Valorization of agro-industrial wastes to produce hydrolytic enzymes by fungal solid state fermentation

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Abstract

Purpose: The aim of this work was to produce hydrolytic enzyme cocktails derived from the fungal solid-state fermentation (SSF) of two different agro-industrial residues: orange peels (ORA) and exhausted sugar beet cossettes (ESBC). These cocktails were used to hydrolyse the residues in order to convert them into fermentable sugars. Their effectiveness compared to commercial enzymes was also studied.

Methods: SSF was carried out on ORA and ESBC for a few days and xylanase, pectinase (exo-polygalacturonase), cellulase and β-glucosidase activities were measured on the extracts obtained. Enzymatic hydrolysis of ORA, ESBC and the solid waste obtained after orange peels SSF were carried out by using different enzymes cocktails, analysing resulting reducing sugars.

Results: High xylanase and pectinase titers were measured on enzymatic extracts from both wastes; being the production higher on ESBC. With regard to the enzymatic hydrolysis of the solid residues, the highest reducing sugar yields were obtained when commercial cellulase was supplemented with the enzymatic extracts obtained in this work by SSF.

Conclusions: ORA and ESBC are adequate substrates for the production of hydrolytic enzyme by SSF. The produced enzymatic extracts might be used as supplement of commercial cellulase in order to hydrolyse lignocellulosic residues, increasing significantly the reducing sugars yield.

1. Introduction

A large number of agro-industrial wastes and by-products, such as exhausted sugar beet cossettes (ESBC), orange peels (ORA), grape pomace or rice husk, are produced by agricultural and agro-food industries. Frequently, these wastes are discarded, however, they can be used to produce a wide range of high-added value by-products such as ethanol, enzymes or biologically active secondary metabolites [1]. In this way, the bioprocessing of these wastes, which are economic and have great availability, can help to solve environmental problems associated with their disposal and, moreover, they can be used as raw materials to obtain high-added value by-products [2]. Different processes have been studied in order to find a more efficient utilization of these materials, being the solid-state fermentation (SSF) an interesting alternative. In fact, it turns out to be a promising technology for waste valorisation, that have been proved to be very efficient in terms of product yields and productivities, low energy consumption, and solving disposal problems [3].

Solid-state fermentation is a three-phase heterogeneous fermentation (solid-liquid-gas), in which solid particles represent the main phase. The majority of the water in the system is absorbed within the moist solid-particles. In this technique, microorganisms grow on the surface of a porous solid substrate with enough moisture to maintain microbial growth and metabolism.

This process is carried out in absence or near-absence of visible water between particles [2]. In the majority of cases, filamentous fungi are used in this process due to they are very efficient and competitive in solid substrates, given their high potential to produce hydrolytic enzymes [4]. Due to their composition, agro-industrial wastes are interesting substrates for SSF processes, given that they are rich in sugars, mainly cellulose, hemicellulose and pectin, which can be assimilated by microorganisms [5, 2]. For this purpose, they have to release previously hydrolytic enzymes to convert these polymeric sugars into more simple ones. In this way, different types of hydrolytic enzymes, such as pectinases, xylanases and cellulases, have been produced by this technique, which might be used in the hydrolysis process of different agro-industrial residues or even solid wastes generated after solid-state fermentation processes. Moreover, resulting simple sugars of enzyme hydrolysis might be fermented to a wide range of high-added value products. Thus, through this sequence of processes, the complete residue can be exploited efficiently [3].

In the present work, enzymes production kinetics by fungal SSF on orange peels and exhausted sugar beet cossettes are studied. Moreover, enzymatic extracts obtained by SSF have been applied for the hydrolysis of both solid wastes, comparing the reducing sugars yields obtained with the corresponding ones for commercial extracts with similar enzymatic activities.

