glucose	1	5.30	0.27	50.94
P + glucose	5	11.67	0.05	4.28
P + glucose	10	10.93	0.13	11.89
P + sucrose	1	5.66	0.27	47.70
P + sucrose	5	7.82	0.07	8.95
P + sucrose	10	10.23	0.09	8.80



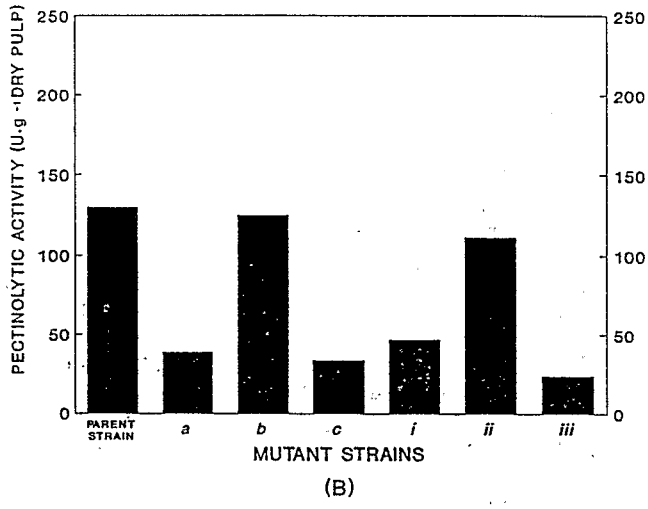


Figure 2 Comparison of pectinase activities of *A. niger* mutants of strain C28B25 isolated on AW99 medium (series *dgrAW99*). (A) Activities in liquid medium (U g^{-1} DW of biomass) in pectin 10 g l^{-1} (■) and pectin 10 g l^{-1} + sucrose 5 g l^{-1} (▨). (B) Activities from coffee pulp (SSF) (U g^{-1} dry pulp)

Mutagenesis and selection of mutants of A. niger *C28B25*

The UV dose for mutagenesis of *A. niger* spores, indicated in the previous section, was found to correspond to a survival rate of approximately 10%.

UV-irradiated spores of *A. niger* C28B25 parental strain were plated in Petri dishes with AW99 selective medium as indicated above. Only from one to three colonies grew per Petri dish within an incubation period of a week. Those mutants were called *dgrAW99*, as indicated above, and labeled as *a, b, c, i, ii,* and *iii*. The apparent mutation rate for this phenotype was 10^{-7}, because the inoculation dose was 1.15×10^7.

Plating UV-treated spores on Petri dishes with AW96 medium resulted in only one fast-growing colony per dish out of 1.5×10^7 inoculated spores. The omission of 2DG in the AW96 medium resulted in many fast-growing colonies, which made selection practically impossible. The use of 0.001 g l^{-1} 2DG and 10.0 g l^{-1} pectin made the isolation of this class of mutants easy. Again the apparent mutation rate of this second phenotype was apparently close to 10^{-7}, indicating a very strong selection pressure in this protocol. These mutants were labeled as *dgrAW96: 1, 2, 3,* and *4*.

Repeated cultures of all *dgr* mutants, with the sole exception of *dgrAW96-3*, showed that 2DG resistance is a very stable mutation of this *A. niger* parental strain. It should be noted, however, that not all wild strains of *A. niger* have been found to be sensitive to 2DG, i.e., CH4 strain is naturally 2DG-resistant.

Characterization of dgrAW99 *mutants*

This type of mutant was grown by the SmF technique with 10 g l^{-1} pectin alone or with 10 g l^{-1} pectin and 5 g l^{-1} sucrose in order to check the degree of CR for each mutant. The results shown in *Figure 2A* indicate two interesting points: (a) mutants *dgrAW99-a,c* and *iii* had an important increase of pectinase production with pectin alone, and (b) mutant *dgrAW99-iii* showed a relatively low level of CR, as compared to pectinase production by the parental strain. More specifically, mutant *dgrAW99-iii* produced pectinase by the SmF technique at a level of 300% with respect to the parental strain. These results agree with previous reports that the *dgr* phenotype is associated with derepression of hydrolase synthesis by *Saccharomyces cerevisiae*[28,29] and *Neurospora crassa*.[30] Other workers[10] have also obtained pectinase-hyperproducing mutants of *A. niger*. However, they required the analysis of 120,000 randomly obtained mutants in order to grow 1,000 strains by the SmF technique, which were tested by viscosimetry in order to isolate a single mutant with only 50% increase of pectinase activity. Therefore, the use of the very strong selection pressure of the AW99 medium seems to be an efficient way of obtaining pectinase-hyperproducing mutants to be grown by the SmF technique.

