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Influence of Produced Water and Light Irradiation on the Composition of Exopolysaccharide Produced by *L. amnigena* Evaluated by Raman Spectroscopy

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ABSTRACT

This study aimed to compare the changes in the composition of the exopolysaccharide (EPS) produced by *Lelliottia amnigena* in culture medium containing distilled water (DW) and dialyzed produced water (DPW) irradiated by either Laser (λ660 nm, 8.0 J/cm^2) or LED (λ630 nm, 12.0 J/cm^2) during bacterial growth using Raman spectroscopy at 1064 nm. The cultures of *L. amnigena* were irradiated at 9- and 12-h, and the EPS obtained from different production protocols were analyzed dehydrated. Raman spectra showed peaks assigned to saccharides from EPS polymer, and principal component analysis revealed differences in the composition of the EPS produced depending on the water used in production and the light source used for irradiation. Remarkably, the presence of acyl groups (acetyl and pyruvyl) in the mannose residues at the group DW and mannose without evidence of acetyl in the irradiated groups; the irradiated groups also presented evidence of carboxylate (succinyl).

1 | Introduction

Produced water (PW) is an unwanted by-product (wastewater) that the petrol industry generates. It occurs when substantial amounts of water in underground rocks are extracted with oil, being the most significant volume of liquid effluent in oil activities. The reservoir's geological formation and geographical location influence its physical, chemical, and biological characteristics [1, 2]. Significant efforts for proper PW recycling have been proposed and triggered [3]. Biological treatment systems for PW have been described in the literature [4]. However, only some studies have aimed to reuse PW as a substrate for generating value-added products actively participating in the circular economy system [5, 6].

The exopolysaccharides (EPSs) produced by selected bacterial species have drawn the attention of the oil industry in the extraction field. The excretion of EPSs is a protective response by the bacteria to adverse and unfavorable conditions to increase their environmental adaptability, often promoting symbiotic relationships and adherence by forming biofilms [7]. EPSs present different physiological functions depending on the

microorganism and ecological circumstances and are also responsible for the adsorption of exogenous compounds and inorganic ions due to their anionic charge [8, 9].

The properties of the EPSs, such as water retention, emulsification, thickening, gelling, and pseudo plastic behaviors, lead to improved fluid flow through porous formations, increased oil production rates by enhancing oil recovery, and promotion of reservoir stabilization [10, 11]. Additionally, EPSs function as efficient, biodegradable alternatives to synthetic polymers, mitigating environmental concerns while offering superior performance in drilling muds and hydraulic fracturing fluids [12].

Carbon sources produce microbial EPS, including by-products and residues [13]. The carbon source type influences the EPS yield, quantity, and composition [14]. Organic nitrogen sources often result in a higher specific bacteria growth rate and increase EPS production [15]. Further, some carbon in nitrogen sources might serve as a substrate for EPS production. It may increase the carbon/nitrogen ratio, thus promoting EPS production [14]. The presence of metal ions such as Cd(II), Cu(II), and Pb(II) may affect the production of EPS [16]. Strains of the species *Enterobacter cloacae* may present resistance to heavy metal ions such as Cr(IV), Cu(II), Zn(II), and Fe(II). They may present changes in the metabolism in the presence of these metals, increasing microbial concentration and EPS production [17, 18].

Polysaccharides are high molecular weight molecules obtained by polymerization of various sugar monomers (such as fucose, glucose, galactose, glucuronic acid, and mannose) connected through glycosidic linkages (–O–) at the hydroxyl group (–OH) of both monomers. The monomers are usually neutral, acid, and/or amino sugars. However, often polysaccharides also contain non-carbohydrate substituents such as organic acyl groups (e.g., acetate, pyruvate, and succinate esters) and inorganic compounds (e.g., phosphate and sulfate) [19, 20], some of which confer an anionic character to these macromolecules. These different charged substituents are responsible for the adsorption sites found in EPSs that allow interactions with ions and other molecules, making these biopolymers attractive in several applications [9].

Examples of EPSs from Gram-negative bacterial species are xanthan gum and colanic acid. Colanic acid is a product of many strains of *Enterobacteriaceae* when grown under suitable conditions. The polymer is formed from hexasaccharide repeat units composed of monosaccharides fucose, glucose, galactose, and glucuronic acid. Still, it is additionally acetylated (acetyl group on the unsubstituted fucose residue of each hexasaccharide repeating unit) and carries pyruvate ketal groups at the galactosyl residue (Figure 1A) [7, 8]. Although the same carbohydrate structure is found in colanic acid from different bacterial species, the acyl substituents may vary [7].

The structure of the EPS produced by *Lelliottia amnigena* (formerly *Enterobacter amnigenus*) is also formed by hexasaccharide repeat units resembling colanic acid, in which the terminal galactosyl residue is replaced by methylated and carboxylated mannose [7, 21] (Figure 1B). The EPSs of *L. amnigena* BPT 165 and *Enterobacter ludwigii* Ez-185-17 are structurally related to each other and to colanic acid, differing in the presence of mannose in place of galactose in the trisaccharide side chain of the former EPS [22]. EPSs containing fucose and mannose are unusual as mannose is an intermediate in fucose biosynthesis at the sugar nucleotide level [7]. However, another example was seen in the disaccharide repeat of exopolysaccharide from *Alcaligenes latus* [23]. Examples of EPSs without mannose include alginate and fucopolysaccharides.

