LIGNIN AS A CARBON SOURCE FOR THE CULTIVATION OF SOME *Rhodotorula* SPECIES

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The paper presents the results obtained in the cultivation of two strains of *Rhodotorula spp*. yeast producing carotenoid pigments, in a medium with different lignin concentrations. Lignins separated from wheat straw (L1) and from Sarkanda grass (L2), provided by Granit Recherche Développement SA, Lausanne, Switzerland, were used for the study. The evolution of yeasts in the culture media in which lignins represented the carbon source was observed as to wet biomass yield, pH and content of carotenoid pigments. At the end of the cultivation process, the residual lignin was recovered by precipitation and subjected to FTIR spectroscopic investigations, to highlight the structural changes caused by these microorganisms. It was found out that biomass yield and carotenoid pigment biosynthesis were influenced by the presence of lignins, which were deeply modified from a structural point of view.

Keywords: *Rhodotorula spp*., lignin, biomass, carotenoid pigments, FTIR spectroscopy

INTRODUCTION

Lignin is one of the main aromatic components characteristic of higher plants. It is chemically bonded to the cellulose and hemicelluloses from the cell walls of plants, and provides structural strength, stiffness and stability to the enzymatic biodegradation of carbohydrates. Lignins are natural polymers with a three-dimensional structure consisting of phenyl propane units, linked together by different types of bonds, some of which cannot be hydrolyzed.[1]

In plants, lignin is synthesized from L-phenylalanine and cinnamic acid through different metabolic pathways,[2] leading to lignin precursors, p-coumaryl-, coniferyl- and sinapyl-alcohols,[3] subsequently transformed into this biopolymer by enzymatic dehydrogenative polymerization. At worldwide level, the lignin resulting from wood and annual pulping or biomass hydrolysis technology could be considered as a material with high-recovery potential, due to its origin from renewable resources and low price.[4]

The characterization of lignin is a very difficult task, as due to its diversity in terms of origin and methods of separation.[5] In the case of lignin, FTIR spectroscopy is recommended as an analytical tool for qualitative assessment of the structural changes caused by the action of chemical and biochemical agents.[6] Compared to conventional chemical analysis, this technique requires a limited number of samples and a short analysis time.[7]

Lignin has a high biological stability as, due to its complex structure, only several types of microorganisms can degrade it.[2] Biodegradation is caused by the enzymes that initiate its extra-cellular oxidative depolymerization. These enzymes, responsible for the generation of non-specific free radicals with high reactivity, attack the C–C bonds connecting the structural units of lignin.[8] Although the literature presents some species of yeast that degrade lignin monomers, such as *Rhodotorula graminis*, *Geotrichum klebahnii*, *Rhodotorula mucilaginosa*, this class of microorganisms is not considered capable of degrading the lignin macromolecule. At the same time, scarce information is available on yeast interaction with lignin.[9]

According to the studies of Chen X. *et al*.,[11] *Rhodotorula spp.* are able to metabolize lignin in the presence or absence of glucose through reactions of oxidative and demethoxylation degradation,[12] for further uses in practical applications. The main products involved in lignin degradation are vanillin, guaiacol, protocatechuic acid and 4-hydroxybenzaldehyde.[11]

Starting from the information available in literature, the present study analyzes two types of lignin from annual plants, as carbon sources for the cultivation of two strains of *Rhodotorula spp*. yeast. The influence of lignin solutions with different concentrations (as a function of their origin) on yield biomass and concentration of carotenoid pigments was studied. Lignin's transformations under the action of microorganisms have been further investigated by FTIR spectroscopy.

EXPERIMENTAL

Materials

Two types of lignin, offered within the Ecobinders European program (FP6), by the Granit Recherche Développement SA Company, Lausanne, Switzerland, were employed, namely, residual lignins isolated through the alkaline delignification of annual plants: wheat straw (L1) and Sarkanda grass (L2). The characteristics of these lignins are presented in Table 1.

