Biochemical Engineering Journal 26 (2005) 100–106

Biochemical Engineering Journal

www.elsevier.com/locate/bej

Hydrolytic enzyme production by *Aspergillus awamori* on grape pomace

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Abstract

Grape pomace, the main waste in the wine industry, has been shown to be the sole nutrient source for solid state fermentation to produce hydrolytic enzymes (cellulases, xylanases and pectinases) using *Aspergillus awamori*. Petri dishes with this natural support inoculated with spores were incubated under static conditions during 7 days and the enzymatic extracts obtained at different time intervals were analysed. The enzymes analyses demonstrated that grape pomace could be competitive with other typical agroindustrial wastes used as substrates in SSF processes.

Keywords: Solid state fermentation; Agroindustrial residues; Grape pomace; *Aspergillus awamori*; Cellulase; Xylanase; Pectinase

1. Introduction

Solid state fermentation (SSF) is a technique that has been well known for centuries [1] and has lately become increasingly important. The technique essentially involves the growth of microorganisms on wet solid supports in the absence (or near absence) of free water. New interest in this technology derives from the fact that it is considered to be an appropriate approach for processes including the bioremediation or the biodegradation of toxic compounds, the detoxification of agricultural wastes, the biotransformation of crops and biopulping, etc. [2–4]. Moreover, SSF has been successfully applied in the preparation of new high value products, such as secondary metabolites, organic acids, pesticides, aromatic compounds, fuels and enzymes [5–9]. The advantages of SSF in comparison to traditional submerged fermentation are better yields, easier recovery of products, the absence of foam formation and smaller reactor volumes. Moreover, contamination risks are significantly reduced due to the low water contents and, consequently, the volume of effluents decreases [10]. It has been shown that for some specific processes, particularly enzyme production, the costs of these techniques are lower and the production higher than submerged cultures [11]. Examples of this situation include cellulase production, which is estimated to be 100 times more economical with SSF [12], and lipase production, which is 78% cheaper with SSF [13]. For this reason, many researchers have recently focused on the production of industrial enzymes and, in particular, on reactor design [14–17], in the search for new solid supports [18–20] or process optimization [21–23].

A wide variety of natural solid supports have been used for SSF, with crops and agroindustrial wastes the most studied; besides immobilizing the microorganisms, these supports supply the main nutrients needed for growth (minerals, vitamins, etc.). In the field of enzyme production, several natural solids have been successfully employed: e.g., wheat, corn, rice, sugar cane and beet, banana waste, potato, tea, coccus, apple and citrus fruits, wheat flours and corn [9]. Of these supports, wheat fibres have been the most widely investigated. However, a very few researchers have published work on grape pomace, which today is a very significant waste product in agriculture industries. Grape pomace is the residue left after juice extraction by pressing grapes in the wine industry. In Spain alone, over 250 million kg of this by-product (constituted by seeds, skin and stem) are used every year either as animal feed (with low nutritional value)

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doi:10.1016/j.bej.2005.04.020

or for ethanol production by fermentation and distillation (low level benefit). This material is under-exploited and most of it is generally disposed in open areas, leading to serious environmental problems. In contrast, the potential utility of this waste for value-added products by SSF is promising. The variety *Palomino fino*, selected for the work described here, has a high carbohydrate content (8% in the seeds, 13% in the skin), with the fibre representing about 50% of the total mass. The principal component of fibre is lignin representing 64% of the fibre in the pips and 59% in the skin. The other majority components of fibre are the hemicelluloses (18% of the fibre in pips and 31% in skin) and cellulose (17.75% and 6% in pips and skin, respectively). The minority component of the fibre is pectin with only a 0.25% of fibre in pips and 4% in skin [24]. Several bioprocesses have been developed that use grape pomace as the raw material for the production of bulk chemicals and fine products by SSF; these processes include the production of citric acid [25], gluconic acid [26], carotenoids [27], xanthan [28] and ethanol [29]. For example, grape pomace has been reported to induce the production of lacase enzyme in a submerged culture with *Trametes versicolor* [30] and has been used as a solid support for the growth of *Trichoderma viride* with SSF [31]. However, this material has never been specifically used as a solid support for enzyme production.

