Journal of Industrial Microbiology & Biotechnology (2001) 26, 271–275
© 2001 Nature Publishing Group 1367-5435/01 $17.00

www.nature.com/jim

Exopectinases produced by *Aspergillus niger* in solid-state and submerged fermentation: a comparative study

G Díaz-Godínez[1], J Soriano-Santos[2], C Augur[3] and G Viniegra-González[2]

[1]*Área de Biotecnología del Centro de Investigación en Ciencias Biológicas, Universidad Autónoma de Tlaxcala, Ixtacuixtla Tlaxala, México;* [2]*Departamento de Biotecnología, Universidad Autónoma Metropolitana, Av. Michoacán y La Purísima, México, DF, CP 09340, México;* [3]*IRD-Mexique, Cicerón 609, Los Morales 11530, México, DF, México*

Exopectinase production by *Aspergillus niger* was compared in submerged fermentation (SmF) and solid-state fermentation (SSF). SSF was carried out using polyurethane foam (PUF) as the solid support. The purpose was to study the effect of sucrose addition (0 or 40 g/l) and water activity level (A_w=0.99 or 0.96) on the level of enzyme activity induced by 15 g/l of pectin. Mycelial growth, as well as extracellular protease production, was also monitored. Sucrose addition in SmF resulted in catabolite repression of exopectinase activity. However, in SSF, an enhancement of enzyme activity was observed. Protease levels were minimal in SSF experiments with sucrose and maximal in SmF without sucrose. Exopectinase yields (IU/g *X*) were negligible in SmF with sucrose. The high levels of exopectinase with sucrose and high A_w in SSF can be explained by a much higher level of biomass production without catabolite repression and with lower protease contamination. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 271–275.

Keywords: exopectinase; solid-state fermentation; polyurethane; enzyme yield

Introduction

Enzymes are important commercial products with an estimated annual sales value between $600 and $700 million [14]. Another estimate [4] indicates a world sales figure of $505 million in 1991 and $1 billion for 2000 with more than 9% annual growth. A large share goes to food and feed enzymes, with a total market of about $483 million in 1995, including "pectinases (which) are of the utmost importance in juice and wine production" [4]. Most of the enzymes are produced by the submerged fermentation (SmF) technique [11]. However, the number of research papers related to the alternative solid-state fermentation (SSF) technique is increasing steadily [1–3,5,7,12,13,15,17–20,22–27]. Furthermore, several studies have been published describing the use of SSF for pectinase production [3,5,7,12,22–24]. Advantages of the latter technique include higher enzyme titers [22,23], higher productivity levels [2], stability of excreted enzymes [2,17], and a low level of catabolite repression [2,15,22,23]. However, such studies have not yet established the nature of the physiological differences between pectinase production in SSF and SmF, e.g., in terms of technical coefficients such as specific growth rate (μ=h^{-1}) and enzyme yield ($Y_{E/X}$=enzyme units/g biomass). Lack of a detailed understanding is due mainly to the biodegradable nature of the solid supports used, such as sugar cane bagasse [1,2], coffee pulp [3,17], and wheat bran [7,12]. In these examples, biomass measurements have been either difficult or impossible to obtain.

Recently, polyurethane foam (PUF) has been used as inert support for enzyme production by the SSF technique [18,26,27], allowing direct measurement of biomass production, together with measurements of substrate uptake and enzyme activity in the culture medium. An important question to address is whether higher enzyme productivity by SSF is related to higher levels of biomass production or to higher enzyme yields (units/g biomass). This paper presents evidence supporting the former by measuring kinetic parameters of biomass production and enzyme excretion using a strain of *Aspergillus niger* cultured on defined liquid medium adsorbed on PUF cubes. Special attention is paid to the effect of environmental factors such as water activity, the proportion of sucrose and pectin in the culture medium, and the presence of protease activity. Differences in stability of exopectinase between SmF and SSF are, in part, due to different levels of protease activities induced by the environmental factors and protein in the crude extract. Such considerations may be of utmost importance when considering future industrial applications.

Materials and methods

Microorganism

A. niger C28B25 [3,5] was propagated in Erlenmeyer flasks at 35°C for 72 h and maintained at 4°C on potato–dextrose agar.

