0273-2289/03/110/0033/$20.00

Effect of Tributylphenyltetraethoxylate on Enzyme Production of *Pleurotus Ostreatus*

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Received February 2002; Revised August 2002; Accepted September 2002

Abstract

The effect of the addition of the nonionic surfactant tributylphenyltetraethoxylate to culture media on pH and extracellular protein content, and on production of β-glucosidase, xylanase, laccase, and manganese-dependent and -independent peroxidases by the edible fungus *Pleurotus ostreatus* was determined. The relationship between fermentation parameters and concentration of surfactant was assessed by multiple linear regression analysis, and the similarities and differences among the fermentation parameters were elucidated by principal component analysis. Calculations proved that except for xylanase all other cultivation parameters were significantly influenced by the surfactant, with the effect higher at higher surfactant concentrations. Surfactant increased the production of β-glucosidase and inhibited laccase and manganese-dependent and -independent peroxidase activities.

Index Entries: Enzyme production; *Pleurotus ostreatus*; nonionic surfactant; principal component analysis.

Introduction

Fungi of the genus *Pleurotus* can cause white rot of lignocellulosic materials. Some species have the capacity to remove lignin preferentially, with limited degradation of cellulose *(1)*, a characteristic relevant to biotechnological delignification processes for feed production *(2)* and paper pulp manufacture *(3)*. The different abilities of individual mushroom

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species to grow and fruit on a particular lignocellulosic substrate are determined by both fungus- and substrate-associated factors *(4)*. These factors include the level of tolerance of the mushroom to potentially toxic phenolic monomers present in lignocellulosic residues *(5,6)*, and the capacity of the mushroom to produce hydrolytic and oxidative enzymes necessary for the degradation of cellulose, hemicellulose, and lignin *(7)*.

Lignin is the earth's second most abundant plant polymer, ranking only behind cellulose in quantity as a natural biopolymer. It is composed primarily of phenylpropanoid monomeric units interconnected by a complex array of stable carbon-carbon and carbon-oxygen bonds *(8)*. Lignin is highly recalcitrant; therefore, its biodegradation is a rate-limiting step in the global carbon cycle. White-rot fungi have evolved a unique mechanism to accomplish lignin degradation using extracellular enzymes for the generation of oxidative radical species *(9)*. These enzymes (lignin peroxidase, manganese-dependent and manganese-independent peroxidase, laccase, and so on) have broad substrate specificities *(10,11)*.

Because of its capacity to degrade wood, *Pleurotus ostreatus*, an edible basidiomycete, is of increasing biotechnological interest. It produces a wide range of extracellular enzymes that enable it to degrade insoluble lignocellulosic substrates into soluble substances that can be uptaken by the mushroom as nutrition. The production of manganese-dependent peroxidase (MnP) and manganese-independent peroxidase (MiP) *(12)* and laccase *(13)* during the complete life cycle of *P. ostreatus* cultivated in liquid media composed of agroindustrial residues as the sole source of carbon and nitrogen has recently been reported.

Surfactants have been frequently used in various biotechnological processes *(14)*. Thus, they have been successfully employed for extraction of proteins *(15)*, and for modification of the activity of a wide variety of enzymes such as glucose oxidase *(16)*, as well as lipases produced by *Trichosporon fermentans (17)* and *Chromobacterium viscosum (18)*.

The objectives of the present study were to measure the activity of diverse enzymes such as β-glucosidase, xylanase, laccase, MnP and MiP produced by *P. ostreatus* during its life cycle in media containing a nonionic surfactant at different concentrations; and to apply various multivariate mathematical statistical methods such as multiple linear regression (MLR) analysis *(19)* and principal component analysis (PCA) *(20)* to elucidate the effect of the nonionic surfactant tributylphenyltetraethoxylate on the fermentation parameters and to assess the production of various enzymes. The results may facilitate a better understanding of the biochemical and biophysical processes governing enzyme production and find potential applications in the biotechnology of enzyme production.

Materials and Methods

Chemicals

Sodium tartarate, ammonium tartarate, hydrogen peroxide, EDTA, glucose (each of proanalysis quality), $MnSO_4$, and potato dextrose agar

(PDA) were purchased from Merck KGaA (Darmstadt, Germany). 2,6-Dimethoxyphenol (99%, GC), *p*-nitrophenyl-β-D-glucopyranoside (99+%), *p*-nitrophenol (98%), 3,4-dimethoxybenzyl alcohol (veratryl alcohol), birchwood xylan, and xylose were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Tributylphenyltetraethoxylate, a nonionic surfactant, was acquired from Hoechst AG (Frankfurt, Germany). Each chemical was used as received. Bovine serum albumin (BSA) was purchased from Reanal (Budapest, Hungary).

