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Growth and Pectinase Production by *Aspergillus* Mexican Strain Protoplast Regenerated Under Acidic Stress

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Abstract

Protoplasts from *Aspergillus* sp. FP-180 and *Aspergillus awamori* NRRL-3112 were released and regenerated at extreme acidic conditions. The best conditions for protoplast release were 0.8 *M* KCI, pH 5.8, and 3 h of digestion using mycelia from 12- to 16-h cultures from either *Aspergillus* sp. FP-180 or *A. awamori* NRRL-3112. The addition of fresh mycelia to an ongoing digestion after 1 h increased protoplast 4.5–5 times. A regeneration efficiency of 90% was attained at pH 6.0, and it was possible to regenerate protoplasts at pH 1.7 with a regeneration efficiency of 0.5% for *Aspergillus* sp. FP-180. The LpH-10 strain, derived from protoplast from *Aspergillus* sp FP-180, was able to regenerate at pH 1.7 and grow at pH values as low as 1.5, values at which the original strain is unable to grow. Regeneration at extreme pH improved the performance of LpH-10 strain. It showed a twofold increase in cell growth at pH 2.0 in liquid culture and a higher pectinolytic activity in relation to that produced by the original strain.

Index Entries: *Aspergillus*; protoplast formation; regeneration under acid stress; protoplast storage; pectinases.

Introduction

Protoplast technology has been used for preparing cell-free extracts and organelles for biochemical studies and, more recently, for genetic

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transformation *(1–4)* and protoplast fusion *(5–7)* and, thus, has become an important tool in strain improvement programs. In any case, the success of fusion and transformation systems depends on the availability of protoplasts in large numbers. The use of either protoplast or spores of various fungi has been reported for high-efficiency transformation with *Agrobacterium tumefaciens (8)*. Although numerous methods have been reported, the formation and regeneration of protoplasts from different fungi is not routine *(9)*. In fact, in many cases protoplasts do not regenerate well, and more research to improve regeneration conditions must be conducted, as is the case for the formation of protoplast from *Rhizoctonia solani (10)*.

Protoplast formation involves a series of functional and structural changes, such as modification in ultrastructure and distribution of organelles, plasma membrane, and cytoskeleton *(1)*. It has been demonstrated that fungal protoplasts possess the ability to repair alterations and to direct the formation of a new cell wall to give a functional and structurally complete cell *(1,11)*. However, not all protoplasts in a given population are able to regenerate owing to the lack of a nucleus or to other defects involving the regeneration process *(11)*.

Environmental conditions also affect the regeneration process. Synthesis of structural polysaccharides, the assembly of microfibrils, and the subsequent organization of the cell wall depend on several external conditions, including pH. The activities of enzymes responsible for polysaccharide synthesis have an optimum pH value so that their efficiency will be modified by changes in the external pH *(12)*. Autoassembly of structural polysaccharides of the cell wall could be modified by microenvironmental conditions and by covalent or hydrogen bond formation among different polymer chains in the cell wall matrix *(12–14)*. Thus, it is clear that there must be a regulatory system to ensure the synthesis of the right enzymes at the right pH. Acid and alkaline phosphatases are examples of such a control *(15,16)*.

Protoplast regeneration could be used to improve production strains. In fact, selection of colonies regenerated form a single protoplast has been used with success to improve enzyme productivity of the cellulase-producing fungus *Robillarda (17)* and for endoglucanase, xylanase, and β-glucosidase from *Aspergillus awamori (18)*. However, as far as we know, there are no reports on the regeneration of protoplasts at extreme acidic conditions.

We previously reported that a strain of *Aspergillus* growing under acidic conditions (pH 2.0–2.5) showed an enhanced pectic enzyme production despite the fact that cell growth was severely affected *(19–21)*. Since pectinases are widely used for fruit and juice extraction and clarification, it could be important to produce enzymes capable of acting at low pH. Thus, the production of enzymes at extreme acidic pH could be an advantage. With the aim of improving growth or production of pectic enzymes by *Aspergilli*, we report an efficient method for the formation of protoplasts from two *Aspergillus* strains and the regeneration of protoplasts from one of these strains under extreme acidic conditions. Conditions for the preser-

vation of protoplasts for further study are evaluated. The production of pectinases, xylanases, and amylases are also compared.

Materials and Methods

Microorganisms

The strains that we used were *A. awamori* NRRL-3112 and *Aspergillus* sp FP-180. The latter is a tropical strain isolated in Mexico from decaying tomato *(21)*. Both strains were propagated and maintained on potato dextrose agar slants at 37°C. The *A. awamori* strain was kindly provided by the Northern Regional Research Laboratory, US Department of Agriculture.