Table 1
Characteristics of lignin samples L1 and L2

Characteristics	L1	L2
Acid-insoluble lignin, %	90	87
Acid-soluble lignin, %	1.0	2.0
COOH, mmole/g	3.8	3.3
Aromatic OH, mmole/g	1.7-1.8	1.8-1.9
OH/C9 (groups chemical method)	1.02	1.07
OH/C9 (FTIR)	1.06	1.05
pH (10% dispersion)	2.7	3.2
Mw	3510	4310
T softening, °C	170	163
Solubility in furfuryl alcohol, %	88.5	84
Solubility in aqueous alkali, %	98.5	98.5
Ash, %	2.5	4.1

Microorganisms

Two different yeast strains of *Rhodotorula spp*., denoted as R1 and R2, selected and purchased by the Biotechnology Applied in Food Industry – Bioaliment Integrated Center for Research and Education, "Dunarea de Jos" University, Galati, were cultivated. Prior to the experiment, the yeast was cultivated in a medium with the following composition: 10 g/L glucose, 5 g/L peptone, 3 g/L malt extract, 3 g/L yeast extract. Fermentation was carried out using a thermostated stirring platform for 48 h, at 27 ºC and 120 rpm. The cells were recovered by centrifugation at 5000 rpm for 15 min, washed twice with distilled water and inoculated on a culture medium containing lignins.

Methods

Preparation of lignin suspensions

Lignin suspensions were prepared in distilled water, of 0.1 and 0.5% concentration, and their pH value was adjusted to 6 using 0.1 N NaOH solutions, which allows some lignin to turn into solution and affords suitable conditions for the growth of microorganisms. After 24 h, the undissolved residue was recovered, to establish the concentration of dissolved lignin at pH 6. After the measurements, the degree of lignin dissolution was of about 17%.

Preparation of culture medium

The culture medium used to cultivate the two yeast strains had the following composition: 1 g/L glucose,[13] 5 g/L KH_2PO_4,[14] 1 g/L $(NH_4)_2SO_4$,[15] 0.05 g/L $MgSO_4 \cdot 7H_2O$,[15] 5 g/L yeast extract.[14] For pH correction, solutions of HCl 6N[14] or NaOH 0.1 N were used.

The culture medium components were dissolved in the solution of lignin, in the concentrations mentioned above, then sterilized at 110 °C for 20 min. After sterilization, the medium was distributed (100 mL) into 250 mL Erlenmeyer flasks and inoculated by the procedure described above.[16]

Fermentation lasted for over 4 days, the amount of wet biomass and the pH value being determined every 24 h. At the end of the experiments, residual lignin was recovered through precipitation from broth, and characterized by FTIR spectroscopy. The amount of wet biomass recovered from the culture medium was used for the extraction of carotenoid pigments.

Lignin recovery from culture medium

The lignin from the culture medium was separated through precipitation with HCl 6N at pH 1. The precipitate was recovered by centrifugation, washed with distilled water and dried. The separated lignin was characterized by FTIR spectroscopy.

Extraction of carotenoid pigments

The amount of wet biomass resulting from centrifugation was treated with 3 mL DMSO, and left at -20 ° C for 24 h,[17] after which it was subjected to sonication for 15 min[18] and centrifuged, the supernatant being recovered in a centrifuge tube. This procedure was performed 3 times, to destroy the whole yeast cell wall. After the treatment with DMSO, residual biomass was treated with acetone until it remained colorless. The phases separated by acetone were mixed with those obtained with DMSO. In the tube containing both phases, 20% NaCl and 2 mL hexane were added, for achieving liquid-liquid extraction, until hexane remained colorless. The hexane phases were collected and brought to the volumetric flask, to report the concentration of total carotenoid pigments. After extraction, the samples were stored at -20 ° C until the UV-VIS analysis.[19]

Analysis techniques

FTIR spectroscopy

FTIR spectroscopy can be applied to monitor the functional changes occurring in the structure of degraded lignin, comparatively with the original samples. The spectra were recorded in KBr pellets in a ratio of 1:200, using an Excalibur FTS-2000 DIGILAB spectrometer. The working parameters were the following: spectral range – 4000-400 cm^{-1}, resolution – 4 cm^{-1}, number of scans -20. FTIR spectra were processed with a specialized program from the *SpectraManager* series.

