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Research Article

Cochineal Waxy Residues as Source of Policosanol: Chemical Hydrolysis and Enzymatic Transesterification

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The aim of this study was to obtain and characterise the long-chain alcohols present in policosanol derived from waste from the production of carminic acid, a natural colouring agent widely used in the food industry. The effectiveness of different methods designed for extraction of policosanol from waxy waste was investigated and its content and composition was determined. Triacontanol was the main component in policosanol produced by chemical processes, and it yields up to 13% by alkaline hydrolysis in water and chloroform extraction. Regarding enzymatic transesterification, policosanol was obtained using lipase *Candida antarctica* recombinant in *Aspergillus niger* (CAL-B*n*) in a reaction medium with toluene. To improve the reaction, different acyl receptors, propanol, butanol, and isopropanol, were tested and molecular sieves were employed to maintain an anhydrous reaction medium. In this case, the policosanol was made up of other long-chain alcohols, but triacontanol was obtained in yields of up to 19% using isopropanol as an acyl receptor. Triacontanol has a great commercial value due to its effect as a promoter of plant growth, and these results contribute to the use and application of this agroindustrial waste in obtaining value-added products.

1. Introduction

The carmine industry, which produces natural red dye food additives, generates a great quantity of waste, which includes wax, cuticles, and cocoons derived from female cochineal insects that are cultivated and maintained on *Opuntia* plants throughout their life cycle (90 days). The insects produce a waxy layer, which is renewed twice (ecdysis) and constitutes around 3% of the weight of the cochineal insect. Cochineal wax is a mixture of esters of cocceric acid and cocceryl alcohol (triacontanol) [1, 2]; therefore, cochineal wax could be a good source of policosanol.

Policosanol is a mixture of long-chain alcohols with a length of 20–36 carbons. It consists mainly of tetracosanol (24C), hexacosanol (26C), octacosanol (28C) and triacontanol (30C). It is found naturally, free or esterified, in different vegetable and animal sources [3–8]. The mixture has been shown to have distinct beneficial effects, such as hypolipemiant, antiatherosclerotic, and cholesterol-lowering as well as antiaggregatory and ergogenic properties [9–12]. Also, triacontanol is a valuable compound because it helps to increase the yields of various agricultural crops [13].

The most widely used procedure for obtaining policosanol esterified in waxes is through the chemical hydrolysis under alkaline conditions, followed by solid-liquid extraction with organic solvents [5, 14–16]. Recently, Ma et al. [17] presented a method via solvent-free reduction from insect wax using $LiAlH_4$. These methods are characterised by the use of substances highly harmful to the environment, requiring high energy expenditure because of the special treatments necessary for the purification of the wastewater generated [18]. On the contrary, the use of immobilised

enzymes in transesterification is considered an ecofriendly process. The enzymatic reaction of transesterification is chemo-, regio-, and stereoselective, as a result of which the products are obtained in high yield and purity, in contrast to chemical hydrolysis, and the method allows the continuous use of the enzyme [19].

Immobilised lipase from the yeast *Candida antarctica*, CAL-B, as well as recombinant lipases expressed in *Aspergillus oryzae* or *Aspergillus niger*, have been reported as efficient catalysts to perform hydrolysis and esterification in organic solvents [20]. Enzyme activity depends on different factors, such as the support type or immobilisation system maintaining the active conformation of the enzyme; other factors include the interaction between the enzyme and substrate, water content, substrate molecular size, amount of organic solvent, and support pore diameter [21, 22].

The water content is essential for the enzymatic reaction to be carried out; an excess affects the conversion rate, so it must be determined specifically for each reaction system [23, 24]. Molecular sieves have been used to control the humidity in the reaction system; however, if the lipases are immobilised on a hydrophilic support, the molecular sieves do not have an impact on the performance of the products [25]. The disadvantage of removing water and using an organic solvent is that the activity of the enzyme is greatly reduced and the reaction time is increased [26].

In this work, chemical and enzymatic transesterification reactions for obtaining policosanol from waxy waste of cochineal insects generated in the carmine industry were compared. First, in chemical reactions, different bases and solvents were evaluated; then, during transesterification, the use of molecular sieves, two lipases, and the effect on the reaction product yields of different alcohols as acyl receptors were analysed.

2. Materials and Methods

2.1. Raw Materials and Conditioning. The materials were provided by Campo Carmín S.P.R. of R.L., located in Morelos, México. Samples of waxy residues corresponding to adult cochineals were collected according to the periods of infestation and crop harvest, which was approximately 90 days after infestation. Lumps, particles, remaining insects, and other impurities were manually removed from the samples. After that, the screening of samples was performed with an electric sieve (Rotate, model RX-2) with mesh sizes 40–100, for 20 min. The finest fraction corresponding to cochineal wax (particles < 0.149 mm) was collected to determine the effectiveness of different policosanol extraction methods. The clean waxy residues were stored at room temperature for further processing.

2.2. Chemical Hydrolysis. Three methods of alkaline hydrolysis were evaluated to investigate their effectiveness in extracting policosanol from waxy residues of cochineal, based on the work performed by Magraner et al. [14] and by Jia and Zhao [15]. Each extraction was run in triplicate.

