0273-2289/02/98-100/0289/$13.00

Use of Steam Explosion Liquor from Sugar Cane Bagasse for Lignin Peroxidase Production by *Phanerochaete chrysosporium*

Maria Antonieta Ferrara,*[,1] Elba P. S. Bon,[2] and Julio Silva Araujo Neto (in memoriam)[3]

[1]*Faculdade de Engenharia Química de Lorena, Lorena, SP, Brazil; (Present address: Far-Manguinhos, FIOCRUZ, Av. Sizenando Nabuco, 100, 21041-250, Rio de Janeiro, Brazil), E-mail: ferrara@far.fiocruz.br;*
[2]*Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; and*
[3]*Escola de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Abstract

The possibility of using two by-products of the sugar cane industry, molasses and bagasse steam explosion liquor (SEL), for lignin peroxidase (LiP) production by *Phanerochaete chrysosporium* was investigated. For comparison, the fungus was initially cultivated in synthetic media containing either glucose, sucrose, xylose, or xylan as sole carbon sources. The effect of veratryl alcohol (VA) was also investigated in relation to the enzyme activity levels. Results showed that sucrose was not metabolized by this fungus, which precluded the use of molasses as a carbon source. Glucose, xylose, and xylan promoted equivalent cell growth. Enzyme levels in the absence of VA were lower than 28 UI/L and in the presence of VA reached 109 IU/L with glucose and 85 IU/L with xylose or xylan. SEL was adequate for *P. chrysosporium* LiP production as LiP activity reached 90 IU/L. When VA was added to this medium, enzyme concentration increased to 155 IU/L.

Index Entries: *Phanerochaete chrysosporium*; lignin peroxidase; steam explosion liquor; sugar cane bagasse.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Lignin depolymerization is catalyzed by extracellular enzymes of white rot basidiomycetes of which *Phanerochaete chrysosporium* has been the most extensively studied. Potential use of these enzymes include pulping and pulp bleaching, degradation of recalcitrant environmental pollutants, increasing digestibility of lignocellulosics, and bioconversion of lignin to higher value products *(1,2)*.

The ligninolytic system, including lignin peroxidase (LiP), is activated during secondary metabolism, regulated by the availability of nutrients. Literature also indicates that LiP activity level is enhanced by the presence of lignin *(3)*, lignin-related compounds, or low-molecular-weight aromatic compounds such as veratryl alcohol (VA) *(4,5)*.

Several attempts to improve LiP production by *P. chrysosporium* included fungus immobilization *(6–9)*, bioreactor operating conditions *(10,11)*, fermentor design *(12)*, and culture conditions, such as agitation, aeration, temperature, addition of activity enhancers *(13)*. Most of these studies have been carried out in chemically defined N-limited media using glucose as carbon source. There are only few studies dealing with utilization of industrial by-products as carbon sources, such as barley straws, rice husks, and kraft lignin *(14)* and corncob *(15)*.

Brazil is the largest world producer of sugar and alcohol from sugar cane. Therefore, sugar cane industry by-products, such as molasses and bagasse, are abundant and low- ost. Sucrose, which is present in large amounts in sugar cane juice and molasses, could be an attractive carbon source for fungus culture and large-scale enzyme production.

Although a great deal of bagasse is burned for energy generation, since 1982 the Brazilian Alcohol Program has generated large surpluses of this by-product, which allowed its increased use as feed stuff. In 1989, at least 12 sugar and ethanol factories produced steam exploded sugar cane bagasse for use as a basic component of cattle feed *(16)*. Steam explosion treatment produces a mixture of exploded bagasse and a brown liquor—steam explosion liquor (SEL)—which can be separated easily. This liquor, as it has some nutritionally undesirable components, may be advantageously isolated. In this work, we suggest the utilization of SEL as a basic component of a low-cost medium for *P. chrysosporium* culture for two reasons: it contains sugars from hemicellulose autohydrolysis, mainly xylose and xylose oligomers *(17,18)*, which could be used as carbon sources by the fungus; and several aromatic compounds are released during the steam explosion treatment *(19)* and may have some affect in enhancing LiP production.

This investigation examined the possibility of using molasses and SEL for LiP production by *P. chrysosporium*. For comparison, the fungus was also cultivated in synthetic media containing either glucose, sucrose, xylose, or xylan as sole carbon sources. The effect of VA was investigated in relation to the enzyme activity level.

Materials and Methods

Chemicals

All chemicals were reagent grade, except xylan (from oat spelts, Sigma X-0376), which was technical grade. Veratryl alcohol (Aldrich) was used without further purification.