2. Material and methods

2.1. Raw material

For SSF process two different residues have been used: orange peels (ORA) and exhausted sugar beet cossettes (ESBC). Their compositions are showed in Table 1 and 2, respectively. Orange peels (ORA) were obtained after juice extraction of oranges (Washington Navel variety) collected at a local market. Samples were collected and stored at 4 ºC until use. They were cut, washed, crushed and dried (40ºC – 48h). Exhausted sugar beet cossettes (ESBC) were provided by the British company AB-Sugar situated in Jerez de la Frontera (Cádiz, South of Spain). Samples were collected and stored at 4 ºC until use. The dried pellets show 6 mm diameter and variable length (10–40 mm) as a consequence of extrusion during pellets production. So, they were hydrated at 3% (w/w) to break the pellet conformation, and then, they were dried at 40 ºC during 24 h.

Both wastes were sterilized in and autoclave (120ºC – 20 min) before SSF.

For hydrolysis processes, the same ORA and ESBC, stored as described above, were used. Furthermore, orange peels waste generated after SSF process (ORASSF) was also used as raw material.

Table 1. Composition of Orange peels [6].

Component	Orange peels
Pectin	14.4 %
Protein	7.9 %
Cellulose	16.2 %
Hemicellulose	13.8 %
Ash	1.7 %
Lignin	1 %
Crude fat	3.9 %
Water soluble material	41.1 %
Glucose	14.6 %
Fructose	15.5 %
Sucrose	10.9 %

Table 2. Composition of exhausted sugar beet cossettes [7]

Component	ESBC
Cellulose	22.7 %
Hemicellulose	36.6 %
Pectin	22.84 %
Lignin	1.16 %
Ash	2.51 %
Others	2.73 %
Others	2.37 %

2.2. Spore production

An industrial strain of *Aspergillus awamori* 2B.361 U2/1 was originally obtained from ABM Chemicals, Woodley, Cheshire. The Commonwealth Mycological Institute classified the particular strain in the *Aspergillus niger* complex because it is a sequential mutant of NRRL 3312, which is a member of the *A. niger* series. Spores of this microorganism were propagated at 30 ºC during 5 days on petri dishes containing a medium compose (g/L) of: 1 peptone, 0.5 yeast extract, 15 agar, 6 xylan, 5 avicel and 1 pectin.

2.3. Solid state fermentation process

SSF process was carried out with 5 grams of ORA or ESBC, depending on the experiment, in static conditions at 30ºC in petri dishes. A scaled-up process was carried out with 100 grams of ORA in roux bottles at the same conditions. A nutrient solution containing (g/L) 2.4 urea, 9.8 g $(NH_4)_2SO_4$, 5.0 KH_2PO_4, 0.001 $FeSO_4 \cdot 7H_2O$, 0.0008 $ZnSO_4 \cdot 7H_2O$, 0.004 $MgSO_4 \cdot 7H_2O$ and 0.001$CuSO_4 \cdot 5H_2O$ at pH 5.0, was added to obtain an initial moisture content of 70% (w/w). Inoculum concentration was adjusted to $1 \cdot 10^7$ spores/g.

After SSF, the content of each petri dish or roux bottle was transferred into an Erlenmeyer flasks with 0.1% (v/v) Tween 80 with a liquid solid ratio of 10%, obtaining the enzyme extract. Subsequently, the mixture was extracted in a rotary shaker at 4ºC and 150 rpm for 30 min. Afterwards, the solid was separated by centrifugation at 10,000 rpm and 4 °C for 10 min and the liquid recovered was used as enzymatic extract.

For SSF in the roux bottles, enzyme extracts were concentrated by freeze-drying (3 days) in order to store them until use in the saccharification processes.

2.4. Enzymes assays

Enzymatic activities of cellulase (FPase, EC 3.2.1.91), xylanase (EC 3.2.1.8), exo-polygalacturonase (exo-pg, EC 3.2.1.67) and β-glucosidase (EC 3.2.1.21) were measured in the enzymatic extracts obtained by SSF and in the commercial preparations described in 2.7. All measurements were carried out in triplicate.

