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Biorefinery of Rice Husk to Obtain Functionalized Bioactive Compounds

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Abstract: The biomass industrialization valorisation of grains and cereals is considered an opportunity for the countries where agro-industrial activity is one of its main economic activities, rendering new higher-value products with a concomitant solution to waste accumulation issues. To that end, in this work we describe and characterise bioactive compounds generation from rice husk by semi-solid fermentation, obtained from 500 g of the material at room temperature and 60% humidity with mixed cultures of Phanerochaete chrysosporium and Gloeophyllum trabeum. The extract was evaluated in different situations: murine mammary tumour cells (4T1), normal cells (NIH 3T3) and in Lactobacillus acidophilus, and Trichoderma harzianum. The results exhibited that the extract inhibited 4T1 cells at concentrations higher than 20μg/mL, but did not inhibit normal cells, and displayed germicide activity after 3 days incubations. We propose that these functionalized compounds have a potential application in industry/agriculture/medicine obtained from rice husk waste.

Keywords: Biorefinery; rice husk; bioactive compounds; valorisation of biomass; veratryl alcohol

1 Introduction

The growing use of biopolymers in different industrial applications positions lignin as one of the polyfunctional polymers with greater applications potential. This potential is highly dependent on the modifications that can be made in the chemical structure of the biopolymer. In this sense, polymers such as cellulose, chitin and chitosan are being applied in medicine and agroindustry, among others. Some industrial applications can be implemented by associated biopolymers used in hybrid composite materials. Fig. 1 schematises some potential products biotransformations derived from lignin [1,2].

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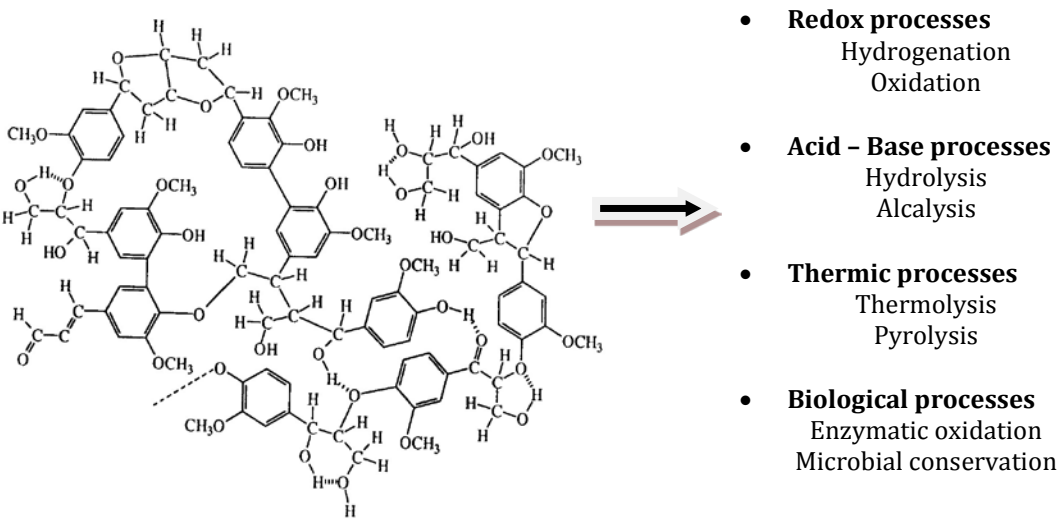

Figure 1: Schematic representation of different processes for lignin transformation [2]

Lignin is a polyfunctional compound that forms a lignin-cellulose complex in wood and plants. The extraction and modifications of its structure can be performed chemically, as they are typically done in industrial processes, in addition to modification by microbiological or enzymatic methods, which are widely studied in research but have less industrial development.

The biodegradation of lignins has been studied using species such as *Coriolus versicolor*, *Phanerochaete chrisosporium* and *Phlebia radiata*, and their enzymes have been characterized in greater detail. Most investigations refer to two aspects: the analytical study of the changes produced in the treated lignins and the search for a common mechanism of action for all these enzymes.

From a chemical point of view, lignin is a biopolymer very resistant to enzymatic hydrolysis compared to other biopolymers. The incubation of lignins with enzymes shows a smaller increase in the molecular weights of the oligomers than those found in the fermentations with microorganisms, possibly because the small fragments are efficiently metabolized and disappear leaving a remnant substrate of high molecular weight [3-5]. Particularly, three mechanisms of degradation caused by basidiomycete fungi are suggested. One of them were the chemical modifications that can be made in the macromolecule of lignin is that of the O_2/C_9 ratio, which offers very significant changes, and is associated with oxidative processes [6]. Some modifications have been described but the mechanism underlying them is still unknown [7]. Generally observed that the methoxyl and the beta-*O*-4 substructures decrease markedly while the content of oxygen and aromatic and aliphatic carboxyl groups increases significantly [8].

These processes consisted in: oxidative cleavage of the propyl side chain between alpha and beta carbons for the formation of benzoic acid; the rupture of the aryl ether bond and modification of the side chain, and the degradation of the aromatic nucleus by an oxidative ring opening [9].

Other studies evaluated some of the possible mechanisms in enzymes of white rot fungi such as lignin peroxidase and laccase. Fig. 2 shows the main bonds present in the macromolecule of lignin and its possible types of rupture of potential commercial interest [2].

