



Evaluation of ionic liquids as alternative solvents for aldolase activity: Use of a new automated SIA methodology



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ABSTRACT

An automated methodology is proposed for the evaluation of a set of ionic liquids (ILs) as alternative reaction media for aldolase based synthetic processes. For that, the effect of traditionally used organic solvents and ILs on the activity of aldolase was studied by means of a novel automated methodology.

The implemented methodology is based on the concept of sequential injection analysis (SIA) and relies on the aldolase based cleavage of D-fructose-1,6 diphosphate (DFDP), to produce dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (G3P). In the presence of FeCl₃, 3-methyl-2-benzothiazoline hydrazine (MBTH) forms a blue cation that can be measured at 670 nm, by combination with G3P. The influence of several parameters such as substrate and enzyme concentration, temperature, delay time and MBTH and FeCl₃ concentration were studied and the optimum reaction conditions were subsequently selected. The developed methodology showed good precision and a relative standard deviation (rsd) that does not exceed 7% also leading to low reagents consumption as well as effluent production.

Resorting to this strategy, the activity of the enzyme was studied in strictly aqueous media and in the presence of dimethylformamide, methanol, bmpyr [Cl], hmim [Cl], bmim [BF₄], emim [BF₄], emim [Ac], bmim [Cl], emim [TfMs], emim [Ms] and Chol [Ac] up to 50%.

The results show that the utilization of ILs as reaction media for aldolase based organic synthesis might present potential advantages over the tested conventional organic solvents. The least toxic IL found in this study was cho [Ac] that causes a reduction of enzyme activity of only 2.7% when used in a concentration of 50%. Generally, it can be concluded that ILs based on choline or short alkyl imidazolium moieties associated with biocompatible anions are the most promising ILs regarding the future inclusion of these solvents in synthetic protocols catalyzed by aldolase.

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1. Introduction

Industrial organic synthesis commonly involves the use of volatile organic compounds (VOCs) in the whole synthetic pathway or in specific steps as well as in the separation and purification of synthesis products [1].

However, VOCs are sometimes difficult to eliminate from the final products and are responsible for contaminations being in these cases referred as “residual solvents” or “organic volatile impurities”. Considering the well-known toxicological issues related to VOCs and the solvent use which is about 80–90% of mass utilization in a typical industrial synthesis, the use of these solvents became a serious concern regarding Green Chemistry

principles [2]. Consequently, manufacturers and scientific entities are developing efforts concerning not only the minimization of VOCs use in industrial synthesis but also the search for alternative solvents with minor environmental and human impact and reduced influence on the properties of the final products [3–5]. The current concerns of both academia and industry on this topic resulted already in several attempts to substitute VOCs by ionic liquids (ILs) in organic synthesis [6–9]. The growing application of ILs in organic chemistry stems from their favorable physico-chemical properties, such as lack of vapor pressure, good thermal and chemical stability and good dissolution properties of both organic and inorganic compounds [6]. Moreover, this strategy is also based on the demonstrated ability of ILs to drastically change the rate and selectivity of organic chemical reactions [8]. Indeed, ILs can have impact on the reactivity of the species involved in organic synthesis due to a fine balance of entropic and enthalpic contributions that involve the interactions inside the IL and

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between the ions and dissolved species. In fact, ILs can interact with solutes through dipolar and dispersion forces and act as strong hydrogen-bond acceptors [8].

Furthermore, the possibility of developing tailor-made ILs for particular purposes offers exciting opportunities in the field of organic chemistry enabling the development of task specific compounds. ILs can also form biphasic systems with classic VOCs which allow the simple isolation of reaction products by extraction of the IL layer. Considering future industrial applications some particularities can be exploited such as their ionic nature that facilitates the immobilization and recycling of many transition metal catalysts.

In the field of organic synthesis, ILs have already attracted increasing attention also as reaction media in enzyme based synthetic processes with enhanced enzyme stability and selectivity and higher rates. Considering that several industrial processes nowadays apply enzyme-catalyzed reactions for the large-scale production of chemicals, this approach is of the most importance from both the economical and safety perspective. The applicability of ILs in biocatalysis has been exploited intensively through the study of several enzymes in ILs with structural variability [10,11]. The polarity of ILs seems to be one of the most important factors affecting enzyme stability and selectivity. High polarity permits the utilization of ILs as reaction media for biotransformations of a wide range of substrates, especially those with high polarity such as carbohydrates and amino acids. Other studies reveal that the viscosity of ILs can also have a strong impact on the enzyme's activity [12], as the high viscosity may produce a decrease in the rate of diffusion of the species involved in the process with reduction of the interaction of the enzyme with its substrate influencing negatively the yield of reaction. The hydrogen bond capacity of ILs has been also considered very important for protein stability and maintenance of its native structure [13]. The classic enzyme thermal unfolding is prevented by the entrapment of enzymes, with small amounts of water, in the hydrogen bond network of ILs [14]. The suppression of side reactions due to the decrease of water in the reaction media has also been associated with an increased yield of enzymatic reactions in the ILs [15].

