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Effect of Carbon Sources on The Extracellular Lignocellulolytic Enzymetic System of *Pleurotus Sajor-Caju*

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Abstract

The edible fungus *Pleurotus sajor-caju* was investigated for its ability to grow on different carbon sources and to produce various ligninolytic and cellulolytic enzymes such as laccase, lignin peroxidase, manganese peroxidase, xylanase and cellulase. The production pattern of these extracellular enzymes was studied during the growth of this fungus in shake cultures for a period of 20 days. The presence of complex polysaccharides containing substrates such as olive mill wastewater (OMW) and wheat straw in the growth medium were found to be favorable for the production of the above extracellular enzymes. Simple carbon sources were rapidly utilized and consumed by the fungus which lead to high biomass formation with low levels of enzymes activity. On the other hand, complex carbon sources exhibited a similar growth rates and a better induction process for more enzyme biosynthesis and production. The maximum enzymatic activities were obtained between 2 and 14 days of culture growth.

الملخص

تم دراسة امكانية انماء الفطر الصالح للاكل من نوع Pleurotus sajor-cajuعلى انواع مختلفة من المصادر الكربونية لانتاج انزيمات متخصصة بتحطيم مادتي السيليولوز واللقنين. كما دراسةعملية انتاج هذه الانزيمات اثناء نمو الفطر في اوساط مخفوقةبشكل متواصل لمدة 20يوم. اظهرت النتائج ان الاوساط المحتوية على سكريات معقدة مثل المياة العادمة للزيتون ودقيق قشر القمح كانت الافضل لانتاج الانزيمات المختلفة. ان استخدام السكريات البسيطة ادى الى استهلاكها السريع من قبل الفطر الذي ادى بالنهاية لانتاج كمية اكبر من الكتلة الحيوية و كميات اقل من الانزيمات. بالمقابل ان استخدام المواد الكربونية المعقدة اظهر نموا مشابها وعملية تحفيزافضل لانتاج الانزيمات. ان اعلى فعالية للانزيمات كانت قد سجلت اثناء الفترة ما بين 2-14 يوما من نمو الفطر.

Keywords: *P. sajor-caju*, lignocellulolytic enzymes, carbon source, shake culture.

1. Introduction

A large fraction of the carbon fixed by photosynthesis each year is deposited as lignocellulose that forms the structural frame work of higher plants and would therefore, forms the renewable resource since in a balanced system the biomass removed for substrate usage can be replaced by replanting and reforestation (Batt, 1991). As population increases, traditional agriculture will be hard-pressed to meet the demand for food and more efficient use of all the carbohydrates available are required (Mitchell *et al*., 2000). With the currently available agricultural waste materials there would be sufficient biomass as feedstock to processes for fuel and food production in biotechnological processes. Biodegradation of these wastes into useful products is an example of biotechnological process that not only uses lignocellulosics as energy feed stock but is also associated in pollution abatement (Wan Mohtar *et al*., 2003).

lignocellulosic materials are degraded by a few number of microorganisms. White rot fungi are considered to be the most promising group of microorganisms that degrade this complex structure (Lechner and Paptinutti, 2006). *Pleurotus sajor caju* has been extensively studied because of its powerful ligninolytic enzymes production (Massadeh and Modallal, 2008; Hameed *et al*. 2005). Generally white rot fungi are so important because they produce extracellular polyphenol oxidases particularly lignin peroxidases, manganese peroxidases and laccases which are highly effective in degrading lignin (Revankar and Lele, 2006). Studies demonstrate that under certain conditions laccase and manganese peroxidases are able to oxidize both the phenolic and non-phenolic substrates (Cabaleiro *et al*., 2006). These enzymes are synthesized during primary and secondary metabolism in response to nitrogen, carbon or sulphur limitation (Cabaleiro and Couto, 2007). Several environmental conditions, such as high oxygen tension, culture age and medium composition,