Microorganism

Phanerochaete chrysosporium (ATCC 24725) was kept on 2% malt agar slants. The inoculum was prepared as described by Kirk et al. *(20)*.

Steam Explosion Liquor of Sugar Cane Bagasse

Sugar cane bagasse from São Martinho sugar and ethanol factory (Pradópolis, SP) was treated by steam explosion at 200°C during 5 min as previously described by Kling et al. *(18)*. The treated material was vacuum filtered and the obtained liquor (SEL) was used to prepare different growth media. SEL presented pH 4.3, 4.4 g/L total reducing sugars (TRS), and 1.6 g/L reducing sugars (RS).

Growth Conditions

Glucose, xylose, xylan, and sucrose cultures were carried out in a chemically defined N-limited media modified after Kirk et al. *(21)*. It contained 10.0 g carbohydrate, 2.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.22 g ammonium tartrate, 0.1 g $CaCl_2$, 1.0 mg thiamine, 1.0 mL trace element solution, and 1000 mL 0.01 *M* sodium phtalate buffer pH 4.5. The trace element solution contained (per liter) 1.0 g NaCl, 0.5 g $MnSO_4 \cdot H_2O$, 0.18 g $CoSO_4 \cdot 7H_2O$, 0.18 g $ZnSO_4 \cdot 7H_2O$, 0.10 g $FeSO_4 \cdot 7H_2O$, 0.02 g $AlK(SO_4)_2 \cdot 12H_2O$, 0.01 g boric acid, 0.01 g Na_2MoO_4, and 0.01 g $CuSO_4 \cdot 5H_2O$.

SEL cultures were conducted on three complex media: A, pure SEL; B, SEL plus all components of the chemically defined medium, except sugar and buffer; C, SEL plus 0.22 g/L ammonium tartrate, 0.2 g/L KH_2PO_4, and 1.0 mg/L thiamine. All media had their pH adjusted to 4.5 with 0.1 *N* NaOH solution.

Fermentations were carried out at 39°C; 125 mL Erlenmeyer flasks containing 10 mL of growth medium were inoculated with 2.5×10^6 spores/mL *(20)*. Wherever stated, VA (0.4 m*M*) was added on the day of inoculation. Cultures were flushed with pure O_2 on the day of inoculation and, from d 3 onward, once a day.

Analytical

The culture supernatant was used for the determination of RS, TRS, ammonium N, and LiP activity. RS were determined in the filtered extracellular fluid by the procedure of Nelson *(22)*, using dextrose as standard. In SEL fermentation, TRS were quantified after hydrolysis with 0.4% H_2SO_4 (0.5 atm, 15 min), neutralization with $CaCO_3$ and filtration. Ammonium N

was determined by the method of Fawcett and Scott *(23)*, using NH_4Cl as standard. For the determination of mycelium dry weight and total mycelial nitrogen, mycelia of three culture flasks were washed and dried at 50°C to constant weight. Total Kjeldahl nitrogen was then determined *(24)* (protein = N × 6.25). LiP activity was assayed according to Tien and Kirk *(25)*.

Results and Discussion

Glucose Fermentations

When *P. chrysosporium* was cultivated on the chemically defined N-limited medium containing glucose as the sole carbon source, ammonium N was exhausted and maximum LiP activity was obtained within 2 and 4 d, respectively. Figure 1 shows average data from four experiments and the overall picture agrees well with published results *(21,26,27)*. As expected, VA did not influence the fermentation time course, although it caused a four-fold increase in the maximum LiP activity, which reached 109 IU/L.

The effect of veratryl alcohol on the ligninolytic system has been previously observed by Leisola et al. *(4)* and Faison and Kirk *(5)*. These authors also observed that several other lignin-related compounds substantially enhanced lignin-degrading enzyme production. Research carried out by Cancel et al. *(28)* indicated that VA acts as a stabilizer of LiP activity and not as an inducer of LiP synthesis.

Sucrose Fermentations

Before testing molasses as a low-cost carbon source for *P. chrysosporium* growth and LiP production, some experiments were carried out with pure sucrose. Although Livernoche et al. *(29)* and Duran et al. *(30)* showed that some white-rot fungi (e.g., *Coriolus versicolor* and *Chrysonilia sitophila*) were able to use sucrose as carbon source, our results showed that this disaccharide could not be metabolized by *P. chrysosporium*, since no growth was observed when this fungus was cultured on a chemically defined N-limited medium containing sucrose as the sole carbon source (Table 1). This finding precluded experiments with molasses.