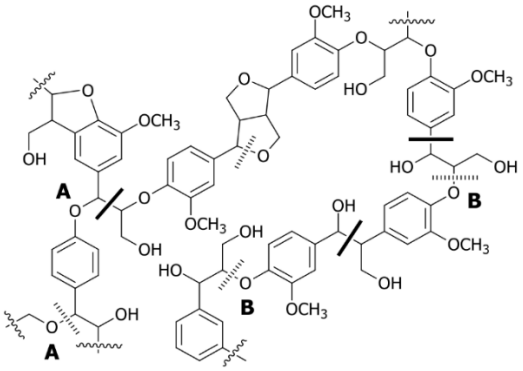

Figure 2: Main links present in the macromolecule of lignin, the possible types of bond C-O [alpha] aryl ether (A) and [beta] aryl ether and the possible type of cleavage C-C (dark bar) and C-O (dotted bar) [2].

The products obtained by biotechnological processes allow obtaining water-soluble extracts of modified functionalized compounds (FC) that have different properties such as germicidal, fungicidal and insecticidal properties. In order to do this, the "functional units", from lignin macromolecules, have been obtained from different sources, using biochemical, microbiological and enzymatic techniques. Some microorganisms are able to use lignin as a substrate causing its degradation by means of induction enzymes. In *Acinetobacter anitartus* enzymes such as manganese peroxidase, laccase and lignin peroxidase (LiP) [10], are described, which are known to depolymerize, oxidize and demethylate.

White rot fungi (*Phanerochaete chrysosporium*) can reduce the lignin content without causing rapid depolymerization in cellulose, while brown rot fungi (*Gloeophyllum trabeum*) preferentially degrade cellulose and hemicellulose. Both are responsible for the demethylation groups increasing the number of FC and hydroxylated aromatic groups. They promote the oxidation of the side chains and cleavages in the aromatic groups.

The microbiological processes, especially the operations with enzymatic extracts, can be considered as large-scale techniques, such as fermentation in solid phase and fermentation of industrial lignin (Kraft) [11-13].

Cytotoxicity assays studies of different compounds are widely used as a predictor of possible effects in humans when the viability of cultured cells is analyzed These types of *in vitro* biological tests are required to determine the survival and proliferation of different cells lines for further investigation studies in humans to different applications including cancer therapies.

In these sense, assays are based on counting cells that include/exclude a dye, measuring released 5aCr protein after cell lysis, measuring the incorporation of radioactive nucleotides ([^{3}H] thymidine or [^{125}I] iodine deoxyuridine) during cell proliferation and using MTT to evaluate cellular metabolism activity [14]. The MTT assay is a rapid, versatile and quantitative method to determine cell growth and survival.

In the present work, the aim was to produce FC, through the enzymatic action of twomixed fungus *Phanerochaete chrysosporium* and *Gloeophyllum trabeum* and in a semi-solid fermentation (SSF) system with rice husk waste. The FC were characterized using assays involving survival and proliferation of different cells lines for potential biomedical applications. In addition to this valorization, the use of this material will allow resolving the annual generation of 108 tonnes of rice husk in the world and about 375,000 tonnes per year in Uruguay [15].

2 Material and Methods

Rice Husk

The raw material was provided by the Uruguayan rice production company COOPAR S.A. Lignin content was determined and the value corresponds to 14% of the lyophilized material (according to the bibliography).

Growth of *Phanerochaete Chrysosporium* and *Gloeophyllum trabeum*

The growth of the fungi was carried out in a liquid medium composed of 40 g/L of maltose, 10 g/L of yeast extract and 1 mL of Kirk's salts in 250 mL of culture medium. The medium was inoculated, and the cultures were maintained for 6 days at 30°C, without agitation. The preservation medium was prepared in duplicate, in potato starch-dextrose-agar and in agar-malt, both kept for 7 days at 25ºC and later stored at 4ºC.

Production of Enzymes

One hundred and fifty mL of medium was prepared with 10 g/L of glucose, 0.2 g/L of yeast extract, 0.5 g/L of ammonium tartrate and 1 mL of Kirk's salts, at pH5. The medium was sterilized for 15 minutes at 121ºC and inoculated with 5 mL of growth medium with each fungus. Each culture was maintained at 37°C for 6 days and the supernatant was analyzed for enzyme activity.

Analysis of Enzymatic Activity

In the culture supernatant, enzymatic activity assays were performed. The samples were analyzed in triplicate. A Shimadzu spectrophotometer (model UV-1800) was used for the determinations.

Lignin Peroxidase

This activity was determined by the Tien and Kirk Method. [10]. A 0.01 M of veratryl alcohol was used as a substrate in sodium tartrate buffer (0.1 M, pH3.0). The reaction was initiated by adding 4 mM H_2O_2 and monitored by measuring the increase in absorbance at 310 nm (ε_{max} = 9300 / M cm).

Methoxyl Hydrolase

The method of measurement was applied on a methoxylated model compound. The reaction was followed by spectrophotometric measurements and the determination of the change in the spectrum. Two mL of 3,4,5-trimethoxybenzaldehyde (Aldrich) 0.1 M, pH4 was taken and incubated with 0.1 mL of the growth fungi supernatant at 30°C. The decrease of the substrate was measured by UV spectrophotometry.

Production of "FC Units" of Lignin

The production of the FC units was carried out in a semi-solid fermentation system for 40 days in a controlled chamber. A volume of 1.5 L of rice husk corresponding to 500 g was inoculated with the propagated fungi *Phanerochaete chrysosporium* and *Gloeophyllum trabeum* which were previously tested by the enzyme production capacity. The semi-solid fermentation process was performed in an open vessel without stirring, inside a controlled oven (humidity: 60-70%; temperature: 30ºC; pH5-6). The activity of the inoculated fungi was controlled by measuring the enzymatic activity in the material of semi-solid fermentation. For this, 2 g of inoculated rice husk was taken and suspended in 10 mL of water. After 1 h, the enzymatic activity was determined in the liquid: lignin peroxidase in an average of 50 EU/mL and methoxyl hydrolase in an average of 15 UE/mL. Forty days after enzymatic action, a volume equal to that occupied by the solid material, 1.5 L, was decanted for two hours and centrifuged and the supernatant was analyzed by UV spectrophotometry determining the FC units and derivatives. The samples were analyzed in triplicate. The equipment used was a spectrophotometer of the Shimadzu brand, model UV-1800.