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were also reported to affect the profiles of its isoenzymes and the level of its activity (Dosoretz *et al.*, 2007). In the present study, *P. sajor caju* has been selected to be used as the source for lignin-degrading enzymes and the fermentation behavior for the enzyme production by this fungus was examined. This paper describes our findings on the influence of various carbon sources on the lignin degrading enzymes production by *P. sajor caju.*

2. Materials and Methods

2.1. Maintenance of P. sajor-caju Culture

A subculture of *P. sajor-caju* was obtained from Plant Pathology and Mycology Research Laboratory at the Faculty of Agriculture/Jordan University of Science and Technology/ Jordan. The fungus was maintained on potato dextrose agar (PDA) Petri plates (9 cm in diameter) and stored at 4 °C till use.

2.2. Medium composition and culture conditions

Shake flask cultures of *P. sajor-caju* were performed at room temperature 28 °C (±2 °C) with continuous agitation at 150 rpm in 250 ml Erlenmeyer flask containing 50 ml medium. The medium employed for fungal growth and metabolism consisted of (g l^{-1}): $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2; $CaCl_2$, 0.3; $MgSO_4$, 0.3; $FeSO_4.7H_2O$, 0.005; $MnSO_4.7H_2O$, 0.0016; $ZnSO_4.7H_2O$, 0.0014; $CoCl_2$, 0.002; protease peptone, 0.75 and Tween 80, 1; with a final pH of 5.5 (Sternberg, 1976). The carbon sources listed in Table 1 were used at a final concentration of 1% (w/v) except for the olive mill wastewater (OMW) that was used at a final concentration of 5% v/v. Olive mill Wastewater used in this study was collected from a three-phase centrifugal olive mill located around Al-Hashimiyah area (Zarqa, Jordan). Wheat straw was chopped and then milled to 1-2 mm particle size. The flasks were then inoculated by a mycelium plug (radius of 10 mm and 2 mm in thickness) cut at the advancing edge of *P. sajor-caju* grown on the solid cultures media. Samples from duplicate flasks were taken periodically, centrifuged at 8000 × g for 20 min at 4°C. The clear supernatant was used for determination of enzyme activity.

2.3. Analytical methods

Laccase activity was determined at pH 5 by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 469 nm for 2 min. 100 μl sample was added to 890 μl of a solution containing 50 mM sodium malonate and 1 mM 2,6-DMP (pH 4.5) in a 1 ml cuvette. The enzyme activity was expressed as units/ml, where 1 unit was defined as 1 mmol of substrate oxidized per minute (Munoz *et al.*, 1997). Lignin peroxidase (Lip) activity was assayed using a method described by Tien and Kirk (1984). The assay mixture contained 2 mM veratryl alcohol, 0.4 Mm H_2O_2 in 50 mM sodium tartrate buffer (pH 6.8) and 0.2 ml of filtered supernatant. Veratryl alcohol oxidation was followed at 310 nm. Manganese peroxidase activity (MnP) was determined according to Giardina *et al.* (2000). The assay mixture contained 0.5 mM $MnSO_4$, 0.5 mM H_2O_2 in 50mM sodium malonate buffer (pH 4.5) and 0.2 ml of crude enzyme preparation. All enzyme activities were expressed in Unit/L. The activity of the extracellular cellulase (endoglucanase) was assayed as described by Mandels *et al*. (1976). Xylanase enzyme activity was detrmined according to the method of Miller *et al*. (1959). The Fungal growth was determined by measuring the dry weight content of mycelia collected at the end of each experiment after media filtration. Dissolved protein concentration was estimated by using Lowry method (Lowry *et al*. 1951).

3. Results and Discussion

Table 1 shows the effect of various carbon sources on the growth and the production of laccase, LiP, and MnP. Based on their structure, the carbon sources were divided into five groups. The fungus grew rapidly in the medium containing monosaccharides, disaccharides, polysaccharides and complex polysaccharides. Nevertheless, low or no growth was observed in the culture supplemented with phenolic compounds which subsequently resulted in lower enzyme production. Stoilova *et al*. (2006) reported that, phenolic groups lowered the growth of different types of microorganisms. Nevertheless, some phenolic compounds stimulated the production of lignin degrading enzymes.

