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Addition of Polyglycol to Old Culture of *Phanerochaete chrysosporium*

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Abstract

Polyglycols increased lignin peroxidase activity in shaken cultures of *Phanerochaete chrysosporium* even when they were added to an old fungus (5 or 10 d after inoculation). The effects depended on the polyglycol mol wts (10^2–10^6 Daltons) as well as on the backbone structure (i.e., poly[ethylene glycol], poly[butylene glycol], poly[propylene glycol]) and terminal groups (i.e., poly[ethylene glycol], poly[ethylene glycol] methyl ether, poly[ethylene glycol] dimethyl ether). The residual quantity of polyglycol in the biomass and in the culture filtrate also varied among different polyglycols. The polyglycols act after being adsorbed to the cell membrane exaggerating the asymmetry of the membrane environment. In old fungus, the incompatibility of polyglycols and glycans decreases the adsorption and the effect of high mol wt polyglycols.

Index Entries: Fungi; lignin peroxidase; *Phanerochaete chrysosporium*; poly(glycol).

Introduction

The white-rot fungus *Phanerochaete chrysosporium* is widely studied for its ability to produce lignin peroxidase (LiP) and manganese peroxidase. These enzymes are involved in lignin degradation and also in oxidation of different xenobiotics *(1,2)*. If stimulatory substances are not added to agitated culture, enzymatic production of the fungus is usually low (about 20 U/L). It has been observed *(3)* that in shaken culture of *P. chrysosporium*, LiP production is markedly enhanced by Tween-80 (polyoxyethylene *[20]* sorbitan monooleate). The effect is the consequence not only of the fast excretion of formed enzymes *(3)* but also of their enhanced production following the enhanced oxygen supply to the cells *(4)*.

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Because $(–CH_2CH_2O–)_n$ is the active moiety of all Tweens, the stimulatory properties of poly(ethylene glycol) (PEG) and some other polyglycols (i.e., poly[butylene glycol] [PBG], poly[propylene glycol] [PPG], poly [ethylene glycol] methyl ether [pegME], poly[ethylene glycol] dimethyl ether [pegDME], and PEG derivatives having amino-terminal groups) were checked. All these polyglycols increased the enzymatic activity in shaken cultures of *P. chrysosporium (5)*. PEG is distributed between the biomass and the culture fluid, and its quantity declines during cultivation *(6,7)*. A PEG of lower molecular weight is degraded by the fungus to a higher degree, but it induces a lower LiP activity than a PEG of higher molecular weight *(7)* and cannot replace glucose as a carbon source.

The stimulatory substance is usually added before inoculation. However, there are studies on addition of Tween-80 to up to 6-d-old fungi *(3,6,8)*. Jäger et al. *(3)* did not find any enzymatic activity when adding Tween-80 on d 6 of cultivation under agitation. Katič et al. *(6)*, as well as Venkatadri and Irvine *(8)*, on the other hand, observed a substantial increase in ligninolytic activity also when Tween-80 was added a few days after inoculation.

To our knowledge, there is no information about the effect of PEG or its derivatives when added to *P. chrysosporium* culture at the time of its maximum LiP activity or at the time when LiP activity disappears, i.e., when it is added to an old fungal culture. The controversial observations that we have cited encouraged further study of these possibilities, since if an old culture could be induced to produce additional quantities of enzymes, this would allow multiple harvesting of enzymes.

The purpose of the present study was to test whether polyglycols increased production of ligninolytic enzymes when added to an old fungal culture.

Materials and Methods

Organism

Subcultures of *P. chrysosporium* MZKI B-223 (ATCC 24725) were prepared every 3 to 4 wk on malt agar slants and maintained in a refrigerator (4°C) until use.

Medium and Culture Conditions

A nitrogen-limited liquid growth medium *(9)* with low (0.02 mmol/L) Mn(II) concentration was prepared, with the pH adjusted to 4.5. A stimulator of LiP production (1.0 g/L) was added either before inoculation or 5 or 10 d after inoculation. The fungus was grown in agitated (200 rpm) 500-mL Erlenmeyer flasks containing 100 mL of the growth medium. The flasks were inoculated with 4×10^6 spores/mL from agar slants, and the cultures were incubated at 32°C. At least three individual flasks were used in each experiment and the mean values are presented.