UV-VIS measurements

Carotenoid pigment concentration was determined by reading sample absorbance at 450 nm, on an UV-VIS spectrometer. Concentration calculation was performed using a standard curve of β-carotene in hexane,[20] and the concentration of carotenoid pigments was expressed in μg pigment/g dry biomass.[19]

RESULTS AND DISCUSSION

Influence of lignin on wet biomass yield

According to previous studies, the yeasts of the *Rhodotorula* genus used in these experiments have proved their ability to assimilate some aromatic compounds, such as vanillic, ferulic and L-malic acid or polyphenols separated from different renewable sources.[16,21] The afore-mentioned compounds are usually associated with lignin degradation (*e.g.* vanillic acid, gallic acid) from plant biomass. However, not all strains tested by Libkind D. *et al.* in 2004 were able to degrade these molecules, which do not occur in a free form in nature, nor are normally encountered in culture media.[22]

Therefore, the present study focused on the behavior of two species of yeast towards the lignins introduced in the culture medium, and their ability to use lignin as a carbon source, while glucose concentration was reduced. Figure 1A presents the yield variation of wet biomass, resulting in the cultivation of strain R1 in environments containing lignin L1, which was found in concentrations of 0.1 and 0.5%. The data obtained show that, at low concentrations of lignin from wheat straw, a lower yield in wet biomass is observed (about 16 g/L at 72 h), which increases to 17 g/L, along with increasing lignin concentration to 0.5%.

The R1 strain behaved very differently in the culture medium in which lignin from grass (L2) was introduced, recording a lower amount of biomass, as compared to the previous case. At a concentration of 0.1% lignin in the culture medium, the highest wet biomass yield is obtained at the end of the fermentation process – approximately 14 g/L wet biomass (Fig. 1B). Further addition of lignin is favorable for the multiplication of microorganisms. Therefore, one can estimate that strain R1 acts differently on the two types of lignin, due to their characteristic structural aspects.

In terms of the R2 strain action upon lignin, a different behavior was observed, comparatively with that of strain R1. Thus, in the first case, it was found that lignin was more rapidly degraded, which resulted in a biomass yield of 12 g/L after the first 24 h, at a 0.1% concentration of L1 lignin. At a concentration of 0.5% L1, a longer time was necessary for the yeasts to accommodate to the existing substrate, after which lignin consumption began, so that, at the end of the experiment, 17 g/L of wet biomass were obtained (Fig. 2A).

Figure 2B shows the results of R2 strain cultivation in a culture medium in which various amounts of lignin from grass (L2) were dissolved. Like in the other experiments, one may also notice the structural features of lignin, and hence, that microorganisms evolve faster at lower lignin concentrations (0.1%), amounts of 15 g/L biomass resulting at the end of the process. At high lignin concentrations, it is more difficult for the yeast to accommodate, while the biomass yield is lower – this concentration is not

recommended for maintaining the growth of the microorganism noted R2.

One can appreciate that grass lignin exhibited a slightly inhibitory character on the two strains of yeast, which resulted in a lower yield in biomass, compared to the one reported for the introduction of the product from wheat straw into the culture.

Variation of pH during yeast cultivation processes

The introduction of wheat straw lignin into the culture medium of yeast R1 does not significantly influence pH variation (Fig. 3A), which ranges between 5 and 6. A slight decrease in pH could be associated with the formation of acidic products through lignin degradation and their subsequent metabolization, the pH value rising back to 6 towards the end of the experiment. The same situation seems to be characteristic of grass lignin (Fig. 3B).

In the first 24 h of R2 strain cultivation, the pH slightly drops; the decline can be also associated with the degradation of glucose present in the culture and with its introduction into the metabolic system (Fig. 3C). Subsequently, the pH approaches 6, yet these variations cannot be correlated with biomass yield. Very low variations in the pH of strain R2, growing in a grass lignin-containing medium, can be observed (Fig. 3D).

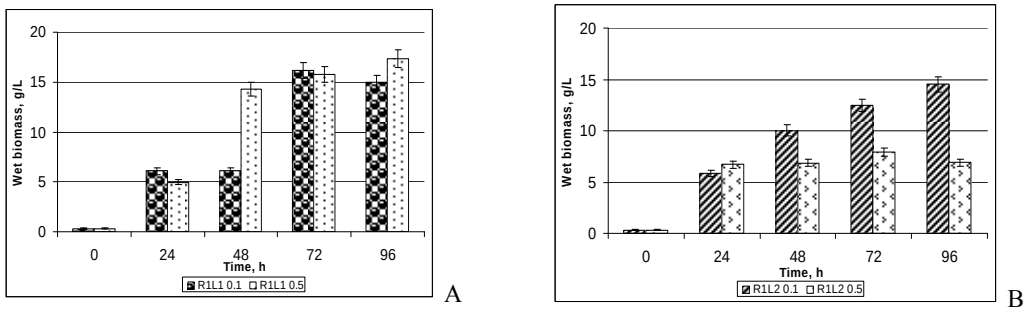

Figure 1: Variation of biomass yield resulting from R1 strain cultivation in L1 and L2 lignin-containing media

