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Short communication

Xylanase and pectinase production by *Aspergillus awamori* on grape pomace in solid state fermentation

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Abstract

The feasibility of using grape pomace for the production of xylanase and exo-polygalacturonase by *Aspergillus awamori* in solid state fermentation has been evaluated. Solid state fermentation experiments indicated that the particle size did not influence the enzyme production. The addition of extra carbon sources and the initial moisture content of the grape pomace were found to have a marked influence on the enzymes yields. Xylanase and exo-PG activities were high at 65% (w/w) initial moisture content and glucose supplementation.

Keywords: Solid state fermentation; Agroindustrial residue; Grape pomace; *Aspergillus awamori*; Xylanase; Polygalacturonase

1. Introduction

Grapes are the most widely cultivated fruit crop in the world. From the world's total production of 60 million tonnes, about 68% of grapes are used for making 29 million tonnes of wine [1]. The manufacturing process generates a lignocellulosic waste called pomace. Grape pomace, or marc, is the residue left after juice extraction by pressing and it is formed from the skins, seeds, and pieces of stem, and constitutes about 16% of the original fruit. In countries like Spain, Italy and France, the annual production of this lignocellulosic residue from the wine industry is about 240 million kilograms.

Grape pomace can be used as animal food, especially in dry seasons when pastures are scarce. However, the use of pomace is limited to 30% of the total food for ruminants due to the very low nutritional value of grape pomace and the presence of anti-nutritional factors such as phenolic components, which inhibit the rumial symbionts [2]. In the production of fertilisers, relatively high levels of phenolic compounds in grape pomace are a problem because they inhibit the germination properties [3]. Therefore, most of this by-product is generally disposed of in open areas, leading to potentially serious environmental problems. Given this situation, it is necessary to look for processes that allow the controlled elimination of this residue or, even better, its industrial reutilization.

In recent years there has been an increasing trend towards efficient utilization and value-addition of agro-industrial residues such as wheat bran, coffee pulp, sugarcane bagasse, apple pomace and others. Biotechnological processes, especially the solid state fermentation (SSF), have contributed enormously to such reutilization. SSF is defined as the fermentation involving solids in the absence (or near absence) of free water. The substrate, however, must contain sufficient moisture to support the growth and metabolism of the microorganisms [4]. The application of agro-industrial residues in SSF bioprocesses not only provides an alternative substrate but also helps to solve some of the pollution problems caused by their accumulation.

In the case of grape pomace, several bioprocesses have been developed for its utilization as a raw material for the production of bulk chemicals and value-added fine products by SSF. As examples, the production of ethanol [5] gluconic acid [6], carotenoids [7], xanthan [8] or citric acid [9] can be cited. In a related area, there is a significant interest in using SSF technique to produce a variety of enzymes, mainly from molds, as indicated by the growing number of research papers published [10], and the marketing and development undertaken by a number of fermentation industries [11]. Several advantages

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are often cited for SSF processes and these include that enzyme titers are higher in SSF than in submerged fermentation [12].

Xylanases (EC 3.2.1.8) are used commercially in the pulp and paper, food and animal feed industries [13] while fungal polygalacturonases (EC 3.2.1.67) are useful in industrial applications such as processing aids for extraction, clarification and deproteinization of fruit juices, for maceration of fruits and vegetables and in the production of oligogalacturonides as functional food components [14].

Previous studies carried out in the University of Cadiz have suggested that the solid grape pomace can be used as an alternative substrate for hydrolytic enzyme production [15]. This investigation is carried out studying the effects of substrate conditioning such as initial moisture content, particle size and the addition of extra carbon sources in the production of xylanase and exopolygalacturonase by *Aspergillus awamori* in SSF.

2. Materials and methods

2.1. Microorganism

A. awamori 2B.361 U2/1, classified by the Commonwealth Mycological Institute as *Aspergillus niger* complex, was propagated and preserved on 5% whole wheat flour and 2% agar slants at 4 °C. Inoculum for the experiments were prepared from fresh flour slants as described earlier [15]. Inoculum concentrations were adjusted to (0.3–5) × 10^5 spores/g solid grape pomace.