Lytic Enzymes

The lytic enzymes used were lytic enzymes from *Trichoderma harzianum* (2, 5, 7.5, and 10 mg/mL), hemicellulase from *Aspergillus niger* (20 and 40 µg/mL), β-glucuronidase from *Helix pomatia* (460 and 690 U/mL), and chitinase from *Streptomyces griseus* (20 and 40 µg/mL), all from Sigma (St. Louis, MO).

Formation and Recuperation of Protoplasts

Mycelium for preparation of protoplasts was grown in 500-mL Erlenmeyer flasks with basal medium (BM) containing (per liter of distilled water): 2.0 g of KH_2PO_4, 2.0 g of K_2HPO_4, 2.0 g of $(NH_4)_2SO_4$, and 10 g of pectin as carbon source. After cultivation in an incubator shaker for 12–24 h at 100 rpm at 37°C, the mycelium was harvested by filtration and washed thoroughly with isotonic NaCl solution (0.85% [w/v]) and resuspended in stabilization buffer (SB) consisting of 0.2 *M* phosphate buffer (pH 6.0) supplemented with the appropriate osmotic stabilizer. As osmotic stabilizer, we used KCl (0.6–1.0 *M*) or sorbitol (0.6–1.2 *M*).

Six to fifteen milligrams of mycelium (dry wt) was treated with the lytic enzyme mixture for 1–3 h at 37°C with mild agitation. Samples were withdrawn at different times, and the protoplasts produced were counted in a Neubauer counting chamber (Boeco, Germany). In some cases, after 1 h of digestion an equal amount of fresh mycelium was added to the digestion mixture and the incubation continued for another 2 h.

After treatment with lytic enzymes, the digestion mixture was filtered through a filter and washed two to three times by centrifugation and resuspended in SB. The effect of centrifugation speed on protoplast integrity and yield was evaluated in a range of 430–720*g* with different concentrations of osmotic stabilizer (0.6–1.0 *M*).

For protoplast preservation, following formation, in the final step of the washing procedure, they were suspended in test tubes containing SB alone or mixed with either 20, 50, or 80% glycerol. The resulting protoplast suspensions were allowed to stand at +4 and –20°C for different time periods. Each day, test tubes were taken from each condition and viable protoplasts counted.

Regeneration of Protoplasts

Regeneration was carried out either on BM with pectin as carbon source (BMP medium) at pH 1.2–6.0 or on glucose-yeast extract agar (GYE) plates at pH 6.0. The additional supplements included osmotic stabilizers and tartaric acid to adjust the pH in BMP agar plates.

Growth and Enzyme Production

For enzyme production, shake-flask experiments were conducted using BM containing 0.4% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.2% K_2HPO_4, and 1% of the appropriate carbon source. Citrus pectin, birchwood xylan, and starch were used as carbon source. Medium was sterilized at 121°C, 15 psi for 20 min. Growth and enzyme production were followed in 500-mL Erlenmeyer flasks containing 200 mL of culture medium and shaken at 100 rpm in an incubator shaker (New Brunswick Scientific) kept at 37°C. The initial pH was adjusted to either 2.0 or 5.0. All flasks were inoculated with a spore suspension to give a final concentration of 1×10^6 spores/mL of culture medium.

Cell growth was determined by dry weight and reported as milligrams per milliliter.

Enzyme Activity Assays

Exo- and endopectinases were assayed as previously reported *(19)*. One unit of exopectinase was defined as the amount of enzyme that released 1 µmol of galacturonic acid in 20 min under the assay conditions. One unit of endopectinase was defined as the amount of enzyme that reduced the viscosity of 1% pectin solution by 50% in 10 min under the assay conditions. Xylanases were assayed by measuring the reducing groups released from xylan by the dinitrosalicylic acid method *(22)*. The reaction mixture consisted of 500 µL of 2% xylan, 400 µL of 0.17 *M* acetate buffer at pH 5.0, and 100 µL of enzymatic sample. The reaction mixture was incubated at 50°C for 10 min. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of xylose in 10 min under the assay conditions. Amylase activity was measured as reported previously *(23)*. Briefly, 0.8 mL of 1.2% starch solution was incubated with 0.1 mL of enzymatic solution for 10 min, after which 0.1 mL of 1 *N* H_2SO_4 was added to stop the reaction. One hundred microliters of this mixture was added to 2.4 mL of 0.4% lugol solution. The resultant solution was read at 620 nm. One unit of amylolytic activity was defined as the amount of enzyme that hydrolyzed 1.0 mg of soluble starch/min under the assay conditions.

