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Research Article

Eco-Friendly Oleogels from Functionalized Kraft Lignin with Laccase SilA from *Streptomyces ipomoeae*: An Opportunity to Replace Commercial Lubricants

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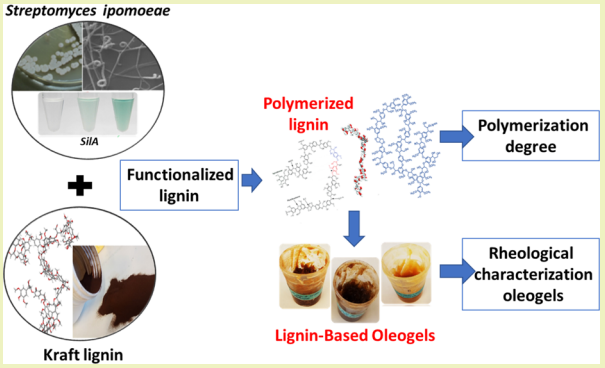
ABSTRACT: This work demonstrates the usefulness of SilA laccase from *Streptomyces ipomoeae* to functionalize Kraft lignin to be used as a thickener in the preparation of biodegradable oleogels for lubricating purposes. First, conditions for the enzymatic reaction were optimized by determining the polymerization degree of lignin along the incubation time. Next, using rheological characteristics as a response function, the Kraft lignin amount and incubation time were optimized to get the best characteristics of functionalized Kraft lignin to be used as a thickener in castor oil oleogels. In both cases, response surface methodology (RSM) with a factorial design 2^3 was applied. The results obtained demonstrated that Kraft lignin was oxidized by recombinant laccase from *S. ipomoeae* (SilA) increasing its reactivity and, as consequence, the molecular weight average. It was estimated that the highest polymerization occurred with a Kraft lignin concentration of 1.25 g/L and an incubation time of 24.0 min. Moreover, the maximum values of the "plateau modulus" for the oleogels within the limits of the studied region were reached with 66.6 g/L of Kraft lignin and a reaction time of 1.98 h. In conclusion, eco-friendly oleogels obtained in this work fulfil the industrial requirements concerning their rheological properties to be considered as an efficient and biodegradable alternative to traditional lubricants containing lithium as the thickener.



KEYWORDS: *Streptomyces laccase, residual lignin valorization, response surface methodology (RSM), sustainable oleogels, rheological characterization, circular economy*

1. INTRODUCTION

Polluting industries are being impelled to substitute polymeric materials derived from oil refining or petrochemical sources with natural resources that are more friendly with the environment. Among products of these industries, it should be noted lubricant greases, that are constituted not only by mineral oils but also thickeners, most of which are non-biodegradable metal soaps.[1]

The thickener content in lubricant greases can range between 3 and 30% of the total weight, so there is a clear need to develop new renewable thickeners that confer adequate technical performance. Biothickeners developed from natural polymers represent an environmentally friendly alternative to synthetic polymers so that the search for new biodegradable thickeners remains as a main biotechnological target. Biopolymers, in comparison with synthetic polymers, render greater biodegradability, prominent recyclability, and reduced process energy requirements.[2] Among the most promising natural polymers for this purpose causes lignin, one of the major components of lignocellulosic biomass, which is considered a good platform to produce high-value chemicals and fuels.[3] Nowadays, the use of lignin as potential feedstock to produce biobased polymers is starting to attract significant attention to make better use of this important natural resource.[4]

This new technological strategy fits well with the "circular economy" concept since upgrading lignin residues into useful raw materials will allow us to re-enter them into the value chain of the production system. Recently, wheat straw-based oleogels have been obtained presenting relatively poor mechanical stability.[5] Thus, for the industrial use of the

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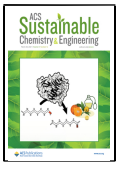

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transformed residual biopolymers such as residual lignins as the raw material, it is necessary to look for appropriate sustainability strategies to increase their reactivity. Oxidative functionalization of these lignins through enzymatic catalysis[6−8] is trying to solve the problems derived from their chemical and/or structural alteration in pulp and paper production technologies. To achieve this objective, the functionalization of residual lignins with microbial enzymes such as laccases seems to be a promising approach.