Mutants selected by the AW99 medium were also

grown by the SSF technique. They all showed a decrease of pectinase production as compared to the parental strain (*Figure 2B*).

Mycelial development by SSF of these mutant strains seemed to be less than that of the wild strain when viewed through a microscope. After 3 days of fermentation, sporulation was not yet visible. Only mutants *dgrAW99-b* and *dgrAW99-ii* seemed to produce mycelium in a similar amount to that of the wild strain.

A separate study[31] found that the main effect of decreasing a_w from 0.99 to 0.95 was to decrease pectinase production of the parental strain (C28B25) and of the *dgrAW99-b* and *iii* mutants when grown either by SmF or SSF techniques.

Apparently the AW99 medium is not suited to the isolation of mutants for the production of pectinase by the SSF.

Characterization of dgrAW96 *mutants*

The four isolated mutants of this class were cultivated by the SmF technique in order to test the CR effect exerted by 5 g l^{-1} sucrose. The results shown in *Figure 3A* indicate that practically all four *dgrAW96* mutants had a diminished CR effect, showing that pectinase production without sucrose (black columns) was also increased as compared to the parental strain level. The increase of SmF pectinase shown in *Figure 3A* was not as important as the increase found in mutants *dgrAW99 a, c,* and *iii* (*Figure 2A*), since only mutant *dgrAW96-4* doubled the reference level. Cultivation of *dgrAW96* mutants by the SSF technique produced higher activity levels in all these strains (*Figure 3B*), and especially by mutants *dgrAW96 1* and *3*, which produced 219 and 221 U g^{-1} DP, respectively. Those observations are in contrast with the decreasing SSF pectinase levels in all *dgrAW99* mutants shown in *Figure 2B*.

Sporulation of *dgrAW96* mutants was clearly observable 2 days after the onset of fermentation. We have verified on Petri dish cultures that the mycelium of these mutant strains grows faster than that of the wild strain on BMM medium supplemented with pectin as the sole carbon source and with 15% of ethylene glycol as water depressant (a_w = 0.96). In a separate study[31] it was found in Petri dishes that decreasing values of a_w, from 0.99 to 0.96, produced by the addition of increasing amounts of ethylene glycol, from 0 to 15%, had the effect of reducing the colonial extension rate and pectinase clearing zones in a very similar way for all types of *A. niger* strains, but it had a different effect on the mycelial density (mg dry biomass cm^{-2}) of different strains, indicating that stable mycelial density is one of the specific features of strains adapted to the SSF technique. Further studies on this subject are still under way in order to best characterize the phenotype of *dgrAW96* mutants.

Comparison between dgrAW99 *and* dgrAW96 *mutants*

In Figure 4 both classes of mutants are compared, plotting pectinase activities produced by the SmF technique versus the pectinase activities produced by the SSF technique. The plot is divided into two domains by the boundary horizontal line corresponding to the parental strain level. This kind of graphic shows that points corresponding to each one of the mutants are plotted in quite different domains: class *dgrAW99* falls in the lower domain, indicating that all these mutants decreased SSF pectinase production and increased SmF pectinase production. Linear regression analysis for this class indicates an inverse correlation between SSF versus SmF activities (R = −0.78, α = 0.005). The meaning of such an inverse relationship between SSF and SmF pectinase production in *dgrAW99* mutants is not yet understood but may be related to the mutation of a specific kind of genetic structure closely related to membrane transport, hydrolase regulation, and metabolite accumulation. This is supported by the

