Efficient Synthesis of Bio-Surfactant from D-Glucose by Immobilized Candida cylindracea Lipase-Catalyzed Reaction in Ionic Liquids

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Abstract—Biotechnology products are compounds with a great applications and the main products are those relating to health, pharmacy, environment. The current work focuses on the optimization of lipase-catalyzed synthesis of a bio-surfactant from D-glucose and lauric acid in ionic liquids. Fifteen reaction conditions solvents were tested in order to find the combination for maximal conversion. The results showed that 1-ethyl-3-methylimidazolium was the best solvent for the synthesis, and adequate for a maximal conversion of 86%.

Index Terms—bio-surfactant, sugar, Candida cylindracea lipase, immobilization, ionic liquids.

I. INTRODUCTION
Sugar esters are nonionic surfactants [1], [2] with many advantages, including in particular health safety, cosmetics and formulation of drugs. The biotechnological way exploiting enzymes synthesis capabilities for sugar esters synthesis has been largely used in the last decade [3], [4]. Lipases constitute the most important group of biocatalysts for biotechnological applications [5]. Lipase-mediated ester synthesis of sugar fatty acid esters is indeed the alternative route to the chemical synthesis. The nature of the solvent [6]-[8]and that of the lipase [9], [10] are parameters which greatly affect the course of the enzymatic reaction. A most prominent problem is the difficulty of dissolving sugars in organic solvents. Numerous researches [11], [12] aim to find solutions in order to find a dual objective of dissolving sugars and preserving the stability of enzymes whose activity is inhibited in an excessively polar environment [13]. In this case, ionic liquids (ILs) turned out to be ideal solvents for enzymatic catalysis to overcome the solubility problem of sugars in organic solvents [14].

Ionic liquids (ILs) have negligible vapor pressure and are extensively used as volatile organic solvents replacements. They are composed from ions and their polarity depends on the nature of the substituent of the cation and on the anion size [15]. Small cations and anions lead to highly polar ILs. On another hand, ILs are liquids at ambient or far below ambient temperature, exhibit hydrophobicity, are thermally stable and can have multiple solvation interactions with organic compounds [16]. All these interesting combination of properties opens the road to a wide range of applications in biocatalysis. The solvent has therefore a significant impact on the enzymatic activity and on the solubility of the sugar, rigorous selection of the solvent and of the lipase can be very beneficial. Furthermore, the performance of enzymes in ILs is improved significantly by modification with solid supports [17]. In this study, we focus on the use of Candida cylindracea lipase immobilized on celite under varying solvent conditions namely ethylmethylketone (EMK) [18], tetrahydrofurane (THF), 2-methyl-2-butanol (2M2B), 1-ethyl-3-méthylimidazolium tétrafluoroborate ([Emim][BF$_4$]), 1-butyl-3-méthylimidazolium tétrafluoroborate ([Bmim][BF$_4$]) to enhance its catalytic activity. Efficiency of different ILs/DMSO and organic solvents/DMSO mixtures at different ratios was also explored in order to improve the reaction conversion.

We also try to explain the evidence of modification of the lipase by X-ray diffraction and scanning electron microscopy [19].

II. MATERIALS AND METHODS
A. Chemicals
Crude lipase from Candida cylindracea and D-(+)-glucose were purchased from Sigma-Aldrich, United States. Celite was purchased from Fluka, Switzerland. Ionic liquids, 1-ethyl-3-méthylimidazolium tétrafluoroborate ([Emim][BF$_4$]) and 1-butyl-3-méthylimidazolium tétrafluoroborate ([Bmim][BF$_4$]) were purchased from Merck, Germany. All other chemicals and solvents used in this research were of analytical grade.

B. Lipase-Catalyzed Synthesis
D(+)-glucose (180 mg, 1mmol) was dissolved in the solvent or a mixture of solvents for one night. After that, lauric acid (200 mg, 1mmol) was added. The biocatalyst and molecular sieves were then incorporated. Aliquots were removed at intervals, filtered and analyzed.
quantitatively by volumetric titration. At the end of the reaction, lipases were removed by filtration and the solvent was evaporated to dryness under reduced pressure. 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 [20].

C. Scanning Electron Microscopy

The surface morphology of the immobilized lipase was characterized by Scanning Electron Microscopy (SEM). Analysis were performed on a FEI QUANTA 250 SEM apparatus with accelerating voltage of 200 V to 30 kV.

III. RESULTS AND DISCUSSION

A. Effectiveness of the Lipase Immobilization

Generally enzymes do not dissolve readily in most ILs. By modifying the form of enzymes in which it is used, like immobilized lipases, solubility as well as activity can be improved. ILs can also induce enzyme conformational changes resulting in inactivation. So the modification of the surface of the lipase is carried out to protect it in order to avoid its inhibition. Immobilized Candida cylindracea lipase was isolated by immobilization of the crude lipase on celite [21].

