Comparison of the production of xylanase and exo-PG using two filamentous fungi by solid state fermentation on grape pomace

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Abstract: The aim of the present work is the study of the production of xylanase and exo-poligalacturonase (exo-PG) using two different filamentous fungi: *Aspergillus awamori* and *Botrytis cinerea*. These enzymes are produced by solid state fermentation on grape pomace. According to the results, *Botrytis cinerea* seemed to give better results for the studied enzymes reaching 1.35 IU/gds for exo-PG and 1.05 IU/gds for xylanase after 15 and 14 days of fermentation respectively.

Key-words: SSF, xylanase, exo-polygalacturonase, grape, pomace

1. Introduction

Solid state fermentation (SSF) is a technique which involves growth of microorganisms on moist solid substrates in absence of free water [1]. This technique has been well known for centuries and has been credited to be responsible for the beginning of fermentation technique in ancient time [2]. It has been employed in Japan and Asia from centuries for the production of some fermented food as koji, tempeh or soya sauce, among others [3]. 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. [4].

Among these processes, the utilization of this technique to produce several enzymes has increased considerable due to it presents several advantages over the traditionally employed submerged fermentation [5].

Grapes are the most widely cultivated fruit crop in the world. 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. The average composition of this medium includes carbohydrates, fibre, fats, proteins and mineral salts [6]. 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 [7].

It´s very importat to look for the best microorganism for the enzyme production. *Aspergillus awamori* has been widely used in the production of such enzymes with SSF in different supports [6], [8],[9] and [10].

On the other hand *Botrytis cinerea* is well known because it causes grey mould even at low temperature, which is considered the most important disease of table grape [11].

2. Materials and methods

2.1. SSF in a synthetic culture media

A series of experiments, by using a typical synthetic culture media used for the growth of fungus, were carried out in order to study the enzyme production on it. This medium contained xylan and pectin as sole carbon sources. In this way, the production of the studied enzymes -xylanases and exopolygalacturonases- would be induced by the presence of those activators.

The synthetic culture medium was composed of peptone (1 g/L), yeast extract (0.5 g/L), agar (15 g/L), xylan (3.5 g/L) and pectin (3.5 g/L).

It was prepared in 100 mL Erlenmeyer flasks by adding 20 mL of the mentioned media. For the fermentation, $4.5 \cdot 10^7$ spores of *A.awamori* were added to each Erlenmeyer studying the enzyme production.
With respect to the extraction of the enzymes, the conditions used were: 15 ml of Tween 80 (0.01%), 30 min of contact solid-solvent, 4ºC and 150 rpm.

2.2. SSF in grape pomace

2.2.1. 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 washed to remove free reducing sugar. Later was dried for another 48 h and sterilised in an autoclave.

2.2.2. Spore suspension

A. awamori NRRL 3312 was propagated on 5% whole-wheat flour and 2% agar slants. On the other hand, *B.cinerea* BCK2 was propagated on petri dishes which contain a solid medium composed of thick soap tomatoe (washed and peeled) and agar (20g/L) (50%w/w).
For the fermentation purposes, spores were resuspended with Tween 80 (0,01%). The quantity of spores suspension used in the experiments was such to have 4.5×10^7 spores/g of solid.

2.2.3. 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 70% and the spore suspension were added. The Petri dishes were incubated under static conditions at 30 °C, in the case of *A. Awamori* and 25ºC in the case of *B.cinerea*, for several days.

2.3. 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 for 30 minutes at 4ºC with a stirring rate of 150 rpm. Conditions of extraction used were optimized in a previous work [7].

2.4. Reducing sugar assay

The reducing sugars remained in the extracts, not consumed by the fungus, were measured by a modification of the dinitrosalicylic acid method [12].

