INTERESTS FOR THE DESIGN OF BIOSOURCED DERIVATIVES BY CHEMO-ENZYMATIC SYNTHESIS

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Abstract

Conventional chemistry is difficult to implement with carbohydrates, the basis of many "green" surfactants. Biocatalysis appear as a possible solution to synthesize biosourced molecules with more sustainable industrial processes that comply with the principles of green chemistry: energy saving, product selectivity, monodisperity, reduction of the use of solvents, with energy eco-efficiency. Enzymes have a great versatility, their active site can receive very variable substrates, in mild conditions and in alternative solvents. Thus, biosourced products such as carbohydrates derived from biomasses such as beet or starch crops, are used as platform entities to obtain high added value products. An optimization of monosaccharide ester synthesis has been done by modulating different parameters. The synthesis of linolenic ester of glucose allowed to determine the interest of using tertiary alcohols as reaction solvent. Reaction parameters of temperature, medium stirring, reaction time, enzyme ratio, desiccant in the reaction medium, and ose:fatty acid ratio were determined after several trials. Moreover, by adding a solubilizing agent at different concentrations, the yields could be improved. The optimal parameters were applied to other fatty acids (FA) and allowed to determine a relative affinity for long hydrocarbon chains (C18 saturated or not) compared to shorter chains. The operating conditions were transposed to transesterification and a better molar yield was obtained. An optimization of this reaction was carried out by modifying the ratio, the reaction time, and the stirring. The results show the importance of the ose:FA ratio of 1:3 against the 1:2, 48h of reaction time and a high agitation, 240 rpm, as well as an activation temperature of catalytic activity between 50°C and 60°C. A variation of the osidic derivative shows an identical affinity for all glucose epimers and this lipase.

Key words: biobased, biocatalysts, chemo-enzymatic, optimization.

INTRODUCTION

Fatty acid esters and sugar esters are non-ionic biosurfactants obtained from naturally renewable resource (Klein et al., 2019). As such, they have the property of reducing surface tension at air/water interfaces and oil/water interfacial tension (Sachdev and Cameotra, 2013). This set of molecules, classically called surfactants, is mainly obtained by chemical means or by microbial means, as with rhamnolipids for example. The use of microorganisms generates a non-negligible cost, particularly for purification, and even more, the small quantities generated are a limiting factor (Nott et al., 2013). The alternative, particularly in terms of the cost of synthesis, would be to turn to so-called classical chemistry syntheses. Nevertheless, classical chemistry is difficult to implement with carbohydrates (Gloster, 2020). Biocatalysis is emerging as a possible solution for designing biosourced

molecules. Over the past two decades, interest in biocatalytic transformations has increased due to an urgent need for more sustainable industrial processes that are consistent with green chemistry principles (Santi et al., 2021). Enzymatic engineering allows the production of high value-added molecules (Benítez-Mateos et al., 2021). Every year, the number of published studies on biocatalysis increases (Nikulin and Švedas, 2021). Enzymatic synthesis will have many advantages compared to chemical synthesis: energy saving, product selectivity, reduced use of pre-sourced solvents. The use of enzymes makes it possible to avoid the undesired obtaining of by-products, as well as the steps of protection and deprotection of hydroxyl groups of the carbohydrate molecules platforms (Perugino, 2004). These steps have a significant cost and complicate the syntheses. Enzymes have great versatility, their active site can receive various substrates, under varying conditions of temperature and alternative solvents. The optimization of a synthesis pathway will require the consideration of relatively different factors, ranging from the conditions necessary for catalytic activity, to those required to make the substrates bioavailable to the enzyme. The main enzymes used are lipases, which catalyse hydrolysis and esterification reactions. Due to the unique properties of lipases, they can be used in several fields.