Table 1: Lignin peroxidase (LiP), Manganese peroxidase (MnP), and Laccase enzymes production by *P. sajor-caju* when grown on various carbon sources.

Carbon sources (1% w/v)	Growth (g/1)	LiP (U/l)	MnP (U/l)	Laccase (U/l)
Monosaccharides				
Glucose	4.1	65	81	ND*
Xylose	3.7	23	29	ND
Disaccharides				
Lactose	2.8	ND	ND	ND
Sucrose	3.1	22	11	ND
Maltose	3.4	26	18	ND
Polysaccharides				
α-cellulose	2.4	31	28	10
Carboxymethyl cellulose	2.5	33	31	18
Soluble starch	2.5	12	21	7
Xylan	2.6	10	18	ND
Lignin indulin	3.1	160	155	120
Complex Polysaccharides				
Wheat straw	4.1	145	158	112
OMW**	3.9	165	170	131
Phenolic compounds				
Phenol	1.1	12	9	21
2,6 Dimethoxyphenol	1.3	20	9	24
Dichlorophenol	ND	ND	ND	20
Dinitrophenol	0.8	8	ND	10
Syringic acid	1.4	22	18	31

* not detected. ** OMW was used at a percentage of 5%.

After 10 days of incubation, the growth of *P. sajor-caju* was similar when utilizing monosaccharides, disaccharides and polysaccharides as a carbon source. However, better growth was observed in the medium supplemented with complex polysaccharides such as OMW or wheat straw. Eventhough fungal growth was satisfactory, monosaccharides and disaccharides showed no significant effect on the biosynthesis of enzymes. The rate of utilization of complex polysaccharides by the fungus was constant, eliminating the feedback inhibition by the products or other intermediates on the fungal growth. The growth and the activity of LiP and MnP were almost similar using either wheat straw or OMW as substrates. However, the production of laccase enzyme was found to be higher in a culture containing OMW than in culture containing wheat straw as a carbon source. Massadeh and Modallal (2008) claimed that OMW was a suitable substrate for the growth of *P.sajor-caju* and its contents induced laccase enzyme production which in turn reduced phenolic compounds present in the wastewater. Nevertheless, it was observed that LiP and MnP were produced by *P. sajor-caju* in the growth medium containing glucose although lignin was not present in the culture medium. However, in the medium containing lignin, significant amounts of LiP, MnP and laccase were produced. The existence of a ligininolytic enzyme system that is synthesized irrespective of the presence of lignin or lingo-cellulose suggests that the ligininolytic system of *P. sajor-caju* may be relatively non-specific.

The time course profile of LiP, MnP, and laccase production was examined in the culture medium containing glucose, lignin, and OMW as substrates (Figure 1). As indicated in this figure, LiP was produced significantly during the first stages of fungal growth on whatever substrate. LiP activity was detected initially on the second day of incubation and increased rapidly to reach a maximum activity after 4 days of incubation. MnP was detected from the first day of incubation and increased slowly, achieving maximum production after 7 days of incubation. At this stage, the production of LiP was declining while laccase enzyme production started a bit later after 7 days of incubation. The maximum laccase activity was observed in all cultures after 12 days of incubation except for glucose supplemented cultures where it was not detected. This result is in agreement with the findings of Tsioulpas *et al*. (2002) and Massadeh and Modallal (2008) where they reported late production of laccase enzyme by *Pleurotus* sp. This result is further supported by the data of fungal growth where it was observed that the *P. sajor-caju* entered stationary phase after 13 days of incubation (except for glucose supplemented cultures) which coincided with the maximum activity of laccase enzyme. These results suggest that the production of these enzymes was necessary for fungal growth survival along its growth phases. When glucose was used as the carbon source, it allowed higher growth rate compared to other substrates, but it ceased early (after 8 days of incubation). Laccase enzyme was not detected in this culture indicating that *P. sajor-caju* did not need this enzyme for its survival.