Xylose and Xylan Fermentations

Data from Livernoche at al. *(29)*, Ander and Eriksson *(31)*, and Kirk et al. *(32)* stated that xylose and xylan could be used as C source for *P. chrysosporium* growth and development of ligninolytic activity ($^{14}CO_2$ evolution from [^{14}C]lignin), although no information concerning culture parameters were given.

Our results showed that enzyme production in the absence of VA was low and, in accordance to the glucose results, increased upon addition of VA, reaching 85 IU/L in xylose and xylan cultures (Figs. 2 and 3). According to data presented in Table 1, both xylose and xylan were as adequate for cell growth as glucose, although normalized enzyme levels were 26 % lower.

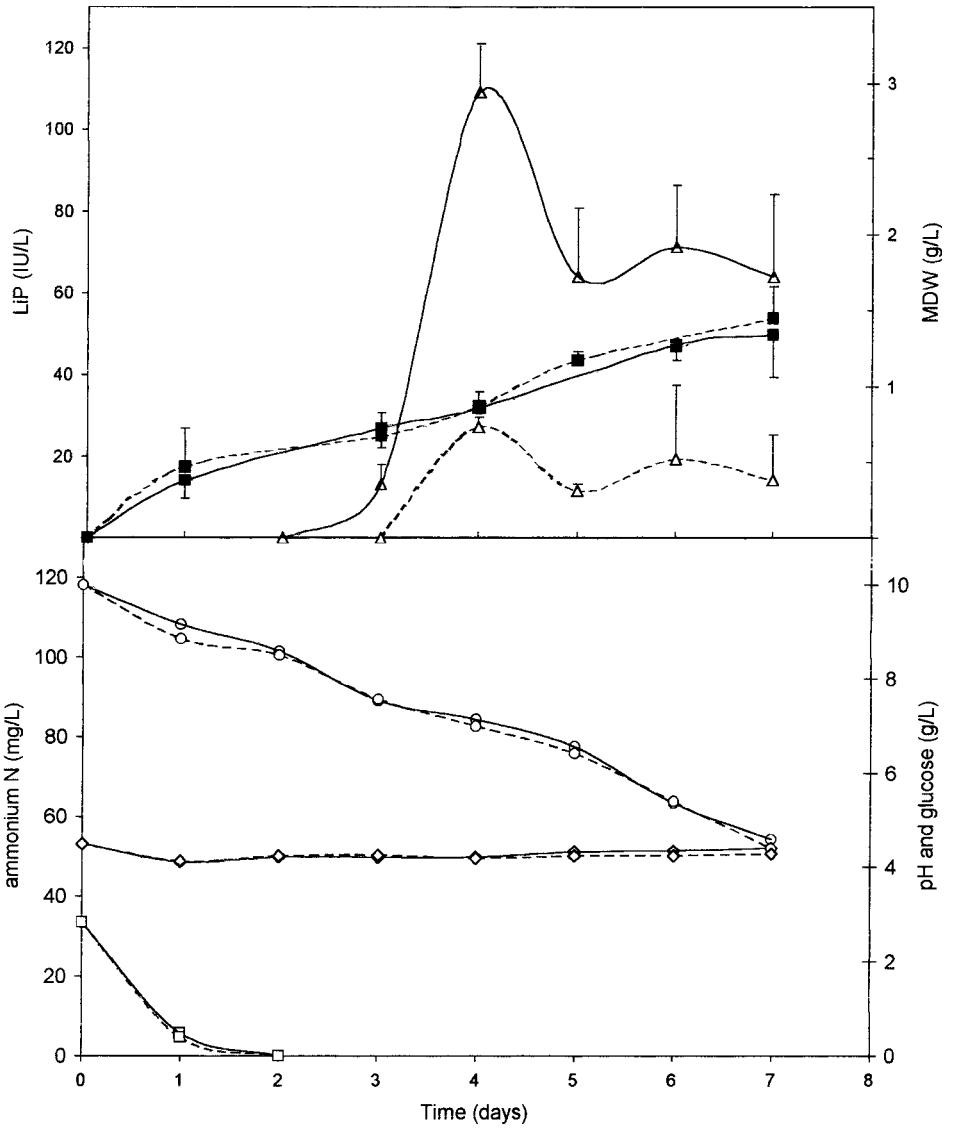

Fig. 1. Time course of *P. chrysosporium* culture on chemically defined N-limited medium containing glucose as C source in the presence (—) and absence (---) of veratryl alcohol. (○) glucose; (□) ammonium N; (◇) pH; (Δ) LiP; (■) mycelium dry weight (MDW).

Considering the fermentation time courses presented in Figs. 2 and 3, enzyme production in the presence of VA was also concomitant to N depletion, within 2 d. Although VA xylose and xylan cultures presented lower enzyme levels in comparison to the VA glucose fermentation, LiP activity was more stable. Peak LiP activity was also observed within 4 d in both cases.