Results

Since the physiologic state of the cell is of crucial importance for protoplast yield, the optimal culture time for mycelial growth for protoplast formation was evaluated. *Aspergillus* sp. FP-180 was cultured for 12, 16, 20,

Table 1
Effect of Culture Time and Lytic Enzyme Concentrations on the Yield of Protoplasts Released From *Aspergillus* sp. FP-180

Culture time (h)	Protoplasts released ($\times 10^7$/mL)					
	Enzyme concentration (mg/mL)[b]					
	5		7.5		10	
	Control	Addition	Control	Addition	Control	Addition
12	2.52	ND	ND	ND	ND	ND
16	2.00	6.50	2.20	10.0	2.30	11.60
20	0.50	0.50	0.60	0.60	0.65	0.70
24	0.012	0.035	0.055	0.06	0.065	0.075

[a]The digestion mixture was incubated at 37°C for 3 h with *T. harzianum* lytic enzymes. The osmotic stabilizer was 0.8 *M* KCI. Mycelium was obtained growing *Aspergillus* sp. FP-180 on BMP at 37°C for 12 h. ND, not detected.

[b]For the control, mycelium concentration was 12 mg/mL as dry wt. For the addition, mycelium concentration was 6.0 mg/mL, and after 1 h of digestion a further 6.0 mg/mL was added to the digestion system.

and 24 h at 37°C and treated with lytic enzymes (Table 1). Maximum protoplast yield was attained at 12 h of culturing. Mycelia from 16 h of growth also gave good results (80% in relation to 12 h of yield), whereas at 20 and 24 h a dramatic decrease in protoplast yield was observed (Table 1).

Increasing the concentration of *T. harzianum* lytic enzymes from 5 to 7.5 and 10 mg/mL produced only a small increase in protoplast yield with the mycelia from different times of cultivation (Table 1). Maximum increase was obtained with older mycelium (24-h culture). However, the final yield of protoplasts was very low. With younger mycelium (16-h culture) the yield was increased by 15% using 10 mg/mL compared with the lowest enzyme concentration (5 mg/mL) used (Table 1). Similar results were obtained using *A. awamori* 3112 (data not shown). In further experiments on protoplast formation, we used mycelium from 12-h cultures.

Surprisingly, the addition of fresh mycelium to the digestion system produced a spectacular increase in the yield of protoplasts released irrespective of the concentration of lytic enzymes used (Table 1). The yield was 3, 4.5, and 5 times higher for 5, 7.5, and 10 mg/mL of *T. harzianum* lytic enzymes, respectively, compared to the control.

Lytic enzymes from *T. harzianum* were tested alone or in combination with hemicellulase from *A. niger*, β-glucoronidase from *H. pomatia*, and/or chitinase from *S. griseus*. These enzymes were added to a digestion system containing 12 mg/mL of mycelium.

The use of *T. harzianum* lytic enzymes at 2.0 mg/mL alone produced good yields of protoplasts from *A. awamori*, reaching 1.9×10^7 protoplasts/mL. This yield was slightly lower than that obtained with 5.0 mg/mL of *T. harzianum* lytic enzymes (Table 1). However, the addition of either hemicellulase (40 g/mL) or β-glucuronidase (460 U/mL) produced an

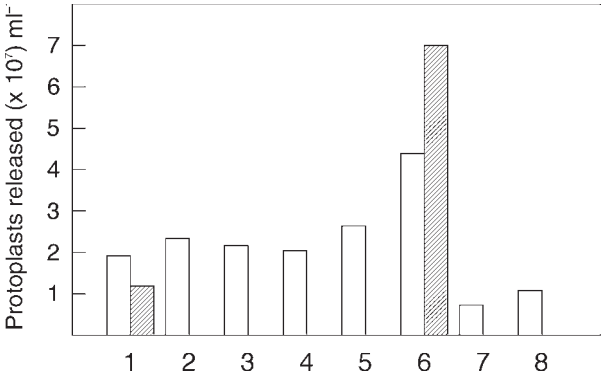

Fig. 1. Effect of different lytic enzyme mixtures on protoplast release with *A. awamori* NRRL-3112 (■) and *Aspergillus* sp. FP-180 (□) at 12 mg/mL final concentration (dry wt basis [w/v]). 1, *T. harzianum* lytic enzymes at 2.0 mg/mL: this condition was taken as control; 2, control plus hemicellulase (40 µg/mL; 3, control plus β-glucuronidase (460 U/mL); 4, control plus chitinase (40 µg/mL); 5, control plus hemicellulase and β-glucuronidase at 40 µg/mL and 460 U/mL, respectively; 6, same as (5) plus chitinase at 40 µg/mL; 7, control with 6.0 mg/mL of mycelium; and 8, same as (5) but with 6.0 mg/mL of mycelium.

increase of 22 and 12%, respectively. With the addition of both enzymes, an increase of 37% was obtained in relation to the use of *T. harzianum* enzymes alone (Fig. 1). The mixture containing *T. harzianum* enzymes plus chitinase (40 µg/mL), hemicellulase (40 µg/mL), and β-glucuronidase (460 U/mL) showed a very impressive effect, yielding more than twice the number of protoplasts than the control (*T. harzianum* lytic enzymes alone; Fig. 1).