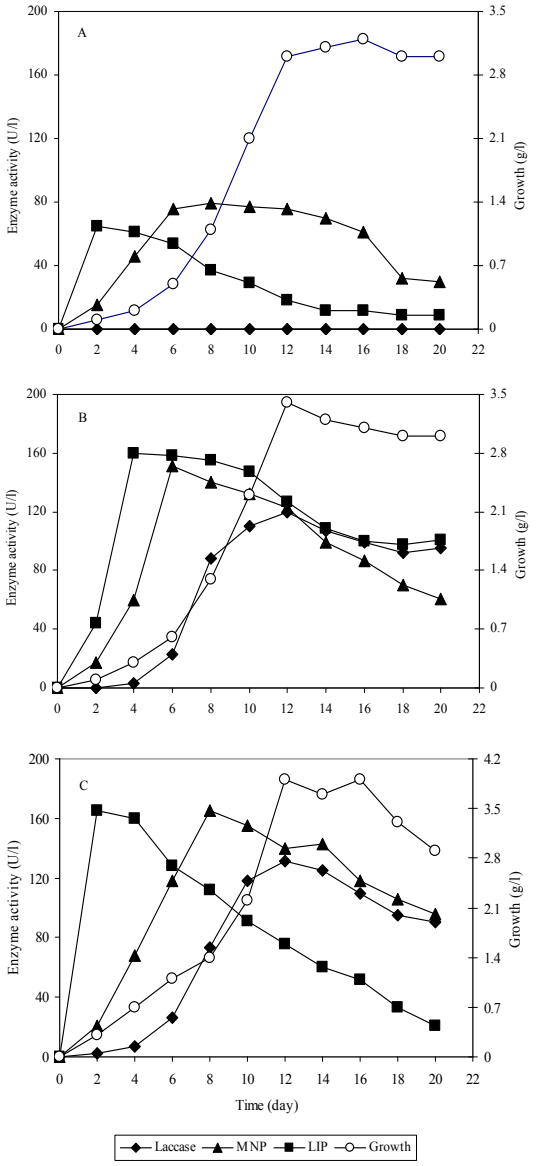

Figure 1: Extracellular ligninolytic enzymes production during the growth of *P. sajor-caju* on (A) glucose, (B) Lignin, and (C) OMW.

The cultures of *P. sajor-caju* grown on wheat straw and OMW as substrates were further analyzed for other extracellular enzymes (Table 2). Cellulase and xylanase enzymes were detected significantly in both cultures employing wheat straw or OMW as substrates which could be due to the high content of cellulosic and hemicellulosic material present in these substrates. Thus, the use of lignin indulin as the substrate was only necessary to enhance the production of LiP, MnP, and laccase while cellulase and xylanase were not detected. These results indicated that the lignin-degrading enzymes are inducible enzymes, and their production is dependent on the varying concentration of the accompanying materials within the lignocellulosic substrates. Therefore, it was clearly shown that the activity of the lignocellulolytic enzymes varied significantly depending upon the proportion of lignocellulolsic materials present in wheat straw, OMW, and lignin indulin.

In conclusion, the production of lignin-degrading enzymes by *P. sajor-caju* depends upon the available substrate (inducer) in the production medium, although it is shown that there were no substrate specificities for enzyme production except the presence of the right inducible carbon source. Complex lignocellulosic substrates such as wheat straw and OMW exhibited similar growth rate, which subsequently resulted in a high production of extracellular enzymes.

Table 2: Effect of complex carbon sources on cellulase and xylanase enzymes production by *P. sajor-caju*.

Carbon source	Dissolved protein (mg/ml)	Xylanase (U/ml)	Cellulase (U/ml)
Lignin	0.6	0.1	ND*
Xylan	0.8	9.1	0.1
α-Cellulose	1.1	2.3	2.7
Wheat Straw	1.3	9.3	1.8
OMW	1.1	9.8	2.2

* not detected.

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