For xylanase, a reaction mixture containing 0.1 mL of xylanase solution and 0.9 mL of xylan suspension (0.5 % w/v Birchwood xylan in 0.1 M acetate buffer, pH 5.0) was incubated at 50 °C for 10 min. For pectinase activity, a reaction mixture containing 0.2 mL of exo-PG solution and 0.8 mL of pectin suspension (0.5 % w/v in 0.1 M acetate buffer, pH 5) was incubated at 45 ºC for 10 min. FPase assay was carried out by incubating 0.5 mL of suitably diluted crude enzyme with 1 mL of citrate buffer (50 mM, pH = 4.8), containing a Whatman No.1 filter paper strip (1 x 6 cm, 50 mg), at 50 °C for 60 min.

Reducing sugars produced after all these reactions were measured by a modification of the dinitrosalicylic acid method (DNS) [8]. A unit of enzyme activity (U) was defined as the amount of enzyme that produces 1 μmol of reducing sugars per minute under the specified conditions of pH and temperature.

β-Glucosidase activity was determined by incubating 0.250 mL of 4 mM p-nitrophenyl β-D-glucopyranoside (p-NPG) and 0.250 mL of acetate buffer (0.1 M, pH = 5) with 0.050 mL of enzymatic extract. The assay was performed at 60 ºC for 10 min, after which 2 mL of Na_2CO_3 2 M were added to stop the reaction and allow the development of the yellow colour of the p-nitrophenolate ion, which was measured at 410 nm. β-glucosidase activity was calculated based on the calibration curve of 4-nitrophenol. One unit of enzyme activity was defined as 1 μmol 4-nitrophenol equivalent released per minute.

2.5. Kinetic model of SSF

Experimental data of temporal enzymes production on ORA and ESBC were adjusted according to the kinetic model purpose by Díaz et al. [2]. This is a simple kinetic model, with only three fitting parameters, that adjusts accurately the production of a wide range of enzymes production in Petri dishes by solid-state fermentation [2]. The model is able to predict temporal enzyme activity in solid-state fermentations on complex media, such as orange peels, grape pomace, among others. Furthermore, it is able to estimate the maxima enzyme activities expected and the time at which these maximum will be reached. The fitting parameters are: the specific growth rate of the fungus (μ, h^{-1}), the enzyme production constant (k_E, U/kg) and the enzyme inhibition constant (k_D, h^{-1}).

2.6. Enzymatic hydrolysis

Hydrolysis experiments were performed in order to compare the effectiveness of the enzyme produced by SSF on ORA with commercial enzymes. Three different solid residues were analysed: ORA, ESBC and ORASSF. Before enzymatic hydrolysis, 6 grams of solid (ORA, ESBC and ORASSF) were autoclaved at 120 ºC for 20 min in a 250 mL erlenmeyer flask containing 50 mL of acetate-phosphate buffer 0.1M at pH 5.0. After that, 10 ml of the same sterilized buffer were added to ESBC and ORASSF, but 30 ml in the case of ORA.

Three different enzymes cocktails were used for the hydrolysis experiments. Commercial cellulase from *Thrichoderma reesei* (Cellulcast®, Sigma) (referred as "Exp. 1"), commercial cellulase supplemented with xylanase from *Thermomyces lanuginosus* (Sigma), exo-polygalacturonase (exo-PG) from *Aspergillus niger* (Sigma) and β-Glucosidase from *Aspergillus niger* (Sigma) (referred as "Exp. 2"), and commercial cellulase supplemented with the enzymatic extract obtained in this work by SSF (referred as "Exp. 3"). The enzyme activities used in these experiments are shown in Table 4.

Enzyme activity	Exp. 1	Exp. 2	Exp. 3
FPase (FPU/g)	1110	1110	1110
Xylanase (UA/g)	-	23.4	23.4
Exo-PG (UA/g)	-	31	31
β-glucosidase (UA/g)	-	0.7	3.25

Table 4. Enzymatic activity used in the hydrolysis processes.