Organism and Culture Conditions

The strain of *P. ostreatus* was obtained from the collection of the University of Medelin (Medelin, Colombia). It was maintained on PDA and cultivated in 400 mL of liquid medium containing 30 g/L of glucose, 0.6 g/L of ammonium tartarate, and 1% (w/v) milled wheat straw. Surfactant at 0, 0.001, 0.005, 0.01, 0.05, and 0.1% (w/v) was added to the fermentation broth. Cultures were sterilized at 121°C for 20 min, cooled to 24 ± 2°C, and inoculated by adding a 1-cm-diameter piece of agar with mycelium. The cultures were incubated at 24 ± 2°C in the dark for 30 d, and then they were maintained at 18 ± 2°C with 12 h of light/d until the growing cycle was completed (60 d). Each fermentation was run in triplicate.

Determination of pH, Extracellular Protein Content, and Enzyme Activities

Samples were taken from the culture medium under sterile conditions at 3, 10, 17, 24, 31, 38, 45, 52, 56, and 60 d of fermentation. They were centrifuged at 20,000*g* for 20 min, and the supernatant was applied for analyses. The pH of the supernatant was also measured. Extracellular protein concentration was determined with the method described in ref. *21* using BSA as standard. The activity of β-glucosidase was determined by visible spectrophotometry (UNICAM 8700 Spectrophotometer; UNICAM Ltd., Cambridge, England) at 430 nm in a 10-mm-long cuvet. The reaction mixture contained 250 µL of *p*-nitrophenyl-β-D-glucopyranoside (1.5 mg/mL), 250 mL of acetate buffer (0.2 *M*, pH 4.0), and 500 µL of culture medium appropriately diluted. After 60 min of incubation at 37°C, the reaction was stopped by adding 1 mL of 0.4 *M* glycine-NaOH buffer (pH 10.8), and the absorption was measured.

Xylanase Activity

The reaction mixture contained 250 µL of sample and 125 µL of 2% birchwood xylan suspended in 50 m*M* sodium acetate buffer (pH 5.0). The mixture was incubated for 30 min at 50°C, and the concentration of reducing sugars was measured by the dinitrosalicylic acid method and expressed as xylose equivalents.

Laccase Activity

The reaction mixture contained 10 m*M* 2,6-dimethoxyphenol in 100 m*M* sodium tartarate (pH 5.0) and 500 µL of extracellular culture fluid

in a 1-mL total volume. The absorbance was measured at 469 nm after 5 min of incubation at 30°C.

Manganese Peroxidase

The reaction mixture contained 50 m*M* sodium tartarate (pH 5.0), 2.5 m*M* 2,6-dimethoxyphenol, 0.1 m*M* $MnSO_4$, and 300 µL of extracellular culture fluid in a 1-mL total volume. The reaction was initiated by adding 80 µL of 0.1 m*M* hydrogen peroxide to the reaction mixture. Absorbance was measured at 469 nm after 5 min of incubation.

Manganese-Independent Peroxidase

The reaction mixture contained 50 m*M* sodium tartarate (pH 4.5), 1 m*M* 2,6-dimethoxyphenol, 1 m*M* EDTA, and 420 µL of extracellular culture fluid in a 1-mL total volume. The reaction was initiated by adding 80 µL of 0.4 m*M* hydrogen peroxide to the reaction mixture. Absorbance was measured at 469 nm after 10 min of incubation. EDTA was added to chelate the possible manganese contamination. Enzyme activity is expressed in nanomoles/(minutes·milliliter). Blanks were prepared by incubating enzyme and substance solutions separately.