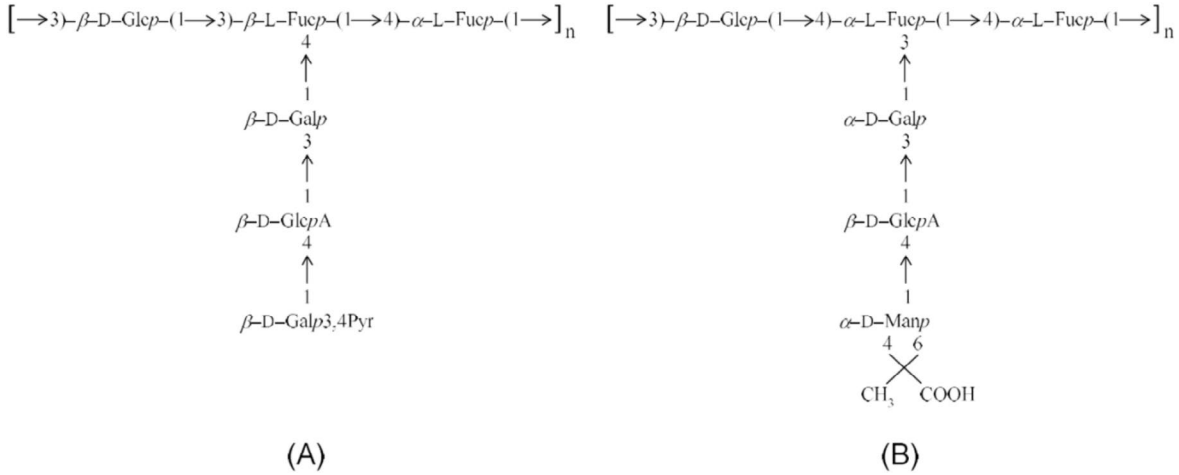

FIGURE 1 | Diagram showing the structural representation of polysaccharides with branched glycosidic linkages, indicating the connections between monosaccharides and the positions of the branches. (A) Structure of the EPS colanic acid produced by *Escherichia coli*, *Salmonella typhimurium*, and *Enterobacter cloacae* [7]. (B) Structure of the EPS produced by *Enterobacter amnigenus* (Cescutti et al., Chart 4 [21]) that resembles the structure of the EPS produced by *Lelliottia amnigena* [7]. Fuc, fucose; Glc, glucose; Gal, galactose; GlcA, glucuronic acid; Man, mannose; Pyr, pyruvyl; *p*, pyranose (cyclic form of a monosaccharide with a six-membered ring: five carbon atoms and one oxygen atom). *α* and *β*—anomers of cyclic monosaccharides. D and L—stereoisomers (molecules with the same molecular formula but differing in spatial orientation). 1 → 3, 1 → 4: carbon 1 of the first monosaccharide bonds to carbon 3 or 4 of the second monosaccharide. 3, 4, 6—substituents are attached at the 3rd, 4th, and 6th carbon positions, respectively. CH_3, methyl group; COOH, carboxyl group.

FucoPol is a high molecular EPS produced by *Enterobacter* A47 (DSM 23139); besides fucose, it contains glucose, galactose, and glucuronic acid, and acyl groups (acetate, pyruvate, and succinate) at the glucuronic acid residue [24–26]. The estimated composition in percentage is fucose (32–37 mol%), glucose (27–37 mol%), galactose (25–26 mol%) and glucuronic acid (9–11 mol%), and acyl groups: pyruvate (9–14 wt.%), acetate (3–8 wt.%) and succinate (2–3 wt.%) [27, 28]. The bacterium of the genus *L. amnigena* can produce a fucopolysaccharide with high molecular weight (> 106 Da) in medium containing glycerol as a carbon source, presenting in its constitution glucose, galactose, glucuronic acid, and mannose in addition to fucose [7, 28–30], and acyl group substituents (pyruvyl, acetyl and succinyl) in the glucuronic acid and/or mannose residue [7, 24, 30]. Figure 1B presents the structure of the EPS produced by *E. amnigenus* from Cescutti et al. [21], which is like the EPS produced by *L. amnigena* from Sutherland [7].

Raman spectroscopy is a method used for both qualitative and quantitative evaluation of the chemical composition of biological compounds and for monitoring biological processes non-destructively [31, 32]. Different biocomposites can be assessed, and Raman spectroscopy has shown both changes in the composition and differences in the structural and molecular conformations of polymers studied, such as EPSs from *Xanthomonas campestris* and *Arthrospira platensis* [33–35]. A previous study showed that the composition of xanthan gum produced by *X. campestris* could be assessed using dispersive Raman spectroscopy (1064 nm excitation), particularly detecting the relative amount of xanthan, acetyl, and pyruvyl substituents in culture mediums containing distilled or produced water in different concentrations [33]. Also, Raman spectroscopy (1064 nm) was employed to evaluate the composition of xanthan produced in media containing distilled or produced (dialyzed or not) water after photo-stimulation with laser or LED, showing that xanthan production increased in the culture with distilled water; in contrast, both pyruvyl and acetyl mannose content went up in the culture with produced dialyzed water [34]. The Raman spectra can also be collected with two common excitation wavenumbers: 532 and 633 nm to monitor in real time the EPS components purified from *A. platensis* with the aim to identify the composition of the saccharides and impurities [35]. Therefore, Raman spectroscopy at near-infrared 1064 nm excitation is suitable for EPS characterization since it can overcome the strong fluorescence in biopolymers when the Raman signal is excited with 785 or 830 nm [36].

It is hypothesized that different culture mediums (distilled water (DW) or dialyzed produced water (DPW)) and photostimulation with Laser or LED light could influence the composition of the EPS produced by *L. amnigena*. Therefore, the objective of this study was to use dispersive Raman spectroscopy at 1064 nm excitation to describe the spectral features of the EPS produced by *L. amnigena* using as culture medium distilled water (DW) and dialyzed produced water (DPW) from a carbonate oil field, irradiated by either Laser (λ660 nm emission) or LED (λ630 nm emission) during bacterial growth, in terms of the Raman features of the saccharides (i.e., the presence of peaks assigned to fucose, glucose, galactose, glucuronic acid, and mannose) and the acyl groups (pyruvyl and acetyl) in the mannose residues and carboxylate (succinyl) in the glucuronic acid and/or mannose residues, thus correlating these Raman spectral features with the probable EPS composition. Also, it is intended to compare the changes in the composition of the produced EPS depending on the type of water (DW or DPW) and irradiation (Laser or LED) employed to obtain the formed polysaccharide through principal component analysis (PCA).