2.7. Sugar determination

Reducing sugars (RS) were measured during hydrolysis process by a modification of the dinitrosalicylic acid method (DNS) [8].

2.8. Hydrolysis rate estimation

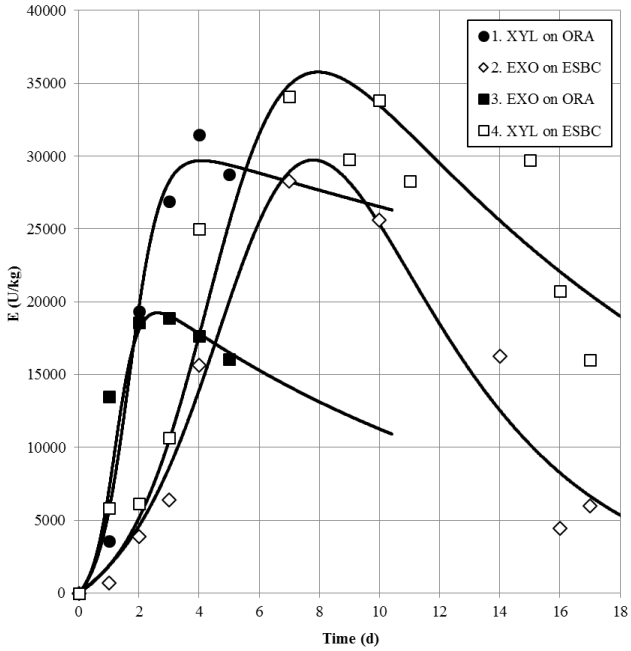
It is known that there are many stages involved in the enzyme hydrolysis process of a solid waste, such as enzymes adsorption, products desorption or enzymatic deactivation, which make the process quite complex. Due to this difficulty, for an easy and fast hydrolysis rate estimation, experimental data were adjusted to a simple first order kinetic model. It is based on the supposition that, at high doses of enzymatic activities, the solid hydrolysis rate is directly proportional to the quantity of solid in the medium. Thus, the general equation is as follows: $G = G_o + G_f\left(1 - e^{-k_h t}\right)$ (Equation 2). Where G is the concentration of RS at each instant time, G_o and G_f the initial and maximum RS concentrations, respectively, and k_h the hydrolysis rate constant [7].

3. Results and discussion

3.1. Solid-state fermentation of orange peels and exhausted sugar beet cossettes

Kinetics of enzymes production by *Aspergillus awamori* fermentation on ORA and ESBC are shown in Figure 1. As it can be observed, enzymatic activities increase through the fermentation time until a maximum is reached. In the case of orange peels, the maximum xylanase activity was 31,000 U/Kg at the 4th day and for exo-PG activity was 17,600 U/kg at the 2nd day. Nevertheless, a maximum of 35,000 U/kg for xylanase at the 10th day and 28,000 U/kg for exo-PG at 7th day were obtained on ESBC. FPase and β-glucosidase activities were also measured, however, the quantity of these activities were negligible and therefore results are not shown.

Figure 1. Enzyme production kinetic of *Aspergillus awamori* on ORA (black) and ESBC (white).



Comparing the two residues, xylanase and exo-PG activities on ESBC are 20% and 76% higher than respective ones for ORA. On the other hand, the times at which the maximum values are reached are higher in the case of ESBC.

Kinetic parameters of the model used to adjust the enzyme production are shown in table 5. As it can be seen, although the highest enzymatic activities are measured on ESBC, the maximum activities values are reached earlier for ORA (higher k_F values). It seems that enzymes produced on ORA are more stable than those produced on ESBC (lower k_d values).

Table 5. Adjusted parameters of SSF by model described in [2], where μ is the specific growth rate (10^{-3} h^{-1}), k_D is the enzyme inhibition constant (10^{-3} h^{-1}) and k_E is the enzyme production constant (U/kg).

