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THE USE OF SPECTRAL MAPPING FOR THE STUDY OF THE ENZYME PRODUCTION OF THE EDIBLE MUSHROOM *PLEUROTUS OSTREATUS*

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The effect of the addition of extracts of agro-industrial wastes to the culture media on the production of β-glucosidase, xylanase, laccase, manganese-dependent and independent peroxidases by the edible fungus *Pleurotus ostreatus* was determined. The relationship between cultivation parameters and the enzyme activities was assessed by spectral mapping technique combined with non-linear mapping. It was proved that extracts enhanced markedly the activities of laccase, manganese-dependent and independent peroxidases. Multivariate mathematical-statistical methods indicated that the enzyme activities were the highest in culture containing pepper extract. It was further demonstrated that the selectivity of the enzyme production was negligible up till 14 days of fermentation and reached the maximum at the 28th day.

Keywords: agro-industrial wastes, enzyme production, *Pleurotus ostreatus*, spectral mapping

Besides their traditional role as energy sources, oligo- and polysaccharides may exert beneficial effect on human health by stimulating specifically the intestinal flora and the digestive system. Edible mushrooms are potential sources of this class of nutrients. Furthermore, edible mushrooms produce polysaccharides with antitumor activity (CHIHARA et al., 1969; MINATO et al., 1999). The antitumor agent was purified and identified as lentinan (CHIHARA et al., 1970) and it has been established that lentinan consists of β-1,3-linked-D-glucan units with β-1,6-branchings (SAITO et al., 1979).

The concept of the application of excess biomass or wastes from agricultural and agroindustrial residues to produce foods is not necessarily new. Because of their capacity to degrade the insoluble components of wood and other wastes such as lignin and lignocellulosic substrates, white rot fungi became of considerable biotechnological interest (EVANS et al., 1994; SCHOBER & TROSCH, 2000). They synthetise a large set of extracellular enzymes both hydrolases and oxigenases which degrade insoluble substrates into soluble compounds of low molecular mass. Mushrooms uptake these compounds and use them as nutrients (BOURBONNAIS & PAICE, 1990; FERRAZ et al., 2000). The various aspects of the enzyme production of *Lentinula edodes* (MORAIS et

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al., 2001a; MORAIS et al., 2001b) and *Pleurotus ostreatus* (MORAIS et al., 2001c; MORAIS et al., 2002) have been recently investigated.

The objectives of the study were the measurement of the activity of laccase, manganese dependent (MnP) and independent peroxidases (MnIP) produced by *Pl. ostreatus* in liquid cultures containing extracts of lignocellulosic agro-residues, and the separation of the strength and selectivity of the effect of the various extracts on the enzyme production by spectral mapping technique (SPM) (LEWI, 1976; LEWI, 1989) followed by non-linear mapping (NMAP) (SAMMON, 1969).

1. Materials and methods

The strain of *Pl. ostreatus* was taken from the collection of the National Agronomical Station (Oeiras, Portugal). It was maintained on potato dextrose agar at 4 °C. Basic medium contained 2 g of ammonium tartarate, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $KCl \cdot 2H_2O$, 0.1 mg of $Na_2B_4O_7$, 0.07 mg of $ZnSO_4 \cdot 7H_2O$, 0.01 mg of $CuSO_4 \cdot 5H_2O$, 0.01 mg of $MnSO_4 \cdot 4H_2O$ and 0.01 mg of $(NH_4)_6MoO_2 \cdot 4H_2O$ per liter. Control contained 10 g of glucose and 1 g of yeast extract in one liter basic medium. Glucose and yeast extract were substituted by the extracts of agro-industrial wastes in the other culture media. The agro-industrial wastes were used to study the effect of complex compounds such as polysaccharides and polyphenols on the production of enzymes involved in the degradation of lignin. The extracts of dry industrial residues of the production of red pepper (*Capsicum annuum*, dry matter 85.7%), potato (91.2%) and tomato (90.3%) were used in the experiments. Extracts were prepared by cutting the raw materials into small pieces (approximately 4 mesh) and mixing with the appropriate amount of tap water (pepper, 350 g l^{-1}; potato 560 g l^{-1}; tomato (345 g l^{-1}). The suspensions were let to stay for 24 h at 18±2 °C without stirring then were filtered with Whatman No. 1 filter paper and centrifuged at 20,000 *g* for 20 min. Media were prepared by adding 15 ml of pepper (total and reducing sugar content 279.2 and 51.2 mg ml^{-1}, respectively), 15 ml of potato (total and reducing sugar content 235.6 and 11.9 mg ml^{-1}, respectively) and 8 ml of tomato extract (total and reducing sugar content 213.9 and 74.9 mg ml^{-1}, respectively) to 985, 985 and 992 ml of basic media. Conditions of fermentation and the determination of enzyme activites have been previously described (MORAIS et al., 2004).