2.5. Enzyme assay

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 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 discusion

3.1. SSF in the synthetic culture media

As it can be observed (Fig. 1), the xylanase activity was kept practically constant during the fermentation, reaching a maximum of 1.12 IU/gds after 16 days of fermentation. Values obtained for exo-PG were smaller than the ones obtained for xylanase. The highest values were produced at the beginning of the

fermentation reaching a peak (0.22 IU/gds) after three days of fermentation.

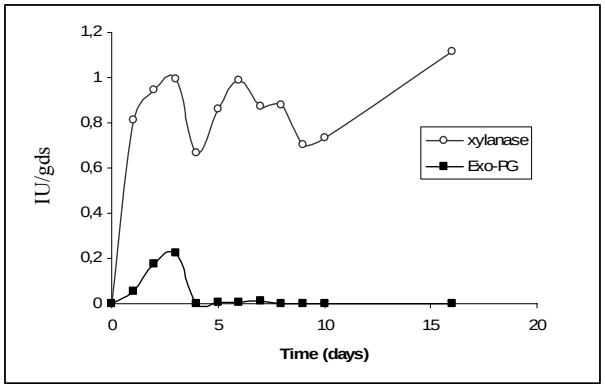

Fig. 1. Xylanase and exo-PG activities measured in the synthetic culture media.

3.2. SSF in grape pomace

Extracts from cultures were assayed to evaluate pH, reducing sugars and xylanases and pectinases activities.

For both fungi, the pH increases as long as the time of fermentation went on (Fig. 2). For *B.cinerea*, at the beginning of the fermentation the pH was 3.9 increasing up to 6.4 after 20 days of fermentation. In the last days, a small fall is produced.

On the other hand the increase of the pH for *A.awamori* is more slight, beginning with 3.9 reaching 5.0 after 14 days of fermentation.

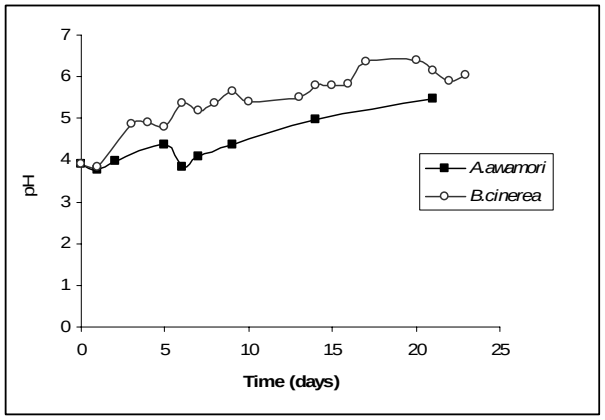

Fig. 2. pH temporal evolution along *A. awamori* and *B.cinerea* SSF on grape pomace

As it was expected, the reducing sugars concentration decreased for both fungi from approximately 0.01 mmol/mL to completely consumption (Fig. 3). Those trends can be explained taking into account that fungus consumes the reducing sugar necessaries for their metabolism.

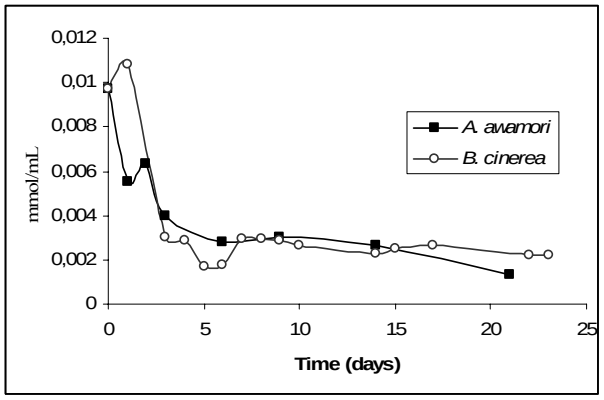

Fig. 3. Reducing sugars temporal evolution along *A. awamori* and *B. cinerea* SSF on grape pomace.