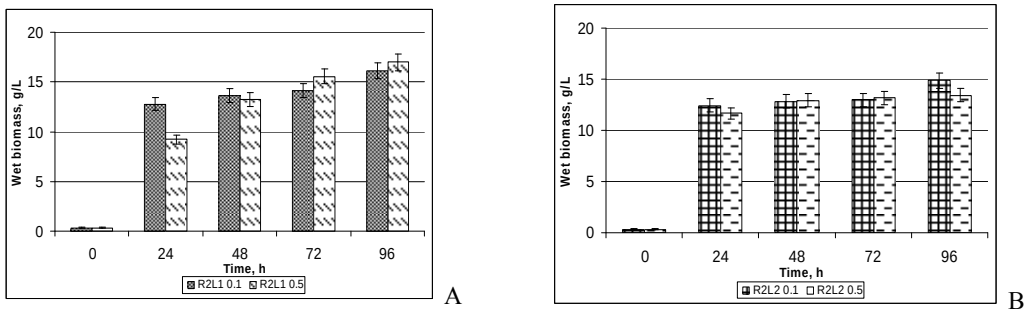

Figure 2: Variation of biomass yield resulting from R2 strain cultivation in L1 and L2 lignin-containing media

Variation in the concentration of carotenoid pigments synthesized by yeast strains

Until recently, carotenoid pigments were extracted from some plants (paprika and saffron processed to dry powder). Hence, the production of natural dyes from plant sources has declined, because of the decrease in plant material, the changes of climatic conditions, and also because of the quality of the final product. The international market for carotenoid pigments has focused more on chemically synthesized compounds, however, the demand for natural products is constantly increasing.[23] Therefore, in recent years, a keen interest has been manifested for carotenoids obtained by microorganism cultivation.

The biosynthesis of carotenoids from different red yeasts recommends these microorganisms as a potential source of pigments, causing a huge interest in biotechnology research,[22] given the

dependence of the quantitative and qualitative composition of carotenoid pigments on species, substrate and growth conditions applied.

The data provided in this study showed the conditions under which lignin may be the used as the main source of carbon for the biosynthesis of carotenoid pigments by the two species of yeast. Thus, it has been found out that strain R1, cultivated in a medium containing lignin L1, does not produce the biomass from which a high content of carotenoid pigments could be extracted (Fig. 4A), the largest amount of pigments being biosynthesized for the fermentation produced in the presence of 0.5% L1 in the culture. Grass lignin has an important inhibitory effect upon the biosynthesis of carotenoid pigments, which explains the very low concentrations recorded at the end of the fermentation processes (Fig. 4B; R1L2 0.1% and R1L2 0.5%).