Method A. Hydrolysis in water by extraction with chloroform. One gram of cochineal wax residue was hydrolysed in 100 ml of NaOH (20% w/v) by stirring for 4 h at 90°C. The mixture was cooled and filtered, and the mud cake separated was extracted with chloroform in a Soxhlet system for 6 h. Finally, the extract was cooled, evaporated at atmospheric pressure, and dried completely in a desiccator with anhydrous sodium sulphate.

Method B. Hydrolysis in alcohol by extraction with ethyl acetate. One gram of cochineal wax residue was hydrolysed in a 100 ml solution of 5 g of KOH diluted in ethanol (25%). The hydrolysis was carried out by stirring for 4 h at 90°C. The mixture was cooled and filtered, and the mud cake separated was extracted with ethyl acetate in a Soxhlet system for 6 h. Finally, the extract was cooled, evaporated at atmospheric pressure, and dried completely in a desiccator with anhydrous sodium sulphate.

Method C. Hydrolysis in water by extraction with hexane. One gram of cochineal wax residue was hydrolysed in 100 ml of NaOH (12% w/v) and the mixture vigorously stirred on a heating plate for 4 h at 90°C. The mixture was cooled and filtered, and the mud cake separated was extracted with hexane in a Soxhlet system for 6 h. Finally, the extract was cooled and evaporated at atmospheric pressure and dried completely in a desiccator with anhydrous sodium sulphate.

2.3. Enzymatic Transesterification Reaction

2.3.1. Wax Purification and Crystallisation. To carry out enzymatic transesterification reactions, the clean waxy residue was purified in a Soxhlet system with cyclohexane for 4 h, followed by extraction with methanol and acetone. The fraction soluble in cyclohexane, corresponding to the wax, was filtered and crystallised, and the fractions soluble in methanol and acetone dried at room temperature. Each resulting powder fraction and the purified wax were characterised by GC-MS.

2.3.2. Enzymatic Transesterification Conditions. Two lipases from *C. antarctica* were used for enzymatic transesterification: CAL-B*o* recombinant from *A. orizae* immobilised on Immobead 150 and CAL-B*n* recombinant expressed in *A. Niger* immobilised on acrylic resin, both from Sigma-Aldrich (México).

Solubility tests were carried out, and toluene was chosen as a good solvent for the wax; this is classified as a type yellow solvent, compared to chloroform, the most common solvent to dissolve policosanol, which is classified as red [27].

The transesterification reaction was carried out as follows: The reaction was performed at 60°C, and 100 mg of enzyme per gram of purified wax was added and dissolved in 5 ml of toluene as a reaction medium; to the mixture was added 250 μl of different alcohols as acyl receptors (propanol, butanol, isopropanol, and terbutanol). Molecular sieves of 3 Å and 4 Å were incorporated in the mix to decrease the negative effect of water on the reaction. This

experiment was realised with continuous orbital stirring or sonication for 2 h for comparison.

The transesterification reactions assisted by sonication were carried out in an ultrasonic bath (Branson, model 1–291) which had a 60 min mechanical timer, a 40 kHz transducer, and heating to 60°C.

2.4. Characterisation and Quantification

2.4.1. FTIR Analysis. The policosanol obtained by chemical hydrolysis and triacontanol standard alcohol was analysed using Fourier transform infrared (FTIR) spectroscopy. A Bruker Vertex 70 FTIR spectrometer with Opus Quant® software (version 6.5) was used to obtain characteristic spectra. The technique of attenuated total reflectance (ATR) was used, with 120 scans per sample and 60 scans for the baseline at 4 cm^{-1} resolution, in a wavenumber range of 400–4000 cm^{-1}.

2.4.2. GC-MS. The characterisation of fractions and the policosanol obtained was carried out with a gas chromatograph (Agilent Technologies Automatic Injector 7890a) coupled to a mass spectrometer (Agilent Technologies 5975C Inert MSD with Triple-Aixs detector). Samples and standards were prepared in chloroform (Cromasol V. Plus, 99.9%, HPLC grade), derivatised with 100 μL of a silanising agent (MSTFA) and heated to 60°C for 15 min [5]. The peaks obtained were characterized, and identification of the long-chain alcohols was carried out by comparison with the mass spectral library [28].

The yield of triacontanol obtained was determined from the mass spectra collected with GC-MS. A standard curve method was adopted to guarantee reliable results. The abundance of the fragment ions, m/z, was taken into account to identify and quantify the triacontanol; the resulting fragment ions were 495, 479, 125.103, 83, and 75.

2.4.3. HPTLC. The enzymatic transesterification reactions were monitored at different times by applying extracts diluted in chloroform in duplicate to chromatographic plates of silica 60 F254 on glass support, incorporating a developer for UV light (CAMAG). A CAMAG-brand HPTLC equipment model I15020060200-0001-2013 was used, integrated with LINOMAT5, ADC2, and VISUALIZER.

Different solvent systems were tested, and a hexane/diethyl ether/acetic acid 85 : 15 : 2 (v/v/v) system was chosen to achieve a optimum separation. Triacontanol standard (Sigma-Aldrich México) was applied for comparison of Rf.

