Biochemical and molecular characterization of South African strains of *Phanerochaete chrysosporium*

THEODORUS H. DE KOKER[1], JIONG ZHAO[1], DAN CULLEN[2] AND BERNARD J. H. JANSE[1]

[1] *Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa*
[2] *Institute for Microbial and Biochemical Technology, U.S. Department Agriculture, Forest Products Laboratory, Madison, Wisconsin, U.S.A.*

Fifty-five strains of *Phanerochaete chrysosporium* were isolated in South Africa, and screened for indicators of ligninolytic activity: lignin peroxidase (LiP), manganese peroxidase (MnP) and glyoxal oxidase (GLOX). MnP-production as a function of time was followed us all strains. Nine strains were selected for quantification of MnP, LiP and GLOX activities. Statistically significant variation in MnP and GLOX activities existed among the different strains. Under low nitrogen, LiP activity of selected strains showed no significant variation, whereas strian PP25 had significantly increased LiP levels under high nitrogen conditions. Probing genomic DNA with the genes encoding lignin peroxidase *(lipD* and *lip1),* manganese peroxidase *(mnp2)* and glyoxal oxidase *(glox)* showed significant genetic diversity with lignin peroxidase and manganese peroxidase probes, but not with the glyoxal oxidase probe.

An estimated 10^{11}-10^{12}t of lignocellulolytic material is produced annually worldwide and 15-36% of this is lignin (Eriksson, Blanchette & Ander, 1990). Next to cellulose, lignin is the most important renewable material, and it constitutes a barrier that must be breached before natural cellulose is available to other enzymes. White-rot basidiomycetes degrade lignin more extensively and rapidly than any other group of organisms, and *Phanerochaete chrysosporium* Burds. is, by far, the most thoroughly studied species.

Although the exact mechanism of lignin degradation is not yet fully understood, lignin peroxidase (LiP), manganese peroxidases (MnP) and laccases have been implicated in the oxidation of lignin by white-rot fungi (Bourbonnais & Paice, 1990; Eriksson *et al.,* 1990). The activities of both peroxidase depend on H_2O_2 which is produced by enzymes such as glyoxal oxidase (GLOX) (Kersten & Kirk. 1987; Kersten, 1990). As lignin peroxidases were first isolated from *P. chrysosporium* this organism has become a model for describing lignin degradation

Dunng the past 15 yr, hundreds of reports have described the physiology, biochemistry and molecular genetics of two strains of *P. chrysosporium,* BKM-F-1767 and ME-446 (for review see Eriksson *et al.,* 1990; Alic & Gold, 1991; Gold & Alic, 1993; Cullen & Kersten, 1996). The same strains are central to numerous studies dealing with organopollutant degradation and biomechanical pulping (for review see Eriksson & Kirk 1985; Kirk & Chang, 1990: Hammel, 1992; Lamar, 1992).

Although limited in scope, several studies have clearly demonstrated phenotypic variation in natural populations of lignin-degrading basidiomycetes (Blanchette *et al.,* 1988,1992; Blanchette, 1991).

Most strains of *P. chrysosporium* have beers isolated in the Northern Hemisphere although we recently described *P. chrysosporium* in South Africa (Zhao, de Koker & Janse, 1995). in this study we evaluate the ligninolytic and genetic diversity among 55 South Africa strains of *P. chrysosporium.* The LiP, MnP and GLOX activities of nine selected strains, as well as strains BKM-F-1767, ME-446 and INA-12 were quantified on agar media. The genetic diversity of the *lip, mnp,* and *glox* genes of these strains was assessed.

MATERIALS AND METHODS

Fungal strains

P. chrysosporium strains BKM-F-1767 and ME-446 were obtained from the Forest Products Laboratory, U.S. Department of Agriculture, Madison, WI. *P. chrysosporium* strain INA-12 was obtained from the Department of Biology, The Chinese University of Hong Kong, Hong Kong. Local strains of *P. chrysosporium* were isolated by plating *Pinus. Eucalyptus* and indigenous wood samples onto 1-5% (w/v) malt extract agar (MEA) plates containing phenol, benomyl and dichloran (Hunt & Cobb, 1971). All the strains were incubated at 37 °C and maintained on 2% (w/v) MEA slants.