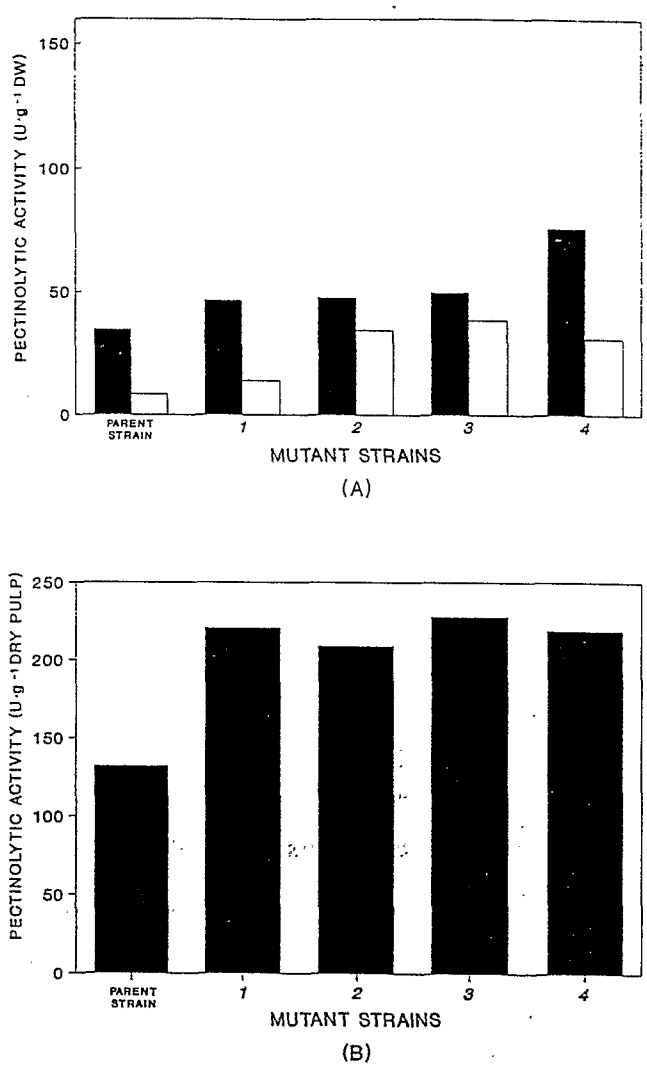


Figure 3 Comparison of pectinase activities of *A. niger* mutants of strain C28B25 isolated on AW96 medium (series *dgrAW96*). (A) Activities in liquid medium (U g^{-1} DW of biomass) in pectin 10 g l^{-1} (■) and pectin 10 g l^{-1} + sucrose 5 g l^{-1} (▨). (B) Activities from coffee pulp (SSF) (U g^{-1} dry pulp)

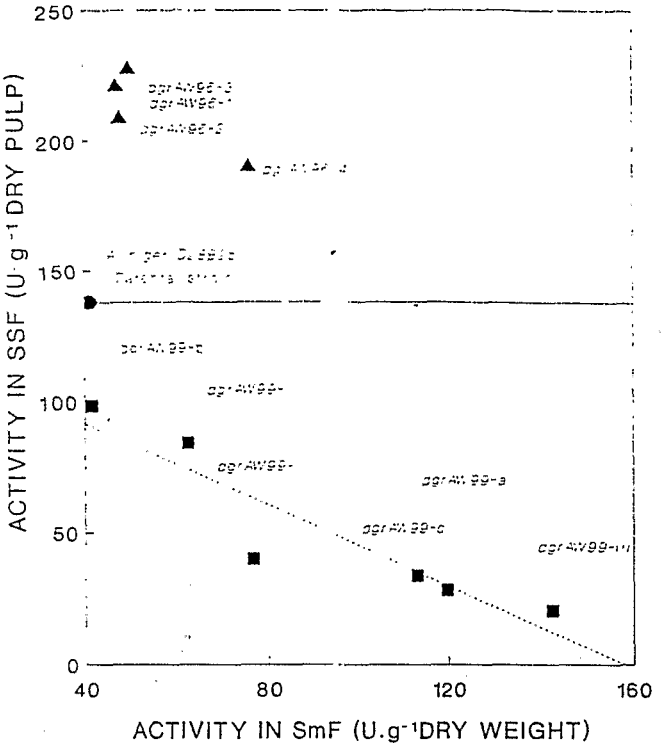


Figure 4 Pectinase activities in submerged culture (SmF) plotted vs pectinase activity in coffee pulp (SSF) for the parental strain (*A. niger* C28B25) and for each of the isolated mutant strains. The plane is divided into two domains, above and below the horizontal line corresponding to the reference strain. (■ ··· ■) Series *dgrAW99*; (▲ ··· ▲) series *dgrAW96*

