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Ionic liquids for the concomitant use in extremophiles lysis and extremozymes extraction

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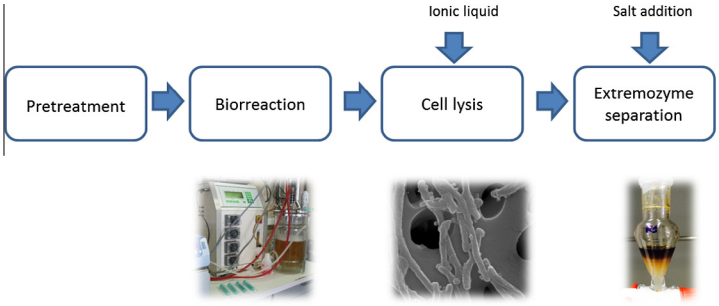
HIGHLIGHTS

- C_{10}MIMCl showed an outstanding lysis ability in cultures of *Thermus thermophilus* HB27.
- Na_2CO_3 and $(NH_4)_2SO_4$ were efficient salting out agents in C_{10}MIMCl aqueous solutions.
- The combination of $(NH_4)_2SO_4$ and C_{10}MIMCl allowed extracting 96% of lipolytic enzymes.

GRAPHICAL ABSTRACT



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ABSTRACT

Ionic liquids have been successfully proposed to modify membrane permeability in cultures of a model extremophilic bacterium *Thermus thermophilus* HB27, which makes up the first time that aqueous solutions of these molten salts are applied in downstream stages of this kind of microorganisms. The presence of 1 g/L of C_{10}MIMCl entails a great solubilisation of cell biomass, thus allowing the release of intracellular and membrane-bound enzyme. The influence on the enzyme activity of two inorganic salts such as Na_2CO_3 and $(NH_4)_2SO_4$, selected on the basis of their high salting out potential and biocompatibility with enzymes, respectively, was investigated. In parallel, their ability to trigger phase segregation was confirmed in the presence of the enzyme crude, leading to very high levels of enzyme extraction (96%). The validity of the strategy was confirmed by operating at bioreactor scale, and the main bioprocess parameters were obtained by modelling the experimental data.

1. Introduction

One of the bottlenecks often faced in biotechnological processes to obtain biocatalysts is focused on the product recovery stages. Although extracellular enzymes expression is desirable, very frequently the targeted product is located intracellularly or membrane-bound. Thus, different methods for cell disruption have been proposed, and can be classified into two main categories: physical and chemical. High-energy consuming mechanical disruption methods, namely cryogenic grinding and sonication, are the most commonly used since chemical ones alone are usually considered less effective (Vandeventer et al., 2011). Therefore, the hunt for efficient alternatives is a must to be tackled by the scientific community, in order to compete with the conventional cell lysis methods. These drawbacks affect to different biotechnological processes. More specifically, extremozymes (enzymes produced by extremophiles) have been the subject of a great interest, as their naturally developed resistance to harsh reaction conditions (chemical agents and extreme values of temperature, pH and salinity) makes them suitable for an array of biocatalytic reactions (Deive et al., 2012a).

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In our group, we have bet in thermophiles, because working at elevated temperatures involves advantages such as reduced viscosities, higher reaction rates, lower resistance to mass transfer, lower risk of contamination and higher solubility of most of the substrates and products (Rothschild and Macinelli, 2001). In this sense lipases (EC 3.1.1.3) have been recognized among the top-three most interesting group of enzymes in terms of sales, with widespread use in industrial sectors as varied as food, drugs, biofuels and detergents, just to mention a few. They typically act on triglycerides to yield diglycerides, monoglycerides, or even up to glycerol. However, in media with low water activity, these versatile enzymes biocatalyze also the synthesis of triglycerides, such as esterification, interesterification, and transesterification reactions (Reetz, 2002). These reactions demand the presence of conventional organic solvents to avoid possible hydrolysis. Nonetheless, their high volatility, bearing detrimental environmental and health effects, has urged the scientific community to search for greener alternatives like ionic liquids. These salts pose a range of benefits, being tunability one of the most appealing for our purposes. The existence of millions of cation/anion combinations ensures a successful outcome for each desired application. In this particular case, the emergence of ionic liquids as catalytic milieu for thermophilic lipase-catalysed reactions opens new opportunities since they allow stable operations at elevated temperatures (higher than 75 °C) without facing any volatility problem.

In this work, we have selected a microorganism from the genus *Thermus* as model thermophile (*Thermus thermophilus* HB27). This genus is well known since an enzyme from *Thermus aquaticus* has been considered an indispensable tool for the development of genetic engineering, due to its use in the polymerase chain reaction. Recently, we have demonstrated the ability of this microorganism to produce lipolytic enzymes (Domínguez et al., 2004).