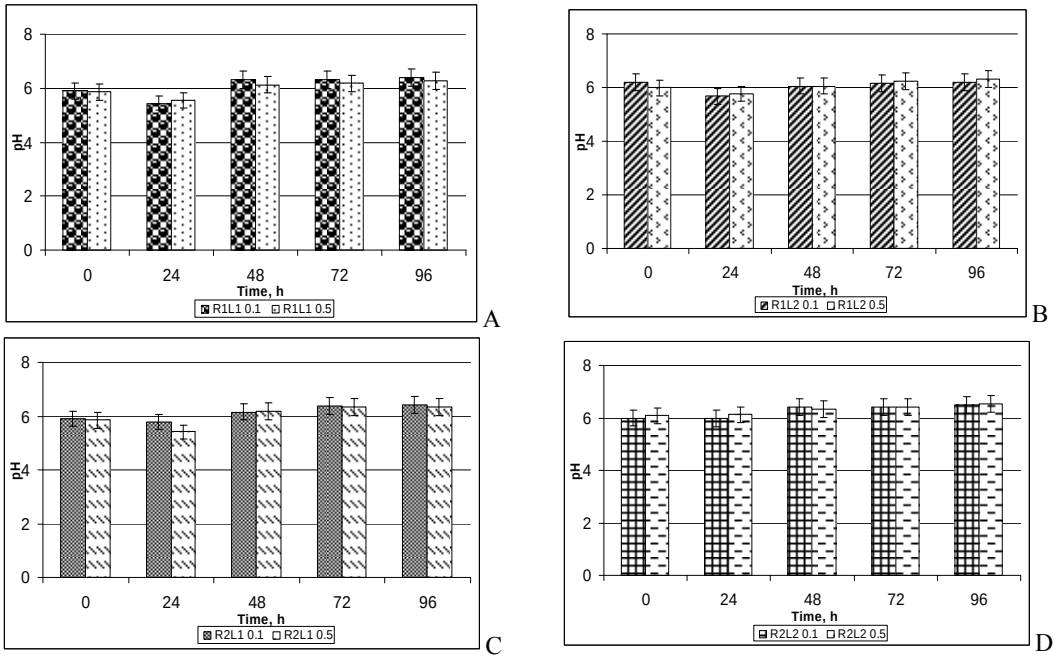

Figure 3: Variation of pH during cultivation of the two microorganisms on lignin substrates: A – R1L1; B – R1L2; C – R2L1; D – R2L2

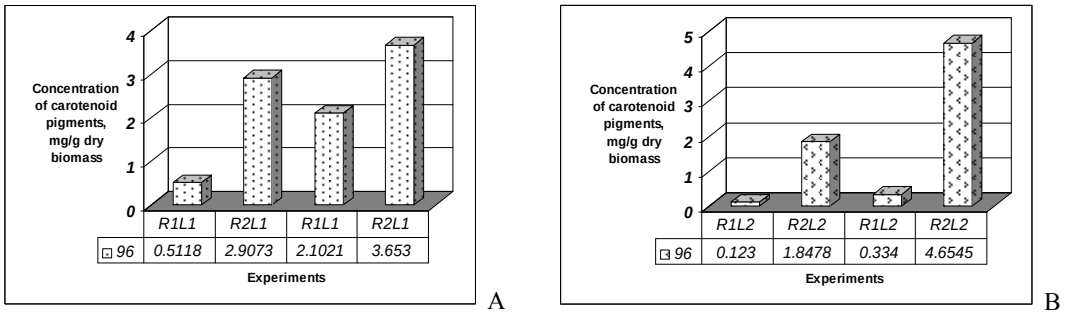

Figure 4: Variation of carotenoid pigments content extracted from biomass resulting at the end of fermentation processes (A – R1L1 and R2L1; B – R1L2 and R2L2)

The R2 strain grown in a medium containing wheat straw lignin produces a positive transformation and a positive assimilation of the products in the process of biosynthesis of carotenoid pigments (Fig. 4A). A maximum concentration is recorded when 0.5% lignin is present in the culture medium. The value obtained is much higher than that found for strain R1, at the same concentration of lignin in the culture medium. The R2 strain grown in media

containing grass lignin (L2) biosynthesizes the maximum amount of pigments at a 0.5% concentration of natural polymer, comparatively with strain R1, whose pigment concentration is very low: 0.334 μg/g dry biomass (Fig. 4B).

Structural changes of lignin under the action of yeast

As generally known, lignin has a very complex structure, which varies with the plant species, method of separation and modifications that may induce particular characteristics. In terms of functional groups, lignin contains in its structure at least three basic types of functional groups: methoxyl, hydroxyl (alcoholic and phenolic) and propane side chain.[4] Thus, after introducing lignins into the culture medium, the yeast enzyme systems act on its structure. All these changes, produced within the hydroxyl, carbonyl, carboxyl, methyl groups, and even in the structures of benzene, syringyl and guaiacyl units, were demonstrated by FTIR spectra.

Figures 5 and 6 present the FTIR spectra recorded for the wheat straw lignin (L1) present in the culture medium in different concentrations, and recovered from it at the end of R1 and R2 yeast cultivation. Table 2 lists the main characteristic absorption bands of the analyzed samples.

The analysis of the recorded spectra shows that, in the 3423-2846 cm^{-1} region, characteristic vibrations of the aromatic C-H bonds, aliphatic and phenolic hydroxyl groups may occur.

The control lignin sample (L1) shows a higher intensity of the absorption peaks in this area because, according to the data listed in Table 2, characteristic signals at 3423 cm^{-1} are present only in control lignin.

Absorption bands at 3337-3246 cm^{-1} are evident only for the samples of lignin recovered from the culture medium of strain R1. The presence of these bands may be due to the influence of the microbial activity in the culture medium on the –OH phenolic groups.