Enzyme	Xylanase		exo-PG	
Waste	ORA	ESBC	ORA	ESBC
μ	80	30	110	25
k_D	0.87	3.11	3.19	9.27
k_E	514.5	824.9	357.9	1116.7
R^2	0.99	0.88	0.83	0.96

Extracts obtained from SSF of ORA in roux bottles (100 g of solid) were freeze-dried and their enzymatic activities measured, obtaining the following enzymatic activity units per gram of lyophilized enzymatic extract: 93 U/g of xylanase, 127 U/g of exo-PG, 57 U/g of FPase and 13 U/g of β-glucosidase.

3.2. Saccharification of agro-industrial wastes

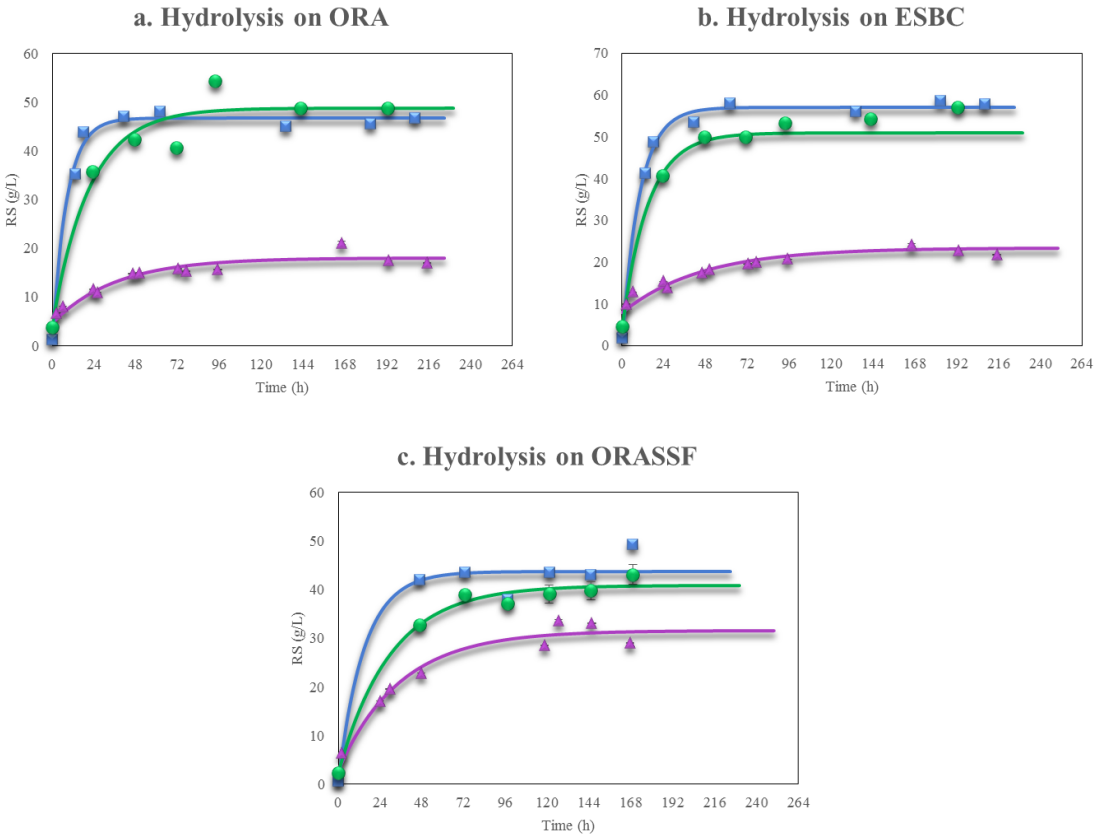
Hydrolysis experiments of different agro-industrial wastes were carried out with the aim to compare the effectiveness of the enzymatic extract produced by SSF on orange peels with a commercial extract prepared with the same activities (cellulase, xylanase, pectinase and β-glucosidase). ORA, ESBC and ORASSF were the agro-industrial wastes hydrolysed in these experiments. The reducing sugar concentrations (g/L) obtained in the medium are shown in Figure 3.

