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Evaluation of the conditions for the extraction of hydrolitic enzymes obtained by solid state fermentation from grape pomace

Ana B. Díaz, Ildefonso Caro, Ignacio de Ory *, Ana Blandino

Department of Chemical Engineering, Food Technology and Environmental Technologies, Faculty of Sciences, University of Cádiz, Pol. R. S. Pedro S/N, Pto., Real-Cádiz, Spain

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Abstract

In this work, conditions for the extraction of xylanase and exo-poligalacturonase, previously produced by solid state fermentation on grape pomace using *Aspergillus awamori*, have been evaluated. The effect of the type and volume of solvent, temperature and time of incubation and stirring rate were studied separately and combined. Furthermore, the application of repetitive extractions was evaluated. Recommended conditions for both enzymes recovery from fermented grape pomace are 50 ml Tween 80 (0.01%), 4 °C and 150 rpm. In this way, xylanase and exo-PG recovery can be increased in 7.7- and 5.5-fold. In addition, the data are used as the base for the theoretical calculation of the total amount of enzyme which could be extracted from the solid.

Keywords: Solid state fermentation; Grape pomace; Extraction conditions; Xylanase; Exo-poligalacturonase

1. Introduction

Solid state fermentation (SSF) is defined as the fermentation involving solids in the absence (or near absence) of free water. The substrate must contain sufficient moisture to support the growth and metabolism of the microorganism. This technique has been employed in Japan and Asia from centuries for the production of some fermented food as koji, tempeh or soya sauce, among others [1]. In the last decade, an increasing interest in the development of such processes has been registered all over the world due to the fact that this type of fermentation has showed to be an appropriate approach for several processes including the bioremediation and biodegradation of toxic compounds, detoxification of agricultural wastes, biotransformation of some cultures and their wastes for the improvement of their nutritional quality, etc. [2]. SSF is also being successfully applied in the production of enzymes, antibiotics, surfactants or biocides and for the production of value-added products from wastes [3].

Solid state fermentation is acquiring a special relevancy in the field of the biotechnological processes as an alternative to the traditional submerged fermentation: SSF processes have lower energy requirements, produce less wastewater and are environmental-friendly as they resolve the problem of solid wastes disposal [4]. Thus, these processes give high product concentrations, avoid the foaming and have lower risks of contamination [5,6]. However, submerged fermentation has clear advantages in the process control and easy recovery of extracellular enzymes, mycelia or spores [7]. Nevertheless, SSF has noticeable lowers costs and requires cheaper raw materials [8,9].

The solid materials used in SSF can be classified into two great categories: inert materials, which only act as an attachment place for the microorganism and non-inert materials, which not only function as an attachment place but also supply some nutrients to the microorganism [10]. These materials are typically ligno-cellulose-based agricultural products or agro-industrial sources such as grains and grain by-products. A number of such substrates have been employed [11]: sugar cane bagasse, wheat, rice and maize brans, wheat straw, rice straw, rice husk, soyhull, banana waste, sugar beet pulp, apple pomace, wheat or corn flours, etc. In the case of grape pomace, several bioprocesses have been developed for its utilization as a raw material for the

* Corresponding author. Tel.: +34 956016554; fax: +34 956016411.
E-mail address: ignacio.deory@uca.es (I. de Ory).

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production of bulk chemicals and value-added fine products by SSF. As examples, the production of a wide variety of interesting compounds such as gibberellic acid, penicillin, citric acid and cheese aromas can be cited [12]. However, studies related to the production of industrial enzymes on grape pomace by solid state fermentation have been hardly reported to date.

This natural substrate (formed from the skins, seeds and pieces of stem) is the main waste from the wine industry, which results of pressing grape to produce juice and finally wine. Grape is 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 winemaking. Grape pomace constitutes about 16% of the original fruit. The average composition of this medium includes carbohydrates, fibre, fats, proteins and mineral salts [13]. The main component of the fibre is lignin and then hemicelluloses, cellulose and pectin. This by-product, with scanty economic profitability and pollutant, can be reduced using SSF and relevant value-added products (as enzymes) can be obtained using an economic technology.