Culture conditions

Media: Two basal liquid media having the same C/N=5.5 ratio were used. The first was labeled NS (no sucrose) having (g/l): pectin with 8% methoxylation, 15; $(NH_4)_2SO_4$, 2.9; K_2HPO_4, 3.0; urea, 1.0; $MgSO_4$–$7H_2O$, 0.066; $FeSO_4$, 0.096; $MnCl_2$, 0.001; $ZnCl_2$, 0.001; $CuSO_4$, 0.001; water up to 1 l. The second was labeled PS (plus sucrose) and contained: pectin, 15; sucrose, 40; $(NH_4)_2SO_4$, 12.6; K_2HPO_4, 6.5; urea, 3.0; $MgSO_4$–$7H_2O$, 0.2; $FeSO_4$, 0.29; $MnCl_2$, 0.001; $ZnCl_2$, 0.001; $CuSO_4$, 0.001. Water up to 1 l was added. Both media were adjusted to an initial pH of 4.5. To depress water activity (A_w=0.96), ethylene glycol was added to 10% (v/v). In all other experiments, A_w=0.995 was measured from vapor pressure at equilibrium. All chemicals were from J.T.

Correspondence: G Viniegra-González, Departamento de Biotecnología, Universidad Autónoma Metropolitana, Av. Michoacán y Purísima DF, CP 09340, México
Received 5 July 2000; accepted 27 January 2001

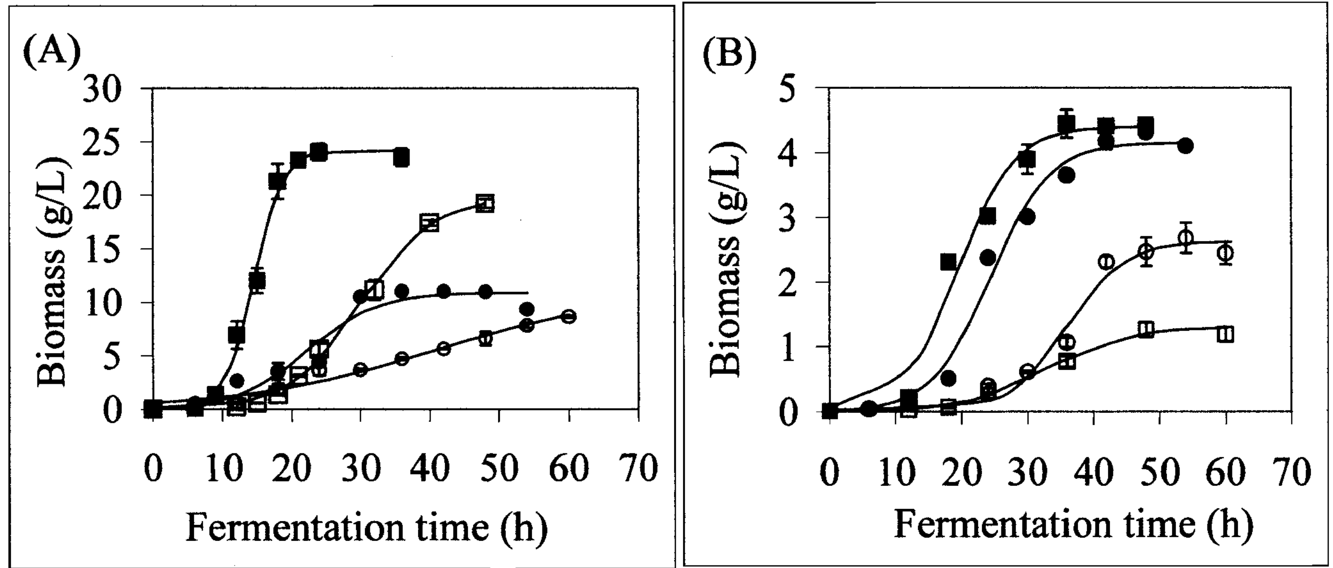

Figure 1 Growth curves of *A. niger* obtained by (◪) SSF and (○) SmF techniques. (A) In the presence of sucrose (PS media) and (B) in the absence of sucrose (NS media). Full symbols correspond to A_w = 0.995 and open symbols correspond to A_w = 0.96. The error bars on the graphs represent three different fermentation runs.

Baker (Mallinckrodt Baker, S.A. de C.V. Edo. de México, México), except pectin which was from Sigma–Aldrich, (Sigma Chemical Co. St. Louis, MO, USA).

