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

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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

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[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

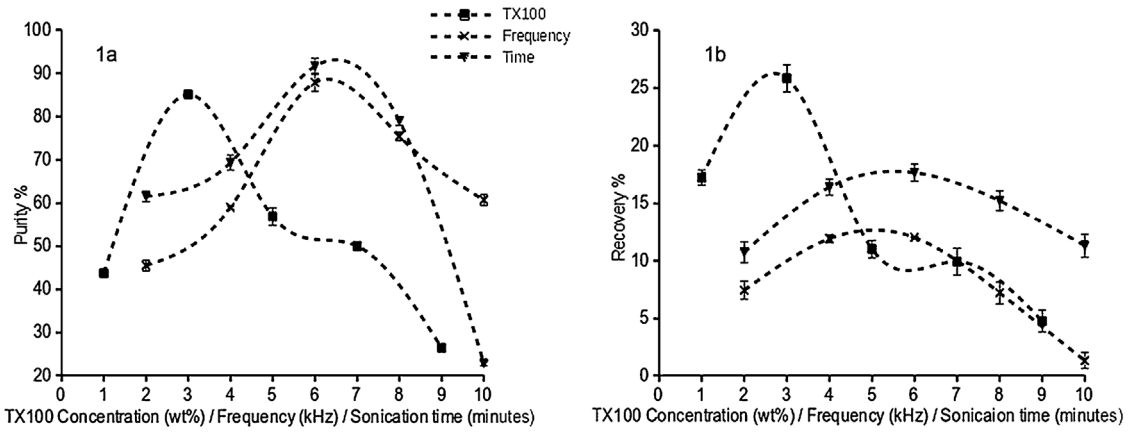

Fig. 1. Effect of TX100 concentration, sonication frequency and sonication time on purity % (a) & recovery % of PHA (b).

value of purity % and recovery % was considered for the analysis and the graphs were plotted by incorporating the standard deviation of each experiment. A maximum standard deviation of 1.8 for purity % and 1.44 for recovery % were noticed in the present work.

TX100 concentration, which gave maximum purity % was fixed for the further extraction studies; effect of sonication frequency on purity % and recovery % was studied by varying the frequency between 2–10 kHz for 3 min and with a pulse interval of 2 s. TX100 concentration and frequency which gave maximum purity of PHA were fixed to study the effect of sonication time between 2–10 min. Mixture of surfactants comprising nonionic surfactant-TX100 (concentration as taken above) was added to other surfactants, nonionic − TX114/anionic − AOT/cationic − CTAB and their effect of varying concentrations (1–5 wt%) was studied. Effect of additives were studied by considering different electrolytes from hofmeister series (Sodium chloride, sodium sulphate, ammonium chloride and ammonium sulphate) and their varied concentrations of 0.1–1 M and Polymer − PEG 4000, 6000 and 8000 (0.1–1 wt%) in the presence of different surfactant mixtures. Overall extraction efficiencies were compared and the system with maximum purity was subjected to chromatographic analysis.

2.3. Chromatographic analysis of sonic wave assisted cloud point extracted PHA

Liquid chromatography Mass spectrometry (LCMS) analysis was performed for the system with highest purity % of PHA obtained via low frequency sonic wave assisted CPE. 20 µl of the same was injected to a reverse phase column, capcell pak C18 MG II type maintained at 40 °C. Mobile phase comprising acetonitrile:water at a ratio of 70:30 (vol:vol) was passed through the column at a flow rate of 1 ml/min. Chromatographic peaks were obtained at 235 nm and the fractionated sample corresponding to the retention time of PHA was passed on to ESI–MS, as programmed. Nitrogen with a flow rate of 1.5 l/min and 15 l/min was used as nebulizing gas and drying gas respectively. MS unit heat block was maintained at 200 °C, while ion interface temperature was maintained at 350 °C. The raw data obtained was processed using LC–MS software and was analyzed for chromatogram and mass peaks. Similar run was also performed for standard PHBV dissolved in chloroform. Chromatograms of standard PHBV and PHA obtained via the current process were compared and studied for their retention time.

3. Results and discussion

Known volume of crude fermentation broth was utilized to determine the amount of PHA present in the broth by performing modified crotonic acid assay protocol [46] Fermentation broth contained 42.35 mg/mL of biomass which encloses 35.97 mg/mL of PHA that accounts to 85% of PHA accumulation in the microbial cells. PHA extracted was observed to be amorphous and viscous in nature even when exposed to atmosphere, viscous nature of the PHA material perpetuated when dissolved in water.

