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Effect of acyl donor chain length and sugar/acyl donor molar ratio on enzymatic synthesis of fatty acid fructose esters

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Abstract

Lipase-catalyzed synthesis of fatty acid sugar esters through direct esterification was performed in 2-methyl 2-butanol as solvent. Fructose and saturated fatty acids were used as substrates and the reaction was catalyzed by immobilized *Candida antarctica* lipase. The effect of the initial fructose/acyl donor molar ratio and the carbon-chain length of the acyl donor as well as their reciprocal interactions on the reaction performance were investigated. For this purpose, an experimental design taking into account variations of the molar ratio (from 1:1 to 1:5) and the carbon-chain length of the fatty acid (from C8 to C18) was employed. Statistical analysis of the data indicated that the two factors as well as their interactions had significant effects on the sugar esters synthesis. The obtained results showed that whatever the molar ratio used, the highest concentration (73 g l^{-1}), fructose and fatty acid conversion yields (100% and 80%, respectively) and initial reaction rate (40 g l^{-1} h^{-1}) were reached when using the C18 fatty acid as acyl donor. Low molar ratios gave the best fatty acid conversion yields and initial reaction rates, whereas the best total sugar ester concentrations and fructose conversion yields were obtained for high molar ratios.

Keywords: Sugar esters; *Candida antarctica* lipase; Direct esterification; Sugar/acyl donor molar ratio; Fatty acid chain length; Experimental design

1. Introduction

Enzymatic catalysis in non-aqueous media has received keen attention in the past decade. This process allows to obtain pure products due to enzyme specificity. Besides, the catalysis is conducted under mild temperature and pH conditions, which minimize side reactions compared to the chemical process [1–5].

Fatty acid sugar esters are one of the main compounds synthesized by means of enzymatic catalysis. These molecules have several applications particularly in the cosmetics and food industry. In fact, these nonionic surfactants can be used as emulsifiers [6] and are the essential gradients of natural aroma [7] in a great variety of food formulations.

Several works have been reported on the enzymatic production of sugar fatty acid esters. The obtained data showed that the performance of the reaction is affected by several variables. Among these, sugar/fatty acid molar ratio and carbon-chain length of the fatty acid seemed to play a major role. Khaled et al. [8] and Scheckermann et al. [9] ob-

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tained optimal yields when using an excess of the fatty acid. However, Coulon et al. [10] reported that fructose oleate concentration and initial rate of the transesterification reaction of fructose with methyl oleate ester arose progressively when the initial sugar/acyl donor molar ratio was varied from 1:1 to 1:5 and then dropped when the acyl donor excess was more than fivefold higher than fructose.

Another key parameter that has been widely studied is the carbon-chain length of the fatty acid. The research led by Cao et al. [11] demonstrated that the highest conversions were always achieved using saturated long carbon-chain fatty acids in the presence of acetone as co-solvent and at a sugar/fatty acid molar ratio of 1:1. Nevertheless, the initial rate of the synthesis of butyl esters catalyzed by pregastric lipase decreased as the carbon-chain length of the fatty acid increased [7].

For all previously cited work, the authors did not mention whether there is any interaction between these two key parameters and whether there respective effects on the reaction performance are not related to these interactions owing to their influence on the medium hydrophobicity.

The present article describes the effect of carbon-chain length of some fatty acids (C8 to C18) and the sugar/fatty acid molar ratio (1:1 to 1:5) as well as their potential interactions on the fatty acid sugar esters enzymatic synthesis catalyzed by *Candida antarctica* lipase. A statistical method using experimental designs was used to quantify these effects and their possible interactions.

2. Materials and methods

2.1. Materials

C. antarctica SP 435 lipase (Novozym, EC 3.1.1.3, 7000 PLU mg^{-1}: propyl laurate units synthesized per gram of catalyst) was purchased from Novo Industries. The enzyme was immobilized on a macroporous acrylic resin. The fatty acids from C8 to C18 (purity > 97%) were from Fluka (Buchs, Switzerland).

All reactions were performed in 2-methyl 2-butanol and with fructose as acyl acceptor, both from Merck (Darmstadt, Germany).