SmF: Erlenmeyer flasks with a nominal volume of 125 ml were filled with 50 ml of liquid medium and inoculated under aseptic conditions with 10^8 spores per gram of carbon source. All cultures were incubated at 35°C using an orbital shaker rotating at 200 rpm.

SSF: Low-density (17 kg/m^3) PUF was cut into cubes (0.5×0.5×0.5 cm^3) and washed twice with boiling water. Erlenmeyer flasks with a nominal volume of 250 ml were filled with 1 g of oven-dried and sterile PUF cubes to which 30 ml of liquid medium was added. Inoculation was made under aseptic conditions with 10^8 spores per gram of carbon source. All cultures were incubated at 35°C.

Separation of biomass and crude extracts in SmF and SSF systems

Enzyme extracts (EEs) obtained by SmF were filtered through Whatman no. 4 paper and subsequently through a Millipore membrane (0.45 μm pore size). Biomass retained on the Whatman paper was dried to constant weight. At selected times, 20 ml of distilled water was added to SSF and the PUF was then compressed in a Büchner funnel. EEs obtained were passed through a Millipore membrane (0.45 μm pore size). The solid retentate with PUF was dried to constant weight. The calculated biomass was the difference between initial and final PUF weights [27]. The pH of the EE was then measured. All experimental data are presented as mean±SD of three separate runs.

Excreted protein

Total extracellular protein excreted was measured in cell-free EE by the Bradford [6] method using bovine serum albumin as standard.

Exopectinase activity

Exopectinase activity in cell-free EE was assayed by quantifying reducing sugars using the DNS (3.5-dinitrosalicylic acid) method [16]. The incubation mixture consisted of 0.1 ml of EE, 0.5 ml of 1.0% pectin solution, and 0.4 ml of acetate buffer pH 4.5 incubated for 15 min at 45°C. Results were expressed as galacturonic acid equivalents. One international unit (IU) of exopectinase activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol galacturonic acid per minute.

Protease activity

Protease activity in cell-free EE was measured by changes in the absorbancy at 520 nm. They were produced by 50 μl of EE and mixed with 850 μl of 0.5% Azocoll (Sigma) in acetate buffer 0.1 M, pH 4.5 after 30 min of incubation at 35°C. The reaction was stopped with 100 μl of 5% trichloroacetic acid. One arbitrary Azocoll unit (AU) of protease activity was defined as the amount of enzyme that released an azo group, increasing by 0.001 the absorbency per minute [8,9].

Estimation of kinetic parameters

Evolution of biomass X=$X(t)$ was followed by the Velhurst–Pearl or logistic equation:

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_{\max}}\right]X \qquad (1)$$

where μ is the maximal specific growth rate and $X_{\max}$ is the maximal (or equilibrium) biomass level achieved when dX/dt=0 for X >0. The solution of Equation 1 is as follows:

$$X = \frac{X_{\max}}{1 + Ce^{-\mu t}} \qquad (2)$$

where C=$(X_{\max} - X_0)/X_0$ with X=X_0 being the initial biomass value.

Estimation of kinetic parameters in the previous equation was done using a non-linear least square fitting program called

Table 1 Growth kinetic parameters of *A. niger* and maximal values of exopectinase activity

Fermentation system	A_w	Media	μ (h^{-1})	X_{max} (g/l)	Exopectinase activity (IU/l)	Specific activity (IU/mg protein)	$Y_{E/X}$ (IU/g X)
SSF	0.995	PS*	0.47	23.9 (0.87)[a]	7150 (450)[a]	382	330
		NS*	0.23	4.4 (0.06)[e]	623 (16.5)[d,e]	29	187
	0.96	PS	0.18	18.5 (1.04)[b]	1911 (141)[b]	91	600
		NS	0.15	1.2 (0.08)[g]	541 (8.1)[e]	33	1129
SmF	0.995	PS	0.19	11.0 (0.12)[c]	355 (0.8)[e]	10	0
		NS	0.22	4.38 (0.06)[e]	1714 (34)[b]	181	534
	0.96	PS	0.07	9.9 (0.47)[d]	876 (50)[d]	24	0
		NS	0.22	2.7 (0.13)[f]	1405 (165)[c]	119	557

Means with the same letter are not significantly different. Numbers in parenthesis correspond to standard errors of three separate experiments.
*PS=40 g/l sucrose, NS=no sucrose. All media had 15 g/l pectin.

