BIOTECHNOLOGICAL MODIFICATION OF LIGNIN

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ABSTRACT

A literature search of organisms capable of degrading lignin was conducted. Four fungi were selected for study and these were Phanerochaete chrysosporium, Chrysosporium pruinosum, Phlebia tremellosus and Trametes versicolor. Other organisms, Pleurotus ostreatus, Pleurotus florida and Lentinus edodes were also tested in preliminary experiments. All cultures were screened for their ability to degrade the lignin component of aspen sawdust and also extracted lignin. This type of screen was followed by analysis of culture filtrates for the presence of ligninase, the marker enzyme for lignin degradation. Phanerochaete chrysosporium and Chrysosporium pruinosum were the most active ligninase producers and were consequently chosen for further studies in fermentors. Considerable efforts were directed to production of ligninase in fermentors. Only when Chrysosporium pruinosum was pre-cultured in a shake flask for 4 days and then transferred to a fermentor could ligninase activity be detected. The enzyme from shake flasks has been concentrated ready for use in bench-scale studies on cell-free depolymerization of lignin.

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1. INTRODUCTION

Previous studies on lignin degradation have been concentrated on optimizing the complete oxidation of lignin in wood or other lignocellulosic wastes, e.g., straw to carbon dioxide (1,2). Very little information was available on the release of lower molecular weight moieties from the lignin polymer. The complete oxidation requires the presence of an actively growing cell and the presence of ligninase in the extracellular medium. Ligninase catalyses the peroxidative cleavage of the aromatic ring of the lignin subunits and also some demethylation of the methoxy phenolic groups. The aim of this project was to identify possible biological agents capable of degrading lignin and to isolate the enzyme systems from these agents. These enzymes would then be tested for the cell-free depolymerization of various lignin polymers extracted in various ways from several sources of steam exploded wood. To facilitate the production of sufficient enzyme for larger trials, it would be necessary to grow up large volumes of cells and for this reason a further goal of the project was to study the production of ligninase in fermentor cultures of the selected biological agents.

2. MATERIALS AND METHODS

2.1 Origin and Maintenance of Organisms

The organisms used in this study were obtained from a variety of sources as shown in Table 1. All organisms except Pleurotus sps. and Lentinus edodes were maintained on slants of Cereal Pablum Agar (Cereal Pablum 100g/L and Bactoagar 20g/L). Pleurotus sps. and Lentinus edodes were maintained on slants of Potato Dextrose Agar (Difco Potato Dextrose Agar, 39g/L and Bactoagar, 5g/L). All strains were subculture from a master slant at two-monthly intervals.

2.2 Growth of Organisms

The liquid growth medium was the same for all fungi and was limiting with respect to the level of nitrogen, i.e., a condition conducive to the induction of ligninase. The medium of Reid (3) was used and contained, in g/L: glucose, 10.0; monosodium glutamate, 1.87; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.1; 2,2-dimethylsuccinic acid, 3.6, adjusted to pH 4.50 prior to autoclaving. When cool to this sterile solution was added sterile thiamine (0.1mL/75mL of a solution containing 75mg thiamine/L), sterile ferric chloride (1.0mL/75mL of a solution containing 0.9g $FeCl_3.6H_2O$/L) and sterile trace elements (0.1mL/75mL of a solution containing 4.95g $ZnSO_4.7H_2O$/L, 3.75g $MnSO_4$/L, 0.75g $CoCl_2.6H_2O$/L and 0.75g $CuSO_4.5H_2O$/L). Later in the studies the level of glutamate was changed to 7.5g/L (high N) or 0.75g/L (low N). In addition, the glutamate was substituted with an equivalent amount of nitrate-N in the form of NH_4NO_3 and/or the glucose was substituted with glycerol at an equivalent carbon content. In later experiments the growth medium was supplemented with Polypropylene glycol 2000 (PPG 2000) at 100ppm, 1.4mM veratryl alcohol at and 0.01% Tween 80.

