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PECTINASE PRODUCTION PROFILE OF *Aspergillus foetidus* IN SOLID STATE CULTURES AT DIFFERENT ACIDITIES

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SUMMARY

Solid-state cultures of a pectinase-producing fungus (*Aspergillus foetidus* NRRL 341) were performed under different acidic conditions. Glass bottles containing 5 g of wheat bran and 7.5 mL of 0.2, 0.3, 0.4, or 0.5 N HCl were autoclaved (15 min, 121°C), inoculated with a spore suspension appropriately diluted to achieve an initial concentration of 4×10^4 spores per gram of wet substrate (with a 60 % humidity, on wet basis) and incubated at 30°C.

Time course of pH and of different pectinase activities were determined in culture extracts. Total pectinase activity (TPA), expressed in terms of viscosimetric units per gram of wet substrate ($VU.g^{-1}$), was affected by the initial culture acidity. The higher the HCl concentration used, the higher the TPA achieved, but after longer cultivation times. On the other hand, when 0.5 HCl was used, no fungal growth was observed. Nevertheless, enzyme productivity increased with culture acidity. When 0.4 HCl was used, TPA reached its maximum after 36 h of cultivation (2,535 $VU.g^{-1}$). With 0.2 and 0.3 N HCl, TPA was the highest at 24 h (733 $VU.g^{-1}$) and at 30 h (1,860 $VU.g^{-1}$) respectively.

The composition of the pectinase pool was also affected by culture acidity. The higher the acidity, the lower the pectinesterase activity and the higher both the polymethylgalacturonate lyase and polygalacturonase activities.

INTRODUCTION

Pectinases are a heterogeneous group of enzymes that degrade pectin-containing substrates. They are widely used in the food industry to improve the cloud stability of fruit and vegetable nectars (Baker and Bruemmer, 1972), for production and clarification fruit juices, and for haze removal from wines (Rombouts and Pilnik, 1978; Bauman, 1981; Kilara, 1982). Industrially, pectinases are produced using both solid-state culture (SSC) and submerged fermentation (SmF) techniques with *Aspergillus* strains (Meyrath and Volavsek, 1975; Sakai *et al.*, 1993). But SSC is generally considered more suitable either for rendering higher enzyme yields than SmF or for obtaining a particular composition of the enzyme mixture (Rombouts and Pilnik, 1980; Ward, 1985).

Information relating to the commercial production of pectinases is very sparse, particularly in the case of SSC; most of the available data are concerned with the optimization of culture conditions in SmF. Production of pectinases in SSC has been previously described (Ghildyal *et al.*, 1981; Budiatman and Lonsane, 1987; Hours *et al.* 1988 a and b; Antier *et al.*, 1993; Berovic and Logar-Derenein, 1993; Solis-Pereira *et al.*, 1993; Acuña-Argüelles *et al.*, 1994). These

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reports deal with factors that affect pectinase production, such as composition of solid media (effect of different carbon sources, supplementation with minerals and nitrogen sources, etc.), effect of steaming on wheat bran, control of temperature during SSC, the use of alternative solid substrates to wheat bran such as apple pomace, coffee pulp and cassava fibrous waste, extraction of the enzyme from moldy bran, and finally culture variables like inoculum size, mixing and water activity. In this paper, we report the preliminary results of the effect of different acidities during thermal pretreatment of the substrate (wheat bran), resulting in different culture initial acidities, on pectinase production and on the composition of the enzyme pool obtained in SSC.

MATERIALS AND METHODS

Microorganism, media and culture conditions. *Aspergillus foetidus* (NRRL 341, ATCC 16878), was maintained on potato-dextrose agar (PDA) under a layer of vaseline at 4°C. Inocula were prepared from 7-day-old slants, with a heavy sporulation, suspended in a 0.01 % Tween 80 solution.