Lipases are active in environments that contain at least two distinct phases, in which all the reactants are distributed between these phases, although their distribution is dynamic as the reaction proceeds. The kinetics of lipase-catalysed reactions are governed by several factors (Stergiou et al., 2013). Optimizing a synthesis will involve varying these to improve yields, or simply to decrease the overall carbon footprint of the final product. Enzymes are generally sold immobilized on polymeric support. This immobilization confers to the lipases a stability to the reuse, to decrease the effects of the thermal variations as well as a maintenance in time of the active conformations of the protein sites. The choice of a reaction solvent is also an element to consider: it is necessary to consider the fact that acylation will progressively increase the solubility of the oses (Chamouleau et al., 2001) in organic solvents. Co-solvents, such as DMSO, pyridine or DMF have the advantage of solubilizing almost all the molecules but inactivate the enzyme (Jia et al., 2010). Another alternative to improve the synthesis of carbohydrate esters by enzymatic reaction is the transesterification. The objective of this work was therefore to discriminate different solvents for glucose esterification reactions and to optimize the synthesis of lauric ester of glucose by transesterification.

MATERIAL AND METHOD

All reagents used had a minimum purity of 98% unless otherwise stated.

Tert-butanol (t-But), 2-methyl-2-butanol (2M2B) were from Janssen (Belgium). Dichloromethane was from Carlo Erba Reagents. Ethyl Acetate (AcOEt), methanol (MeOH) and petroleum ether were from VWR (France). Molecular sieve 3Å (8-12 mesh, preactivated by drying 4h at 250 ◦C before use, then dried overtime at 105°C), diméthylsulfoxide (DMSO), glucose monohydrate, glucose, rhamnose, galactose, mannose, lauric acid (C12), ethyl laurate (C12Et) and vinyl laurate (C12V) were purchased from Sigma-Aldrich (USA). Xylose was obtained from TCI.
Acrylic lipase (EC 3.1.1.3), ≥5,000 U/g, recombinant, expressed in *Aspergillus niger*, was purchased from Sigma-Aldrich (USA).

Esterification protocol:
Molecular sieve (10% w/v), ose (1eq) and a fatty chain (3eq) were stirred together in t-But or 2M2B. 20% DMSO (v/v) was added next. The reaction mixture was stirred and heated to the target temperature in a closed thermostatically agitator. After 1 hour of heating and a homogeneous medium observed, a sample was taken in order to carry out TLC kinetics. The enzyme was finally added with a ratio of 1% (w/v). The syntheses were always conducted with a negative control, without enzyme.

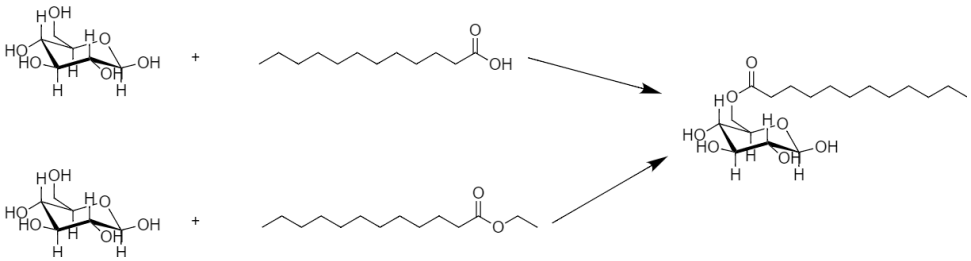

Fig. 1 Desired products from the synthesis of lauric ester of glucose by esterification (C12) and transesterification (C12Et)

Purification of the product:
After synthesis, the reaction media were immediately cooled to 4°C. The samples were then stored at -18°C before being purified. They were then filtered on paper to recover enzyme and sieve. The enzyme was washed with MeOH or t-But and stored at 4°C. The organic solvents, tertiary alcohols, were then evaporated under reduced pressure. A liquid extraction with H_2O and dichloromethane was then performed. The organic phase was recovered and evaporated. The oil-like crude product, mixture of the starting fatty chain of the desired biosurfactant, was then purified by flash chromatography with a mobile phase consisting of a variable ratio of petroleum ether, ethyl acetate and methanol. The isolated product was then characterized by IR-FT and lyophilized.