2 | Materials and Methods

2.1 | Group Studies, Bacterial Growth, and EPS Production

2.1.1 | Group Studies to Produce the EPS

The study was divided into four groups with different water types used in the culture medium and the type of irradiation of the culture medium according to Table 1. The EPS production was performed in triplicate in each group study.

2.1.2 | Produced Water

During primary recovery activities, the produced water (PW) was collected in a carbonate oil field (located in Sergipe state, Brazil). The produced water was further dialyzed, generating the dialyzed produced water (DPW). The water's physical–chemical characteristics and metal content and the dialysis process applied to the water were previously described by Pinheiro et al. [34] using the method described by the American Public Health Association (APHA) [37].

2.1.3 | Bacterial Strain

The microbial culture of *L. amnigena* comb. nov. (taxonomic reclassification from *E. amnigenus*) was obtained from the collection of cultures of microorganisms from the Laboratory of Biotechnology and Ecology of Microorganisms (LABEM) of the Institute of Health Sciences (ICS), Federal University of Bahia

TABLE 1 | Group studies depend on the type of water used as a culture medium and the irradiation to obtain the EPS from *Lelliottia amnigena* species.

	Irradiation		Production medium	
Groups	**Laser**	**LED**	**DW**	**DPW**
EPS produced in DW[a]	(–)	(–)	(+)	(–)
EPS produced in DPW	(–)	(–)	(–)	(+)
EPS produced in DPW + Laser	(+)	(–)	(–)	(+)
EPS produced in DPW + LED	(–)	(+)	(–)	(+)

Abbreviations: DPW, dialyzed produced water; DW, dialyzed water; EPS, exopolysaccharide.
[a]The group DW was considered a control.

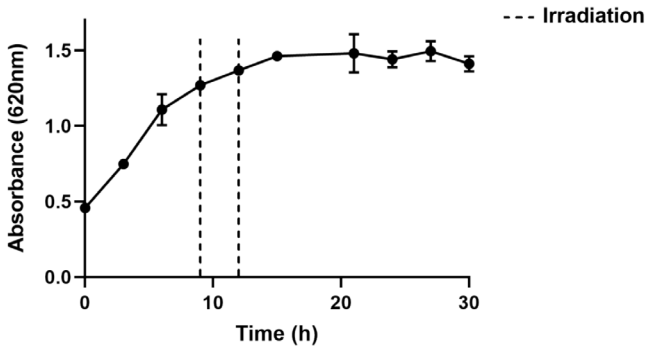

FIGURE 2 | The growth curve of *L. amnigena* was obtained by measuring the light attenuation due to the absorption at 620 nm and the interval times when the photo-stimulation with Laser and LED was done during the exponential growth phase at 9 h and stationary phase at 12 h.

TABLE 2 | Irradiation parameters used in the *L. amnigena* culture to produce the EPS.

Parameters	Laser	LED
Wavelength (nm)	660	630 ± 2
Spot size (cm^2)	0.040	0.50
Output power (mW)	40	140
Irradiation time (s)	200	180
Energy density (J/cm^2)	8.0	12.0

(UFBA), Salvador, BA Brazil. For long-term storage, the cultures were kept in an ultra-freezer at −73°C (−80°C Series TSX, Thermo Electron Corp., Bartlesville, OK, USA) in cryogenic tubes containing Hogness medium (33.0 g/L potassium phosphate dibasic; 9.0 g/L potassium phosphate monobasic; 2.25 g/L sodium citrate; 0.45 g/L magnesium sulfate heptahydrate; 4.5 g/L ammonium sulfate; and 22.0 g/L glycerol).

2.1.4 | Production of Exopolysaccharide

The preparation of the microbial inoculum was performed by inoculating a cryotube with cryoconservated strain (1.0 mL) in 20 mL of trypticase soy broth medium (Becton Dickinson GmbH, Heidelberg, Germany) and incubation for 24 h at 30°C under orbital agitation of 150 rpm (model I26 Incubator Shaker Series, New Brunswick Scientific Co, San Diego, CA, USA). The re-inoculation of 10% (v/v) was conducted after 24 h using 4 mL of inoculum in 36 mL of TSB medium in 250 mL Erlenmeyers and incubated under the same conditions (30°C and 150 rpm) for 16 h. After this time, 10 mL (4.2×10^8 cells) of the initial culture was inoculated in 90 mL of modified mineral salt medium (MSM) [29]. The MSM medium consisted of (w/v): 0.277% sodium phosphate dibasic, 0.10% potassium phosphate monobasic, 0.00713% calcium nitrate tetrahydrate, 0.10% ammonium sulfate, 0.020% magnesium sulfate heptahydrate, 0.050% yeast extract, 2.5% sucrose, 2.0% glycerol. Depending on the study group, the MSM medium was prepared using DPW or DW. The pH was corrected to 7.0 with a 5.0 M NaOH sodium hydroxide solution and pasteurized by incubation at 65°C for 30 min. This was followed by 44 h incubation at 30°C under orbital agitation of 250 rpm (model I26 Incubator Shaker Series).

After the production phase, the produced EPS was centrifuged at 10,000 *g* (model 5804 R centrifuge, Eppendorf SE, Hamburg, Germany) for 30 min at 4°C. Later, 150 mL of 99.5% ethyl alcohol at 4°C was added to the supernatant and packaged at 4°C for 16 h for EPS insolubilization. The insolubilized material was recovered by centrifugation at 10,000 *g* (model 5804 R centrifuge) for 30 min at 4°C. The EPS was sent for drying in an oven at 30°C until it reached constant weight, measured with an analytical balance (model ATY224, Shimadzu Co., Kyoto, Japan).