Given the composition of grape pomace, the enzymes cellulase, xylanase and pectinase were selected to test the production with SSF. Cellulase is the general term for a group of enzymes consisting of endo-1,4-β-D-glucanase (EC 3.2.1.4), exo-1,4-β-glucanase (EC 3.2.1.91) and β-D-glucosidase (EC 3.2.1.21). These enzymes are employed in feed, fuel and chemical industries for the processing of lignocellulosic materials. These enzymes, together with xylanase (EC 3.2.1.8), are very common in the textile industry. The pectinase group is formed by five enzymes – pectinesterase (EC 3.1.1.11), endo-polygalacturonase (EC 3.2.1.15), exo-polygalacturonase (EC 3.2.1.67), pectin liase (EC 4.2.2.10) and petate liase (EC 4.2.2.2) – and is commonly used in the production of juices and fruit extracts [9]. Finally, *Aspergillus awamori* has been widely used in the production of such enzymes with SSF on other solid supports, such as wheat grain [32–34].

2. Materials and methods

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 5% whole-wheat flour and 2% agar slants at 4 °C.

Spores stored on flour slants were washed with 0.01% (v/v) Tween 80 solution (10 ml). The spore solution (0.5 ml) was spread on the surface of the solid flour medium (100 ml) in 500 ml Erlenmeyer flasks, and the inoculated Erlenmeyer flasks were incubated at 30 °C for 5 days. After the incubation period, 0.01% (v/v) Tween 80 solution (100 ml) was added to the flasks and the spores were suspended by gentle shaking. The number of spores was later counted in an Improved Neubauer Counting Chamber (Assistent-Germany; BDH).

2.2. Grape pomace

Industrial white grape pomace (*P. fino* variety) grown in the Jerez-Xeres-Sherry area, in south-western Spain, was used as the sole nutrient source for the fermentation studies and the production of hydrolytic enzymes. Samples of freshly pressed white virgin marc were collected from a local wine cellar and stored at −24 °C. For any given series of experiments, sub-samples (250 g) were taken and dried in an oven at 60 °C for 48 h. The solid was then milled in a commercial mill and sieved. The mean diameter (D_{50}, the diameter below which 50% of the particles fall) of the milled pomace was 0.74 mm.

2.2.1. Chemical analysis

After a preparatory separation of the stems, the chemical content was analysed using the following techniques:

- *Moisture and ash content*: A known weight of each sample was heated to 90 °C in an oven for 24 h. The dried samples were weighed again and the moisture content was calculated. Ashing was performed by calcination of the samples in a muffle furnace at 660 °C for 6 h.
- *Glucose*: A Beckmann glucose analyser was used to calculate the glucose concentration in the grape pomace.
- *Free amino nitrogen*: This parameter was determined by the ninhydrin colorimetric method.
- *Total nitrogen*: This parameter was measured as Total Kjeldahl Nitrogen (TKN) by the Nessler method.
- *Phosphorus*: The determination of phosphorus in grape pomace samples relied on the destruction of the samples by acid digestion and the colorimetric determination (430 nm) of the resulting phosphate.

2.3. Fermentation procedure

The production of hydrolytic enzymes by *A. awamori* in SSF was evaluated in grape pomace using petri dishes (9 cm in diameter). Prior to inoculation, the solid was sterilized in an autoclave for 20 min at 120 °C and 1.2 atm. To each plate 10 g of sterilized solid was added, the required volume of spore suspension to obtain a final spore concentration of 5×10^5 spores/g, and the appropriate amount of water needed to adjust the moisture to the desired level (60%).

The petri dishes were incubated under static conditions at 30 °C during 7 days and samples in triplicate were withdrawn at different time intervals (0 h, 11 h, 17 h, 22 h, 24 h, 48 h, 72 h, 96 h, 120 h and 168 h).