Evaluation of Fermentation Kinetics by Multivariate Mathematical-Statistical Methods

The simultaneous effect of fermentation time and the concentration of the nonionic surfactants on the fermentation parameters (pH; concentration of extracellular protein in the culture media; activity of β-glucosidase, xylanase, laccase, MiP, and MnP produced by the fungi) was elucidated by MLR analysis. Calculation was carried out seven times. The dependent variables were separate from the seven fermentation parameters listed; and the independent variables were in each instance the fermentation time and concentration of the surfactant. The significance level for acceptance of an independent variable was set at 95%. The similarities and differences among the fermentation parameters were assessed by PCA. The 7 parameters were the variables, and the 10 sampling times and 6 concentrations of surfactant were the observations (altogether 60 observations). The level of variance explained was set at 95%. Since the resulting matrix of PC loadings is also multidimensional, its dimensionality was reduced to two by rotation around two axes and by the nonlinear mapping (NLMAP) technique *(22)*. Iteration of the NLMAP was carried out to the point when the difference between the last two iterations was lower than 10^{-8}.

Software for PCA and NLMAP were prepared by Dr. Barna Bordás (Plant Protection Institute of Hungarian Academy of Sciences, Budapest, Hungary). Software for MLR was purchased from Compudrug (Budapest, Hungary).

Results and Discussion

It was established that *P. ostreatus* grew in each medium; however, the viability of the fungal mycelium was affected by the surfactant. This fact

Table 1
Effect of Fermentation Time and Concentration of Tributylphenyltetraethoxylate on pH of Culture Media[a]

Fermentation time (d)	Concentration of tributylphenyltetraethoxylate (% [w/v])					
	0	0.001	0.005	0.01	0.05	0.1
3	5.24	4.78	5.31	5.43	4.30	4.00
10	5.10	4.18	5.04	4.95	3.91	3.71
17	4.79	4.13	4.76	4.84	3.80	3.42
24	4.83	4.05	4.59	4.84	3.62	3.22
31	4.92	3.98	4.49	4.92	3.39	3.09
38	4.96	3.92	4.43	4.89	3.48	3.11
45	5.01	3.92	4.38	4.88	3.37	3.08
52	5.05	3.86	4.26	4.84	3.39	3.05
56	5.14	3.85	4.41	4.82	3.42	3.14
60	5.11	3.78	4.22	4.79	3.37	3.05

[a]Initial pH = 5.90.

Table 2
Effect of Fermentation Time and Concentration of Tributylphenyltetraethoxylate on Concentration of Extracellular Protein in Culture Media (μg/mL)[a]

Fermentation time (d)	Concentration of tributylphenyltetraethoxylate (% [w/v])					
	0	0.001	0.005	0.01	0.05	0.1
3	545	561	501	475	374	407
10	504	422	434	423	384	404
17	372	265	235	259	233	237
24	334	339	226	325	175	364
31	404	465	480	339	447	374
38	423	382	239	481	310	367
45	361	450	350	398	387	337
52	376	296	258	433	198	302
56	339	435	377	426	222	331
60	352	522	493	357	448	447

[a]Initial concentration of extracellular proteins = 696 μg/mL.

indicated that the presence of surfactant influences the growth of fungi even at very low concentrations. The pH and concentration of extracellular protein in the culture media at various sampling times and concentrations of surfactant are compiled in Tables 1 and 2, respectively. The differences among the pH and protein concentration suggest that not only the fermentation time but also the presence of surfactant exerted a marked influence on these two parameters.

Table 3
Effect of Fermentation Time and Concentration of Tributylphenyltetraethoxylate on Activity of β-Glucosidase (U/mL) Produced by *P. ostreatus*

Fermentation time (d)	Concentration of tributylphenyltetraethoxylate (% [w/v])					
	0	0.001	0.005	0.01	0.05	0.1
3	6.53	9.31	8.09	6.98	7.10	7.07
10	9.14	10.77	9.19	8.32	9.19	8.90
17	10.65	11.46	10.54	9.49	10.90	10.59
24	12.40	11.47	10.54	9.49	10.90	11.99
31	15.61	12.81	12.58	11.99	14.03	14.67
38	18.46	12.93	13.92	13.33	18.07	16.83
45	20.39	14.32	15.20	14.50	18.00	19.98
52	23.36	15.02	16.71	15.90	20.33	22.49
56	24.06	15.43	18.81	16.07	20.85	23.94
60	24.82	16.31	18.52	16.54	24.76	24.76

Table 4
Effect of Fermentation Time and Concentration of Tributylphenyltetraethoxylate on Activity of Xylanase (U/mL) Produced by *P. ostreatus*

