CHEMICAL CHANGES OF KRAFT LIGNIN AND
SOME ENZYMES PRODUCED BY THE WHITE-ROT FUNGUS
CORIOLOPSIS GALLICA

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INTRODUCTION

A great variety of soil microorganisms, among which bacteria
and fungi are included, take part in the transformation and
degradation of plant materials. White-rot fungi are well known
degraders of wood structural components, including lignin. Using
Phanerochaete chrysosporium it has been shown that the lignino-
lytic activity takes place during secondary metabolism, when
cultures starve of nitrogen, sulphur and carbohydrates (Jeffries
et al., 1981; Kirk, 1983), this occurs mainly in fungi during
the autolytic period.

Lignin is a complex molecule, highly resistant to biogical
degradation, constituted by phenylpropane units linked by carbon-
carbon and diaryl ether bonds. A complex enzymatic system con-
stituted by intra and extracellular enzymes is involved in
lignin degradation. In the present work, the white-rot fungus
Coriolopsis gallica was grown for a long period of time in a
liquid medium with and without indulin with the aim of studying
the activities of some extracellular enzymes more or less im-
plicated in the lignin degradation, as well as the resulting
chemical changes in the molecule of this polymer using infrared
spectrophotometry.

MATERIAL AND METHODS

Organism. C. gallica B-3 was isolated from eucalyptus trunk
tree by Dr. C. Saíz-Jiménez.

Lignin. Indulin (pine kraft lignin) was used in these experiments. It was repeatedly washed with distilled water in order to eliminate soluble low molecular weight compounds and other impurities.

Medium and culture conditions. The fungus was grown in a modified medium (Reyes and Byrde, 1973) as follows (g/l): glucose (anhydrous), 2.5; ammonium tartrate, 0.5; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; yeast extract, 0.5; trace elements, 1 ml. The medium was distributed in 20 ml-amounts in conical flasks of 100 ml capacity, and steam sterilized three consecutive days, 20 min each day. Each flask was inoculated with a circular fragment of a 10-12 days old culture of C. gallica grown on malt-agar at 25°C. The flasks were stationary incubated at 25 ^{O}C. This was repeated twice, and culture fluid separated from mycelium by filtration through Whatman No. 1 filter paper on a Büchner funnel and the mycelium washed with distilled water and dried at 70 ^{O}C to constant weight. Culture fluid was made up to initial volume by the addition of distilled water and analysed.

Fungal culture on inert support in the presence of indulin. The technique of Ander et al. (1981) slightly modified was used. Aliquots of 20 ml of culture medium were poured into Petri dishes. Separately, each inert glass fibre disc was impregnated with 20 mg indulin dissolved in 2 ml of a mixture acetone: water (80:20, v/v), the acetone being evaporated at 45 ^{O}C and the support disc placed in the Petri dish, inoculating it afterwards with a 10 mm diameter fungal plug. Two Petri dishes were taken each time. The experiment was repeated twice.

Analytical methods. Total reducing substances were determined according to Somogyi (1945) in conjunction with that of Nelson (1944). Soluble proteins in culture fluid were estimated by the Lowry et al.(1951) method.

Enzyme assays. Laccase (E.C.1.10.3.2.) activity in culture fluid was determined with 0.1 M guaiacol (Merck) at 30 ^{O}C in presence of 0.05 M citrate-phosphate-borate buffer pH 5.0 by the method of Kirk and Kelman (1965). One enzyme unit is the amount of enzyme that oxidizes 1 µmol guaiacol per min and sample[x].

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[x] sample. The amount of culture fluid contained in a flask or Petri dish

Peroxidase (E.C.1.11.1.7.) activity was measured in the same
way as laccase, except that the assay mixture also contained
1 ml of H_2O_2 (final concentration 15 mM) without buffer (Ander
and Eriksson, 1975). One enzyme unit is the amount of enzyme
that oxidizes 1 μmol guaiacol per min and sample. 1,3-β-glucanase
(E.C.3.2.1.6.) was measured by a technique based on the method
of McLelland et al. (1970), using as substrate a solution of
1 mg laminarin/ml at 37 ^{O}C. One unit of enzyme activity was de-
fined as the amount which liberates 1 μmol glucose per min and
sample at 37 ^{O}C. The specific activity for each one of the
studied enzymes expresses the enzyme activity per mg of culture-
filtrate protein.
Degree of autolysis. The degree of autolysis is defined as the
loss (%) in the mycelium dry weight between the incubation day
of maximum growth to the day in which the sample is taken.
IR spectroscopy. Infrared spectra were obtained in KBr discs
using a Perking Elmer 377 spectrophotometer.