To inoculate the cultures either a spore suspension (for Phanerochaete chrysosporium and Chrysosporium pruinosum) or a homogenized mycelial suspension (for Phlebia tremellosus and Trametes versicolor) was used. Spore suspensions were prepared by adding 10mL 0.9% NaCl containining 0.01% Tween 80 to a slant of the fungus and scraping the surface of the slant. The spore concentration of this suspension was taken by counting in a haemocytometer. An aliquot of this suspension was added to the growth medium such that the final spore concentartion in the medium was 5×10^5 spores per mL. For the non-sporulating fungi, the mycelial mat was removed from the slant with a sterile spatula and added to 30mL 0.9% NaCl + 0.01% Tween 80 solution in a homogenization cup of a Waring blender. The mycelia was homogenized for a total of 30 seconds, 3 x 10 second bursts with 5 second breaks. The homogenized mycelial suspension was added at the rate of 7.5mL per 100mL growth medium. Growth medium inoculated with spores or mycelial fragments are referred to as "preinoculated medium".

In studies where the degradation of wood sawdust or extracted lignin was tested the former was added at a concentration of 10g/100ml medium and lignin at a concentration of 2.5g/100mL. Preweighed tubes (in triplicate) containing a known amount of substrate were prepared and these were

TABLE 1

LIST OF ORGANISMS USED IN THIS STUDY

ORGANISM	DESIGNATION	ORIGIN
Trametes versicolor	ATCC 12679	American Type Culture Collection, Rockville, MD.
Phlebia tremellosus	PRL 2845	Dr. I. D. Reid, Biotechnology Research Institute, Montreal.
Phanerochaete chrysosporium	UAMH 3642	University of Alberta Mould and Herbarium, Devonian Gardens, Edmonton.
Chrysosporium pruinosum	UAMH 4521	University of Alberta Mould and Herbarium, Devonian Gardens, Edmonton.
Pleurotus ostreatus	FC-284	Dr. S. Davies, Alberta Environmental Centre, Vegreville, Alberta.
Pleurotus florida	FC-26	Dr. S. Davies, Alberta Environmental Centre, Vegreville, Alberta.
Lentinus edodes	ARC-1	Isolated by Dr. A. Jones from fruiting body.

sterilized dry. Three tubes containing no wood or lignin were also sterilized and these served as controls to estimate the extent of fungal growth. To these samples was added the "preinoculated medium". All tubes were incubated with or without shaking at room temperature for up to 21 days. At this time the contents of the tubes were filtered through preweighed Whatman #4 filter paper and the residue dried in an oven at 70°C overnight. After equilibration at room temperature for 1 hour the filter papers and residue were weighed. Wood or lignin loss was calculated using the following equation:

Weight loss of lignin or wood = Wt. of original lignin/wood - [Wt. of lignin/wood - Wt. cells in control)

$$\% \text{ Weight Loss} = \frac{\text{Average Weight Loss}}{\text{Average Weight of Original Lignin/Wood}} \times 100$$

2.3 Growth of *Phanerochaete chrysosporium* and *Chrysosporium pruinosum* in Fermentors

These two fungi were selected for growth in fermentors as they produced the highest level of ligninase. As larger quantities of enzyme would be required for further studies on lignin depolymerization, a readily available large-scale supply of the enzyme would be required. The growth medium used had the same basic composition as described above. The fermentors used were 2L New Brunswick Multigen fermentors with an operating volume of 1.5L. Aeration was supplied at 2L air per minute and agitation was at 200rpm. All fermentors were incubated at 26°C for a period of 14 days. Samples of the culture were taken at various times after 8 days of growth and analysed for ligninase activity.

2.4 Assay of Ligninase Activity

Ligninase activity was measured by following the oxidation of verAtryl alcohol which was detected spectrophotometrically by the increase in absorption at 310nm (4). The assay mixture (in a final volume of 2.0mL) contained 1.0mL 0.3M sodium tartrate buffer, pH 3.0; 0.3mL 4mM veratryl alcohol, 0.1-0.5mL culture supernatant (source of ligninase) and 0.1-0.4mL water in a 3.0mL cuvette. A blank reference cuvette lacking hydrogen peroxide was also set up. The reaction was started by addition of 0.1mL 0.54mM hydrogen peroxide to the test cuvette and the increase in absorption at 310nm was followed over a period of 3 min. Enzyme activity is expressed as umoles veratryl alcohol oxidized per minute per mL supernatant and was calculated using a molar extinction coefficient of 9300 for veratryl alcohol.

2.5 Materials

All chemicals used were of the highest grade and obtained from Sigma Chemical Company or Fisher Scientific. Samples of Aspen wood chips and lignin were obtained from the Forest Products Testing Department of the Alberta Research Council. The lignin samples were NaOH extracted lignin from Exploded Aspen wood residue (615psi, 48sec); NaOH extracted lignin from exploded Aspen wood (613psi, 45sec); ethanol extracted lignin from exploded Aspen wood (612psi, 45sec) and Tomlinite.