The stationary cultures were incubated at 24±2 °C in the dark for 32 days without artificial aeration. Each cultivation was run in triplicate. Samples were taken from the culture medium under sterile conditions at day 0, 3.5, 7.0, 10.5, 14, 17.5, 21.0, 24.5, 28.0 and 31.5 of cultivation and were centrifuged at 20,000 *g* for 20 min, and the supernatant was employed for the analyses. Besides enzyme activities the concentrations of total phenolics, total and reducing sugars and pH were also determined. As the enzyme activities were expressed in unit activity per ml, the biomass of mycelia was not determined.

The effect of fermentation days and the addition of agro-industrial wastes on the pH and nutritive value of the media were elucidated by stepwise regression analysis (SRA) (MAGER, 1982).

In order to extract maximal information from the original data SPM was carried out twice using the original data matrix (SPM1) and its transverse (SPM2). The dimensionality of SPM selectivity maps has been reduced to two by NLMAP. SRA was applied for the elucidation of the relationship between the strength and selectivity of the overall enzyme production and the activity of the individual enzymes and sampling times.

2. Results and discussion

The differences between the replicate determinations of pH and the spectrophotometric measurements were low with the coefficient of variation being 1.2–1.7% and 1.9–2.6% for pH and spectrophotometry, respectively. However, the enzyme production showed considerable deviations between the replicate fermentations; the coefficient of variation reached even 8%.

The results indicate that both pH and the concentration of nutrients show marked dependence on the time of fermentation and on the composition of media (Table 1). The cultures became more alkaline during the fermentation process which may influence the enzyme production. The quantity of both total and reducing sugars markedly decreased during the fermentation indicating that not only reducing but also other sugar species can be used by *Pl. ostreatus*. SRA found significant relationships between the dependent and independent variables in each instance (Table 2). This finding indicates that other parameters not included in the calculations may influence the relationships. The activity of enzymes generally increases with increasing fermentation time (Table 3). It was also established that the composition of culture media exerts a considerable influence on the activity of the enzymes with the effect markedly depending on the type of enzyme, too. The potency values clearly demonstrate that the enzyme activities are monotonously growing during the fermentation. The differences between the potency values of laccase, MnP and MnIp are relatively low indicating that *Pl. ostreatus* produces these enzymes in commensurable amount. The distribution of points on the two-dimensional non-linear spectral map calculated from the original data matrix demonstrates that the selectivity of enzyme production is very low in the first 14 days of fermentation (corresponding points are very near to each other in cluster A in Fig. 1). The highest differences in the selectivity of laccase, MnP and MnIP activities were observed at the 28th day (points 8, 17 and 26); the point corresponding to MnIP deviating most markedly from the others. This finding suggests that the composition of culture media has the highest effect on the selectivity of MnIP production. The activity of β-glucosidase and xylanase production did not show marked selectivity during the whole fermentation process.