2.2. Solid state fermentation (SSF)

2.2.1. Fermentation conditions

Grape pomace used as the solid substrate for the production of hydrolytic enzymes was from the variety Palomino Fino (this grape variety is the most common one used for producing Sherry wines). Typical batches of grape pomace were collected in plastic bags from a local wine cellar just after pressing and then stored at −24 °C until required. For any given series of experiments, sub-samples (500 g) were removed and dried in an oven at 60 °C for 48 h. The solid mass was then milled and sieved before autoclaving at 121 °C for 20 min.

The required amount of spore suspension was poured into disposable Petri dishes (9 cm diameter) and the solid fermentation was started by adding 10 g of the sterilized solid substrate. The pH was not controlled and the plates were incubated under static conditions at 30 °C. Two plates were withdrawn for analysis at the desired intervals. The initial moisture content of all substrates was adjusted to 60% (w/w).

2.2.2. Effect of particle size

The effect of particle size of the solid substrate on the production of xylanase and exo-polygalacturonase was studied by growing the fungus in three different milled stocks (WP, F1 and F2). WP (Whole Pomace) was the whole milled pomace without subsequent separation with an average particle diameter of 0.74 mm. F1 consisted of particles from WP that were larger than 1 mm with a maximum particle size of 1.6 mm. Fraction F2 was composed of particles smaller that 1 mm with minimum size of 0.063 mm.

2.2.3. Effect of initial moisture

In order to understand the effect of water availability, substrate swelling and the oxygen diffusion, different moisture levels were tested (45–80%, on dry weight basis). In all cases WP was used as the solid substrate. Sterile distilled water was used as the moistening agent.

2.2.4. Effect of supplementation with carbon sources

The effect of supplementation of carbon sources on the production of the two enzymes was also assessed. For the first set of experiments, the whole grape pomace medium (WP) was supplemented with 2–8% (w/v) glucose. For the second set of experiments, 1% (w/w) apple pectin (Sigma) or 1% (w/w) birchwood xylan (Sigma) were added to the grape pomace.

2.3. Sample preparation

All the fermented samples were extracted with distilled water (50 ml) and mixed for 30 min on a rotary shaker at 200 rpm and 30 °C. The resulting solid suspensions were centrifuged at 10,000 rpm for 10 min and the supernatant was used for the measurement of pH, xylanases and exopolygalacturonase assays and determining reducing sugars (R.S.).

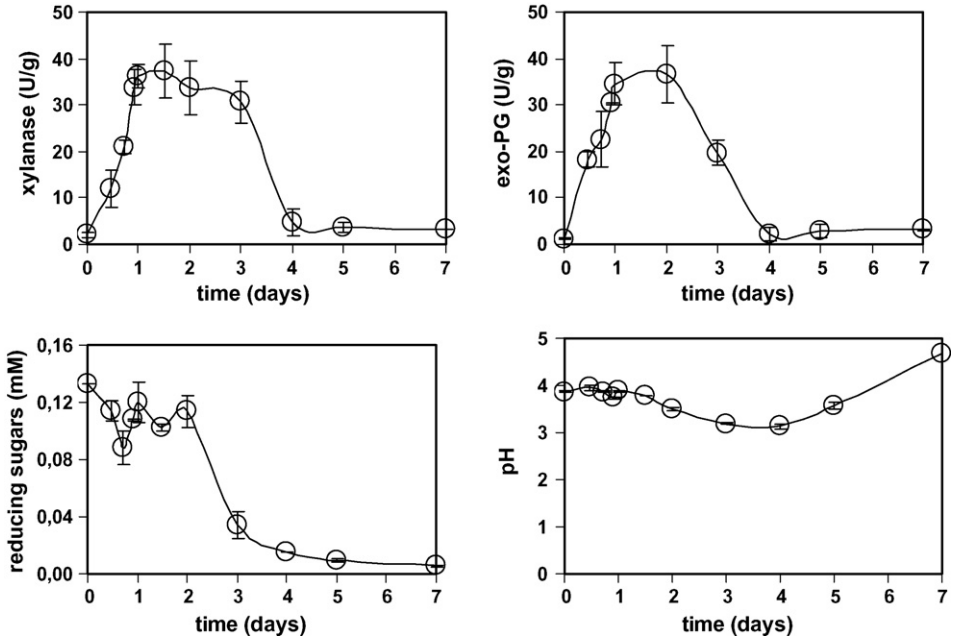

Fig. 1. Kinetics of xylanase and exo-polygalacturonase activities, reducing sugars concentration and pH during SSF of grape pomace by *Aspergillus awamori* on F2 medium.