RESULTS AND DISCUSSION

The first experiments were performed to test various solvents that can allow the formation of the desired product (as described in the conditions described in Figure 2). The experiment was led with heptane, acetone, ethanol, water, isobutanol, isoamyl alcohol, as well as co-solvents heptane/acetone, ethanol/water, hexane/THF. The characterization of reaction media by TLC allowed to highlight that isobutanol and isoamyl alcohol, although complicated to evaporate, could be good candidates. Particular attention was paid to the solubility of the initial mixture. Glucose and more generally, the oses, are only very slightly soluble in isoamyl alcohol and isobutanol. Therefore, based on this fact, the use of ethanol seems to be the best candidate to solubilize all the reagents. The infrared spectra obtained show the formation of an ester, hence an enzymatic activity, but also the absence of glucose free hydroxyl groups, located around 3300 cm^{-1} on IR-FT spectra. The molecules obtained were therefore alcohol esters. Ethanol is indeed a very good substrate for the this Lipase, expressed in Aspergillus Niger. However, non-substrate solvents for lipases are mainly tertiary alcohols but weakly solubilize oses. The best reaction solvent among t-But and 2M2B was determined, as shown in Figure 2. DMSO was added in 20% (v/v) proportion to increase the solubility of starting raw materials.

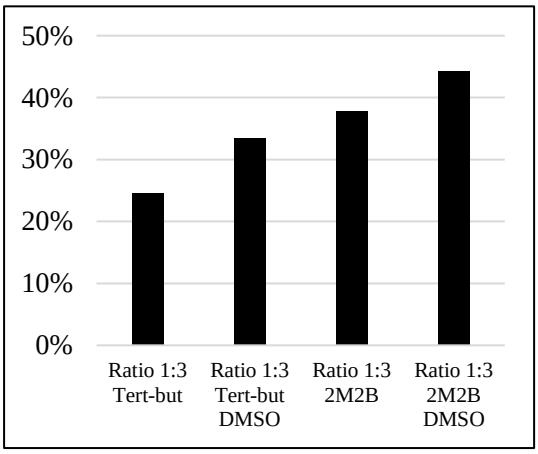

Figure 2: Effect of solvent on molar yield for the synthesis of oleic ester of glucose with fatty acid – 56°C – 72h – 240rpm

The results obtained here distinguish a highly positive effect of DMSO when it represents 20% of the final concentration. 2M2B seems to be a better reaction solvent. Triplicates performed on this synthesis confirm the results with an average of 26% yield in tert-butanol, 39% when 20% DMSO is added, and 32% and 45% yield in 2M2B and 2M2B with DMSO.

Then, the objective of the work was to extrapolate the results obtained to other fatty acids. First, the interest was to see if the 2M2B/DMSO mixture was still the best mixture. Glucose has a solubility, at 60°C, of 2.40 g/L in

2M2B, and 2.30 g/L in t-But. The solubility of glucose is increased by a factor of 5 in 2M2B when the DMSO concentration is close to 20% at 60°C (Tsavas et al., 2002). DMSO would potentially induce a conformational change in the tryptophan residues of lipase. Studies report a non-essential activating effect, not leading to structural changes in lipase (Mangiagalli et al., 2020). Further study will confirm one of theses hypotheses. Secondly, the objective was to determine a potential affinaty for some chain. The preferred solvent is 2M2B with 20% DMSO with 45% for the oleic glucose ester (Figure 3).
Moreover, the yields were higher with long chain (C18:1) and unsaturated compared to shorter chain (C12) and saturated. Without lipase, a product was obtained, probably a sugar ester, hypothesis validated by IR-FT.