Table 1. pH and the concentration of nutrients in the culture media at various fermentation times

pH	Total phenolic ($\mu g\ ml^{-1}$)	Total sugar ($\mu g\ ml^{-1}$)	Reducing sugars ($\mu g\ ml^{-1}$)	Potato	Control	Pepper	Tomato	Days
4.41	15.14	3534	178	1	0	0	0	0
4.52	1.73	10097	10125	0	1	0	0	0
4.43	54.13	4188	768	0	0	1	0	0
4.57	37.98	9030	599	0	0	0	1	0
4.64	7.85	3207	131	1	0	0	0	3.5
4.68	14.33	10117	9202	0	1	0	0	3.5
4.70	47.17	2941	707	0	0	1	0	3.5
4.61	29.89	4277	565	0	0	1	0	3.5
4.59	11.31	1040	21	1	0	0	0	7.0
4.65	30.32	9528	7549	0	1	0	0	7.0
4.67	53.64	1366	523	0	0	1	0	7.0
4.68	33.56	1711	471	0	0	0	1	7.0
4.73	5.69	850	5	1	0	0	0	10.5
4.63	28.81	8363	6853	0	1	0	0	10.5
4.93	42.20	1034	440	0	0	1	0	10.5
4.98	23.19	998	255	0	0	0	1	10.5
4.87	8.72	621	4	1	0	0	0	14.0
4.61	20.60	8010	6546	0	1	0	0	14.0
4.76	34.86	994	433	0	0	1	0	14.0
5.36	34.42	940	249	0	0	0	1	14.0
5.23	56.89	266	4	1	0	0	0	17.5
4.64	66.62	7614	6291	0	1	0	0	17.5
4.86	126.02	958	303	0	0	1	0	17.5
5.88	56.35	912	248	0	0	0	1	17.5
5.26	69.32	244	1	1	0	0	0	21.0
4.92	59.60	7504	5870	0	1	0	0	21.0
5.18	131.42	798	252	0	0	1	0	21.0
6.08	76.34	869	225	0	0	0	1	21.0
5.48	45.55	221	2	1	0	0	0	24.5
4.59	93.62	6778	5714	0	1	0	0	24.5
7.46	110.90	691	236	0	0	1	0	24.5
8.01	89.84	802	179	0	0	0	1	24.5
5.58	69.32	212	1	1	0	0	0	28.0
4.56	171.93	6359	5709	0	1	0	0	28.0
8.31	218.91	602	184	0	0	1	0	28.0
8.15	252.93	718	175	0	0	0	1	28.0
6.05	95.78	192	1	1	0	0	0	31.5
4.22	104.96	5105	3798	0	1	0	0	31.5
8.46	119.54	459	113	0	0	1	0	31.5
8.55	72.02	483	97	0	0	0	1	31.5

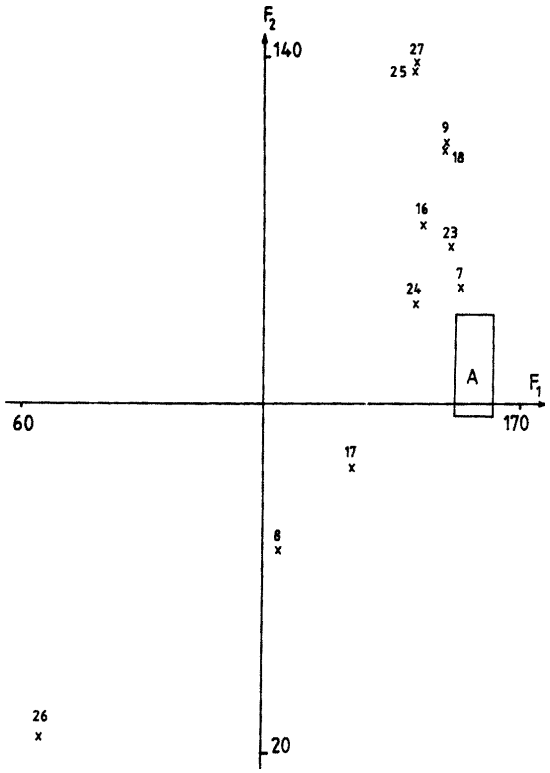

Fig. 1. Selectivity of the enzyme production at various sampling times taking into consideration simultaneously each composition of culture media. Two-dimensional non-linear selectivity map. Number of iterations: 347; maximal error: $8.60.10^{-4}$. Points refer to enzyme activities in Table 1. All other points are concentrated in cluster A

The potency values characterising the culture media indicate that the lowest enzyme production was found in the control (181.65) followed by cultures containing potato (1015.86), tomato (1772.25) and pepper extracts (2280.80). This result proves that the addition of various agro-industrial wastes to the culture media considerably enhances the enzyme production. The data in the two-dimensional non-linear spectral map of culture media illustrate that the selectivity of culture containing potato extract is similar to that of control, while the selectivities of culture with pepper and tomato extracts deviate markedly (Fig. 2).