3. Results and Discussion

3.1. Chemical Hydrolysis. FTIR signals allowed monitoring of the hydrolysis reaction, so that it was also possible to distinguish changes in band patterns between the raw material and the policosanol obtained. The most significant changes related to the disappearance of the 1731 cm^{-1} signal, which indicated the rupture of the ester groups (COO^-) and the increase in the 1704 cm^{-1} band, signifying the presence of acid group (–COOH) (Figure 1(a)). Acids and long-chain alcohols are products of the hydrolysis reaction, so changes in these signals indicate good hydrolysis efficiency.

The FTIR spectra of the policosanol extracts were compared with triacontanol standard (Figure 1(b)). The triacontanol standard showed characteristic bands at 3294 (OH bond), 2916 (–CH links), 1461 (C–OH primary alcohol bonds), 1062 (–CO), and 729 cm^{-1}, which is indicative of the presence of at least four methylene groups ($–CH_2–$). The signals found were similar in all extracts and are consistent with those found in the characterisation of the policosanol obtained from *Agave furcroydes* L. wax [3].

However, the FTIR analysis did not allow characterisation and quantification of the policosanol obtained, so we resorted to analysis by GC-MS. The results obtained showed that triacontanol was the major long-chain alcohol present in these extracts, and unquantifiable octacosanol and hexacosanol traces were detected. Hydrolysis by method A resulted in the highest yield of policosanol 134.00 ± 16.58 g/kg of cochineal wax, compared to methods B (49.95 ± 14.21 g/kg) and C (14.25 ± 4.31 g/kg), although method A had the lowest purity (29%) (Table 1). The chloroform used in method A is a solvent commonly used to dissolve policosanol in chromatographic analysis [5, 29], and it also improves extraction, but is not very selective. On the contrary, the hexane used in method C, with polarity index of 0.1 compared to chloroform (4.1) and ethyl acetate (4.4), could be more selective for policosanol. Triacontanol is a nonpolar alcohol due to its long-chain structure of 30 carbons, and so could be more related to hexane. Notwithstanding, hexane was not sufficiently selective, so policosanol of 65% purity was obtained (Table 1). To obtain a high yield of policosanol and higher purity, this method could be complemented with extractions by solvents of different polarities. In the following experiments, methanol and acetone were tested for wax purification and showed good selectivity for long-chain alcohols.

3.2. Enzymatic Transesterification

3.2.1. Chemical Purification. For the enzymatic reaction, the finest fraction (particles < 0.149 mm) of the total cochineal wax residue was subjected to chemical purification to obtain pure wax with a yield of 28%. During wax purification, it was observed that fractions soluble in methanol and acetone contained long-chain alcohols, in comparison to the purified wax, as can be seen in the HPTLC plate shown in Figure 2. This means that methanol and acetone extractions were effective in the separation or extraction of long-chain alcohols. By characterising these fractions by GC-MS, we can conclude that the methanolic fraction composition was as follows: octacosanol, triacontanol, untriacontanol, dotriacontanol, and tritriacontanol, and the fraction obtained from acetone was found to be composed only of triacontanol (Figure 2).

3.2.2. Enzymatic Transesterification Reaction under Sonication. Once the wax was purified, it was used for the

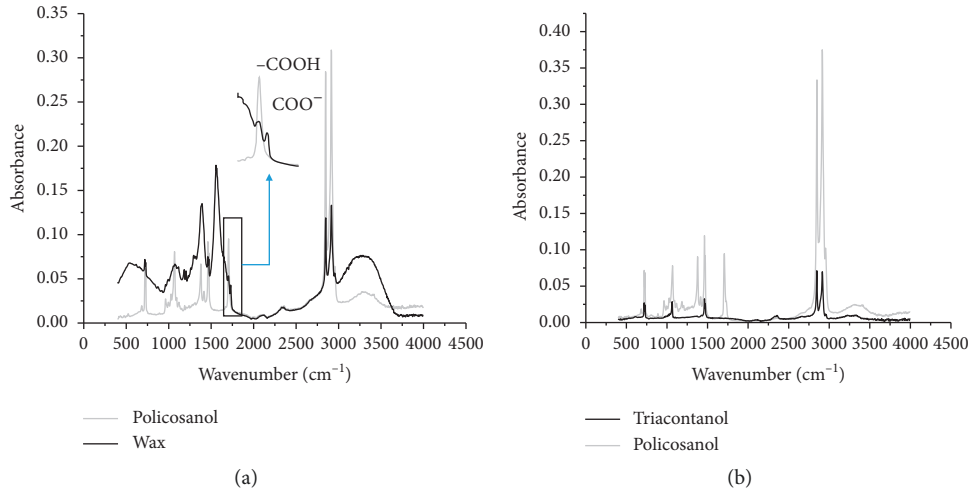

Figure 1: FTIR spectra of (a) policosanol and cochineal wax and (b) policosanol and triacontanol standard.

Table 1: Composition and quantification of policosanol in g/kg of cochineal wax obtained by chemical hydrolysis methods.

