Journal of Chromatography B 1060 (2017) 207–214

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Low frequency sonic waves assisted cloud point extraction of polyhydroxyalkanoate from *Cupriavidus necator*

Sivananth Murugesan, Regupathi Iyyaswami*

Department of Chemical Engineering, National Institute of Technology Karnataka, Surathkal, Mangalore 575025, India

ARTICLE INFO

Keywords:
Sonication
Cloud point extraction
Cupriavidus necator
Polyhydroxyalkanoate

ABSTRACT

Low frequency sonic waves, less than 10 kHz were introduced to assist cloud point extraction of polyhydroxyalkanoate from *Cupriavidus necator* present within the crude broth. Process parameters including surfactant system variables and sonication parameters were studied for their effect on extraction efficiency. Introduction of low frequency sonic waves assists in the dissolution of microbial cell wall by the surfactant micelles and release of cellular content, polyhydroxyalkanoate granules released were encapsulated by the micelle core which was confirmed by crotonic acid assay. In addition, sonic waves resulted in the separation of homogeneous surfactant and broth mixture into two distinct phases, top aqueous phase and polyhydroxyalkanoate enriched bottom surfactant rich phase. Mixed surfactant systems showed higher extraction efficiency compared to that of individual Triton X-100 concentrations, owing to increase in the hydrophobicity of the micellar core and its interaction with polyhydroxyalkanoate. Addition of salts to the mixed surfactant system induces screening of charged surfactant head groups and reduces inter-micellar repulsion, presence of ammonium ions lead to electrostatic repulsion and weaker cation sodium enhances the formation of micellar network. Addition of polyethylene glycol 8000 resulted in increasing interaction with the surfactant tails of the micelle core there by reducing the purity of polyhydroxyalkanoate.

1. Introduction

Over the past few years, as the necessity towards sustainable separation process has outgrown, research and development on novel purification techniques, integration of separation processes and their feasibility have been extensively explored in the field of downstream processing. Liquid-Liquid Extraction (LLE), an industrially employed conventional separation process has had its paradigm shift towards green chemistry in the last decade, owing to adverse effects of usage of replenishable petrochemical based solvents and their global environmental issues on its disposal after usage [1,2]. Surfactant based LLE has attained major attention towards separation of both hydrophilic and hydrophobic solutes from the feed stream [3], while reverse micellar extraction involves the usage of organic solvents and surfactants for phase formation and separation; cloud point extraction, a potent aqueous biphasic separation system is considered to be eco-friendly and sustainable for various reasons [4,5]. When an aqueous surfactant system is subjected to temperature variation, surfactant monomers are completely solubilized at a particular temperature named as kraft point and with further variation in temperature, this homogeneous system becomes turbid and results in the formation of two phases comprising of top aqueous phase, wherein hydrophilic solutes get partitioned and a bottom surfactant rich micelle phase (coacervate phase) that encapsulates hydrophobic solutes [4]. The phase transformation takes places as a result of dehydration of surfactant tails, causing structural deformation and formation of micellar network that partitions as a separate phase from the bulk liquid, the temperature at which phase transformation begins is denoted as cloud point temperature. Cloud point extraction (CPE) is a solvent free aqueous based separation process and accounts to various advantages such as ease in operation, recycling of used surfactants and scale up. Based on surfactant type and its concentration, presence of additives including cosurfactant, salts, polymer, cloud point temperature of the system varies. However, maintaining high temperatures while operating large feed volumes is difficult, that in turn has an effect on the overall operation and maintenance cost. To overcome these issues during scale up of CPE process and as a potent alternative, external forces such as microwave [6], co-precipitation [7], magnetic field [8], sonication [9], stirring [10] have been applied and studied in inducing cloud point systems and for their extraction of solutes from feed stream.

Ultrasonication assisted cloud point extraction (UACPE) process was innovated by inducing CPE systems in the presence of sonic waves

* Corresponding author.
E-mail address: regupathi@nitk.ac.in (R. Iyyaswami).

http://dx.doi.org/10.1016/j.jchromb.2017.06.009
Received 3 April 2017; Received in revised form 27 May 2017; Accepted 4 June 2017
Available online 06 June 2017
1570-0232/

[11,12]. Introduction of sonic waves in a fluid results in the formation of micro bubbles, which usually grow and implode as an effect of alternative compression and rarefaction (cavitation). Bubbles are usually formed at a range of few nanometers to micrometer that vary with the effect of operational and system parameters [13]. Explosion of microbubbles lead to adiabatic release of gas trapped inside that increases the system's temperature upto 5000 K and about 2000 atmospheric pressure within the liquid medium [14]. Transient cavitation mostly occurs in the presence of gas or vapor that results in uneven oscillation of bubbles and release of high temperatures and pressures which often denature the biomolecules. Stable cavitation is very much suitable for the separation of solutes from biological feeds and are experienced at low frequencies, as even bubbles are created with an uniform oscillation that exert shear stress on the solute molecules [15]. Generation of bubbles and their size decides the extraction efficiency of an UACPE process and are generally influenced by source of sonication, usually a tip type sonicator produces larger bubbles and are highly efficient compared to that of the bath type sonicators. Sonication parameters such as input power in terms of frequency, sonication duration and intervals also decide bubble size and its generation. Apart from these, variable system parameters like presence of additives and pH of the feed solution have also been reported to play a vital role on the efficiency of sonication assisted extraction process. Presence of charged molecules and surfactants lower the surface tension of the solution to a larger extent, which also alters the bubble properties: size, stability, adsorption, rupture and density [16]. Presence of sonic waves in a surfactant systems cause a structural rearrangement of surfactants within a micelle, as the micelles reshape their extraction efficiency also varies. Unlike external heat induced CPE, microbubbles induced by the sonic waves within the liquid medium are entrapped between the micelles, such microbubbles implode as a result of micelle reptation releasing high temperature and pressure. This abundant release of energy leads to structural transformation of surfactants within the micelle [17] and the replacement of water between the micelles leads to micelle-micelle interaction that in turn leads to formation of bottom micellar phase and top aqueous phase.