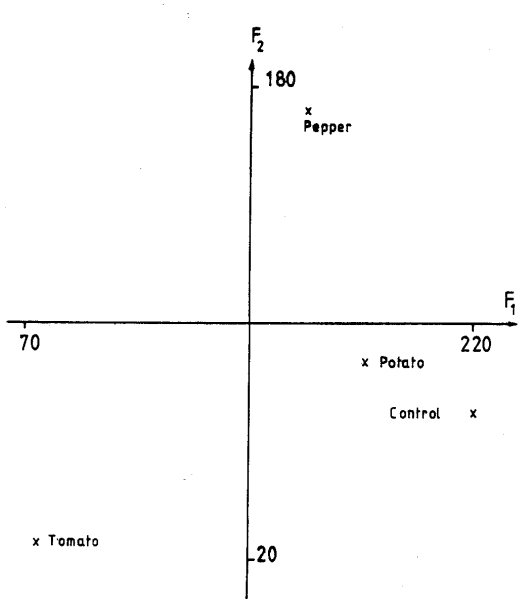

Fig. 2. Selectivity of the enzyme production in various culture media taking into consideration simultaneously each enzyme and each sampling time. Number of iterations: 61; maximal error: $5.29.10^{-4}$

Table 2. Parameters of significant relationships between the pH and concentration of nutrients and the fermentation time (days), and composition of media. Results of stepwise regression analysis (n=40)

Parameters	Equation I	Equation II	Equation III	Equation IV
a	4.69	–2.28	1505	689
b_1	–0.85	37.13	21000	6517
s_{b1}	0.32	13.85	1317	319
b_2	–1.33	3.74	–	–30.0
s_{b2}	0.32	0.60	–	13.7
b_3	0.08	–	–	–
s_{b3}	0.01	–	–	–
b_1' (%)	21.11	29.91	–	90.93
b_2' (%)	33.17	70.09	–	9.07
b_3' (%)	45.7	–	–	–
r^2 (%)	61.46	55.75	86.99	91.94
$F_{calc.}$	19.14	23.31	254.06	211.12
$F_{99.9\%}$	7.05	8.77	12.61	8.77

I: pH = $a+b_1$.Potato extract $+b_2$.Control $+b_3$.days
II: Concentration of total phenolics = $a+b_1$.Pepper extract $+b_2$.days
III: Concentration of total sugars = $a+b_1$.Control
IV: Concentration of reducing sugars = $a+b_1$.Control $+b_2$.days

Table 3. Enzyme activities of *Pleurotus ostreatus* grown on various cultures (nanomol min^{-1} ml^{-1})

