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Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentations

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Abstract. A study was made to compare the production of pectinase by *Aspergillus niger* CH4 in solid-state (SSF) and submerged (SmF) fermentations. Production of endo- (endo-p) and exo-pectinase (exo-p) by SSF was not reduced when glucose, sucrose or galacturonic acid (up to 10%) were added to a culture medium containing pectin. Moreover, both activities increased when concentrations of the carbon sources were also increased. In SmF, these activities were strongly decreased when glucose or sucrose (3%) was added to culture medium containing pectin. The addition of galacturonic acid affected endo-p activity production to a lesser extend than exo-p. Final endo-p and exo-p activities in SSF were three and 11 times higher, respectively, than those obtained in SmF. The overall productivities of SSF were 18.8 and 4.9 times higher for endo-p and exo-p, respectively, than those in SmF. These results indicate that regulatory phenomena, such as induction-repression or activation-inhibition, related to pectinase synthesis by *A. niger* CH4 are different in the two types of fermentation.

Introduction

Pectinases are a group of enzymes that catalyse the breakdown of pectin-containing substances and can be produced either by submerged (Fogarty and Ward 1974) or solid-state (Siessere and Said 1989) fermentation procedures. These enzymes are widely used in the industrial processing of fruits and vegetables since they decrease the viscosity of juices and facilitate extraction, maceration, liquefaction, filtration and clarification processes (Robbins 1968; Brawman 1981).

Submerged fermentation (SmF) systems have been extensively used for the production of high-priced materials and for the study of biochemical and physiological aspects of the synthesis of microbial metabolites. On the other hand, solid-state fermentations (SSF) have been used to improve the production of microbial metabolites such as proteins (Mitchell et al. 1988), enzymes (Nisio et al. 1979) and ethanol (Kargi and Curme 1985). However, there are insufficient fundamental studies on the physiology of the microorganisms involved as well as their regulatory systems when applied to this second kind of fermentation system. A previous comparative report (Trejo-Hernández et al. 1991) on pectinase production from sucrose and pectin by SSF and SmF techniques with *Aspergillus niger* CH4 found a greater enzyme yield for SSF.

Pectinase produced by SmF with moulds of the genera *Aspergillus* and *Fusarium* are induced by pectin or by some of its derivatives (Zetelaki 1976; Perley and Page 1971). More specifically, polygalacturonase and pectinesterase synthesis by *A. niger* is induced by galacturonic or polygalacturonic acid and at the transcription level (Maldonado et al. 1989). Pectinase production by moulds can also be controlled by catabolite repression exerted by a readily assimilated carbon source (Siessere and Said 1989; Fogarty and Kelly 1983). In *A. niger* 26, glucose represses polymethylgalacturonase synthesis; repression is reversed when cAMP is added to the fermentation medium (Angelova et al. 1987). Polygalacturonase synthesis by *A. niger* is also repressed by glucose during transcription and translation (Kertezs 1955; Torakazu et al. 1975). All this knowledge on the regulatory aspects of pectinase production by moulds has been generated by SmF studies. However, there is a lack of information about the regulatory aspects of pectinase synthesis in SSF.

The objective of the present work was to compare the overall physiological behaviour of mould pectinase production in solid and liquid fermentations. More specifically, we have evaluated the effect of the addition of glucose, sucrose and galacturonic acid on the activities of pectinase produced by SmF and SSF systems.

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Materials and methods

Microorganism. A strain of *A. niger* CH4, donated by Dr. Carlos Huitrón (Biotechnology Department, IIBM, UNAM, México) was used. It was propagated on potato-dextrose-agar (PDA) slants at 35° C and transferred monthly. For short-term storage, slants were maintained at 4° C. Stock cultures were maintained in spore suspensions frozen in 25% glycerol. Inocula were prepared on 250-ml flasks with 40 ml PDA medium. After 72 h incubation at 35° C, 50 ml Tween 80 (0.05%) was added. Spores were suspended under agitation with a magnetic stirrer and counted in a Neubauer chamber.

Submerged fermentations (SmF). The fermentation medium contained (%, w/v): $(NH_4)_2SO_4$, 0.66; KH_2PO_4, 0.35; $FeSO_4$, 0.015; and $MgSO_4 \cdot 7H_2O$, 0.01. Pectin (3%) and 3% pectin supplemented with either sucrose, glucose or galacturonic acid (3%, w/v) were used as carbon sources. Liquid media were sterilized for 15 min at 121° C. The initial pH was adjusted to 4.5 with 0.5 M NaOH solution. An inoculum of 10^7 spores per flask was used. Fermentations were carried out in 150-ml flasks containing 50 ml medium on a rotatory shaker at 200 rpm at 35° C.