2.4. Enzyme assays

2.4.1. Extraction

The whole contents of each petri dish (10 g of fermented material) were mixed with 50 ml of distilled water and incubated for 30 min at 30 °C and at 220 rpm in a rotary shaker. The suspension was then centrifuged at 10000 rpm for 10 min and the resulting liquid – the enzymatic extract – was stored at −20 °C until required for subsequent enzymatic analysis.

2.4.2. Analysis

- *Reducing sugars* were measured by the dinitrosalicylic acid (DNS) method described by Miller [35]. Results were expressed as glucose concentration using a calibration curve.
- *Cellulase activity* was assayed using carboxymethyl cellulose 1% (w/v) dissolved in 50 mM glycine/NaOH buffer (pH 9) as a substrate. To 1 ml of carboxymethyl cellulose solution, 0.5 ml of appropriately diluted enzyme was added. The resulting solution was incubated at 50 °C for 10 min. The reaction was stopped by the addition of 2 ml of 0.3 M trichloroacetic acid (TCA). The reducing sugars concentration was then determined by the DNS method. One enzyme activity unit was defined as the amount of enzyme that released 1 μmol of glucose per minute under the assayed conditions.
- *Xylanase activity* was assayed using Birchwood xylan (Sigma) as a substrate. A 1.5 ml reaction mixture, containing 0.5 ml of appropriately diluted enzyme solution and 1 ml of a 0.5% (w/v) suspension of xylan in 0.05 M citrate buffer, was made at pH 5.4. The mixture was incubated at 50 °C for 10 min and the reaction stopped by the addition of 2 ml of TCA (0.3N) The reducing sugars produced were assayed by the DNS method using D-xylose as the standard. A unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of reducing sugars per minute at pH 5.4 and at 50 °C.
- *Exo-polygalacturonase activity* was assayed by measuring the release of reducing sugars by DNS method. In this case, D-galacturonic acid was used as the standard. In a test tube, 1 ml of 0.5% pectin (from apple fruit) in 0.1 M acetate buffer (pH 5) was added to 0.5 ml of the diluted enzyme solution. After incubation for 10 min at 45 °C, the reaction was stopped by the addition of 2 ml of TCA (0.3N) and the released reducing sugars were measured by the DNS method. One unit of exo-polygalacturonase activity was defined as the activity that liberates 1 μmol of D-galacturonic acid per min at 45 °C and pH 5.
- *Endo-polygalacturonase activity* was measured viscometrically using a Cannon-Fenske routine viscosimeter. A solution of 15 ml of 0.5% pectin (from apple fruit) in 0.1 M acetate buffer (pH 5) was incubated in a water bath at 45 °C. After the pectin solution had tempered, 5 ml of the enzymatic extract was added and the mixture was incubated for 10 min. The reaction was stopped by boiling for 5 min. The activity was calculated by measuring the viscosity reduction of the sample against a blank. The blank solution was made by adding 5 ml of the enzymatic extract to 15 ml of 0.5% pectin and immediately boiling for 5 min to denaturalize the enzymes. One endo-pectinase unit was defined as the amount of enzyme that reduces the viscosity of the pectin solution by 50% per minute under the conditions stated above (50 °C and pH 5).

All the above measurements were made in triplicate, and the enzymatic activities were expressed as activity units per gram of dry substrate (IU/gds).

3. Results and discussion

3.1. Chemical composition

Application of the techniques outlined in Section 2.2.1 enabled the analysis of the chemical content in terms of ash, total sugar, nitrogen, phosphorus, amino nitrogen and glucose in the grape pomace (Table 1). Attending to the composition, this natural medium is a potentially good support for fungus growth with SSF.

3.2. Enzyme production

The production of xylanase, cellulose and pectinase (endo-polygalacturonase and exo-polygalacturonase) were studied by growing the fungus on petri dishes, as discussed in Section 2.3.

An electron micrograph (×2000) was obtained on a sample from the second day (Fig. 1). The picture shows the grouped spores of *A. awamori* with a rough surface texture.

The evolution of enzymatic activity, reducing sugars and pH on using *A. awamori* on grape pomace as a fermentation medium is plotted in Fig. 2.