Table 1
Polyglycols Tested

Polyglycol	Chemical structure	Molecular weight (Daltons)
PEG	$H(OCH_2CH_2)_nOH$	106–1,000,000
pegME	$CH_3(OCH_2CH_2)_nOH$	2000
pegDME	$CH_3(OCH_2CH_2)_nOCH_3$	2000
PPG	$H(OCH(CH_3)CH_2)_nOH$	2025
PBG	$H(OCH_2CH_2CH_2CH_2)_nOH$	2000
Tween-80		1308

Stimulatory Substances (Polyglycols)

Substances that stimulated the production of ligninolytic enzymes when added before inoculation *(5)* were used. PEGs with different mol wts (10^2–10^6 Daltons) and several other substances with average mol wts of about 2000 Daltons were tested (Table 1). The aqueous solution of the stimulatory substance was sterilized and added to the culture on the specified day after inoculation. PPG and PBG (more hydrophobic, insoluble in water) were not sterilized before addition into the growth medium, since LiP activity induced by nonsterilized PEG with a molecular weight of a similar range was <8% lower compared with that obtained by a sterilized polymer *(10)* and, additionally, no contamination problem was observed.

Enzymatic Activity

LiP activity in the culture filtrate was determined according to Tien and Kirk *(11)* using veratryl alcohol as a substrate. The method was modified *(12)* so that the final concentrations of veratryl alcohol and H_2O_2 were 2 and 1 m*M*, respectively. Measurement was performed kinetically and the ligninolytic activity was calculated from the highest slope of absorbance vs time. One unit of LiP activity (U) is defined as 1 μmol of veratraldehyde formed during 1 min. The relative standard deviation was 3% at the LiP activity of 600 U/L, and 6% at 200 U/L.

For determination of intrapellet LiP activity *(13)*, expressed in units per gram, 0.3 g of washed and blotted pellets was suspended in 8.7 mL of buffer solution (50 m*M* sodium tartrate, pH 3.0) used for LiP determination in the culture filtrate. The reaction was initiated by the addition of 0.3 mL of H_2O_2. After 30, 60, 90, and 120 s, the pellets were filtered off as fast as possible, and the absorbance of the filtrate at 310 nm was measured by the kinetic method for 20 s and extrapolated to the filtration starting time.

Determination of Amount of Polyglycol

At the end of cultivation, the biomass was separated from the growth medium. It was washed with ice-cold buffer and gently mixed in methanol for 1 h. The solid matter was removed and the solvent evaporated. From the solid residue, as well as from the growth medium, polyglycol was extracted with chloroform, dried, and weighed. Its identity was checked by performing thin-layer chromatography and Fourier transform infrared spectroscopy *(6)*.

The percentage of degraded polyglycol (DPg) and the apparent distribution coefficient (ADC) for the polyglycol between the biomass and the culture filtrate were calculated as follows:

$$\mathrm{DPg} = 100 - (\mathrm{PgB} + \mathrm{PgC})$$

$$\mathrm{ADC} = \frac{\mathrm{PgB}}{\mathrm{PgC}}$$

in which PgB is the polyglycol in the biomass (% [w/w]) and PgC is the polyglycol in the culture filtrate (% [w/w]).

Because Tween-80 contains approx 80% of the hydrophilic moiety, which is its active constituent *(6)*, its metabolized portion was calculated regarding its initial amount in Tween-80 (0.8 g/L).

Results and Discussion

When Tween-80 was added as stimulatory substance before inoculation, the maximum LiP activity was observed on d 5 or 6. It could be concluded that on d 5 the fungus was in the stage of secondary metabolism and had optimal conditions for LiP production. To understand how stimulatory substances affect the excretion of LiP when they are added to an old culture, they were added 5 d or more after inoculation.

In 5-d-old fungi growing in the growth medium without a stimulatory substance, there was no intrapellet LiP activity. After intrapellet LiP measurement, PEG 10,000 (the best stimulatory substance among PEGs; cf. ref. *5*) was added to the growth medium. In spite of maceration and nonsterile work at intrapellet LiP measurement, maximum LiP activity (200–240 U/L) appeared 35–50 h after the addition of PEG 10,000 (data not shown).