Culture conditions

A modified semirefined medium (DMS) of Buswell, Cai & Chang (1995) was used in this study. To a litre of the basal medium 0-1 g yeast extract 25 mg thiamine. HCl and 1 ml of a trace element solution was added. Trace element solution

contained (l^{-1}): 4·8 g $FeC_6H_5O_7.5H_2O$, 2·64 g $ZnSO_4.7H_2O$, 2·0 g $MnCl_2 4H_2O$, 0-4 g $CoCl_2.6H_2O$ and 0·4 g $CuSO_4.5H_2O$. Nitrogen (NH_4NO_3 and L-asparagine) was added to a final concentration of 2·4 and 24 mM for low (LN) and high nitrogen (HN) media, respectively. The pH was adjusted to 4·5 with 2 M KOH. In solid medium, agar (Biolab, South Africa) was added to a find concentration of 12 g l^{-1}. All strains were incubated at 37°.

Growth rate

The radial growth rate on MEA at 37° was measured. Inoculum consisted of a 5 mm agar plug of a 5-d-old culture grown on MEA at 37°.

Qualitative screening

Phenol oxidase activity was determined with tannic acid as substrate (Bavendamm, 1928). Drop tests for laccase. extracellular oxidase and peroxidase activity were done as described by Nobles (1965), Stalpers (1978) and Molitoris (1978). Drop tests were done with 5-d-old cultures grown on both HN and LN DMS agar media. Manganese peroxidase (MnP) activity was assayed on DMS agar plates supplemented with 0·02% phenol red (Boominathan *et al.*, 1990). General ligninolytic activity was rated on DMS plates supplemented with 0·2% Poly R-478 (Sigma) (Gold, Glenn & Alic,1988). All plates were inoculated with 7-d-old cultures and incubated at 37°.

Quantitative enzyme assays

MnP activity from decolonized zones on LN Poly R-478 agar plates was determined on days 3, 5 and 7 for all 55 strains according to the method of Zhao, de Koker & Janse (1996). One unit of MnP-activity was defined as 1 µmol of Mn^{2+} oxidized to Mn^{3+} min^{-1} ($\varepsilon_{210} = 8{\cdot}1 \times 10^3 M^{-1} cm^{-1}$) (Gold & Glen 1988). Lignin peroxidase and GLOX activities were determined for nine selected strains, as well as BKM-F-1767, ME-446 and INA-12. Lignin peroxidase activity from decolonized zones on low and high nitrogen Azure B plates was determined on days 3, 4 and 5, as described by Zhao et al. (1996). One unit of LiP-activity was defined as 1 µmol of veratryl alcohol oxidized to 1 µmol veratraldehyde min^{-1} ($\varepsilon_{310} = 9{\cdot}3 \times 10^3 M^{-1} cm^{-1}$) Glyoxal oxidase activity was determined using the phenol red method described by Zhao & Janse (1996). One unit of oxidase activity was defined as 1 µmol of H_2O_2 min^{-1} ($\varepsilon_{510} = 2{\cdot}2 \times 10^4 M^{-1} cm^{-1}$) (Kersten & Kirk 1987).

DNA isolation and Southern hybridization

High molecular weight chromosomal DNA of all 55 shains of *P. chrysosporium* as well as strain BKM-F-1767 was isolated as described by Raeder & Broda (1968), digested with *Hin*d III. size fractionated in 0·8% agarose and transferred to Nytran (Schleicher & Schuell). Membranes were probed with *glox* (Kersten & Cullen 1993), *mnp2* (Bogan *et al.*, 1996). *lipl1* and *lipD* (Cullen & Kersten 1996) clones, previously isolated from strain BKM-F-1767. Based on initial RFLPs observed in *Hin*d III digestion, representative strains were also subjected to digestion with *Xba* I/*Bam*H I and probed. Probes were nick-translated to > 5×10^8 dpm g^{-1}. Hybridizations were at 42° in 35% formamide/7% (w/v) SDS/0·25 M Na^+/l mM EDTA with > 3×10^6 dpm of probe ml^{-1}. Following final 0·25 M Na^+ washing at 42° blots were exposed to Kodak XAR film with amplifying screen for 1-7 d.