The behaviour of *A. awamori* in producing xylanase, cellulase and exo-polygaracturonase followed similar patterns. Enzymatic activity was not detected at the beginning of the fermentation, suggesting that the enzymes were produced only by the fungus and that the enzyme content of the pomace was negligible. The trends show a sharp increase in the activity of these enzymes in the early stages of incubation with a maximum value attained within the first 24 h. A re-

Table 1
Chemical analysis of grape pomace.

Chemical analysis	
Component	Composition (w/w) db (%)
Moisture	7.66
Ash	6.20
Glucose	7.13
Nitrogen	1.5
Protein ($N \times 6.25$)	9.32
Phosphorus	0.14

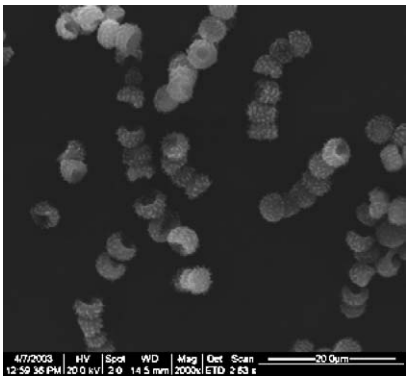

Fig. 1. Electron micrograph (×2000) of spores of *A. awamori* under SSF conditions.

duction in the enzyme activity was observed after the first few days of fermentation.

The xylanase activity (Fig. 2a) reached a maximum value after 24 h of fermentation (40.4 ± 15.6 IU/gds) after which a reduction in its activity was observed. On the seventh day, an activity of only 3.5 IU/gds was measured. Comparing the xylanase production on grape pomace with the corresponding ones for other agricultural residues, it seems that grape pomace is a good potential substrate for the synthesis of this enzyme. Thus, when *Penicillium decumbens* was grown on a mixture of 90% corn straw and 10% wheat bran moistened with 25 ml of a mineral solution, 13.59 IU/gds of xylanase were measured after 4 days of culture at 28 °C [36]. Couri et al. [37] also studied the production of xylanase by *A. niger* using different agroindustrial residues – mango peel and wheat bran – as the solid substrate. For those experiments, 40 g of the sterile fermented solid containing a moisture level of around 60% was incubated at 32 °C. When wheat bran was used as the substrate, xylanase activities around 100 IU/gds were reached after 72 h of incubation. When mango peel was used, maximum xylanase activity (50.4 IU/gds) was reached after 24 h of fermentation. In the same line, the production of xylan-degrading enzymes by a