The viability of this solid as support for the production of hydrolytic enzymes by solid state fermentation has been previously studied by the authors. Specifically, synthesis of xylanase (EC 3.2.1.8) and exo-polygalacturonase (exo-PG) (EC 3.2.1.67) using *Aspergillus awamori* was tested with good results [13]. The hyphal mode of fungal growth and their good tolerance to low water activity make fungi efficient in natural microflora for the bioconversion of the solid substrate. The hydrolytic enzymes obtained degrade polysaccharides of the cellular wall like celluloses, hemicelluloses and pectins, having relevant applications in textile industry, the production of juices and fruit extracts and the pulp, paper and animal feed industries [14,15].

In such processes, after a static fermentation with the fungi growing on the solid surface, an extraction step with a liquid solvent is required in order to obtain the enzymes. Many variables of the extraction can affect the quantities of product obtained (activity units of the hydrolytic enzymes): type of solvent, temperature, time of extraction, etc. Optimizing such conditions becomes a relevant key to increase and improve the whole process efficiency.

2. Materials and methods

2.1. Solid state fermentation

- *Natural medium*. White grape pomace from the Xerez-Sheres-Sherry area (*Palomino Fino* variety) was used as natural substrate for the solid state fermentation. This solid was stored at −20 °C for its conservation. For the fermentation, the grape pomace was introduced in an oven during 48 h to obtain a dry solid. Subsequently, it was milled, sieved (to study the particle distribution) and sterilised in an autoclave.
- *Spore suspension*. *A. awamori* NRRL 3312 was propagated and stored on 5% whole-wheat flour and 2% agar slants at 4 °C. For the fermentation purposes, it was resuspended with Tween 80. The quantity of spores suspension used in the experiments was such to have 4.5×10^5 spores/g of solid.
- *Fermentation procedure*. The fermentation was carried out in Petri dishes with 9 cm of diameter. In each dish, 10 g of sterilized solid, the exact amount of water to fit a moisture content of 60% and the spore suspension were added. The Petri dishes were incubated under static conditions at 30 °C for 1 day. Each experiment was developed in triplicate.

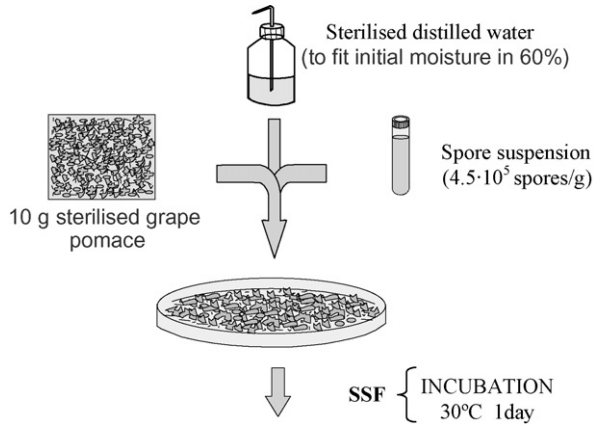

Fig. 1. Scheme of the solid state fermentation procedure.

Fig. 1 shows a scheme of the fermentation procedure.

2.2. Extraction conditions

The content of each Petri dish was spilled in Erlenmeyer flasks containing 50 ml of solvent, and then introduced in a rotary shaker. Different variables concerning the extraction were studied in the present work: type of solvent, extraction temperature, time of extraction, stirring rate, volume of solvent and number of extraction steps. Every variable is studied separately, maintaining the rest in the standard values: 50 ml of distilled water, 30 min of shaking, 30 °C and 200 rpm. The suspension resulting after every extraction was centrifuged (4000 rpm, 15 min) and the supernatant stored at −20 °C until required for the enzymatic analysis.