	Policosanol composition			Policosanol yield (%)	Policosanol purity (%)
	Triacontanol	Octacosanol	Hexacosanol		
Method A	134.00 ± 16.58^{a}	Traces	Traces	47	29.0
Method B	49.95 ± 14.21^{b}	Traces	Traces	10	51.0
Method C	14.25 ± 4.31^{c}	Traces	Traces	2	69.0

(Tukey $P < 0.05$).

evaluation of two enzymes in the transesterification reaction under sonication: lipase *C. antarctica* recombinant in *A. orizae* (CAL-B*o*) and lipase *C. antarctica* recombinant in *A. niger* (CAL-B*n*). The results obtained by HPTLC showed that CAL-B*o* showed no catalytic activity in any of the combinations of organic solvents, possibly due to the immobilisation support to which the enzyme was attached. Lipase *Candida* CAL-B*o* is immobilised in support Immobead 150, which is formed of methacrylate copolymers with epoxy functions, with an average particle size of 150–300 μm and presents a wide pore size range, with pores in the micropore and mesopore region [30]. In contrast, CAL-B*n* is immobilised on a macroporous acrylic poly resin (methyl methacrylate-*co*-divinylbenzene), having an average particle size of 315–1000 μm and a pore diameter of ~150 Å [31, 32]. Both supports show hydrophobic properties; however, the pore size of the enzyme CAL-B*o* (12–20 Å) is smaller than that of CAL-B*n* (~150 Å) and the pore size distribution is not homogeneous [30]. This characteristic is determinant for the enzyme-substrate coupling; a small pore size or inhomogeneous pore size allows only the smallest substrates to penetrate. The bigger substrates would clog the channels, resulting in a weaker affinity of the enzyme for the substrate by diffusion limitation in small pores [33, 34].

It has already been shown that the lipase enzyme of *C. antarctica* has good catalytic activity with toluene as a reaction medium at 60°C [32], and the use of alcohols as acyl receptors in the transesterification reaction has been proposed because they have a less negative effect on lipase stability [35]; nevertheless, more polar alcohols, such as methanol, change the polarity of the medium, decreasing the stability of the enzyme, while high concentration can lead to serious inactivation of the enzyme [35, 36].

It has been reported that the use of terbutanol as a cosolvent in enzymatic reactions helps to diminish the negative polar effect of methanol in transesterification reactions and therefore favours reuse of the enzyme [37]. In this case, the use of terbutanol as an acyl receptor did not allow obtaining policosanol, in contrast to butanol and isopropanol, which showed advantages in the enzymatic transesterification reaction and therefore obtaining of policosanol. It was found that lipases show different preferences for primary or secondary alcohols; Nelson et al. [38] found *C. antarctica* was suitable for secondary alcohols such as isopropanol or 2-butanol as acyl receptors, with 80% conversion. Alcohols could be modifying the organic system differently; it is well known that lipases show higher activity in hydrophobic than in hydrophilic solvents due to the three-dimensional structure of the enzyme being affected by the balance between hydrophobic interactions, electrostatic charge interactions, hydrogen bonding, disulphide linkages, and van der Waals forces with organic solvents. Solvents that can penetrate into the active sites of the enzyme can cause unfolding of proteins to occur due to disturbances in these forces [39]. As a result, transesterification reactions in organic solvents are strongly dependent on the polarity of the reaction medium [40]. Alcohol addition modifies the polarity of the organic system and affects solubility; maintaining solubility is essential for the transesterification