Cavitation as a result of introducing sonic waves, leads to increased permeability of membranes and its thinning, physical parameters of the microbe, growth status and presence of outer cell membrane (lipopolysaccharide and protein layer in gram negative bacteria) determines the efficiency of sonication [47]. Low frequency ultrasound offers increased sonochemical destruction of living cells, increased extraction of cellular content. Presence of surfactant in the feed mixture and introduction of sonic waves causes enhanced penetration of surfactant monomers into the lipopolysaccharide layer of gram negative bacteria and its dissolution, on further presence of sonic waves, cell permeability or cell wall disruption is possible and improvised mass transfer of cellular contents into the surrounding medium [30]. Ultrasonic probe tip is chiefly employed for homogenization process owing to their increased shear force and lower radical formation.

3.1. Effect of individual surfactant − TX100

Initially, sonication assisted CPE was performed at 8 kHz of sonication frequency operated for 3 min with 2 s pulse interval. Effect of individual surfactant was studied by considering TX100 and its varying concentrations between 1–10 wt%. Fig. 1a & b represents the purity % and recovery % of PHA obtained respectively. It is inferred from the graph that purity % and recovery % was found to increase with increasing TX100 concentration and at higher concentrations of TX100 both purity and recovery of PHA declined. Maximum purity % of 84.7 with a PHA recovery % of 26.1 was obtained in the presence of 3 wt% of TX100.

Earlier reports suggest that the addition of surfactant, they form a thin film along the bubble surface creating a no-slip boundary condition, increases microstreaming of the bubble until bubble implosion. Increase in microstreaming capacity, enhances the mass transfer across the bubble surface. During continuous compression and rarefaction of bubbles, surfactant density in a bubble increases during compression which inclines the mass transfer resistance, expansion of bubble leads to decrease in surfactant density and mass transfer resistance [48]. Higher concentrations of surfactants have been found to increase the growth rate of a bubble as a result of early onset of surface oscillations that creates microstreaming in its vicinity.

During cavitation, surfactants are adsorbed on the bubble surface on the vapor-water interface and diminish the bubble coalescence rate which results in the formation of uniform sized bubbles stabilized by micelles [48–50]. Surfactant type (charge and chain length) determines the bubble size, with increasing concentration of surfactants bubble size was found to increase by rectified diffusion and also lead to decrease in

surface tension as stated by [51]. Hydrophobic interaction between PHA and the surfactant micelles increases with increasing surfactant concentration, which is strong enough to form micelle-PHA complexes [52] and settle down in the bottom coacervate phase and most of the hydrophilic cellular impurities are present in the top aqueous phase. However, high surfactant concentrations results in increase in HLB value of the system [53] which enhances micelle-protein interactions that leads to the reduction in purity % and recovery %. Similar results have been reported on the effect of surfactant concentration on the extraction of estrogen from human urine samples by employing Tergitol 6 in UACPE [12] and the extraction of polybrominated diphenyl ethers using TX114 [54].

3.2. Effect of sonication frequency

Effect of sonication frequency on the purity % and recovery % was studied in the presence of TX100 at a fixed concentration of 3 wt% and a sonication time of 3 min with 2 s interval, the results obtained are represented as Fig. 1a & b. From the graphs it is observed, as the frequency increased from 2 to 6 kHz; PHA purity was found to increase and reached a maximum of 88.23% at 6 kHz. However, with further increase in sonication frequency, purity % was found to decline. Frequencies > 6 kHz leads to generation of increased number of bubbles in a shorter span around the sonic wave source that are not good enough to form void and collapse. Earlier reports suggest that the viscosity of micelle phase increases with increasing frequency. Long wavelengths are propagated at low frequencies creating large sized microbubbles while smaller microbubbles are created at high frequencies as a result of shorter wavelength. Presence of large sized microbubbles lead to higher shear compared to that of smaller sized microbubbles created at high frequencies [17]. Thus, low frequencies result in adiabatic implode which can disrupt the microbial cell and aid in the release of cellular components into the surrounding medium. Presence of TX100 in the solution reduces the surface tension and intense the effect of cavitation on the microbial cell surface for rupture and cell leakage [55]. From Fig. 1b, it is to be noted that increased frequency of sonic waves lowered the recovery of PHA from the medium. It was also observed that the volume of coacervate phase decreases at frequencies > 6 kHz as a result of repulsion of scattered water molecules among the micelles and leading to micelle-micelle interaction. These stronger micellar interactions attract cellular impurities thereby declining the PHA recovery.

