Preparation and Evaluation of Xylose Palmitate Ester Using *Candida antarctica* Lipase B as A Reusable Biocatalyst

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THE activity and stability of lipase B from *Candida antarctica* were investigated in direct enzymatic esterification of D-xylose with palmitic acid using ethylmethylketone as reaction media. The xylose palmitate was obtained under the optimized conditions, 1:1 molar ratio of xylose: palmitic acid, at 250 rpm and 60°C. Effect of the use of Butanol to wash the immobilized lipase before the enzyme reuse was evaluated. The lipase could be successfully reused four times without loss of activity. Various physicochemical characteristics of 1-O-hexadecanoyl-D- xylopyranose were also studied.

Keywords: Xylose, Lipase, Reuse, Activity, Surface active properties.

Introduction

Lipase-catalyzed reactions in organic medium have been extensively exploited for a wide range of applications in biotechnology [1,2]. Sugar Fatty acid esters (SFAE) are non-ionic surfactants [3] that can be synthesized in a single enzymatic reaction step by esterification reaction catalyzed by enzymes [4] such as lipases in non-aqueous systems [5,6]. Because of their properties of amphiphilic nature, odorless, tasteless, among others, SFAE are widely used as flavor enhancers in the food industry and fragrances in the cosmetic industry [7].

The aim of this paper was the preparation of xylose-palmitic acid ester by using immobilized *Candida antarctica* lipase B as a biocatalyst. To the best of our knowledge, the direct enzymatic esterification of D-(+)-xylose and free palmitic acid with *Candida antarctica* lipase B has never been investigated up to now. Immobilized *Candida antarctica* lipase B (CAL B), performed significantly better than many other lipases in carrying out the esterification of sugar and fatty acids. C12–C18 straight chain fatty acids are suitable acyl donors for CAL B [8]. Xylose palmitate ester was obtained using renewable substrate [9]. D-(+)-xylose is a constitutive component of arabinoxylans and other naturally occurring polysaccharides, such lignocellulose which are natural source for xylose. Using renewables as raw materials makes the conversion processes fully ecologically friendly and the lipase-catalyzed synthesis also has the advantage that the product may be accepted as a natural product [10]. Furthermore, stability and activity of CAL B were studied in repetitive cycle reuses [11]. Butanol was used to wash the recycled lipase. Influence of solvent structure on the activity and stability of the lipase were studied. In this lipase-recycling system, the lipase was reused to synthesize the surfactant under the same conditions with a less loss of activity. In this paper we also report surface-tension data for the synthesized surfactant. Critical micellar concentration and surface tension value of the amphiphilic compound were determined and obtained parameter values were discussed.

Materials and Methods

Chemicals and Reagents

Novozym 435 (*Candida antarctica* type B lipase immobilized on acrylic resin) was provided by Novo Nordisk (Bagsvaerd, Denmark). Xylose (Sigma-Aldrich) and palmitic acid from Merck were tested as starting materials. All other chemicals used in this work were of analytical grade and used without further purification.

Xylose Palmitate Synthesis

D-xylose (200 mg) was first dissolved in one volume of ethyl methyl ketone for one night. After that, palmitic acid was added, the mixture equilibrated for 15 min and *Candida antarctica* lipase B finally incorporated. Aliquots were

removed at intervals, filtered and analyzed qualitatively by thin layer chromatography and quantitatively by volumetric titration.

Analysis

The sugar content was quantified by calculating the residual fatty acid amount in the reaction mixture. Samples were analyzed by volumetric method. 0.1 g of sample of reaction mixture was diluted in 20 mL of 0.1 wt % phenolphthalein solution in absolute ethanol and then titrated with standardized sodium hydroxide solution of 0.1 M in water.

Surface tension measurements

Surface tension values were obtained at 25°C, using the IT concept Tracker drop tensiometer. The data obtained were plotted against various concentration of surfactant solution in water. The value of CMC was determined from the plot of surface tension versus concentration. The surface parameters, maximum surface excess (Γ), surface area occupied by molecule at air-water interface (A), efficiency in surface tension reduction (γ), and the micelle packing parameter (P) were calculated from the slope of the decrease in surface tension with the increasing concentration of surface active solution.