Silica gel $20 \times 20 \times 0.5$ cm^3 thin-layer chromatography (TLC) plates were from Merck.

2.2. Fatty acid sugar esters synthesis

Fructose (0.139 M) was dissolved in 1 l of 2-methyl 2-butanol at 60°C. Total solubilization of the fructose prior to initiation of the reaction was not necessary. As the reaction proceeded, additional fructose dissolved in response to conversion of the soluble fructose to its ester form.

The acyl donor concentration was adjusted to obtain a given sugar/fatty acid molar ratio in solution. Reactions were started by addition of 10 g l^{-1} Novozym. The mixture was incubated in a double jacket batch reactor with a stirring rate fixed at 200 rpm and a vacuum of 200 mbar.

Aliquots of the reaction mixture were removed at timed intervals and the reaction was followed until the production of fatty acid sugar esters stopped.

2.3. Analytical procedures

2.3.1. TLC analysis

This method was used to monitor the reaction and allowed a qualitative evaluation of the product concentration. It was performed using silica gel plates eluted with chloroform/methanol/acetic acid/water mixture (80/15/8/2, v/v/v/v).

Compounds were revealed by spraying the plate with α-naphthol solution (1.59 g of α-naphthol was dissolved in 51 ml of ethanol and then added to 4 ml of water and 6.5 ml of sulfuric acid 18 M). The products were obtained by carbonization at 105°C (5.5 min) and quantified at 545 nm by photodensitometry using a Shimadzu CS-9000 apparatus (Kyoto, Japan).

2.3.2. High-performance liquid chromatography (HPLC) analysis

The product concentration was quantified by HPLC. Analyses were carried out with a Merck system (Lachrom, Merck) equipped with a scattering mass light detector (Eurosep, DDL31). A Purospher® RP-18e column 5 μm (250×4.0 mm^2, Merck) was used. The separation of the different components of the reaction medium is performed

using a gradient of methanol and water at a flow rate of 1 ml min^{-1} at 55°C. The concentrations of products were determined from the peak area.

2.4. Experimental design

Factors considered important were fructose/fatty acid molar ratio and the carbon-chain length of the fatty acid. Response surface methodology (RSM) was used to optimize the reaction factors. RSM is a process of locating an optimal value in a higher-order model. A second-order polynomial model of the form:

$$\eta = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \beta_{12} X_1 X_2$$

where η is the value of the response, X_i ($i = 1$–2) is the coded value of the factors, β_0 is a constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{12} is the interaction coefficient, was fitted to both the concentration, conversion yields and initial rate.

The performance of the enzymatic synthesis of the fatty acid sugar esters was evaluated by analyzing four responses: (1) initial reaction rate (Ri, g l^{-1} h^{-1}); (2) the concentration of total sugar esters (TSE, g l^{-1}); (3) the fatty acid conversion yield (YFA, %); (4) the fructose conversion yield (YFrc, %).

The initial rate (Ri, g l^{-1} h^{-1}) was calculated from the value of the formation rate of sugar fatty acid esters in 30 min, which is within the linear reaction range. The yield was calculated based on the conversion of either fructose (YFrc, %) or the fatty acid (YFA, %) to the corresponding fructose fatty acid ester after reaching equilibrium.

2.4.1. Data analysis and optimization

Modde 4.0 (UMETRI AB) was used to fit the quadratic response surface model to the experimental data.

Optimization of the reaction conditions in terms of fructose/fatty acid molar ratio and fatty acid chain length was carried out using the predictive models from RSM. The synthesis of the fatty acid sugar esters was carried out at the predicted optimal conditions. The observed responses obtained at these conditions were analyzed and compared to the predictive values.

3. Results and discussion

The time course of the reaction showed an increase in the total sugar esters concentration during the first hours of incubation, then the reaction reached a plateau. The time required to reach this plateau was dependent upon the operating variables (i.e., carbon-chain length of the fatty acid and the fructose/fatty acid molar ratio). The total sugar esters concentration, conversion yields and the initial rate of the reaction were used as the responses in the experimental design, and the effects of the operating variables on these responses were investigated. The correlation between factors and responses was determined by statistical analysis.