Table 1
Mycelial Concentration and LiP Activity as Related to Mycelial Nitrogen in *P. chrysosporium* culture at d 4 (day of Maximum LiP Activity)[a]

Carbon Source	Mycelial dry weight (g/L)	Mycelium N (g/L)	LiP activity (IU/g mycelial N)
Glucose	0.86 ± 0.02	0.030	3640
Sucrose	0	0	0
Xylose	0.87 ± 0.07	0.031	2720
Xylan	n.d.[b]	0.032	2660

[a]Cultures were grown on chemically defined N-limited medium amended with 0.4 mM veratryl alcohol.
[b]n.d. - not determined.

Xylose consumption rate (Fig. 2) was not so different from that of glucose (Fig.1). Xylan consumption could not be measured since this polysaccharide was not completely soluble in the culture medium.

Sugar Cane Bagasse Steam Explosion Liquor Fermentations

Figure 4 shows the results obtained when *P. chrysosporium* was cultured on nutrient enriched SEL (medium B). As observed with synthetic media, ammonium N was exhausted within 2 d of fermentation. LiP activity was detected in the culture supernatant 2 d after N depletion and peaked at d 5. Although enzyme activity was delayed in relation to N exhaustion, the maximum LiP activity achieved (90 IU/L) was similar to that obtained on synthetic medium supplemented with VA, suggesting that some components of SEL stimulate LiP activity. After d 5, activity decreased rapidly. Mycelial growth, as measured by mycelial N, reached a maximum at d 2. The increase in mycelial dry weight on the following days, also observed on synthetic media (Fig. 1), indicates production of polysaccharides by the fungus.

Our data indicate that not only the sugars, but also SEL acidic components were used as carbon sources by the fungus. This is supported by the high yield factor $Y_{X/S}$ (mycelium weight produced/total sugar consumed) of 2.7 in comparison to the glucose synthetic medium culture yield of 0.4 and by the pH increase at d 1. (In chemically defined medium, pH always decreased in the first day to 4.0–4.2 and then slowly increased to 4.3–4.5, as shown in Figs. 1–3.) Besides sugars (mainly xylose and xylose oligomers, but also arabinose and glucose), bagasse steam explosion liquor contains acetic acid and furfural *(17)*. In addition, the identification of the chemical composition of steam exploded poplar liquor indicated the presence of *p*-hydroxybenzoic acid, siringic acid, *p*-hydroxybenzaldehide, and vanillin *(19)*. Assuming a certain degree of similarity between SEL and poplar liquor, it could be considered that the consumption of these compounds by *P. chrysosporium* would explain the low sugar consumption and pH increase

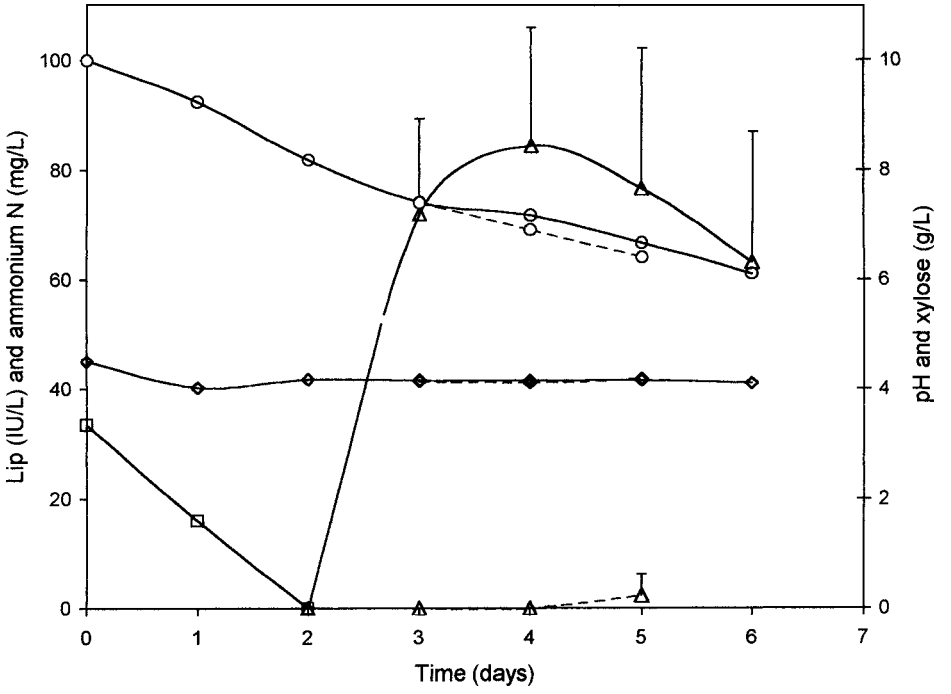

Fig. 2. Time course of *P. chrysosporium* culture on chemically defined N-limited medium containing xylose as C source in the presence (—) and absence (- -) of veratryl alcohol: (○) xylose; (□) ammonium N; (◇) pH; (Δ) LiP.