- *Type of solvent*. The solvents tested were: distilled water, buffer citrate 0.005 M (pH 5.4), buffer acetate 0.1 M (pH 5.0), NaCl 0.1 M and Tween 80 (0.1%).
- *Extraction temperature*. The studied temperatures were 4, 20, 30 and 40 °C.
- *Time of extraction*. The studied times were 15, 30, 45 and 60 min.
- *Stirring rate*. The speeds evaluated were 0, 100, 150, 200 and 250 rpm.
- *Volume of solvent*. About 50, 100, 200, 300, 400 and 500 ml of distilled water were tested under standard conditions.
- *Number of extraction steps*. Consecutive extractions using the initial fermented solid and renewing the solvent (distilled water and standard conditions) were carried out. After every single extraction step, the flask is centrifuged to separate the solid and the liquid. Then, new 50 ml of distilled water are added to the solid for the next extraction. This procedure was repeated six times, thus obtaining six extracts.

2.3. Enzyme assays

The enzymatic activities of xylanase and exo-polygalacturonase (exo-PG) in the different extracts obtained were assayed. For the xylanase, the reaction mixture containing 0.5 ml of the appropriately diluted enzymatic extract and 1 ml of xylan suspension (0.5% (w/w) Birchwood xylan in 0.05 M citrate buffer, pH 5.4) was incubated at 50 °C for 10 min, and the reaction was stopped by the addition of 2 ml of 0.3N TCA (trichloroacetic acid). The reducing sugars produced were measured by a modification of the dinitrosalicylic acid method [16] using D-xylose as the standard. A unit of enzyme activity (IU) was defined as the amount of enzyme producing 1 μmol of reducing sugars per minute.

Exo-polygalacturonase activity was evaluated adding 0.5 ml of the diluted enzymatic extract to 1 ml of the pectin solution (0.5% pectin in 0.1 M acetate buffer, pH 5.0). Samples were incubated at 45 °C for 10 min and the reaction was stopped by the addition of 2 ml of 0.3N TCA. The reducing groups in the enzymatic extract were determined by the DNS method. A unit of exo-PG activity was defined as the amount of enzyme that produced 1 μmol of D-galacturonic acid per minute under the conditions described above.

All the measurements were made in triplicate and the results expressed as reduced sugars using a calibration curve.

3. Results and discussion

The results obtained in the recovery of xylanase and exo-polygalacturonase from the fermented grape pomace at different extraction conditions are shown in Table 1. They are all expressed in activity units per gram of dry solid (IU/gds). As every experiment was made in triplicate, the average values and the confidence limits for a probability of 95% are also shown.

3.1. Type of solvent

The results obtained in the extraction of xylanase and exo-PG by using distilled water, buffer citrate 0.005 M (pH 5.4), buffer acetate 0.1 M (pH 5.0), NaCl 0.1 M or Tween 80 (0.1%) at standard conditions (50 ml of solvent, 30 min of shaking, 30 °C and 200 rpm) are shown in Table 1.

Tween 80 (0.1%), a non-ionic surfactant, appears as the best solvent for the extraction of xylanase, reaching 28.5 IU/gds. The worst solvent for the extraction of xylanase was the buffer acetate, differing in 30% with the maximum value.

The best solvents for the recovery of exo-PG were NaCl (0.1 M) and Tween 80 (0.1%). With these two solvents, the enzymatic activities recovered were 42.5 and 40.7 IU/gds, respectively. On the contrary, distilled water offers the lowest results, differing in 21% with the maximum value.

In conclusion, Tween 80 seems to be the most adequate solvent for the improvement of the extraction of both enzymes. These results can be supported with bibliography data, which report that surfactants increase cell permeability, thus increasing the export of several molecules across the cell membrane [17]. Nevertheless, the use of distilled water (the cheapest solvent of all the tested) also gives good results.