The viability of different hydrophilic ionic liquids to be used in lipolytic enzymes recovery stages after thermophile cultivation has been investigated. The basis for this selection lies in both the polarity and hydrophobicity conferred by the combination of different cations. These properties may determine the degree of interaction with the microbial membrane. Thus, different imidazolium-based ionic liquids have been reported to dissolve microalga membranes (Fujita et al., 2013), cellulose and even raw biomass (Sun et al., 2011). Based on their high values of solvatochromic Kamlet–Taft parameters (β) (Jessop et al., 2012), we have selected 1-ethyl-3methyl imidazolium (C_2MIM) cation combined with diethyl phosphate, hydrogen sulphate, ethylsulphate and chloride. Additionally, the alkylchain length in the cation was also modified to assess the importance of the molecule hydrophobicity, and C_nMIMCl ionic liquids (n = 6 and 10) were also selected.

The presence of this kind of hydrophilic ionic liquids as lysis agent may also be harnessed for extraction and biocatalytic milieu. In the last years, the addition of high density charge salts to aqueous solutions of ionic liquids has been a subject in the limelight, since their ability to further phase segregation has exhaustively investigated by different research groups, as excellently reviewed by Freire et al. (2012). This separation technique, named aqueous biphasic systems (ABS) is thus the result of a complex competition between ionic liquids and salts for the water molecules. Different advantages have justified their application for the extraction of a portfolio of compounds with industrial interest, such as their mild operating conditions, short segregation time, or their low energy requirements (Freire et al., 2010) Among the different uses, the extraction of biomolecules such as lipases has been highlighted in different research works (Deive et al., 2011a; Ventura et al., 2012).

In view of these considerations, two inorganic salts (Na_2CO_3 and $(NH_4)_2SO_4$) have been selected in this work to salt out the ionic liquid and the lipase produced by *T. thermophilus* HB27, taking into account criteria such as their segregation ability and their biocompatibility with the enzyme. Finally, the proposed strategy will be implemented at bioreactor scale to check its validity.

2. Experimental methods

2.1. Chemicals

Ionic liquids $C_2MIMC_2SO_4$, $C_2MIM(C_2H_5)_2PO_4$ and C_6MIMCl (all of them with purity higher than 99%), and $C_2MIMHSO_4$, C_2MIMCl, and $C_{10}MIMCl$ (with purity higher than 98%) were purchased from IoLiTec. They were all subjected to vacuum ($2{\cdot}10^{-1}$ Pa) and moderate temperature (343.15 K) for several days to remove possible traces of solvents and moisture, always prior to their use. The ionic liquids were kept in bottles under inert atmosphere until use. The inorganic salts Na_2CO_3 and $(NH_4)_2SO_4$ (with purity higher than 99%) were supplied by VWR and Scharlau, respectively.

2.2. Microorganism and culture conditions

T. thermophilus HB27 was kindly provided by Dr. J. Berenguer (Universidad Autónoma, Madrid, Spain). The microorganism was grown in a liquid medium containing (g/L, in distilled water) 8 trypticase, 4 yeast extract and 3 NaCl. The medium was autoclaved at 121 °C for 20 min. Thermophile cultures were carried out in 250-mL Erlenmeyer flasks with 50 mL of medium. The flasks were inoculated (3%) with previously obtained cell pellets, and incubated in an orbital shaker at 70 °C, 100 rpm for 30 h with cellulose stoppers. Ionic liquids were added after 24 h of culture.

The bioprocess scale-up was carried out in a 5-L stirred tank bioreactor (Biostat B, Braun, Germany) containing 3 L of medium. It operated at 70 °C, 500 rpm of agitation, and 0.33 vvm of aeration. The bioreactor was inoculated with actively growing cells from 24 h-Erlenmeyer flask cultures (3% v/v). Samples (10 mL) were taken at regular times. The values shown in figures and tables correspond to mean values with a standard deviation (S.D.) lower than 15%.

2.3. Sample preparation

Cells were harvested by centrifugation (10 min, 5000×g) and suspended in a 2 mL Tris/HCl buffer 50 mM pH 7.5, containing 25 mM EDTA and 25 mM NaCl. The supernatant was reserved for extracellular enzyme analysis. The cell suspension was freezed for 24 h and then sonicated in two cycles of 2 min at 50% of the maximum power (Branson Sonifier, model 250). The procedure was carried out in an ice bath, and a 2 min cooling time was allowed between cycles. Then, the mixture was centrifuged for 10 min at 5 °C and 5000×g. The supernatant and the sediment were separated for the measurement of intracellular lipolytic activity and of membrane lipolytic activity, respectively.

2.4. Aqueous phase segregation

The immiscibility region in ABS was determined by the cloud point method in a jacketed glass vessel containing a magnetic stirrer at 298.15 K (Albertsson, 1986). The selected inorganic salts and water were alternatively added to the mixtures until turbidity and a clear solution was detected, respectively. The composition of all the components was quantified by weighting in an analytical Sartorius Cubis MSA balance (125P-100-DA, ±10–5 g).