Laccases (EC 1.10.3.2) are multicopper oxidoreductases (MCOs) widely distributed in nature.[9,10] The fact of not needing cofactors together with their oxidative capacity makes them an interesting tool for the functionalization of lignin.[11] Bacterial laccases, mainly from *Streptomyces*, have been recently demonstrated to be key enzymes in the lignin solubilization by these microorganisms.[12,13] These enzymes gained attention in recent years because of their advantages to act at a wide range of pH and temperature and in the presence of high concentration of salts and inhibitors, in contrast with most of fungal laccases.[14,15] The recombinant laccase from *Streptomyces ipomoeae* (SilA) has shown to have a biotechnological value in the degradation of textile dyes that are harmful to the environment,[16] degradation of emerging pollutants (quinolones),[17] and degradation of Kraft pulp *Eucalyptus*, thus increasing the degree of whiteness in a biowhitening process.[11] Moreover, Moya et al.[18] demonstrated that *S. ipomoeae* SilA laccase has potential to be applied in the polymerization of industrial waste lignins under alkaline conditions, opening new possibilities to the application of SilA for the valorization of industrial lignins.

In this work, we explore the ability of the laccase SilA from *S. ipomoeae* CECT 3341 to generate high molecular weight lignin polymers from Kraft lignin to be used as thickeners in the formulation of biodegradable oleogels, inducing cross-linking with hexamethylene diisocyanate (HDI) in castor oil. To validate the results, response surface methodology (RSM) was applied, allowing us to establish optimal polymerization conditions to elaborate oleogels whose rheological characteristics were compared with those of commercial lubricants.

2. MATERIALS AND METHODS

2.1. Production of SilA Recombinant Laccase. The enzyme selected for this study was the recombinant laccase (SilA) from *S. ipomoeae* CECT 3341.[16] For the production of SilA, the strain of *Escherichia coli* BL21 + plasmid pET28A, in which the SilA gene was previously cloned and overexpressed, was used. Transformed *E. coli* cells were incubated at 37 °C for 24 h in a solid LB (Luria−Bertani) medium supplemented with kanamycin at a rate of 25 μg/mL. After incubation, a pre-inoculum of LB supplemented with the same antibiotic was performed, and the culture was incubated as described above until an optical density (OD, 600 nm) of 0.5−1 (exponential phase) was reached. Laccase expression was induced by adding 1 mM isopropyl β-D-L-thiogalactopyranoside (IPTG) (99% purity degree; Sigma-Aldrich). Then, the culture was incubated at 28 °C for 2 h to induce SilA expression and the cells were recovered by centrifugation (12,439*g*, 20 min, 4 °C) (Beckman-Coulter, USA). The cell pellet was resuspended in phosphate buffered saline (PBS). The cell suspension was centrifuged, and the supernatant was separated from the cell debris.

2.2. Determination of Laccase Activity. Laccase activity was determined by measuring the oxidation of 5 mM 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; 98% purity degree, Sigma-Aldrich) in 50 mM acetate buffer, pH 4.5. The increase in the absorbance at 436 nm was monitored at room temperature in a 2001 Hitachi spectrophotometer (Japan) considering a molar extinction coefficient of 29,300 M^{-1} cm^{-1} for oxidized ABTS.[19] International units of enzymatic activity (μmol/min) were used.

2.3. Evaluation of the Ability of the Laccase SilA to Polymerize Kraft Lignin. *2.3.1. Preparation of the Enzyme Reaction Mixtures for Polymerization.* Before enzymatic treatment, Kraft lignin (*Eucalyptus* Kraft lignin; Sigma-Aldrich) was dissolved in a solution of NaOH (0.1 N). For the enzymatic reaction, 1 mL of the following mixtures was prepared containing phosphate buffer (50 μM), laccase SilA (1 U/g of lignin), and lignin at different concentrations (0.25, 0.75, and 1.25 g/L). The mixtures were incubated at different times of incubation (10, 20, and 30 min) at 45 °C.[16,19] The final pH of each sample was adjusted to pH 7−8 with 50 mM phosphate buffer.

