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Agro-food Wastes for the Release of Phyto-Chemicals and the Production of Enzymes by Solid State Fermentation Using *Pleurotus ostreatus*

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The agro-food wastes represent a very important resource to recover chemical compounds with high added value that can be used in several industrial sectors and to the energetic production by biomass. This is a good method to value the agro-food wastes that are often undervalued. The hydrolitic demolition by lignocellulosic enzymes is one of the most studied approach for recovering phyto-chemicals from the cell wall of plants. Enzymes are perfect to this role, because they can be used in blind conditions resulting cheap and profitable.
In this study, the concentration of reducing sugar (glucose) and total polyphenols (catechin), released in solution after the hydrolysis with lignocellulosic enzymes, were determined. The enzymes (extract enzymes) used are previously obtained, by growing the fungus *Pleurotus ostreatus* in solid state fermentations in presence of four separated agro-food wastes: grape stalks, grape seed, wheat straw and wheat bran. For the hydrolysis, the same biomass was used as substrates doing different combinations of substrate and enzymes extract (from different sources).
The enzyme extracts from grapes stalks and wheat bran promoted the release of reducing sugars that were naturally rich in cellulase, pectinase xylanase activities. The wheat bran was the only matrix able to be hydrolyzed by all the enzyme extracts, probably due to the fact that its arabinoxylan structures are hydrolysable from the xylanase activities found in all the enzyme extracts.
The increases on the polyphenols concentration during the hydrolytic reaction in oenological wastes were observed in the matrices rich in polyphenols such as grape seed and grape straw, even if the enzyme from the wheat bran was able to increase the content of polyphenols of 84 % compared to the initial one.

1. Introduction

In Europe, the agricultural wastes represent a significant potential for the development of biorefineries in different sectors. For example, in 2004 the Italian production of grains was about 22,1 million tons, and screw about 9 million tons, producing respectively 11 and 0,9 million tons of by-products but only a small part of these are converted into other products (ISTAT, 2004). The accumulation in large amounts of this type of biomass, every year resulting not only in the deterioration of the environment but also in the loss of material potentially useful for the transformation and the development of numerous products of high added value (Nigam and Pandey, 2009).
New research in biochemistry and biotechnology applications allow us to consider these issues in a new approach, enhancing the waste as a resource that in many cases has a high potential for use, also economically sustainable. The by-products of vegetable origin are rich in phyto-compounds, which have recently found a great interest in the food, nutraceutical, cosmeceutical and pharmaceutical (Di Gioia et al., 2007). Unfortunately, these compounds are difficult to access due to the complex plant matrix.
The plant cell wall consists mainly of polysaccharides and to a lesser extent from glycoproteins, phenolic esters (coumaric and ferulic acid), minerals and enzymes. The main polysaccharide constituting the cell wall are cellulose, hemicelluloses and pectins. The by-products of vegetable origin are characterized by specific phyto-compounds with antioxidant activity called polyphenols, which find a great interest in the market as antioxidant products for human health both as nutraceuticals and cosmeceuticals that as, in perspective, even

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in the pharmaceutical field. Polyphenols exhibit a wide range of beneficial biological properties acting as antioxidants and antiinflammatory that can be exploited for vascular diseases (Casazza et al., 2015) and are also widely studied for its effects in the production of biogas (Battista et al., 2014).
Enzymatic hydrolysis presents a number of advantages over chemical hydrolysis. The main advantages are specificity, control of the hydrolysis degree, mild action conditions (mild temperature) and minimum formation of byproducts, and main disadvantages are the high costs of the enzymatic complex and the need for pretreatment to achieve efficient conversion rates (Clemente, 2000). The enzymes being biological catalysts, are able to degrade this complex plant structure and to reduce the long polymer chains that compose it, releasing the substances of interest related to them. The recovery of substances can be significantly increased by using enzymes for the degradation of plant walls, representing a valid alternative to the traditional procedures (Zuorro et al., 2014). This is a valid alternative to the use of chemicals for the extraction of phyto-compounds which is usually characterized by a high environmental impact.
In this work it was evaluated the possibility of using agro-food by-products (grape stalks, grape seeds, wheat straw and wheat bran), to produce in solid state fermentation enzyme extracts suitable to hydrolyze the same or other vegetable matrices, releasing in solution reducing sugars and polyphenols.