The FC extracts were lyophilized in a Biobase BK-FD10S vacuum freeze dryer and stored at 4ºC.

3 Characterization of the Obtained Compounds

Determination of FC

Quantification of FC obtained from the rice husk sample was carried out, using the Folin & Ciocalteu method [16-18], using tannic acid as the standard for the calibration curve. Equipment: Visible ultraviolet

spectrophotometer (UV-Vis), (Shimadzu model UV-1800).

HPLC-UV-MS Analysis

The extract was dissolved in water at a concentration of 5 mg/mL. For HPLC-MS analysis, it was further diluted 1 in 10 with an injection of 5 µL.

Analysis were performed using a HPLC instrument (Agilent 1200, Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, an auto sampler, a diode-array detector, a binary pump and a thermostated column oven, coupled to an ion trap mass spectrometer (Esquire 6000, Bruker Daltonik GmbH, Bremen, Germany) and UV detection (280 nm). Samples were analyzed using a reversed-phase C_{18} analytical column (Zorbax Eclipse Plus 18, Agilent, 100 mm length, 3 mm diameter and 3.5 µm particle size), maintained at 30°C. The mobile phase consisted of ultrapure water (A) and a 4:1 mixture of acetonitrile and ultrapure water (B). Flow rate was 0.4 mL/min split in a 1:1 ratio before introduction to the mass spectrometer.

The chromatographic method consisted of an isocratic step of 5% B for 3 min, then a linear gradient from 5 to 100% B in 17 min and an isocratic step of 100% B for 2 min, going back to 5% B in 1 min and 4 min to re-equilibrate, with a total run time of 27 min.

Mass spectrometry analysis was performed in positive and negative-ion mode. The electrospray source conditions were as follows: endplate off set voltage -500 V, capillary voltage 4000 V, nebulizer 30 psi, dry gas flow 8.0 L/min, and dry gas temperature 350°C. Nitrogen was used as drying and nebulizing gas. Mass detection was performed in full scan mode in positive and negative alternating polarity mode. Acquisition and data analysis were performed with Compass 1.3 for Esquire/HCT series software (version 6.2, Buker Daltonik GmbH, Bremen, Germany). Tandem mass spectrometry analysis was performed in positive-ion mode.

UV-Vis Study

The samples were analyzed in triplicate. The equipment used was a spectrophotometer of the Shimadzu brand, model UV-1800.

FTIR Study

The Nicolet 6700 equipment with diamond ATR (Thermo Scientific) was used to analyse the samples. Experimental conditions: 16 scans, resolution 4, sample gain 8.0, optical velocity 0.6329 and aperture 100.

^{1}H NMR Study

The spectrums were recorded, at 30ºC, in Bruker DPX-400 (400 MHz) spectrometer, employing deuterium oxide (D_2O) as solvent, and with a number of scans of 64. The chemical proton shifts are expressed in ppm (δ) employing TMS (tetramethylsilane) as reference.

Cytotoxicity Test

Cytotoxicity was assessed in 4T1 mouse mammary tumour cells (ATCC) and NIH 3T3 embryonic skin fibroblast cells (ATCC). This study was carried out using the MTT cell viability assay, according to the method described by Mosmann [14]. Particularly, 7.5×10^3 4T1 cells or 15×10^3 NHI 3T3 were seeded in a 96-well plate and after 24 h the cells were treated with different concentrations of the functionalised compound (20, 40, 60, 80, 100, 150 µg / mL).

At 48 h of incubation of the two cell lines MTT was added. Absorbance at 570 nm was measured with a Varioskan multimode flash microplate reader (Thermo Scientific). The cell viability percentages of different treatments were calculated considering the absorbance of the control as 100% viability. Data was analysed by Student's *t*-test.

Germicidal Activity Tests

Incorporation of FC in Microcapsules in A Membrane System

For the application of the FC we used microcapsules of polyvinyl alcohol obtained by the Applied Nuclear Techniques on Biochemistry and Biotechnology laboratory from the Nuclear Research Centre (CIN) [19, 20].

The Germicidal Activity was Assayed by Two Different Cultures

For bacterial control we used a plate culture with YPD, and a (1 cm × 1 cm) of polyvinyl microcapsules in a membrane system containing lyophilised FC (0.0306 g) in the centre of the plate. We inoculated *Lactobacillus acidophilus* during 5 days of growth. The assay consists in:

(a) *Lactobacillus acidophilus* growth in YPD medium without FC for 5 days

(b) *Lactobacillus acidophilus* growth in YPD medium and applied the FC in microspheres inside of membranes (1 cm × 1 cm) at the 3 day until 5 days.

(c) *Lactobacillus acidophilus* growth in YPD medium and applied the FC in microspheres inside of membranes (1 cm × 1 cm) at the 0 day until 5 days. This is the assay for the inhibition.