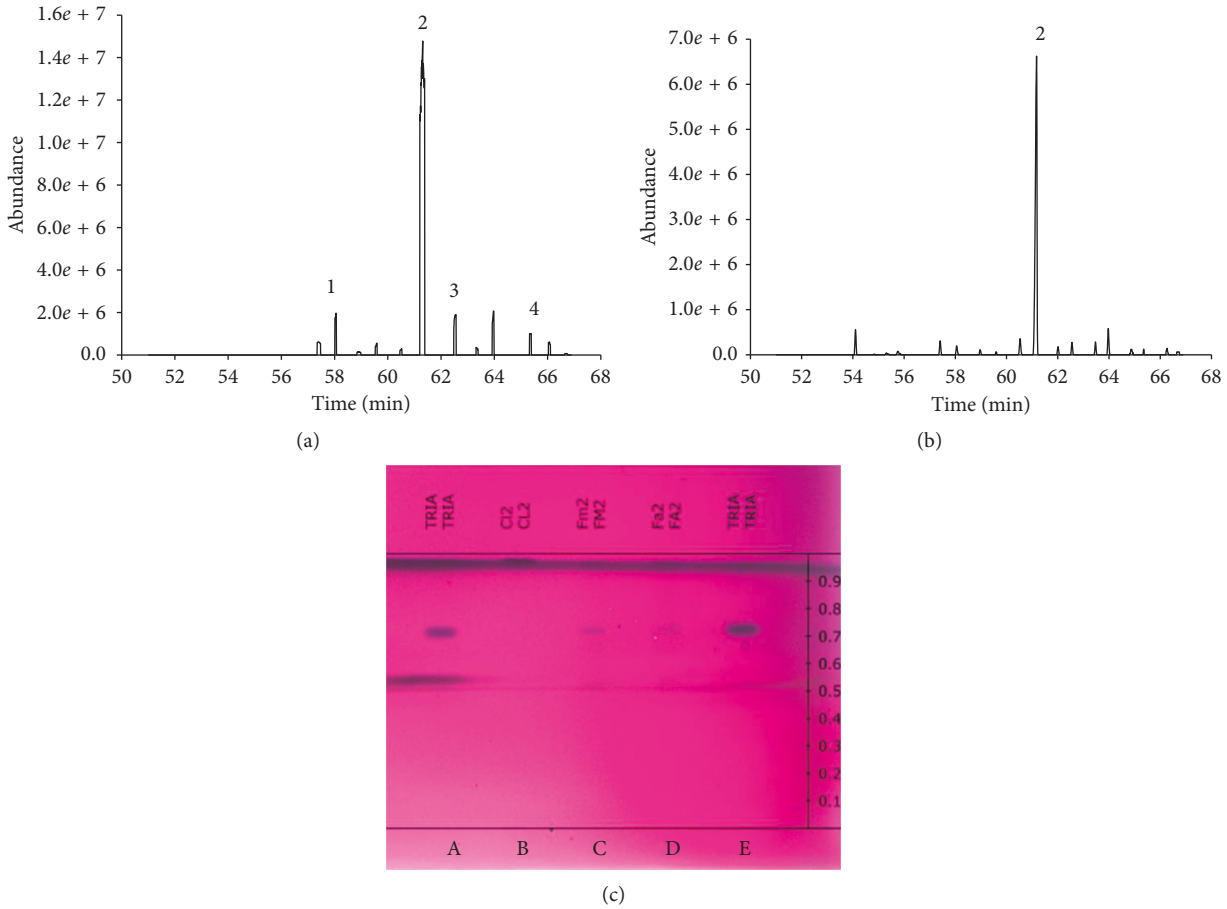

Figure 2: HPTLC chromatoplate: lanes A and E correspond to the triacontanol standard (Rf 0.73); lane B corresponds to the purified wax obtained by extraction with cyclohexane and cleaning with methanol and acetone; lane C is the methanolic fraction; and lane D is the acetonic fraction. Chromatograms of methanolic (a) and acetonic (b) fractions: peaks corresponding to 1, octacosanol; 2, triacontanol; 3, untriacontanol; and 4, tritriacontanol.

reaction to proceed, since it has been observed that inhibition by lower-chain alcohols is often due to alcohol insolubility [41]. Solvents are used to protect the enzyme from denaturation by alcohols by increasing alcohol solubility [41]. In this case, toluene as reaction medium in combination with isopropanol and butanol could improve solubility, favouring transesterification.

Regarding the composition of the policosanol obtained by the different reactions, this depended on the acyl receptor used. When propanol was added to the reaction medium, a policosanol consisting of tritriacontanol and triacontanol was obtained (Figure 3); when isopropanol was added, a policosanol comprising triacontanol, untriacontanol, and tritriacontanol was obtained (Figure 4).

Otherwise, it has been proved that sonication assists transesterification reactions by improving the yield within a shorter reaction time. It is an efficient mixing tool that provides sufficient activation energy to initiate the reaction [42], minimises the molar ratio of alcohol to oil, and reduces energy consumption compared to the conventional mechanical stirring method [43, 44].

A commercial lipase immobilised on acrylic resin, Novozym 435 from *C. Antarctica* lipase, was used as a biocatalyst in the system, and it was found that the enzymatic activity was enhanced with the assistance of low-frequency and mild-energy ultrasonic sound waves. In this case, enzymatic transesterification under sonication was carried out at 40 kHz, a lower frequency. The appearance of the reaction medium did not show good solubility. It was higher when butanol was added, causing problems in characterisation and quantification of the sample. Nevertheless, the activity of the enzyme was not diminished during the 2 h of reaction, as can be seen in the HPTLC plates. Orbital agitation reactions was performance so carry out an adequate quantification of triacontanol content.

3.2.3. Enzymatic Transesterification Reaction under Orbital Agitation. The reaction under orbital agitation (Figure 5) did not proceed as quickly as under sonication (Figures 3 and 4). The appearance of bands with similar Rf (0.53) to the triacontanol in the plates developed under sonication (Figures 3 and 4) can be observed from the first minutes, compared to the plates developed from transesterification reactions under orbital agitation (Figure 5). When propanol was used as the acyl receptor in enzymatic transesterification,

