Evaluation of Filamentous Fungi and Inducers for the Production of Endo-Polygalacturonase by Solid State Fermentation

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Five strains of filamentous fungi (*Aspergillus niger* strains NRRL 3122 and T0005007-2, *Aspergillus oryzae* CCT 3940, *Aspergillus awamori* NRRL 3112 and a *Trichoderma* sp.) were compared for their capacity to produce endo-polygalacturonase (endo-PG) in solid state fermentation. Maximum pectinolytic activity was reached in 72 h of growth, the best two fungal strains being *A. niger* T0005007-2 and *A. oryzae* CCT 3940. Three types of commercial purified pectin and four of unprocessed pectin (tangerine, orange, Tahiti lime and sweet lime rind) were used to assess the effect of pectin on the production of endo-PG by *A. niger* T0005007-2. Maximum pectinolytic activity was achieved using 6 and 10% (w/w) of purified pectin as inducer. Depending on the origin of the commercial pectin used as inducer, maximum endo-PG levels varied from 223 to 876 units per gram of dry medium (one endo-PG unit (U) was defined as the quantity of enzyme which caused a reduction in viscosity of 50% in a 1% w/v solution of pectin in 30 min), indicating that care should be taken when choosing this component of the medium. When the crude pectins were used as inducers at the same concentration as purified pectin, maximum endo-PG activities were 250–300 units/g. However, by increasing the amount of Tahiti lime rind to 50% (w/w) maximum endo-PG was 919 U/g, thus opening up the possibility of a low cost medium for endo-PG production.

Introduction

Pectinases are a group of enzymes that attack and de-polymerize pectin by hydrolysis and trans-elimination as well as by de-esterification reactions which hydrolyze the ester bond between the carboxyl and methyl of groups pectin (Ceci and Lozano, 1998).

Various genera of microorganisms can produce pectinase, but the filamentous fungi *Aspergillus niger* is most used because it is classified as 'generally regarded as safe' (GRAS) by the United States Food and Drugs Administration (USFDA), which has approved its use in the food industry (Pariza and Foster, 1983). Other fungi, such as *Aspergillus oryzae* (Ueda *et al*., 1982), *Thermoascus aurantiacus* (Martins *et al.*, 2002) and *Penicillium expansu*m (Alkorta *et al*., 1998; Fernandes-Salomão *et al*., 1996) are also sometimes used.

Preparations of pectinolytic enzymes have many industrial applications, but are mainly used in the food industry in operations like clarification of fruit juices and wines and the extraction of vegetable oils (Whitaker, 1994; Demir *et al.*, 2001). In the textile industry pectinases are sometimes used in the treatment of natural fibers, such as linen and ramie fibers (Baracat *et al*., 1991).

Solid state fermentation is generally defined as the cultivation of microorganisms on solid materials in the absence or near-absence of free water (Sanzo *et al*., 2001). This process has several advantages, including the ability to reach high product concentrations and the production of less liquid effluents, although the control of pH, temperature and oxygen tension can be difficult (Cannel and Moo-Young, 1980; Costa *et al.*, 1998; Castilho *et al.*, 2000). Pectinase production by *Aspergillus* strains has been observed to be higher in solid state fermentation than in submerged process (Solis-Pereira *et al*., 1996; Maldonado *et al.*, 1998). Acuña-Arguelles *et al*. (1995) have reported that *A. niger* produces distinct physiological re-

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sponses depending on the fermentation technique used, and that there are advantages to the production of pectinase using solid state processes as compared to submersed processes.

The synthesis of pectinase is induced or stimulated by the presence of pectin, and for economic reasons this is normally supplied by adding sugar beet or apple bagasse or citrus fruit rind and wheat bran to the culture medium (Rombouts and Pilnik, 1980). This paper describes the selection of a fungus with a good capacity for the production of endo-polygalacturonase (endo-PG), a pectinolytic enzyme that hydrolyzes the internal $\alpha(1\rightarrow4)$ linkage between D-galacturonic acid units of pectin, and compares the effect of different types and concentrations of pectin as inducers in solid state fermentation.

