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SYNTHESIS OF SUGAR ESTERS USING MAIZE SEED LIPASE AS A BIOCATALYST

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This work is devoted to the extraction of a lipase from maize seeds obtained at low cost without extensive purification technology and used as a biocatalyst in the synthesis of various sugar esters as potential biosurfactants in the detergent industry. Indeed, several reactions have been carried out between different sugars - namely glucose, galactose, saccharose, lyxose, fructose and sorbitol - and stearic acid in a ratio of 2.5:1 in hexane at 50 °C. Kinetic monitoring of the target reactions was carried out by volumetric analysis in order to determine the conversions (C). The best results obtained were C = 76, 71 and 67% in the presence of glucose, lyxose and galactose, respectively. In addition, HPLC and TLC analyses revealed that for every sugar used two types of esters formed.

Keywords: biocatalysis, plant extract, maize seeds, sugar ester, biosurfactant

1. Introduction

Chemicals are essential for healthy living and modern comforts, moreover, are at the heart of many industrial processes for the manufacture of important products [1]. Among these products, surfactants play an important role in several fields such as in the food, cosmetics and detergent industries [2]. These applications depend on the structure of such compounds which can be anionic, cationic, amphoteric or nonionic [1]. Sugar esters are non-toxic, biodegradable and nonionic surfactants [2]. Their synthesis is carried out chemically, resulting in particular in the production of a significant amount of byproducts that must be eliminated, necessitating relatively high purification costs and extreme conditions amongst other drawbacks. These problems can be avoided by opting for a much more specific synthetic route based on the use of enzymes, mainly lipases [3]. These biocatalysts are widespread in animals, microbes and plants [4]. The high cost of commercial lipases, mainly of microbial origin, limits the possibility for this process to be scaled up to an industrial level [1]. As an example, for the synthesis of glucose fatty acid esters, commercial immobilized lipases (Novozym 435, Lipozyme RM IM and Lipozyme TL IM) have been used as biocatalysts [5]-[6] but the purification process required as well as their immobilization, loss of activity and the high cost of these lipases are the main drawbacks for the production of such sugar esters on an industrial scale. Plant biomass represents an alternative as a potential source of inexpensive lipases that are very abundant and easily exploitable [7]-[8]. Indeed, seed lipases have already been used successfully in the synthesis of sugar esters [9]. This enzymatic reaction in non-conventional media continuously produces water which strongly affects enzyme activity. To maintain the reaction parameters, the water activity (a_w) should remain constant [10]. In lipase-catalyzed esterification reactions, water activity is a major factor because a sufficient amount of water must be present to retain the active structure of the enzyme, however, if in excess in the reaction medium, the lipase will catalyze a competing hydrolysis reaction [11]. In addition, a_w can influence the rate and equilibrium yield as well as the stability of enzymes [12]. The objective of this work is to use a plant extract from maize seeds (MSL) to catalyze the synthesis of sugar esters for the first time, without using a desiccant. To date, MSL has only been used in the synthesis of low molecular weight flavor esters [13].

2. Experimental

2.1. Material

Maize (Zea mays) was purchased from the local market. Substrates (glucose, saccharose, lyxose, sorbitol, galactose, fructose and stearic acid) as well as solvents were purchased from Sigma-Aldrich, while all other chemicals and reagents were of analytical grade.

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2.2. General Procedures

The course of the reactions was followed by thin-layer chromatography (TLC) (pre-coated Silica Gel 60 F254 sheets manufactured by E. Merck) and hexane:chloroform (1:1, v/v) was used for elution. The different spots were identified using a solution of basic potassium permanganate (10 g of potassium carbonate, 1.5 g of potassium permanganate in 150 mL of water and 1.25 mL of a 10% aqueous sodium hydroxide solution). FT-IR analysis was performed using a InfraLUM FT-08 spectrometer and the obtained spectra were compared to those of stearic acid and different sugars. An Agilent 1260 instrument as well as an Agilent ZORBAX Carbohydrate Analysis Column were used for the HPLC analysis of all reaction mixtures and products.

2.3. Lipase Extraction

10 g of seeds were soaked in water for 12 h at room temperature before being collected and placed on moist absorbent paper to facilitate germination. The seeds were then spread out and covered with a second layer of cotton and watered on a daily basis. After five days, the germinated seeds were dried at room temperature then grounded with a domestic blender. The obtained seed powder was incubated and continuously stirred in 30 ml of cold acetone for 12 h. After incubation, the mixture was filtered through filter paper with a diameter of 150 µm before being washed 5 to 7 times with 20 ml of cold acetone until the solution became clear. Finally, the residue was dried and the powder (crude MSL) stored at 4 °C.