The 1710-1600 cm^{-1} area includes the ether links and carbonyl groups related to the aromatic link. Thus, at the level of these signals, only small differences in the intensity of the absorption bands are recorded. Significant changes occur in the 1330-1370 cm^{-1} absorption range, where guaiacyl and syringyl vibration units are present in the lignin structure in different ratios, as depending on plant species, on the changes in its structure and on the production process. The presence of absorption bands in the 1300-900 cm^{-1} range is considered specific to the C-O links of secondary alcohols, esters or ethers, as well as to the C-C bonds and their distortion, very low differences being observed in the intensity peaks. The conclusion to be drawn is that the R1 strains enzyme systems do not attack these bonds.

The same changes can be observed for the L1 lignin recovered from the culture medium of strain R2 (Fig. 6). The 2835-2938 cm^{-1} range[24,25] is an area in which the absorption of the corresponding vibration of the methoxyl groups linked to the aromatic ring and of the aliphatic methyl groups occurs, and the vibration bonds of the specific –CO links of unconjugated ketone, carbonyl and ether groups are present. For the control sample (L1), (Fig. 6), one may observe very well-defined peaks. For the lignin samples recovered from the culture broth, this area is affected by a decreasing peak intensity, up to complete disappearance (Table 2).

These observations could be correlated with the demethoxylation of the lignin aromatic rings by the enzyme systems characteristic of yeast strains R1 and R2, or by the attack on the –C-O bond of the lignin L1 monomers. Similarly with the previous case, in terms of the spectral data recorded for lignin L2, one can observe significant changes, caused by these two strains. Figures 7 and 8 and Table 2 present in detail the changes produced in the structure of grass lignin.

Table 2
FTIR absorption bands characteristic of the studied lignin samples

	Samples									
	L1	R1L1 0.1%	R1L1 0.5%	R2L1 0.1%	R2L1 0.5%	L2	R1L2 0.1%	R1L2 0.5%	R2L2 0.1%	R2L2 0.5%
Absorptio	3412	3244	3396	3257	3238	3369	-	-	3265	-
	-	-	3215	-	-	-	3261	3126	-	3115
	2939	2937	2945	2939	2941	2933	2933	2931	2931	2941

2848	-	-	-	-	2850	-	-	-	-
1707	-	1703	-	-	1701	-	1695	-	-
1600	1658	1616	1654	1654	1647	1654	1651	1674	1699
1512	-	-	-	-	-	-	-	1651	1652
-	1514	1512	1517	1514	1512	1516	1514	1516	1514
1462	1456	1458	1454	1456	1460	1456	1458	1458	1460
1425	-	1423	1408	1408	1425	1429	1425	1423	1408
1328	-	-	-	-1	1328	-	1332	1330	-
1265	1274	-	1278	127	1265	1273	-	-	1269
1220	1222	1217	-	1215	1220	1220	1219	1220	1220
-	1168	1163	1166	1161	-	1172	-	1166	1141
1124	-	1124	-	1188	1124	1122	1124	1124	1128
1033	1078	-	1076	1080	1035	1076	1035	1060	1078

L1 – wheat straw lignin; L2 – Sarkanda grass lignin; R1L1 0.1% – R1 strain grown in culture medium containing 0.1% L1 lignin; R1L1 0.5% – R1 strain grown in culture medium containing 0.5% L1 lignin; R1L2 0.1% – R1 strain grown in culture medium containing 0.1% L2 lignin; R1L2 0.5% – R1 strain grown in culture medium containing 0.5% L2 lignin; R2L1 0.1% – R2 strain grown in culture medium containing 0.1% L1 lignin; R2L1 0.5% – R2 strain grown in culture medium containing 0.5% L1 lignin; R2L2 0.1% – R2 strain grown in culture medium containing 0.1% L2 lignin; R2L2 0.5% – R2 strain grown in culture medium containing 0.5% L2 lignin