observation that *dgr* mutants of *Neurospora crassa* have increased sugar transport rates and hydrolase production[30] and, additionally, by the observation that mold adaptation to hypertonicity is done by intracellular accumulation of glycerol, erythrol, and glucose.[32]

The second class, *dgrAW96*, falls in the upper domain of *Figure 4*, indicating that this class of mutations is related to an increase of SSF and SmF activities, but with a less clear inverse relation between them.

Thus, pectinase systems measured by viscosimetry seem to express themselves in dissimilar ways at different values of a_w and may be regulated by distinct sets of genes. Some authors[29,30,33,34] have reported that many 2DG-resistant mutants appear to have pleiotropic regulatory alterations in the activity of some enzymes (hydrolases, permeases, glycolytic enzymes, etc.). In this mutagenesis program, the surplus selection pressure due to the combined action of the low a_w and the presence of 2DG favors the selection of a certain type of derepressed mutant adapted specifically to SSF. In this form, the low value of a_w in the AW96 medium proves to be the key factor for the selection of these type of strains.

Conclusions

This is, as far as we know, the first protocol suggested for the selective isolation of hydrolase-producing mold mutants best suited to the SSF technique.

The use of low water activity as a selective factor for 2DG-resistant mutants seems to help isolate a very special class of mold mutants called *dgrAW96*. These mutants are, in general, derepressed, have enhanced pectinase production by SmF and SSF techniques, and may have quite different genetic and physiological properties from those of the *dgrAW99* class mentioned below.

Selection of 2DG-resistant mutants in culture media with high water activity seems to favor the isolation of a different class of mutants, called *dgrAW99*, which have the remarkable property of increased pectinase production by SmF and inversely reduced pectinase production by SSF. This protocol seems to be very efficient in order to increase the levels of SmF pectinase production by mutagenesis.

Further study of both classes of mutants of *Aspergillus niger* C28B25 seems to be quite promising to try to understand the genetic and physiological basis for the adaptation of molds to SSF.

Acknowledgements

We would like to thank Franck Boccas for his technical assistance in the preliminary evaluation of the pectinase production of the strains in our collection. This project was partially funded by the Mexican National Council of Science and Technology (CONACyT) and also by ORSTOM and UAM.

References

1 Martínez-Carrera, D., Morales, P. and Sobal, M. in *I Seminario Internacional sobre Biotecnología en la Agroindustria Cafetalera* (Roussos, S., Licona, F. R. and Gutiérrez, R. M., eds.) Jalapa, Mexico, 1989, pp. 177–184
2 Pulgarin, C., Schwitzguebel, J. P. and Tabacchi, R. *Biofutur.* 1991, **6**, 43–50
3 Aguirre, F., Maldonado, O., Rolz, C., Menchú, J. F., Espinosa, R. and De Cabrera, S. *Chem. Technol.* 1976, **6**, 636–640
4 Peñaloza, W., Molina, M. R., Gómez Brenes, R. and Bressani, R. *Appl. Environ. Microbiol.* 1985, **49**, 388–393
5 Favela-Torres, E., Huerta, S., Roussos, S. and Gutierrez, M. in *I Seminario Internacional sobre Biotecnología en la Agroindustria Cafetalera* (Roussos, S., Licona, F. R. and Gutiérrez, R. M., eds.) Jalapa, Mexico, 1989, pp. 145–151
6 Zuluaga Vasco, J. in *I Seminario Internacional sobre Biotecnología en la Agroindustria Cafetalera* (Roussos, S., Licona, F. R. and Gutiérrez, R. M., eds.) Jalapa, Mexico, 1989, pp. 63–76
7 Trejo-Hernández, M. R., Oriol, E., López-Canales, A., Roussos, S.,Viniegra-González, G. and Raimbault, M. *Micol. Neotrop. Apl.* 1991, **4**, 49–62
8 Fogarty, W. M. and Kelly, C. T. in *Microbial Enzymes and Biotechnology* (Fogarty, W. M., ed.) Applied Science Publishers, London, 1983, pp. 131–182
9 Ilzuck, Z. *Acta Microbiol. Polon.* 1976, **25**, 401–411
10 Leuchtenberger, A., Mayer, G. and Rutloff, H. *Zentralbl. Mikrobiol.* 1990, **145**, 167–170
11 Bailey, M. J. and Pessa, E. *Enzyme Microb. Technol.* 1990, **12**, 266–271
12 Jain, S., Sarriche, S., Durand, H. and Tiraby, G. *Enzyme Microb. Technol.* 1990, **12**, 691–696
13 Jain, S., Durand, H. and Tiraby, G. *Appl. Microbiol. Biotechnol.* 1990, **34**, 308–312
14 Durrands, P. K. and Cooper, R. M. *Appl. Microbiol. Biotechnol.* 1988, **28**, 463–467
15 Rombouts, F. M. and Pilnik, W. in *Economic Microbiology,*