Cultivation was carried out in preweighed 250 mL cylindrical screw-capped glass bottles containing a mixture of 5 g of wheat bran and 7.5 mL of 0.2, 0.3, 0.4, or 0.5 N HCl. Medium containing bottles were autoclaved at 121°C for 15 min. After cooling, each bottle was weighed in order to measure any water loss during autoclaving, which was corrected during inoculation whenever necessary. The initial water content (on wet weight basis) after inoculation was 60 %. Spore concentration in the inocula was appropriately diluted in order to achieve an initial concentration of 4×10^4 spores per gram of wet substrate. The contents were carefully mixed and the bottles were placed in a humid cultivation chamber with a gentle circulation of air at 30°C, under static conditions.

Chemical analysis. The composition of wheat bran was determined according to the AOAC Official Methods of Analysis (1984).

Medium-containing bottles were prepared by using water or 0.2, 0.3, or 0.4 N HCl and autoclaved as mentioned above. After cooling, total soluble solids (TSS) were extracted with H_2O, filtered and clarified by centrifugation. TSS were determined in an aliquot of the extract after drying at 60°C until constant weight was reached. Total soluble reducing carbohydrates (TSRC, as glucose) and true glucose contents were also determined in another aliquot of the extract by the dinitrosalicylic acid method (Miller, 1959) and using a glucose-oxidase commercial kit (Wiener, Argentina), respectively. In all cases, results are expressed as milligrams per gram of wheat bran.

Processing of SSC samples. Three bottles were examined at one time, and the mean of the three values is given here. After cultivation, cultures were suspended with 40 mL of water, stirred for 10 min and the pH was measured. Then 40 mL of 0.4 N acetate buffer (AcB), pH 4.0, was added and the mixture was left to stand for 10 min. The suspension was filtered through a plastic mesh and the solid material caught in the mesh was extracted again with 20 mL of 0.2 N AcB, pH 4.0, and filtered. Both extracts were pooled, clarified by centrifugation (5,000 × *g*, 10 min) and brought to a total volume of 100 mL with 0.2 N AcB, pH 4.0. The solution obtained was thoroughly dialyzed against 0.2 N AcB, pH 4.0, at 4°C, to remove low molecular weight substances interfering in the enzyme assays, and kept frozen until used (hereafter referred to as solution for analysis, SA).

Enzyme assays. Total pectinase activity (TPA) was determined at 30°C by following the kinetics of the viscosity drop (Mill and Tuttobello, 1961), using a polynomial regression program. The assay mixture consisted of 1 mL of 0.2 N AcB, pH 4.0, containing 2.5 % apple pectin and 1 % of NaCl and 4 mL of an adequate dilution of the SA. Commercial food grade apple pectin (uronic anhydride content of about 73 %, methoxyl content of 7.0 %) was thoroughly washed with a 70 % (v/v) ethanol-0.05 N HCl solution to remove soluble sugars. One unit of TPA, expressed in terms of viscosimetric units (VU), was defined as the activity that causes a 50 % drop in viscosity of the assay mixture in 10 min under these reaction conditions.

Polygalacturonic acid (PGA)-degrading activity (polygalacturonase, PGase) including both endo- and exo-PGase forms (EC 3.2.1.15

and EC 3.2.1.67 respectively) was assayed by measuring the reducing groups released from PGA from oranges (Sigma Chemical Co., St. Louis, Mo.) by the method of Somogyi-Nelson (1952) with D-galacturonic acid monohydrate (Sigma) as the standard. The reaction was performed at 30°C for 60 min in a total volume of 1.0 mL containing 0.09 % PGA and the SA diluted appropriately in 0.2 N AcB, pH 4.0. One unit of PGase activity was defined as the activity that liberates reducing groups corresponding to 1 μmol of D-galacturonic acid per min under these reaction conditions.