"Solver" present in Excel electronic sheet (Microsoft). The coefficient $Y_{E/X}$ was estimated as the slope of the initial linear correlation between E (IU/l) and X (g X/l).

Results

Biomass production

Figure 1 shows biomass production by *A. niger* as a function of fermentation time. SSF had higher production of biomass than SmF, independent of the presence or absence of sucrose or the different levels of A_w. However, the effect of ethylene glycol on biomass production for SSF was stronger than for SmF. Growth curves were fitted by Equation 1 with high correlation coefficients (R^2>0.98) showing that the model was adequate for this process. Highest values of X_{max} (around 20 g/l) were obtained in SSF in the presence of sucrose with A_w=0.99 (Table 1). In the absence of sucrose, biomass was greatly reduced whether produced by the SmF or SSF technique and independently of A_w (Table 1, Figure 1). A similar effect was found for μ: sucrose addition in SSF with A_w=0.99 had μ=0.47 h^{-1}, as compared to μ=0.23 h^{-1} without sucrose. Addition of sucrose to the SmF system did not enhance the rate of growth but increased values of X_{max}, although the highest values of X_{max} in SSF experiments show that *A. niger* can use high levels of sugar more efficiently when cultured on solid support than in submerged culture. This result is very similar to the one obtained with three different strains of *A. niger* grown on PUF or shake flasks and supplied with 100 g/l of sucrose [21], which seems to be the case when initial substrate concentration is higher than 50 g/l (unpublished results). However, depression of water activity by ethylene glycol affected, to a greater extent, the fast-growing cultures in SSF with sucrose than the slow-growing cultures in SmF.

Exopectinase activity

Figure 2A shows the profiles of extracellular exopectinase titers obtained with sucrose addition. In the SSF system, sucrose addition led to a high level of exopectinase activity (E_{max}≈7000 IU/l) when compared to exopectinase activity in SmF (E_{max}≈2000 IU/l).

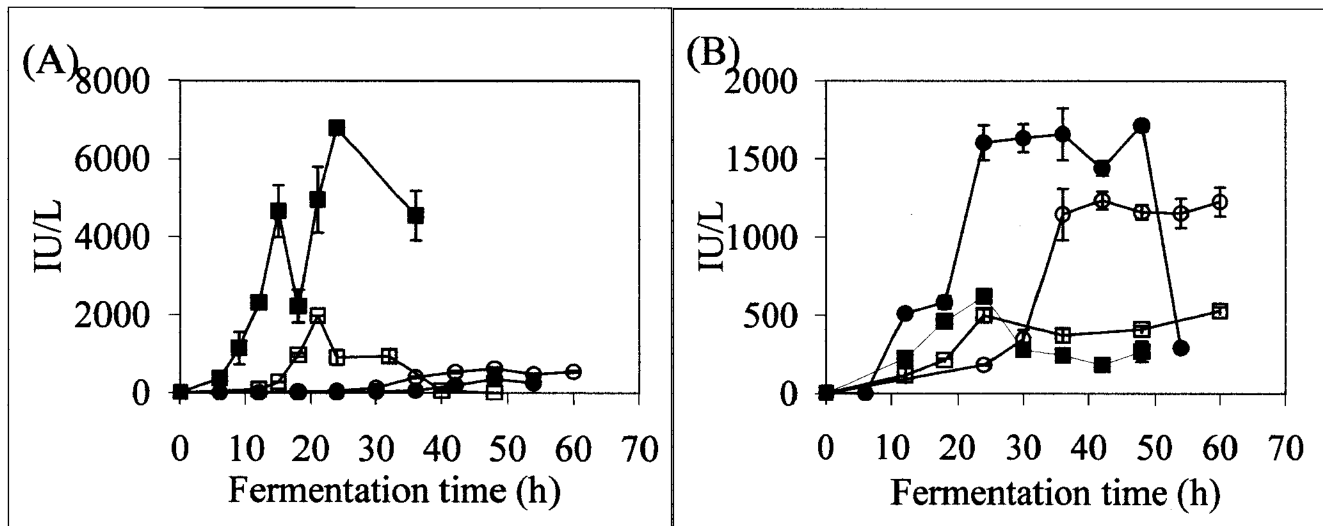

Figure 2 Comparative titers of exopectinase activity produced by *A. niger* in (◪) SSF and (○) SmF techniques. (A) In the presence of sucrose (PS media) and (B) in the absence of sucrose (NS media). Full symbols correspond to A_w = 0.995 and open symbols correspond to A_w = 0.96. The error bars on the graphs represent three different fermentation runs.