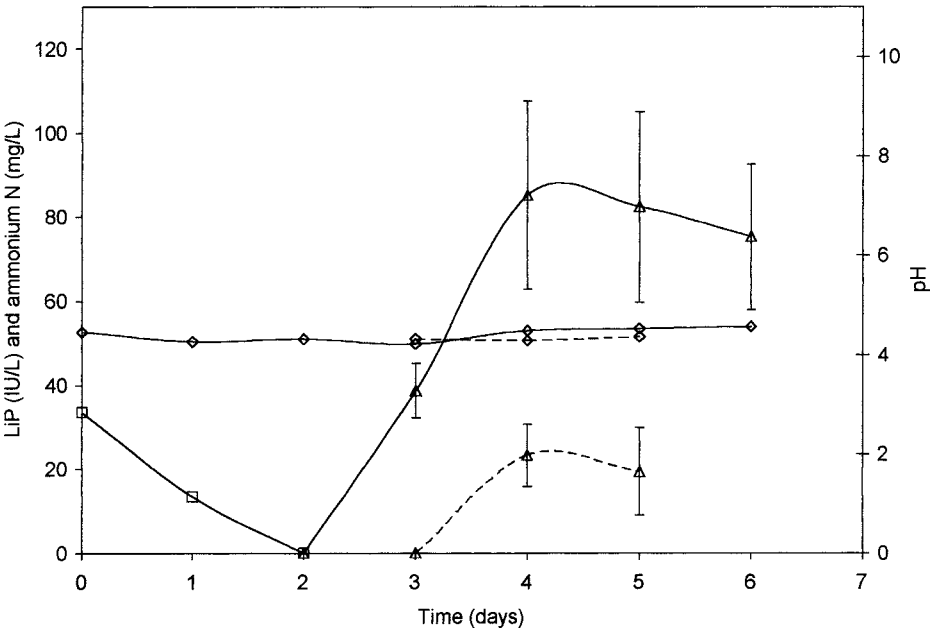

Fig. 3. Time course of *P. chrysosporium* culture on chemically defined N-limited medium containing xylan as C source in the presence (—) and absence (- -) of veratryl alcohol: (□) ammonium N; (◇) pH; (Δ) LiP.