Results and Discussion

Xylose palmitate synthesis pathway

Xylose palmitate ester (1-*O*-hexadecanoyl-D-xylopyranose) was prepared by esterification of the corresponding sugar. The reaction was carried out in ethylmethylketone at 60 ºC with xylose: palmitic acid ratio: 1:1 and catalyzed with *Candida antartica* lipase B (CAL B), the most frequently used enzyme for sugar ester synthesis in organic solvent [12]. The progress of esterification was monitored by determining the residual acid content by titration [13]. After 72 h, the enzyme was removed by filtration and the solvent evaporated. The product was isolated on a silica gel column eluted with (dichloromethane: methanol) (9:1 v/v). The fractions containing xylose palmitate ester were combined, solvent removed by evaporation, and after drying under vacuum, the desired product was obtained as a white solid in 45% yield.

1-*O*- hexadecanoyl- D-xylopyranose was obtained under the optimized conditions [14]. Higher temperature, 60° C, favored the conversion. The maximum conversion of xylose palmitate obtained was 65% after 72 h reaction time in EMK with(xylose/palmitic acid) molar ratio of (1/1) at 60° C. According to the literature, a suitable temperature range for synthesis using CAL B appears to be between 30°C and 70°C although in specific cases a temperature of up to 80°C may be used [15]. In esterification of glucose [16] with palmitic acid (hexadecanoic acid), the conversion rate attained a maximum at a substrate molar ratio of (1/1). Further increase in glucose concentration reduced the conversion rate. This reduction was associated with the substrate inhibition of the enzyme. Figure 1 shows the reaction kinetics for xylose palmitate ester production.
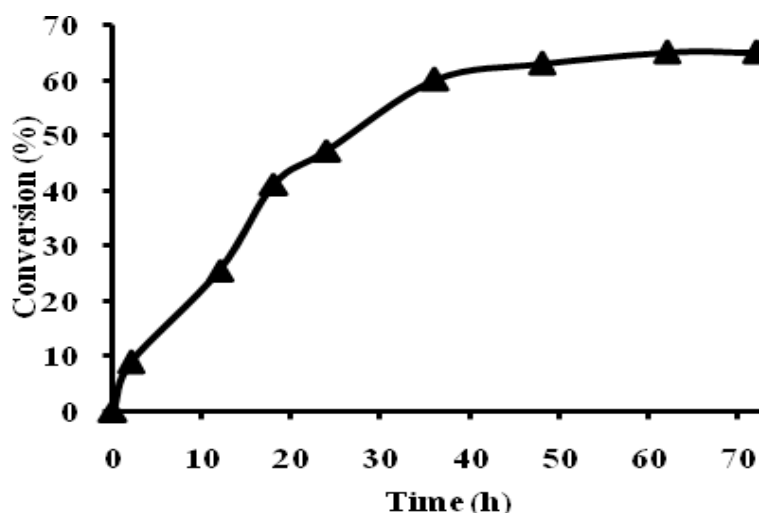
Reaction conditions:1.33 mmol D-xylose, 1.33 mmol palmitic acid, 5 mL ethyl methyl ketone, 30 mg CAL B at 60 ºC, 30 mg molecular sieves (4Aº), 250 rpm.

Fig. 1. Reaction kinetics for xylose palmitate production.

Infrared spectroscopy is generally used to estimate the formation of esters during the enzymatic modification of sugars [17]. The spectrum of purified xylose palmitate shows the characteristic vibrations of the ester group between 1710-1740 cm^{-1}. Combination bands of OCH and COH (1463,1149 cm^{-1}) and C–H (2960, 2850 cm^{-1}) can be observed. The intense peaks at 3000-3600 cm^{-1} are assigned to the OH group.

The enzymatic production is advantageous over a chemical synthesis, as the regiospecificity of fatty acid chain length achieved by the lipase can be exploited to generate pure product with desired properties. ^{1}H NMR is generally used for the determination of the regioisomers formed. Mono-ester was obtained using *Candida antartica* lipase B. The ^{1}H NMR spectrum data for 1-*O*-hexadecanoyl-D-xylopyranose was as follow: (DMSO-d$_6$, 250 MHz), δ (ppm): 0.85 (t, 6H, H-16′), 1.25 (m, 48H, H-4′-15′), 1.50 (m, 4H, H3′) 2.27 (m, 4H, H-2′), 3.20-4.00 (m, 6H, H-4β, H-4α, H-2α, H-2β, H-3β, H-3α), 4.4-4.5 (m, 2H, H-5β, H-5α), 4.6-4.8 (m, 8H, 8 OH), 5.1 (d, 1H, H-1α), 5.65 (d, H-1β).