2.1.5 | Irradiation of Cultures

The cultures of *L. amnigena* were irradiated at 9- and 12-h according to its growth curve in Figure 2. This growth curve was obtained experimentally and the times of 9- and 12-h were based on the times of the exponential growth phase and stationary phase, respectively. Irradiation was applied during this period to extend the exponential phase, during which the bacterium undergoes binary fission, that is, active cell division. Prolonging this phase increases the number of microorganisms and may enhance the yield of exopolysaccharides. Two photo-stimulation devices were used: Laser (Twin Flex, MMoptics São Carlos, SP, Brazil, λ660 nm, 70 mW, spot area 0.5 cm^2) and LED (FisioLED, MMoptics São Carlos, SP, Brazil, λ630 nm, 150 mW, Spot area 0.5 cm^2). These 630 and 66 m nm wavelengths correspond to the absorption spectrum of cytochrome C oxidase [38], an active component in oxidative phosphorylation and, consequently, in ATP production. The irradiation parameters for the groups are described in Table 2. Both light sources have been calibrated to ensure comparable irradiation parameters and are used in continuous emission mode (focal length of 1.0 mm, divergence angle close to zero).

2.2 | Raman Spectroscopy and Data Analysis

2.2.1 | Spectrum Collection

Raman spectra were acquired in a dispersive Raman spectrometer (model Cora 5700, Anton Paar GmbH, Graz, Austria) composed of a laser diode (1064 nm excitation wavelength with 450 mW adjustable power), a spectrograph with a highly efficient volume phase grating and ultra-sensitive CCD camera made up of an InGaAs matrix detector. The spectral resolution ranges from 12 to 17 cm^{-1} in the 400 to 2000 cm^{-1} spectral range. The main advantage of the Raman spectrometer with 1064 nm excitation is that the Raman scattering can be detected in organic molecules with extremely low fluorescence emission; therefore, it is suitable for measuring fluorescent saccharide molecules such as EPS. Previous studies showed that high signal-to-noise ratio spectra could be obtained from saccharides such as xanthan gum using 1064 nm Raman spectrometers, and these spectra could evidence peaks of acyl groups (pyruvyl and acetyl) in the mannose residues [39, 40]. Before sample measurements, the Raman spectrometer's calibration was verified using factory verification standards.

The EPS obtained from different production protocols was analyzed in solid (dehydrated) form. The samples were placed in the sample holder, and the instrument's focus was optimized to produce a high Raman signal. Each sample was measured in triplicate using the laser power of 450 mW, with 10 s of exposure time and ten scan counts for each spectrum. Then, the raw spectra were submitted to pre-processing to remove the baseline by the "mpoly" routine that fits and subtracts the baseline [39], and the mean spectra of each group (as described in Table 1) were calculated for comparison purposes.

Spectra of EPS and basal biochemical compounds that are known to be in the composition of EPS were also obtained. Samples of the saccharides fucose, glucose, galactose, glucuronic acid, and mannose were purchased from Sigma-Aldrich (F2252, G5500, G0750, G5269 and M2069, respectively, Sigma-Aldrich Brasil, São Paulo, SP, Brazil) and the spectrum of each compound "as is" was obtained. These spectra were intended to identify the features of each compound in the observed EPS spectrum and to verify changes in the composition of EPS after the irradiation protocol.

2.2.2 | Exploratory Analysis by PCA

The collected Raman spectra were initially subjected to exploratory analysis by PCA to identify the spectral features related to the effect of the photo-stimulation and the type of water used to obtain the EPS. PCA is a multivariate technique suitable to identify spectral features in the samples that depend on the photo-stimulation used in each group [37, 39]. In this study, the PCA could reveal differences in the amount of EPS produced and changes in its composition since different water compositions used in the bacteria's culture medium and different irradiation protocols used in the groups could lead to changes in the produced EPS under these conditions.

The PCA variables, the principal component scores (Scores), and loadings (PCs) can show the relevant spectral variations that occur in the samples [40] due to the differences in the production of EPS by the bacteria. The Scores are vectors that represent the spectral data projected to the new principal component space (which presents new axes with the maximum variance of the original data in this space) and resemble Raman spectra, thus showing features in the form of bands with peaks in the same positions of the main compounds of the samples under study; the PCs can be interpreted as the "intensities" of the scores in each sample [41, 42] (in fact the PC it is the projection of each spectral data into the score—the cosine projections). Therefore, PCA can show differences in the composition of EPS in each sample group. The Raman features of EPS can be identified in each Score and correlated with the saccharides' features; the corresponding PC can be used to show the "amount" of each compound in each group. Other EPS compounds that may be present in a particular saccharide residue during production, such as acyl groups (pyruvyl and acetyl) and carboxylate (succinyl), could be identified by verifying the presence of peaks of such compounds in any Score and correlating these peaks with the literature, then assigning the high intensity of the respective PC as a meaning of the presence of these compounds in a group.

2.2.3 | Statistical Analysis

Since different variables were evaluated simultaneously (type of water used as a culture medium and use of photo-stimulation), a two-way ANOVA with a balanced design was conducted. The ANOVA general linear model (GLM) was used to verify if these two variables influenced the outcome of the process. The GLM was applied to the results of the PCA and the intensity of Raman peaks of interest related to EPS production, such as pyruvyl and acetyl peaks. In this model, the outcome was evaluated depending on the predictor variables: type of water (distilled water—DW or dialyzed produced water—DPW) and the photo-stimulation (None, Laser, or LED), which were transformed into a "dummy variable." Statistical analyses were conducted with a p-value to reject the null hypothesis ($p < 0.05$), and the models' adequacy was evaluated with the adjusted R^2. Statistical analysis was performed using Minitab software (version 18.0, Minitab Inc., Belo Horizonte, MG, Brazil).

3 | Results and Discussion

3.1 | Raman Spectra of the EPS Produced by L. Amnigena

Figure 3 presents the Raman spectra of the EPS polymer produced by *L. amnigena* with different culture mediums containing DW and DPW, irradiated or not with Laser or LED, obtained with 1064 nm excitation. The key features presented in the spectra can be assigned to saccharides, which are to be expected in the EPS produced by *L. amnigena*: [28] fucose, glucose, galactose, glucuronic acid, and mannose.