2. Material and methods

2.1 Enzymatic Hydrolysis

The substrate was treated thermomechanically, graining 10 g of substrate and autoclaving at 120 °C for 20 minutes with 70 ml of distilled water (optimal ratio determined in previous studies to maximize the release of the metabolites in these conditions). After the treatment, the hydrolysis was made with substrate, supernatant liquid to the substrate (after the heat treatment) and enzyme extract (produced in solid state fermentation) used in the proportions 1: 5: 2. The hydrolysis was made for 45 hours at 25°C by measuring the reducing sugars and the total polyphenols at 0, 2, 4, 24 and 45 hours from the beginning of the addition of enzyme.

2.2 Solid state fermentation and recovery of the enzyme extract

The solid state fermentation was prepared with 50 grams of dry weight of substrate (wheat bran, wheat straw, and grape seed) and 9.1g of dry weight of grape stalks (50 g wet weight) as such in a Pyrex bottle of 500 ml with a cotton cap were wetted with 50 ml of distilled water and sterilized by autoclaving at 120 °C for 20 minutes. This substrate was inoculated using 9 g of *Pleurotus ostreatus* grown on malt extract agar. In this study a commercial strain of *Pleurotus ostreatus* was purchased from Azienda Agricola Funghi Mara ®, BO.
The fermentation takes place at 25 °C, in absence of light for a period of 28 days and were monitored taking samples every 7 days. The enzyme extract was recovered every 7 days, opening the fermenter in the sterile hood and a specific quantity of water was added. The substrate was put into a screw extruder with cutting blade and extrusion holes of 5 mm diameter. The extract was recovered pressing and filtering the substrate. A new quantity of water was added to bring the humidity necessary for the subsequent fermentation. The enzyme extract (free-water) was centrifuged for 5 minutes at 13,000 rpm to remove the solid fraction (Masutti et al., 2014).

2.3 Reducing sugar

The reducing sugars (glucose) released after the enzyme hydrolyzing was determined using 0.4 ml of sample was added in 0.6 ml of 3,5-dinitrosalicylic acid (DNSA), boiled for 7 minutes in boiling water, the samples were cooled and centrifuged for 5 minutes at 13,000 rpm. The chromophore group created by the reaction between DNSA and reducing sugars, was detected by spectrophotometer at 550 nm against a control done in the same way as samples, but with 4 ml of distilled water and 0.6 ml of DNSA. The method was described by Bailey et al., (1992).

2.4 Total polyphenols

The total polyphenols (catechin) released after the enzyme hydrolyzing was determined following the method described by Folin and Ciocalteau (1927), by spectrophotometer at 700 nm.

3. Results and discussions

The enzyme extract used in this work has been done previously using grape stalks, grape seeds, wheat straw and wheat bran used separately in four solid state fermentations with the extrusion process in the presence of *Pleurotus ostreatus* for the production of hydrolytic enzymes. The extrusion process was used to the enzyme production because improves the diffusion of oxygen, the surface area of the substrate and the permeability of water as the extraction solvent (Masutti et al., 2014).
The enzyme extracts recovered from these fermentations were used on the same matrix to evaluate their potential through hydrolytic release in solution of reducing sugars (glucose) and total polyphenols

(catechins).The hydrolysis was made for 45 hours at 25°C by measuring the reducing sugars and the total polyphenols at 0, 2, 4, 24 and 45 hours from the beginning of the addition of enzyme.
The zero time refers to the measure before the addition of enzyme extract. Both for the calculation of reducing sugars and of polyphenols were summed values calculated separately for the enzyme extract and the treated substrate thermo-mechanically returning them to the volume added. The activities are determined as amount of glucose released normalized unit of volume.
In Table 1, the concentration of the reducing sugars released in solution after enzymatic treatment was maximum by the addition of the enzyme extract derived from the grapes stalks and wheat bran consistently with the value of the assets identified in previous tests of fermentation with *Pleurotus ostreatus*. The matrices treated with enzyme extract coming from the grapes stalks, start from a greater initial quantity of reducing sugars, coming just from the enzyme extract contains a concentration of reducing sugars in higher than the other.
We have observed that the wheat bran the only matrix was hydrolyzed by all the enzyme extracts. This is probably due to the fact that the arabinoxylans structures of wheat bran are easily hydrolyzed by the xylanase activities generally found in all the enzyme extracts tested (Masutti et al., 2014). However, the highest released of reducing sugars on the substrate of wheat bran (640%) was obtained by the action of enzyme extract derived from the fermentation of wheat bran. It is reasonable to think that during the fermentation in solid state on wheat bran to obtain the enzyme extract used, the fungal species has probably produced the enzyme activity most suitable to degrade this substrate, making it ideal for the hydrolysis of wheat bran.
Finally, it can be seen in almost all cases, the production peak is observed at the end of the first drawdown (24 hours after the addition of enzyme extract), this could be due to the fact that as time goes by reducing sugars released, they are consumed and / or degraded in part by external agents, since the hydrolysis was not made in a totally aseptic environment.