Table 1
Recovery of xylanase and exo-polygalacturonase (IU/gds) from fermented grape pomace at different extraction conditions

	Xylanase (IU/gds)	Exo-PG (IU/gds)
Solvent		
Distilled water	17.1 ± 5.0	33.4 ± 11.3
NaCl	23.0 ± 0.9	42.5 ± 5.1
Buffer citrate	22.1 ± 2.6	37.2 ± 6.5
Buffer acetate	20.0 ± 5.2	38.8 ± 0.5
Tween 80	28.5 ± 1.0	40.7 ± 3.3
Temperature (°C)		
4	31.6 ± 1.2	26.7 ± 0.5
20	31.7 ± 1.8	37.1 ± 2.9
30	22.3 ± 3.3	34.0 ± 8.1
40	12.9 ± 0.8	27.0 ± 2.9
Time of contact (min)		
15	23.7 ± 1.3	27.7 ± 0.5
30	26.4 ± 1.0	27.6 ± 1.6
45	23.6 ± 4.6	24.2 ± 1.2
60	25.8 ± 1.2	31.2 ± 2.7
Stirring rate (rpm)		
0	20.8 ± 1.9	26.4 ± 3.9
10	16.4 ± 8.1	21.3 ± 7.5
150	27.2 ± 2.3	35.0 ± 3.9
200	21.9 ± 3.1	37.6 ± 1.6
250	9.8 ± 3.1	40.2 ± 2.6

3.2. Extraction temperature

Although the optimum temperature for the production of xylanase is 50 °C [5], the optimum temperature for the extraction is within the range 4–20 °C, reaching maximum enzyme activities around 32 IU/gds. Nevertheless, 4 °C must be considered a most adequate temperature in order to guarantee thermal stability of both enzymes. For the exo-PG, the optimum extraction temperature is near to 20 °C, with an enzyme activity of 38 IU/gds. In both cases, the lowest enzymatic activity was obtained at 40 °C. These facts can be explained accepting that, at high temperatures, a number of compounds present in the grape pomace can be extracted, producing the saturation of the solvent and making the enzyme extraction less efficient. Results described later in Section 3.4 prove this saturation of the solvent.

3.3. Time of extraction

According to the results in Table 1, no significant differences are observed in the recovery of both xylanse and pectinase when different times of contact solid-solvent were tested. Thus, in the case of xylanase, the maximum and minimum values differ in 11% and for the exo-PG in 23%. This could lead to the conclusion that in 15 min a complete saturation of the solvent with the enzymes and other components extracted from the pomace is produced.

3.4. Stirring rate

In the case of exo-PG, at the range of speeds tested, as the stirring rate increases the enzyme recovery is also increased, reaching a maximum of 40.2 IU/gds when the maximum speed (250 rpm) was applied. This trend is predictable, because of the increasing in the homogenisation level in the liquid phase obtained at higher stirring rates. However, the optimum speed for the extraction of xylanase is 150 rpm, with an activity of 27.2 IU/gds, and later decreasing to 9.8 IU/gds when the stirring rate is 250 rpm. This kind of behaviour might be explained attending to the particular structure of xylanase [18].

3.5. Volume of solvent

Six different experiments were carried out, using the same amount of solid and extracting with different volumes of solvent (distilled water): 50, 100, 200, 300, 400 and 500 ml during 30 min, at 30 °C and 200 rpm. Fig. 2 shows the results.

For both enzymes, as the volume of solvent is increased more activity is extracted. Nevertheless, at the conditions assayed the maximum enzyme extraction is not reached. As it can be seen in Fig. 2, despite the volume of solvent is increased the enzyme extraction never levels off. Attending to a better utilisation of the

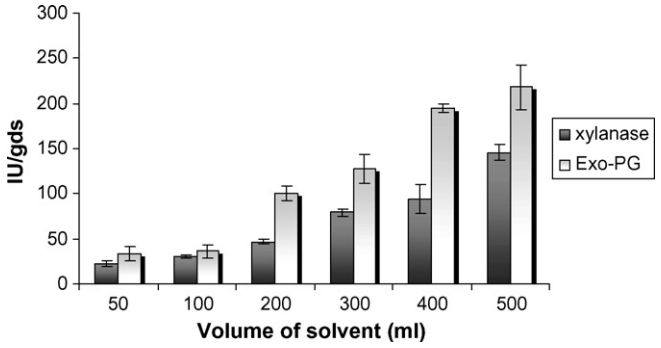

Fig. 2. Repetitive extraction of xylanase and exo-PG (IU/gds) from the solid with different volumes of solvent (distilled water).

solid and a more rational extraction, a subsequent experiment by using repeated extractions was carried out.