3.3. Effect of sonication time

TX100 concentration of 3 wt% and sonication frequency of 6 kHz was fixed to study the effect of sonication time which was varied between 2–10 min. From Fig. 1a, it is inferred that the purity % was maximum at shorter sonication time (6 min) and with further increase in sonication time, purity of PHA obtained was found to decrease. Similar results were observed for the effect of sonication time on recovery of PHA from the fermentation broth. Exposure of sonic waves for longer durations ensures increased cell rupture and leakage [56,57]. However the heat generated leads to the denaturation of protein and breakage of PHA chain length. These denatured proteins further precipitate out in to the micellar phase which leads to lower the purity and yield of PHA.

3.4. Effect of fermentation broth pH

When the broth pH was altered from acidity to basicity, the purity % was found to increase initially and with further increase towards basic pH, purity and recovery of PHA was found to decline as represented in Fig. 2. The maximum PHA purity and recovery was achieved at a pH value of 5. Further increase in the pH to basicity leas to the reduction of purity due to the precipitation of the cellular proteins. However, the PHA molecules were not extracted to the micellar phase due to the reduction in the attractive force and the effect of pH on bubble

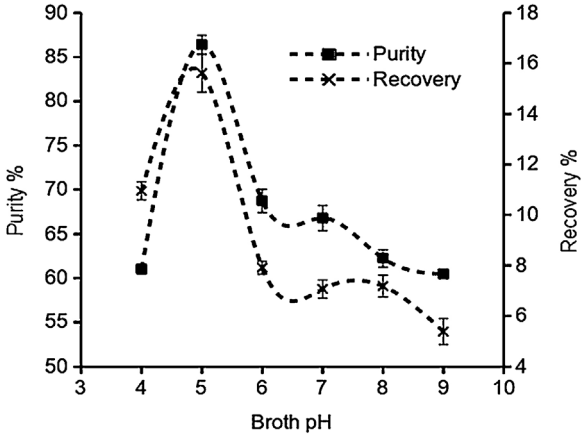

Fig. 2. Effect of fermentation broth pH on purity % and recovery %.

parameters is negligible unlike its effect on cellular proteins at higher pH. Though, nonionic surfactant based micelles exert hydrophobic bonding with solutes, acidic pH causes protonation of amino acids thereby increasing the hydrophilicity of the proteins, which causes a repulsion of the same from the coacervate phase to the top aqueous phase. However, the increase in the pH towards basicity leads to the exposure of hydrophobic domains of the protein and consequently their interaction with the micelles leads to the precipitation of proteins in the micellar phase. At higher pH, the cellular proteins are involved in stronger hydrophobic interaction with the micelles than the solute PHA. Further the membrane proteins are mostly negatively charged while few others are positively charged owing to the change in pH around the pI of the proteins. Thus the change in hydrophobicity of cellular protein with base pH ultimately reduces the purity and recovery of PHA due to the precipitation of proteins in to the micellar phase.

3.5. Effect of mixed surfactants

Though, PHA purity was found to increase, recovery % was considerably low at different concentration of TX100 due to the lower interactive forces between the micelles and solute PHA. However, the hydrophobic and ionic interactive forces may be enhanced by incorporating mixed surfactants and additives (salts and polymers), respectively for the micelle formation. Hence, different combinations of mixed surfactants (TX100 + AOT, TX100 + CTAB and TX100 + TX114) were studied to enhance the recovery. Effect of mixed surfactants was studied by adding varying wt% of AOT/CTAB/TX114 with TX100 which was fixed at a concentration of 3 wt%. Fig. 3 indicates that purity and recovery % was found to decrease with increasing concentrations of AOT and CTAB; while the purity of PHA was found to increase initially with increasing concentrations of TX114 and further increase in TX114 concentration lead to a steady decline in the PHA purity. Owing to neutral charged head groups and hydrophobicity exerted by TX100 tails, its interaction with the other surfactants in the mixture is purely based on hydrophobic interactions. Thus, TX100 + TX114 surfactant mixture imparts strong hydrophobic interactions with that of PHA; while, the presence of charged surfactant head group (AOT and CTAB) in the mixture TX100 + AOT and TX100 + CTAB imparts electrostatic interaction between micelles and the solute molecules within the system [58]. Additionally, HLB value of surfactant mixtures are different than that of HLB value of individual TX100 concentration, which is as a result of mixing different surfactants at various concentrations [53]. As a result increasing concentration of TX114 in the surfactant mixture of TX100 + TX114 resulted in a maximum purity of 89.98%. Even though the purity was found to improve due to the existence of electrostatic interaction between the solute and surfactant head groups present in the micelle by adding mixed surfactant, it fails to improve the recovery of the PHA. The effect of