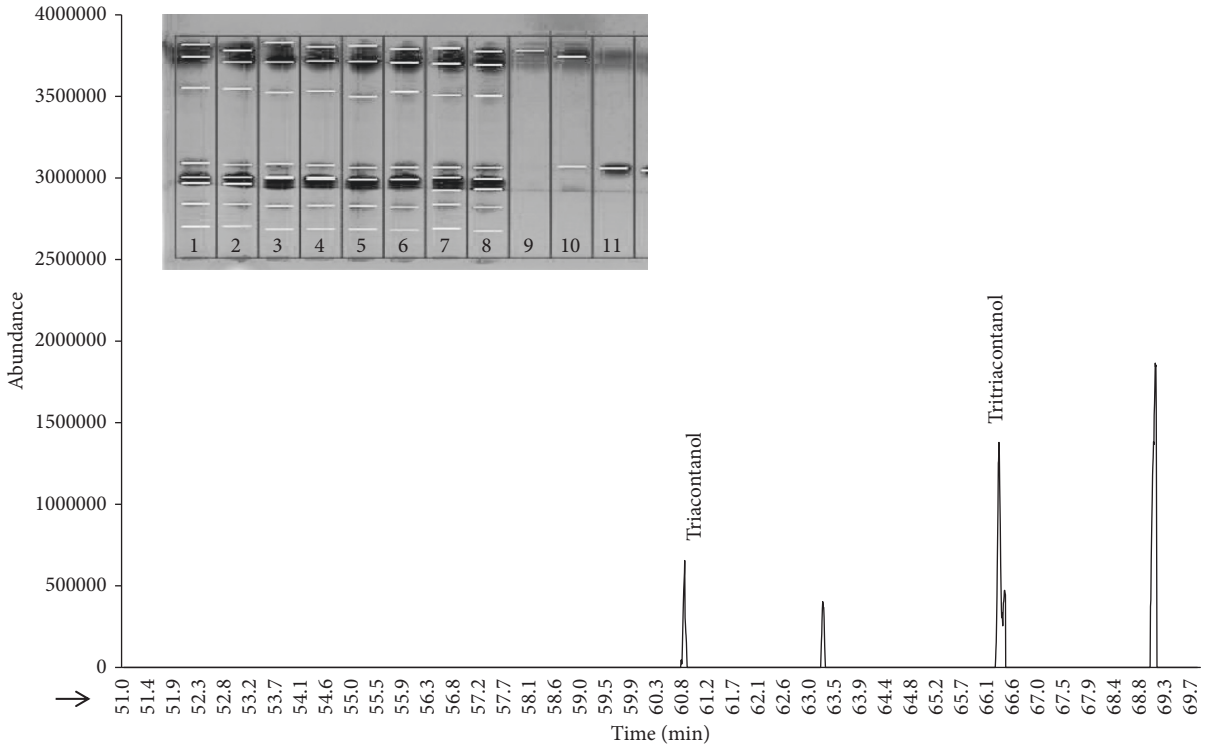

Figure 3: Policosanol composition in toluene medium, using propanol as the acyl receptor, in the enzymatic transesterification under sonication. HPTLC chromatoplate shows products of the reaction over time from lanes 1 to 8; lane 9 is the reaction target without enzyme; lane10 is the purified wax; and lane 11 is the triacontanol standard (30C).

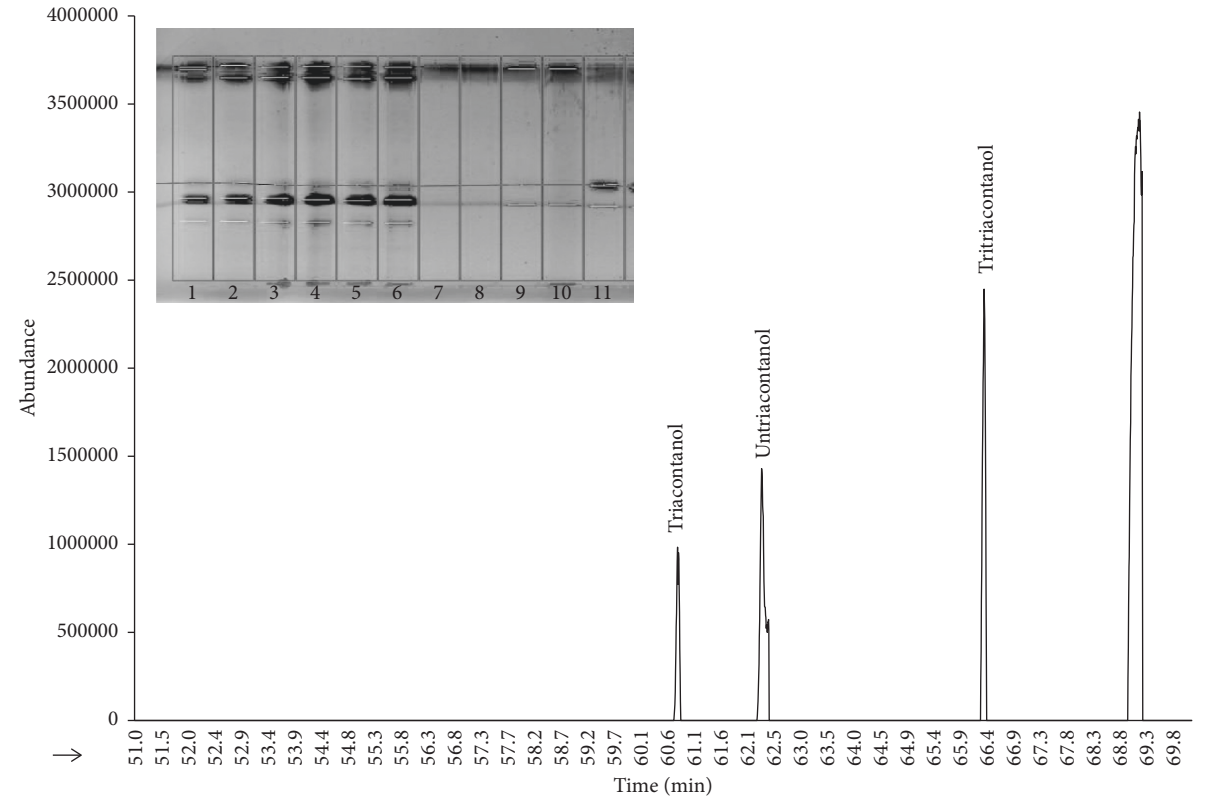

Figure 4: Policosanol composition using isopropanol as acyl receptor in toluene medium from enzymatic transesterification assisted by sonication. HPTLC chromatoplate shows products of the reaction over time from lane 1 to 6; lanes 7 and 8 are the reaction target without enzyme; lanes 9 and 10 are the purified wax; and lane 11 is the triacontanol standard (30C).