The effect of chitinase was similar when the amount of mycelium was 6.0 mg/mL (dry wt). A yield increase from 0.72×10^7 protoplasts/mL (without chitinase) to 1.08×10^7 protoplasts/mL in the presence of the enzyme was recorded (Fig. 1). With *Aspergillus* sp. FP-180, we evaluated the use of *T. harzianum* alone and the mixture of all four enzymes. The effect of this mixture produced nearly six times more protoplasts in relation to the control (Fig. 1). The final concentration of the lytic cocktail was 2.0 mg/mL of *T. harzianum* lytic enzymes, 40 µg/mL of hemicellulase, 40 µg/mL of chitinase, and 460 U/mL of β-glucuronidase. The high concentration of the latter enzymes did not result in important differences except for chitinase. However, the yield obtained is not high enough to support the use of three times more enzyme in the digestion mixture (data not shown).

The effect of mycelial concentration on protoplast yield using the final four-enzyme cocktail was also tested on *A. awamori*. Mycelium concentrations used were 6, 8, 10, 12, and 15 mg/mL and the yields obtained were 1.05×10^7, 3.84×10^7, 7.25×10^7, 9.60×10^7, and 13.8×10^7 protoplasts/mL, respectively. As can be seen, the yield of protoplasts released increased as the mycelium concentration increased. Maximum yield was attained at the highest mycelial concentration used. However, recovery was more difficult at high mycelium concentration because the proportion of undigested

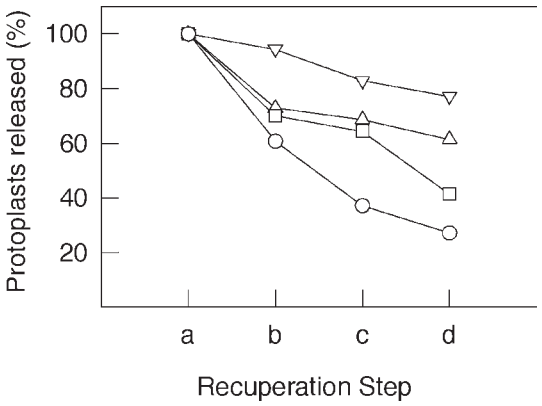

Fig. 2. Effect of differences in centrifugal speed and concentration of osmotic stabilizer (KCI) on protoplast recovery from *Aspergillus* sp. FP-180. (○) Centrifugation at 760*g* and 0.6 *M* KCl; (□) centrifugation at 430*g* and 0.6 *M* KCI; (△) centrifugation at 430*g* and 0.8 *M* KCI; and (▽) centrifugation at 520*g* and 1.0 *M* KCI. a–d represent successive washing steps.

mycelium was very high. A concentration of 10–12 mg/mL was considered optimal.

Another important factor for protoplast formation and regeneration is the osmotic stabilizer used. Here the effects of KCI and sorbitol were tested. The best yield was obtained using 0.8 *M* KCI (9.6×10^7 protoplasts/mL). However, 0.6 *M* KCI gave almost the same yield (9.18×10^7 protoplasts/mL), 95% in relation to 0.8 *M* KCI, whereas at higher concentrations a considerable reduction was observed (4.38×10^7 protoplasts/mL). Similar results were obtained using sorbitol, and the optimal concentration was also found to be 0.8 *M* (data not shown). The latter results were obtained with the *A. awamori* strain.

Since protoplasts are very labile structures and manipulation after their formation could be deleterious, the effect of washing conditions during recovery was evaluated (Fig. 2). We found that centrifugal speed and the concentration of the osmotic stabilizer were very important (Fig. 2). The best concentration of KCI for protoplast formation was 0.8 *M*; however, for the subsequent recovery, it had to be substituted by 1.0 *M* KCI (Fig. 2). A rapid decrease in protoplasts was obtained with 0.6 *M* KCI at the high centrifugal speed. A reduction in the centrifugal speed gave better yield, which was slightly improved by an increase in KCI concentration to 0.8 *M*. However, the best results were obtained with 1.0 M KCI and an intermediate centrifugal speed, 520*g* (Fig. 2).