Fermentation time (d)	Concentration of tributylphenyltetraethoxylate (% [w/v])					
	0	0.001	0.005	0.01	0.05	0.1
3	5.71	6.55	8.52	6.54	7.10	6.60
10	7.20	7.08	6.72	8.03	7.44	7.20
17	10.35	8.45	7.97	10.53	8.92	8.27
24	12.67	9.34	9.70	12.07	9.70	9.70
31	14.57	11.83	12.73	12.09	10.41	12.13
38	15.40	12.61	13.97	14.87	12.19	13.56
45	16.94	14.21	14.63	16.05	13.74	14.75
52	17.36	14.86	15.46	16.59	14.69	15.22
56	17.96	16.77	17.84	17.54	14.69	15.64
60	17.96	16.94	17.66	17.84	15.16	16.99

The values of enzyme activities produced by *P. ostreatus* are compiled in Tables 3–7. The activity values show high variations indicating that both the concentration of surfactant and fermentation time modify equally the enzyme production. It has been found that the activity of laccase *(23)* and MnP *(24)* is higher than that observed in simple glucose-tartarate culture media.

The parameters of MLR analyses describing the dependence of fermentation parameters on the concentration of surfactant and fermentation

Table 5
Effect of Fermentation Time and Concentration of Tributylphenyltetraethoxylate on Activity of Laccase (U/mL) Produced by *P. ostreatus*

Fermentation time (d)	Concentration of tributylphenyltetraethoxylate (% [w/v])					
	0	0.001	0.005	0.01	0.05	0.1
3	1.57	0.02	0.48	1.44	0.38	0.04
10	8.98	0.49	6.56	5.59	0.98	0.35
17	9.47	1.07	5.61	4.84	0.32	0.23
24	13.21	0.17	6.93	6.29	0.30	0.16
31	16.15	0.53	5.88	12.38	0.08	0.17
38	17.38	0.43	7.65	14.38	0.04	0.04
45	19.15	0.78	8.80	14.78	0.10	0.01
52	19.30	0.24	7.63	15.83	0.15	0.10
56	21.30	0.10	7.93	18.01	0.01	0
60	20.12	0.48	8.71	19.85	0.05	0.02

Table 6
Effect of Fermentation Time and Concentration of Tributylphenyltetraethoxylate on Activity of MiP (U/mL) Produced by *P. ostreatus*

Fermentation time (d)	Concentration of tributylphenyltetraethoxylate (% [w/v])					
	0	0.001	0.005	0.01	0.05	0.1
3	2.68	0.01	1.64	3.10	0.84	0.02
10	6.25	0.35	1.71	6.69	0.82	0.18
17	7.24	1.56	3.66	9.42	0.81	0.22
24	7.45	1.63	3.78	9.53	0.50	0.26
31	7.70	1.88	5.08	10.54	0.05	0.15
38	8.18	0.97	5.60	10.77	0.02	0.06
45	8.50	1.24	5.73	10.82	0.28	0.03
52	8.50	1.07	6.03	11.53	0.02	0.01
56	8.73	0.51	6.19	11.87	0.02	0.02
60	8.79	1.52	7.72	12.06	0.01	0.01

time are given in Table 8. The relationship between the fermentation parameters and fermentation time was significant in each instance; with the exception of xylanase production, all other parameters also depended significantly on the concentration of surfactant in the culture media (see $F_{calc.}$ values in Table 8). The ratio of variance explained by the independent variables (see r^2 values in Table 8) showed considerable deviations, varying between 86 and 17%. This finding indicates that other parameters not included in the calculations may exert a marked impact on the fermen-

Table 7
Effect of Fermentation Time and Concentration of Tributylphenyltetraethoxylate on Activity of MnP (U/mL) Produced by *P. ostreatus*

Fermentation time (d)	Concentration of tributylphenyltetraethoxylate (% [w/v])					
	0	0.001	0.005	0.01	0.05	0.1
3	3.24	0.02	0.48	3.52	0.41	0.06
10	3.45	0.70	3.13	10.47	1.32	0.46
17	4.36	1.60	4.12	11.39	0.95	0.49
24	4.67	1.00	6.25	11.55	1.02	0.72
31	7.52	0.82	8.09	12.98	1.13	0.94
38	10.35	1.37	8.12	14.04	0.20	0.17
45	11.89	0.47	8.65	15.15	0	0.01
52	13.30	1.14	9.05	16.75	0.34	0.55
56	13.88	0.82	10.03	19.40	0.24	0.04
60	14.99	2.05	10.18	20.88	0.07	0.04