Table 1: Concentration of reducing sugar released from the substrates (grape stalks, grape seed, wheat straw and wheat bran) treated with 4 different enzyme extracts, taken at different times of 0, 24 and 45 hours - (Increase or decrease percentage calculated on the greater increase than the initial time).

Substrate	Enzyme extract	Concentration of reducing sugar (µmol/ml) 0 h	24 h	45 h	Increase (%)
Grape stalk	Grape stalk	2.9 E+01	6.7 E+01	6.8 E+01	130
	Grape seed	1.2 E+01	5.6 E+00	5.4 E+00	---
	Wheat straw	1.4 E+01	7.0 E+00	7.1 E+00	---
	Wheat bran	7.1 E+00	1.1 E+01	8.1 E+00	54
Grape seed	Grape stalk	3.6 E+01	8.5 E+01	7.5 E+01	140
	Grape seed	1.9 E+01	1.0 E+01	1.0 E+01	---
	Wheat straw	2.1 E+01	1.6 E+01	1.5 E+01	---
	Wheat bran	1.4 E+01	3.1 E+01	2.5 E+01	120
Wheat straw	Grape stalk	3.0 E+01	7.3 E+01	7.6 E+01	150
	Grape seed	1.3 E+01	8.0 E+00	7.9 E+00	---
	Wheat straw	1.4 E+01	1.0 E+01	9.8 E+00	---
	Wheat bran	7.9 E+00	2.4 E+01	2.1 E+01	210
Wheat bran	Grape stalk	3.2 E+01	7.5 E+01	5.7 E+01	140
	Grape seed	1.5 E+01	2.0 E+01	2.0 E+01	33
	Wheat straw	1.6 E+01	2.5 E+01	2.5 E+01	49
	Wheat bran	9.8 E+00	7.2 E+01	5.8 E+01	640

In Table 2, you can check the increase over time of the concentrations of total polyphenols released into solution, due to the action of hydrolytic enzyme extracts of different origins on the selected plant matrices. They are observed predominantly increments of the concentration with respect to time zero of polyphenols from wine by-products that release quantities most relevant in solution in respect to the other biomass. This is due to both the nature of the vegetable matrices that are rich in polyphenols, which also to the characteristics

of the enzyme extracts that are able to hydrolyze the vegetable structure to release these substances in solution.

By analyzing the substrate of grape seeds, can be observed as the concentration present before adding the enzyme extract is already very high compared to other substrates. The grape seeds release in solution polyphenolic components that are released during the thermo-mechanical treatment. It is however seen as the addition of the extract enzyme from the wheat bran promotes a further production of more than 84 % and instead the other enzyme extracts are not able to increase the release of polyphenols in solution. The enzyme extract produced from grape seeds in solid state fermentation, is found to be the most suitable for hydrolyzing the grape stalks and wheat bran increasing the release of total polyphenols in solution of 3300 % and 210 % respectively; while, a fairly low release in polyphenols was observed in presence of wheat straw and wheat bran. However, It can be noted that the extract enzymatic by fermentation of wheat straw showed a greater effect with respect to the other enzyme extracts tested, increasing by 200 % the initial concentration of released polyphenols, when it was used for hydrolyzing wheat straw.

The results obtained in Table 1 and 2 were resumed in a synoptic Table 3. It then observes that the enzyme extracts which promote the release of reducing sugars are those derived from the fermentation of grape stalks and wheat bran which are naturally rich in cellulase, xylanase and pectinase activities (Masutti et al., 2014). In particular, the enzyme extract of grape stalks was able to release high concentrations of reducing sugars from the grape stalks and grape seeds due to the action of the pectinase while the extract from wheat bran mainly acted on wheat straw and wheat bran being reached of cellulase and xylanase activities. Generally, the enzyme extracts from grape seeds, wheat straw and wheat bran showed the release of higher quantities of phenolic compounds.