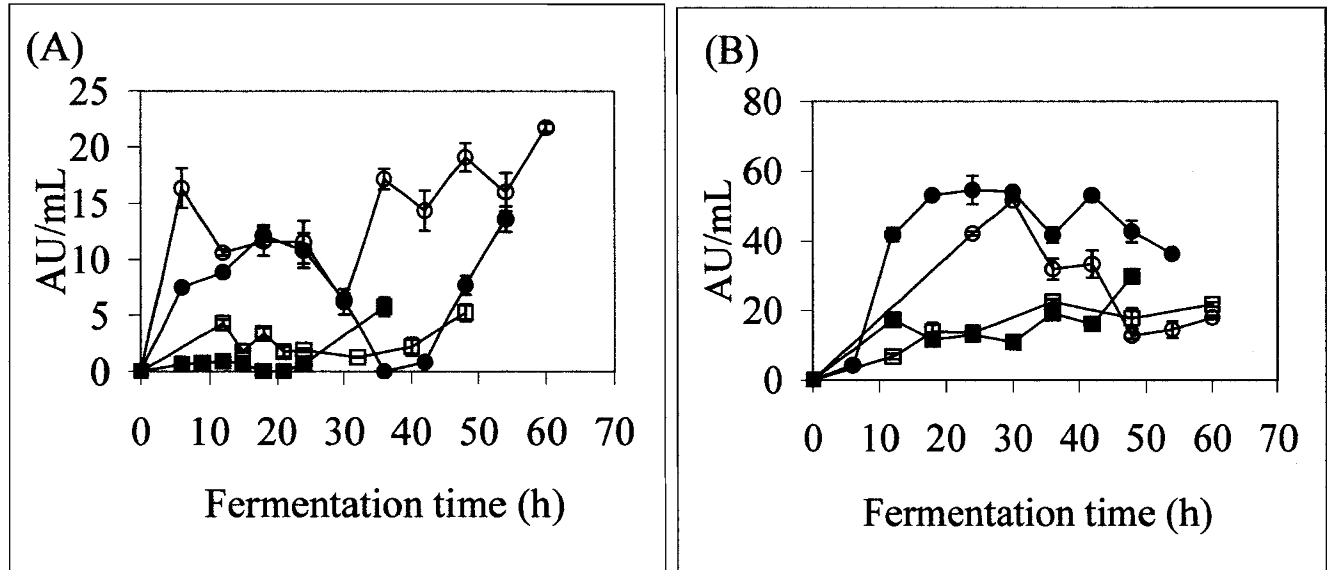

Figure 3 Protease production by *A. niger* in (◪) SmF and (○) SmF techniques. (A) In the presence of sucrose (PS media) and (B) in the absence of sucrose (NS media). Full symbols correspond to A_w = 0.995 and open symbols correspond to A_w = 0.96. The error bars on the graphs represent three different fermentation runs.

Addition of ethylene glycol (A_w=0.96) decreased maximum exopectinase levels in all cases. Analysis of variance (ANOVA) of E_{max} data showed again, as in biomass production, that exopectinase production was enhanced by high levels of water activity and by the use of SSF over SmF. It is worth noticing that in the SmF system with sucrose addition, a repressive effect was observed. However, in SSF, the effect was the opposite (Figure 2, Table 1). Exopectinase production had a linear correlation (R^2>0.98) with the level of biomass during the initial period of fermentation (X<$X_{max}/2$), which roughly corresponds to the exponential phase of growth. The value of the slope was designated $Y_{E/X}$ (IU/g X) and was a measure of the productivity of exopectinase by the fungal cells. Table 1 shows that $Y_{E/X}$ was in SSF (with or without sucrose, and at the two A_w levels) equal or lower than $Y_{E/X}$ in SmF (without sucrose), suggesting that increases in exopectinase production by the SSF system were related to better fungal growth but not to higher productivity of exopectinase. This is important because it has been the subject of speculation as to why SSF produces higher levels of enzymes [2,19,22]. The specific activity (SA) of excreted exopectinase was expressed as international units per milligram of excreted protein, as shown in Table 1. Exopectinase specific activity was highest in SSF in the presence of sucrose and with a water activity of 0.995. This indicates that *A. niger* grown by the SSF technique on PUF produces a purer pectinase extract as compared to the experiments done by the SmF technique.