RESULTS

Isolations and growth rate

Fifty-five strains of *P. chrysosporium* were isolated in South Africa. Two were from indigenous wood samples (KKP10, KKP16), six from *Pinus* spp. and 47 from *Eucalyptus* spp. Radial growth rate showed little variation (31·7-39·7 mm d^1) among the strains (data not shown).

Qualitative screening

Poly R-478 decolonization results are shown in Table 1. Nitrogen concentration of the medium did not affect the weak positive laccase activity observed for all the strains with the method of Molitoris (1978) (data not shown). In contrast, four strains were negative for laccase activity under high nitrogen concentrations when the method of Stalpers (1978) was used Extracellular oxidase activity was observed in all the strains under both LN and HN, while 10 strains showed no peroxidase activity after 5 d on LN DMS media (data not shown). On HN DMS media only eight strains showed a positive peroxidase reaction. Three of these strains (PP25, PC83, BG64B) also showed fast decolonization of Poly R-478 under HN.

Mangasasse peroxidase, lignin peroxidase and glyoxal oxidase activity on agar plates

In Table I and Fig. 1 MnP, LiP and GLOX activities of selected strains are shown as a function of time. All 55 strains showed MnP-activity within the range 0·04-0·23 U g^{-1} agar media. LiP-activities were between 0·029 and 0·078 U g^{-1}, except for strain PP25 on HN media (0·138 U g^{-1}). GLOX activity was between 0·042 and 0·13 U g^{-1}.

Southern hybridizations

RFLP patterns of *Xba* I/*Bam*H I digested genomic DNA from selected strains are shown in Fig. 2 *(a-c).* The *lipl1* blot was characterized by the presence of up to seven bands, of homology that varied in size between 500 and 9000 bp, whereas the *mnp2* blot showed at least four bands that varied in size from 3000 to 9000 bp. All strains showed three bands when *glox* was used as probe and they varied in size from 500 to 3000 bp.

Table 1. Poly R-478 decolorization and MnP, LiP and GLOX activity of selected strains of *P. chrysosporium*

	Poly R-478*	MnP activity†	LiP activity Low N†	LiP activity High N†	GLOX activity†
BKM-F-1767	4 (4)	0·059 ± 0·013 (7)	0·054 ± 0·023 (5)	0·034 ± 0·005 (5)	0·015 ± 0·002 (4)
ME-446	4 (4)	0·130 ± 0·043 (5)	0·042 ± 0·013 (4)	0·051 ± 0·018 (4)	0·013 ± 0·003 (6)
INA-12	4 (4)	0·062 ± 0·024 (5)	0·040 ± 0·018 (4)	0·040 ± 0·017 (3)	0·019 ± 0·008 (7)
KKP10	4 (4)	0·080 ± 0·032 (3)	0·045 ± 0·020 (3)	0·049 ± 0·029 (3)	0·020 ± 0·003 (6)
KKP16	3 (4)	0·086 ± 0·015 (7)	0·049 ± 0·019 (4)	0·063 ± 0·065 (4)	0·022 ± 0·004 (4)
PP25	1 (1·5)	0·180 ± 0·038 (3)	0·049 ± 0·027 (4)	0·138 ± 0·013 (5)	0·022 ± 0·015 (6)
BG64B	2 (2)	0·111 ± 0·007 (5)	0·029 ± 0·005 (3)	0·061 ± 0·036 (5)	0·029 ± 0·003 (7)
BG80	2 (2)	0·167 ± 0·016 (5)	0·051 ± 0·022 (4)	0·058 ± 0·013 (4)	0·021 ± 0·002 (7)
PC79A	4 (4)	0·120 ± 0·038 (3)	0·057 ± 0·049 (4)	0·044 ± 0·005 (3)	0·042 ± 0·005 (7)
PC84	2 (2)	0·090 ± 0·018 (7)	0·050 ± 0·022 (4)	0·038 ± 0·011 (5)	0·013 ± 0·006 (3)
WP24	2 (3)	0·095 ± 0·0086 (3)	0·072 ± 0·015 (4)	0·055 ± 0·004 (3)	0·016 ± 0·002 (5)
H15A	2 (4)	0·130 ± 0·035 (5)	0·041 ± 0·031 (3)	0·078 ± 0·019 (4)	0·016 ± 0·004 (3)

