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Production of Enzymes from Rice Husks and Wheat Straw in Solid State Fermentation

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In Europe, the agricultural wastes represent a significant potential for the development of biorefineries in different sectors such as cereals. Recovery of phytochemicals as well as the energetic valorization of the plant matrixes needs the demolition of the wall cell plants. Hydrolitic demolition by lignocellulosic enzymes is one of the most studied approach. White rot fungi such as *Pleurotus ostreatus* produce a wide range of extracellular enzymes to degrade complex lignocellulosic substrates into soluble substances that can be used as nutrients.
The objective of this study is to induce the production of lignocellulosic enzymes through the growth *Pleurotus ostreatus* in solid state fermentation using agro-food wastes as substrates: rice husks and wheat straw. The activities of cellulase, xylanase, peroxidase, laccase, and arylesterase are determinate by specific colorimetric assays.
All trials showed a scarce productivity of cellulase while all the detected enzymatic activities resulted higher using rice husk as a substrate than in presence of wheat straw. *Pleurotus ostreatus* exhibited a prevalent production of arylesterase activity and, in particular, the contemporary presence of significant xylanase and feruloilesterase activities was probably due to the typical ferulic bond and diferulic bridge in the heteroxylane structure of monocot's plant cell walls such as rice and wheat. Moreover, in terms of yields arylesterase activities for both substrates, are prevalent on other activities.
Our findings showed that the enzymatic production was strictly dependent to the periodic removal of the produced enzymes. The development of new solid state bioreactor design for a steady state production of enzymes from *Pleurotus ostreatus* could open an interesting industrial approach.

1. Introduction

In Europe, the agricultural wastes represent a significant potential for the development of biorefineries in different sectors such as cereals. The residues from the processing of grains are about 11 million tons/year of dry basis (Di Blasi et al., 1997). Recovery of phytochemicals as well as the energetic valorization of the plant matrixes needs the demolition of the wall cell plants. Over the past 30 years, it has become clear that research on the degradation of lignocellulose by fungi may lead to other industrial applications (Crawford and Crawford, 1980). Hydrolitic demolition by lignocellulosic enzymes is one of the most studied approach. In recent years, Solid state fermentation (SSF) has received more and more interest from researchers, since several studies for enzymes (Pandey et al., 1999), flavours (Ferron et al., 1996), colorants (Johns and Stuart, 1991) and other substances of interest to the food industry have shown that SSF can give higher yields (Tsuchiya et al., 1994) or better product characteristics than submerged fermentation (SmF) (Acuña-Argüelles et al., 1995). Using of agro-industrial residues as substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or not-utilized residues. Solid state fermentation has been defined as the

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fermentation process which involves solid matrix and is carried out in absence or near absence of free water; although, the substrate must possess enough moisture to support growth and metabolism of the microorganism. The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients that allows the development of the microorganisms. Fungi and yeast were termed as suitable microorganisms for SSF according to the theoretical concept of water activity, where as bacteria have been considered unsuitable (Chinn et al., 2007). On one hand, by utilizing the low cost agricultural residues, SSF adds on to economic feasibility of the process (Robinson and Nigam, 2003) and on other hand it solves the problem of its disposal which otherwise cause pollution (Singhania et al., 2009).

Enzymes are among the most important industrial products obtained for human needs by microbial sources; in fact, a large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or the other. Solid state fermentation shows increasing interest for the production of enzymes, specially where the raw fermented waters may be used directly as enzyme sources (Pandey et al., 1999).

White rot fungi such as *Pleurotus ostreatus* produce a wide range of extracellular enzymes to degrade complex lignocellulosic substrates into soluble substances that can be used as nutrients (Giardina et al., 1995; Marzullo et al., 1995). The objective of this study is to induce the production of lignocellulosic enzymes through the growth *Pleurotus ostreatus* in SSF using agro-food wastes as substrates: rice husks and wheat straw.