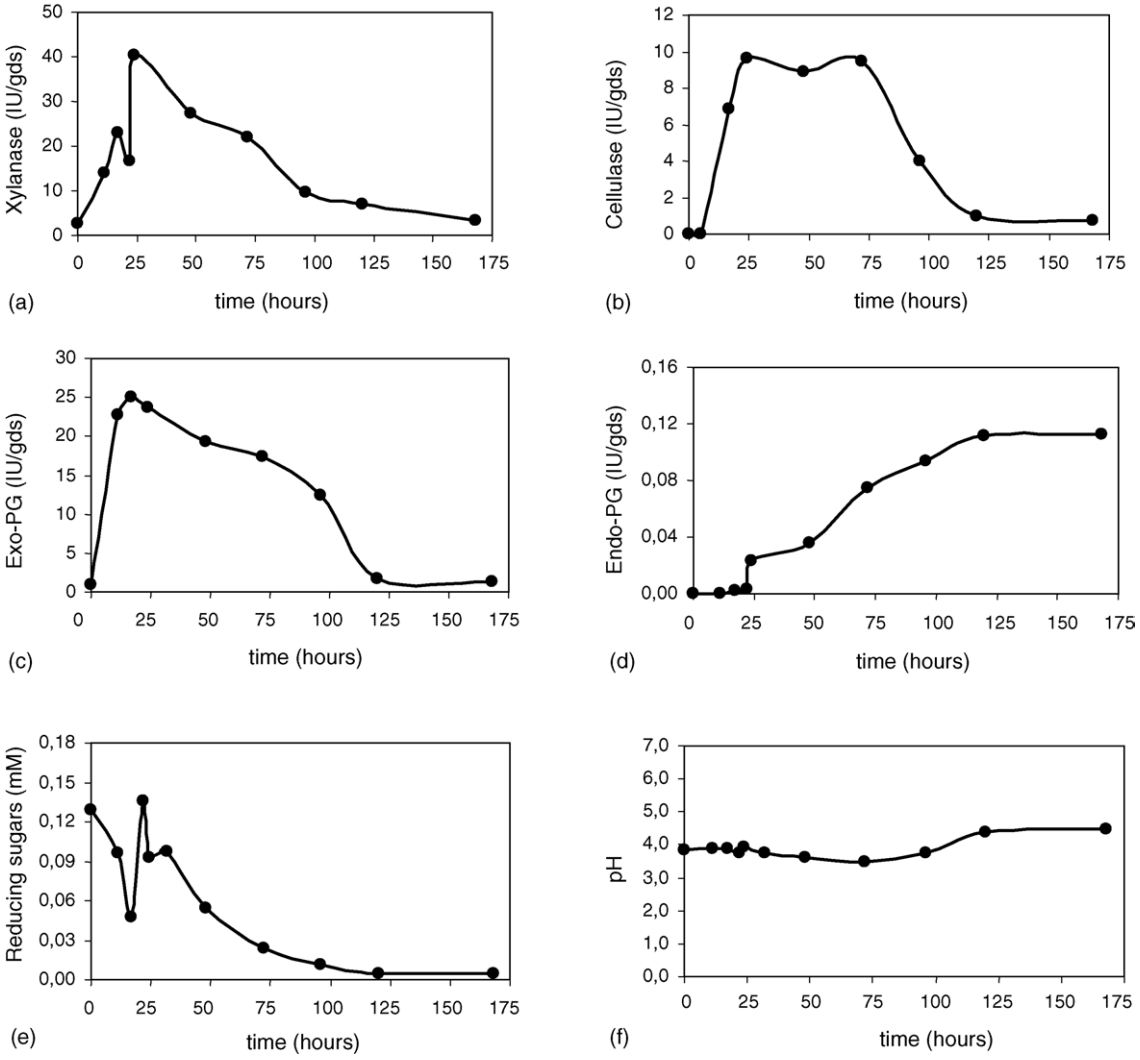

Fig. 2. Evolution of the enzymatic activity of xylanase (a), cellulase (b), exo-PG (c), endo-PG (d), concentration of reducing sugars (e) and pH (f) produced by *A. awamori* on grape pomace.

koji mold, *Aspergillus oryzae* RIB 128, has been tested on dried wheat bran, rice bran and orange peel [38]. The highest productivity – about 60 IU/gds – was reached when wheat bran was used as the substrate. For those experiments, the moisture content of the solid was adjusted to 67%, and solid state cultures were grown at 30 °C for 4 days. The production of xylanase by the bacterial strain *Bacillus licheniformis* has been reported by Archana and Satyanarayana [39]. In this case, a maximum activity of 16.8 IU/gds was measured after 72 h of inoculating the strain on 10 g of wheat bran with 25 ml of a mineral solution, and incubated at 50 °C.

Cellulases (Fig. 2b) were not excreted until 17 h after the inoculation, with the highest value reached within the first 24 h. This activity (9.6 ± 0.76 IU/gds) remained constant until the third day. The activity subsequently decreased to 1.0 IU/gds on the fifth day. A previous study described in the literature shows that different agricultural wastes containing lignin inhibit cellulase activity on cellulose [40]. This observation could also explain the lower values for the enzyme studied here. In order to evaluate the goodness of grape pomace as substrate for the synthesis of cellulase, different agricultural residues reported in literature have been compared considering endoglucanase activity or CMCase. Yang et al. [36] reported the growth of *P. decumbens* on a mixture of 90% corn straw and 10% wheat bran (moistened with a mineral solution) and produced a maximum of 9.05 IU of cellulase per gram of dry substrate, after 4 days of culture at 28 °C. Jecu [41] obtained a maximum production for this enzyme of 296 IU/gds using a milled mix of wheat straw and wheat bran (9:1) with an initial humidity of 74% using *A. niger*, grown during 96 h of incubation at 30 °C. Krishna [42] grew *Bacillus subtilis* on banana wastes in order to measure CMCase activity and obtained 9.6 IU/gds after 72 h (moisture 70%; pH 7.0) at 35 °C with additional mineral nutrients. The same author tested SSF with wheat bran, rice bran and rice straw obtaining 4–10-fold lower activities with the same microorganism. Jha et al. [43] used soyhull (material produced during soybean processing) with added urea (2%, w/w) and the fungus *Phanerochaete chrysosporium* to enhance cellulase yields of 74.8 IU/gds at 25 °C.