For the three residues, it can be observed an increment of the maximum reducing sugars concentration reached when cellulase was supplemented with exo-PG, xylanase and β-glucosidase. Moreover, this value was higher for the hydrolysis carried out with enzymatic extracts obtained in this work. Also, the hydrolysis rate for the three wastes is higher when cellulase was supplemented with enzymes cocktail obtained in this work.

In the case of ORA, a 100% of hydrolysis yield is obtained when cellulase is supplemented with the enzymatic extract or with commercial enzymes. However, in the case of ESBC and ORASSF, this yield is slightly higher when cellulase is supplemented with the enzymatic extract in comparison with values measured for the commercial extract. Similar results have been found in literature [7], where hydrolysis of ESBC were carried out with commercial enzymes.

Comparing the hydrolysis yields of ORA with those of ORASSF, it can be observed an increment in the final reducing sugars obtained with the second one, when this residue is hydrolysed only with commercial cellulase. This effect could be the result of hemicellulose and pectin degradation by the fungus, leaving cellulose more accessible to the enzymes attack in the hydrolysis process. Nevertheless, this does not happen when cellulase is supplemented with the other enzymes. In this case, reducing sugars measured were lower for ORASSF due to part of hemicellulose and pectin were consumed by fungus, not being available during the hydrolysis process.

Figure 3. Hydrolysis on ORA (a), ESBC (b) and ORASSF (c) with commercial cellulase (triangle), with commercial cellulase supplemented with enzymatic extract obtained by SSF (square) and commercial cellulase supplemented with enzymatic commercial extract (circle).



The estimated kinetic parameters for the hydrolysis process are shown in Table 6.

Table 6. Hydrolysis rate constant (k_h in h^{-1}), maximum reducing sugars concentration (G_f in g/L) and reducing sugars yield (Y_G in %) for different enzymatic cocktails used for ORA, ESBC and ORASSF hydrolysis.

Waste	Enzymatic cocktail	G_f	k_h	Y_G	r^2
ORA	Exp. 1	17.978	0.028	37.0	0.92
	Exp. 2	48.693	0.047	100.1	0.95
	Exp. 3	46.665	0.120	95.9	0.99
ESBC	Exp. 1	23.299	0.022	25.5	0.92
	Exp. 2	50.916	0.067	55.7	1.00
	Exp. 3	57.101	0.098	62.5	0.99
ORASSF	Exp. 1	31.524	0.028	64.8	0.97
	Exp. 2	40.797	0.034	83.9	0.99
	Exp. 3	43.656	0.067	89.8	0.96

4. Conclusions

In the present work, two agro-industrials residues have been used as nutrient source for the production of hydrolytic enzymes. They can be applied in the exploitation of lignocellulosic residues in order to obtain high-added value bio-products such as fuels, chemicals, and so on. Results demonstrated that orange peels and exhausted sugar beet cossettes are good natural media for the growth of *Aspergillus awamori* by SSF and for the production of hydrolytic enzymes. They contain all the principal nutrients required for the fungi metabolism. Moreover, the reduced cost of both materials make them promising raw materials for SSF processes.

The enzymatic extract obtained by SSF on both wastes are composed of a broad range of hydrolytic enzymes, mainly xylanases and pectinases. Although its FPase activity was very low, this enzymatic extract can be used as a supplement cocktail of cellulose for saccharification processes of lignocellulosic material. Thus, the cost of the hydrolysis stage might be significantly reduced.

Enzymatic extract obtained from solid state fermentation of orange peels, provided higher hydrolysis yield of all residues tested in this work than a commercial enzymatic cocktail with the same enzymatic activities.

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