RESULTS

    C. gallica obtained its maximum weight at the 13th day of
incubation, coinciding with the exhaustion of the carbon source.
The degree of autolysis amounted to 85%, 76 days after inocu-
lation (Fig. 1). Small changes in the value of the pH were ob-
served during incubation time, the lower value coinciding with
the initiation of the autolytic phase of growth. Proteins in
culture fluid slightly increased their content at the end of
the period of time here studied.
    Laccase in flasks culture (without indulin) was only detect-
ed in small amounts in the first 17 days of incubation; reaching
thereafter the highest activity (25.1 mU/mg) at 48 days of in-
cubation. On the other hand, laccase in Petri dish cultures
could also be detected during the first days of the fungal growth,
until reaching two peaks of activity during autolysis (Fig.2a).
    Peroxidase, from flask cultures, could only be detected in
very small amounts until the 26th day of incubation, its activ-
ity being around the value 2 mU/mg throughout the period
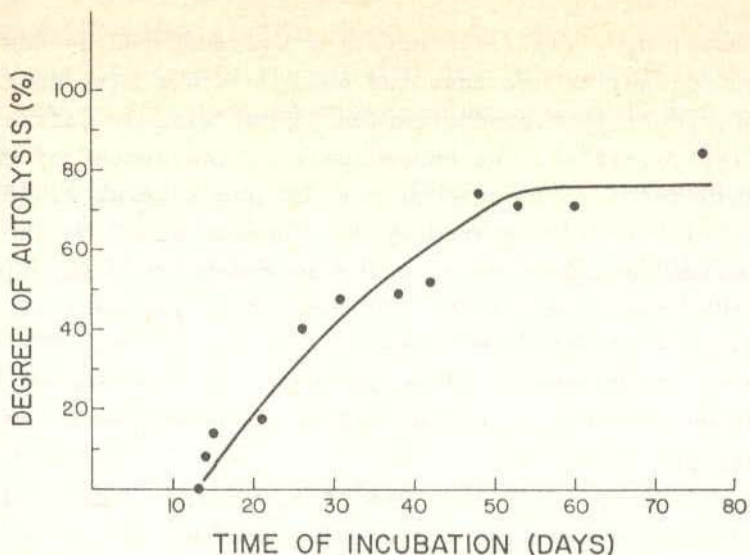studied. In the Petri dishes (in presence of indulin), the syn-

493

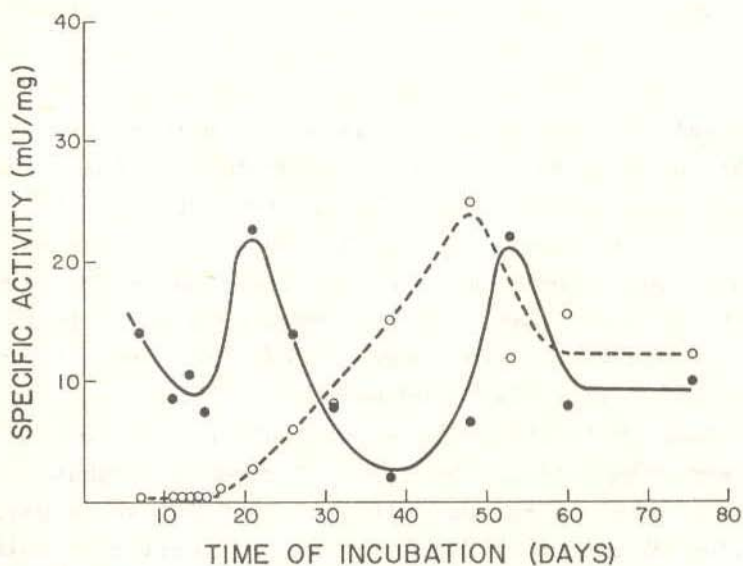*Fig.1.*Variations in the degree of autolysis of C. gallica mycelium during 76 days, in flask culture