Solid-state fermentations (SSF). The fermentation medium contained (%, w/w dry matter): $(NH_4)_2SO_4$, 4.2; KH_2PO_4, 2.17; urea, 1.0; $FeSO_4$, 0.1; $MgSO_4 \cdot 7H_2O$; 0.07; and sugar cane bagasse (SCB) pith as support, 77. Pectin at 3.5, 5.5 and 10% and 5.5% pectin supplemented with either sucrose, glucose or galacturonic acid at 3.5, 7 and 10% were used. Medium constituents were dissolved in 150 ml distilled water. The solution was heated at 70° C for 15 min; the SCB was separately sterilized for 15 min at 121° C. After cooling, both fractions were mixed and inoculated with a spore suspension (2×10^7 spores/g dry matter). The initial pH and moisture values were 4.5 and 70%, respectively. Glass columns (3.5 cm × 27 cm) were packed with 50 g of the inoculated material at an apparent density of 0.3 g/cm^3 and were incubated in a water bath at 35° C. The aeration rate was 0.4 l air/g dry matter per hour. Fermentations were carried out in a system reported by Raimbault and Alazard (1980). It should be noted that differences in media composition for SmF and SSF were due to independent nutritional requirements optimized by previous work (E. Favela, unpublished results).

Enzyme extraction. For SSF, 20 g fermented material was mixed with 20 ml distilled water and immediately pressed at 87 kg/cm^2 in a hydraulic press. The liquid extract was filtered (Millipore, 0.45 µm) and kept at 4° C for enzymatic assays. Previous work (Trejo-Hernández et al. 1991) indicated that single extraction procedures yielded approximately 80% of total extractable pectinase. For SmF, liquid samples were filtered (Millipore, 0.45 µm) and kept at 4° C for further assays.

Analytical methods. Polygalacturonase activities were measured at 45° C by viscometry for endo-pectinase (endo-p) and by the release of reducing sugars for exo-pectinase (exo-p). For viscometry, 1 ml of a suitably diluted sample was mixed with 18 ml of 2% pectin in 0.1 M acetate buffer, pH 4.5. Reduction in viscosity was followed with a rotational viscosimeter (Brookfield Engineering Laboratories, USA). One endo-p unit (U) was defined as the amount of enzyme that reduces the viscosity of the solution by 50% per minute under the conditions mentioned above. For measuring reducing sugars, samples were dialysed against 0.1 M acetate buffer, pH 4.5; 0.3 ml of a suitably diluted sample was added to a solution containing 1 ml of 0.9% of the substrate and 0.7 ml of 0.1 M acetate buffer, pH 4.5; samples were incubated at 45° C for 30 min; reducing sugars were determined by the dinitrosalicylic (DNS) acid method (Miller 1959) using galacturonic acid as reference. One exo-p unit (U) was defined as the quantity of enzyme that liberates one micromole of galacturonic acid per minute under the conditions mentioned above.

In SmF, biomass was measured after filtration (Millipore, 0.45 µm) and dried until constant weight at 60° C. All fermentations and assays were carried out in duplicate. The results shown are mean values. In SSF, biomass could not be measured accurately by using Lowry or Kjeldahl techniques due to the interference of bagasse in protein measurements.

Results and discussion

Effect of the addition of sucrose, glucose or galacturonic acid on pectinase production in SmF

In order to study the effect on the production of pectinase by *A. niger* CH4, pectin supplemented with either glucose, sucrose or galacturonic acid as carbon sources was used. The results are shown in Figs. 1 and 2. When pectin was used as the sole carbon source, the production of endo-p and exo-p increased to 0.80 U/ml and 2.1 U/ml, respectively (Fig. 1A and B). Both activities decreased rapidly after 72 h of fermentation. Endo-p was not detected at 96 h of culture and exo-p reached 0.85 U/ml at 120 h. This decrease (Fig. 2C) may be pH-related; previous work (unpublished results) showed that above pH 6.5, endo-p and exo-p produced by *A. niger* CH4 under the same culture conditions were unstable.