Polymethylgalacturonate lyase (pectin-lyase, PLase; EC 4.2.2.10) activity was assayed by monitoring the increase in absorbance at 235 nm as described by Albersheim (1966). The reaction was carried out at 30°C in a total volume of 1.0 mL containing 0.1 % apple pectin and the SA diluted appropriately in 0.2 N AcB, pH 4.0. One unit of PLase activity was defined as the activity that causes an increase in absorbance of 1.0 per min at 235 nm under these reaction conditions.

Pectinesterase (PEase; EC 3.1.1.11) activity was determined by monitoring the decrease in absorbance of the reaction mixture containing bromocresol green at 617 nm due to carboxyl groups being released in apple pectin according to Vilariño *et al.* (1993). The reaction mixture contained 1.95 mL of the pectin-dye solution (1 vol. of 0.017 g/100 mL bromocresol green solution and 10 vols. of 0.5 % apple pectin in distilled water, adjusted to pH 5.1 with 1 N NaOH) and 50 μL SA. One unit of PEase activity was defined as the activity required to release 1 μEq of carboxyl groups per min at 30°C using D-galacturonic acid as the standard.

In all cases, enzyme activities are expressed in terms of enzymatic units per gram of solid substrate (wet).

RESULTS AND DISCUSSION

The composition of wheat bran used was determined and yielded the following results (% w/w): dry matter, 88.9; crude protein (N × 6.25) 15.7; crude fat, 6.1; soluble reducing carbohydrates, 0.9; starch, 38.5; ash, 2.8 and crude fiber, 23.8.

Chemical analysis of wheat bran samples after thermal treatment with water or with 0.2, 0.3, and 0.4 N HCl was performed and the results are shown in Table 1.

Table 1: Effect of acidity during thermal pretreatment (121°C, 15 min) of wheat bran on the solubilized solids.

Treatment	TSS ($mg.g^{-1}$)	TSRC ($mg.g^{-1}$)	Glucose ($mg.g^{-1}$)
Water	131	11	3
0.2 N HCl	264	61	8
0.3 N HCl	337	123	13
0.4 N HCl	399	187	19

TSS: Total soluble solids. TSRC: Total soluble reducing carbohydrates (expressed as equivalent amounts of glucose). All data are expressed per gram of wheat bran. See text for details.

The higher the acidity during thermal pretreatment, the higher both the amount of TSS and the ratio TSRC/TSS. The amount of glucose released also increased with acidity and the ratio glucose to TSRC remained almost constant (aprox. 0.1) in the acid treated samples. According to these results, the thermal pretreatment of wheat bran under the acidic conditions tested improved its amenability to microbial assimilation by forming smaller permeable molecules.

The effect of different concentrations of HCl during thermal pretreatment of the substrate (wheat bran) on pectinase production in SSC using *Aspergillus foetidus* was studied. When 0.5 N HCl was used, no fungal growth was observed, probably due to the high acidity of the culture medium (initial pH of the culture extract was 1.8). Non germinated fungal spores were found in microscopic observations of these culture samples. Other HCl concentrations tested were 0.2, 0.3 and 0.4 N, hereafter called cultures under weak, medium, and strong acidic conditions (WAC, MAC, and SAC respectively).

Figure 1 shows changes of pH and TPA in culture extracts with time. The pH of the culture extracts was much affected by the concentration of HCl used during thermal pretreatment; WAC, MAC, and SAC culture extracts had initial pH values of 4.0, 3.0, and 2.3, respectively. Moreover, the pH values in culture extracts under the different acidic conditions tested

showed no substantial variations with time. In all cases, a small increase of around 0.2 to 0.4 units of pH was observed at those cultivation times when the highest enzyme productivity occurred.