Fig.2a Changes in the specific activity of laccase in the culture fluid of C. gallica during 76 days of incubation. -o-o-. flask culture; -●-●-. Petri dishes each containing a glass fibre disc impregnated with 20 mg indulin
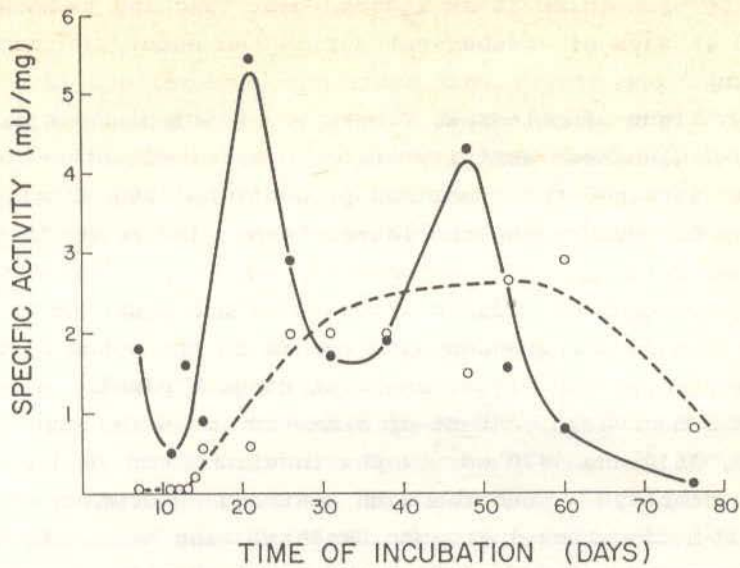
Fig.2b Changes in the specific activity of peroxidase in the
culture fluid of C. gallica during 76 days of incubation.
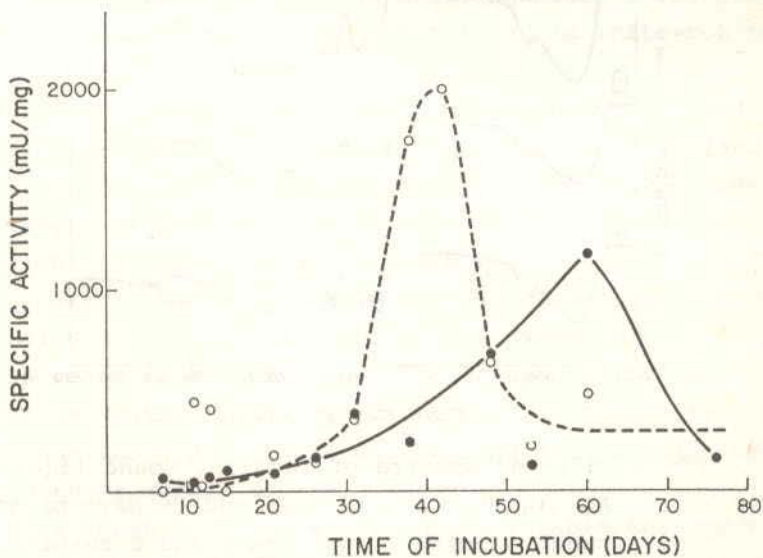-O-O-, flask culture; ●-●- , Petri dishes (with indulin)

Fig.2c Changes in the specific activity of 1,3-β-glucanase in the
culture fluid of C. gallica during 76 days of incubation.
-O-O-, flask culture; -●-●-. Petri dishes (with indulin)

thesis of peroxidase is an induced one, reaching two maxima (21 and 48 days of incubation) during the autolytic period (Fig. 2b).

Both kinds of cultures, flasks and Petri dishes, produced a low 1,3-β-glucanase activity prior to autolysis, the former cultures attained their maximum production (2005 mU/mg) at the 42nd day of incubation, the latter being 1192 mU/mg at the 60th day (Fig. 2c).

The spectrum of indulin after 38 days of culture with C. gallica showed differences with regard to the sound lignin. Thus, the lower intensity of the skeletal rings breathing modes indicates a decreased content of aromatic rings (absorption bands at 1595, 1510 and 1420 cm^{-1}). The indulin after 76 days of culture displays a spectrum with a strong absorption at 1725 cm^{-1} due to C=O of carboxyl groups. Skeletal ring breathing modes also decreased with regard to the sound indulin. Further, absorptions of alcoholic hydroxyls are weaker (Fig. 3).
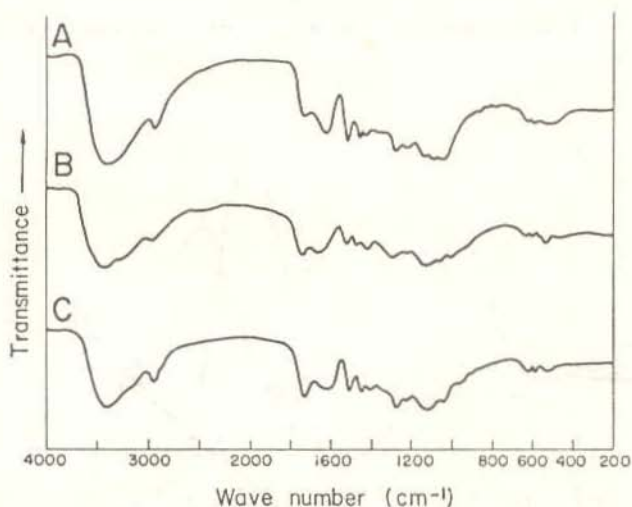