When either sucrose, glucose or galacturonic acid was added to the medium the pectinolytic activity profiles were different from those obtained with pectin as the sole carbon source (Fig. 1A and B). The addition of sucrose or glucose to the pectin medium had an inhibitory effect on the synthesis of both endo-p and exo-p. Endo-p production was reduced more than that of exo-p. Reduction of both types of enzyme activities in the presence of sugars seems to be related to the addition of

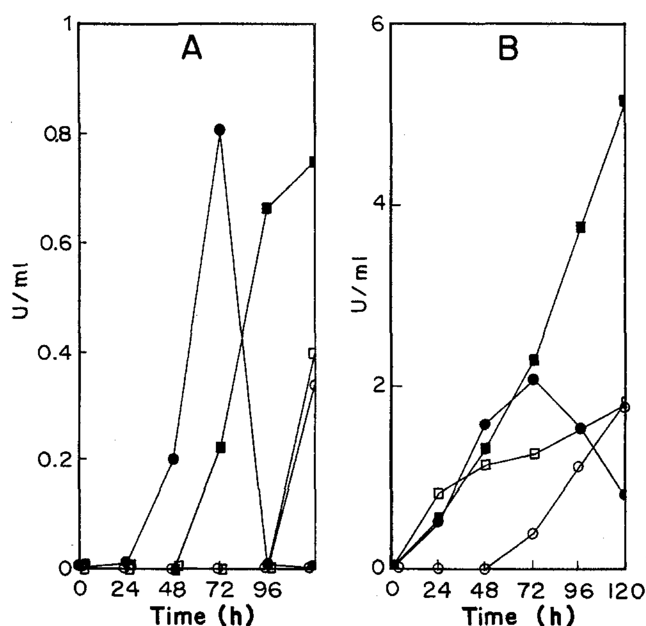

Fig. 1A,B. Pectinase production by submerged fermentation (SmF) with various carbon sources: ●, pectin; ○, pectin + glucose; ■, pectin + galacturonic acid; □, pectin + sucrose. **A** Endo-pectinases. **B** Exo-pectinases

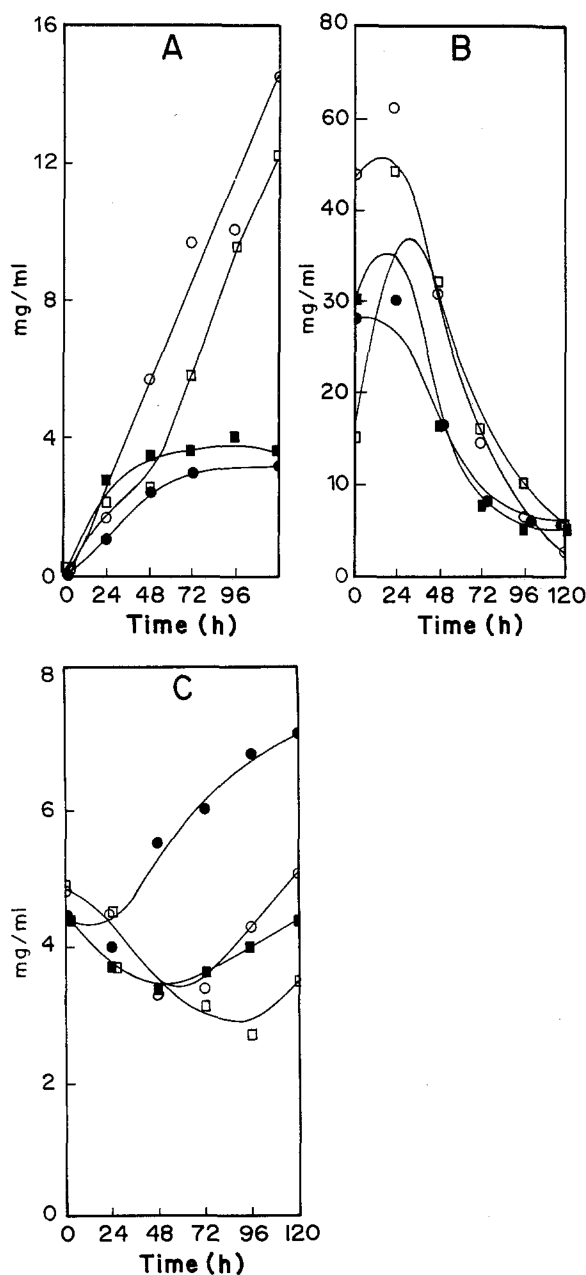

Fig. 2A–C. Biomass production (**A**), reducing sugar consumption (**B**) and pH change (**C**) during pectinase production by SmF: symbols as in Fig. 1