2.3.2. Optimization of the Conditions for the Enzyme Reaction for Polymerization. The efficiency of the enzymatic treatment was assessed by determining the degree of polymerization of lignin along the incubation time. For this, the lignin polymers in control solution and treated with SilA were separated according to their molecular mass. The molecular mass distribution of the control and treated lignin polymers was analyzed by exclusion liquid chromatography in an HPLC 1260 Infinity Binary (Agilent; USA) equipped with a 50 × 7.5 mm Polargel-M Guard pre-column (p/n PL1117-1800) and a Polargel-M 300 × 7.5 mm column (p/n PL1117-6800) in series, coupled to a diode array detector (DAD). Phosphate buffer (50 mM, pH 8) was used as the mobile phase with a flow of 1 mL/min and a column temperature of 25 °C. From each sample, 20 μL of aliquots was taken using an Agilent 1200 Infinity autoinjector. Elution chromatographic profiles were monitored at 254 nm for 20 min of run time. Moreover, to determine the optimal conditions of enzyme reaction for the polymerization of Kraft lignin with SilA, response surface methodology (RSM) with a factorial design 2^3 was applied. For the statistical optimization, the input variables used were Kraft lignin concentration (0.25, 0.75, and 1.25 g/L) and time of incubation (10, 20, and 30 min) being the output variable (response function) the degree of lignin polymerization achieved.

The obtained response values were used to estimate the model coefficients by means of the statistical package Statgraphics Centurion XV. Student's *t*-test and variance analysis (ANOVA) were also applied for the sake of reliability and accuracy of findings.

2.4. Evaluation of the Effect of Functionalized Kraft Lignin with SilA on the Preparation of Oleogels. *2.4.1. Preliminary Assays for Oleogel Preparation.* For this purpose, enzyme reaction mixtures (100 mL) containing 100 μM phosphate buffer (pH 8), distilled water, Kraft lignin (80 g/L), and laccase (1 U/g) were incubated at 45 °C for different times (1, 2, and 3 h). The functionalized samples once acidified with HCl at pH 2 for lignin precipitation were incubated at 4 °C overnight. After 24 h, all samples were centrifuged (Beckman-Coulter, USA) at 12,439*g* for 20 min. The different functionalized lignins were dried at 60 °C for 48 h. Controls were prepared under the same conditions using a heat-inactivated enzyme (100 °C, 20 min).

The functionalized lignins were previously ground using a mortar to avoid large clusters obtained during processing. Then, lignin (4 g) was added to a 100 mL stainless steel reactor, together with castor oil (14 g) and 2 g of hexamethylene diisocyanate (HDI) (99% purity degree, Sigma-Aldrich). The mixture was stirred for 24 h using a RW20 IKA agitator (Germany) coupled with an anchor impeller. The vessel was maintained at 24 °C using a water bath. Once the processing was finished, the oleogels were kept at room temperature for 1 month to ensure that the free diisocyanate reacted and the sample could not undergo any internal structural change.

2.4.2. Optimization of Oleogel Preparation Conditions through RSM Application. To optimize the conditions to obtain oleogels from functionalized Kraft lignins with competitive rheological characteristics, response surface methodology (RSM) with a factorial design 2^3 was used. This technique allowed us to identify the factors that influence the process and to obtain a quadratic polynomial model representing the responses obtained. A central composite design (CCD) with two independent variables, Kraft lignin concentration (50, 75, and 100 g/L) and time of incubation (1, 2, and 3 h), was

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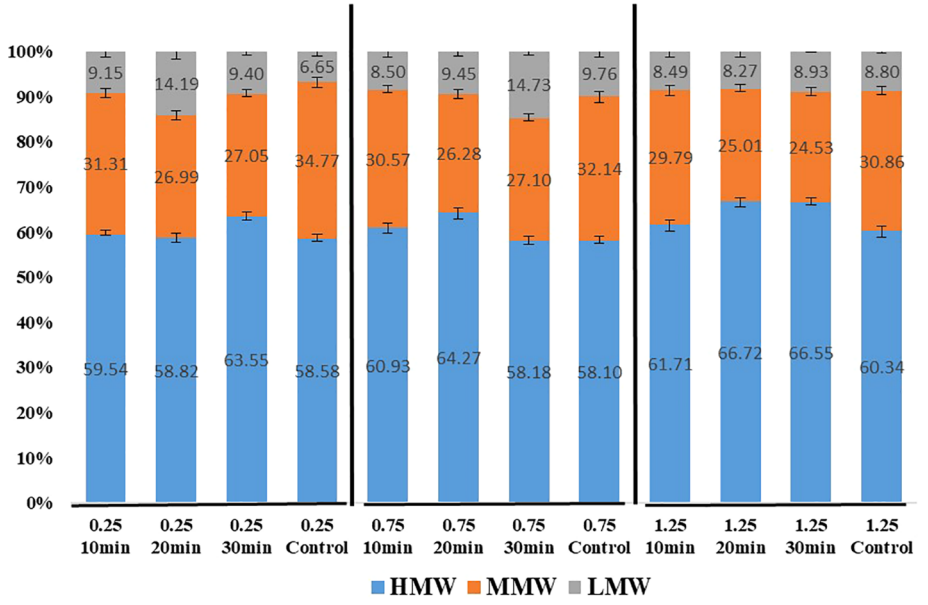