* Day on which decolorization started with values in parentheses for results under HN.

† U g^{-1} solid agar medium are means for triplicate values, and values in parentheses are the day on which maximum activity occurred.

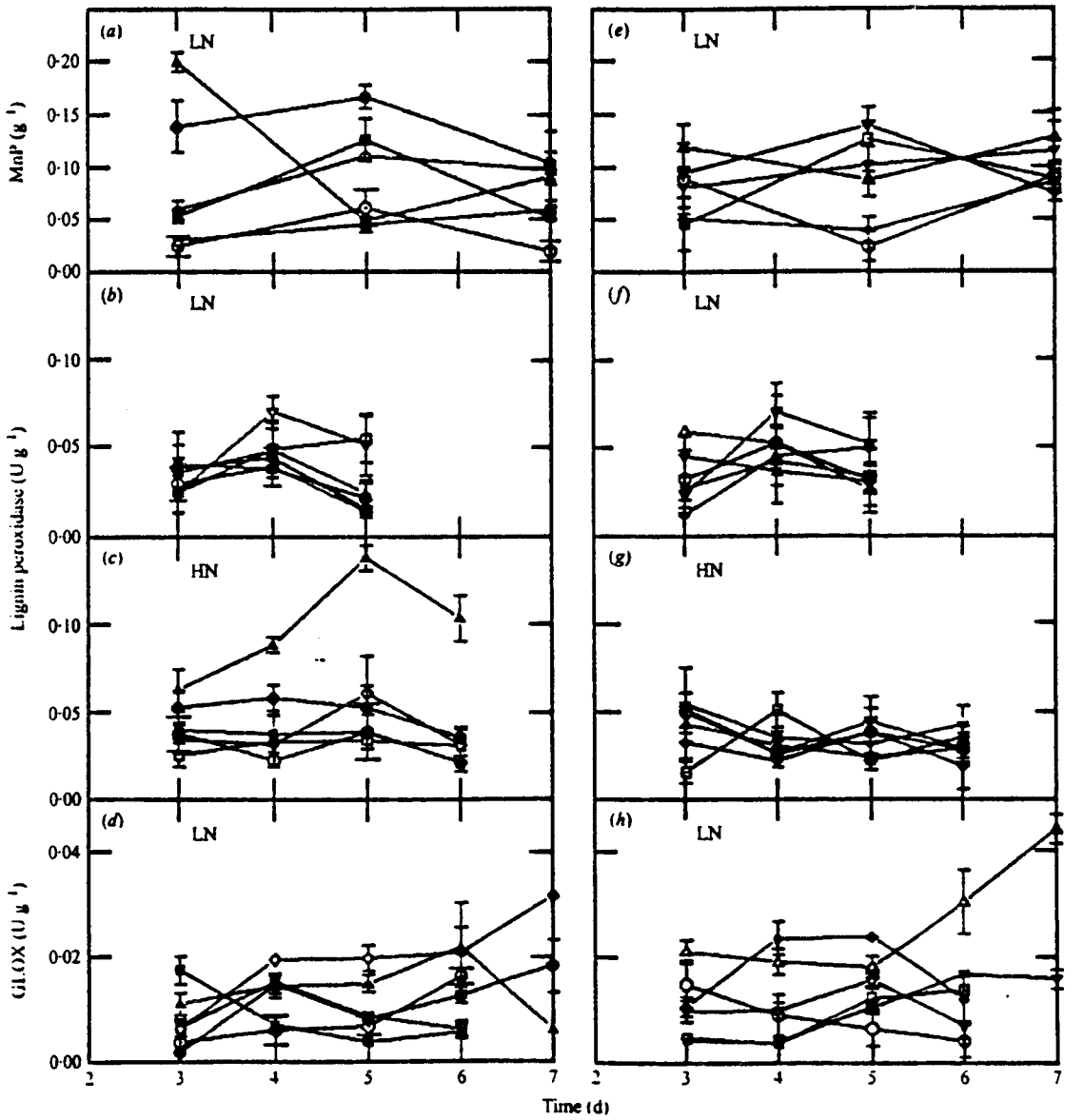

Fig. 1. The course for *(a,c)*. manganese peroxidase activity (LN; *(b,f)*, lignin peroxidase activity (LN); *(c,g)*, lignin peroxidase activity (HN); and *(d,h)*, glyoxal oxidase activity (LN) for *P. chrysosporium* strains BKM-F-1767 (❑), PP25 (▲). BG80 (●) H15A (■), BG64B (◊), INA-12 (○), KKP10 (▼), KKP16 (◆), PC79A(△), PC84 (⊙), WP24 (▽) and ME-446 (⊡). Error bars give standard errors.

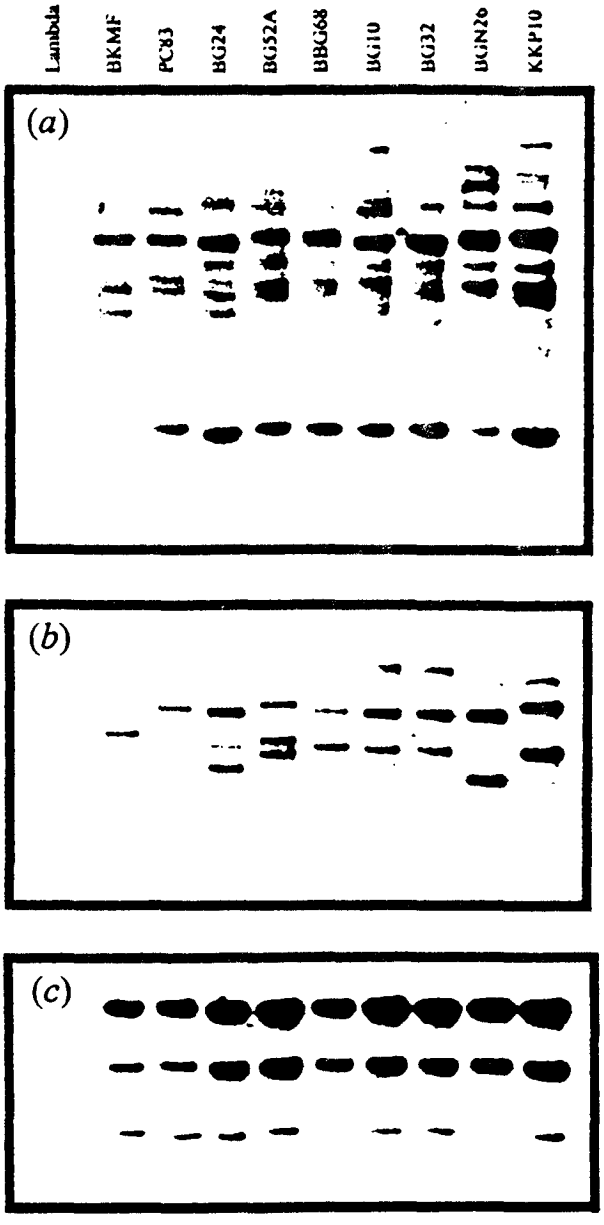

Fig. 2. RFLP patterns of *Xba* I/*Bam*H I digested chromosomal DNA. of selected strains, probed with *(a) lip1:. (b) mnp2* and *(c) glox.* The negative control Lambda DNA size marker with EcoR I and *Hind* III.