free sugars in the medium because the production of exo-p and endo-p (U/ml) was smaller and slower when glucose, sucrose or galacturonic acid were added as compared to sole pectin SmF (Fig. 2B). Similar studies (Aguilar and Huitrón 1990) carried out with different carbon sources (glycerol, sucrose, fructose and galacturonic acid) demonstrated that exo-p activity appeared earlier than endo-p. Furthermore, the same authors showed that endo-p is more sensitive to catabolic repression than is exo-p activity. When galacturonic acid was added to the pectin medium, values of 0.75 U/ml (endo-p) and 5.2 U/ml (exo-p) were obtained at 120 h of culture (Fig. 1). This stimulatory effect on pectinase production by *Aspergillus* sp. was reported previously (Aguilar and Huitrón 1986). Maximum biomass concentrations of 3.2, 3.6, 12.22 and 15.24 mg/ml were obtained with pectin, pectin-galacturonic acid, pectin-sucrose and pectin-glucose media, respectively (Fig. 2A). It has been recently demonstrated (Aguilar and Huitrón 1990) that sucrose and glucose are more efficiently used by *Aspergillus* sp. for growth than is galacturonic acid.

Results obtained in SmF (Figs. 1 and 2) indicated that the reduction or delay of enzyme endo-p and exo-p activities produced by *A. niger* CH4 by the addition of sugars (Fig. 1A and B) seemed to be correlated to the peak levels of free sugars in the medium (Fig. 2B). That is, glucose and sucrose produced higher levels of reducing groups (Fig. 2B) as compared to only pectin and pectin plus galacturonic acid. However, even when the final level of exo-p was similar (galacturonic acid and pectin vs only pectin), enzyme production was delayed (120 h vs 72 h in Fig. 1A). Moreover, this antagonistic effect of free sugars was found to be stronger for the endo-p activity than for exo-pectinase activities (Fig. 1A and B) since this type of activity was reduced by the addition of sugars or galacturonic acid. In relation to the positive effect on exo-p by adding galacturonic acid to the medium (Fig. 1A), it was previously reported that inductive or repressive effects of galacturonic acid on the synthesis of pectinase depend on its concentration in the culture medium (Aguilar and Huitrón 1986). In this case, the lack of information on enzyme titres cannot support a firm conclusion on the nature of regulatory action involved, i.e., induction-repression, or activation-inhibition, or both. It is interesting to see in Figs. 1, 2A, and 2B that biomass production was faster and greater with sugar addition (glucose and sucrose) than with solely pectin or pectin plus galacturonic acid, which is in agreement to the idea that glucose or sucrose are preferred substrates to pectin or galacturonic acid for mycelial growth. Changes of pH with time were very similar with all runs of SmF except for only pectin (Fig. 2C), where the final pH was above 7.0, whereas all others remained in the range 3.0–5.0. Perhaps such an increase in pH with only pectin was a factor causing the decrease in exo-p activity after the peak at 72 h (Figs. 1A and 2C).

Effect of the addition of sucrose, glucose and galacturonic acid on pectinase production in SSF

In order to study this effect, 3.5, 7.0 and 10.0% (w/w) of either galacturonic acid, sucrose or glucose were added to medium containing 5.5% pectin. Endo-p (Fig. 3) and exo-p (Fig. 4) SSF activities were stimulated by any of those additions when compared to SSF cultures containing only pectin. This finding is in sharp contrast with the general antagonistic effect of the addition of sugars of galacturonic acid to pectin described with SmF. Final endo-p (Fig. 3C) and exo-p (Fig. 4C) activities were almost the same when glucose, at the three concentrations tested, was added to the culture medium, representing more than twice the activities obtained with pectin as sole carbon source. Exo-p activity was more stimulated by glucose in SSF. Similar results were ob-