Figure 1. Percentages of high, medium, and low molecular weight (HMW, MMW, and LMW) fractions in samples containing different concentrations of Kraft lignin treated with SilA at different times of incubation, compared with controls. Range of molecular mass distribution: HMW (180−120 kDa), MMW (120−20 kDa), and LMW (20−2 kDa).

employed. The response functions measured were the rheological characteristics of oleogels. All assays were carried out at the same pH and temperature conditions as above described, and 1 U/g of laccase. was used.

The regression coefficient of the response polynomials was estimated by a multiple linear regression model, Student's *t*-test, and analysis of variance. The Statgraphics Centurion XV program was used to analyze the data and obtain the response surface plots from the polynomials.

2.4.3. Rheological Characterization of the Oleogels. Linear viscoelastic behavior was analyzed by frequency sweeps in both ARES (Rheometric Scientific, UK) and Mars (Thermo Scientific, Germany). Serrated plate-plate geometries of 25 and 20 mm in diameter were used, respectively. The frequency sweeps were attained between 100 and 0.03 rad/s. The linear viscoelastic range was obtained using strain or stress sweeps.

To get only one value for comparison, the "plateau modulus" (Gn^0) was focused,[20] which was calculated as the value of the storage modulus (G′) where the loss tangent was minimum.[5,21]

3. RESULTS AND DISCUSSION

3.1. Statistical Optimization for Functionalization and Polymerization of Kraft Lignin by the SilA Laccase. The elution chromatographic profiles of functionalized Kraft lignins obtained at 254 nm are shown in Figure 1. In all cases, an increase in the absorbance peaks of the samples treated with SilA both in the range of high and low molecular weight was observed (HMW and LMW, respectively). Indeed, a decrease in the absorbance peaks in the range established for the polymers of the medium molecular weight (MMW) was detected. It is important to remark that the possible contribution of the enzymatic extract to the absorbance peaks of HMW was discarded after proving by HPLC-DAD that the absorbance of an enzymatic dose of 1 U/mL was lower than that corresponding to the lignin samples treated with SilA laccase.

In SilA-treated samples containing 1.25 g/L of Kraft lignin, the highest increase of the HMW polymer fraction was observed after 20 and 30 min of incubation, representing 66.72 and 66.55% of the total area, respectively, compared with the untreated controls (60.34%). In a similar way, these enzymatic treatments produced the greatest decrease in the MMW fraction percentages and the lowest increase of the LMW fraction percentages (Figure 1).

The results obtained demonstrated that Kraft lignin was oxidized by the bacterial SilA laccase increasing its reactivity and, as consequence, the molecular weight average. The ability of fungal laccases to oxidize lignin from lignocellulosic residues and other lignin derivatives such as lignosulfonates was previously described.[22,23] To our knowledge, there are no references on bacterial laccases that have proven capable of polymerizing residual lignins to obtain eco-friendly value-added products with potential industrial applications. Nevertheless, previous studies carried out with laccase SilA pointed out the ability of this enzyme to polymerize SECO lignin achieving oligomers of until six units as well as several technical lignins under alkaline conditions.[18]

Nowadays, it is increasing interest to set up enzymatic processes as friendly tools for the development of polymers from lignocellulosic materials and/or different forms of residual lignins derived from different pulping processes. These transformed polymers offer a wide range of applications as textile fibers, composite boards, oleogels, packaging materials, etc.[24−27]

The effectiveness of the SilA treatment on Kraft lignin polymerization was evaluated using RSM methodology. Once we obtained the data of the factorial design, the regression model, describing the correlation existing among the independent variables and the response, showed a good adjustment. The investigation results showed high accuracy of the model, signifying the model validation under the tested conditions.

