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Enhance hydrolytic enzymes production by *Aspergillus awamori* on supplemented grape pomace

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ABSTRACT

The disposal of grape pomace by wine cellars leads to serious environmental problems. In this work we have evaluated the potential utility of this waste as substrate of fermentation for the production of several hydrolytic enzymes commonly used in the clarification processes in wine cellars and juices industries. Our results have demonstrated that the synthesis of exo-polygalacturonase (exo-PG), xylanase and cellullase by solid state fermentation (SSF) on a mixture of washed grape pomace and orange peels (a natural source of pectin, cellulose and hemicellulose), supplemented with a nutrient solution, increased compared to whole grape pomace. For experiments carried out with suspensions of the same mixed substrates in submerged fermentation (SmF), higher values for pectinase and xylanase were reached but with significant lower exo-PG activity per mL of extract. The activities of these enzymes on mixtures of grape pomace and orange peels, in both SSF and SmF, were similar or even higher to those produced using other agro-industrial wastes, demonstrating therefore its potential utility as an alternative substrate for the production of enzymatic extracts for clarification purposes.

Keywords: Grape pomace; Orange peels; Solid state fermentation; Xylanase; Pectinase; Cellulase

1. Introduction

Solid state fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence (or near absence) of free water (Mitchell et al., 2002; Pandey, 2003; Sharma et al., 2008). This technique offers the possibility of processing agro-industrial residues that can be used, for instance, as substrates for enzymes production helping in this way to minimize the pollution.

Pectinases constitute a group of enzymes which degrade the pectin present in most plants. These enzymes are mainly used in food industries for clarification and extraction of fruit juices. Although several types can be found, polygalacturonases are the most abundant and studied ones, representing around 25% of the industrial enzymes sales (Jayani et al., 2005). *Aspergillus niger* pectinases are the most widely used in industries because this strain possesses GRAS (Generally Regarded As Safe) status so that metabolites produced by this strain can be safely used (Gummadi and Panda, 2003).

In most cases, agroindustrial wastes used to produce these enzymes by SSF or submerged fermentation (SmF) do not contain all the necessary nutrients for this purpose, or maybe they are available in sub-optimal concentrations. In these cases, the substrate must be supplemented to stimulate or improve the enzyme production by adding extra carbon sources or nitrogen sources (Galiotou-Panayotou and Kapantai, 1993; Patil and Dayanand, 2006). Supplementation can also be carried out with the adjustment of the initial moisture content of the residue using a solution containing mineral salts or combining the solid with another residues (Khandeparkar and Bhosle, 2006; Papinutti and Forchiassin, 2007). Thus, mixtures of wheat and orange bagasse or cotton bran with orange pulp have been successfully tested for SSF (Martin et al., 2007).

Grape pomace is the residue left after juice extraction from the grapes in the wine making industry (Botella et al., 2005). In Spain, over 250 million kg of this by-product (constituted by seeds, skin and stem) are generated and re-used as animal feed or nutritive ingredient, in the production of

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citric acid and ethanol by fermentation, in the extraction of anthocyanins, etc. (Francis, 1992; Ana et al., 1995; Lu and Yeap, 1999). This material is under-exploited and most of it is generally disposed in open areas, leading to serious environmental problems. By contrast, the potential utility of this waste for value-added products by SSF is promising. Our research is focused in the revalorization of grape pomace using it as a substrate for the production of hydrolytic enzymes such as xylanase, exo-polygalacturonase (exo-PG) and CMC-ase (a type of cellulase), which are commonly used together to facilitate clarification processes in wine cellars and juice industries (Botella et al., 2007). However, grape pomace composition changes from season to season and depends on types of grapes, weather conditions, etc., and, therefore, no reproducible enzymatic productivities are achieved when different grape pomaces are used as solid substrates for fermentations. For this reason, in this work we have established a protocol for the adjustment of the nutrients composition of grape pomace in order to enhance the production of pectinases (exo-PG) and other lateral activities (xylanase and CMC-ase). This process involves the washing of the solid substrate in order to remove excess of reducing sugars and the supplementation with a solution rich in mineral salts and nitrogen sources and with orange peels, which constitute a natural source of pectin, cellulose and hemicellulose.

2. Methods and materials

2.1. Spore production

A. awamori 2B.361 U2/1, classified by the Commonwealth Mycological Institute as *Aspergillus niger* complex, was propagated and stored on slants which contained a synthetic medium for fungal growth composed of (g/L): 1 peptone, 0.5 yeast extract, 15 agar, 6 xylan and 1 pectin. This medium contains xylan and pectin as sole carbon sources to induce the production of xylanase and exo-polygalacturonase.