α/β: 40/60, Rf: 0.45 (CH$_2$Cl$_2$/MeOH: 9/1).

The ^{13}C NMR spectrum data for 1-*O*-hexadecanoyl-D-xylopyranose was as follow: (DMSO-d$_6$, 250 MHz), δ (ppm): 14.87 (C16′), 22.01 (C15′), 25 (C14′), 28.35-31 (C13'-3'), 34.38-35 (C2'α,β), 69.54 (C5$_α$), 74.00 (C5$_β$), 71.05 (C4$_α$), 70.50 (C4$_β$), 73.36 (C3$_α$), 76.65 (C3$_β$), 72.69 (C2$_α$), 75.18 (C2$_β$), 95.78 (C1$_α$), 96.76 (C1$_β$), 175.83 (C1'$_α$), 176 (C1'$_β$).

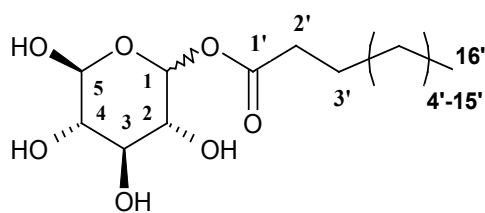**Fig. 2. 1-O-hexadecanoyl-D-xylopyranose.**

Enzyme reuse
Sugar fatty acid esters production catalyzed by immobilized lipases offers the possibility of easy reuse of the catalyst [18] which is very important to minimize costs and to make this process economically feasible. In this study, the reuse of immobilized *Candida antartica* lipase B was investigated in enzymatic esterification of D-xylose with palmitic acid. After the esterification reaction, the lipase was separated from the reaction medium by means of simple filtration. The recovered biocatalyst was then washed with 20 mL of Butanol (log P = 0.88). Differences in solvent polarity have been used to explain their effect on the catalytic activity and specificity of reused CAL B [19]. Highest enzyme activities and stabilities are generally observed in solvents with high log P values.

Table 1 shows the conversions attained after the lipase washing with Butanol. According to this table, the relative fatty acid conversion attained after four cycle reuse is 60,48% at 60°C. The catalytic performance of CAL B to produce xylose palmitate ester during four consecutive cycles was observed. *Candida antarctica* lipase B was stable, retaining most of its original catalytic activity after consecutive uses. 98,84% and 91.11% of the original enzymatic activity of CAL B was still retained respectively after the second and the fourth cycles of the lipase reuse. It can be concluded that in most reuse assays, the behavior of CAL B was stable and the lipase could be reused for four cycles while maintaining its stability and activity.

Physicochemical Properties
Surfactants from renewable resources [20] are biodegradable and exhibit low toxicity (green surfactants) compared with those synthesized

TABLE 1. Enzyme reuse in the esterification reaction of xylose with palmitic acid

Used	Conversion (%)	Specific activity (µmols·min^{-1}·g^{-1})
1	65	8.67
2	63,42	8.57
3	62	8.25
4	60,48	7.90

from petroleum [21,22]. SFAE surfactants make up a very important group of surface active compounds due to advantages with regard to performance, consumer's health and environmental compatibility [23]. Various surface measurements were estimated, specifically Critical Micellar Concentration (CMC), effectiveness of surface tension reduction (γ$_{CMC}$), surface excess concentration (Γ), and area per molecule at the interface (A).

CMC and γ_{CMC} were obtained graphically from Fig. 3. 1-*O*-hexadecanoyl-D-xylopyranose exhibit excellent surfactant properties characterized by a surface tension of 38 mN/m for a CMC of 0.01 mmol/L.
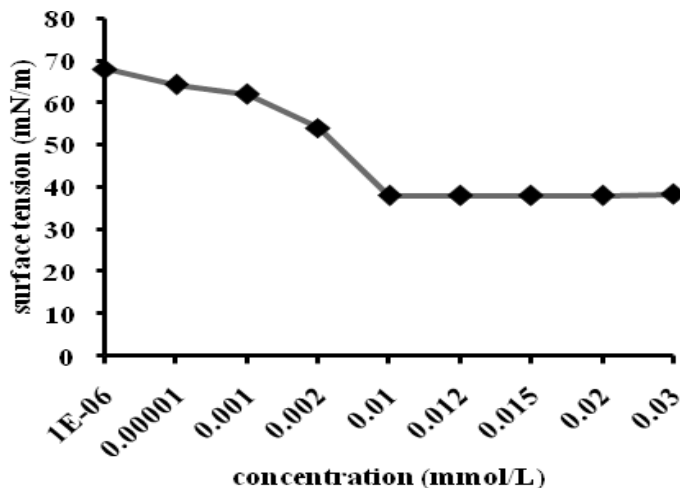