The analysis of the results showed that there are no statistically significant differences ($p > 0.05$) regarding the percentage of the HMW fraction achieved in all the conditions assayed. The quadratic regression equation in terms of the coded factor for the Kraft lignin polymerization model in the HMW fraction is given as follows

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$$\text{lignin polymerization (HMW)} = 55.1582 + 0.681208 \times \text{rt} - 6.60333 \times \text{C} - 0.0152667 \times \text{rt}^2 + 0.0415 \times \text{rt} \times \text{C} + 6.75333 \times \text{C}^2$$

Once we obtained the adjusted polynomials for dependent variables, dimensional 3D graphs were established for the HMW fraction to investigate the optimal effect of each parameter and their interaction on Kraft lignin polymerization with SilA laccase (Figure 2). From this response surface plot, it

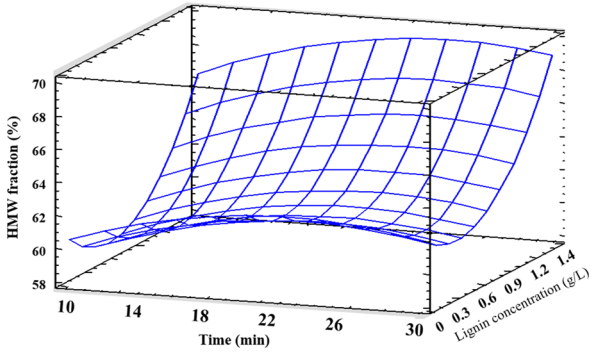

Figure 2. Response surface combined effect of Kraft lignin concentration and the reaction time of SilA laccase on the HMW fraction obtained by lignin polymerization.

was possible to obtain a critical zone, where the combination of the optimal values of the variables studied can be located to obtain maximum polymerization. Thus, to achieve the highest polymerization, the optimal concentration of Kraft lignin was estimated at 1.25 g/L and an incubation time at 24.0 min.

Functionalization of lignin by enzymatic treatment and subsequent polymerization is a convenient way to improve its properties for biotechnological use.[6] Then, once the lignin polymerizing capacity of laccase was demonstrated, we tried to verify that the properties of oleogels are better with laccase-polymerized lignin than with untreated lignin as the thickener. To check the oleogels' rheological properties, the required experiments were carried out using a factorial design 2^3, maintaining 1 U laccase.

3.2. Optimal Conditions for Oleogel Obtention Evaluated through Their Rheological Properties. Because of the absence of data about the obtention of oleogels from functionalized lignin with laccase and to check the efficiency of the laccase in the functionalization of Kraft lignin, preliminary assays were developed to know in what extent the different variables tested could affect their quality. Kraft lignin treated with inactivated laccase was used as control.

Results obtained showed that oleogels prepared from Kraft lignin (80 g/L) functionalized with 1 U/g of SilA laccase and 3 h of incubation presented the highest viscoelastic functions with values of 4 orders of decimal magnitude greater than the control, which implies a hardening of oleogels giving similar characteristics to the commercial greases containing lithium as the thickener.[27] Figure 3 shows the mechanical spectra of the oleogels as a function of the treatment of lignin with laccase.

The lineal viscoelasticity observed allows us to confirm that highly structured oleogels were obtained where the values of storage modulus G′ were always considerably higher than those of loss modulus G″ over the entire frequency range

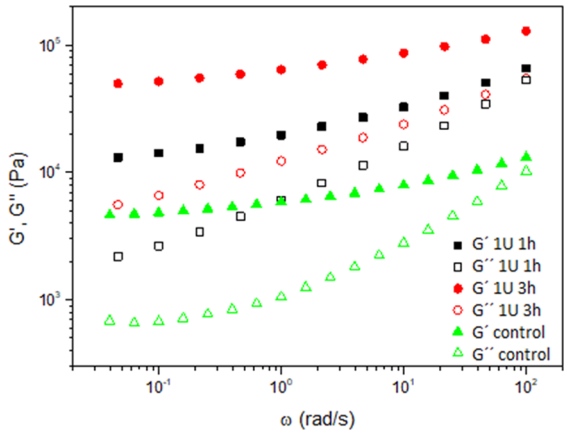

Figure 3. Evolution of the storage (G′) and loss (G″) moduli with frequency for oleogels prepared from functionalized lignin with 1 U laccase at different times of incubation.

