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Recovery of *Bacillus cereus* cyclodextrin glycosyltransferase using ionic liquid-based aqueous two-phase system

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ABSTRACT

Ionic liquids-based aqueous two-phase system (ILATPS) offers immediate phase separation and thus reduces the overall processing time, is significantly advantageous as compared to conventional ATPS such as polymer/polymer ATPS and polymer/salt ATPS. In this study, ILATPSs composed of imidazolium-based ionic liquid (IL) and salt were experimentally evaluated for their efficiencies in recovering *Bacillus cereus* cyclodextrin glycosyltransferase (CGTase) from fermentation broth. The phase-forming behavior of 1-ethyl-3-methylimidazolium tetrafluroborate, (Emim)BF_4/sodium citrate ILATPS and (Emim)BF_4/sodium carbonate ILATPS were first studied by constructing the binodal curves. Effects of the ILs concentration, pH value, feedstock loading, and addition of sodium chloride (NaCl) on the recovery of CGTase in ILATPS were investigated. The optimum conditions for the recovery of CGTase were obtained in an ILATPS consisting of 35% (w/w) (Emim)BF_4, 18% (w/w) sodium carbonate and 3% (w/w) NaCl. Experimental results showed that 78% of CGTase could be recovered in the IL-rich phase in single-step purification with a purification fold (P_F) of 15.4. The high P_F indicates that this ILATPS is feasible to be applied in the recovery and separation of CGTase from the fermentation broth.

1. Introduction

Ionic liquids (ILs) are potential green extraction solvents for various biomaterials due to their unique features such as high chemical and physical stability, negligible vapor pressure, and also non-flammability. The relatively lower viscosity of ILs as compared to the polymer-based phase-forming reagents (e.g. PEGs, dextran, etc.) offers rapid phase separation in ATPS construction [1–4]. Besides, ILs is suggested to be an attractive ATPS-forming component because of the ability to ensure a clean manufacturing process due to the negligible vapor pressure [5]. ILATPSs have been used in the extraction of small organic molecules, proteins and other biomaterials [3,5–7]. ILATPSs are capable of overcoming the limitations of conventional ATPSs by adopting a more environmentally benign ATPS. Other advantages of these ILATPSs include enzyme stabilization, high selectivity of product or substrate in ATPS, and negligible emulsion formation [5]. However, ILs is rather expensive as compared to other ATPS phase-forming components, thus the recycling of ILs is significantly important for the sustainable downstream processing of the biomaterials [8]. The extremely low vapor pressure featured by ILs enables them to be easily recycled with least energy consumption, and thus minimizes the cost of the ILATPSs [9]. ILATPSs have become more attractive when several strategies for recycling of ILs have been established (e.g. ultrafiltration [10], dialysis, pervaporation [11] and supercritical CO_2), where the potency of recycling of ionic liquids have been affirmed. Recent studies also showed that ILs can be recycled through electrodialysis (membrane

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separation) and different types of distillations such as vacuum evaporation, column distillation and molecular distillation [12–14].

ILATPSs are usually formed by using hydrophilic ILs instead of hydrophobic ILs because hydrophobic ILs are relatively more expensive and environmentally disadvantageous as compared to hydrophilic ILs. Hydrophilic ILs are unable to be directly applied in the ATPS formation due to the complete mutual solubility. They often required the presence of an aqueous solution of water-structuring salts (i.e. promoters for the water structure) or known as kosmotropes in order to be salted out as a separated aqueous phase in ATPS [15,16]. Citrate salt was suggested as a promising candidate for promoting for the phase separation of ILATPS owing to its biodegradability and non-toxicity. It can be readily disposed into biological wastewater treatment plants, results in least environmental impact factor [17].

Cyclodextrin glycosyltransferase, CGTase (E.C. 2.4.1.19) is a hydrolytic enzyme used in the starch hydrolysis for the synthesis of cyclodextrins (CDs) [18]. Bacterial CGTase often involved in the production of three major types of CDs, namely α-CD, β-CD and γ-CD at different ratio. Besides, large-ring CDs consisted of nine and more glucose units can also be produced by CGTase [19]. CDs are cyclic oligosaccharides contain an apolar cavity that is capable to structure inclusion complexes with various guest molecules and changed their physiochemical properties [20]. CDs are widely utilized in various industries owing to the unique structure of CDs and their derivatives [20–22]. As a consequence of the increasing demands of CDs in various industries, it is vital to enhance the recovery and purification strategies of CGTase.