The spectra of the saccharides are seen in Figure 4. Most of the peaks overlap due to the similarities of the carbohydrate's Raman spectra [43], suggesting minor differences in the composition of the produced EPSs. The peaks in Figure 3 that can be assigned to a particular saccharide (peak position in the saccharide is seen in parenthesis) are the peaks of fucose at 448 (436), 1265 (1275), and 1456 (1456) cm^{-1}, the peaks of glucose at 527 (516), 768 (768), 924 (914), 1074 (1074), 1113 (1123) and 1339 (1330) cm^{-1}, the peaks of galactose at 1074 (1064) and 1265 (1247) cm^{-1}, the peaks of glucuronic acid at 448 (459), 1074 (1034), 1113 (1113), 1339 (1366) and 1727 (1702) cm^{-1}, and the peaks of mannose at 527 (527), 1074 (1084) and 1113 (1132) cm^{-1}. Exploratory analysis by PCA was performed to unveil minor but relevant differences in the composition of the EPS produced by different culture media and irradiation or not with Laser or LED, particularly the possible presence of peaks referred to as acyl groups (acetyl, pyruvyl, and succinyl) in the mannose residues.

3.2 | Exploratory Analysis by PCA

The exploratory analysis by PCA is seen in Figure 5 (principal components variables Scores—spectral variances, and PCs—intensities of these variances in each spectrum) revealed differences in the composition of the EPS produced depending on the water and the light source used for irradiation during production. The assignments of the Raman features seen in both Figures 3 and 4 in terms of the saccharides produced in the DPW

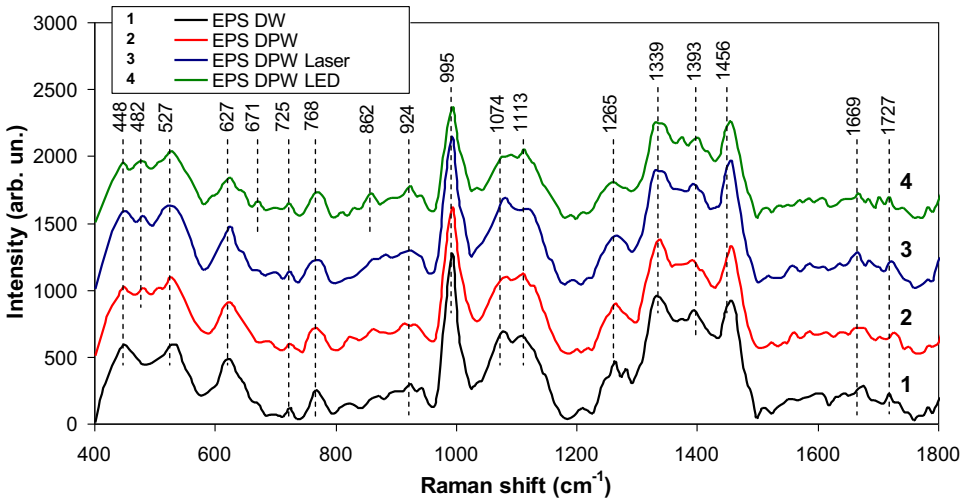

FIGURE 3 | Mean Raman spectra of the EPS polymer produced by *L. amnigena* under different culture conditions: Distilled water (DW), distilled produced water (DPW), distilled produced water irradiated with laser (DPW Laser), and distilled produced water irradiated with LED (DPW LED). Peak labels correspond to saccharide assignments detailed in the text.

groups compared to the DW (control) group show a probable difference in the amount of the EPS, suggesting the presence of acyl groups in some saccharide residues (mainly acetylated and pyruvylated mannose). The description of the spectral features seen in the PCA Scores (Figure 5 and in the following paragraphs) is tentative and may be confirmed by further incremental studies.

The statistical analysis showed that PC1, PC2, and PC3 variables did not present significant differences between the groups (ANOVA, $p = 0.113$, $p = 0.231$, and $p = 0.336$, respectively). Despite the not significant difference between the PC intensities, the spectral features seen in Score 1, Score 2, and Score 3 may be related to differences in the amount of EPS produced in each group, the relative amount of a particular saccharide residue produced (mannose), and the presence of acyl groups in the residues in several samples of each experimental group.

Score 1 showed spectral features that are common to all samples and could be assigned to the saccharides found in the EPS [44] in positions like the ones found in the mean spectra of the groups in Figure 3 and the spectra of pure compounds in Figure 4: fucose, glucose, galactose, glucuronic acid, and mannose. The spectral features at 627, 995, 1393, and 1669 cm^{-1} can be assigned to acetyl (627, 995, and 1669 cm^{-1}) [45] and pyruvyl (627, 995, 1393, and 1669 cm^{-1}) [46] in several samples. These acyl modifications are typically introduced during EPS biosynthesis by enzymes such as acyltransferases [47] depending on the environmental conditions. Despite not being significant, PC1 showed that the groups DW and DPW Laser may present similar amounts of EPS, and the groups DPW and DPW LED may present decreased amounts of EPS.

Score 2 exhibited positive peaks at 527 and 984 cm^{-1} assigned to mannose. Despite not being significant, PC2 showed that the groups DPW and DPW Laser presented an increased amount of mannose compared to the groups DW and DPW LED, with the group DPW LED presenting no change and the group DW presenting a reduced amount of mannose. This suggests that the presence of produced water as a carbon source may promote the incorporation of mannose into the EPS structure, particularly when not combined with LED irradiation.

Score 3 featured positive peaks at 459 cm^{-1} that could be assigned to glucuronic acid or mannose, at 605 and 948 cm^{-1} that could be assigned to acetyl [45], and a negative feature at 872 cm^{-1} that may be assigned to pyruvyl [46]. Although not statistically significant, PC3 suggested that the groups DW and DPW LED may have higher levels of acetylated saccharides (acetylated mannose or glucuronic acid), whereas the group DPW may present increased pyruvylation at the mannose residue as already observed in the Score 2/PC2. To confirm the possible pyruvylated mannose production, data analysis showed a high (negative) correlation between the mean PC2 and mean PC3 of all groups (mannose features in Score 2/PC2 correlated to pyruvyl features in Score 3/PC3, Pearson's correlation coefficient $r = -0.927$, plot not shown), suggesting that pyruvylation may increase as mannose content rises, particularly in the group DPW.