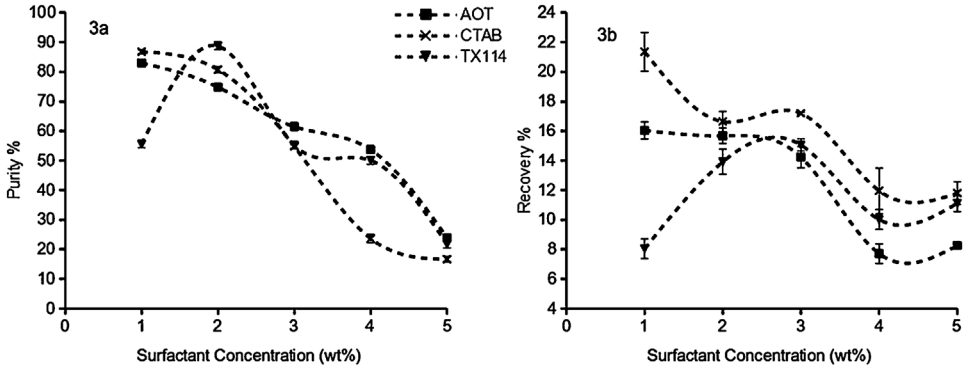

Fig. 3. Effect of mixed surfactant concentrations in the presence of 3 wt % TX100 on purity % and recovery % of PHA.

mixed surfactant results indicated that the stronger electrostatic and hydrophobic forces are necessary to attach the PHA molecule on the micelle surface. Hence, the further experiments are designed to improve the electrostatic force by considering the electrolyte salts and hydrophobic force by mixing the polymers in the system as additives.

3.6. Effect of additives

Effect of additives were studied by adding different electrolytes (Na_2SO_4, NaCl, $(NH_4)_2SO_4$, NH_4Cl) and polymer of different molecular weights (PEG 4000, PEG 6000, PEG 8000) at varying concentrations. The mixed surfactant systems (TX100 + AOT, TX100 + CTAB, TX100 + TX114) and their respective concentrations which derived maximum PHA purity was used to study the effect of additives. Fig. 4a, represents that maximum purity was obtained in the presence of sodium sulphate for TX100 (3 wt%) + TX114 (2 wt%), while variation in recovery of PHA is represented as Fig. 4b. Maximum PHA purity of 94.28% was obtained with the addition of 0.1 M sodium sulphate in the presence of TX100 (3 wt%) + TX114 (2 wt%) surfactant mixture. In case of TX100 + AOT mixture, increasing concentration of sodium sulphate from 0.1 to 1 M lead to increase in the purity of PHA; while PHA purity was found to decrease with increasing sodium sulphate concentration for TX100 + CTAB and TX100 + TX114 systems. Presence of electrolytes, enhance the cavitation effect on hydrophobic surfaces compared to hydrophilic surfaces [58–60], as a result of which increased cell rupture and leakage takes place. Stronger cations induce the surface tension and there by decline the extraction efficiency during ultrasonication assisted extraction process while weaker cations decreases the surface tension of the system and induce the extraction of solute into the coacervate phase. However, most of the salts reduce the bubble coalescence rate while a few have no effect [59,60]. Thus a combined effect of surfactants, electrolytes and ultrasonication affect the purification of PHA from the broth.