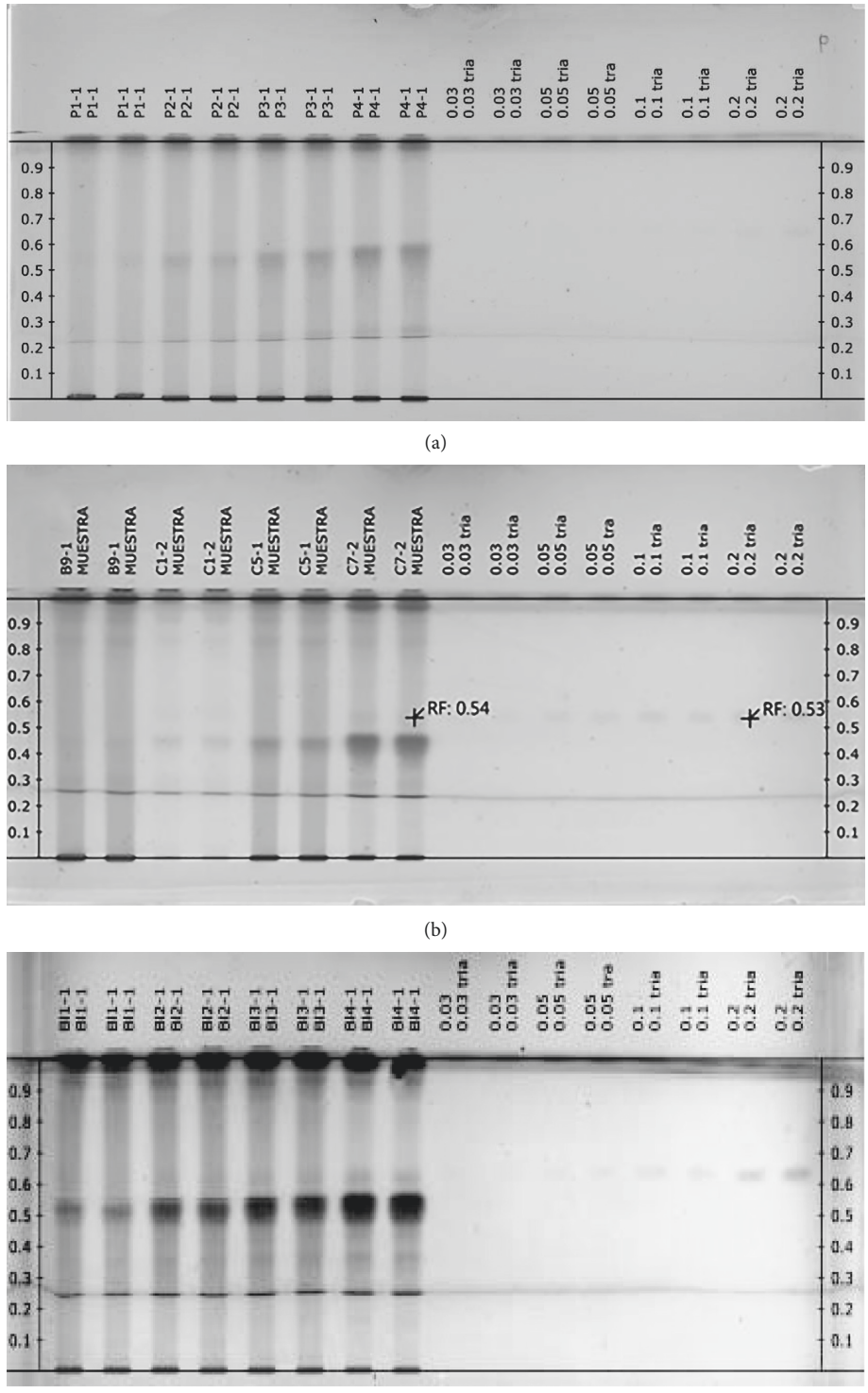

Figure 5: HPTLC chromatoplates. Products of the reaction with (a) propanol, (b) isopropanol, and (c) butanol as acyl receptors and toluene as a reaction medium under orbital agitation.

policosanol was not detected (Figure 5(a)), in comparison to reactions performed under sonication (Figure 3). Policosanol was obtained only after adding isopropanol and butanol as acyl receptors under orbital agitation (Figures 5(b) and 5(c)).