3.1. Statistical analysis

Table 1 gives the analysis of variance and regression coefficients of full polynomial models representing relationships between factors and responses.

Table 1
Analysis of variance and regression coefficients of full polynomial models representing relationships between fatty acid conversion yield (YFA), fructose conversion yield (YFrc), total sugar ester concentration (TSE) and initial rate (Ri) and independent variables of carbon-chain length of the fatty acid ($i = 1$) and fructose/fatty acid molar ratio ($i = 2$)

Coefficient	*P*-value[a]			
	YFA	YFrc	TSE	Ri
β_0	1.612e−21	4.746e−25	6.904e−23	2.152e−03
β_1	5.156e−09	1.239e−09	1.695e−17	0.028
β_2	2.032e−12	1.174e−12	2.031e−12	0.547
β_{11}	0.651	0.015	1.165e−03	0.488
β_{22}	0.281	1.893e−05	1.544e−05	0.328
β_{12}	9.060e−06	0.089	0.041	0.146
R^2 of the model[b]	0.97	0.95	0.97	0.81

[a] Probability of observing a value of the test statistics that is at least as inconsistent with the null hypothesis as the value of the statistics actually observed.

[b] The fraction of variation of the response explained by the model.

The two process variables indicated that the four responses (YFA, YFrc, SET and Ri) can be well described by polynomial models with satisfactory coefficients of determination R^2. Moreover, the statistical analysis showed that both factors had significant effects on most responses (P-value < 0.05). Indeed, the effect of carbon-chain length of the fatty acid (β_1) is significant on all responses, whereas the molar ratio (β_2) has a large effect on three of the responses (YFA, YFrc and TSE) and insignificant effect on the initial reaction rate. Quadratic effects of carbon-chain length of the fatty acid (β_{11}) and fructose/fatty acid molar ratio (β_{22}) are significant in the case of YFrc and TSE. Finally, the model showed a two-factor interaction for YFA and TSE.

The relationships between reaction factors and responses could be best understood by examining the isoresponse surfaces. The isoresponse representations are generated by plotting contours of the response vs. both factors.

3.2. Evolution of the fatty acid conversion

Fig. 1 shows that the best fatty acid conversions are obtained for long carbon-chain fatty acids and equimolar proportions of fructose and fatty acid. As an illustration, these proportions gave about 80 % fatty acid conversion for stearic acid against only 46% when caprylic acid was used as an acyl donor. The regiospecificity of lipases may be responsible for this enhanced reactivity towards long carbon-chain fatty acids. These results are in accordance with those reported by Cao et al. [11] who suggested that most lipases have a preference for lipophilic substrates.

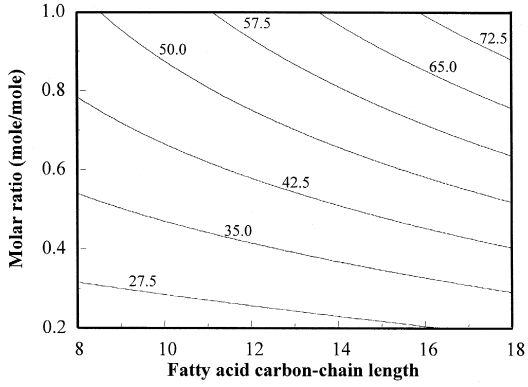

Fig.1. Isoresponse contour plots showing the effect of fatty acid carbon-chain length and fructose/fatty acid initial molar ratio on fatty acid conversion yield (%).

We also noticed that for a given fatty acid, YFA increased with the fructose/fatty acid molar ratio until 1:1 and then reached a plateau, which is due to a limitation by the fatty acid. The level of this plateau decreased for short carbon-chain fatty acids. Likewise, Khaled et al. [8] observed the same profile during the lipozyme-catalyzed synthesis of oleate fructose.

A lower molar ratio, less than 1:1, provoked a decrease in the fatty acid conversion yield. Hence, YFA of stearic acid dropped from 80% to 26 % when varying the initial molar ratio from 1:1 to 1:5. This evolution could be attributed to an inhibition of the *C. antarctica* lipase by the acyl donor concentrations or is due to a limitation by fructose concentration in the medium. The inhibition by the substrate, at low molar ratios, has already been expressed by Coulon et al. [10] and Mutua and Akoh [12].