PEGs of different mol wts from 10^2 to 10^6 (diethylene glycol [DEG], PEG of mol wts from 900 to 1,000,000 Daltons) were added to the culture 5 d after inoculation. During the first 6 h after addition of PEG, LiP activity in the culture filtrate was negligible, indicating that PEG does not cause the release of enzymes from the cells. However, PEGs of mol wts from 900 to 1,000,000 Daltons caused maximum LiP activity to appear 2 to 3 d after their addition. DEG did not induce LiP activity when the fungus was old. Normalized data, expressed as $\mathrm{LiP}_i/\mathrm{LiP}_{tw80}$, where LiP_i is the corresponding LiP activity and LiP_{tw80} is maximum LiP activity measured when Tween-80 was added, show that PEG 10,000 gave the best results, even better than Tween-80 (Fig. 1). It was interesting to observe that PEG 900 followed it and was even better than Tween-80. PEG 100,000 and PEG 1,000,000 gave rise in this case to lower activity than Tween-80.

The results of comparison of maximum LiP activity when PEGs were added either before inoculation or to 5-d-old fungi are presented in Fig. 2.

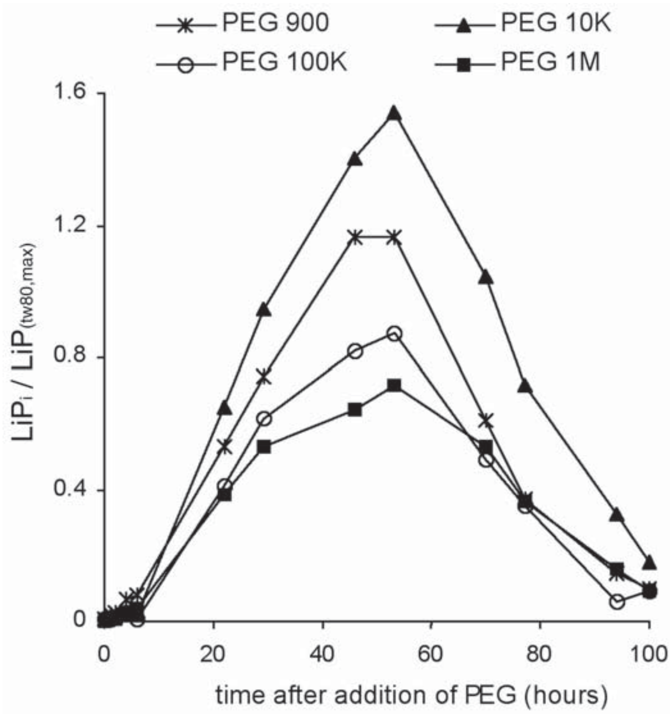

Fig. 1. LiP activity induced by PEGs, normalized to the maximum LiP activity observed on addition of Tween-80 ($LiP_i/LiP_{tw80,max}$). PEGs with different molecular weights were added to 5-d-old fungi. Replicates containing Tween-80 reached maximum LiP activities (500–600 U/L) about 50 h after polyglycol addition.

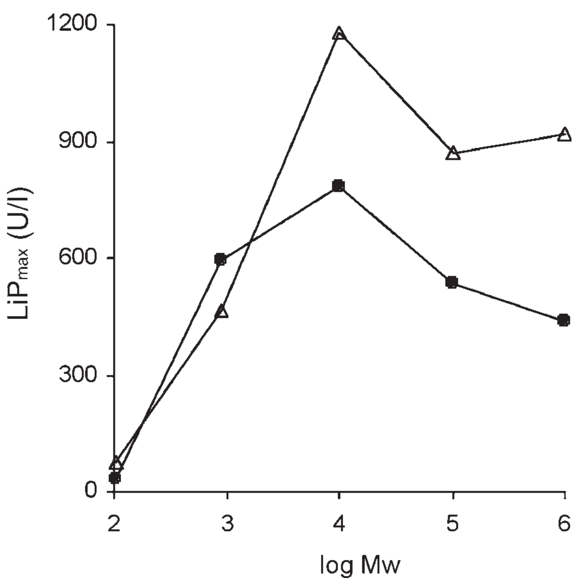

Fig. 2. Comparison of maximum LiP activities observed when PEGs of different molecular weights (Mw) were added either before inoculation (—△—) or to 5-d-old fungi (—●—).