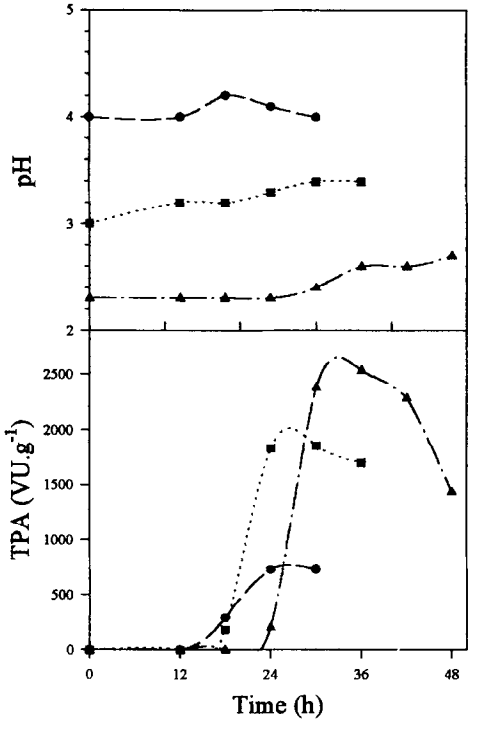

Fig. 1: Time course of pH and total pectinase activity (TPA) in solid state culture extracts. ●, ■ and ▲ correspond to WAC, MAC and SAC (see text above).

TPA was strongly affected by culture acidity. Under the acidities tested, SSC yielded different enzyme production profiles. The higher the culture acidity, the higher the TPA achieved, but cultivation times to achieve maximum TPA were extended. In the SAC culture, TPA was maximum at 36 h of process (2,535 $VU.g^{-1}$). In WAC and MAC cultures, the TPA was highest at 24 h (733 $VU.g^{-1}$) and at 30 h (1,860 $VU.g^{-1}$), respectively. Moreover, enzyme productivity, expressed as $VU.g^{-1}$ per hour of culture, increased with culture acidity. WAC, MAC, and SAC cultures rendered 30, 62, and 70 $VU.g^{-1}.h^{-1}$ when TPA was the highest in each case.

A specific determination of the different enzymes involved in pectin degradation was made. Figure 2 shows changes with time in PEase, PGase, and PLase activities in culture extracts. The composition of the pectinase pool was also affected by the culture acidity. The higher the acidity, the lower the maximum titres of PEase activity and the higher both the PGase and PLase activities.

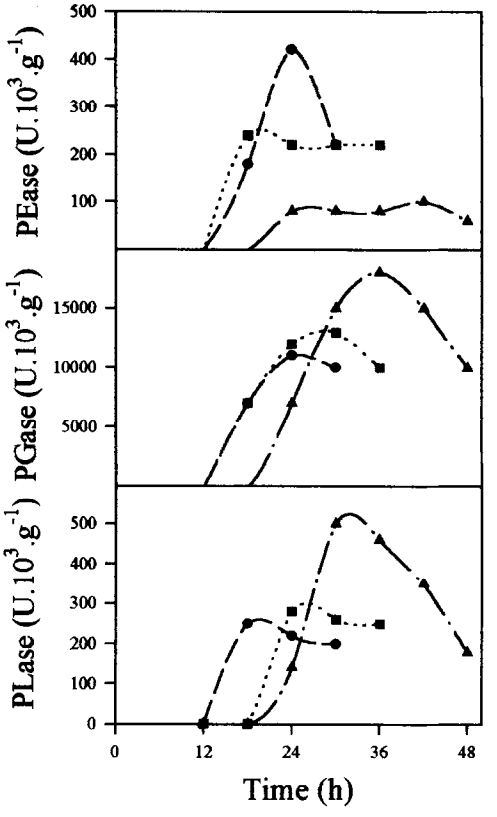

Fig. 2: Time course of different pectinase activities in solid state culture extracts. ●, ■ and ▲ correspond to WAC, MAC and SAC (see text).