Materials and Methods

Microorganisms

The five filamentous fungi assessed were: *Aspergillus oryzae* CCT 3940 (Universidade Estadual de Campinas, Brazil), *Aspergillus niger* NRRL 3122, *A. niger* T0005007-2 (Universidad de Salta, Argentina), *Aspergillus awamori* NRRL 3112 and a strain of *Trichoderma sp* isolated by our group, all of which were maintained on potato-dextrose agar (PDA) and replicated monthly.

Media

Wheat bran (Moinho Santa Lúcia, Passo Fundo, Brazil) was used as the support and main carbon source for all media, to which was added 45 ml of a mineral salts (MS) solution containing (% w/v) KH_2PO_4, 0.2; $MgSO_4$, 0.1; $(NH_4)_2SO_4$, 0.4; $FeSo_4$, 6.3×10^{-4}; $MnSO_4$, 1.0×10^{-4} and $ZnSO_4$, 6.2×10^{-4} for each 100 g of bran (Bertolin *et al.*, 2001), to produce basic wheat bran medium (WB). Purified or unprocessed pectin were added to WB medium to serve as pectinase inducer, as detailed below. The initial moisture of all media was adjusted to 63% by the addition of distilled water. All media were autoclaved at 121 °C for 30 min.

For strain selection experiments, all the above strains were grown in WB medium enriched with 6% (w/w) of apple pectin (SIGMA, St. Louis, USA) (Dartora *et al.*, 2000). Further assays were conducted with this medium, with the selected strain *A. niger* T0005007-2, to evaluate the best way to prepare the culture media: Method 1 – apple pectin was mixed with WB, the MS solution added, the moisture adjusted with distilled water and the media sterilized; Method 2 – apple pectin was added to the MS solution and this and the WB media sterilized separately, the moisture being adjusted after autoclaving using sterilized distilled water; Method 3 – The MS solution was added to the WB along with half the quantity of distilled water needed to adjust the moisture, the apple pectin scattered over the humid media and the remaining distilled water added before sterilization.

Aspergillus niger strain T0005007-2 was also used in experiments with purified and unprocessed pectins. To assess purified pectinase inducers, we used SIGMA apple pectin and two types of citrus pectin produced in Brazil, citrus pectin A (INLAB, São Paulo, Brazil) and citrus pectin B (DELAWARE, Porto Alegre, Brazil) added to WB media at concentrations of 2, 4, 6, 8 and 10% (w/w). To assess unprocessed inducers we used the dried and ground rind from tangerine (*Citrus deliciosa* Tenore), orange (*Citrus sinensis* Osbeck), Tahiti lime (*Citrus latifolia* Tanaka) and sweet lime (*Citrus limettioides* Tanaka) added to WB media at concentrations of 2, 4, 6, 8, 10% (w/w). *C. latifolia* based medium was also prepared with 30, 40 and 50% (w/w) rind.

Preparation of spore suspension

For each fungi, 50 ml of PDA was placed in 1000 ml flasks and inoculated by adding 0.2 ml of spore suspension produced by adding 10 ml of water to PDA slants of the appropriate fungi. The flasks were incubated at 30 °C for 5 days. The spore suspension was obtained using sterile water and adjusted to 1×10^7 spores/ml.

Culture conditions

The experiments were carried out in 250 ml flasks each containing 14 g of WB medium which itself contained different types and quantities of pectin as inducer. Controls contained no pectin. The flasks were inoculated with 1 ml of spore suspension (1×10^7 spores/ml). This procedure was repeated for each of the fungi. The flasks were statically incubated at 30 °C in a humidified atmosphere. For each sample point – after 0, 14, 24, 40,

48, 63, 72 and 96 h of cultivation – two flasks were removed and analyzed.

Analytical methods

Moisture of culture media was determined according to A.O.A.C. (1997). For quantification of the total carbohydrate (TC) content in the different media, the samples were hydrolyzed with concentrated HCI in an autoclave at 121 °C for 15 min, alkalinized to pH 14 with 40% (w/v) NaOH and treated with potassium ferricyanide and cupric sulfate solutions to remove proteins, after which the concentration of soluble carbohydrate was estimated by the 3,5 dinitrosalicilic acid (DNS) method (Miller, 1959) as total reducing sugars. Soluble carbohydrate (SC) present in the culture media was measured as free reducing sugars by the DNS method without prior hydrolysis of the samples. Both TC and SC in this paper are expressed as mg of reducing sugars per g of dry medium.