Addition of salts to a surfactant solution shields the electrical layer formed by the charged surfactant head groups, reduces the surface oscillation of a bubble. This effect reduces the microstreaming of the bubbles and as a result less shear force acts upon the microbial cell surface and its cell breakage. In the presence of AOT (negatively charged surfactant) within TX100 + AOT mixture, sodium ions interact with the surfactant head group forming Gouy Chapman layer [61], while free sulphate interacts with the negative sites on the protein and repels them into the top aqueous phase. However, in the case of CTAB (positively charged) within TX100 + CTAB mixture, surfactant head group interacts with sulphate and as a result negatively charged cellular impurities settle down over the coacervate phase. The interaction between TX100 + TX114 micelles and sodium sulphate is based on hydrophobicity, where charged ion species involves in hydrogen bonding with surfactant head groups and tails. Increasing concentration of salt leads to precipitation of proteins and cellular impurities which settle over coacervate phase that results in lower purity and recovery of PHA. Larger ionic radius of ammonium ions enables it's interaction with the surfactant as well as cellular impurities compared to smaller ionic radius of sodium [62,63]. As a result, presence of ammonium and combination of anionic species lead to reduction in the purity and recovery of PHA.

Results obtained by studying the effect of polymer (PEG) molecular weight and its varying concentrations are represented as Fig. 5a & b, respectively. Addition of 1 wt% of PEG 8000 resulted in maximum purity, while maximum recovery. Introduction of polymer to micelle system leads to micelle-polymer interaction via hydrophobic interaction that in turn alters partitioning of PHA into the coacervate phase. Higher concentrations and molecular weights of PEG in the micelle system impart a strong hydrophobic interactions with hydrophobic PHA molecules and repulse the protein into the top aqueous phase thereby increasing the purity of PHA. Presence of polymer reduces the surface tension of the solution that enhances cavitation and generation of even sized bubbles that implode and improvises cell disruption and leakage. Higher concentrations and molecular weights of PEG in the micelle system impart a strong hydrophobic interactions with hydrophobic PHA molecules and repulse the protein into the top aqueous

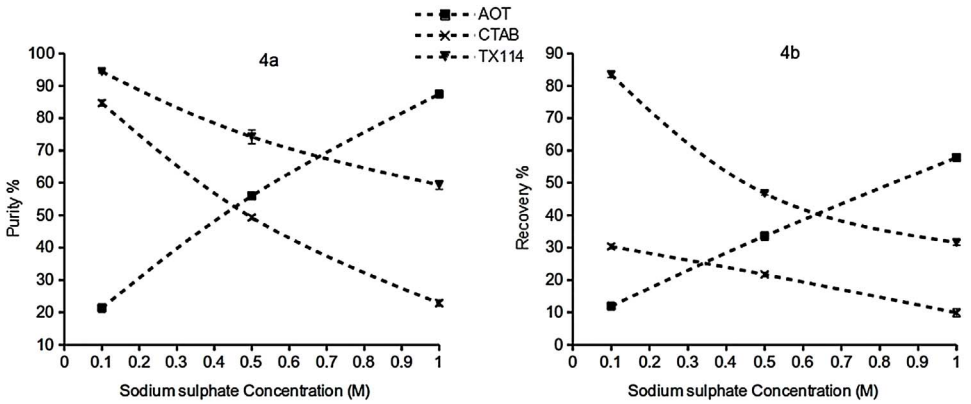

Fig. 4. Effect of sodium sulphate and its varying concentrations on purity % (a) and recovery of PHA (b).

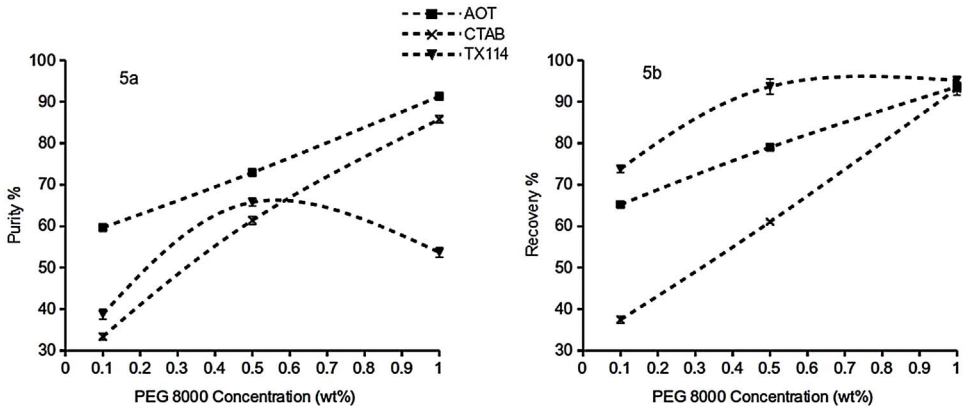

Fig. 5. Effect of PEG 8000 and its varying concentrations on purity % (a) and recovery % PHA (b).