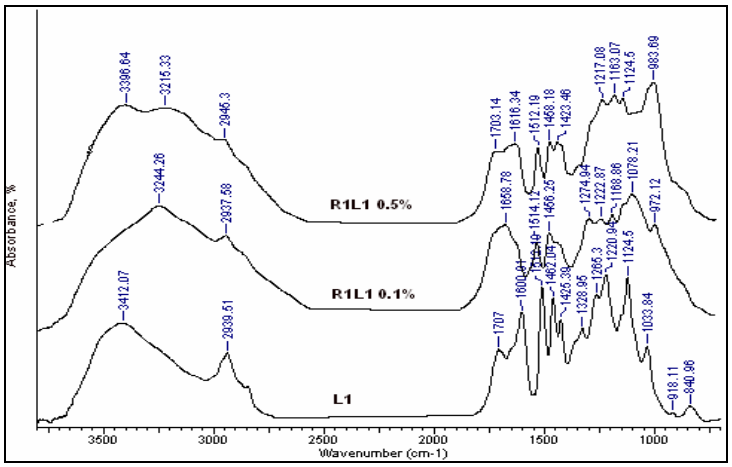

Figure 5: FTIR spectra recorded for lignins recovered from the culture media of R1L1 0.1% and R1L1 0.5% *versus* the spectrum of control lignin sample (L1)

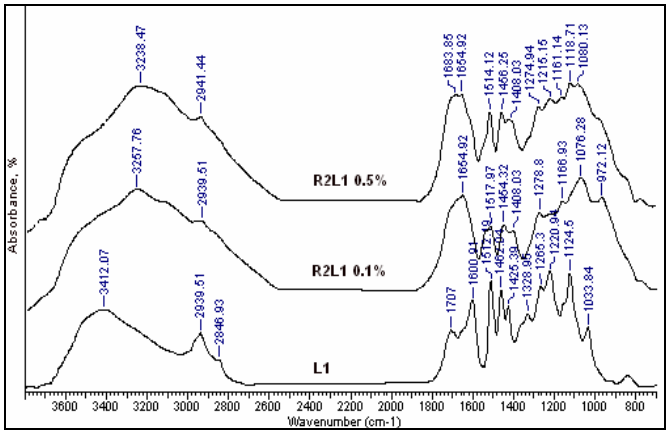

Figure 6: FTIR spectra recorded for lignins recovered from the culture media of R2L1 0.1% and R2L1 0.5% *versus* the spectrum of control lignin sample (L1)

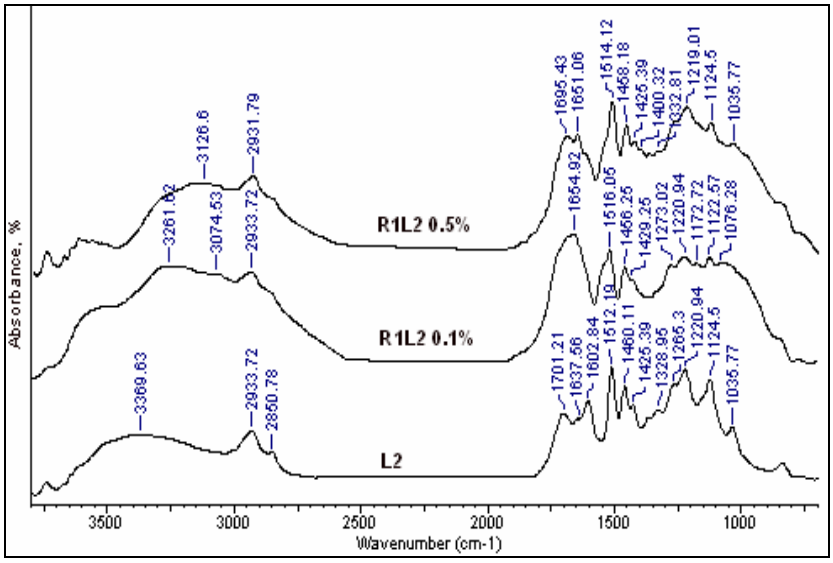

Figure 7: FTIR spectra recorded for grass lignins recovered from the culture media R1L2 0.1% and R1L2 0.5% *versus* the spectrum of control lignin sample (L2)