In the quantification carried out by GC-MS, it was possible to establish a relationship of triacontanol content with time. In Figure 6, it can be seen that in the transesterification reaction carried out with butanol as acyl receptor, the highest amount of triacontanol was found after

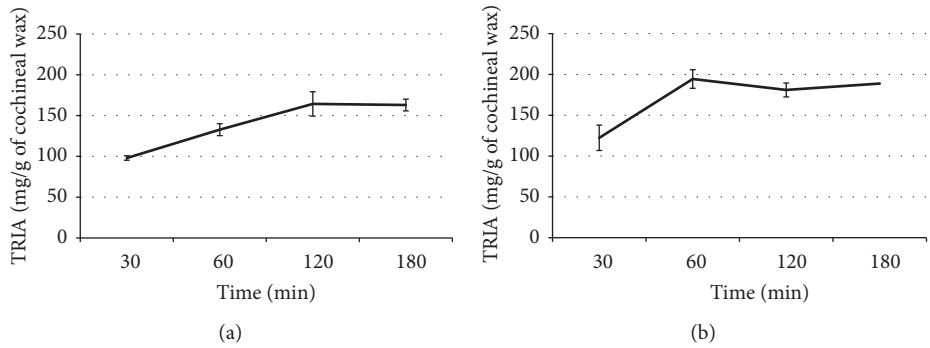

Figure 6: Enzymatic transesterification reaction assisted by orbital agitation with (a) butanol and (b) isopropanol as acyl receptor, in toluene reaction medium.

120 min (164.3 ± 14.9 mg/g of cochineal wax). However, when isopropanol was used as an acyl receptor, the maximum content of triacontanol was found after 60 min (194.4 ± 11.4 mg/g of cochineal wax).

Molecular sieves were used to create a water-free medium, especially in reactions where methanol was used as an acyl receptor, since this alcohol, in high doses, causes inhibition of the reaction. The effect of 3 Å and 4 Å molecular sieves on the enzymatic transesterification for policosanol procurement was analysed, and it was found that the use of molecular sieves had no significant effect on yields of triacontanol in any of the reactions (Figure 7). These results coincide with those of Hsu et al. [25], where the use of molecular sieves had a null effect on enzymes of *T. lanugilosa* and *C. antarctica* immobilised on silica granules; however, the use of molecular sieves favours the reuse of the enzyme in a semicontinuous process [45].

Triacontanol possesses commercial value due to its effect as growth promoter in plants. In this work, policosanol obtained from cochineal wax by both methods—alkaline hydrolysis and enzymatic transesterification—yielded triacontanol. Triacontanol yields in the enzymatic reactions were approximately 19%, compared to chemical hydrolysis, where the highest yields by method A were close to 13%. Although it would be worth conducting technical economic studies to evaluate the cost-benefit of each method, the proposed methods of alkaline hydrolysis do not require the chemical purification of cochineal wax, instead the enzymatic transesterification reactions raised in this work use an enzyme catalogued as a green product (CAL-B*n*), and in addition, solvents catalogued as type yellow are used.

The concentration of triacontanol obtained from cochineal wax is higher compared to that obtained from *Ericerus pela* insect wax, where yields ranged from 1.6 to 2.5% of triacontanol by chemical hydrolysis [15] and 4.9% by a method of solvent-free reduction using $LiAlH_4$ [17]. Other wax sources from agroindustrial waste, such as cuticle and leaves of cane, sesame seeds, and wheat, present triacontanol contents in values of mg per kilogram of wax (<1%) [4–6]. Irmak et al. [5] obtained a policosanol from beeswax, with a content of triacontanol of <1%, and there are reports of up to 200 g/kg of beeswax (20%) [15]. However, the disadvantage of policosanol from these sources is the octacosanol content,

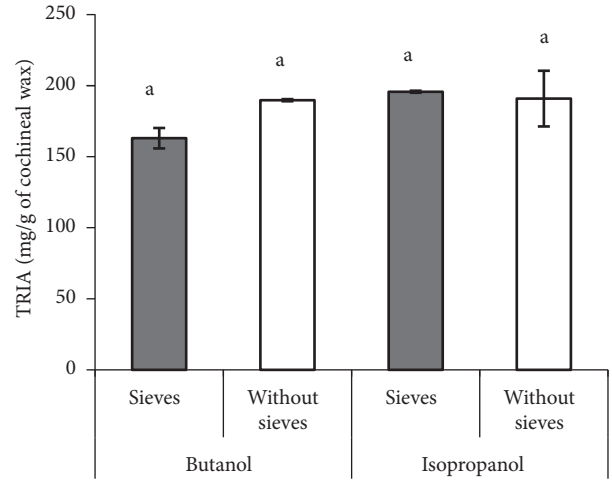

Figure 7: Effect of molecular sieves on triacontanol (TRIA) content obtained in transesterification reactions with butanol and isopropanol as acyl receptors in toluene medium using orbital agitation ($P < 0.05$; Tukey).

which is considered an inhibitor of the growth-promoting effect of triacontanol [46, 47]. Therefore, the use and application of triacontanol from these policosanol sources requires an additional process of purification. Triacontanol from cochineal wax could be applied directly to plants, since it is usually applied in the form of extracts in commercial products.

4. Conclusions

These results show an option to valorise the waste generated by the carmine industry. The policosanol obtained was composed mainly of triacontanol, an alcohol with great commercial value due to its properties as plant growth promoter. Triacontanol yields of up to 13% were attained through chemical hydrolysis and up to 19% by a novel method of enzymatic transesterification. Enzymatic transesterification was carried out with lipase *Candida antarctica* (CAL-B*n*) in a reaction medium with toluene, molecular sieves, and different acyl receptors. This ecofriendly method

can be applied to other wax sources to improve policosanol extraction.

The policosanol obtained presented high yields of triacontanol in relation to the most common sources used for this purpose, with the advantage that cochineal wax is a waste with no profitable application so far.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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