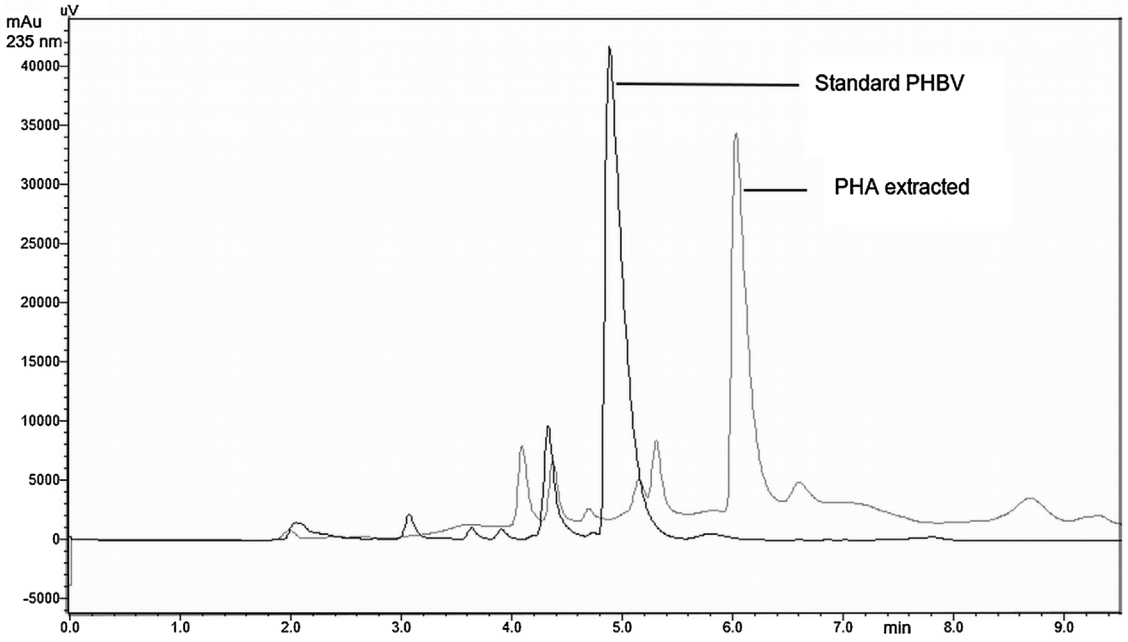

Fig. 6. Comparative chromatogram of Standard PHBV and UACPE sample with highest purity of PHA.