3. RESULTS AND DISCUSSION

3.1 Screening of Fungal Strains for Degradation of Aspen Wood and Lignin

Initially the four white-rot fungi were tested for their ability to degrade aspen wood and the various lignin samples. It must be noted that the time-frame for each of these experiments was 17-21 days. This is necessary as lignin degradation is a slow process and to ensure that statistically valid weight losses are observed. In addition, the incubation conditions had not been optimized for each fungus and thus the rates of lignin oxidation will vary for each organism under the standard conditions used in these assays. The results (Table 2) show that all fungi tested are able to degrade natural aspen wood, steam exploded aspen wood and the various types of lignin obtained from aspen wood. Some differences are seen from sample type to sample type. The natural wood was less susceptible to degradation than the isolated lignin. This is not too surprising as the lignin molecule is more readily exposed in the free lignin compared to that in the natural wood where it is masked from attack by ligninases by the presence of cellulose. Of the lignin preparations, the NaOH extracted lignin appears to be the most amenable to oxidation and, in some cases (Chrysosporium pruinosum, Phlebia tremellosus and Trametes versicolor), greater than 25% degradation was observed. For this reason NaOH extracted lignin was used in further studies on lignin degradation and ligninase production by these organisms.

Three other organisms, Pleurotus ostreatus, Pleurotus florida and Lentinus edodes were tested for their ability to degrade lignin. These organisms are representatives of the Basidiomycetes and are interesting in that their fruiting bodies (mushrooms) are edible. They, like the other four strains, are white-rot fungi. The results of a preliminary test with these fungi are shown in Table 3. Only Lentinus edodes showed any significant degradation of either wood or Tomlinite and may deserve further study in the future (5).

3.2 Production of Ligninase by Shake Flask Cultures of Fungi

As the production of ligninase is of prime importance in the degradation of lignin and the preparation of any cell-free biological system for lignin depolymerization, efforts were directed to studies on the production of ligninase in larger-scale incubations. The appearance of ligninase was initially followed in media containing NaOH extracted lignin in the presence/absence of 1.4mM veratryl alcohol. The latter was added as it had been shown to be an inducer of ligninase in cultures of Phan. chrysosporium (7). The presence of veratryl alcohol was not found to stimulate the degradation of lignin or the production of ligninase in shake flask cultures. This was not surprising as it had recently been shown that the addition of Polypropylene glycol 2000 was necessary for the production and release of ligninase by shake cultures of Phan. chrysosporium (6). Therefore, all future experiments also included the addition of PPG 2000 to liquid shake cultures of these organisms. In the presence of NaOH lignin, no ligninase activity could be detected after an incubation time of 14 days for any of the organisms. The experiment was repeated in the presence and

TABLE 2

COMPARISON OF THE DEGRADATION OF ASPEN WOOD AND LIGNIN BY VARIOUS FUNGI

SUBSTRATE	WEIGHT LOSS IN THE PRESENCE OF			
	PHANEROCHAETE CHRYSOSPORIUM	CHRYSOSPORIUM PRUINOSUM	PHLEBIA TREMELLOSUS	TRAMETES VERSICOLOR
Aspen wood	4.59%	4.20%	3.08%	3.13%
Steam exploded wood, 475psi, 3sec.	4.06%	1.56%	1.45%	1.48%
NaOH lignin, 612psi, 45sec	12.90%	27.60%	27.17%	24.08%
Alcohol lignin, 612psi, 45sec.	3.09%	7.30%	6.98%	4.55%
Tomlinite, hardwood Kraft lignin.	6.29%	5.70%	5.19%	0.75%