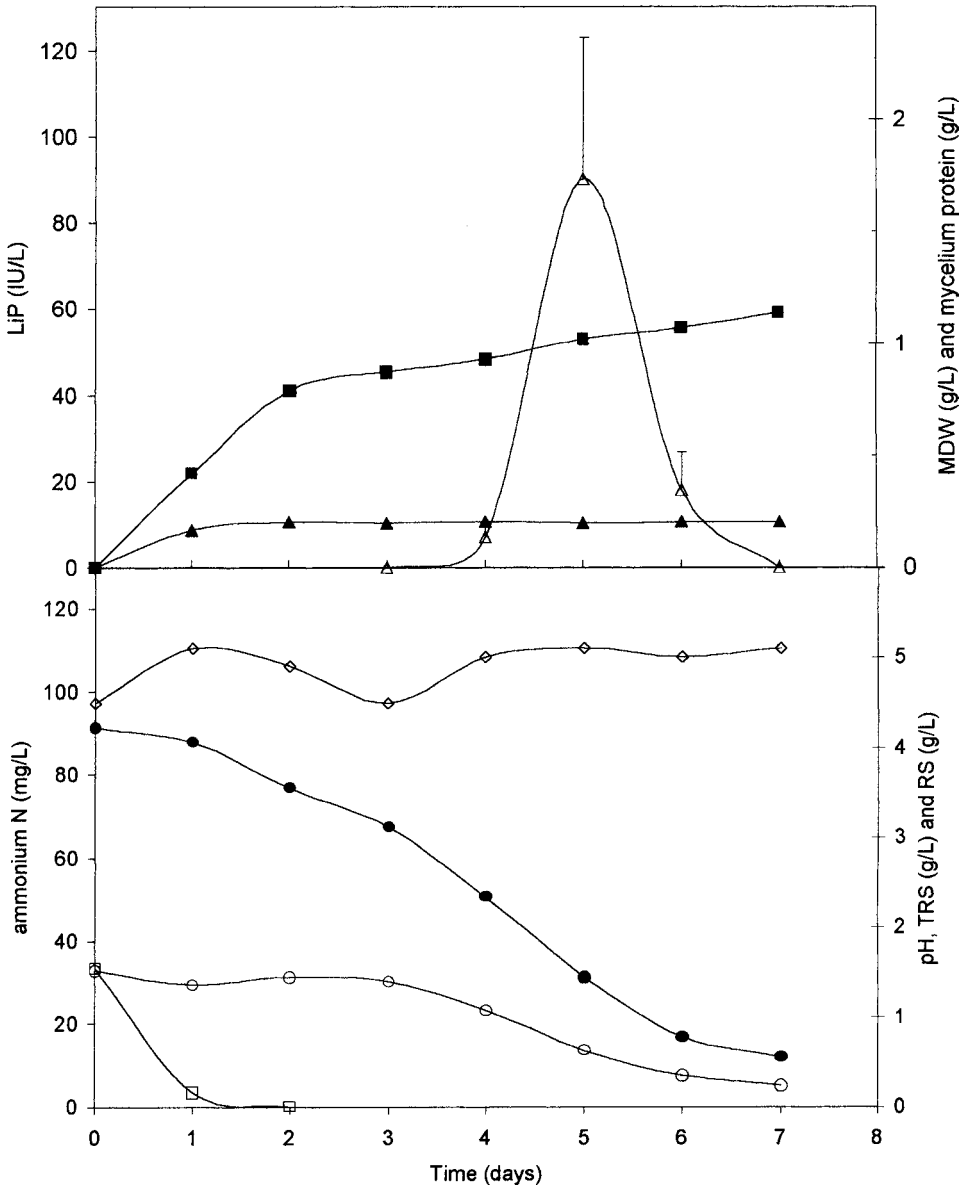

Fig. 4. Time course of *P. chrysosporium* culture on a nutrient enriched steam explosion liquor of sugar cane bagasse (medium B): (○) reducing sugar (RS); (●) total reducing sugar (TRS); (□) ammonium N; (◇) pH; (Δ) LiP; (■) mycelium dry weight (MDW); (▲) mycelium protein.

in the fermentation early stages. Moreover, in accordance to this possibility, the UV absorption spectrum of culture filtrates as a function of fermentation time (Fig. 5) would suggest a consumption of lignin-derived aromatic compounds as can be seen by the decrease in 205 nm and 280 nm peaks, characteristics of lignin compounds *(33)*. Indeed, it has been reported that