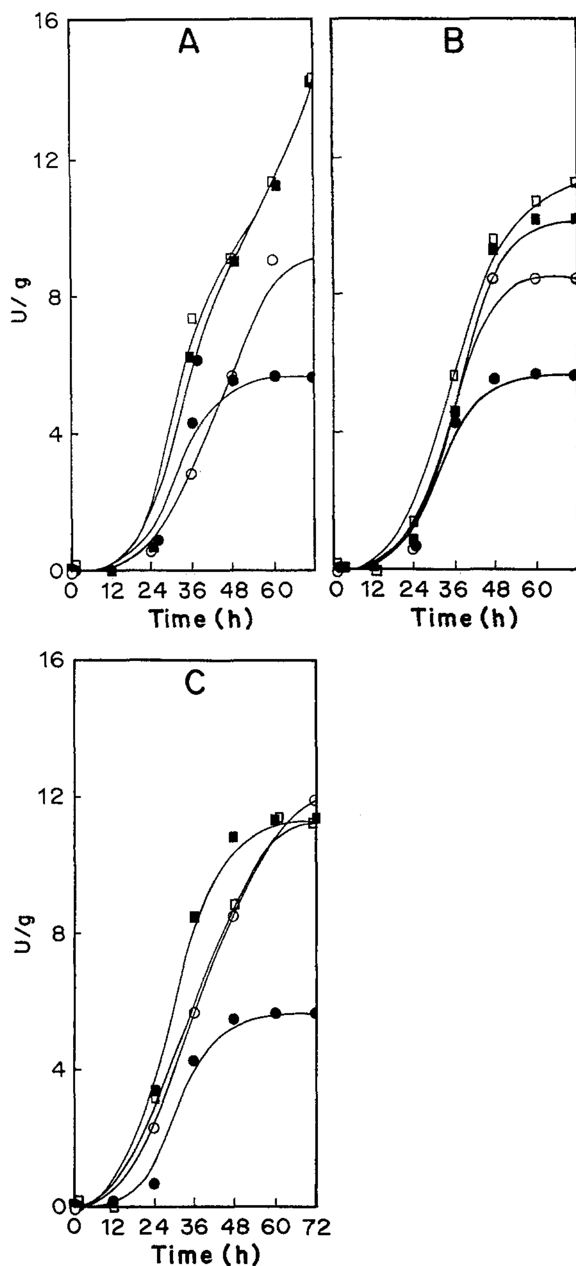

Fig. 3A–C. Endo-pectinase production by solid state fermentation (SSF). Effect of adding carbon sources at different concentrations to basal medium containing 5.5% pectin: ●, 0%; ○, 3.5%; ■, 7.0%; □, 10.0%. **A** Galacturonic acid. **B** Sucrose. **C** Glucose

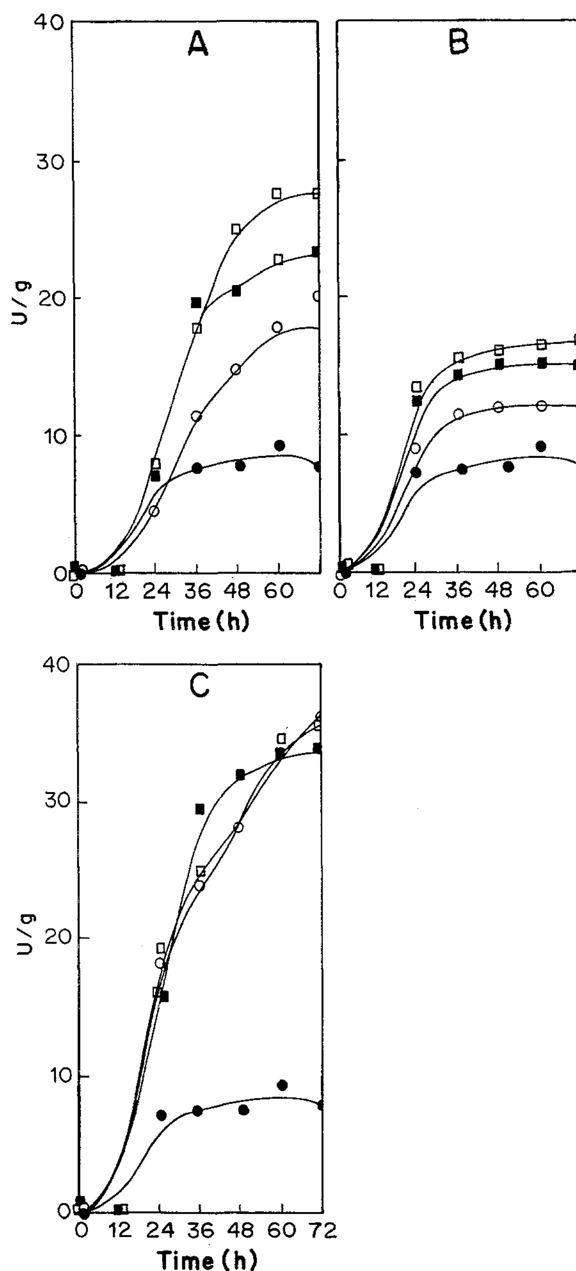

Fig. 4A–C. Exo-pectinase production by SSF. Effect of adding carbon sources at different concentrations to basal medium containing 5.5% pectin: symbols as in Fig. 3. **A** Galacturonic acid. **B** Sucrose. **C** Glucose