Endo-PG was extracted from the media by suspending 1 g of dry sample in 15 ml of sodium acetate buffer (pH 4.0) contained in 125 ml flasks agitated at 100 rev/min, the crude enzyme solution being filtered through Whatman no. 41 filterpaper. The endo-PG activity of the filtrate was determined by adding 2 ml of filtrate to 8 ml of a 1% (w/v) apple pectin (SIGMA, USA) solution and measuring the viscosity in a RVDV-II viscometer (Brookfield Engineering, Middleboro, USA). One endo-PG unit (U) was defined as the quantity of enzyme which caused a reduction in viscosity of 50% in 30 min of reaction under standard conditions (Gainvors *et al.*, 2000). Endo-PG activity in this work is expressed as units per gram of dry medium (U/g).

Results and Discussion

Selection of fungi strain with highest endo-PG production

Figure 1 shows the time course of total carbohydrate (TC) and soluble carbohydrate (SC) concentrations, both measured as reducing sugars, and endo-polygalacturonase activity (endo-PG), during the cultivation of different fungi on WB medium with 6% (w/w) SIGMA apple pectin. In general, the TC level decreased until it reached a plateau at which there was a relatively high concentration of carbohydrate still available to the fungi. This unused carbohydrate may have resulted, probably, from the fact that mycelial growth occurred predominantly on the surface of the solid medium and the fungal mycelia may not have extend far into the medium due to the limitation of oxygen. Additionally, some other fact could be involved, such as limitation of another nutrient, change in pH or production of a toxic metabolite.

With respect to the variation in the concentration of soluble carbohydrate (SC) with time, from Fig. 1, one can see that there was an accumulation of reducing sugars in the medium during the initial phase of cultivation which varied according to the fungal strain, probably because at this stage the rate of starch hydrolysis was greater than the rate of sugar consumption. After SC levels reached their maximum values the level dropped, agreeing with previous work by Cavalitto *et al.* (1996). The peak SC values for *A. niger* T0005007-2 and *A. oryzae* were lower than those of the other fungi, which may have been due to the relatively low production of amyloglycosidase by these two fungi. Although *A. awamori* had the highest SC production rate in the early stages of the experiment it was not an efficient producer of endo-PG.

Figure 1 also shows that maximum enzymatic activity occurred at about 72 h, the highest endo-PG activities being produced by *A. niger* T0005007-2 and *A. oryzae.* Working with the same media components as was used in our study, but using submerged culture, Malvessi (2000) found high endo-PG activities with *A. oryzae* CCT3940 but insignificant titers of these enzymes with *A. niger* T0005007-2, showing the difficulty involved in comparing submerse and solid-state enzyme production. We decided to use *A. niger* T00050072 for the inducer evaluation experiments using different types of pectin because *A. niger* strains are generally regarded as safe by the USFDA.

Assessment of inducers using A. niger T0005007-2

The way the media was prepared influenced the results of the process and it is probable that such an effect would be even more important on an industrial scale. After 72 h of solid state culture of *A. niger* T0005007-2 in 6% (w/w) SIGMA apple