Ultrasonication is applied in various fields ranging from petrochemical, mining and metallurgical fields towards extraction of suspended solids from the feed. Apart, ultrasonication is predominantly used in the field of food, pharmaceutical and cosmetic industries towards varied applications, specifically targeting the stabilization of emulsions [18] used to increase the shelf life of the product. In biotechnology/biochemical Engineering, sonicating waves are used for cell disruption [19], especially focusing on selective release of intracellular proteins [20], sludge treatment [21–23], Enhancing transesterification reactions for the production of biofuels [24–26], enzyme extraction [27] enzyme catalyzed waste treatment process [28,29], Reduction of moisture in fruit extracts [30], Crystallization [31,32], Biosensors [33,34], ultrasonication assisted extraction of biocompounds [35–43].

Polyhydroxyalkanoate (PHA) are biopolyesters, fermentatively synthesized by microbes in the presence of excess carbon source and limited nitrogen or sulphur or oxygen or phosphorous source prevailing in the medium [44]. PHA is currently explored as a potent alternative to chemically synthesized commercial plastics and has been utilized for varied applications in various sizes and shapes [44]. Solvent extraction is employed to carry out large scale extraction of PHA from the medium, utilization of hazardous chemicals not only impart environmental threat but also result in breakage of polymeric bonds and loss of nativity of biopolymer being separated. As mechanical disruption and aqueous biphasic extraction as individual separation techniques for extraction of PHA has been discussed for their efficiency [45], a process integrated unit operation, ultrasonication assisted cloud point extraction of polyhydroxyalkanoate from *Cupriavidus necator* was developed and studied.

2. Materials and methods

2.1. Materials

Surfactants -Triton X-100 (TX100), Triton X-114 (TX114), Dioctyl sodium sulfosuccinate (AOT), cetyltrimethyl ammonium bromide (CTAB), Polymers- Polyethylene glycol (PEG) 4000, 6000 & 8000, and standard Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (12%) were purchased from Sigma Aldrich, India. Sodium sulphate (Na_2SO_4), sodium chloride (NaCl), ammonium sulphate ($(NH_4)_2SO_4$) and ammonium chloride (NH_4Cl) were purchased from CDH, India. HPLC grade acetonitrile and concentrated sulphuric acid (H_2SO_4) (98%) were purchased from Merck, India. Deionized water was used during the protocols and the experiments were conducted at 30 °C, unless and otherwise stated.

Sonics vibra-cell VCX 130, USA was used for ultrasonication studies; the sonicating unit contains a 6 × 113 mm (diameter × height) probe tip made up of autoclavable titanium alloy with a net power output of 130 W, and frequency of 20 kHz. LABINDIA analytical UV 3000 + UV/Vis spectrophotometer, India was used for the UV spectral analysis and Shimadzu HPLC LCMS 2020, Japan was used for chromatographic analysis. The whole set up is places inside a sound proof wooden box as a protective measure to prevent harmful effect of sonic waves and to maintain isothermal conditions within the unit.

Cupriavidus necator DSM 428 procured from MTCC, IMTECH Chandigarh, India was used for the production of PHA by submerged batch fermentation under limited ammonium sulphate as nitrogen source and abundant crude glycerol obtained from biodiesel industry was used as carbon source in the medium. PHA accumulation in the biomass was estimated by subjecting a known volume of fermentation broth to low speed homogenization for 10 min and the sample was subjected to modified crotonic acid assay protocol [46]. The fermentation broth after incubation was used as such for the extraction protocol.

2.2. Extraction protocol

A total volume of 10 ml of the feed mixture containing fermentation broth and surfactant solution was taken in pre-weighed, graduated centrifuge tube. Equal volume (3 ml) of fermentation broth contained 42.35 mg/mL of biomass which encloses 35.97 mg/mL of PHA was maintained in all the experiments, while varied volume of surfactant solution added to the feed based on its concentration studied. After addition of required volume of fermentation broth and surfactant solutions, the remaining volume was adjusted to 10 ml using deionized water. Effect of TX100 as an individual surfactant on low frequency sonic wave assisted CPE of PHA from fermentation broth was studied by varying the concentration between 1–10 weight % (wt%). The tubes were subjected to ultrasonication at an initial frequency of 8 kHz for 3 min with a pulse interval of 2 s and were observed for initiation of cloudiness and two phase formation. After two phase formation, the tubes were centrifuged at 5000 rpm for 10 min and the obtained pellet was oven dried at 100 °C for one hour. Tubes were cooled down to room temperature and their respective post weights were recorded, difference in pre-weight and post-weight of the tubes denote biomass cell dry weight (CDW). Pellet obtained was re-suspended in chloroform and the same was subjected for crotonic acid assay according to modified protocol [46]. Purity % (Eq. (1)) and recovery % (Eq. (2)) of PHA were calculated.

$$\text{Purity } \% = \frac{\text{PHA extracted}}{\text{Biomass(CDW)}} \times 100 \quad (1)$$

$$\text{Recovery } \% = \frac{\text{PHA extracted}}{\text{Initial PHA}} \times 100 \quad (2)$$

All the experiments were performed in triplicate and the average