The results obtained in the recovery of xylanase and exo-polygalacturonase from the fermented grape pomace are shown in figures 4 and 5. They are all expressed in activity units per gram of dry solid (IU/gds).

The shape of the curves obtained for the production of xylanase and exo-PG by *B.cinerea* is quite similar, unless higher values were measured for exo-PG in most of the days (Fig. 4). After six days of fermentation, an increase in the enzymatic production was measured for both xylanase and exo-PG, reaching 0.53 IU/gds for xylanase the ninth day and 0.73 IU/gds for exo-PG the eighth day. Between 14th and 15th day, a peak was observed in the production of both enzymes. The value for exo-PG was up to 1.35 IU/gds after 15 days of fermentation whereas for xylanase was up to 1.06 IU/gds after 14 days.

As it can be observed in figure 5, the enzymatic production by *A. awamori* was lower than the one obtained for *B. cinerea.* However, as it was said before, the activities obtained for exo-pG were higher. For both enzymes, after the fifth day of fermentation, an increase in the production was observed. The maximum activity measured for xylanase was detected after 21 days of fermentation with an activity of 0.43 IU/gds. For exo-PG the maximum activity was reached at the 20th day of fermentation with a value of 0.53 IU/gds.

If figure 3 is compared with figures 4 and 5, it can be mentioned that the production of the

enzymes started after 5 days of fermentation, which is the time when the reduced sugar are practically consumed.

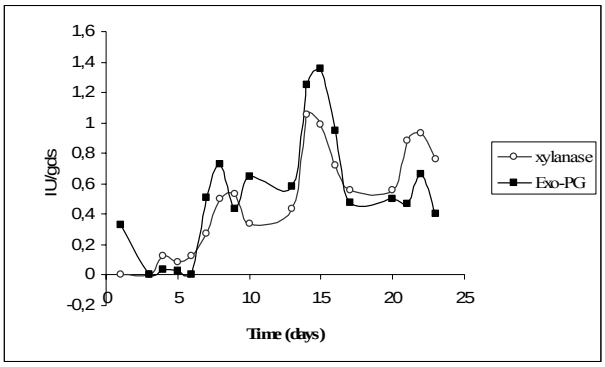

Fig. 4. Xylanase and exo-PG activities measured along SSF of *B.cinerea* on grape pomace.

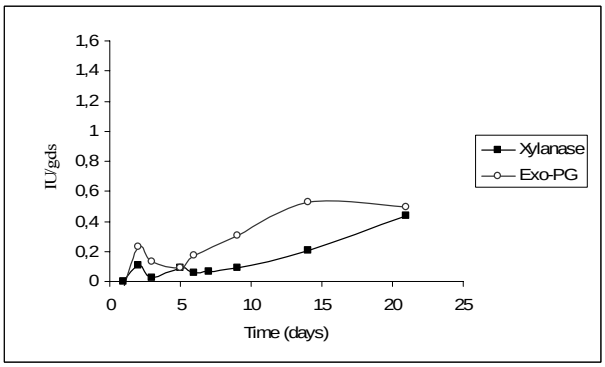

Fig. 5. Xylanase and exo-PG activities measured along SSF of *A. awamori* on grape pomace.

4. Conclusions

B. cinerea gives the best results for the production of the studied enzymes. For xylanase, the maximum activity obtained was 2.43 times higher than the one measured for *A. awamori,* reaching 1.06 IU/gds. The maximum enzyme activity during *B. cinerea* SSF was detected after 14 days of fermentation, whereas it was delayed to 21 days of fermentation for *A. awamori.*

On the other hand, the highest activity obtained for exo-PG was 2.55 times higher than the one obtained for *A. awamori* reaching a value of 1.35 IU/gds.

The results obtained for the synthetic media culture have similar order of magnitude, or even a little smaller, than the ones obtained for grape pomace. So,it can be concluded that grape pomace has an enormous potential as a substrate for the production of high-value hydrolytic enzymes.

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