Table 2
Normalized Maximum LiP Activity (LiP_i/LiP_{tw80})
of Various Polyglycols With Mol Wts of About 2000 Daltons[a]

	Time of substance addition (d)		
Polyglycol	0	5	10
PEG	0.63	1.43	0.81
pegME	0.85	0.84	0.99
pegDME	0.74	1.05	0.76
PPG	0.66	1.17	0.86
PBG	0.38	0.75	0.42
Tween-80[b]	1 (430)	1 (360)	1 (320)

[a]Polyglycols were added either before inoculation or to 5- or 10-d-old fungi.
[b]Absolute values (in U/L) are given in parentheses.

The maximum LiP activity increased with increasing molecular weight of PEG up to a certain level. The highest LiP activity was obtained with PEG 10,000, regardless of the time of addition. When PEG was added to the growth medium before inoculation, greater enzymatic activities were observed than in cases in which PEG was added to old culture, with PEG 900 as an exception. Since this effect is molecular weight dependent, it can be concluded that the diffusion of PEG is less limited in a young fungus than in an old one.

Some chemicals with mol wts of about 2000 Daltons and that are chemically related to PEG (i.e., PBG, PPG, pegME, pegDME), were also tested. They were added to the growth medium either before inoculation or on d 5 or 10 after inoculation, respectively.

When Tween-80 was added before inoculation, LiP activity first appeared on d 3 and the maximum was reached on d 6 after addition. On addition of polyglycols, LiP first appeared on d 5 after addition. There were some differences with respect to the day of maximum LiP activity. Hydrophilic polyglycols (PEG, pegME, and pegDME) caused greater LiP activity and in a shorter time (maximum on d 7). On addition of more hydrophobic polyglycols (PPG and PBG), a lower LiP activity was observed, especially with PBG, and the maximum LiP activity appeared later (on d 8 or 9). On addition of any polyglycol to 5- or 10-d-old fungi, the maximum LiP activity was observed 70–100 and 20–60 h after their addition, respectively. As with addition of polyglycol on d 5, and also in the case of addition on d 10, the lowest LiP activity was observed with PBG (Table 2).

The distribution of polyglycols between the biomass and growth medium was also determined (Table 3). Extraction was performed at the end of cultivation when enzymatic activity had decreased (i.e., 7–10 d after addition of polyglycol). On later addition of polyglycols, PPG and PBG were added directly into the Erlenmeyer flask. Therefore, direct comparison with hydrophilic polyglycols cannot be made. When hydrophilic polyglycols

Table 3
Amount of Residual Polyglycol in Biomass (B) and Culture Filtrate (CF) at Different Times of Addition[a]

	Residual polyglycol (%)[b]					
	Time of polyglycol addition, after inoculation (d)					
	0		5		10	
Polyglycol	B	CF	B	CF	B	CF
PEG	8.0	68.6	3.3	64.0	3.3	76.6
pegME	9.7	79.1	1.7	65.6	4.0	77.8
pegDME	9.9	63.3	2.3	59.6	2.8	85.8
PPG	11.9	48.9	17.2	34.6	7.5	22.5
PBG	5.2	6.1	51.0	6.0	47.8	8.5
Tween-80	9.1	31.0	4.6	31.2	2.1	22.8

[a]The mol wts of tested polyglycols were about 2000 Daltons, except Tween-80 (control).
[b]The amount of residual polyglycol is expressed as the percentage of the initial amount of polyglycol in the growth medium (1 g/L).

Table 4
Maximum LiP Activity (LiP_{max}), Percentage of Degraded Polyglycol (DPg), and Apparent Distribution Coefficient (ADC) for Polyglycol Between Biomass and Culture Filtrate[a]

Polyglycol	LiP_{max} (U/L)	DPg (%)	ADC
PEG	270	23.4	0.117
pegME	370	11.2	0.115
pegDME	320	26.8	0.156
PPG	290	39.2	0.243
PBG	160	88.7	0.848
Tween-80	430	50.0	0.294

[a]Different polyglycols with mol wts of about 2000 Daltons were added before inoculation.

(PEG, pegME, pegDME, and Tween-80) were added on d 5 or 10, their amounts in the biomass were lower than on addition before inoculation.