Fig. 3 Infrared spectra of indulin. Sound lignin. A; Lignin after 38 days and 76 days of incubation with C. gallica B and C respectively

DISCUSSION

Several studies have confirmed that lignin and lignosulphonates are degraded by white-rot fungi (Ander and Eriksson, 1975; Hiroi and Eriksson, 1976) and phenol oxidases are important in this process (Ander and Eriksson, 1978). In cultures of C. gallica without lignin we have observed that the secretion of laccase and peroxidase progressively increases during autolysis, until a maximum value is reached. In Petri dish cultures in which the production of phenol oxidases is induced, their appearance takes place during the log and stationary phases, the two highest values of activity appearing during autolysis (Fig. 2). This bimodal distribution, previously observed by Ferm and Cowling (1972) suggests that, at least in a first step, they oxidize the free phenolic hydroxyl groups of lignin, later, the action of other enzymes acting on the oxidized substrates would induce a further increase in the phenol-oxidizing enzymes secretion (Fig.2). In this sense it is possible to indicate that a laccase-glucose quinone oxido-reductase cycle (Green, 1977) and laccase-cellobiose quinone oxidoreductase cycle (Westermark and Eriksson, 1974) have been postulated in white-rot fungi (Iwahara, 1983).

1,3-β-glucanase is excreted by many white-rot fungi (Reese and Mandels, 1959). This enzyme is present in autolyzed cultures, as well as in the cell wall of several fungi (Pérez-Leblic et al. (1982). We have observed in C. gallica that 1,3-β-glucanase is present in small amounts in culture fluid from the first days of incubation, the activity increasing as autolysis proceeds, coinciding with the exhaustion of the carbon source (Fig. 2c). This increase in activity could be attributed to the fact that the fungus synthetizes a greater amount of this enzyme in order to degrade the 1,3-β-glucan of the fungal cell wall, therefore being used as energy source.

It is known that the easily metabolizable carbohydrates can act as repressers for the hydrolytic enzyme synthesis (Ander and Eriksson, 1975). The appearance of 1,3-β-glucanase in cultures of C. gallica in the pre-autolysis would indicate that glucose does not completely repress the action of the enzyme.

Taking into account the complexity of the lignin molecule, it is reasonable to assume that besides the enzymes studied here, some other enzymes will take part in the splitting of aryl-alkyl ether linkages, ring cleavage, etc., participating in the degradative process.

Infrared spectrophotometry has been found useful for the characterization of lignins of diverse origin, as well as to detect changes in the chemical structure of lignins after degradation by fungi (Kirk and Chang, 1974). In general, from the infrared spectra data it can be concluded that a degradation of indulin occurs during the autolytic phase of growth. Main alteration of the lignin molecule is oxidation of the side-chain, which explains the decrease of the alcoholic hydroxyl absorption and the increase of carboxyl, and cleavage of aromatic rings. In a similar way, an oxidation of the C_3-alkyl chain at the $C\alpha$ and C_β positions, and cleavage in the side-chain, mainly between $C\alpha$ and C_β has been suggested for the biodegradation of lignin by Coriolus versicolor (Saíz-Jiménez and de Leeuw, 1984). Further studies on the chemical characterization of sound and degraded lignins are in progress.

SUMMARY

The excretion of extracellular enzymes and the degradation of indulin (pine kraft lignin) by the fungus Coriolopsis gallica were studied. By using a lignin-impregnated glass fibre disc which simulated natural conditions, the fungus excreted phenol oxidases during the log phase of growth and reached two activity maxima in the autolytic phase. However, in absence of indulin the fungus had a different behaviour with respect to phenol oxidases. It was concluded that in the extracellular enzymatic system at least two enzymes, laccase and peroxidase, were involved in the indulin degradation. Evidence of structural changes in the indulin molecule was obtained by infrared spectrophotometry.

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