3.6. Repeated extractions

The efficiency of extraction of both enzymes from the fermented pomace with distilled water was carried out in six cycles of 30 min each. About 50 ml of distilled water was added in each cycle to the same fermented solid. Results in Fig. 3 show the enzymatic activities measured in the enzymatic extracts after every single step of extraction.

Results indicate that, after four extractions, xylanase contained in the fermented solid is almost exhausted. However, in the case of exo-PG, after the sixth stage of extraction more enzyme could be still separated from the fermented solid.

These results can be used as the base for the theoretical study of the total quantity of enzyme which could be extracted from the solid. For this aim, results shown in Fig. 3 were previously converted into IU/ml of solvent for each step of extraction (see Fig. 4). Later, as extraction in solid state fermentation is a process of dissolution and leaching in a heterogeneous liquid–solid system, the process kinetics may be expected to follow an exponential form:

$$E = A\,\mathrm{e}^{-kV} \tag{1}$$

with E is the enzyme extracted in every extraction step (in activity units per ml of solvent, IU/ml), A the parameter related with the quantity extracted in the first drop of solvent (when

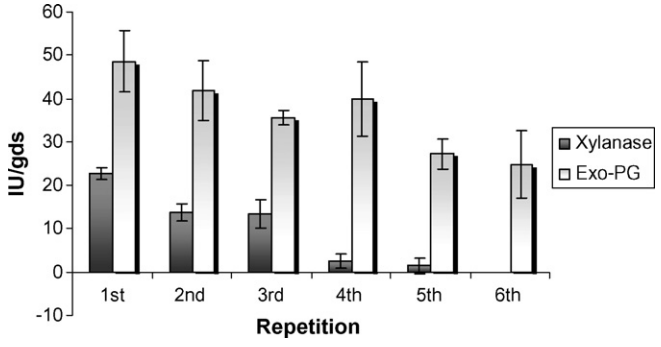

Fig. 3. Extraction of xylanase and exo-PG (IU/gds) from the solid with repetitive extractions, using the same volume of solvent (distilled water).

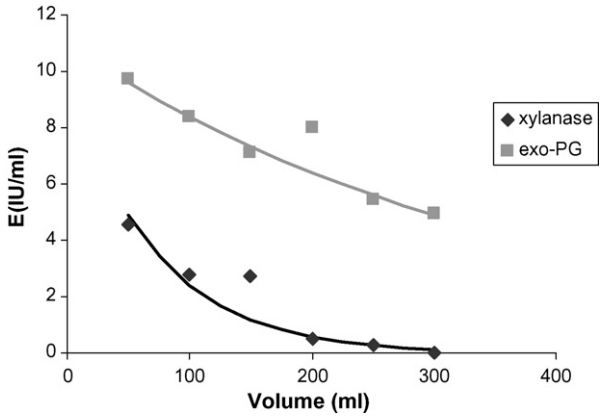

Fig. 4. Activity units of xylanase and exo-PG (IU/ml) extracted in every extraction step vs. accumulated volume of solvent. Lines correspond to the theoretical extraction sequences fitted to the experimental data.