2.4. Hydrolytic Activity

The assay of the lipase activity was carried out using a previously described titrimetric method [14]. In a flask, 5 g of olive oil and 2.5 ml of hexane were stirred at 50 °C for 15 mins. 1 g of the crude MSL was added to the mixture and left to react for 1 h while continuously stirred. Having been incubated, 25 ml of acetone:ethanol (1:1 v/v) was added to stop the reaction. A 0.1M solution of NaOH was used to quantify the amount of released free fatty acids (FFAs) using phenolphthalein as an indicator before the specific activity (*SA*) of the enzyme was calculated according to the following formula:

$$SA = \frac{n_{FFA}\ (\mu mol)}{x(mg)\times t(mn)} \qquad (1)$$

where n_{FFA} denotes the number of moles of free fatty acids, x stands for the mass of the lipase extract and t represents the reaction time.

The lipase extraction experiments and the corresponding lipase activity measurements were carried out in triplicate and the average standard deviation of which was approximately 3%.

2.5. General Procedure for the Esterification of Sugars

The sugar (glucose, galactose, saccharose, sorbitol, lyxose or fructose) and the fatty acid (stearic acid) were dissolved in 5 ml of hexane at 50 °C. Having been stirred for 30 minutes, 0.375g of MSL was added to the mixture. 0.2 g samples were taken from the reaction medium before adding the enzyme and every 24 h after its addition for the following 72 h. The reaction was then stopped, and the mixture filtrated using filter paper with a diameter of 60 mm to eliminate the MSL before the hexane was eliminated using a rotary evaporator.

The amount of unreacted fatty acids was determined by titration according to the following procedure: 0.2 g of the reaction mixture was diluted in 5 ml of a 0.1% solution of phenolphthalein in ethanol. A 0.1 M solution of NaOH was then added until a pale pink color was observed. The conversion (*C%*) was calculated according to the following formula:

$$C\% = \frac{(V_i - V)\times[NaOH]}{n_{SA}} \times 100\% \qquad (2)$$

where V_i denotes the volume of NaOH consumed during the reaction before the enzyme (control) was added, V stands for the volume of NaOH consumed during the different reactions at different intervals after adding the enzyme, *[NaOH]* represents the concentration of NaOH and n_{SA} refers to the molar quantity of stearic acid.

All esterification reactions and the corresponding conversion measurements were repeated twice.

3. Results and discussion

3.1. Lipase Extraction

Although lipases are widely distributed within plants, they are found mainly in seeds, which are generally rich in triacylglycerol that serves as an energy source for the newly emerging plant. During germination, the lipid reserves in the albumen or cotyledons of these seeds begin to disappear since the lipase facilitates the hydrolysis of these nutrient reserves. Indeed, during germination, lipase activity is high [15]. In this work, maize seeds were used as a source of lipase and the enzyme extract was obtained after five successive steps, namely soaking in water, germination, drying, crushing and then defatting [14]. The filtration residue of the defatted maize seeds was dried at room temperature before being stored at 4 °C. This MSL was used for the synthesis of sugar esters.

3.2. Hydrolytic Activity

The hydrolysis test was carried out in the presence of the prepared MSL using olive oil as a substrate according to the protocol described in the Experimental section. The MSL had a specific activity of 0.0015 UI/mg under the

Table 1: Effect of different reaction conditions on the conversion

Entry	Molar Ratio (glucose:stearic acid)	T (°C)	Conversion (%)		
			24h	48h	72h
1	1:1	50	18	54	59
2	1.25:1	50	17	58	64
3	1:1.25	50	32	54	54
4	1.25:1	30	15	30	35
5	1.25:1	40	17	40	41
6	1.25:1	60	20	49	51
7	2:1	50	33	61	70
8	2.5:1	50	50	74	76
9	3:1	50	43	62	68

used operating conditions. It should be noted that a reaction was carried out under the same conditions in the absence of the enzyme but no hydrolytic activity was observed.

3.3. Choice of Operating Conditions

Many studies have been carried out to determine the ideal conditions for the synthesis of sugar esters by primarily focusing on the sugar:acid molar ratio, type of the solvent, nature and amount of the enzyme as well as temperature [16]. Many articles on the synthesis of enzymes have focused on glucose fatty acid esters and especially glucose stearate using stearic acid as a natural fatty acid [17]. It has also been reported that the best solvents used in this biocatalysis are tetrahydrofuran, acetone, tert-butanol and n-hexane [16],[18]. The optimum temperature of the MSL is 50 °C according to [19]. The quantity of MSL used in this work (*Figure 1*) was 0.375 g in accordance with the optimized amount used in a previous work (data not shown).