DISCUSSION

Enzymic characterization

Qualitative screening of 55 strains of P. chrysosporium indicated variation in the ligninolytic activities among these strains (Table 1). Preliminary results on agar plates indicated uniform LiP production among the strains, whereas MnP and GLOX activities varied. MnP-production for all 55 strains, as a function of time, showed statistitiy significant differences regarding maximum MnP levels. Except for six strains, the South African strains produced higher levels of MnP when compared to strain BKM-F-l767.

Activity of strain PP25 peaked *ca* 4 times higher than BKM-F-1767 (Fig. la). Peak MnP activity occured on day 3 for 18 strains, day 5 for 24 strains and day 7 for 13 strains. The Lip and GLOX activities of nine strains with the highest MnP levels, together with BKM-F-1767, ME-446 and INA-12 were studied further (Table 1 and Fig. 1).

As was suggested by the preliminary study, no statistically significant differences could be detected in LiP activity among the selected strains in LN medium (Fig. 1*b, f).* However, when HN medium was used, certain strains showed an increase in LiP production (Fig. 1*c).* The production of LiP by strain BKM-F-1767 in liquid cultures has been shown to be regulated by C, N and S concentrations (Eriksson *et al.,* 1990; Gold & Alic 1993). Most of the strains we tested peaked later and/or showed lower LiP activity on HN media (Table 1). The overall pattern of LiP production was the same for all the strains including strain BKM-F-1767, thereby suggesting that LiP, MnP and GLOX activities are also produced during secondary metabolism in our strains as was reported for BKM-F-1767 (reviewed by Cullen & Kersten, 1996). Kersten (1990) indicated that GLOX is activated by LiP activity. We observed that on LN media an increase in LiP activity from day 3 to 4 was usually mirrored by an increase in GLOX activity. However, an increase or decrease in LiP activity from day 4 to 5 was not always mirrored by GLOX activity. As discussed by Kersten (1990), GLOX is a minor component of the ligninolytic system in terms of protein content, but has a higher k_{cat} for its substrate than LiP for veratryl alcohol. Thus, once present, the activity of GLOX appears to match the catalytic activity of LiP. Furthermore, Zhao & Janse (1996) showed that in the same culture various H_2O_2-producing enzymes did not peak at the same time.

Of all the enzymes studied, GLOX levels showed the greatest variation between strains (Table 1. Fig. 1*d, h).* Strains PC79A BC64B, BG80, KKP16 and KKP10 produced more GLOX than BKM-F-1767. In three of these strains, however, the GLOX levels peaked only after 7 d incubation. Strain PC79A produced the most GLOX. Thus, differences in enzyme levels and production patterns suggested possible differential regulation of the *glox* genes in different strains.

Genetic characterization

In the intensely studied laboratory strains BKM-F-1767 and ME-446, LiPs and MnPs are encoded by complex families of structurally related genes (reviewed by Gold & Alic, 1993; Cullen & Kersten, 1996). In contrast a single *glox* gene has been identified in these laboratory strains (Kersten *et al.,* 1995; P. J. Kersten & D. Cullen, unpublished results). The multiplicity of *lip* and *mnp* signals among South Africa strains indicates that these peroxidase gene families are a common feature of *P. chrysosporium* strains. Moreover, band sizes are conserved in several instances (Fig. 2*a-c),* suggesting considerable conservation of nucleotide sequences in and around these genes. The *glox* banding patterns were completely conserved among all the strains studied. Some variation between strains (probably allelic) was detected when genomic DNA was digested with *Kpn* I and probed with *glox* (data not shown).

In conclusion, our data demonstrate both enzymic and molecular variation among *P. chrysosporium* strains. Strains vary little with regard to the number and structure of genes

so differences in levels of enzyme activity are probably due to differential regulation of their genes. We have also related a strain which produces relatively high yields of LiP under HN conditions, and which also has elevated levels of LiP, MnP and GLOX when compared to strain BKM-F-1767 under LN conditions. We are further characterizing this strain in an attempt to understand the differential regulation of the *lip* genes.

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