TABLE 3

DEGRADATION OF ASPEN WOOD AND TOMLINITE
BY OTHER BASIDIOMYCETES

SUBSTRATE	WEIGHT LOSS IN THE PRESENCE OF		
	PLEUROTUS OSTREATUS	PLEUROTUS FLORIDA	LENTINUS EDODES
Aspen wood	No loss	No loss	4.24%
Tomlinite	0.98%	2.58%	10.88%

absence of lignin and the results are shown in Table 4. Only cultures of Phan. chrysosporium and Chrys. pruinosum showed any ligninase activity. The enzyme appeared in the medium after 9-11 days of growth in the absence of lignin and 12-13 days in the presence of lignin. The maximum activity appeared in cultures of Chrys. pruinosum. Activities for both cultures were comparable to those reported in the literature. It must be noted that although triplicate flasks of each culture were prepared, not all flasks showed ligninase activity. The type and size of pellet seemed to affect the production of enzyme. This has been reported by other workers. The presence of lignin appeared to induce higher levels of enzyme in cultures of Phan. chrysosporium. No ligninase activity could be detected in filtrates of Phl. tremellosus or Ir. versicolor even after 22 days of growth. In view of the inability to detect ligninase in the latter organisms work on these organisms was discontinued.

The production of ligninase was further tested under a variety of growth conditions whereby the levels and type of carbon and nitrogen sources used changed. An initial experiment was performed to determine whether the level of nitrogen, as sodium glutamate, had any marked effect on the level of ligninase. The results are shown in Table 5. It is seen that only under conditions of nitrogen deficiency was ligninase activity detected in extracts of Phan. chrysosporium and Chrys. pruinosum. The presence of lignin again caused an increase in the time required for enzyme appearance in Chrys. pruinosum. In view of these preliminary shake flask experiments and the effect of lignin on the appearance of ligninase, it was decided to perform all other shake flasks and fermentor studies in a medium lacking lignin. In addition, as the presence of low nitrogen gave a more reproducible induction of ligninase, a medium containing 0.75g monosodium glutamate /L was used in all cases. At this point in the studies it was noted that the stability of ligninase in cultures was limited and further incubation in the presence of the cells resulted in a rapid decrease in activity. This phenomenon is probably due to the presence of proteolytic enzymes being induced on nitrogen starvation. It was decided to test the effect of storing active cultures at a lower temperature, e.g., $4^{o}C$. When this was performed it was found that activity could be stabilized for up to 7 days with only a minor loss (5%) of activity. This is of importance for the future use of the enzyme in studies on lignin degradation.

In another series of experiments, the effect of varying the type of nitrogen source and/or type of carbon source on ligninase activity was also tested. Therefore glycerol was substituted for glucose as carbon source and ammonium nitrate was substituted for monosodium glutamate. All shake flasks were incubated for 13-14 days and samples of culture were removed at days 11, 12, 13 and 14 for assay of ligninase activity. The results are shown in Tables 6 and 7. It is seen that ligninase was induced under all conditions of growth. In the case of Chrys. pruinosum, growth on glcerol/monosodium glutamate appeared to give a higher yield of enzyme. Production of ligninase by Phan. chrysosporium was similar under all conditions but was delayed in the glucose/ammonium nitrate medium. These results seem to suggest that all these growth conditions could be used successfully for ligninase production and it may be interest to test such media in fermentors in an attempt to stimulate induction under these conditions of growth.

TABLE 4

EFFECT OF ADDITION OF PPG 2000 (100PPM) AND VERATRYL ALCOHOL ON THE INDUCTION OF LIGNINASE

CULTURE	CONDITIONS	TIME OF INCUBATION	LIGNINASE ACTIVITY (UMOL/MIN/ML CULTURE)
Phanerochaete chrysosporium	No PPG, no veratryl alcohol	14 days	0.000
	+PPG + veratryl alcohol	14 days	0.019 (flask 1) 0.034 (flask 2)
	+PPG + veratryl alcohol + lignin	14 days	0.041 (flask 1) 0.044 (flask 2) 0.066 (flask 3)
Chrysosporium pruinosum	No PPG, no veratryl alcohol	14 days	0.000
	+PPG + veratryl alcohol	14 days	0.161 (flask 1) 0.233 (flask 2)
	+PPG + veratryl alcohol + lignin	14 days	0.002 (flask 1) 0.002 (flask 2)

TABLE 5

EFFECT OF LEVEL OF NITROGEN SOURCE CONCENTRATION ON INDUCTION OF LIGNINASE IN CULTURES OF *PHANEROCHAETE CHRYSOSPORIUM* AND *CHRYSOSPORIUM PRUINOSUM*

CULTURE	CONDITIONS	TIME OF INCUBATION	LIGNINASE ACTIVITY (UMOL/MIN/ML CULTURE)
Phanerochaete chrysosporium	0.75g glutamate	16 days	0.036
	0.75g glutamate	20 days	0.003
	7.50g glutamate	16 days	0.000
Chrysosporium pruinosum	0.75g glutamate	16 days	0.076
	0.75g glutamate + lignin	20 days	0.094
	7.50g glutamate	16 days	0.000