served for pectinase production by submerged fermentation by the plant pathogen *Botrytis cinerea* (Leone and Van den Heuvel 1986). Other work dealing with pectinase production in SmF has also shown that the addition of glucose to fungi growing on a pectic substrate caused a strong reduction in pectinase production (Aguilar and Huitrón 1987). When sucrose or galacturonic acid were added to the pectin-containing medium, endo-p and exo-p activities increased with increasing initial substrate concentration (Figs. 3 and 4). The stimulatory effect of galacturonic acid on the activities of endo-p and exo-p in SSF was higher than that observed for sucrose. Exo-p activity increased with increasing substrate concentration, with greater increase occurring with galacturonic acid than with sucrose (Fig. 4A). The stimulatory effect of galacturonic acid in submerged fermentation, depending on its concentration, has been previously reported (Aguilar and Huitrón 1990).

Addition of galacturonic acid or glucose was associated with initial (24 h) high rates of substrate consumption, which increased in proportion to the carbohydrate concentration (Fig 5A and B). When sucrose was added to pectin-containing media, a small increase in reducing groups was observed (Fig. 5B). However, the levels of sugars after 24 h (Fig. 4) were below 52 mg/g dry mass (equivalent to 9 mg/ml of absorbed liquid) and

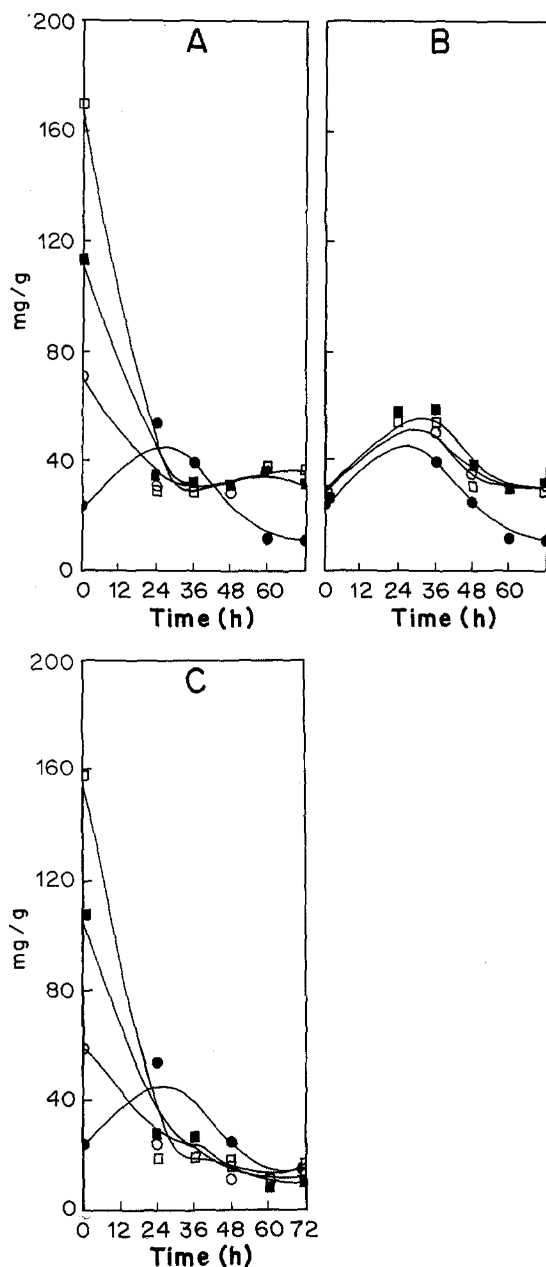

Fig. 5A–C. Reducing sugar profiles during pectinase production by SSF. Effect of adding carbon sources at differential concentrations to basal medium containing 5.5% pectin: symbols as in Fig. 3. **A** Galacturonic acid. **B** Sucrose. **C** Glucose

certainly smaller than the levels found in SmF (above 20 mg/ml). This supports the existence of physiological differences between SSF and SmF. It is worth noticing that the level of 20 mg/ml (2%) has been found to be associated with repression of the synthesis of polygalacturonases (Angelova et al. 1987) and polymethylgalacturonases (Torakazu et al. 1975) in other studies with *A. niger* in SmF. Those differences in sugar metabolism between SSF and SmF may be associated with different patterns of pectinase production for each fermentation technique.