The characteristics of the final preparation for immobilized lipase are listed in Table I.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Specific Activity [22] (µmols of acid.min⁻¹.g⁻¹)</th>
<th>Protein content [23] (%)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free CCL</td>
<td>3880</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Celite-CCL</td>
<td>13260</td>
<td>32.5</td>
<td>58</td>
</tr>
</tbody>
</table>

The efficiency of lipase immobilization was demonstrated by scanning electron microscopy. Fig. 1 shows the SEM photograph of the free CCL. The SEM results indicate that significant amounts of the lipase were embedded on the support due to the porous morphology of the celite (Fig. 2). The combination of the porous material to the lipase changed the morphology of CCL, as shown in Fig. 3. Different spots related to the lipase on the surface of the virgin celite are visible on the SEM photograph (Fig. 3) and are the proof of the adsorption of CCL on porous celite. The lipase from Candida cylindracea was well integrated on celite.

B. Effect of Solvents

The activity and stability of celite-immobilized lipase from Candida cylindracea were investigated in the esterification of D-glucose with lauric acid using different reaction media. It should be noted that native lipase (CCL) does not show significant esterification activity in the selected solvents.

Firstly, the enzymatic esterifications in pure organic solvents and pure ILs was studied. A suitable solvent is required to dissolve the starting sugar. A preliminary solvent screening in organic solvents such as EMK [18], 2M2B, and THF was explored. The maximum conversion achieved was 45% in EMK (Fig. 4). This may be due to the low solubility of glucose in the selected organic solvents.

For performing biotransformations involving highly polar substrates such as sugars, ILs can be used as alternative reaction media. According to Reichardt’s scale [24], ILs containing [BF₄⁻] anions are considered as moderately polar solvents and can be used as reaction media.
media to enhance the reactivity and stability of enzymes [25]. Other ILs like those with chloride or dicyanamide anions have been reported to be good solvents for sugar dissolution, but most enzymes are inhibited in these ionic liquids [26].

Analysis of enzymatic reactions in the ILs revealed that celite-immobilized CCL exhibited excellent catalytic performance while the native enzyme suspended in ILs showed no activity. Hydrophobicity and alkyl chain length of [Bmim][BF₄] slightly affected enzyme activity, and the relatively hydrophobic IL [Emim][BF₄] was the preferred medium for enzymatic reactions (Fig. 4). The hydrophobic [Emim][BF₄] leads to a slightly better conversion (55%) than the more hydrophobic [Bmim][BF₄] (48%).

Enzyme activity was much higher in [Emim][BF₄] than in conventional organic solvents, and excellent activity was associated with unique properties of this IL such as hydrophobicity and polarity.

DMSO is a good and preferred solvent in due to its high polarity (log Pow = -1.35) giving high solubility rate for dissolving sugars [27]. It was introduced into the solvent system as a solubilizing agent and a co-solvent. Efficiency of different organic solvents or ILs/DMSO mixtures was also explored in order to improve the solubility of sugar [28] and to avoid the problem of mass transfer leading to lower reaction conversions.

To obtain optimized enzyme activity and stability, mixture of organic solvents, ILs and DMSO at different ratios such as organic solvent or ILs/DMSO: 1ml/20μl or 1ml/50μl was used as a reaction medium for lipase-catalyzed synthesis of glucose ester. When using 20 μl DMSO conversion decrease for almost all organic solvents. A slight increase in conversion is observed in the case of ILs and the best result (61%) was obtained when the reaction is done in ([Emim][BF₄]) (Fig. 5). With addition of 50 μl DMSO there is a significant increase in conversion (86%) in the case of ([Emim][BF₄]) (Fig. 6). For organic solvents the conversion does not exceed 50%. The use of two different ratios of DMSO (20 μl and 50 μl) as co-solvent is more effective since sugar was dissolved after short time of stirring. Visually we observed completely dissolution of glucose when using 50 μl of DMSO. On another hand, this quantity remains small to keep the hydrophobicity of the medium in order to maintain the enzymatic activity. Indeed, hydrophobic medium is essential to protect the water layer around protein molecules which increase stability of enzyme towards denaturing. In terms of activity and stability conversion increased as ratio of DMSO increased reasonably.

Among solvent tested (organic solvent and ILs), the highest activity of lipase was observed in [Emim][BF₄]/DMSO:1/50.

IV. CONCLUSION

We concluded that in transformations for biotechnology applications carried out with highly polar substrates like sugars, celite-immobilized lipase from Candida cylindracea showed an ideal catalysis performance with respect to activity and stability when tested in ILs as the reaction media. This facile and efficient greener synthesis approach can be employed to
overcome the problem of glucose solubility and low enzyme stability in order to support sustainable chemistry.

**CONFLICT OF INTEREST**

The authors report no conflicts of interest.

**AUTHOR CONTRIBUTIONS**

Chahra Bidjou-Haïour conducted the research, and wrote the manuscript. Nacer Rezgui did the laboratory work and analyzed the data. All authors had approved the final version.

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**REFERENCES**


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Nacer Rezgui was born in Tebessa, Algeria on July 12, 1984. His research is involved with the development of bio-surfactants based on sugars by the enzymatic and chemical routes. He is now studying at the Department of Chemistry, University of Annaba, Algeria, for getting Doctoral degree.