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 (α_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 α_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 α_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 cerasises 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 In particular, we have studied pectinase production by solid-state fermentation (SSF) using this by-product as a fermentable substrate.⁴ According to Pulgarín et al.,² coffee pulp contains around 6.5% pectin, and Zuluaga⁵ 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 SSF⁴ process. Troje 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 Kelly⁸ 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. occitanus*,¹²,¹³ or *Verticillium 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. Shankaranaand 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-
neous distribution of solutes, the matrix structure due to substrate porosity, and adsorption forces can be recognized as key factors in SSF. 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. Oriol et al. 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. 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$_2$PO$_4$ 1.3, Na$_2$HPO$_4$ 0.60, (NH$_4$)$_2$SO$_4$ 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-O-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 for each repetition.

System-Decagon device. For the analysis of samples obtained for enzyme assays. The pectinase activity (presumably to be mainly of the type, poly-1, α-D-galacturonic glycanohydrolase, E.C. 3.2.1.152) 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 Viscometer. Thus 18 ml of the pectin solution and 1 ml of the enzyme

Erlenmeyer flask cultures. Fifty milliliters of BMM in 250-ml flasks containing 10 g l^-1 pectin were inoculated with 5 × 10⁶ 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, and maintained at 25°C within a thermostated water bath. Each 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 × 10⁶ 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 dry-weight 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 6000g for 5 min. Supernatants were used for enzyme assays. The pectinase activity (presumably to be mainly of the type, poly-1, α-D-galacturonic glycanohydrolase, E.C. 3.2.1.152) 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 Viscometer. 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

<table>
<thead>
<tr>
<th>H/Ci &gt; 1</th>
<th>C, = 0</th>
<th>H/Ci = 1</th>
<th>A' &lt; 0</th>
<th>A' = 0</th>
<th>A' &gt; 0</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested</td>
<td>12</td>
<td>117</td>
<td>97</td>
<td>7*</td>
<td>15*</td>
<td>248</td>
</tr>
<tr>
<td>(%)</td>
<td>(5)</td>
<td>(47)</td>
<td>(39)</td>
<td>(3)</td>
<td>(8)</td>
<td>(100)</td>
</tr>
</tbody>
</table>

C, Diameter of colony i
H, Diameter of hydrolyzed zone around colony i
Cref and Href Reference values (A. niger CH4)
* Strains selected to be cultivated by SSF

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

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, = 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/Ci = 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 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, but it has not been understood as yet.
Table 2  A. niger C28825 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.

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Biomass (g l⁻¹)</th>
<th>Pectinolytic activity (U ml⁻¹)</th>
<th>(U g DW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin (P)</td>
<td>6.30</td>
<td>0.22</td>
<td>34.92</td>
</tr>
<tr>
<td>P + glucose</td>
<td>5.30</td>
<td>0.27</td>
<td>50.94</td>
</tr>
<tr>
<td>P + glucose 5</td>
<td>11.67</td>
<td>0.05</td>
<td>4.28</td>
</tr>
<tr>
<td>P + glucose 10</td>
<td>10.93</td>
<td>0.13</td>
<td>11.89</td>
</tr>
<tr>
<td>P + sucrose</td>
<td>5.66</td>
<td>0.07</td>
<td>8.95</td>
</tr>
<tr>
<td>P + sucrose 5</td>
<td>7.82</td>
<td>0.07</td>
<td>8.80</td>
</tr>
<tr>
<td>P + sucrose 10</td>
<td>10.23</td>
<td>0.09</td>
<td>8.80</td>
</tr>
</tbody>
</table>

Mutagenesis and selection of mutants of A. niger C28825

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 C28825 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 x 10⁷.

Plating UV-treated spores on Petri dishes with AW96 medium resulted in only one fast-growing colony per dish out of 1.5 x 10⁷ inoculated spores. The omission of 2DG in the AW96 medium resulted in many fast-growing colonies, which made selection practically impossible. The use of 6.601 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 and Neurospora crassa. Other workers 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 found that the main effect of decreasing $a_w$ from 0.99 to 0.95 was to decrease pectinase production of the parental strain (C28825) 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 SmF pectinase shown in 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, 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 dgrA W99 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 and SmF pectinase production in dgrAW99 mutants. 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
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 dgrAW99. These mutants are, in general, derepressed, have enhanced pectinase production by SmF andSSF 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.

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