Fig. 3. Surface tension measurements for 1-O-hexadecanoyl-D-xylopyranose in water.

calculated from:

$$\Gamma = \frac{1}{nRT}\left[\frac{\partial \gamma}{\partial \ln C}\right] \qquad (1)$$

Where:
γ: surface tension (N.m^{-1}).
Γ: surface excess concentration (mol.m^{-2}).
C: concentration of solution (mol.L^{-1}).
R: universal gas constant (R=8. 314 J.mol^{-1}.K^{-1}).
T: température (K).
n: constant of dissociation; n = 1 for nonionic surfactant.

The area per molecule, calculated from the formula (2) given below is 23 Å^2.

$$A = \frac{1}{Na\,\Gamma} \qquad (2)$$

A: area per molecule at the interface (m^2).
N: Avogadro's number (N = 6.023 10^{23}).
Γ: surface excess concentration (mol/m^2).

After the CMC, monomers of surfactants form aggregates called micelles. The micelle packing parameter P is calculated from (3). The equation is utilized to predict molecular self-assembly in surfactant solutions. Most probably, the value obtained for P (Table 2) is due to the length of hydrocarbon chain

and related to rodlike micelle of the adsorbed surfactant molecules.

$$P = \frac{V}{A.\,l_t} \qquad (3)$$

Where:
V: volume of the surfactant hydrocarbon chain (Å^3).
l_t: hydrocarbon chain length (Å).

The data (Table 2) revealed various advantages and potentials of 1-*O*-hexadecanoyl-D-xylopyranose as a surfactant. According to Griffin [25], the HLB (Hydrophilic Lipophilic Balance) is a number on the scale of 1 to 40. It is used to predict what type of surfactant properties a molecular structure will provide. HLB is
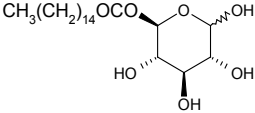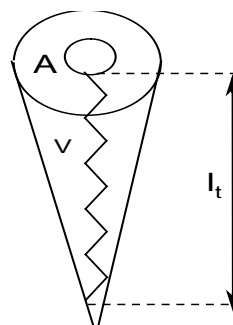

Fig. 4. Schematic illustration of surfactant volume in an aggregate.

TABLE 2. Physicochemical properties of 1-O-hexadecanoyl-D-xylopyranose

Compound	HLB	CMC (mmol.L^1)	γ_{CMC} (mN.m^{-1})	Γ (mol.m^{-2})	A (Å^2)	P (m^3)
	9,41	0,01	38	$7{,}01{.}10^{-4}$	23	0,92

calculated from fractional ratio of hydrophobic part to hydrophilic part of surfactant [26]. Weight percentage of each type of group on a molecule predicts what behavior the molecular structure will exhibit [27]. 1-*O*-hexadecanoyl-D-xylopyranose has an intermediate HLB number (Table 2) and behaves as an oil-in-water emulsifier. 1-*O*-hexadecanoyl-D-xylopyranose is also characterized by a low CMC and appears very hydrophobic. A long hydrocarbon chain makes the surfactant more effective. The low value of the interfacial tension indicates the ability of using this surfactant in several applications. It can act as an emulsifier that will allow a compound that is normally insoluble to dissolve. The obtained values for surface excess concentration and the area per molecule at the interface indicate that it is characterized by good surface-active and self-aggregation properties. Due to surface activity and emulsifying capacity 1-*O*-hexadecanoyl-D-xylopyranose is promising for applications in food industry [28,29].

Conclusion

Here we present the first report of xylose palmitic acid ester synthesis by enzymatic method with CAL B. Conversion of 65% is achieved in EMK and more than 90 % of initial activity of the lipase was still be retained after four cycle reuse. Surface tension measurements allow the determination of several important parameters. The most important feature of any given surfactant is its ability to reduce the surface tension of a liquid medium. 1-*O*- hexadecanoyl -D- xylopyranose behaves as a nonionic-single chain surfactant. The obtained results in this paper could be further applied to predict the thickness and stability of emulsions, or the size of the rodlike micelles in solutions.

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