As fungal control we used a plate culture with PDA and a polyvinyl microcapsule in a membrane system (1 cm × 1 cm) containing lyophilised FC (0.0306 g) in the centre of the plate. We inoculated *Trichoderma harzianum* during 7 days. The assay consists in:

(a) *Trichoderma harzianum* growth in PDA medium without FC

(b) *Trichoderma harzianum* growth in PDA medium and applied the FC in microspheres inside of membranes (1 cm × 1 cm) after 5 days of growth.

(c) *Trichoderma harzianum* growth in PDA medium and applied the FC in microspheres inside of membranes (1 cm × 1 cm) during 7 days.

The different inhibition of growth in *Lactobacillus acidophilus* and *Trichoderma harzianum* were calculated according the growth of the control as 100%. An analysis of the relationships between variables (intra-site relationships) was conducted using correlation analysis (n = 3).

4 Results and Discussion

Protein concentrations of 1.2 mg/mL were obtained in the filtrates of enzyme production by mixed cultures of *Gloeophyllum trabeum* and *Phanerochaete chrysosporium.* The enzymatic activities obtained are shown in Tab. 1. Enzymatic activity of lignin peroxidase and methoxyl hydrolase was determined, being higher for lignin peroxidase.

Table 1: Enzymatic activity obtained in liquid system of *Gloeophyllum trabeum* and *Phanerochaete chrysosporium*

Enzyme	*G. trabeum* and *P. chrysosporium*	UE/mL
Lignin peroxidase	+	50
Methoxyl hydrolase	+	15

Solid state fermentation (SSF) was carried on 500 g of rice husk, with an enzymatic extract of 65 UE/mL, with a specific activity of 0.78 UE/mg of proteins.

Particullarly, 50 g of rice husk in SSF was lixiviated with 200 mL of water and were obtained 200 mL of aqueous extract, equivalent to 6.12 g FC (determined after the lyophilization).

The performance of the process reveals a 61.2 gram that corresponds at 12% of FC in the rice husk and the 87% of the content of lignin (14% lignin in rice husk).

The products of the rice husk treatment were lyophilized and characterized by HPLC and spectroscopic methods. HPLC analysis showed the presence of only one main product (higher than 98%, Fig. 3), with the same retention time as 3,4-dimethoxybenzyl alcohol (veratryl alcohol), i.e., $t_{R,\ standard}$ = 10.6 min, $t_{R,\ extract}$ = 10.7 min.

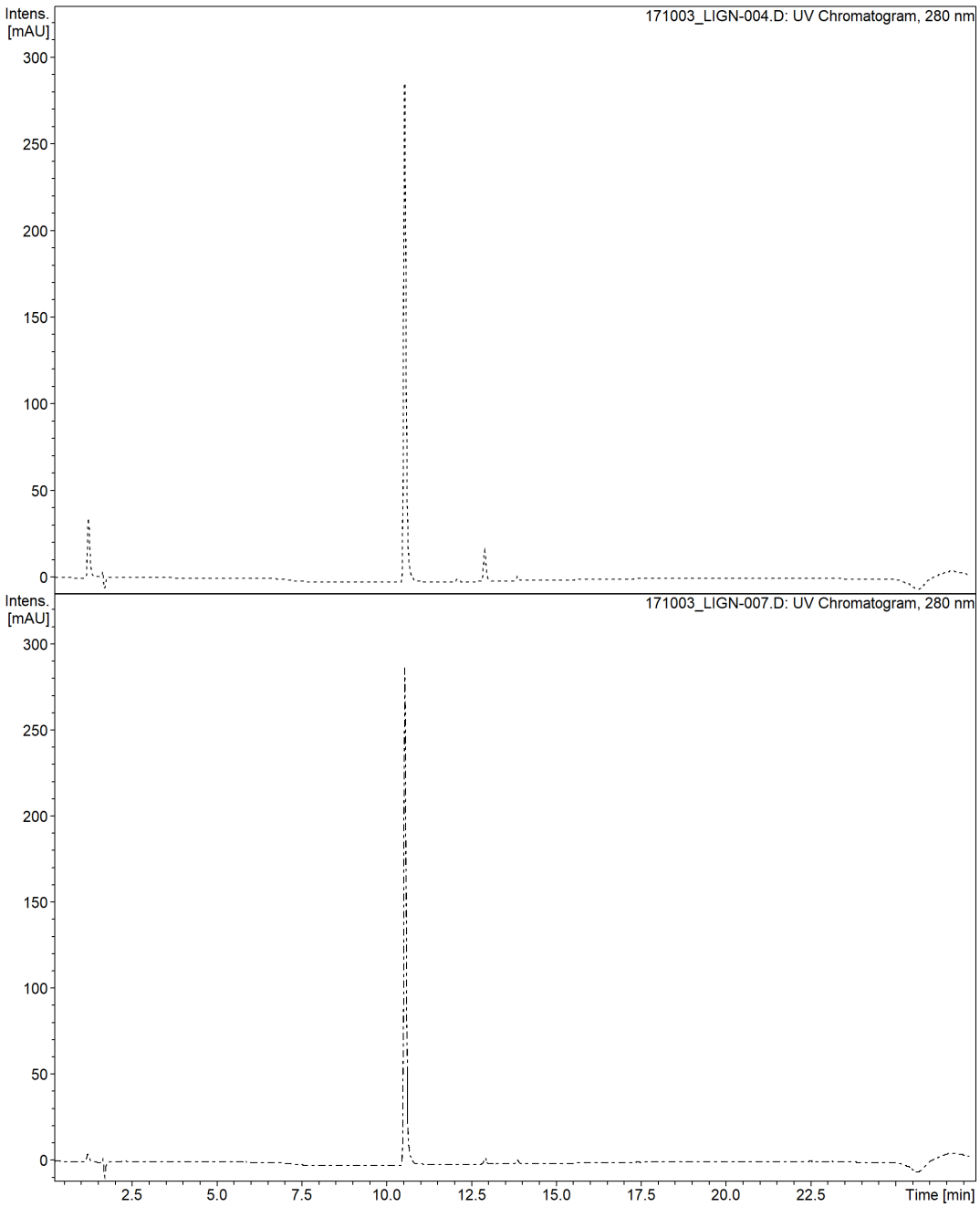

Figure 3: HPLC analyses at UV-Vis at 280 nm. Up: lyophilised extract; Down: standard of veratryl alcohol. For experimental conditions see “Materials and Methods” section