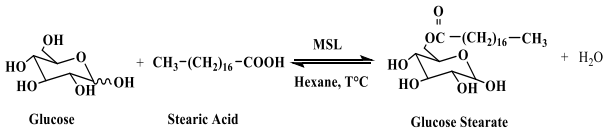

Figure 1: Synthesis of glucose stearate
Reaction conditions: D-glucose:stearic acid (different molar ratio), 0.375g of MSL in 5 ml of hexane at different temperatures

According to the literature, esterification reactions are carried out using different sugar:acid molar ratios [16]. Therefore, in order to determine the best molar ratio, three reactions were performed as a starting study. The first one was performed with equimolar quantity of the two reactants (1:1), the second with a small excess of sugar (1.25:1) and the third with a small excess of acid (1:1.25) all at 50 °C in hexane in the presence of 0.375 g of MSL. The obtained results (*Table 1:* entries 1-3) indicate that the conversion progresses from 17% after 24 h to 64% after 72 h when the 1.25:1 molar ratio was applied. On the other hand, for the other reaction with an excess of acid, a maximum conversion rate of 54% was reached after 48 h. The results with an equimolar quantity of the two reactants, namely 1:1, were no better than in the case of the 1.25:1 reaction.

3.4. Effect of Temperature

To determine the ideal temperature for this synthesis, three reactions were carried out with the molar ratio of 1.25:1 at 30, 40 and 60 °C (*Table 1:* entries 4-6). The conversions obtained were compared to those at 50 °C (*Table 1:* entry 2). The results show that the conversion of stearic acid in the reaction carried out at 50 °C was better than in the other reactions, so the ideal temperature expected for this synthesis is 50 °C [19].

3.5. Effect of the Sugar:Stearic Acid Ratio

According to the results obtained previously concerning the effect of the molar ratio, a slight excess of sugar yielded better conversion rates. Therefore, a reaction with a greater excess of sugar was studied. For this purpose, the synthesis of glucose stearate was carried out with molar ratios of sugar:stearic acid of 2:1, 2.5:1 and 3:1 at 50 °C in hexane (*Table 1:* entries 7-9). The best results were obtained when using a ratio of 2.5:1, when the conversion increased from 50% after 24 h to 76% after 72 h. A larger excess of sugar seems to affect the enzyme activity.

These results suggest that the ideal conditions for the synthesis of sugar esters are 2.5 eq. of sugar, 1 eq. of stearic acid and 0.375 g of MSL in hexane at 50 °C.

3.6. Synthesis of Sugar Esters and Kinetic Study

This reaction occurs between stearic acid and various sugars - namely glucose, galactose, saccharose, lyxose, fructose and sorbitol - catalyzed by MSL whilst being stirred by a magnetic stirrer in hexane at 50 °C (*Figure 2*). The reactions were monitored by a volumetric analysis (see the Experimental section).

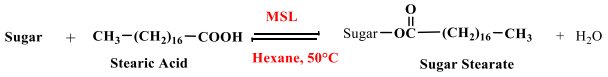

Figure 2: Esterification of sugars with stearic acid in the presence of MSL
Reaction conditions: 2.5 eq. of sugar, 1 eq. of stearic acid, 0.375g of MSL and 5 ml of hexane at 50 °C

During the different reactions, samples of the reaction mixture were taken every 24 h in order to follow the esterification by a volumetric analysis with a basic 0.1M NaOH solution to determine the amount of unreacted acid and, therefore, calculate the conversion. The kinetic monitoring of these esterification reactions catalyzed by MSL in hexane and the conversion obtained for the different reactions are shown in *Figure 3*.

The time dependence of esterification reactions over 72 h is shown in *Figure 3.* The conversions of stearic acid gradually increase over time. During the first 24 hours, the initial rate is faster for the esterification

Table 2: Frontal ratios Rf of the formed mono- and di-esters (1 and 2)

Sugar	R_f
Glucose	$R_f1 = 0.57$ $R_f2 = 0.92$
Galactose	$R_f1 = 0.58$ $R_f2 = 0.90$
Saccharose	$R_f1 = 0.52$ $R_f2 = 0.88$
Lyxose	$R_f1 = 0.59$ $R_f2 = 0.87$
Fructose	$R_f1 = 0.51$ $R_f2 = 0.84$
Sorbitol	$R_f1 = 0.54$ $R_f2 = 0.85$

with glucose compared to with the other sugars. After 24 h, the reaction rate gradually increases to reach a maximum conversion. Except for lyxose, the reactions reach equilibrium after approximately 48 h. Even though these are multiphase reactions with difficult mass transfers, organic solvents reduce the activity and stability of lipases. After 72 hours, the best conversions of 76, 71 and 67% were obtained with glucose, lyxose and galactose compared to 51, 38 and 37% with saccharose, sorbitol and fructose, respectively. Furthermore, no correlation was observed between the sugar structure and the obtained conversions. It should be noted that for each synthesis carried out previously with the different sugars, the reaction was performed in the absence of an enzyme and no conversion was observed.