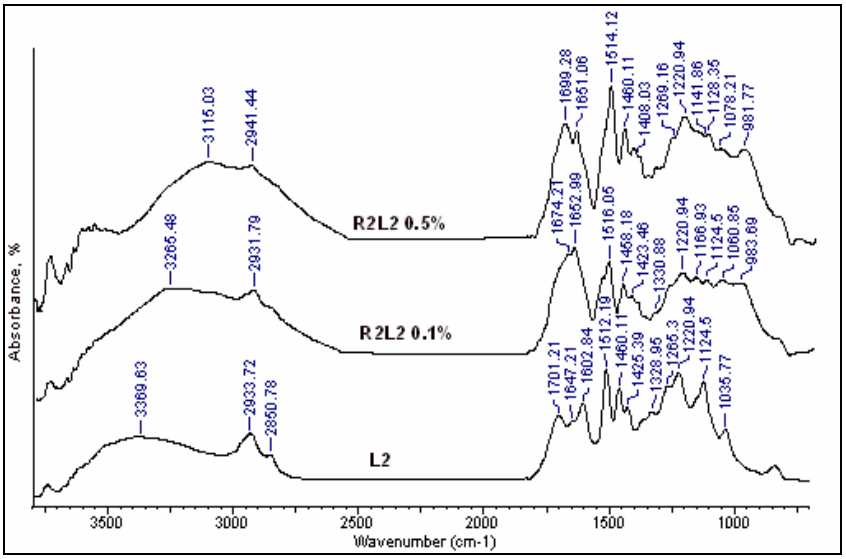

Figure 8: FTIR spectra recorded for grass lignins recovered from the culture media of R2L2 0.1% and R2L2 0.5% *versus* the spectrum of control lignin sample (L2)

In the 3370-3255 cm^{-1} area, absorption bands are present only in the reference lignin and in that isolated from the culture medium, with a concentration of 0.1%. At lignin concentrations of 0.5%, these bands are absent, probably because of the enzymes that degrade the –C-H bonds from the aromatic rings.

However, no specific signals from the phenolic –O-H groups, evidencing significant changes in the lignin structure, are present. The 2850 cm^{-1} band is characteristic of the methoxyl groups linked to the aromatic ring, aliphatic methyl groups and to the vibrations of unconjugated -C-O links specific to carbonyl groups. Possibly, significant changes occur due to lignin concentration in the medium, or to the demethoxylation processes of lignin under enzymatic action, since these signals are missing in most samples.

At 1710 cm^{-1}, valence vibration absorption, characteristic of ether, carbonyl and carboxyl groups is recorded. Changes in the intensity of these peaks are observed in the recovered lignin samples, which could be caused by their oxidation reactions induced by the enzyme systems of the two yeast strains. The 1590-1500 cm^{-1} range corresponds to the vibrations of the -C=C and –C-C- bonds of the aromatic ring, so that peak intensity changes are observed in this area for the lignin samples under study, comparatively with the control (Figs. 7 and 8). These changes are probably due to the enzymatic attack on the aromatic rings, once known that[26] the enzymes synthesized by the yeast attack directly the aromatic ring of the lignin structure, even prior to hydroxylation. The presence of absorption bands in the 1300-900 cm^{-1} area is considered specific to the –C-O links of the secondary alcohols, ester or ether, and to the distortions of the -C-C- bonds (Figs. 7, 8; Table 2).

At the level of these links, various changes occur, so that the intensity of the absorption bands either decreases or increases.

The conclusion of the study is that the two types of lignin introduced in the culture medium under fermentation undergo structural and functional changes caused by the enzymes biosynthesized by the two microorganisms. All these changes are characteristic of a series of reactions, such as oxidation, dehydrogenation and demethoxylation of the macromolecular units of lignin.

CONCLUSIONS

The above-discussed data show that the two microorganisms act differently on the lignins used as a carbon source. Thus, the two types of lignins studied are suitable for strain R1 in terms of biomass yield, while they inhibit the biosynthesis of carotenoid pigments.

Strain R2 grown in culture media containing lignins from annual plants (L1 and L2) biosynthesizes large quantities of carotenoid pigments, at a concentration of the aromatic polymer of 0.5%, although the yield in wet biomass is lower than that for the R1 strain during the fermentation processes and the pH records no significant changes.

The two strains of *Rhodotorula* yeast synthesize enzymes capable of undermining the lignin macromolecules, forming low molecular mass products subsequently used as carbon and energy sources.

The changes produced in the structure of lignin occur in the methoxyl and hydroxyl groups, as evidenced by FTIR spectroscopy. Oxidation reactions occur in the aromatic ring and side chains, leading to the emergence of characteristic functional groups, such as carbonyl and carboxyl.

Thus, the lignins introduced in the culture medium for the cultivation of *Rhodotorula spp*. yeasts are convenient for increasing the biomass yield and for the biosynthesis of carotenoid compounds.

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