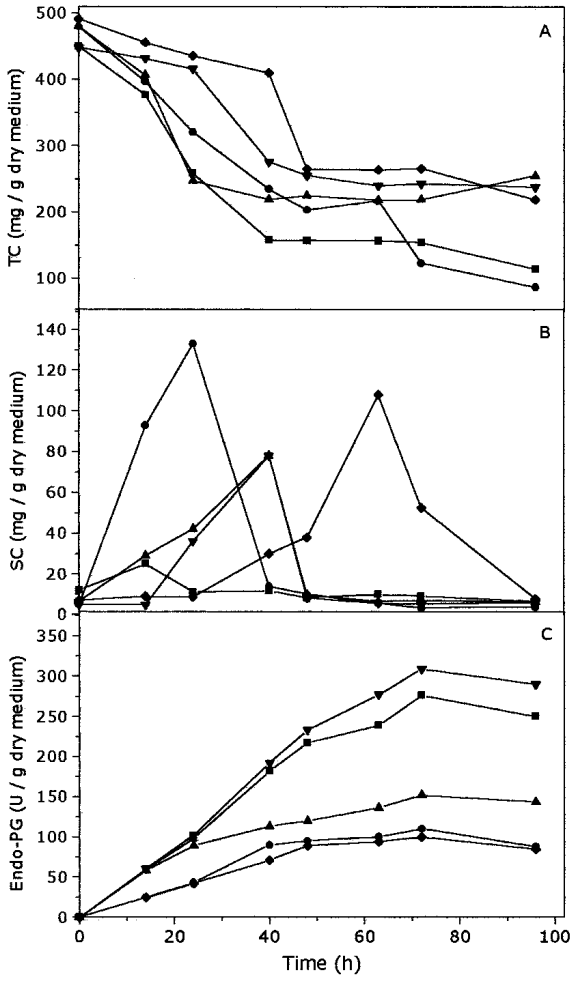

Fig. 1. Variation in the concentrations of total carbohydrate (TC) [A] and soluble carbohydrate (SC) [B], and in endo-polygalacturonase (endo-PG) activity [C] with time during the cultivation of different filamentous fungi in 6% (w/w) SIGMA apple pectin medium. (▲) *Aspergillus niger* ATCC 3122; (●) *A. awamori* NRRL 3112; (■); *A. oryzae* CCT 3940; (▼) *A. niger* T0005007-2; (◆) *Trichoderma sp.*

pectin medium prepared by the three different methods proposed, the following maximum endo-PG activities were achieved: Method 1, 328 U/g; Method 2, 219 U/g; Method 3, 760 U/g. It appears that the most important factor in media preparation is the homogenous distribution of the inducer, which should be distributed in such a way as to avoid the formation of lumps which may limit oxygen diffusion and produce regions where there are insufficient concentrations of pectin to initiate endo-PG production.

The effect of different types and concentrations of inducers on endo-PG production by *A. niger* T0005007-2 was evaluated after 72 h of cultivation, when the maximum endo-PG value was reached. For any commercial pectin tested, the maximum activities were achieved with WB medium containing 6–10% (w/w) inducer. The highest endo-PG value, over 850 U/g, was obtained with SIGMA apple pectin, while medium containing commercial citrus pectin A also presented good results with a maximum activity of just under 700 U/g. The commercial pectin B presented lower enzyme activity (233 U/g), with results similar to, or lower than, those seen with unprocessed pectin. This variation in endo-PG production is perhaps due to the fact that pectins are polysaccharides of very variable structure, being made up of different monomers with various types of ramification and levels of esterification (Ceci and Lozano, 1998). These results indicate that care must be taken to the choice of commercial pectin used as endo-PG inducer.

WB medium containing 8–10% (w/w) of Tahiti lime, orange or tangerine rind produced maximum endo-PG activities of 250–300 U/g, whereas the same amount of sweet lime rind led to a maximum endo-PG activity of only 180 U/g. In addition to the reasons discussed in relation to purified pectins, the lower endo PG obtained with unprocessed inducers may have been related to content of pectin present in these materials, which in the case of orange and Tahiti lime rinds is only about 30% (Rombouts and Pilnik, 1996). Ceci and Lozano (1998) have also pointed out that it is also possible that the low purity of such pectin sources makes it difficult for a microorganism to gain access to the inducer, resulting in lower pectinase production.

Further experiments were carried out using WB medium containing 10, 30, 40 and 50% (w/w) Tahiti lime rind. The maximum endo-PG activities, after 72 h of solid state culture of *A. niger* T0005007-2, were 275, 475, 755, and 919 U/g, respectively. These results indicate that Tahiti lime rind can be used as an effective inducer for the production of endo-PG by this fungus, since the highest pectinolytic activity was comparable to, or higher than, that obtained using purified pectin.

As no significant difference was observed in endo-PG production in medium containing between 6 and 10% (w/w) Tahiti lime rind, the data for these last group of experiments appear to be somewhat contradictory. Probably this may have been due to the small difference (4%) between the concentration of inducer in the first set of runs and from the relatively low pectin content of this material (about 30%). The fact remains that *A. niger* produced over 900 U/g of endo-PG in medium containing 50% (w/w) Tahiti lime rind, demonstrating that it is possible to formulate a low-cost medium for the production of endo-polygalacturonase by solid state fermentation.

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