All results shown below represent the average of three sets of experiments and error bars indicate the 95% confidence limit.

2.4. Analytical methods

The exopolygalacturonase and xylanase activities were estimated by determining reducing sugars released during hydrolysis of pectin and birchwood xylan, respectively [15]. The reducing sugars produced were quantified by a modification of the dinitrosalicylic acid method [16]. The enzymatic activities (U) were defined as the amount of enzyme required to produce 1 μmol of product per minute per gramme of grape pomace.

The reducing groups in the enzymatic extract were determined by the DNS method. The results were expressed as glucose using a calibration curve. The pH of the enzymatic extract was recorded using a pH meter. The moisture contents of the solid substrates were determined from the loss in weight after heating at 90 °C for 24 h.

All the above experiments were done in triplicate.

3. Results and discussion

3.1. Effect of the particle size of the milled whole pomace

It can be seen from Fig. 1 that xylanase activity increased from the beginning of fermentation and attained a peak after 36 h of incubation, it declined thereafter. While the activity of exopolygalacturonase reached a maximum value after 48 h of fermentation and then fell sharply.

Two trends have been clearly observed in the evolution of the reducing sugars. During the first 2 days, a slight decline in reducing sugars was observed. After the second day, the reducing sugars decreased by a factor of almost 20. When the reducing sugars were almost exhausted after 4 days, enzymes levels were very low.

The pH decreased during the first 3 days of fermentation, possibly due to microbial production of organic acids, and reached 3.1. When the concentration of soluble reducing sugars was very low, the pH increased, probably due to microbial assimilation of organic acids. Similar pH trends have been observed by others using *A. awamori* on wheat grains [17], wheat bran [18] and during enzyme production by *A. awamori* [19–21].

It can be seen from the results that the differences in enzyme titres attained on grape pomace of varied particle size are not significant, indicating that the particle size did not significantly affect the enzyme production.

3.2. Effect of initial moisture

Both enzyme activities were less low when the moisture content was higher or lower than 65%. The low enzyme activity at high substrate moisture levels could be attributed to the decreased porosity, alteration in particle structure, gummy texture, lower oxygen transfer or increased formation of aerial hyphae. Likewise, lower moisture levels lead to reduced diffusion of the nutrients in the solid substrate, lower degree of swelling and higher water tension. The optimum moisture contents for xylanase production by *Trichoderma longibrachiatum* and *Aspergillus tereus* were 55 and 75%, respectively [22]. In two *Aspergillus* species, a high enzyme production was attained at 40–50% moisture using dry koji as a substrate [23] and 70% when sugar cane bagasse as a support was used [24].

3.3. Effect of supplementation with carbon sources

When 6% glucose was added as an extra carbon source, the production of xylanase and exo-PG increased significantly. However, at 8% (w/w) both enzymatic activities declined. Although grape pomace contains sufficient nutrients to support the production of xylanase and exo-PG by *A. awamori*, the addition of glucose at certain concentrations exerted a positive effect on the enzyme synthesis, but was repressive when added in excess. It has been reported earlier that xylanase production by *Aspergillus tamarii* on sugar cane bagasse or corn cob was suppressed by glucose supplementation. In contrast, when wheat bran was used as the solid substrate, xylanase was resistant to catabolic repression even at 10% (w/w) glucose concentration [25].

Xylanase production declined when xylan or pectin were added, while exo-PG levels increased. A similar observation was made by Kashyap and co-workers in *Bacillus* sp. [26]. Blandino et al. reported higher production of exo-PG by *A. awamori* on whole wheat flour when pectin was added [27].

4. Conclusions

Grape pomace has an enormous potential as a substrate for the production of high-value hydrolytic enzymes. Particle size did not have a major effect on the enzyme production. Moisture content and the addition of extra carbon sources were found to have exerted a marked influence on the yield of both enzymes.

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