No.	Enzyme	Sampling time (day)	Control	Composition of culture		
				Potato extract	Pepper extract	Tomato extract
1	Laccase	3.5	0.2	3.3	5.1	3.0
2		7.0	1.0	5.4	28.6	16.9
3		10.5	1.7	18.7	45.4	29.5
4		14.0	2.6	40.3	75.2	29.8
5		17.5	3.3	61.4	88.1	85.5
6		21.0	5.6	165.1	237.7	117.9
7		24.5	9.1	232.3	248.6	137.9
8		28.0	9.3	323.0	685.9	367.7
9		31.5	32.7	335.7	1694.8	2094.0
10	Mnp	3.5	0.5	5.2	8.9	6.3
11		7.0	1.5	17.1	32.5	22.0
12		10.5	3.1	40.0	55.0	28.6
13		14.0	5.9	56.4	60.0	34.2
14		17.5	7.0	278.2	87.1	162.1
15		21.0	8.7	353.2	297.6	200.8
16		24.5	12.0	502.7	379.0	220.2
17		28.0	24.2	535.0	1319.9	387.1
18		31.5	57.0	2250	1747.3	1470.4
19	MnIP	3.5	0.1	3.2	4.2	3.6
20		7.0	0.6	10.9	17.4	13.0
21		10.5	1.6	18.6	22.5	12.6
22		14.0	1.7	20.9	23.5	12.9
23		17.5	2.9	373.6	326.4	172.8
24		21.0	6.9	416.7	789.2	241.1
25		24.5	9.6	419.1	1020.6	266.4
26		28.0	13.4	649.0	2188.9	273.1
27		31.0	14.7	777.6	2306.5	4389.9
28	β-glucosidase	3.5	0.0	0.0	0.0	0.0
29		7.0	13.8	15.2	18.7	15.9
30		10.5	13.5	18.7	21.9	14.9
31		14.0	15.6	23.3	34.1	18.0
32		17.5	47.4	123.8	98.4	108.1
33		21.0	68.7	162.3	131.7	123.4
34		24.5	95.8	185.9	134.3	147.8
35		28.0	100.2	186.8	442.1	166.6
36		31.5	104.6	200.3	593.4	444.7
37	Xylanase	3.5	0.0	0.2	0.0	0.1
38		7.0	2.5	0.4	0.6	1.1
39		10.5	38.6	0.4	0.8	1.3
40		14.0	53.6	0.6	0.9	2.4
41		17.5	58.9	0.8	2.2	5.7
42		21.0	73.2	1.2	3.5	6.6
43		24.5	86.7	1.3	4.5	6.8
44		28.0	97.4	2.2	5.4	10.5
45		31.5	111.3	3.5	11.6	12.3

SRA found significant relationships between the strength and selectivity of the overall enzyme production and the activity of the individual enzymes and sampling times (Tables 4, 5). The fermentation time exerts the highest effect on both the potency and selectivity of enzyme production.

Table 4. Activity of enzymes at various sampling times taking into consideration simultaneously each composition of culture media. Potency values are (arbitrary units) calculated by spectral mapping

Sampling time (day)	Laccase	MnP	MnIP	β-Glucosidase	Xylanase
3.5	5.82	10.42	5.55	0.0	0.16
7.0	32.93	37.37	21.52	31.88	2.51
10.5	72.98	62.33	27.16	34.50	28.04
14.0	90.95	78.25	30.06	45.52	38.51
17.5	212.08	268.04	808.46	251.10	63.36
21.0	265.04	367.70	926.70	276.02	26.90
24.5	548.83	1124.48	1415.32	418.81	50.65
28.0	1303.73	1204.57	2887.45	367.84	39.74
31.5	1088.71	1172.45	1490.69	520.87	54.94

Table 5. Parameters of significant relationships between the strength (potency) and selectivity (coordinates of two-dimensional non-linear selectivity map) of the overall enzyme production and the activity of the individual enzymes and sampling times (n=45)

Parameters	Equation I	Equation II
a	–341.9	176.6
b_1	40.5	–0.79
S_{b1}	6.49	0.25
b_2	479.5	–14.0
S_{b2}	151.4	5.63
b_3	–332.5	–
S_{b3}	151.4	–
b_1' (%)	52.97	55.94
b_2' (%)	27.77	44.06
b_3' (%)	19.26	–
r^2 (%)	58.77	27.81
$F_{calc.}$	19.48	8.09
$F_{99\%}$	4.31	5.15

I: Potency = $a+b_1$.Sampling time + b_2.MnIP activity + b_3.xylanase activity
II: First coordinate of the selectivity map = $a+b_1$.Sampling time +b_2.MnIP activity
(III: Second coordinate of the selectivity map = no significant correlation; data not shown)

3. Conclusions

It can be concluded from the results that the addition of agro-industrial wastes to the culture media of *Pl. ostreatus* increases considerably the production of laccase, MnP and MnlP, and moderately enhances the production of β-glucosidase. Spectral mapping technique followed by two-dimensional non-linear mapping proved that the enzyme production was the highest in the presence of pepper extract followed by tomato and potato extracts. The selectivity of the enzyme activities was very low up till 14 days of fermentation and reached the maximum at the 28th day.

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