tation parameters. The normalized slope values (b'_i) suggest that the relative importance of fermentation time and surfactant concentration is similar for pH; protein concentration; and laccase, MiP, and MnP production; however, they are considerably different for the other parameters. The acidity of media was higher at increasing fermentation time and enhanced concentration of the surfactant (Table 8), suggesting that the surfactant intensifies production of acids by *P. ostreatus*. Unfortunately, the individual acids have not been identified; therefore, the biochemical procedure underlying acid production cannot be elucidated in detail. The amount of extracellular proteins was significantly lower at higher fermentation time and surfactant concentration (Table 8). This result demonstrates that the uptake of extracellular proteins by the fungi or their decomposition was also influenced by fermentation time and surfactant concentration. As expected, the activity of each enzyme was higher at longer fermentation times. The effect was highest for β-glucosidase and lowest for MiP. Interestingly, surfactant influenced differently the production of enzymes. It was ineffective for xylanase, enhanced the production of β-glucosidase, and reduced the activity of the other enzymes. This behavior can be tentatively explained by the supposition that the surfactant binds to the enzyme protein *(25)*. The binding modifies the protein structure *(26)*, resulting in enhanced or decreased activity.

These results further demonstrate that the application of surfactants in biotechnology may have both adversary and beneficial effects. However, at present, our knowledge of the effect of a surfactant on enzyme activity cannot be predicted with acceptable accuracy.

The results of PCA are given in Table 9; the most important PC loadings are underlined. Three PCs explain the overwhelming majority of vari-

Table 8
Parameters of Linear Relationships Between pH, Extracellular Concentration of Proteins in Culture Media (μg/mL), Activities of Enzymes Produced by *P. ostreatus*, and Fermentation Time (d, b_1) and Concentration of Tributylphenyltetraethoxylate (% [w/v], b_2)

	Dependent variable (n = 66)						
Parameter	pH	Protein concentration	β-Glucosidase activity	Xylanase activity	Laccase activity	MiP activity	MnP activity
a	5.37	475.9	3.99	4.22	3.56	3.15	3.19
b_1 (d)	-1.91×10^{-2}	–1.95	0.28	0.23	0.13	6.31×10^{-2}	0.11
s_{b1}	3.26×10^{-3}	0.69	0.02	0.01	0.03	1.93×10^{-2}	0.03
b_2 (% [w/v])	–14.08	–802.8	21.04	—	–85.32	–56.12	–70.86
s_{b2}	1.82	383.5	9.16	—	18.34	10.75	15.56
b'_1 (%)	43.03	57.44	88.15	—	45.27	38.52	47.31
b'_2 (%)	56.97	42.56	11.85	—	54.73	61.48	52.69
r^2 (%)	59.96	17.71	82.50	86.78	36.66	37.59	37.30
$F_{calc.}$	47.18	6.59	148.47	420.10	18.24	18.98	18.74

Table 9
Similarities and Dissimilarities Among Fermentation Parameters[a]

No. of PC	Eigenvalue	Variance explained (%)	Total variance explained (%)
1	3.68	52.50	52.50
2	1.97	28.15	80.65
3	0.95	13.53	94.18

PC loadings

	No. of PC[b]		
Parameter	1	2	3
pH	0.70	–0.64	0.00
Concentration of extracellular protein	0.10	–0.36	0.92
β-glucosidase	0.29	0.90	0.19
Xylanase	0.57	0.77	0.18
Laccase	0.96	–0.01	–0.06
MiP	0.96	–0.16	–0.10
MnP	0.96	–0.05	–0.10

[a]Results of PCA.
[b]The most important PC loadings are underlined.