Microbial Enzymes and Bioconversions Vol. 5 (Rose, A. H., ed.) Academic Press, New York, 1980, pp. 227–282
16 Ward, O. P. in *Comprehensive Biotechnology Vol. 3* (Blanch, H. W., Drew, S. and Wang, D. I., eds.) Pergamon Press, Oxford, 1985, pp. 819–835
17 Shankaranand, V. S., Ramesh, M. V. and Lonsane, B. K. *Proc. Biochem.* 1992, **27**, 33–36
18 Ghildyal, N. P., Ramakrishna, S. V., Nirmala Devi, P., Lonsane, B. K. and Asthana, H. N. *J. Fd. Sci. Technol.* 1981, **18**, 248–251
19 Guilbert, S. in *Solid-State Fermentation in Bioconversion of Agroindustrial Raw Materials* (Raimbault, M., ed.) Proceedings of the seminar ORSTROM-Montpellier, France, 1988, pp. 73–82
20 Oriol, E. Doctoral thesis. INSA Toulouse, France, 1987, pp. 1–115
21 Grajek, W. and Gervais, P. *Enzyme Microb. Technol.* 1987, **9**, 658–662
22 Aquiáhuatl, M. A., Raimbault, M., Roussos, S. and Trejo, M. R. in *Solid-State Fermentation in Bioconversion of Agroindustrial Raw Materials* (Raimbault, M. ed.) Proceedings of the Seminar ORSTOM-Montpellier, France, 1988, 13–26
23 Hankin, L. and Anagnostakis, S. L. *Mycologya* 1975, **67**, 597–607
24 Raimbault, M. and Alazard, D. *Eur. J. Appl. Microbiol. Biotechnol.* 1980, **9**, 199–209
25 Leuchtenberger, A. and Mayer, G. *Enzyme Microb. Technol.* 1992, **14**, 18–22
26 Keen, N. T. and Horton, J. C. *Can. J. Microbiol.* 1966, **12**, 443–453.
27 Aguilar, G. and Huitron, C. *Enzyme Microb. Technol.* 1987, **9**, 690–696.
28 Heredia, C. F. and Sols, A. *Biochem. Biophys. Acta* 1964, **86**, 224–228
29 Zimmermann, F. K. and Scheel, I. *Mol. Gen. Genet.* 1971, **154**, 75–82
30 Allen, K. E., McNally, M., Lowendorf, H. S., Slayman, C. W. and Free, S. J. *J. Bacteriol.* 1989, **171**, 53–58
31 Solís, S., Favela, E., Mercado, E. and Viniegra, G. in *IV Congreso Nacional de Biotecnología y Bioingeniería*. Merida, Mexico, 1991, FE17C
32 Beever, R. E. and Laracy, E. P. *J. Bacteriol.* 1986, **168**, 1358–1365.
33 Entian, K. D. *Mol. Gen. Genet.* 1981, **184**, 278–282
34 Entian, K. D. and Mecke, D. *J. Biol. Chem.* 1982, **257**, 1870–1874