3.3. Evolution of the fructose conversion

As described in Fig. 2, whatever the initial molar ratio value, the fructose conversion decreased when the carbon-chain fatty acid was shortened. The same results were attained by Sin et al. [13]. This result confirms the assumption that *C. antarctica* lipase is more specific towards long-chain fatty acids. However, when using lipozyme as catalyst, Chahid et al. [14] demonstrated that the yield of fructose esters dramatically decreased as the acyl group was lengthened.

Additionally, the best fructose conversions were obtained in presence of an excess of the fatty acid in the medium. Indeed, maximal fructose conversion was reached when stearic acid and a molar ratio of 1:5 were used. In these conditions, the limiting reagent is the fructose. Therefore, the low fatty acid conversion yields previously mentioned are due to a limitation by substrate concentration rather than an inhibitory effect on the enzyme. Arcos et al. [15] also reported that nearly total conversion of glucose was achieved when low sugar/fatty acid molar ratios were used ($< 1{:}3$). Consequently, under these condi-

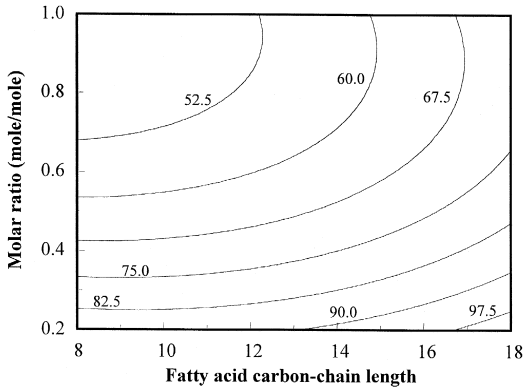

Fig. 2. Isoresponse contour plots showing the effect of fatty acid carbon-chain length and fructose/fatty acid initial molar ratio on fructose conversion yield (%).

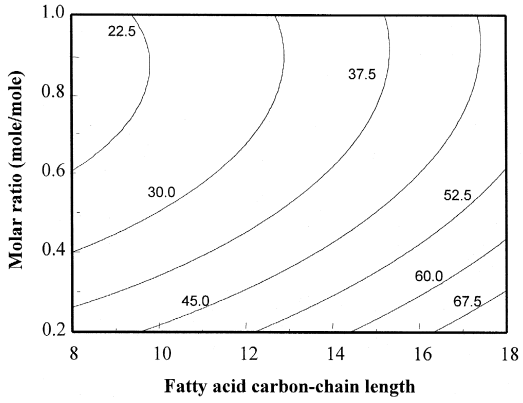

Fig. 3. Isoresponse contour plots showing the effect of fatty acid carbon-chain length and fructose/fatty acid initial molar ratio on total sugar ester concentration (g l^{-1}).

tions, optimization of this process requires a fed-batch reactor with periodic readjustment of the fructose content to at least its solubility in the solvent used.

3.4. Evolution of the total sugar esters concentration

Whatever the operating conditions tested (molar ratio and fatty acid chain length), both mono- and diesters were produced. However, the monoester/diester ratio varies depending on the set points of the two studied parameters. Monoesters are composed of a mixture of α- and β-anomers of 1-acyl fructopyranose, 1 and 6-acyl fructofuranose [16] while the diester is a 1,6-diacylfructofuranose [15].

Similar to the fructose conversion, the best sugar ester concentrations were reached for long carbon-chain fatty acids (C16–C18) and low initial molar ratios. For a fixed set point of the initial molar ratio, total sugar ester concentration dropped when the carbon-chain length of the fatty acid decreased (Fig. 3). In fact, about 73 g l^{-1} of total sugar esters were obtained when stearic acid was used against only 41.5 g l^{-1} with caprylic acid at a molar ratio of 1:5. This result confirms once again the specificity of *C. antarctica* lipase towards long carbon-chain fatty acids. It can also be due, as it was suggested by Selmi et al. [17], to the fact that short- and medium-chain fatty acids reaction media (C8–C14) are more polar, leading to a lower rate of water elimination during the fatty acid sugar esters synthesis.