Spores stored on synthetic medium slants were washed with 2 mL of 0.9% NaCl. The spore solution (0.2 mL) was spread on the surface of 25 mL synthetic medium in eight petri dishes, and incubated at 30 °C for 5 days. After the incubation period, 0.5 mL of 0.9% NaCl solution was added to the petri dishes to collect the cells. The number of spores was counted in an Improved Neubauer Counting Chamber.

2.2. Solid substrates for fermentations

White grape pomace from the Xerez–Sheres–Sherry area in Spain (*Palomino Fino* variety) was used as natural substrate for the SSF experiments. Different samples of white virgin pomace were collected. The pomace was obtained from a local wine cellar after pressing the grapes and stored at −20 °C until use. For any given series of experiments, sub-samples (250 g) were taken and defrosted to ambient temperature. The solid was dried in an oven (60 °C for 48 h), milled and sieved (56.3% of the total mass of particles was over 1 mm in diameter). Then it was washed several times with distilled water to reduce its high reducing sugars content. After this, the solid was dried again and finally sterilised in an autoclave for 20 min at 120 °C and 1.2 atm.

Orange peels (*Washington Navel* variety) were obtained after juice extraction from oranges collected at a local market. Samples were stored at −20 °C. Before their use in SSF experiments, orange peels were defrosted, milled (62.8% of their mass was constituted of particles over 1 mm in diameter) and extensively washed in order to remove all water soluble compounds. Solid was dried at 60 °C for 48 h and finally sterilized in an autoclave (20 min, 120 °C, 1.2 atm). When mixtures of grape pomace and orange peels were used, both residues were conditioned separately as it has been previously described, mixed in a 1:1 proportion and sterilised in an autoclave at prescribed conditions.

2.3. Solid state fermentations

Fermentations were carried out in static conditions at 30 °C. The required amount of spore suspension and the exact amount of a nutrient solution, to obtain an initial moisture content of 70%, were poured into disposable Petri dishes (9 cm diameter). Inoculums concentrations were adjusted to 4.5×10^8 spores/g solid substrate (grape pomace, orange peels or a mixture of them). After this, 10 g of the sterilized solid substrate were added to each one. Every experiment was developed in duplicate.

The nutrient solution had the same basic components (g/L): 2.4 urea, 9.8 $(NH_4)_2SO_4$, 5.0 KH_2PO_4, 0.001 $FeSO_4{\cdot}7H_2O$, 0.0008 $ZnSO_4{\cdot}7H_2O$, 0.004 $MgSO_4{\cdot}7H_2O$, 0.001 $CuSO_4{\cdot}5H_2O$ and 11.5 pectin. Depending on the experiment, pectin was omitted from the formulation of the nutrient solution (see Table 1). The pH of these solutions was adjusted to 5 using HCl 0.1 M.

2.4. Extraction conditions

After fermentation, the content of each Petri dish was transferred into Erlenmeyer flasks containing 50 mL of Tween 80 (0.01%, v/v) and then stirred in a rotary shaker (150 rpm, 30 min, 4 °C). These extraction conditions were optimized in a previous work (Díaz et al., 2007). When mixtures of grape pomace and orange peels were used as solid substrate for fermentations, 70 mL of solvent were added instead. The suspension resulting after the extraction was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant obtained – the enzymatic extract – was stored at −20 °C until required for analysis.

2.5. Submerged fermentations (SmF)

The substrate for fermentations was a mixture of washed white grape pomace and orange peels 1:1 (w/w) suspended in the nutrient solution without pectin (see Table 1) at pH 3. The proportion solid–solution used in the fermentation was of 20 g/L.

The solid and the solution were sterilized separately in the autoclave.

To obtain the inoculum, a volume of 100 mL of solution composed of (g/L) 2.4 urea, 9.8 $(NH_4)_2SO_4$, 5.0 KH_2PO_4, 0.001 $FeSO_4{\cdot}7H_2O$, 0.0008 $ZnSO_4{\cdot}7H_2O$, 0.004 $MgSO_4{\cdot}7H_2O$, 0.001 $CuSO_4{\cdot}5H_2O$, 1 peptone, 0.5 yeast extract, 3.5 xylan, 3.5 pectin and 50 glucose was put into a 500 mL Erlenmeyer flask and inoculated with 3×10^7 spores. The flask was incubated in a rotary shaker at 30 °C and 200 rpm for 5 days. After this time the pellets obtained were filtered through a nytal cloth and were used to inoculate the fermentor.

2.5.1. Stirred tank batch reactor

The reactor consisted on a 5 L vessel (2.5 L work volume), which has probes for pH and temperature control. The pH was maintained at 3 by the addition of 1 M HCl. The vessel