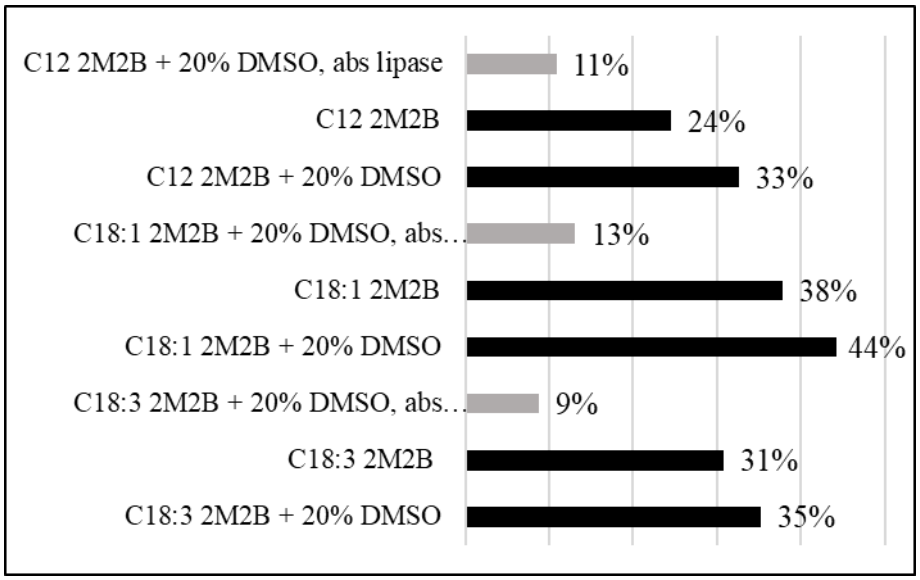

Fig. 3 Effect of DMSO on molar yield – 56°C -72h – 240 rpm (abs means without), light grey for negative control, black for synthesis with lipase.

The work then focused on transesterification, comparing the best conditions, namely 2M2B with DMSO, at 56°C, 240rpm, during 72h. Two ratios were tested and 3 fatty chains were compared: lauric acid for esterification and ethyl laurate and vinyl laurate for transesterification (figure 4).

In their native medium, lipases use to catalyze the hydrolysis reaction of esters. When lipases are used in organic solvents, the reverse reaction is performed. If the neoformed water is not eliminated as it is formed, the equilibrium of the reaction is shifted towards the hydrolysis of glucose esters.

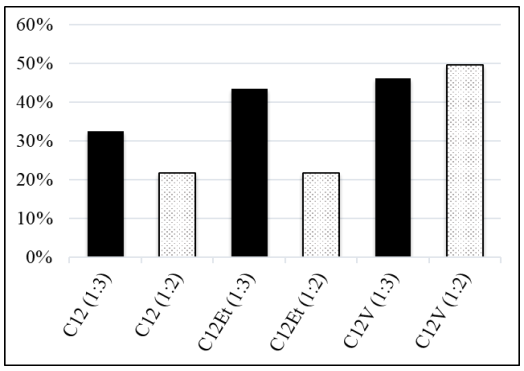

Fig. 4 Molar yield for the variation of ratio for the synthesis of ester lauric glucose

Transesterification is an interesting alternative to fatty acid esterification because, in addition to the fact that alcohol esters are more reactive than fatty acids, the by-product formed is an alcohol and is therefore easier to eliminate.

Vinyl esters are generally preferred over ethyl and methyl esters. The vinyl alcohol formed is tautomerized to acetaldehyde, which is volatile and therefore expected to be easily removed. Nevertheless, acetaldehyde could react with the free amine groups of the lysine residues leading to the inactivation of lipases (van den Broek and Boeriu, 2013). Here, comparable results are obtained with ethyl laurate for a ratio of 1:3 and vinyl laurate for each ratio. For both products, the same profile is obtained by FTIR (cm^{-1}): 3308(O-H), 1730(ester C=O), 1171(C-O), 2850, 2919(aliphatic C-H).

Therefore, it seemed interesting to optimize the lauric glucose ester synthesis by transesterification for the other parameters, namely the synthesis time t, the temperature T and finally the stirring. As for the solvent condition 2M2B+20% DMSO and the ratio (1:3), they were set based on the previous results. For each condition assessed, the other parameters were all fixed. This experimentation allowed to highlight the role of each parameter (figure 5).