Regeneration of protoplasts was evaluated at different pH values (Fig. 3). Regeneration efficiency on BMP was near 90% at pH 5.8. Lowering the pH of the regeneration medium caused a progressive decrease in efficiency with the largest reduction between pH 2.5 and 2.0 (Fig. 3). A colony from the regeneration plate at pH 1.7 was selected and streaked out on the same medium and the same pH. The strain was cultured for various gen-

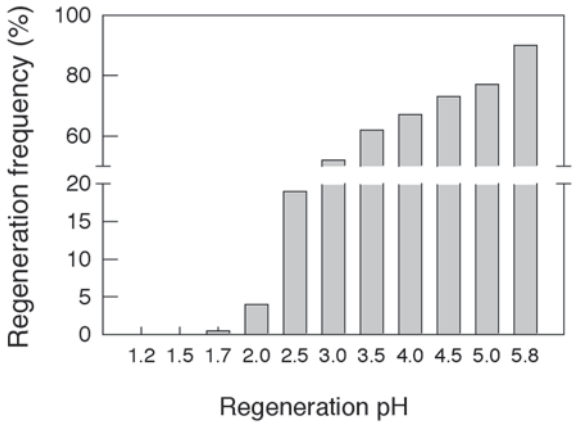

Fig. 3. Effect of pH on regeneration efficiency of *Aspergillus* sp. FP-180. Protoplasts were regenerated on BMP at different pH values; final pH was adjusted by the addition of 10% tartaric acid solution. Plates were incubated at 37°C for 7 d.

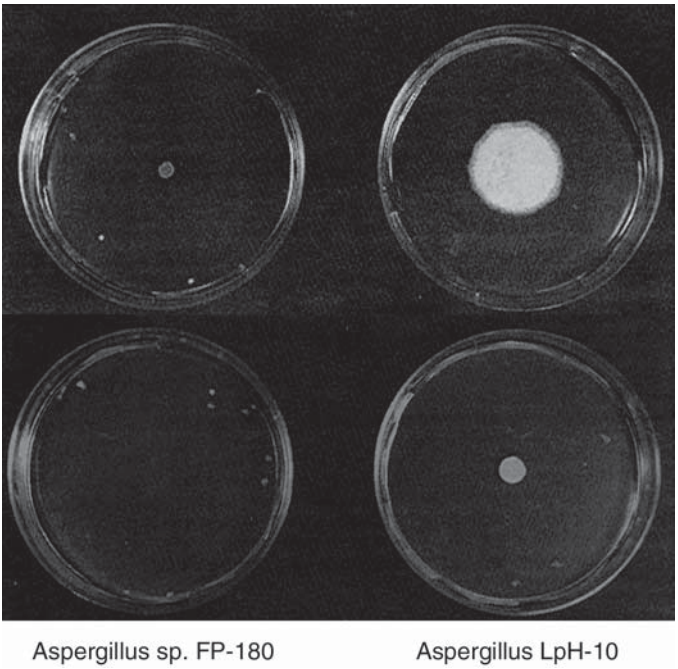

Fig. 4. Growth of *Aspergillus* sp. FP-180 *(left)* and regenerated strain LpH-10 *(right)* on pectin agar plates at pH 2.5 *(top)* and 1.5 *(bottom)*. Culture was carried out at 37°C for 3 d.

erations with (BMP, pH 1.7) and without selecting pressure (GYE, pH 6.0). This strain was designated LpH-10 and was able to grow at pH 1.5 in solid medium (Fig. 4). The parental strain, *Aspergillus* sp. FP-180 was unable to grow under these conditions, and at pH 2.5 the LpH-10 strain grew about four times faster than *Aspergillus* sp. FP-180 (Fig. 4).

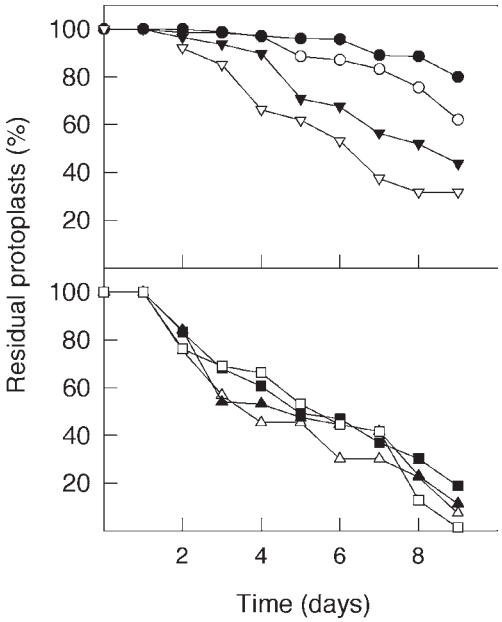

Fig. 5. Preservation of *Aspergillus* sp. FP-180 protoplasts at +4°C (open symbols) and –20°C (closed symbols) with SB alone (○, ●) or with SB plus 20% (▽, ▼), 50% (△, ▲), and 80% (□, ■) (w/v) glycerol. Protoplasts were maintained at +4 and –20°C for 20 d.