When PBG was added before inoculation, its amount in the biomass as well as in the growth medium was the smallest; most of the PBG was degraded (Table 4). In the case of other polyglycols, <50% was degraded. Among the tested substances, PBG was an exception also when comparing the maximum LiP activity (the lowest) and the apparent distribution coefficient between the biomass and the culture filtrate (the greatest). Therefore, greater amounts (up to 8 g/L) of PBG were added before inoculation, but the results were not good: on addition of 3 g/L of PBG, the maximum LiP activity was lower than with an addition of 1 g/L, and on addition of

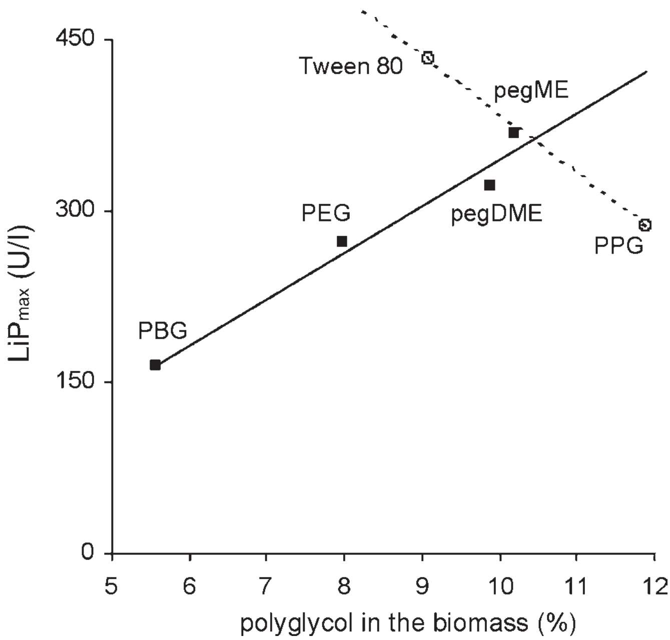

Fig. 3. Maximum LiP activity caused by polyglycols with a mol wt of about 2000 Daltons in correlation to residual substance in biomass. (—■—) Polyglycols with a linear chain; (– –○– –) branched polyglycols.

8 g/L, no LiP activity was observed. The results of the comparison of the maximum LiP activity and the polyglycol quantity in the biomass are presented in Fig. 3. The linear polyglycols are on the solid line, and the branched polyglycols are on the dotted line. In both cases, among linear polyglycols as well as branched polyglycols, the lowest maximum LiP activities were observed on addition of hydrophobic polyglycols (PPG and PBG).

The mechanism of action of polyglycols seems to be indirect *(5,7)*. Polyglycols that cause increased production of LiPs in *P. chrysosporium (5–7)* do not penetrate the cell membrane *(14,15)*, but the possibility that they can enter the periplasmic space of fungi cannot be ruled out *(16)*. They become adsorbed to the cell membrane *(17)*, usually flat to the surface *(18)*, exaggerating the asymmetry of the membrane environment *(5)*. The means by which the contact of polyglycols with the cell membrane and the higher asymmetry of the membrane environment provoke an increased synthesis of LiPs is not yet known. In any case, an induction period of at least 6 h is observed.

Differences in the effect of polyglycols in young vs old cultures of *P. chrysosporium*, on the other hand, can be explained by the differences in the cultures' metabolic status as well as by the incompatibility of polyglycols and glycans. In old culture, the cells of *P. chrysosporium* are surrounded by the glycan sheath, whereas during the first few days of growth they are not. Solutions of polyglycols and glycans of sufficiently high molecular weights are not compatible, and this incompatibility leads to phase separation *(19)*. Because of this incompatibility, old culture allows the high-molecular-weight polyglycols less chance to diffuse through the glycan sheath and cell wall to the cell membrane and to become adsorbed there than young culture devoid of the glycan sheath. This is one of the possible explanations for the effects presented in Fig. 2 and Table 3.

Conclusion

PEG and some chemically related polymers enhance LiP production in shaken cultures of *P. chrysosporium*, not only if they are added into the growth medium before inoculation, but also if they are added at the time of maximum LiP production (around d 5 after inoculation) and even if they are added when the LiP production ceases (around d 10 after inoculation). They act not by releasing formed LiPs from the cells, but by inducing the process that leads to the synthesis of enzymes. The older the culture, the lower the amount of hydrophilic polymers diffused to the cells. Above a threshold of about 1000 Daltons, the higher the molecular weight of the polyglycol, the lower enhancement of lignin production it causes in an old culture.

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