In the field of chemical synthesis, one of the most attractive approaches is the chiral Lewis acid-catalyzed aldol reaction which is considered an important C–C bond-forming reaction being widely used for the production of hydroxyl ketones frequently found in many biologically active compounds and drugs. Hence, developing catalysts for aldol reactions that control the stereochemistry at the newly formed C–C bond independently from the structure of the starting material is of utmost importance. Aldolases are amongst the most promising catalysts for aldol reactions [16,17]. These enzymes catalyze reversibly and stereoselectively the aldol addition of a donor component (i.e., nucleophile), through an enolate or enamine generated at the active site of the enzyme, onto an acceptor component (i.e., electrophile) [18]. Usually these enzymes show high specificity towards the donor substrate and tolerate a broad range of acceptor substrates, making them an attractive tool for the asymmetric synthesis of several compounds such as carbohydrates, amino acids, and their analogs [16,19,20]. Aldolase catalyzed synthetic processes are usually performed in VOCs like methanol or dimethylformamide with the drawbacks exposed above [21,22]. In this context, and considering the above mentioned advantages of biocatalysis in ILs, an automated methodology for the evaluation of aldolase activity in ILs and organic solvents was developed aiming at finding greener solvents for aldolase based synthesis.

The evaluation of aldolase activity has been performed till now recurring to laborious batch techniques, mostly with the purpose of its determination in serum, that involve multiple steps and operations [23] or resort to several enzymes [24,25], which would

disturb the purpose of evaluating the effect of solvents on the aldolase activity. So, for the developed methodology a novel reaction, which was explored herein for the first time, centered on the cleavage of DFDP by aldolase followed by the oxidative reaction between G3P and MBTH in the presence of FeCl_3 .

Keeping in mind the advantages of automation, this determination was implemented in a flow system, based in sequential injection analysis (SIA), a flow technique that has already proved to be an adequate tool for the implementation of bioassays in aqueous media [26] and ILs [27–30]. The use of computer software enables precise control of the reaction conditions through the sampling of chemicals into the system and propelling the sequenced zones to the reactor and detector in a reproducible way. Low sample and reagent consumption and minimal waste generation are remarkable features of this technique, attending the principles of green chemistry applied for the development of clean analytical methods.

Ultimately, the developed work involved a comparative study of the aldolase activity in dimethylformamide and methanol and ILs, to evaluate ILs as an alternative media to conventional organic solvents for aldolase catalyzed organic synthesis.

2. Experimental section

2.1. Materials

All chemicals were of analytical reagent grade with no further purification. High purity water (Milli-Q plus system) with specific conductivity less than $0.1 \mu\text{S cm}^{-1}$ was used for the preparation of solutions and as carrier in the flow system.

Acid 3-(N-Morpholino) propanesulfonic sodium salt (Mops), D-fructose-1,6-diphosphate (DFDP), 3-methyl-2-benzothiazoline hydrazine (MBTH), FeCl_3 , methanol, 1-butyl-1-methylpyrrolidinium chloride (bmpyr [Cl]), 1-hexyl-3-methylimidazolium chloride (hmim [Cl]), 1-butyl-3-methylimidazolium tetrafluoroborate (bmim [BF_4]), 1-ethyl-3-methylimidazolium tetrafluoroborate (emim [BF_4]), 1-ethyl-3-methylimidazolium acetate (emim [Ac]), 1-butyl-3-methylimidazolium chloride (bmim [Cl]), 1-ethyl-3-methylimidazolium trifluoromethanesulfonate (emim [TfMs]), 1-ethyl-3-methylimidazolium methanesulfonate (emim [Ms]), choline acetate (chol [Ac]) were purchased from Sigma-Aldrich Cooperation. All the tested ILs were stored at room temperature in a carefully controlled anhydrous environment.

Solutions of DFDP ($7\text{--}30 \mu\text{mol L}^{-1}$), MBTH 0.05% and aldolase from a rabbit muscle (Sigma, EC 4.1.2.13) 1 mg mL^{-1} were prepared daily in Mops buffer (25 mmol L^{-1} , pH 7). Also daily, a solution of FeCl_3 0.1% was prepared in HCl 30 mmol L^{-1} .

In the inhibitions studies, a series of DFDP ($7\text{--}30 \mu\text{mol L}^{-1}$) solutions were prepared in Mops buffer (25 mmol L^{-1} , pH 7) and in the presence of dimethylformamide (Romil Chemicals), methanol (Atom Scientific), bmpyr [Cl], hmim [Cl], bmim [BF_4], emim [BF_4], emim [Ac], bmim [Cl], emim [TfMS], emim [MS] and chol [Ac] with final concentrations of 5%, 20% and 50% (Fig. 1).

2.2. Flow manifold and instrumentation

The SIA flow system (Fig. 2) consisted of a Gilson Minipuls peristaltic pump, equipped with a PVC pumping tube (1.02 mm) and a Crison module that incorporates an 8-port selection valve. A polymethylmethacrylate-mixing chamber (Fig. 2, MC) of ca. $400 \mu\text{L}$ (including a magnetic stirrer bar), thermostated at 30°C and connected to one of the inlets of the selection valve, was placed on a magnetic stirrer. The chamber is coupled to the system through one of its lateral connectors; the other lateral connector is closed internally so that the excess of solutions is forced to go out

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