The findings put in evidence that the different substrates induce in a different way the amount of enzymes produced. On the basis, the different enzyme extracts presented the optimal conditions for the hydrolysis of the same matrices used by the fungus.

Table 2: Concentration of total polyphenols released from the substrates (grape stalks, grape seeds, wheat straw and wheat bran) treated with 4 different enzyme extracts, taken at different times of 0, 24 and 45 hours - (Increase or decrease percentage calculated on the greater increase than the initial time).

Substrate	Enzyme extract	Concentration of *total polyphenols* (µmol/ml) 0 h	24 h	45 h	Increase (%)
Grape stalk	Grape stalk	3.5 E+03	6.1 E+03	4.4 E+03	73
	Grape seed	1.2 E+02	1.6 E+03	4.1 E+03	3300
	Wheat straw	2.5 E+02	2.3 E+02	8.4 E+02	230
	Wheat bran	3.6 E+02	4.1 E+02	2.6 E+03	610
Grape seed	Grape stalk	6.1 E+03	5.9 E+03	4.9 E+03	---
	Grape seed	2.7 E+03	6.5 E+02	4.5 E+02	---
	Wheat straw	2.8 E+03	1.1 E+03	2.0 E+03	---
	Wheat bran	2.9 E+03	1.9 E+03	5.4 E+03	85
Wheat straw	Grape stalk	4.2 E+03	4.0 E+03	4.7 E+03	12
	Grape seed	7.6 E+02	3.8 E+02	9.5 E+02	25
	Wheat straw	8.9 E+02	1.1 E+03	2.7 E+03	200
	Wheat bran	1.0 E+03	1.6 E+03	8.8 E+02	60
Wheat bran	Grape stalk	3.5 E+03	3.2 E+03	3.5 E+03	---
	Grape seed	1.4 E+02	2.3 E+02	4.1 E+02	210
	Wheat straw	2.7 E+02	5.7 E+01	2.8 E+02	6.2
	Wheat bran	3.7 E+02	6.0 E+02	9.6 E+02	160

The *Pleurotus ostreatus* was demonstrated to induce the production of specific enzymes depending on the substrate used for the growth of the mycelia. All the enzymatic extracts were able to release reducing sugars in solution, in fact, the wheat bran was the only substrate to be always hydrolysed. This phenomena was explained by the presence of xylanase activities which were generally determined in more or less abundant

amounts in all the enzymatic extracts; even if, the corn bran was mostly hydrolyzed by the enzymatic extract produced in a solid state fermentation keeping the wheat bran as a substrate of growth. In this way, reasonable to think that during fermentation, the fungal species has probably produced the enzyme activity most suitable to degrade this substrate.

Table 3: The enzymatic extract most applicable to the hydrolysis of agro-food by-products, determined based on the percentage increase of the concentration of reducing sugars and total polyphenols in solution.

ENZYME EXTRACT	SUBSTRATES			
	Grape stalk	Grape seed	Wheat straw	Wheat bran
Grape stalk	Reducing sugar	Reducing sugar	--------	--------
Grape seed	Polyphenols	--------	--------	Polyphenols
Wheat straw	--------	--------	Polyphenols	--------
Wheat bran	--------	Polyphenols	Reducing sugar	Reducing sugar

4. Conclusions

These claims show an orginal development about the selection of the substrates for the production of specific enzymes by *Pleurotus ostreatus* able to make the demolition of the plants wall cell for the valorisation of the agro-food by-products. The cost of the industrial enzymes is usually a limiting factor for a valorization process. The findings showed that the solid state fermentation fed by the same substrate to be hydrolized could represent an important enhancing for optimize and reduce the cost for the enzyme production.
The release of reducing sugars indicates hydrolysis of vegetable matrices via the enzymatic action of the extracts produce in solid state fermentation using separately the same plant matrices. The polymer chains that make up the complex plant matrix, are degraded in the process releasing in solution different substances of interest such as polyphenols that can be recovered as chemicals with high added value enhancing agro-food by-products and contributing to their eventual disposal. In fact, the recovery of biophenols is also preparatory to subsequent stages fermentative treatment of biomass for the production of biofuels. The microbial biotransformation processes, in fact, are generally inhibited by the presence of polyphenols such as caffeic acid and p-coumaric acid, which can be reduced by enzymatic activity. These features combined with an anti-microbial known phyto-toxicity complicates disposal of these wastes which, therefore, represent a cost for the different companies.

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