The PC4 and PC5 variables presented statistically significant differences between the groups (ANOVA, $p < 0.01$ and $p < 0.05$, respectively). Score 4 showed positive peaks at 516 and 703 cm^{-1} and negative peaks at 413, 820, and 1084 cm^{-1} that may be assigned to mannose, and negative peaks at 605 and 995 cm^{-1} that may be assigned to pyruvyl [46]. The analysis of PC4 suggests a significant increase of pyruvylated mannose in the group DW compared to the groups DPW Laser and DPW LED, as pyruvyl and mannose features are in the same PCA variable, and a significant increase in mannose residues in groups DPW Laser and DPW LED compared to group DW, corroborating the (not significant) increase in mannose in these two irradiated groups observed in the Score 2/PC2. These observations suggest that light irradiation may increase the production of EPS with high mannose content while reducing pyruvylation, possibly due to the modulation of enzymatic activity induced by light. Additionally, the presence of carboxylate features in Score 5 (below) supports the possibility that the mannose residues in these irradiated groups are succinylated rather than pyruvylated.

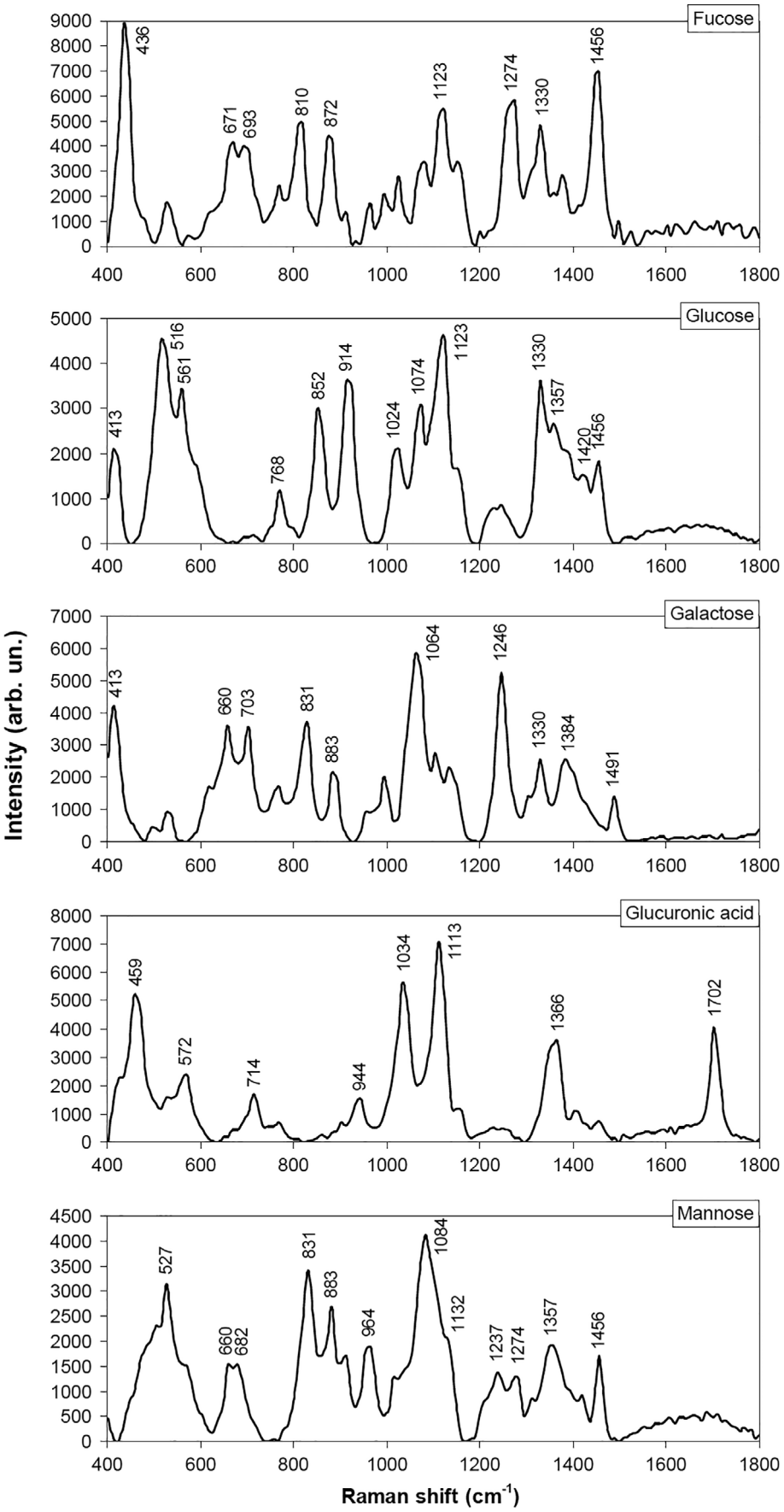

FIGURE 4 | Raman spectra of the isolated saccharide compounds expected in EPS from *L. amnigena*: Fucose, glucose, galactose, glucuronic acid, and mannose. These reference spectra were used for peak assignment in the EPS samples.