2. Material and methods

2.1 Solid state fermenter

Thirty grams of rice husks or wheat straw 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 min. This substrate was inoculated using 9 g of *Pleurotus ostreatus* grow on malt extract agar. The fermentation takes place at 25 °C, in absence of light for a period of 28 days. Three different fermenters were monitored taking samples at different frequency of time with different time every 7 days and 14 days as well after 28 days, respectively. About the sampling, fermenter was opened in the sterile hood and a specific quantity of water necessary to maintain free-water was added. The fermented substrate was mixed and all the free-water was recovered by a sterile pipette. The sample was centrifuged for 5 min at 13,000 rpm to remove the solid fraction. The third fermenter was, instead, opened after 14 days for the addition of water to maintain moisture into the substrate.

2.2 Determination of cellulase activities

The cellulase activities were determined following the method described by Poincelot and Day (1972), using functionalized cellulose. The glucose released was detected by spectrophotometer at 595 nm.

2.3 Determination of peroxidase activities

The peroxidase activity was determined following the method described by Setti et al., (1998), using an oxidative coupling reaction of MBTH (3-methyl 2-benzothiazolinone hydrazone) and methoxyphenols at 30 °C producing red-coloured azo-dye compounds detected by spectrophotometer at 502 nm.

2.4 Determination of Laccase activities

The laccase activity was determined following the method described by Setti et al., (1999), using an oxidative coupling reaction between MBTH and methoxyphenols at 30 °C producing red colored azo-dye compounds detected by spectrophotometer at 502 nm.

2.5 Determination of xylanase activities

The xylanase activities was determined as reducing sugars following the method described by Bailey et al., (1992), using ADNS (3,5-dinitrosalicylic acid) at 30 °C, detected by spectrophotometer at 502 nm.

2.6 Determination of arylesterase activities

To determine the arylesterase activity, in a quartz cuvette were added 0.9 mL of buffer, 0.1 mL of methyl ferulate or methyl caffeate and 0.05 mL of the extract. Immediately after adding the extract, is reading a time drive at a spectrophotometer at 335 nm, in which was detected the disappearance of

methyl ferulate or methyl caffeate. The method was modified with respect to that reported by Giuliani et al., (2001).

3. Results and discussions

The SSF with rice husks and wheat straw showed an absence or low cellulose activities while the other enzymatic activities were significantly accumulated over the time (Figure 1 and Figure 2). The total recovered activities in rice husk were higher than that obtained with wheat straw, except for the xylanase activity in which the yield was quite similar (Table 1 and Table 2). Moreover, the yield per dry substrate was higher when the frequency of the sampling was higher every 7 with respect to 14 and 28 days. It is mean that the production of the enzyme seems to be induced when the produced enzyme is frequently removed from the substrate. One can therefore assume a mechanism in equilibrium between the biosynthesis of the enzyme and the free enzyme in solution.
All the trials showed that the concentration of total enzymes activities (produced after 28 days) per ml of free-water (Table 3 and Table 4) was higher in samples with a frequency of 7 days except for peroxidase and xylanase activity of rice husk as well the xylanase and feruloil-esterase of wheat straw.
These higher enzymatic activities were probably affected by both the addition of fresh water and the mixing for the sampling.
The prevalent enzymatic acitivity for both the substrate was arylesterase and, in particular, the feruloil-esterase activity. The structure and composition of cereal cell walls show complex matrixes of polysaccharides (arabinoxylans and heteroxylan) and glicoproteins (Rose, 2003). In the cell wall of cereals, the hydroxycinnamic acids play a structural role in maintaining the integrity of the wall. Ferulic acid is the major cinnamic acid of the walls in the heteroxylane structure of monocot's plant cell walls such as rice and wheat in which the relevant amount of eterified ferulic acid decreases during maturation, with the formation of ester-ether and ester-ester bonds between wall polymers. Knowing the composition of the major hemicelluloses on cereals, xylanases and arylesterases are then required for the efficient breakdown of the polymers as we carried out in our findings (Faulds et al., 2003).