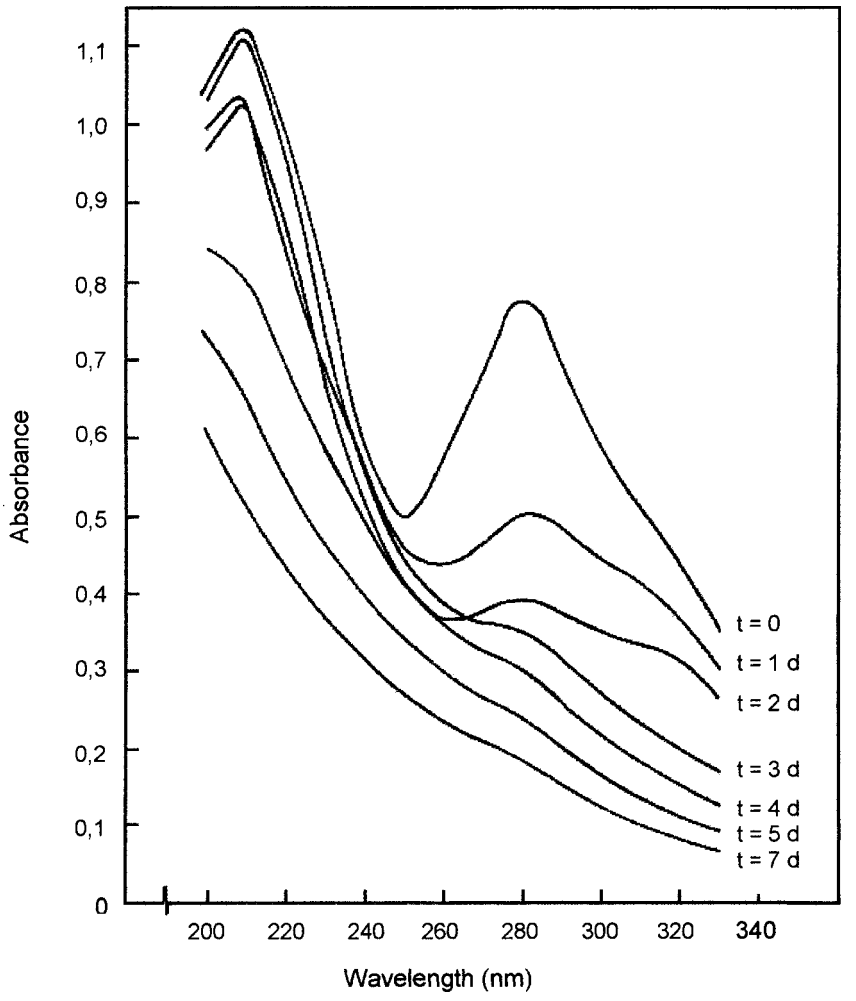

Fig. 5. UV absorption spectra of culture supernatants of *P. chrysosporium* on nutrient enriched steam explosion liquor of sugar cane bagasse (medium B) as a function of time (dilution: 1:50).

the aforementioned compounds are metabolized by *P. chrysosporium (31)*. As LiP activity was only detected after 3 d of fermentation, this drop in UV absorbance could not be related to LiP degradation of the lignin derived low molecular weight compounds.

In an attempt to evaluate the efficacy of SEL components in enhancing LiP activity levels, some experiments were carried out using nutrient enriched SEL (medium B) containing 0.4 m*M* VA. Results (Tables 2 and 3) were very similar to those in the absence of VA (see also Fig. 4), except that enzyme levels were substantially higher in the presence of VA. This finding suggests that either SEL components were not at optimum concentration or that these components and VA have different roles in stimulating LiP

Table 2
Characteristics of *P. chrysosporium* Culture on Nutrient-Enriched SEL (Medium B), Amended with 0.4 m*M* Veratryl Alcohol

Time (d)	TRS (g/L)	pH	LiP activity (IU/L)
0	4.3	4.5	0
4	2.4	5.0	50.9 ± 37.9
5	1.6	5.1	155.0 ± 6.4
6	0.8	5.1	55.5 ± 13.4

Table 3
Mycelial Concentration and LiP Activity as Related to Mycelial Nitrogen in *P. chrysosporium* culture at d 5 (day of Maximum LiP Activity)[a]

Conditions	Mycelial dry weight (g/L)	Mycelium N (g/L)	LiP activity (IU/g mycelial N)
Absence of VA	1.02	0.032	2813
Presence of VA	0.94	0.033	4697

[a]Cultures were grown on nutrient enriched SEL (medium B).

Table 4
Characteristics of *P. chrysosporium* Culture on SEL as a Function of Nutrient Supplementation, after 5 d of Fermentation (Day of Maximum LiP Activity)[a]

Characteristics	Medium A (SEL without addition of any nutrient)	Medium B (nutrient enriched SEL)[b]	Medium C (SEL plus N, P, and thiamin)
TRS (g/L)	4.4	1.4	1.3
pH	4.5	5.1	5.2
LiP activity (IU/L)	0	90.2 ± 32.6	88.7 ± 15.2
Mycelial dry weight (g/L)	0	0.93	0.92
Mycelium N (g/L)	0	0.033	0.033

[a]At beginning: pH = 4.5 and TRS = 4.4 g/L.
[b]Addition of all of the components of the synthetic medium, except sugar and buffer.

activity. LiP production profiles in SEL cultures in the absence and presence of VA showed a sharp peak. From the data collected in this article it would be difficult to explain this apparently enzyme less stability in comparison to synthetic medium fermentations.

Experiments concerning nutritional requirements (Table 4) show that the amendment of SEL with N, P, and thiamine (medium C) is sufficient for *P. chrysosporium* growth and LiP production. The fungus did not grow in pure SEL (medium A).

Steam explosion liquor is toxic to several microorganisms, including baker's yeasts, cellulolytic fungi, and several bacteria *(19, 34)*, and the

inhibitory effects were attributed to lignin and carbohydrate degradation products. However, *P. chrysosporium* growth was not adversely affected by the presence of these inhibiting compounds. Moreover, these compounds seem to increase LiP production. These data open the possibility of using a by-product of the sugar cane industry in *P. chrysosporium* LiP production. It has the advantage of being low cost, selective due to its toxicity to other microorganisms, and adequate source of carbon and enhancers of enzyme activity. Future work would include the replacement of pure thiamine by some low-cost industrial source and the fermentation scale-up using free or immobilized mycelium, aiming at increasing enzyme activity levels.