Protease activity

The kinetics of protease production by *A. niger* in SmF and SSF is shown in Figure 3. Protease production, regardless of the experimental conditions, was sharply lower in SSF. Moreover, the presence of sucrose in the media further decreased protease production (Figure 3). Hence, the SSF technique may offer additional advantages in terms of exopectinase stability because of reduced proteolysis.

Discussion

The biomass of *A. niger* grown by SSF could be measured directly, thanks to the use of PUF as an inert carrier. The level of X_{max} in SSF was higher than was obtained in SmF under equivalent conditions and with the same media. This may be because of the higher availability of oxygen in the solid-to-air interphase, since it is well known that oxygen dissolves poorly in water (6 mg/l). In all cases, residual sugars were minimal. In addition, the estimated maintenance coefficients were higher for the SmF system than for the SSF system (data not shown). In the presence of 40 g/l of sucrose, no catabolite repression was observed in SSF, but did occur in SmF, confirming previous observations that in SSF there is a resistance to catabolite repression of pectinase as reported by other authors [22,23] in SSF on sugarcane bagasse. Unpublished results obtained by Díaz-Godínez showed no inhibition of exopectinase activity in SSF when 40, 60, 80, or 100 g/l of sucrose was used. The SmF system produced more exopectinase activity per gram of biomass ($Y_{E/X}$) than SSF (Table 1). However, the maximal values of exopectinase activity and X_{max} were obtained in SSF. Hence, the high titers of exopectinase activity obtained by the SSF technique were related to the amount of biomass and not to intrinsic differences in enzyme yield. Whatever the experimental conditions (SSF or SmF; with or without sucrose), the addition of 10% ethylene glycol (A_w=0.96) resulted in lower biomass production and lower exopectinase activity. Similar results have been reported [1], and this confirms the importance of high A_w levels on growth and enzyme production. SmF resulted in production of more protease activity than SSF, but the presence of sucrose in media reduced protease activity in both systems. Perhaps, stress conditions induced protease activity in *A. niger*, and such stress conditions were milder in the presence of sucrose at high A_w and using the SSF technique. There are reports on inhibition of protease activity by immobilization of *A. niger* [10]. In summary, we now have reasons to hypothesize that mass transfer phenomena of oxygen and substrates in solid supports are fundamental factors that

account for differences of mold physiology compared to stirred liquid fermentations. Higher oxygen levels at the solid-to-air interphase would support faster growth with higher oxygen demand and very slow diffusion of substrate in the absorbed liquid, producing local substrate and product gradients of concentration. At any rate, a practical consequence of our work is to stress the advantage of SSF with supplemented concentrated culture broths in order to achieve high pectinase titers with low protease activity in the spent liquor.

Acknowledgements

This work was partially financed by an agreement between Universidad Autónoma Metrpolitana (México) and Institut of Recherche pour le Développement (IRD, France) and by a grant from Consejo Nacional de Ciencia y Technología (CONACYT, México) to G. Díaz-Godínez.