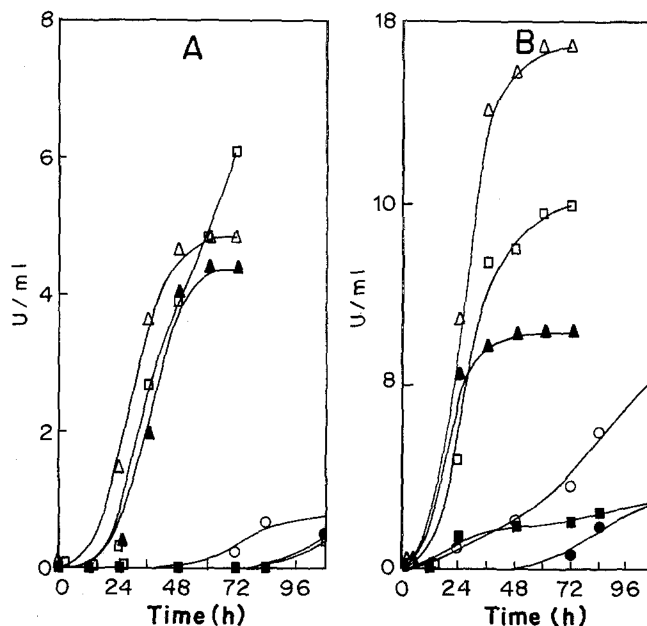

Fig. 6. Endo- (**A**) and exo-pectinase (**B**) production with various carbon sources by SmF (●, glucose; ○, galacturonic acid; ■, sucrose) and SSF (□, glucose; ▲, galacturonic acid; △, sucrose)

Comparison between SmF and SSF

The effect of the carbon source on pectinase production in both cultures is shown in Fig. 6. In order to validate this comparison, the results are those that corresponded to media with similar pectin (3%, w/v) concentration and 3% (w/v) of each of the carbon sources studied separately. Enzymatic activities are reported as units per millilitre of liquid in the culture medium. The fact that maximal activity in SmF was attained at 120 h, in contrast with a shorter time in SSF (48–72 h), allowed the estimatation that SSF endo-p productivity (U/g wet mass per hour) was 14 times higher than SmF endo-p productivity (U/ml per hour). The differences in productivity were due on one hand to the higher enzymatic activities obtained by SSF and on the other hand to the shorter times required for enzyme production. This is clearly observed in Fig. 6, where the kinetics of endo-p (Fig. 6A) and exo-p (Fig. 6B) produced with sucrose, glucose and galacturonic acid are presented for both type of cultures. The differences in the effect of adding sugars or galacturonic acid on endo-p production were higher when pectinase was produced by SmF. The kinetics of exo-p production by SSF were also found to be faster and with higher enzymatic activities for the three carbon sources tested in comparison with those obtained in SmF. It should be noted that pectinase enzymes were not completely extracted from bagasse (see Materials and methods). Therefore, the present productivity estimations favour SSF over SmF techniques.

It is interesting to look at the differences in the rates of sugar consumption with both types of cultures. In SSF, sugar uptake started at 16 h of culture, in such a way that at 24 h more than 90% of the initial sugar was

consumed. In SmF, 90% of sugar consumption was attained after 96 h of culture.

The results presented in this work show that the type of fermentation system used considerably affects the rate of pectinase production by *A. niger* CH4. At high sugar concentrations pectinase production was stimulated in SSF, but in SmF high concentrations of sugars diminished pectinase synthesis, suggesting a catabolic repression in the latter system. The differences found between both kinds of fermentation systems could be related to the inherent differences in mixing and nutrient diffusion between solid and liquid fermentation. Nevertheless, these results give some advantages for the use of SSF, since apparently it is less catabolite repressed that the SmF system, and confirm earlier observations (Trejo-Hernández et al. 1991) that solid fermentation provides a system with higher productivity due to larger enzyme yields and shorter fermentation times.

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