In relation to the preservation of protoplasts, different storage methods were evaluated. The addition of glycerol at various concentrations to the SB and the use of refrigeration (+4°C) and conventional freezing (–20°C) were assessed.

As shown in Fig. 5, the best results were obtained when protoplasts were maintained at –20°C with SB alone. Under this condition, protoplasts could be maintained almost 5 d without loss of viability. Contrary to our expectations, the use of glycerol did not improve the cold resistance of the protoplasts, showing a more rapid loss at the higher concentration. In all cases, freezing at –20°C gave better results (Fig. 5).

Dry weights obtained for strains LpH-10 and FP-180 were 4.5 and 3.37 mg/mL at pH 5.0, respectively, and 1.41 and 0.66 mg/mL at pH 2.0, respectively. As can be seen, growth of the LpH-10 strain was higher than that of the original strain FP-180, despite the initial pH used for growth. This is more evident at pH 2.0. In fact, in the latter case, about two times higher growth was obtained for the LpH-10 strain. The data obtained in liquid culture are in agreement with those obtained on solid medium (Fig. 4).

Pectinase activity was evaluated for both strains (Table 2). Higher exopectinase activity was produced by the LpH-10 strain at both pH values, growing on pectin as a sole carbon source (Table 2). The data from Table 2 are expressed as specific activity, so the total activity in units per milliliter is higher by a factor of two when both strains are grown at pH 2.0. At pH 5.0, exopectinase activity was also higher for LpH-10; however,

Table 2
Enzymatic Activities Produced
by *Aspergillus* sp. FP-180 and *Aspergillus* sp. LpH-101

	Pectinase							
	Exo (U/mg)		Endo (U/mg)		Xylanase (U/mg)		Amylase (U/mg)	
Strain	pH 2.0	pH 5.0	pH 2.0	pH 5.0	pH 2.0	pH 5.0	pH 2.0	pH 5.0
Aspergillus sp. FP-180	14.4	3.7	1.54	0.18	ND	17.3	2.1	8.0
Aspergillus sp. LpH-10	16.0	4.1	1.54	0.18	ND	6.1	ND	4.6

[a]Activities were measured from 72 h cell-free filtrates. Activity is expressed in U per mg of dry weight. ND, not detected under assay conditions.

minor differences were found. Interestingly, specific endopectinase activity was higher at pH 2.0 than at 5.0, but no differences between both strains were found (Table 2). Since the growth of LpH-10 was higher irrespective of growth pH, this strain produces more total activity at both pH values evaluated, about 2 and 1.4 times more activity for pH 2.0 and 5.0, respectively.

Since pectin was used as a selecting substrate during protoplast regeneration, it could be expected that there is some alteration of its production. Thus, the production of other enzymes was also tested. The production of xylanase, amylases, and invertase was evaluated. No xylanase activity was produced at pH 2.0 by any strain. At pH 5.0, the LpH-10 strain grew better than FP-180, while xylanase production showed an opposite profile. LpH-10 produced about 65% less specific activity than that of the original FP-180 strain (Table 2).

Amylase production was also better for the original strain, and the production was higher at pH 5.0. The LpH-10 strain did not produce amylases at pH 2.0, and at pH 5.0 the specific activity was slightly lower than that obtained with the original strain. It is important that the LpH-10 strain grew better at both pH values.

Discussion

It is clear that several factors are important in determining protoplast release from filamentous fungi including lytic enzymes, digestion time, culture age, microbial strain, osmotic stabilizer, and manipulation during recovery. From our results presented here, it is clear that best yields were obtained with cultures of midexponential phase (12–16 h). Lower yields were obtained with older mycelium. This resistance to cell wall digestion by older mycelium has been observed by other researchers *(9,11,24,25)*. The fact that mycelium from late exponential- or stationary-phase culture is difficult to digest is not completely understood. Some researchers suggest

that during exponential growth phase, endogenous enzymes are at a high enough concentration that they can act cooperatively and complete the action of lytic enzymes used for digestion *(10,26)*. Others have suggested that the more complex structure of the mature cell wall in older mycelia, such as the deposition of α-1,3 glucan observed in *A. nidulans (24)*, is responsible for the lower yields obtained. It is believed that in some regions of hyphae of younger active mycelia, there is an incomplete cell wall structure. This is particularly true at the hyphal tip, and because of the continuous extension of hyphae an easier breakdown of this incomplete structure could be carried out. Furthermore, better access of lytic enzymes to the corresponding cell wall components is possible. This is supported by the fact that apical protoplasts are faster to obtain than those from distal mycelium. In fact, in 1 h we obtained a good yield from the apex of 12-h-old mycelia. Indeed, newly germinated conidia (6–8 h of culture) with an average hyphal extension of 70 µm were more easily digested than older mycelia, and higher yields could be obtained: 2.37×10^7 protoplasts/mL for recently germinated conidia compared with 0.5×10^7 and 0.012×10^7 for 20- and 24-h-old mycelia, respectively (unpublished results). The nascent wall of the spore germling *(12)* is thus very sensitive to the lytic enzymes used, further supporting the idea of susceptibility of the new cell wall to digestion at the growing hyphal apices.