References

1. Reid, I. D. (1995), *Can. J. Bot.* **73,** S1011–S1018.
2. Breen, A. and Singleton, F. L. (1999), *Curr. Opin. Biotechnol.* **10,** 252–258.
3. Ulmer, D. C., Leisola, M. S. A., and Fiechter, A. (1984), *J. Biotechnol.* **1,** 13–24.
4. Leisola, M. S. A., Ulmer, D. C., Waldner, R., and Fiechter, A. (1984), *J. Biotechnol.* **1,** 331–339.
5. Faison, B. D. and Kirk, T. K. (1985), *Appl. Environm. Microbiol.* **49,** 299–304.
6. Linko, S. (1988), *J. Biotechnol.* **8,** 163–170.
7. Gerin, P. A., Ashter, M., and Rouxhet, P. G. (1997), *Enzyme Microb. Technol.* **20,** 294–300.
8. Buckley, K. B. and Dobson, D. W. (1998), *Biotechnol. Lett.* **20,** 301–316.
9. Carvalho, M. E. A., Monteiro, M. C., Bon, E. P. S., and Sant'Anna Jr., G. L. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 955–966.
10. Ruckenstein, E. and Wang, X. B. (1994), *Biotechnol. Bioeng.* **44,** 79–86.
11. Feijoo, G., Dosoretz, C. and Lema, J. M. (1995), *J. Biotechnol.* **42,** 247–253.
12. Rivela, I., Couto, S. R., and Sanroman, A. (2000), *Biotechnol. Lett.* **22,** 1443–1447.
13. Bosco, F., Ruggeri, B., and Sassi, G. (1999), *Microbios* **98,** 35–49.
14. Darah, I. and Ibrahim, C. O. (1996), *As. Pac. J. Mol.Biol. Biotechnol.* **4,** 154–166.
15. Couto, S. R., Rivela, I., and Sanromán, A. (2001), *J. Chem. Technol. Biotechnol.* **76,** 78–82.
16. Carvalho Neto, C. C. (1989), Natron Consultoria e Projetos S/A, Rio de Janeiro, Brazil.
17. Dekker, R. F. H., Karageorge, H., and Wallis, A. F. A. (1987), *Biocatalysis* **1,** 47–61.
18. Kling, S. H., Carvalho Neto, C. C., Ferrara, M. A.; Torres, J. C. R., Magalhães, D. B., and Ryu, D. D. Y. (1987), *Biotechnol. Bioeng.* **29,** 1035–1039.
19. Ando, S., Arai, I., Kiyoto, K., and Hanai, S. (1986), *J. Ferment. Technol.* **64,** 567–570.
20. Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. (1978), *Arch. Microbiol.* **117,** 277–285.
21. Kirk, T. K., Croan, S., Tien, M., Murtagh, K. E., and Farrel, R. l. (1986), *Enzyme Microb. Technol.* **8,** 27–32.
22. Nelson, N. (1944), *J. Biol. Chem.* **153,** 375–380.
23. Fawcett, J. K. and Scott, J. E. (1960), *J. Clin Pathol.* **13,** 156–159.
24. Niederl, J. B. and Niederl, V. (1942), *Micromethods of Quantitative Organic Analyses*, John Wiley & Sons, NY.
25. Tien, M. and Kirk, T. K. (1984), *Proc. Natl. Acad. Sci. USA* **81,** 2280–2284.
26. Keyser, P., Kirk, T. K., and Zeikus, J. G. (1978), *J. Bacteriol.* **135,** 790–797.
27. Jeffries, T. W., Choi, S., and Kirk, T. K. (1981), *Appl. Environ Microbiol.* **42,** 290–296.
28. Cancel, A. M., Orth, A. B., and Tien M. (1993), *Appl. Environ. Microbiol.* **59,** 2909–2913.
29. Livernoche, D., Jurasek, L., Desrochers, M., and Veliky, I. A. (1981), *Biotechnol. Lett.* **3,** 701–706.
30. Duran, N., Rodriguez, J., Gomez, E., Campos, E., and Baeza, J. (1988), *Biotechnol. Bioeng.* **31,** 215–219.

31. Ander, P. and Eriksson, K. E. (1975), *Sven. Papperstidn.* **18,** 643–652.
32. Kirk, T. K., Connors, W. J., and Zeikus, J. G. (1976), *Appl. Environm. Microbiol.* **32,** 192–194.
33. Jashenkar, H. and Fiechter, A. (1983), *Adv. Biochem. Eng. Biotechnol.* **27,** 119–178.
34. Nishkawa, N. K., Suctlife, R., and Saddler, J. N. (1988), *Appl. Microbiol. Biotechnol.* **27,** 549–552.