studied. However, the values of the linear viscoelastic functions clearly increase when lignin is polymerized with laccase giving higher values of G′ and G″, resulting in a qualitatively and quantitatively similar linear viscoelastic response to that found in traditional lithium soap-based lubricating greases. These commercial lubricants maintained G′ standard values since 10^4–10^5 Pa, which are similar to those corresponding to commercial lubricants containing mineral oils and metallic soaps.[28,29]

Oleogels obtained in this preliminary assay from functionalized residual lignin were physically stable and exhibited viscoelastic responses that are typical for thickeners usually present in traditional lubricant greases. This behavior is usually attributed, at least partially, to the cross-linking of lignin-based radicals generated as a consequence of their oxidation by SilA.[18,30] This phenomenon gives increase to a highly structured material that allows greater oil confinement, greater availability of the NCO-lignin complex for the subsequent reaction with castor oil, and a stronger arrangement due to the increase of hydrogen bonds joined to the covalent linkages in the structure.[5,31,32]

To get a deeper knowledge on the influence of the lignin functionalization degree achieved with the laccase on the oleogels, the plateau modulus (Gn^0) was estimated. Gn^0, the characteristic parameter of the plateau region, defined as the extrapolation of the contribution of the level of entanglements to G′ at high frequencies, may be considered as a measure of the aggregation number among the dispersed structural units or the density of physical entanglements and, consequently, is related to the strength of the microstructural network.[33]

The Gn^0 values obtained for oleogels containing 50, 75, and 100 g/L of lignin after 1, 2, and 3 h of reaction time resulted quite different and non-linear probably due to the different enzymatic reaction progress and of the generation of active groups. The maximum value of Gn^0 (35,515 Pa) corresponds to the oleogels obtained with 75 g/L of lignin functionalized with SilA after 1 h of enzymatic reaction.

Based on the success of these results, a model was designed by the RMS technique to optimize the conditions for obtaining competitive oleogels based on the viscoelastic parameters to be used as thickeners in the industry. Once we performed the statistical analysis, the results obtained did not show significant differences ($p > 0.05$) between the rheological characteristics of oleogels (viscoelasticity) under the different conditions

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assayed. The adjusted polynomial of the data allowed us to obtain the response surface that shows the behavior of the viscoelasticity of oleogels as a function of the variables studied (Figure 4).

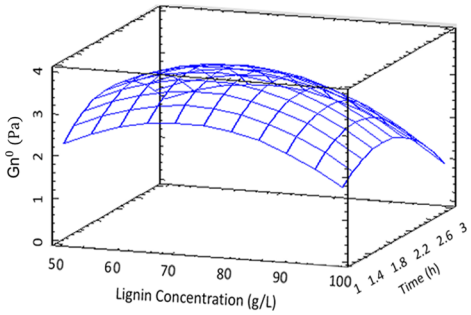

Figure 4. Three-dimensional (3D) response surface and contour plot showing the mutual interaction of Kraft lignin concentration (50−100 g/L) and time of incubation (1−3 h) on viscoelasticity of oleogels.

As shown in the figure, it is evident that the presence of a peak on the surface obtains a maximum point for the variables studied. Therefore, the maximum values of Gn^0 were coincidental with a lignin concentration of 66.6 g/L of Kraft lignin and a reaction time of 1.98 h.

4. CONCLUSIONS

In summary, the rheological properties of oleogels obtained in this work from a laccase-treated residual lignin fulfil the industrial requirements to be considered as an efficient and biodegradable alternative to traditional lubricants. Moreover, the bacterial laccase SilA has demonstrated its effectiveness for lignin valorization improving its reactivity and making easier its transformation in high-added value products. The eco-friendly oleogels obtained proved to be suitable as thickeners for industrial purposes because they could competitively replace metals actually present in commercial greases.

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Notes

The authors declare no competing financial interest.

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