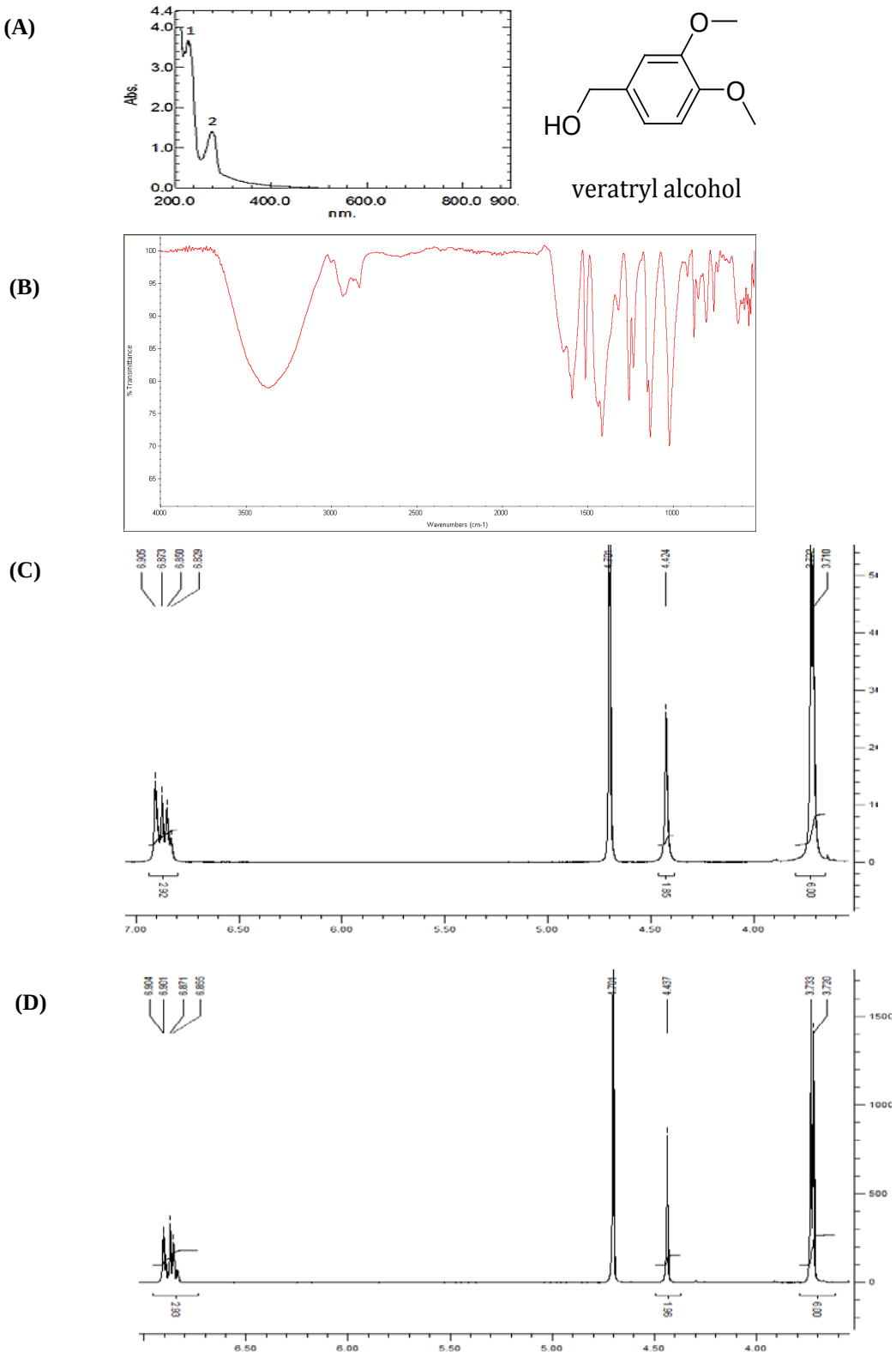

Figure 4: Spectroscopic characterization of the lyophilized extract of SSF. UV-Vis (left A) and structure of main identified product (right A); FTIR (B); ^{1}H NMR (C). The ^{1}H NMR for standard of veratryl alcohol is included (D). ^{1}H NMR spectra showing the 3 to 7 ppm region

The extract was further characterised by different spectroscopic techniques and mass spectrometry. The visible ultraviolet spectra (Fig. 4(A)) showed a signal at 276 nm that is typical for benzylic alcohols [20, 21]. The FTIR showed characteristic absorptions of OH moiety, between 3200-3500 cm^{-1}, C-H moieties between 2800-3000 cm^{-1} and 1300-1650 cm^{-1} (Fig. 4(B)). The ^{1}H NMR spectra showed the characteristic chemical displacement of veratryl alcohol (Fig. 4(C)) that were confirmed performing the ^{1}H NMR spectra of standard sample (Fig. 4(D)). Finally, the MS spectrum confirmed the structure of the main product of lyophilised extract as veratryl alcohol (Fig. 5).