Furthermore, when using low molar ratios, the excess of the fatty acid in the medium increases fructose conversion and thus, the final sugar ester concentrations. This profile could be explained by the fact that an excess of substrate shifts the equilibrium in favor of sugar esters synthesis rather than their hydrolysis.

3.5. Evolution of the initial rate of the reaction

It can be observed from Fig. 4 that the initial rate of the reaction was strongly dependent on the fatty acid carbon-chain length and fructose/fatty acid molar ratio. The best initial rates were reached with

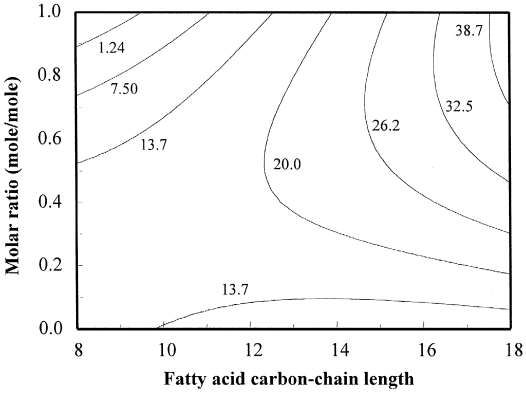

Fig. 4. Isoresponse contour plots showing the effect of fatty acid carbon-chain length and fructose/fatty acid initial molar ratio on the initial reaction rate (g l^{-1} h^{-1}).

long carbon-chain fatty acids and high molar ratios. Moreover, depending on the fatty acid used, optimal values of the initial reaction rates can be reached in two domains. In the case of short carbon-chain fatty acids, low molar ratios induce high initial rates, while for long carbon-chain fatty acids, the highest initial rates are obtained with high molar ratios. As an illustration, at a fructose/fatty acid molar ratio of 1:1, stearic acid gave about 40 g l^{-1} h^{-1}, whereas only 1 g l^{-1} h^{-1} was obtained with caprylic acid. Accordingly, increasing the acyl donor concentration (i.e., low molar ratios) seems to promote the catalytic activity of the lipase for short carbon-chain fatty acids, whereas it leads to a loss of activity when long carbon-chain fatty acids are used. The variation of the lipase activity can be attributed to the evolution of the medium hydrophobicity, which is closely related to the two variables. In fact, Uppenberg et al. [18] reported that the active site of *C. antarctica* lipase is accessible to the substrates through a narrow hydrophobic channel formed by three parts, helices α_5 and α_{10} and a loop region. As *C. antarctica* lipase shows an interfacial activation, the variation of the hydrophobicity of the medium can control the substrate entry to the active site. This phenomenon seems to be controlled by the arrangement of the high mobile helix region mentioned above. So the loss of activity observed with long carbon-chain fatty acids and molar ratios less than 1:1 could be explained by the saturation of the enzyme site by the high quantities of the fatty acid which hinder the fructose entrance. This variation can also be attributed to the medium viscosity. Humeau et al. [19] reported similar results during the lipase-catalyzed synthesis of ascorbyl palmitate. For low carbon-chain fatty acids, the interfacial activation of the enzyme site may necessitate high quantities of the acyl donor. Consequently, the highest initial rates were obtained only when using an excess of the fatty acid.

4. Conclusions

Both initial fructose/fatty acid molar ratio and carbon-chain length of the fatty acid affect the performance of the enzymatic synthesis of fructose fatty acid esters. Moreover, significant effects of the two-factor interaction on the fatty acid conversion yield and the total sugar esters concentration, and, to a lesser extent, the fructose consumption, were observed. Low molar ratios provoked an increase of the fructose consumption and total sugar ester concentrations. Conversely, it negatively affected fatty acid conversions. The effect of the molar ratio on the initial reaction rate was dependent on the fatty acid carbon-chain length. The best performances of the reaction were obtained with stearic acid. For a given fatty acid, the highest yields and concentrations were attained by an adequate adjustment of the molar ratio. These results can be explained by the enzyme specificity towards long carbon-chain fatty acids and the variation of the medium hydrophobicity, thus affecting the lipase activity.

Acknowledgements

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