The effectiveness of KCI as an osmotic stabilizer was demonstrated, as was the significance of KCl concentration. It was interesting to observe that a different molarity of the solution favored protoplast isolation compared to regeneration. This difference may reflect the sensitivity of lytic enzymes to the salt. Sorbitol was equally good, and at 0.8 *M* we did not find differences on protoplast regeneration efficiency using either stabilizer in agar plates containing BMP at pH 6.0. In both cases, nearly 90% regeneration was attained for protoplasts of *Aspergillus* sp. FP-180.

During recovery, a higher concentration of KCI (1.0 *M*) was found to improve protoplast integrity. However, since we could reduce centrifugal force to maintain a good level of protoplast recovery, we decided to use 0.8 *M* KCI for the entire protocol. The best concentration of stabilizer, either KCI or sorbitol, found here was 0.8 *M*, which is similar to that of earlier reports *(9,27)*.

T. harzianum lytic enzymes from different suppliers and laboratories have been used extensively for protoplast formation, and they have been shown to be very efficient, giving high yields *(24,27–29)*. In our case, the addition of complementary enzymes such as β-glucuronidase, hemicellulase, and particularly chitinase was needed for optimal protoplast formation from *Aspergillus* sp. FP-180. This suggested that the hyphal wall of this fungus might have a higher chitin content. The effect of chitinase on *A. awamori* was more subtle but also positive. The addition of more mycelium to an established lytic digestion significantly increased the yield of protoplasts by up to five times. This increase could be the result of liberation of different endogenous enzymes from the first digested mycelium.

Since at 1 h of lytic treatment protoplasts are formed mainly from the hyphal apex and this region is more active, it is highly probable that endogenous enzymes act synergistically with lytic enzymes to digest the new added mycelia more efficiently. Another possibility is that the liberation of autolysins may have an important effect. It has been reported that autolysins from *A. nidulans* can hydrolyze the wall from apical regions at a higher rate than that from lateral regions during autodigestion *(30)*. Fungal autolysis is a consequence of the disruption of normal metabolic activity and loss of membrane function with subsequent activation of cellular hydrolases *(31)*. Similarly, nutrient starvation (carbon and/or nitrogen) has also been shown to activate autolysis *(30–32)*. Conditions for protoplast formation (high salt concentration, no carbon source present during lytic treatment) may resemble stress conditions that can act as important factors to favor autolysin activation.

Regeneration efficiencies reported in our work were high not only at a neutral or mild acidic pH, but also at lower pH values. Regeneration reported for other fungal strains is lower than described here. A regeneration efficiency of protoplasts from *Trichoderma viride* was about 30% *(3)*, while for *Trichoderma reesei* and *Penicillium chrysogenum* values of 52 and 28%, respectively, were obtained *(33)*. Other investigators reported protoplast regeneration frequencies of 10–20% for *A. awamori (6)* and for *Neurospora crassa*, *Flammulina velutipes*, and *Aspergillus oryzae (25)*; 75–87% for *Rhizoctonia solani (10)*, and 47–70% for *Trichosporum cutaneum (28)*. In all these cases, the pH investigated for regeneration was in the range of 5.8–6.0. We were able to regenerate protoplasts from *Aspergillus* sp. FP-180 at a pH as low as 1.7. In the case of *A. awamori* a regeneration efficiency of 20–30% was obtained at pH values in the range 4.0–6.0. However, at pH values below 4.0, regeneration was not observed under the conditions tested for the latter strain (data not shown).

As far as we know, there are no previous reports about regeneration of protoplasts of any microorganism at extreme acidic pH values. The strain LpH-10 derived from protoplasts of *Aspergillus* sp. FP-180 regenerated at pH 1.7 showed a better growth at pH 2.5, and it was also able to grow at pH values as low as 1.5.

The change in cell growth ability and in the enzyme production profile of the regenerated strain is indicative that the regeneration process under acidic conditions could be used as a strategy for the selection of improved strains able to grow and produce enzymes.