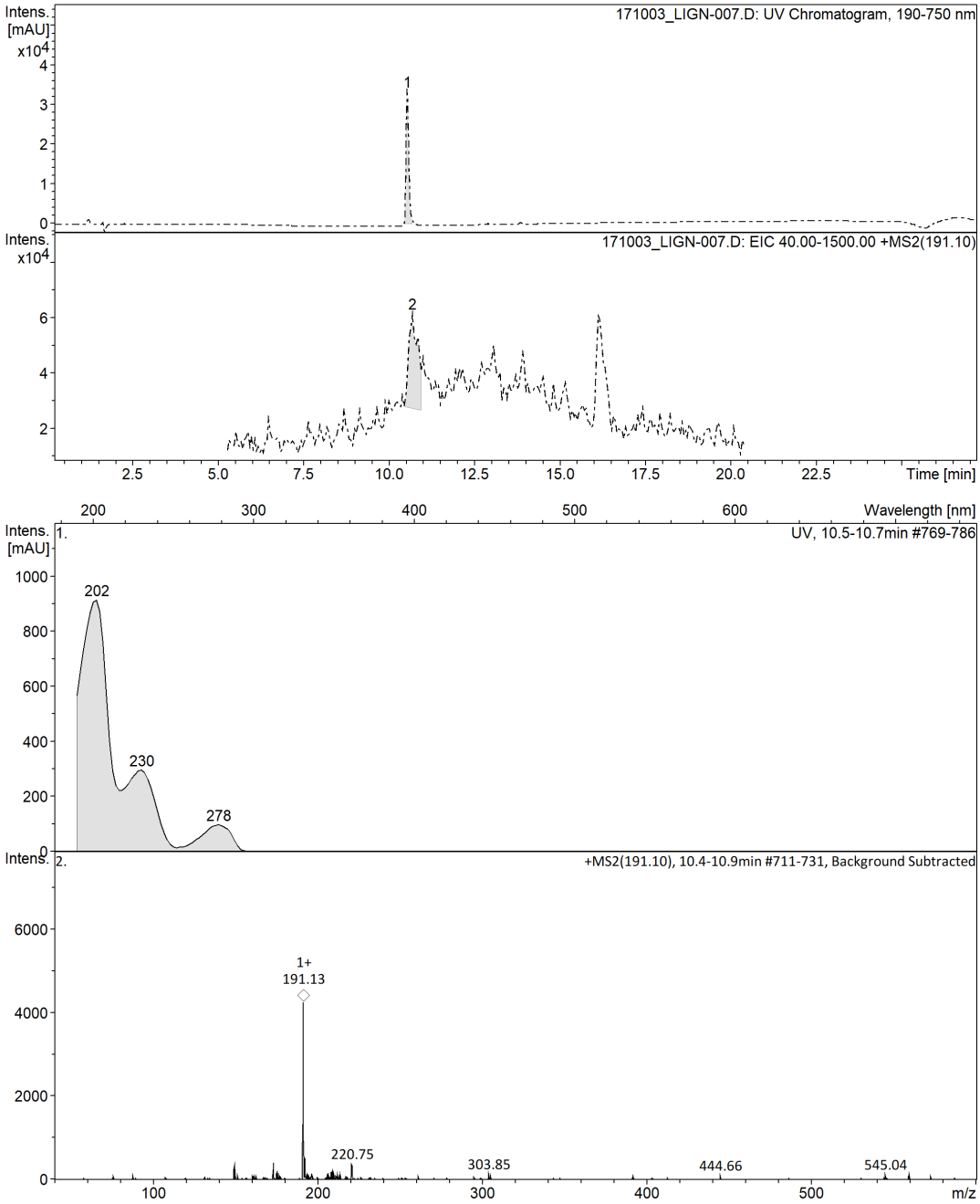

Figure 5: Mass spectra of lyophilised extract. [$M^{+\cdot}$ + Na] for veratryl alcohol: expected: 191.07 u, obtained: 191.15 u

To valorise the potential use of the product of biorefinement of rice husk in industry, agriculture or medicine, it was evaluated as a cytotoxic entity and as a germicide.

The tumoral cells viability treated with the lyophilised extract showed interesting results (Fig. 6). The viability of NIH 3T3 cells was affected at concentrations above 80 µg/mL (Fig. 6), with a slight dose-response tendency at higher concentrations.

In experiments with 4T1 cancer cells, a cytotoxicity effect was observed in concentrations above 20 µg/mL (Fig. 6).

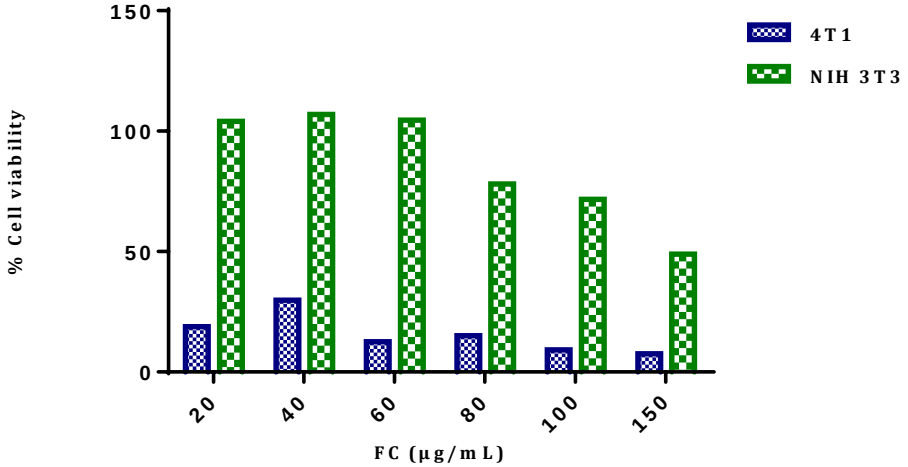

Figure 6: Cell viability of 4T1 and NIH 3T3 cells treated with different concentrations (µg/mL) of FC for 48 hours

The foils polymeric membrane incorporated microspheres and FC revealed a germicidal activity in *Lactobacillus acidophilus* and *Trichoderma harzianum* (Figs. 7 and 8).