The exo-polygalacturonase (exo-PG) activity (Fig. 2c) reached a maximum of 25.0 ± 9.61 IU/gds between 11.2 and 24 h and thereafter decreased until reached a value of 1.7 IU/gds on the fifth day. This enzyme is produced earlier with a high activity value. However, endo-polygalacturonase (endo-PG) activity (Fig. 2d) did not appear during the first 24 h and then increased progressively, stabilising with an activity of 0.113 ± 0.0033 IU/gds between the fifth and the seventh day. Pectinase production in grape pomace will be compared with the data reported for the production of this enzyme using *A. awamori* on whole wheat [44]. In the latter case, a maximum exo-PG activity of 9.18 IU/gds was found. The results obtained with grape pomace showed that the activity measured was almost 2.5 times higher than using wheat as a substrate. In the case of endo-PG, the difference is even more marked: maximum activity of 0.014 IU/gds in whole wheat and 0.113 IU/gds in grape pomace. On the basis of these results, it can be seen that grape pomace is a more effective medium than wheat for the production of pectinase enzymes. Since the microorganism and the conditions of cultivation were the same, the differences outlined above may be well related to the chemical compositions of the media – principally the fibre content (2.7% in wheat and 40% in grape pomace) – which affects the fungal growth and the enzyme synthesis.

The behaviour of the reducing sugars shows a peculiar trend (Fig. 2e). During the first phase of growth, the microorganism consumed the free sugar contained in the substrate. After 22 h, the total concentration of free reducing sugars was very low (less than 0.05 M) and the fungi began to use their hydrolytic enzymes to obtain other carbon sources. The concentration grew and a peak was reached after 24 h. Later the sugars were consumed and the concentration decreased exponentially, coinciding with a fall in the concentration of xylanase, cellulase and exo-PG. This situation could suggest that for these three enzymes, the high concentration of sugars favours the enzymatic production. In contrast, the production of endo-PG would show a catabolic repression when the reducing sugar concentration in the medium is high and this would explain the increasing trend observed for this enzyme.

The graph representing the pH (Fig. 2f) shows a constant value of around 3.8 during the first 24 h and a slight decrease thereafter. This decrease could be linked to the release of organic acids during the growth of fungi. After the third day, the pH increases as the reducing sugar level is very low and this increase is due to the utilisation of organic acids as carbon sources when the sugar is limited.

4. Conclusions

- Grape pomace, the main polluting waste from the wine industry, is a good natural medium for SSF. Its chemical composition is rich in the main nutrients required for the growth of a wide range of microorganisms. The low cost of this material makes it potentially promising for such applications.
- The growth behaviour of *A. awamori* on grape pomace as the sole nutrient source for producing xylanase, cellulase and exo-polygaracturonase followed similar patterns. A rapid rise in the activity of these enzymes was observed in the early stages of incubation (within the first 24 h). Xylanase and exo-polygalacturonase activities were high compared with corresponding values in the literature, showing good future prospects for industrial applications. Cellulase activity is inhibited.
- Endo-polygalacturonase shows a catabolic repression when the reducing sugar concentration in the medium is high (during the first few hours) and its activity increases when the reducing sugars decrease.

Acknowledgement

The authors wish to thank the "Ministerio de Ciencia y Tecnología" of Spain for financial support (PPQ2002-00358).

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