It is therefore possible to conclude that a longer synthesis time is not necessarily synonymous with a higher yield of sugar ester. Indeed, it appears that, for a reaction time beyond 48h, the yield (50%) is lower than for 48h reaction (71%). This is probably due to a destruction of the neoformed product during the synthesis, the lauric ester of glucose becomes the preferred substrate of the lipase instead of glucose. Regarding the temperature, it is obvious that there is a plateau of catalytic activation between 50 and 60°C. Regarding stirring speed, it is interesting to note that it has a very important effect on the reaction efficiency, mainly on the probability that the enzyme, here immobilized on the resin, meets the reactants. Best results were obtained for a minimum agitation of 240 rpm.

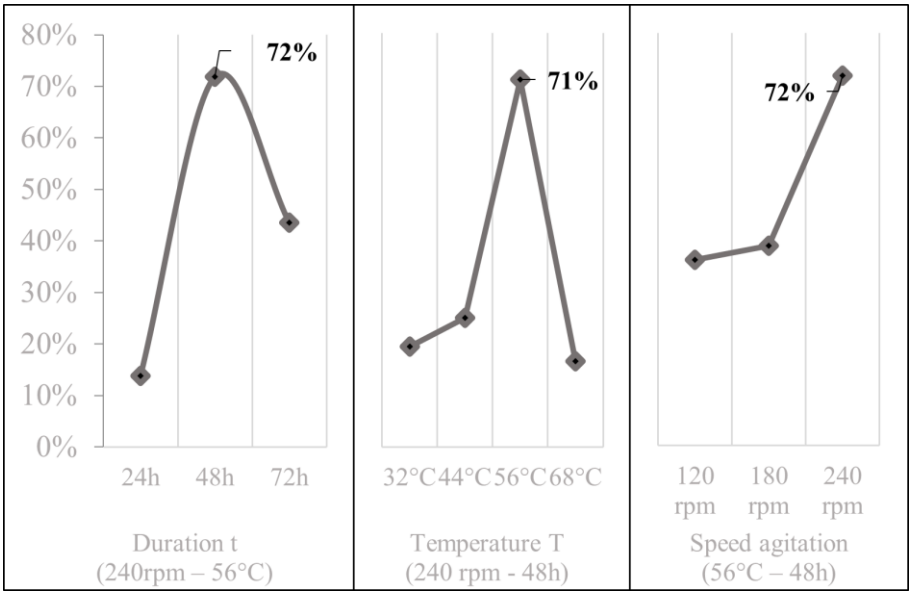

Fig. 5: Molar Yield obtained for the optimization of production of lauric ester glucose

Simultaneously, the objective was to synthesize hexose esters using the previously optimized reaction conditions: transesterification with ethyl laurate in ratio 1:3, 2M2B + 20% DMSO as solvent, 56°C reaction temperature, stirring at 240 rpm, 72 hours reaction time. The yields are similar for the epimers of glucose, namely mannose and galactose. For xylose, the monodisperity of the reactions is different. Indeed, besides the fact that the yield is lower, about 30%, 24% of monoester are obtained, but also 6% of polyester. The monodisperity of the reaction is lost, probably due to the cyclic furanose/pyranose form of xylose. For rhamnose, the negative control, same in all aspects but without enzyme, allows to highlight the catalytic activity of DMSO. The yields are similar for both samples, with and without enzyme, which means that the lipase does not accept rhamnose as a substrate. In fact, for glucose for example, the addition of the ester bond is done exclusively in position 6 of the pyranose ring, on the CH_2OH. As rhamnose is a deoxyose, the lipase does not seem to recognize this substrate.

CONCLUSIONS

This work has highlighted the interest of using enzymes in the context of sugar ester synthesis. Biocatalysis appears as an interesting alternative to classical chemistry. In addition to the green chemistry aspects, enzymatic catalysis allows to obtain syntheses easier to implement, with a relative monodisperity, a great versatility in the substrates and especially a great possibility of optimization, with many parameters on which it is possible to change, with the aim of producing more, with a lower carbon footprint.

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