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References

1. Peberdy, J. F. (1979), *Annu. Rev. Microbiol.* **33,** 21–39.
2. Deane, E. E., Whipps, J. M., Lynch, J. M., and Peberdy, J. F. (1999), *Enzyme Microb. Technol.* **24,** 419–424.
3. Manczinger, L., Komonyi, O., Antal, Z., and Ferenczy, L. (1997), *J. Microbiol. Methods* **29,** 207–210.
4. Thammarongtham, C., Turner, G., Moir, A. J., Tanticharoen, M., and Cheevadhanarak, S. (2001), *J. Mol. Microbiol. Biotechnol.* **3(4),** 611–617
5. Pe'er, S. and Chet, I. (1990), *Can. J. Microbiol.* **36,** 6–9.
6. Ogawa, K., Ohara, H., and Toyoma, N. (1998), *Agric. Biol. Chem.* **52,** 337–342.
7. Ushijima, S., Nakadai, T., and Uchida, K. (1990), *Agric. Biol. Chem.* **54,** 1667–1676.
8. de Groot, M. J. A., Bundock, P., Hooykaas, P. J. J., and Beijersbergen, G. M. (1998), *Nat. Biotechnol.* **16,** 839–842.
9. Anjani Kumari, J. and Panda, T. (1992), *Enzyme Microb. Technol.* **14,** 241–248.
10. Robinson, H. L. and Deacon J. W. (2001), *Mycol. Res.* **105,** 1295–1303.
11. Necas, O. and Svodova, A. (1985), in *Fungal Protoplasts: Applications in Biochemistry and Genetics*, Peberdy, J. F. and Ferenczy, L., eds., Marcel-Dekker, New York, pp. 115–133.
12. Ruiz-Herrera, J. (1991), *Fungal Cell Wall: Structure, Synthesis, and Assembly*, CRC Press, Boca Raton, FL.
13. Burnett, J. H. (1979), in *Fungal Walls and Hyphal Growth*, Burnett, J. H. and Trinci, A. P. J., eds., Cambridge University Press, London, pp. 1–25.
14. Wessels, J. G. H., Mol, P. C., Sietsma, J. H., and Vermeulen, C. A. (1990), in *Biochemistry of Cell Walls and Membranes in Fungi*, Kunh, P. J., Trinci, A. P. J., Jung, M. J., Goosey, M. W., and Copping, L. G., eds., Springer-Verlag, Berlin, pp. 81–95.
15. Caddick, M. X., Brownlee, A. G., and Arst, H. N. (1986), *Mol. Gen. Genet.* **203,** 346–353.
16. Denison, S. H., Oreja, M., and Arst, H. N. (1995), *J. Biol. Chem.* **207,** 28,519–28,522.
17. Kuwabara, H., Magae, Y., Kashiwagi, Y., Okada, G., and Sasaki, T. (1989), *Enzynme Microb. Technol.* **11,** 696–699.
18. Marinova, D. N. (1994), *Lett. Appl. Microbiol.* **18,** 30, 31.
19. Aguilar, G. and Huitron, C. (1990), *Biotechnol. Lett.* **12,** 655–660.
20. Trejo-Aguilar, B. A., Visser, J., and Aguilar, G. (1996), in *Pectin and Pectinases*, Visser, J. and Voragen, A. G. J., eds., Elsevier Science, Amsterdam, pp. 915–920.
21. Delgado, L. (1998), MSc thesis, Universidad Nacional Autonoma de México, D. F. México.
22. Miller, L. (1959), *Anal. Chem.* **31,** 426–428.
23. Aguilar, G., Morlon-Guyot, J., Trejo-Aguilar, B., and Guyot, J. P. (2000), *Enzyme Microb. Technol.* **27,** 406–413.
24. Collings, A., Davis, B., and Mills, J. (1998), *Microbios* **53,** 197–210.
25. Kitamoto, Y., Mori, N., Yamamoto, M., Ohiwa, T., and Ichikawa, Y. (1988), *Appl. Microbiol. Biotechnol.* **28,** 445–450.
26. Bartinki-Garcia, S. and Lippman, E. (1969), *Science* **165,** 302–304.
27. Sandhu, D. K., Wadhwa, V., and Bagga, P. S. (1989), *Enzyme Microb. Technol.* **11,** 21–25.
28. Liu, W. and Zhu, W. M. (2000), *Process Biochem.* **35,** 659–664.
29. Peberdy, J. F. (1985), in *Fungal Protoplasts: Application in Biochemistry and Genetics*, Peberdy, J. F. and Ferenczy, L., eds., Marcel-Dekker, New York, pp. 31–44.
30. Polacheck, I. and Rosenberger, R. F. (1978), *J. Bacteriol.* **135,** 741–747.
31. White, S., Berry, D. R., and McNeil, B. (1999), *J. Biotechnol.* **75,** 173–185.
32. Harvey, L. M., McNeil, B., Berry, D. R., and White, S. (1998), *Enzyme Microb. Technol.* **22,** 446–458.
33. Lakshmi, B. R. and Chandra, T. S. (1993), *Enzyme Microb. Techol.* **15,** 699–702.