Lactobacillus acidophilus stopped growth when exposed at 5 days of growth (Fig. 7(C)).

In *Trichoderma harzianum* a total inhibition was observed when the culture was exposed at the inicial time, whereas the effect was only partial partially when it was exposed after 5 days of growth (Fig. 8).

The germicidal activities shown by the FC could have application in agriculture and biomedicine.

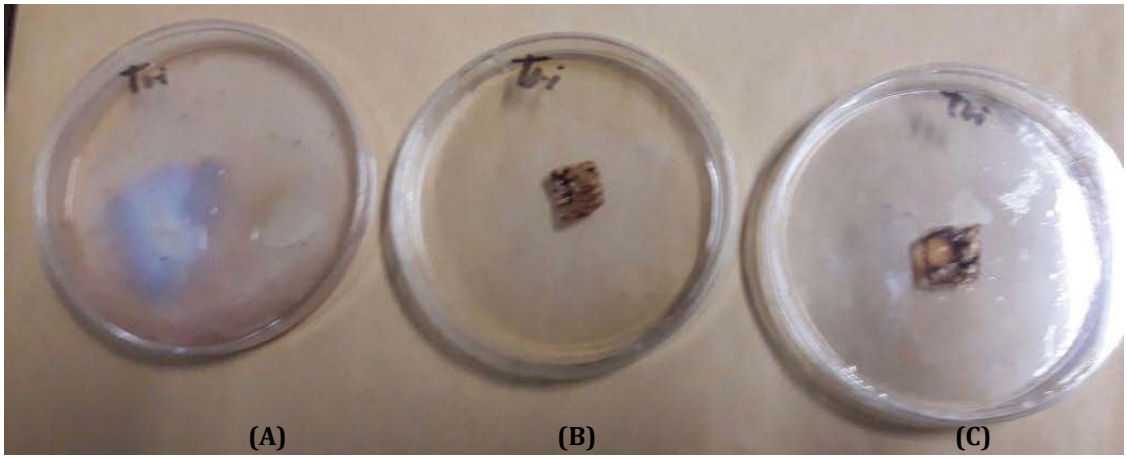

Figure 7: Cultures containing FC as inhibition systems in *Lactobacillus acidophilus* bacteria of 5 days of growth. (A) bacteria growth in YPD medium without FC, (B) YPD medium of bacteria, bacteria and foils polymeric membrane incorporated microspheres and FC at 3 days of growth until 5 days and (C) YPD medium, bacteria and foils polymeric membrane incorporated microspheres at time 0 of growth

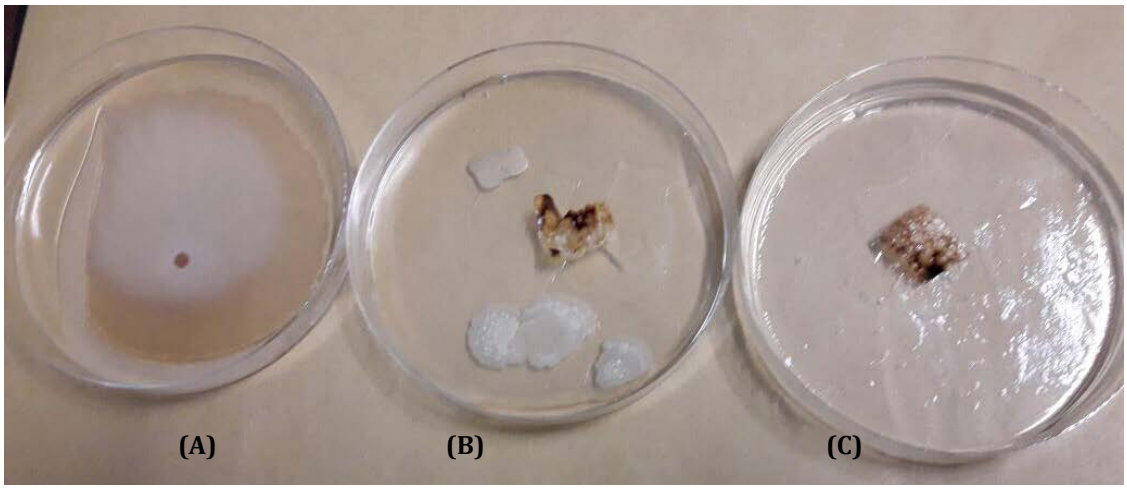

Figure 8: Cultures containing FC as inhibition systems in *Trichoderma harzianum* at 7 days of growth. (A) Trichoderma harzianum growth in PDA medium without FC, (B) exposition of polymeric membrane incorporated microspheres and FC in PDA medium after 5 days growth of fungi and (C) PDA medium, *Trichoderma harzianum* and foils polymeric membrane incorporated microspheres and FC at time 0 of growth

5 Conclusion

This study has shown that it is feasible to prepare specific enzymatic extracts capable of directing the degradation of lignin towards oligomers or monomers that exhibit variable and functional structural characteristics.