References

1 Acuña-Argüelles ME, M Gutiérrez-Rojas, G Viniegra-González and E Favela-Torres. 1994. Effect of water activity on exo-pectinase production by *Aspergillus niger* on solid-state fermentation. *Biotechnol Lett* 16: 23–28.
2 Acuña-Argüelles ME, M Gutiérrez-Rojas, G Viniegra-González and E Favela-Torres. 1995. Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid-state fermentation. *Appl Microbiol Biotechnol* 43: 808–814.
3 Antier P, A Minjares, S Roussos, M Raimbault and G Viniegra. 1993. Pectinase-hyperproducing mutants of *Aspergillus niger* C28B25 for solid-state fermentation of coffee pulp. *Enzyme Microb Technol* 15: 254–260.
4 Biotechnology Support Services. 2000. http://www.biotechsupportindia.com/market.htm.
5 Boccas F, SS Roussos, M Gutierrez, L Serrano and G Viniegra-González. 1994. Production of pectinase from coffee pulp in solid-state fermentation system: selection of wild fungal isolate of high potency by a simple three-step screening technique. *J Food Sci Technol* 31: 22–26.
6 Bradford MM. 1977. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72: 248.
7 Cavalitto SF, JA Arcas and RA Hours. 1996. Pectinase production profile of *Aspergillus foetidus* in solid-state cultures at different acidities. *Biotechnol Lett* 18: 251–256.
8 Chavira R Jr, TJ Burnett and JH Hageman. 1984. Assaying proteinases with azocoll. *Anal Biochem* 136: 446–450.
9 Dosoretz C, H-C Chen and H Grethlein. 1990. Effect of environmental conditions on extracellular protease activity in lignolytic cultures of *Phanerochaete chysosporium*. *Appl Environ Microbiol* 56 (2): 395–400.
10 Fang L, L Wenqing, R Darin, G Tingyue and MY Murray. 1998. Inhibition of extracellular protease secretion by *Aspergillus niger* using cell immobilization. *Biotechnol Lett* 20 (6): 539–542.
11 Harvey LM and B McNeil. 1993. Liquid fermentation systems and product recovery of *Aspergillus*. In: JE Smith (ed.), Biotechnology Handbooks 7. Plenum Press, New York.
12 Hours RA and T Sakai. 1994. Protopectinase production in solid state culture of *Aspergillus awamori. Biotechnol Lett* 16 (7): 721–726.
13 Hesseltine CW. 1972. Biotechnology report solid state fermentations. *Biotechnol Bioeng* 14: 517–532.
14 Layman P. 1990. Promising new markets emerging for commercial enzymes. *Chem Eng News* 68: 17–18.
15 Maldonado MC, AM Strasser and DA Callieri. 1989. Regulatory aspects of the synthesis of polygalacturonase and pectinesterase by *Aspergillus niger* sp. *Sciences Des Aliments* 9: 101–110.
16 Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31: 426–428.
17 Minjares-Carranco A, B Trejo-Aguilar, G Aguilar and G Viniegra-González. 1997. Physiological comparison between pectinase-producing mutants of *Aspergillus niger* adapted either to solid-state fermentation or submerged fermentation. *Enzyme Microb Technol* 21: 25–31.
18 Murado MA, MP González, A Torrado and LM Pastrana. 1997. Amylase production by solid-state culture of *Aspergillus oryzae* on polyurethane foams. Some mechanistic approaches from an empirical model. *Process Biochem* 32: 35–42.
19 Pandey A, P Sevakumar, CR Soccol and P Nigam. 1999. Solid-state fermentation for the production of industrial enzymes. *Curr Sci* 77: 149–162.
20 Qadeer MA, J Iqbal, SK Aziz and H Hassan. 1985. Solid-substrate fermentation for pectinase production by *Aspergillus foetidus*. *Pak J Sci Ind Res* 28: 179–181.
21 Romero-Gómez S, C Augur and G Viniegra-González. 2000. Invertase production by *Aspergillus niger* in submerged and solid-state fermentation. *Biotechnol Lett* 22: 1255–1258.
22 Solís-Pereyra S, E Favela-Torres, G Viniegra-González and M Gutiérrez-Rojas. 1993. Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid-state fermentations. *Appl Microbiol Biotechnol* 39: 36–41.
23 Solís-Pereyra S, E Favela-Torres, M Gutiérrez-Rojas, S Roussos, G Saucedo-Castañeda, P Gunasekaran and G Viniegra-González. 1996. Production of pectinases by *Aspergillus niger* in solid-state fermentation at high initial glucose concentrations. *World J Microbiol Biotechnol* 12: 1–4.
24 Trejo-Hernández MR, E Oriol, A López-Canales, S Roussos, G Viniegra-González and M Raimbault. 1991. Producción de pectinasas de *Aspergillus niger* por fermentación sólida sobre soporte. *Micol Neotrop Appl* 4: 49–62.
25 Viniegra-González G. 1997. Strategies for the selection of mold strains geared to produce enzymes in solid substrates. In: Galindo E and OT Ramírez (Eds.), *Advances in Bioprocess Engineering II*. Kluwer Academic Publishing, Netherlands, pp. 123–135.
26 Zhu Y, W Knol, JP Smits and J Bol. 1994. A novel solid-state fermentation system using polyurethane foam as inert carrier. *Biotechnology* 16: 643–648.
27 Zhu Y, W Knol, JP Smits and J Bol. 1996. Medium optimization for nuclease P1 production by *Penicillium citrinum* in solid-state fermentation using polyurethane foam as inert carrier. *Enzyme Microb Technol* 18: 108–112.