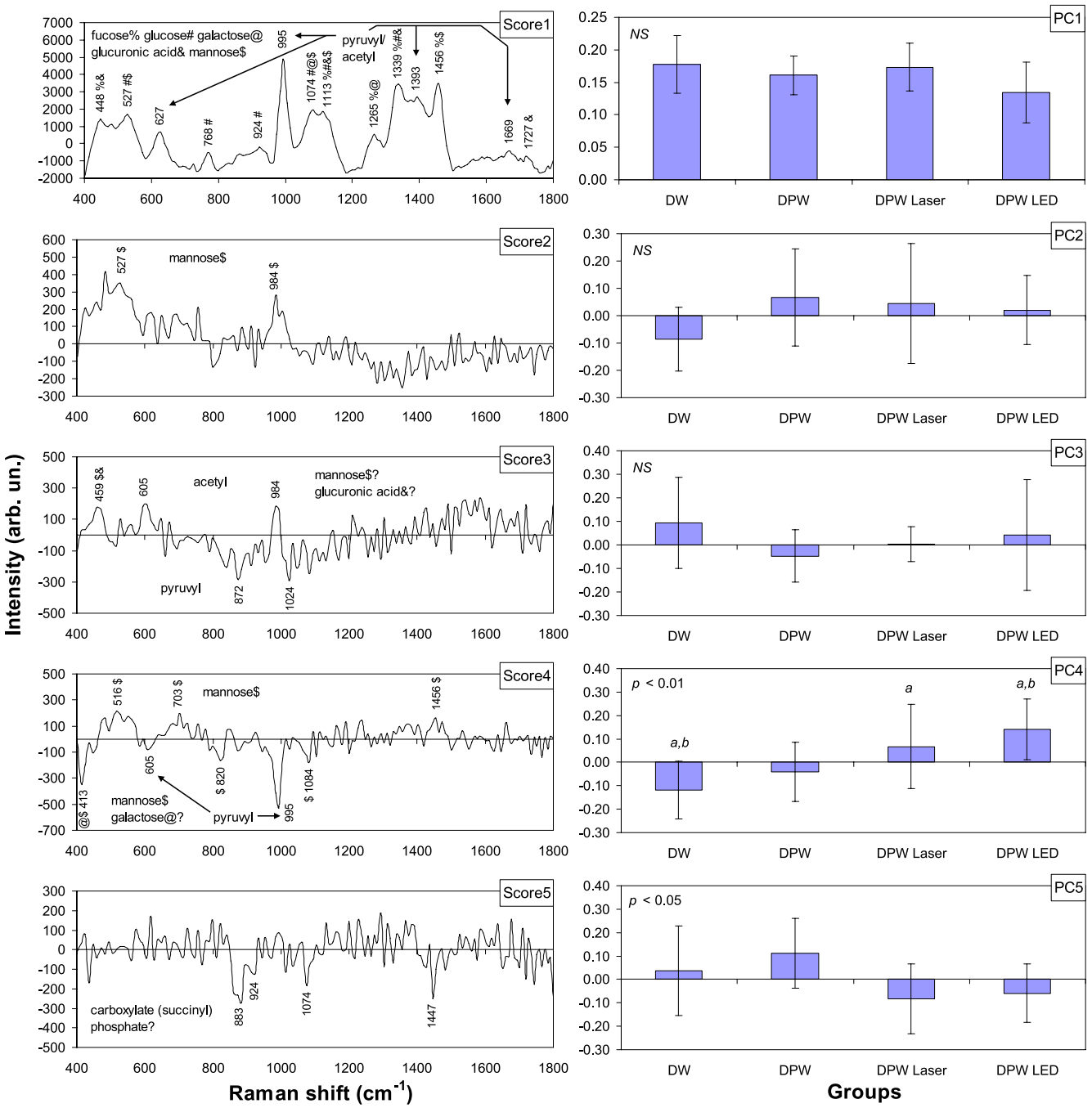

FIGURE 5 | PCA Scores and PC plots derived from Raman spectra of EPS samples. Spectral features in each Score plot are labeled with saccharide or acyl group assignments. PC values indicate the relative mean intensity of each spectral feature per group. Statistically significant PCs (PC4 and PC5) are indicated with *p*-values.

Score 5 showed negative peaks at 883, 924, 1074, and 1447 cm^{-1}, which may correspond to carboxylate groups [48] (succinyl esters) [49]. The negative PC5 values in the irradiated groups (DPW Laser and DPW LED) suggest increased carboxylation (succinylation) in response to irradiation. This may occur at either mannose or glucuronic acid residues and appears to accompany the mannose accumulation at the trisaccharide side chain in these irradiated groups as identified in Score 4/PC4.

The PCA results suggest that the EPS produced by *L. amnigena* varies depending on both the carbon source (distilled vs. produced water used as culture medium) and the presence and type of irradiation (Laser or LED) used to stimulate EPS production. Despite the lack of statistical significance in the first three components, consistent trends were identified. First, the groups DW and DPW Laser may produce more total EPS, with the group DW showing stronger features of acetylated and pyruvylated mannose. Second, the use of produced water (group DPW) appears to promote pyruvylation of mannose. Third, the use of light irradiation (groups DPW Laser and DPW LED) appears to induce the bacteria to produce and incorporate more mannose without acetylation or pyruvylation, suggesting that the enzymes promoting esterification were inactivated or absent, but with evidence of carboxylate (succinyl) substitution.

These results suggest that environmental conditions, particularly water composition and irradiation, modulate the activity of EPS-modifying enzymes, leading to distinct saccharide modifications. Figure 6 presents a schematic diagram summarizing the possible esterification processes that affect mannose residues, including acetylation, pyruvylation, and succinylation.

A study by Sampaio et al. [33] also found that in the production of the EPS xanthan gum, the extensions of the chemical modifications within the mannose group increased with the use of produced water. In the work conducted by Pinheiro et al. [34], it was reported that LED irradiation increased the pyruvylation of mannose residues in xanthan gum production, as verified in the present study. Pyruvate ketals are very common substituents in EPS from Gram-negative bacteria, and the end of the side chain in the EPS produced by *E. amnigenus* is like xanthan gum from *X. campestris* [21].

3.3 | Statistical Analysis—ANOVA General Linear Model

To evaluate the influence of water source and irradiation treatment on EPS composition, a general linear model (GLM) analysis using ANOVA was applied to the PCA loadings (PCs). Table 3 summarizes the statistical outcomes for each PC.

PC1, PC2, PC3, and PC6 did not exhibit statistically significant differences ($p > 0.05$) for either water source or irradiation. However, PC4 showed significant effects for both the type of water ($F = 10.18$, $p = 0.003$) and irradiation ($F = 8.03$, $p = 0.001$), while PC5 revealed a significant effect of irradiation ($F = 3.95$, $p = 0.029$). These results support the interpretation from Section 3.2 that both environmental factors, especially the light source, play a role in modulating EPS composition.