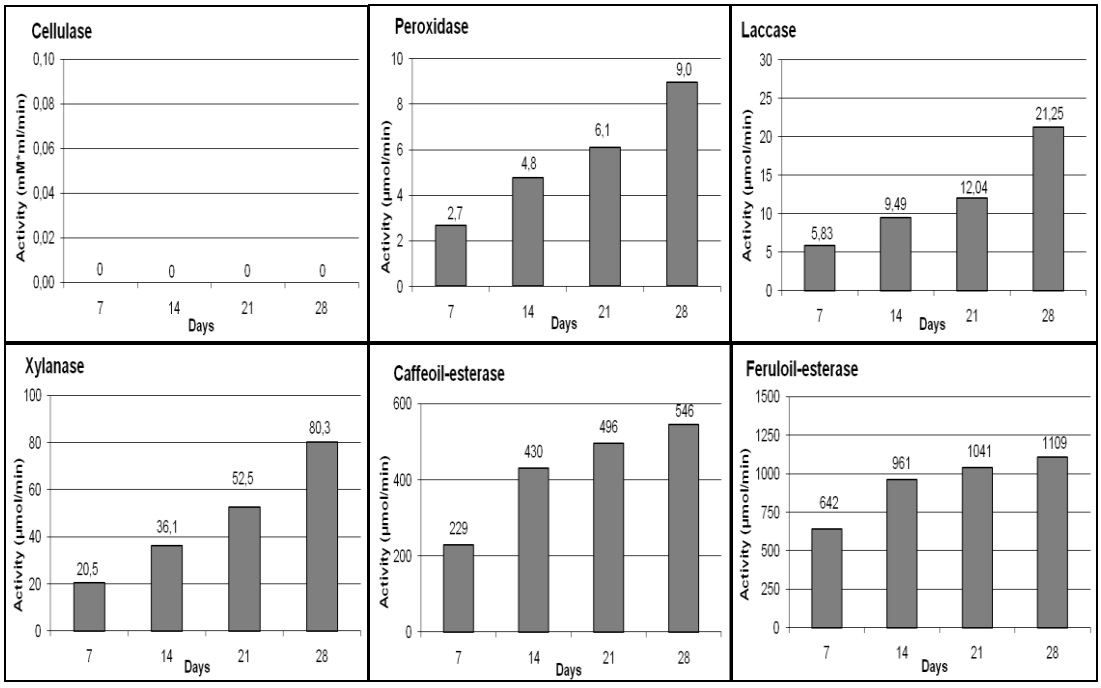

Figure 1: Rice husks – Total activity of the water waste collected every 7 days

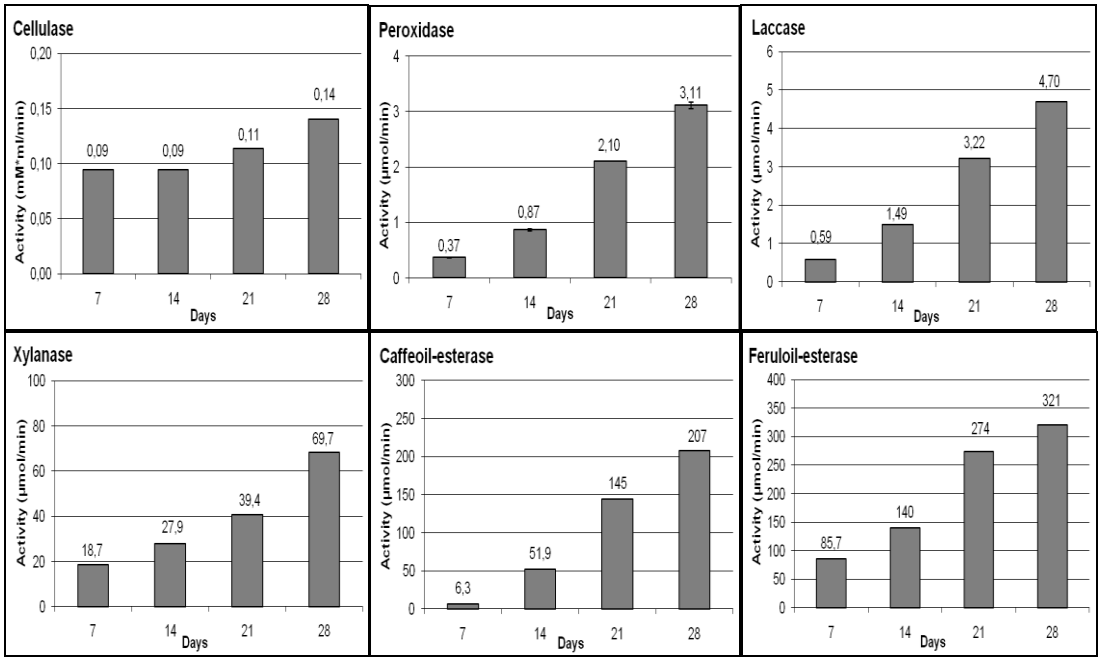

Figure 2: Wheat straw– Total activity of the water waste collected every 7 days

Table 1: Rice husks - Yield per gram of dry weight

Enzymatic activity	Every 7 d	Every 14 d	After 28 d
Cellulase (mM·mL/min)	--	--	--
Laccase (µmol/min)	0.3	0.1	0.1
Peroxidase (µmol/min)	0.7	0.2	0.4
Xylanase (µmol/min)	2.7	1.1	1.1
Caffeoil-esterase (µmol/min)	18.2	1.7	1.9
Feruloil-esterase (µmol/min)	37	2.5	2.9

Table 2: Wheat straw - Yield per gram of dry weight

Enzymatic activity	Every 7 d	Every 14 d	After 28 d
Cellulase (mM·mL/min)	--	--	--
Laccase (µmol/min)	0.1	0.03	--
Peroxidase (µmol/min)	0.16	0.05	0.01
Xylanase (µmol/min)	2.32	0.59	0.27
Caffeoil-esterase (µmol/min)	6.91	2.40	0.22
Feruloil-esterase (µmol/min)	10.7	5.45	0.20

Table 3: Rice husks - Activity per ml of water waste

Enzymatic activity	Every 7 d	Every 14 d	After 28 d
Cellulase (mM·mL/min)	--	--	--
Laccase (µmol/min)	0.18	0.17	0.12
Peroxidase (µmol/min)	0.43	0.50	0.35
Xylanase (µmol/min)	1.64	2.27	1.1
Caffeoil-esterase (µmol/min)	11.1	3.7	1.89
Feruloil-esterase (µmol/min)	22.6	5.4	2.94

Table 4: Wheat straw - Activity per ml of water waste

Enzymatic activity	Every 7 d	Every 14 d	After 28 d
Cellulase (mM·mL/min)	--	--	--
Laccase (µmol/min)	0.09	0.06	0.03
Peroxidase (µmol/min)	0.13	0.09	0.07
Xylanase (µmol/min)	1.99	1.11	1.99
Caffeoil-esterase (µmol/min)	5.92	4.50	1.68
Feruloil-esterase (µmol/min)	9.16	10.2	1.47

4. Conclusions

The enzyme production by *Pleurotus ostreatus* in SSF showed a significant dependence on the frequency of sampling. This has allowed the identification of a phenomenon is inversely proportional to the production of enzyme concentration in the free-water waste.
The design of the bioreactor undoubtedly plays a key role in enzyme production as well as the oxygenation of the substrate by the mechanical process of mixing for the recovery of the added water for the sampling.
The development of an industrial bioreactor must take into account the effects of oxygenation of the matrix, the additional of solvent and the mechanical actions on the same.
A continuous process of solid state fermenter is a complex application, however, further improvements could lead to increased enzyme production increasing the frequency of sampling to approach the maximum potential production.

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