In this paper, TPA is considered as a sort of global enzymatic degradation activity of pectin, in which different enzyme activities are involved, namely hydrolases and transeliminases. In the first case, pectin degradation is performed by the action of PEase which catalyzes de-esterification of methoxyl groups in pectin, followed by the action of PGase, either endo- or exo-type. Polygalacturonate lyases (either endo- or exo-

type, EC 4.2.2.2 and EC 4.2.2.9, respectively) are not considered here because *A. foetidus* does not produce these enzymes, as previously demonstrated (Hours *et al.*, 1988 a). The alternative mechanism for pectin degradation is the cleavage of α-1,4-glycosidic linkages by PLase in the highly methoxylated regions of pectin, which have not been previously de-esterified by the PEase present in the reaction mixture, resulting in a galacturonide with an unsaturated bond between C_4 and C_5 at the nonreducing end of the galacturonic acid formed. Both hydrolytic and transeliminative degradation of pectin produce a reduction of viscosity in pectin-containing solutions. Although no distinction between the different pectolytic enzymes involved in pectin degradation can be derived from TPA data, from the practical point of view, the global determination of a pectolytic pool by this method is a simple and useful test to characterize culture samples.

The production of pectinases by different molds shows induction and repression phenomena. Catabolite repression exerted by readily assimilated carbon sources controls the production of different pectic enzymes (Torakazu *et al.* 1975; Fogarty and Kelly, 1983; Angelova *et al.*, 1987; Sissere and Said, 1989). Leuchtenberger *et al.* (1989) reported that the regulation of PGase and PEase synthesis in SmF of *A. niger* was influenced by the nature and concentration of the carbon and energy source and by the fermentation time. The ratio PGase/PEase increased with sugar concentration. However, there is a lack of information about the regulatory aspects of pectinase synthesis in SSC.

It is known that the synthesis of extracellular enzymes by several microorganisms is regulated by the pH value of the culture. Particularly, in the case of fungal strains, Caddick *et al.* (1986) have found that in SmF of *A. nidulans*, the number and relative amounts of enzymes that can be secreted (e.g. acid phosphatase, alkaline phosphatase, and acid phosphodiesterase) and of certain permeases (e.g. that for γ-amino-*n*-butyrate) are controlled by the pH of the culture medium. In addition, a report on PGase production in SmF of *Rhizopus nigricans* at different culture pHs showed maximum values of enzyme activity when the initial pH was 3.0, and decreasing values as the initial pH increased (Ros *et al.*, 1992). Similar results were found in our case when *A. foetidus* was grown at different culture acidities in SSC.

Protopectinases (PPases) are a heterogeneous group of enzymes that release water-soluble pectin from the insoluble protopectin in plant tissues by restricted degradation of the substrate (Sakai *et al.* 1993). The effect of the same medium and culture acidities tested here in SSC of *A. awamori* on the production of PPases was recently reported (Hours *et al.*, 1994). The profile of the two PPase activities detected was strongly influenced by culture acidity. The higher the culture acidity, the higher the ratio between PPase activity on lemon protopectin to PPase activity on apple protopectin.

The amount of TSRC and glucose increased with initial culture acidity due to the acid catalyzed hydrolysis of biopolymers (mainly starch) present in wheat bran. Catabolite repression due to the high soluble sugar content in citric acid production by fungal cultures carried out in SSC was shown to be overcome due to the particular characteristics of this cultivation system (Shankaranand *et al.*, 1992). In this sense, diffusion of low molecular weight metabolites through the solid matrix of the substrate could control their assimilation. The same phenomenon could be expected in fungal pectinase production in SSC to explain the increase in both PGase and PLase activities. The reason why PEase activity declined with culture acidity still remains unknown.

Depending on the particular aim of the fruit and vegetable processing technology, pectolytic enzyme preparations with different relative proportions of PEase, PGase and PLase are needed. In this sense, pectolytic pools with low PEase and high PLase activity contents are useful, for example, for fruit juice clarification without a simultaneous methanol production (Ishii and Yokotsuka, 1973; Szajer and Szajer, 1982). Therefore, the possibility of (partially) controlling the composition of the pectolytic pool according to the culture acidity in SSC has significant possibilities in practice. Finally, the high acidity of SSC at SAC that yields low PEase activity is a convenient culture condition to facilitate the maintenance of the asepsis

during fermentation.

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