3.7. TLC Analysis

According to the literature, mono-esters of sugars have been identified by this method with a frontal ratio of approximately 0.50 using a mixture of hexane and chloroform in a ratio of 1:1 as an eluent [20].

For all the reactions, the TLC plate analyses indicate the formation of two new products which are the expected mono-esters with an R_f of 0.50-0.59 [20] and probably sugar di-esters. Since the latter are less polar, their R_f are between 0.84 and 0.90 (*Table 2*). The general profile of the different chromatograms is illustrated in *Figure 4*.

According to a study by Cao et al. [21], selectivity is often considered to be the main advantage of the lipase-catalyzed syntheses of sugar esters. It has been observed that selectivity is influenced by the type of lipase, sugar structure, organic solvent, fatty acid chain and solubility of the products in the liquid phase. The probable positions of esterification of the used sugars [2, 21-24] are shown in *Figure 5*.

The IR spectra of the different products display an absorption band at 1740 cm^{-1} attributed to the carbonyl group of the ester, indicating the formation of sugar stearates [25]. Interestingly, by monitoring the different esterification reactions by HPLC, the production of two new compounds for all the reactions is clearly indicated

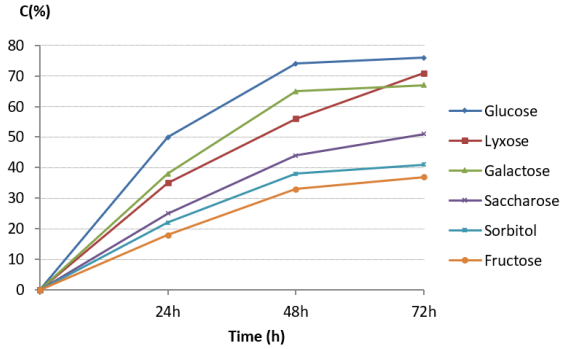

Figure 3: Kinetic monitoring of the different esterification reactions in the presence of MSL (C% = f (t))

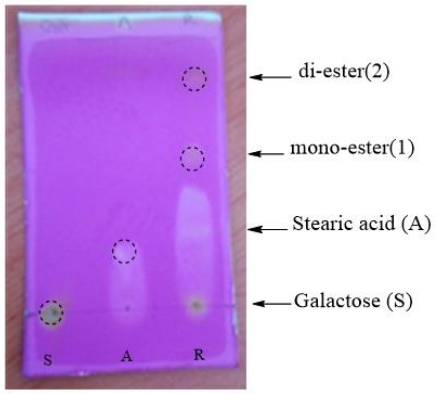

Figure 4: Thin-layer chromatography for the reaction between galactose and stearic acid
Lanes (S), (A) and (R) correspond to the sugar used, stearic acid and the reaction mixture, respectively

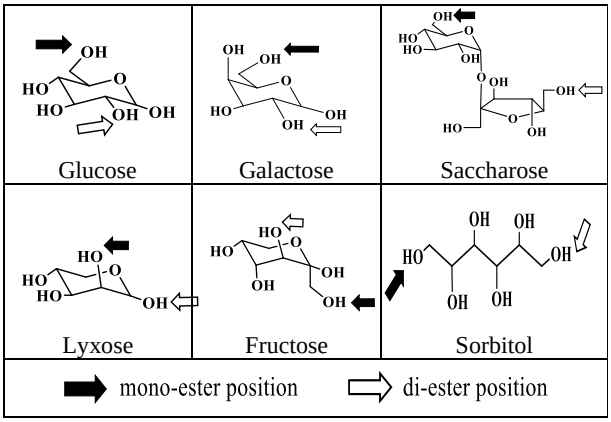

Figure 5: Preferred positions of esterification for the different sugars

(data not shown). The identification of these esters, especially the di-esters, that is, the position of the ester bond, is under investigation.

4. Conclusions

The facile extraction of lipase from maize seeds was used to catalyze the esterification of stearic acid in the presence of different sugars, namely glucose, galactose,

saccharose, lyxose, fructose and sorbitol. A preliminary study was carried out in order to determine the right reaction conditions for the synthesis of sugar esters, that is, a molar ratio of sugar:acid of 2.5:1 at 50 °C using hexane as an organic solvent. The kinetic monitoring of target reactions by volumetric analysis facilitated the determination of the conversions for the various prepared sugar esters. Indeed, the best conversions of 76 and 71% were obtained for glucose and lyxose, respectively, after a reaction time of 72 h. In addition, TLC and HPLC analyses suggested the formation of mono- and di-esters for all sugars used. The results showed that the extract of maize seeds contains one or more lipases, the catalytic activity of which can be exploited without prior purification for the synthesis of sugar esters. Despite the advantages of the enzymatic route, it remains a challenge to convince manufacturers of these interesting biotechnological properties in order to substitute the chemical route with the enzymatic one.

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