In previous studies, PEG/citrate ATPS and ethylene oxide-propylene oxide (EOPO)/phosphate ATPS were developed for the recovery of *Bacillus cereus* CGTase from fermentation broth with P_F of 16.3 and 13.1 respectively [23,24]. Though PEG/citrate ATPS showed high P_F of CGTase, it required longer settling time and centrifugation process to attain the phase separation which is disadvantageous for large-scale production of CGTase. Besides, recycling of PEG often coupled with complicated procedures that increase the overall operation costs. Thus, application of ILATPS in the recovery of *B. cereus* CGTase was deployed in this study with an aim to improve the existing polymer/salt ATPS in the context of cost-efficiency. Moreover, it was suggested that the ILATPS is able to improve the purification efficiency of CGTase through enhancement of CGTase selectivity and preservation of the biological activity in ILATPS [5]. At present, there are very limited studies reporting on the use of ILATPS for the purification of protein. To date, reports on the purification of fermented *B. cereus* CGTase using the ILATPS are still unavailable. In this study, 1-ethyl-3-methylimidazolium tetrafluroborate, (Emim)BF_4 has been selected as the phase-forming component in ILATPS for the recovery of CGTase. The effects of different types of salts on the phase separation and partition coefficient of CGTase have been investigated. The feasibility and purification efficiency of the ILATPSs on the recovery of *B. cereus* CGTase were studied. The working parameters of ATPS such as concentrations of phase components, feedstock loading, addition of neutral salt, NaCl have been evaluated for the optimum recovery of CGTase.

2. Materials and method

2.1. Materials

(Emim)BF_4 (CAS: 143314-16-3) was obtained from Fluka Co. (USA). Bicinchoninic acid solution (BCA), α-, β-, and γ-CDs were purchased from Sigma Chemical Co. (MO, USA). Phenolphthalein, sodium chloride, sodium citrate and sodium carbonate were purchased from Merck (Darmstadt, Germany). All of these chemicals were of analytical grades except for (Emim)BF_4 which was of chemical grade.

2.2. B. cereus cultivation and CGTase production

B. cereus was cultivated in a 500-mL shake flask containing 100 mL of the medium as described in a previous publication [23]. A 10% inoculated culture medium was grown under aerobic condition at 37 °C for 30 h with continuous agitation (250 rpm). The crude feedstock containing CGTase was prepared by subjecting the fermentation broth to centrifugation at 4000 rpm for 15 min. The supernatant was stored at 4 °C.

2.3. CGTase activity assay

CGTase activity was evaluated spectrophotometrically (550 nm) using phenolphthalein method [25,26] with some modifications. 25 μL of enzyme sample (either the crude enzyme or the enzyme samples withdrawn from ILATPS) was added into 750 μL substrate solution [1% (w/v) starch in 0.05 M Tris–HCl buffer pH 8.0] and incubated at 55 °C for 15 min. 375 μL of 0.15 M NaOH was then added into the reaction mixture for enzyme deactivation. Finally, 100 μL of 0.02% (w/v) phenolphthalein reagent was added. The amount of the β-CD produced was determined by using a standard curve of β-CD. One unit of CGTase activity was defined as the amount of CGTase producing 1 μmol of β-CD per min under the assay conditions.

2.4. Bicinchoninic acid assay (BCA assay)

Total protein concentration was measured using BCA assay [27] and calculated using a bovine serum albumin (BSA) standard curve. In a microtiter plate, 200 μL of BCA working reagent was added into the well containing 50 μL samples. The microtiter plate was then incubated at 37 °C for 30 min followed by the measurement of absorbance at the wavelength of 562 nm. To avoid the interference of IL, a blank sample (i.e. an ILATPS containing same phase composition without the feedstock) was prepared.

2.5. Ilatps

2.5.1. Phase diagram

Binodal curves, denoting the two-phase formation as described by Albertsson [1] were constructed based on turbidometric titration method [28]. Known concentrations of IL and salts were mixed to form several ILATPSs in each tube with different total composition. The resulting mixture was turbid initially due to the immiscibility of the two phase-forming components. Distilled water was added drop-wise into the mixture and mixed well until the turbidity was clear. Disappearance of the turbidity indicated the attainment of critical point at which the resulted mixture was homogeneous. The binodal node was determined by measuring the weight of distilled water added for the turbidity to disappear. The final concentrations of IL and salts were calculated and the binodal curves of ILATPSs were plotted at varying concentrations of ILs and salts.

2.5.2. Partitioning of the enzyme CGTase

A 10-g of ILATPS was prepared by transferring appropriate amounts of IL, salts, 20% (w/w) crude CGTase and distilled water into a 15-mL centrifuge tube. The mixture was then gently stirred and followed by a centrifugation at 2000 rpm for 1 min for complete phase separation. V_R of each ILATPS was measured. Samples from top and bottom phases were analyzed using CGTase activity assay and BCA assay.