TABLE 6

PRODUCTION OF LIGNINASE BY PHANEROCHAETE CHRYSOSPORIUM UNDER VARIOUS GROWTH CONDITIONS

GROWTH CONDITIONS		LIGNINASE (UMOL/MIN/ML CULTURE) AT DAY 11	DAY 13
Glucose + monosodium glutamate	1.	0.001	0.015
	2.	0.069	0.027
	3.	0.051	0.044
Glycerol + monosodium glutamate	1.	0.041	0.019
	2.	0.042	0.043
	3.	0.045	0.015
Glucose + ammonium nitrate	1.	0.000	0.020
	2.	0.000	0.027
Glycerol + ammonium nitrate	1.	0.048	0.039
	2.	0.021	0.036

TABLE 7

PRODUCTION OF LIGNINASE BY CHRYSOSPORIUM PRUINOSUM UNDER VARIOUS GROWTH CONDITIONS

GROWTH CONDITIONS		LIGNINASE (UMOL/MIN/ML CULTURE) AT DAY 10	DAY 12
Glucose + monosodium glutamate	1.	0.090	0.046
	2.	0.101	0.039
	3.	0.112	0.052
Glycerol + monosodium glutamate	1.	0.133	0.039
	2.	0.105	0.066
	3.	0.200	0.196
Glucose + ammonium nitrate	1.	0.066	0.039
	2.	0.082	0.056
	3.	0.097	0.054
Glycerol + ammonium nitrate	1.	0.051	0.032
	2.	0.071	0.009
	3.	0.018	0.063

produced ligninase in the stirred tank reactor. The time course of induction coincided very closely with that observed in the shake flask controls showing that the mechanical stress from the fermentor was having no adverse effect. The level of ligninase activity in the fermentor was almost twice that in the shake flask but this is probably due to the higher biomass concentration in the fermentor. The time course of ligninase induction and decline suggests that great care must be taken in harvesting the cultures at the maximum activity or that some feeding strategy must be devised to prevent the degradation of the enzyme by proteases. The enzyme was separated from the cells and will be concentrated for further analysis of lignin depolymerization in a cell-free system. This experiment is also being repeated.

Further experimentation is therefore necessary to delineate the operating parameters for production of ligninase in stirred tank reactors. It would be of great importance to devise an induction system, e.g., either by nutrient modification which would be independent of mechanical alteration or to minimise the detrimental effect of mechanical stress on the initial stages of spore germination and *in situ* pellet formation in the stirred tank. The latter would eliminate the troublesome step of transferring a pre-grown culture to the fermentor. This becomes extremely problematic if larger scale fermentations are planned. An alternative to this pre-treatment may be immobilization of the spores prior to germination. In this way the spores can be protected from mechanical effects because they will be encapsulated within a polysaccharide matrix or held within a inert particle. Actually some workers have found immobilized *Phan. chrysosporium* to have very active ligninase production (9,10,11). A film of *Phan. chrysosporium* (basically a natural immobilized preparation) has also been used effectively to decolourize Kraft bleaching effluents (12,13).

4. CONCLUSIONS

1. A survey of the literature has shown that a variety of organisms are capable of degrading lignin.

2. Of the fungi chosen only Phanerochaete chrysosporium and Chrysosporium pruinosum reproducibly produced ligninase in shake flask cultures.

3. The presence of PPG 2000, veratryl alcohol and Tween 80 was necessary for the production of ligninase in shake flask cultures.

4. Titres of ligninase were comparable to those reported by other workers.

5. A variety of operating conditions were tested, e.g., effect of inoculum size, mode of aeration and agitation, no ligninase activity could be detected in stirred tank reactors for either Phan. chrysosporium or Chrys. pruinosum.

6. Pre-incubation of Chrys. pruinosum spores in a shake flask prior to transfer to a fermentor resulted in an induction of ligninase that parallelled induction in a shake flask.

7. It appears that mechanical stress on initial spore germination/pellet development is the major contributing factor to the inability to induce ligninase in fermentors.

8. Chrys. pruinosum gives higher yields of ligninase than does Phan. chrysosporium in both shake flasks and fermentors. Chrys. pruinosum also is more reproducible in its production of ligninase.

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