The aim of this work was to valorise the rice husk, a very relevant waste in Uruguay, through biotechnological processes to obtain compounds with bioactivity. To this end, we obtained 61% of veratryl alcohol of the transformation of lignin from rice husk a 10%.

In the biological behaviour of the FC, we could remark that it does not affect normal cells (NIH 3T3) at concentrations below 60 µg/mL, but in breast cancer cells (4T1) it affects the cell viability at the all studied doses range (20 to 150 µg/mL) revealing its potential activity as an anti cancer drug.

Additionally, the germicidal activity (against *Lactobacillus acidophilus* and *Trichoderma harzianum*) showed total inhibition when we exposed at 0 time of growth in bacteria and fungi that reveals a potential application in medicine and agroindustry.

References

1. Lopretti, M., Gandini, A. (2012). New polymeric materials derived from renewable sources. *INNOTEC, 7*, 59-63.
2. Chávez-Sifontes, M., Domine, M. E. (2013). Lignin, structure and applications: depolymerization methods for obtaining aromatic derivatives of industrial interest. *Avances en Ciencias e Ingeniería, 4(4)*, 15-46.
3. Lopretti, M., Carlomagno, M., Gervacio, S., Gandomenico, A. (2004). Enzymatic production of phenols from wastes of the paper industry. *Biomass in the Energy Industry*, 1951-1954.
4. Lopretti Correa, M., Esquivel Alfaro, M., Madrigal Carballo, S., Corrales Ureña, Y., Vega-Baudrit, J. (2017). Preliminary study of the production of hybrid compounds of chitosan and polyphenols derived from lignin from agroindustry and shrimp fishery. *Revista Científica, 27(1)*, 32-42.
5. Gelbrich, J. (2009). *Bacterial Wood degradation-a study of chemical changes in wood and growth conditions of bacteria*. Springer Verlag.
6. Jellison, J., Connoly, J., Goodell, B., Doyie, B. (1997). The role of cations in the biodegradation of

wood by the brown rot fungi. *International Biodeterioration & Biodegradation, 39,* 165-179.

7. Agosin, E., Blanchete, R. (1990). Characterization of “palo podrido”, a natural process of delignification in wood. *Appied and Enviromental Microbiology, 11,* 511-517.
8. Gandini, A. (2002). Les lignines et leur utilisation dans les matériaux macromoléculaires. *Initiation à la chimie et à la physicochimie macromoléculaires, 13,* 57-62.
9. Enoki, A., Itakura, S., Tanaka, H. (1997). The involvement of extracellular substances for reducing molecular oxygen to hydroxyl radical and ferric iron to ferrous iron in wood degradation by wood decay fungi. *Journal of Biotechnol, 53,* 265-272.
10. Tien, M., Kirk, T. K. (1988). Lignin peroxidase of Phanerochaete Chrysosporium. *Methods in Enzymology, 161,* 238-249.
11. Yoshinori, K., Hiraku, O., Yoshiharu, M. (2003). Application of chitin and chitosan derivatives in the pharmaceutical field. *Current Pharmaceutical Biotecnology, 4,* 303-309.
12. Senay, A., Nursevin, Ö. (2003). Immobilization of catalase into chemically crosslinked chitosan beads. *Enzyme and Microbial Technology, 32,* 889-894.
13. Ferreira, P., Medina, M., Guillén, F., Martínez, M. J., Van Berkel, W. J. H. et al. (2005). Spectral and catalytic properties of aryl-alcohol oxidase, a fungal flavoenzyme acting on polyunsaturated alcohols. Biochemical Journal, 389, 731-738.
14. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65(1-2),* 55-63.
15. Behak, L., Núñez, W. P. (2013). Effect of burning temperature on alkaline reactivity of rice husk ash with lime, *Road Mater Pavement, 14(3),* 570-585.
16. Folin, O. (1927). Tyrosine and tryptophan determination in proteins, *Journal of Biological Chemistry, 73,* 649-673.
17. Lowry, O., Rosehrough, N. J., Farr, A., Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Biological Chemistry, 193(1),* 265-275.
18. Prior, R. L., Wu, X., Schaich, K. J. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53(10),* 4290-302.
19. Lopretti, M., Olazabal, L. (2014). Microencapsulación de microorganismos *Kluyveromyces Marxianus* en diferentes sistemas y materiales y evaluación de su actividad biológica en la producción de bioetanol a partir de materiales lignocelulósicos, (Microencapsulation of *Kluyveromyces Marxianus* microorganisms in different systems and materials, and evaluation of the biological activity in bioethanol production from lignocellulosic materials). *Revista Iberoamericana de Polímeros, 15(1),* 55-65.
20. Olivera, A., Barreiro, F., Lopretti, M. (2014). Proteínas de actividad biológica microencapsuladas, ultraestructura y distribución de carga (Bioactive proteins: microencapsulation, utrastructure and charge distribution). *Revista Iberoamericana de Polímeros, 15(4),* 219-228.
21. Alvarado, R., Solera, F., Vega-Baudrit, J. (2014). Síntesis sonoquímica de nanopartículas de óxido de cinc y de plata estabilizadas con quitosano. Evaluación de su actividad antimicrobiana (Sonochemical synthesis of zinc and silver oxides nanoparticules stabilized with chitosan: Antimicrobial activity evaluation.). *Revista Iberoamericana de Polímeros, 15(3),* 134-148.
22. Xiaochen, W., Shouwu, G., Jingyan, Z. (2015). Selective oxidation of veratryl alcohol with composites of Au nanoparticles and graphene quantum dots as catalysts. *Chemical Communications, 51,* 6318-6321.