Spectral features associated with PC4 indicate a significant increase in pyruvylated and acetylated mannose in the DW group, whereas irradiated groups (DPW Laser and DPW LED) showed increased mannose content with minimal acylation and evidence of carboxylation (succinylation). These findings align with PCA-based interpretations and confirm that both irradiation and carbon source influence the composition of the EPS.

3.4 | Final Remarks

Raman spectroscopy with 1064 nm excitation proved to be an effective tool for detecting biochemical modifications in EPS, particularly in identifying acylated saccharide residues such as acetylated, pyruvylated, and succinylated mannose and glucuronic acid. Unlike shorter wavelengths, the 1064 nm laser minimized fluorescence interference and enhanced spectral clarity, making it well suited for studying biological polymers like EPS.

The PCA results indicate that the water composition and light exposure significantly influence the enzymatic pathways involved in EPS modification. Produced water appears to increase the availability of mannose in the EPS structure, while light irradiation modulates acylation patterns by reducing acetyl and

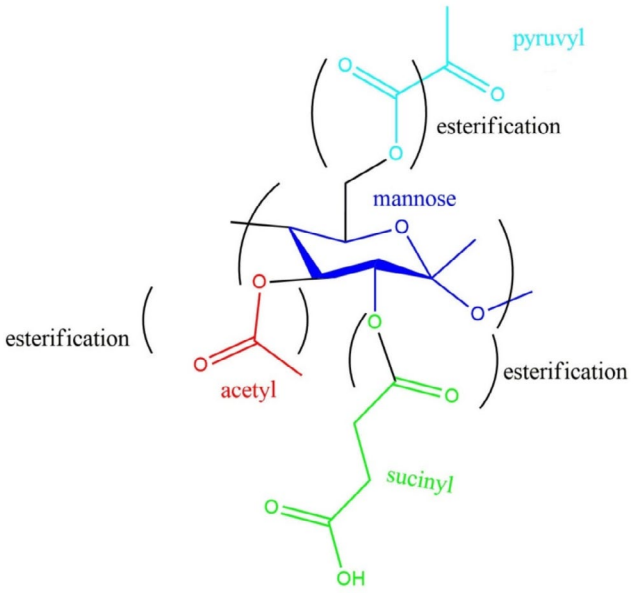

FIGURE 6 | Schematic representation of possible esterification reactions affecting, for instance, the mannose residue in the EPS, including acetylation, pyruvylation, and succinylation. These modifications depend on environmental conditions and the activity of specific bacterial enzymes.

TABLE 3 | Summary of the ANOVA GLM applied to the intensities of the principal component coefficients.

Principal component (PC) loading	Variable	Adjusted SS	*F*	*p*
PC1	Type of water[a]	0.002052	1.8	0.284
	Irradiation[b]	0.008965	2.84	0.072
PC2	Type of water[a]	0.07502	2.77	0.105
	Irradiation[b]	0.01858	0.31	0.733
PC3	Type of water[a]	0.04492	1.63	0.210
	Irradiation[b]	0.007116	0.12	0.887
PC4	Type of water[a]	0.2291	10.18	**0.003**
	Irradiation[b]	0.3255	8.03	**0.001**
PC5	Type of water[a]	0.03691	1.30	0.262
	Irradiation[b]	0.1931	3.95	**0.029**
PC6	Type of water[a]	0.07651	2.82	0.102
	Irradiation[b]	0.1099	2.04	0.146

[a]Distilled water (DW) or deionized produced water (DPW).
[b]None, laser, or LED.

pyruvyl content and increasing carboxylation (e.g., succinyl groups). These findings have implications for optimizing EPS production with tailored functional properties, particularly for industrial applications requiring specific rheological or chemical characteristics.

Previous studies also support these observations. Sampaio et al. (2020) [33] reported increased mannose modification in xanthan gum when produced water was used. Similarly, Pinheiro et al. [34] observed that LED irradiation enhanced pyruvylation in xanthan production. As in those studies, the results here presented suggest that light and type of water can be used to modulate EPS biosynthesis in *L. amnigena*, whose EPS structure shares similarities with that of *X. campestris*.

4 | Conclusion

In this study, Raman spectroscopy with 1064 nm excitation was applied to evaluate the effect of produced water and irradiation by laser or LED on the composition of EPS produced by *L. amnigena*. The spectra of the groups DW, DPW, DPW Laser, and DPW LED showed spectral features assigned to the main components of the EPS: fucose, glucose, galactose, glucuronic acid, and mannose. The spectral features of the acyl groups (acetyl and pyruvyl) and carboxylate (succinyl) in the saccharide residues were also seen.

The PCA could reveal fundamental differences in the amount of EPS produced. In summary, the produced EPS with acetylated and pyruvylated residues was significantly higher in the groups that used distilled water (DW); residues without acetyl (only pyruvyl) were higher in the groups that used dialyzed produced water and were irradiated (DPW Laser and DPW LED). Carboxylate (succinyl) was significantly higher in the groups irradiated (DPW Laser and DPW LED) ($p < 0.05$). Moreover, EPS levels were elevated in the group treated with distilled water (DW) compared to the dialyzed produced water under both light conditions (DPW Laser and DPW LED). Still, this increase was not significant ($p = 0.113$).

Raman spectroscopy at 1064 nm offers a powerful tool for accurately characterizing the composition of the produced EPS, particularly the presence of acyl groups.

Author Contributions

Anna Paula Lima Teixeira da Silva: investigation. Luiz Guilherme Pinheiro Soares: investigation. Lars Duarte Gulberg: investigation. Pedro Jorge Louro Crugeira: investigation. Paulo Fernando de Almeida: investigation. Adjaci Uchoa Fernandes: validation, writing – review and editing. Landulfo Silveira Jr.: conceptualization, formal analysis, investigation, writing – original draft. Antonio Luiz Barbosa Pinheiro: conceptualization, validation, formal analysis, investigation, supervision, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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