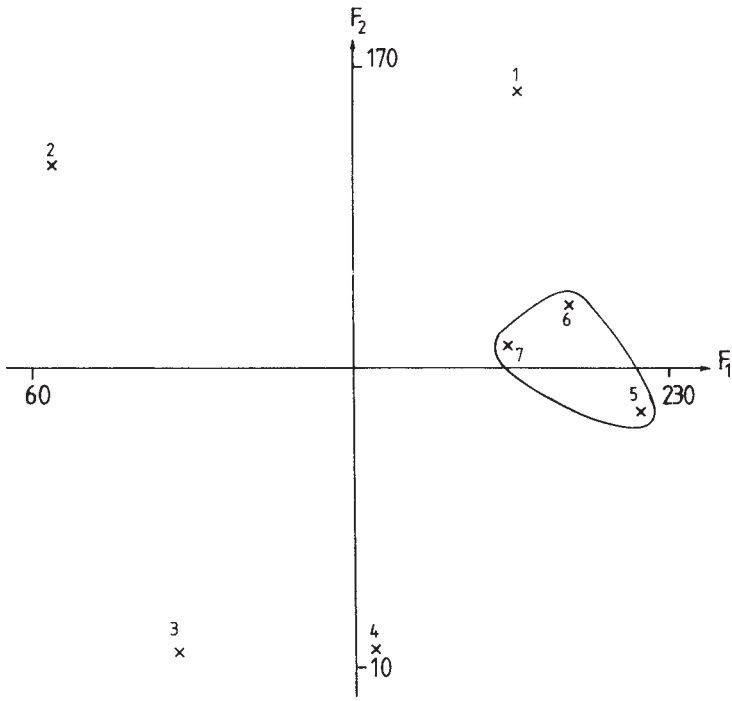

Fig. 1. Similarities and dissimilarities among fermentation parameters. A two-dimensional nonlinear map of PC loadings is shown. Number of iterations: 53; maximal error: 9.94×10^{-3}. Numbers refer to fermentation parameters in Table 8.

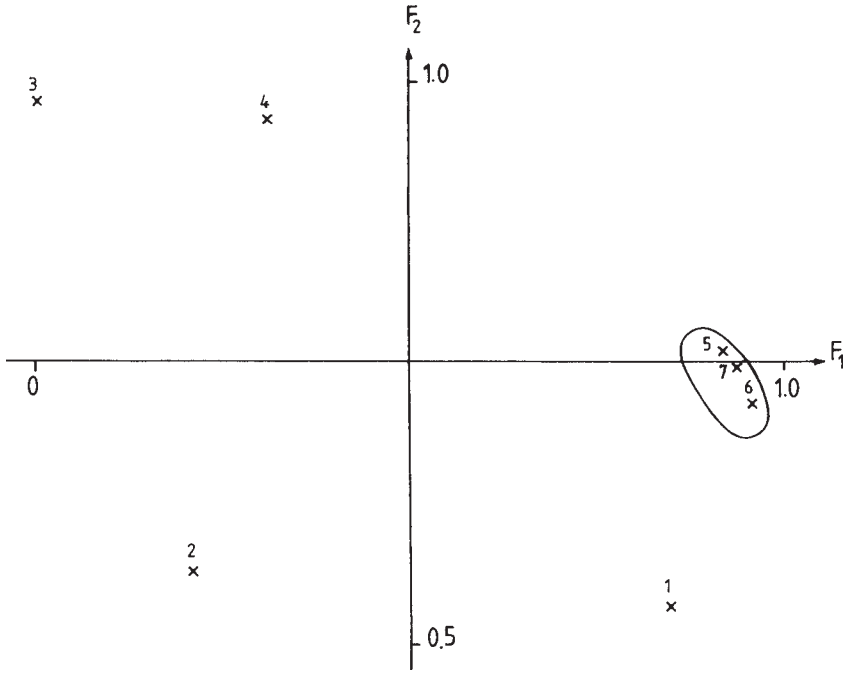

Fig. 2. Similarities and dissimilarities among fermentation parameters. The results of varimax rotation around two axes are shown. Numbers refer to fermentation parameters in Table 8.

ance suggesting that the original seven variables can be substituted with three theoretical ones with only 5.82% loss of information. Unfortunately, PCA does not define these theoretical (background) variables as concrete physicochemical or enzymologic entities; it only indicates their mathematical possibility. The majority of fermentation parameters have high loadings in the first PC, indicating their basic similarity. Plots of the two-dimensional nonlinear map and of varimax rotation around two axes are depicted in Figs. 1 and 2, respectively. The maps are very similar to each other, proving that both methods can be used equally for the reduction of dimensionality of the matrix of PC loadings. The distribution of the fermentation parameters on the maps clearly shows that the effect of fermentation time and concentration of the surfactant is highly different for pH (point 1) and the quantity of extracellular protein (point 2). The activities of β-glucosidase and xylanase form a loose group suggesting their resemblance. Other enzyme activities are very close to each other, proving their very high similarity.

It can be concluded from the data that tributylphenyltetraethoxylate, a nonionic surfactant, exerts a marked influence on the majority of the fermentation parameters of *P. ostreatus*. Modification of the fermentation process by the surfactant can be exploited for the rational design of biotechnological processes of enzyme production.

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