V tends to zero) (IU/ml), k the parameter related with the conditions of extraction (ml^{-1}) and V is the volume of used solvent (accumulated) (ml). Linearizing the Eq. (1):

$$\ln(E) = \ln(A) - kV \tag{2}$$

Experimental parameters A and k can be obtained for each enzyme:

- Xylanase

$$A = 10.07\,\mathrm{IU/ml}, \qquad k = 0.0144\,\mathrm{ml}^{-1}$$

- Exo-PG:

$$A = 10.98\,\mathrm{IU/ml}, \qquad k = 0.0027\,\mathrm{ml}^{-1}$$

The area under the exponential (curves in Fig. 4) indicates the total quantity of the enzyme, I (in IU), contained in the solid (10 g):

$$I = \int_{V=0}^{V\to\infty} A\,\mathrm{e}^{-kV}\,\mathrm{d}V = \frac{A}{k} \tag{3}$$

This mathematical expression allows calculating a general equation to obtain the total quantity of enzyme that could be extracted (I), knowing the value of the enzyme activity obtained in the first extraction (E_1):

$$I = \frac{A}{k} = \frac{\left(\frac{E_1}{\mathrm{e}^{-kV_1}}\right)}{k} = E_1\frac{\mathrm{e}^{kV_1}}{k} = E_1 f, \quad f = \frac{\mathrm{e}^{kV_1}}{k} \tag{4}$$

where I is the total quantity of enzyme in the solid (IU); E_1 the quantity of enzyme obtained in the first extraction (IU/ml); f is the factor depending on the extraction conditions (ml).

For each extraction sequence, by knowing the k-value deduced from Eq. (2), and V_1 (50 ml) it is possible to calculate the value of the f factor. So, for the xylanase, $f = 2.85$ ml and for the exo-PG, $f = 8.48$ ml.

Then, with the experimental value of the f factor obtained in each case it is possible to evaluate the total amount of each

enzyme that could be extracted from a solid sample, only knowing the amount of enzyme extracted in the first step. This procedure can be used in order to establish the economical profitability of the enzyme production process.

3.7. Combination of relevant extraction conditions

Once studied the effect of every single variable on the extraction of xylanase and exo-PG, a combination of optimum conditions regarding to type of solvent, stirring rate and incubation temperature was assayed for each enzyme. The optimum conditions for the extraction of xylanase were 50 ml of Tween 80 (0.01%), 150 rpm and 4 °C. In the case of exo-PG the selected conditions were 50 ml of NaCl (0.1 M), 250 rpm and 20 °C. For both enzymes, time of incubation did not affect the efficiency of extraction, as it was pointed in Section 3.3, so 30 min was selected as incubation time. The results obtained showed that, comparing the enzymatic activities with those obtained with standard values (50 ml of distilled water, 30 min of shaking, 30 °C and 200 rpm), xylanase recovery was 7.7 times higher and exo-PG recovery was 5.5 times higher when optimum conditions for xylanase were assayed. On the other hand, xylanase recovery was 7.0 times higher and exo-PG recovery was 3.5 times higher when optimum conditions for exo-PG were tested.

Therefore, optimum conditions for xylanase extraction are the most suitable for the obtaining of both enzymes. The fact that exo-PG can be obtained more efficiently using the optimum conditions for xylanase can be explained considering that 4 °C is a more appropriate temperature for maintaining enzyme stability. In addition, Tween 80 was also a proper solvent for exo-PG extraction. So, recommended conditions for both enzymes recovery from fermented grape pomace are 50 ml Tween 80 (0.01%), 4 °C and 150 rpm.

4. Conclusions

- Optimizing conditions for the extraction of xylanase and exo-poligalacturonase from grape pomace, previously used as the natural medium for the growth of *A. awamori* by SSF, becomes a relevant key for the improvement of the enzyme production system.
- Tween 80 (0.1%), a non-ionic surfactant, appears to be the best solvent for the extraction among the tested.
- The optimum temperature for the extraction of both enzymes is 4 °C, decreasing at higher temperatures.
- Time of extraction (between 15 and 60 min) is not significant for the process yield.
- Increasing stirring rate leads to an improvement of exo-PG extraction, but 150 rpm is the maximum recommended for xylanase.
- Increasing the amount of solvent leads to an increasing of the enzymatic activity extracted. So, attending to a better utilisation of the solid, repetitive extractions are recommended.
- Recommended conditions for both enzymes recovery from fermented grape pomace are 50 ml Tween 80 (0.01%), 4 °C and 150 rpm.

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