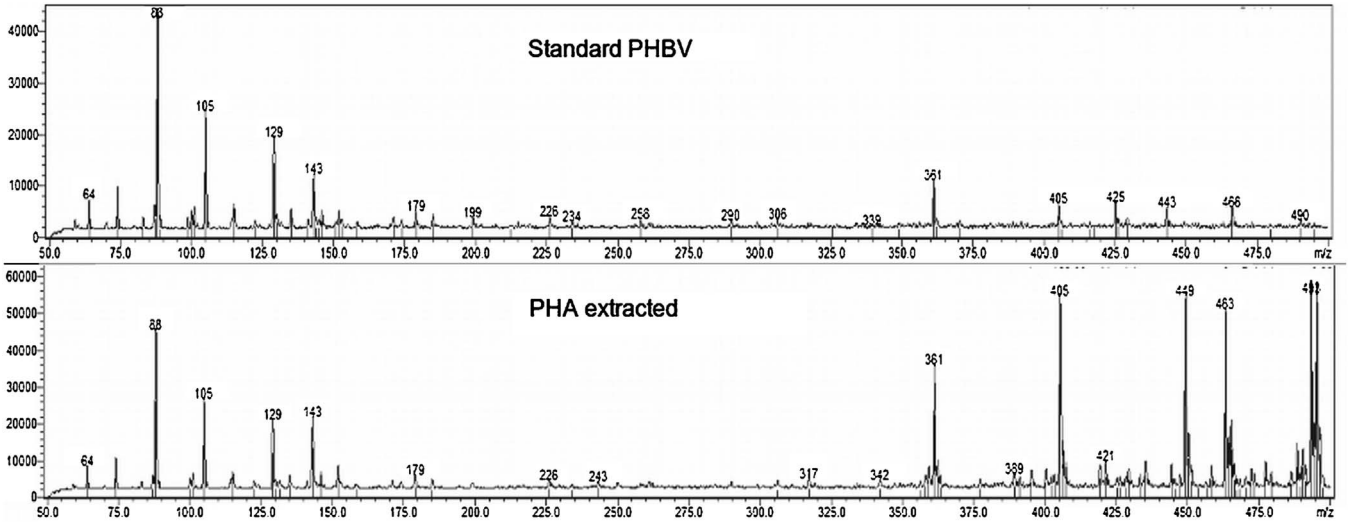

Fig. 7. Mass spectrum – positive *m/z* peaks of standard PHBV (a) and PHA extracted with maximum purity (b).

phase thereby increasing the purity of PHA. However, at lower concentrations and molecular weights of PEG the hydrophobic interaction is relatively low with proteins [64,65] Conversely, the presence of free PEG molecules present in the system at higher PEG concentration competes with PHA in the formation of stable polymer- surfactant complexes, explained by necklace bead model [52] that settle down as coacervate phase and reduce the recovery of PHA extracted.

3.7. Chromatographic analysis of purified sample

LCMS analysis of the sample which gave maximum purity of PHA (3 wt% TX100 + 3 wt% TX114, broth pH − 5, in the presence of 0.1 M sodium sulphate performed at 6 kHz of sonication frequency for 6 min) was performed in a reverse phase HPLC column. 20 µl of the sample was injected to the column run with the appropriate conditions as mentioned above and the obtained chromatograms for standard PHBV and sonication assisted CPE extracted PHA were compared as shown in Fig. 6. It is inferred from the peaks obtained, that the retention time of the standard PHBV peak with the highest intensity was around ~4.8 min while that of the extracted PHA sample with maximum purity was about ~6.2 min.

Positive *m/z* peaks obtained during mass spectral analysis of standard PHBV and PHA chromatographic peaks are represented in supplementary material. Different *m/z* peaks represent the oligomers of PHBV & PHA molecule that are obtained as a result of partial pyrolysis during ionization within the MS unit. Pyrolysis of polymer samples results in oligomer formation that are random in their structure and so the base peak shifts according to the ions that are generated. Occurrence of increasing *m/z* peaks represents the formation of monomer to oligomeric units (dimer, trimer, tetramer and so on). It is deduced from Fig. 7, that most of the peaks fall on the same *m/z* value; however, pyrolysis resulted in highest base peak at 88 *m/z* for standard PHBV while it was 495 *m/z* for extracted PHA sample. The *m/z* peaks < 400 *m/z* in case of extracted PHA denote that the molecular weight of PHA extracted is higher than that of standard PHBV.

4. Conclusion

Process integration of ultrasonication with cloud point extraction lead to development of an adiabatic micellar extraction system which reduces the operational cost to a great extent and also inflates the extraction efficiency. Considering the advantages of UACPE towards separation of metal ions [66], it can actively be extended towards separation of any hydrophobic solutes from biological feed as described in this article. UACPE apart from enhancing specificity based extraction, it also retains the nativity of the solute, which is vital in the separation of any bioproduct. PHA from *Cupriavidus necator* was purified by low frequency sonic waves assisted CPE in the presence of mixed surfactants with an overall purity of 94.34%, higher than purity of 92.49% obtained by heat induced cloud point extraction of PHA using nonionic surfactants [67] and the current sonication assisted process design enhanced the usage of ionic surfactants, whose cloud point temperature (> 100 °C) is usually difficult to maintain. The effect of sonic waves on biopolymer are screened by the presence of micelles and hence the nativity of the polymer is assumed to be unaltered which could be confirmed with further physiochemical and application based studies. Sonication assisted CPE of biopolymer from the source is first of its kind research, setting a standard towards separation of any such polymer from the source without the necessity to perform complex separation process/equipment.

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Conflict of interest

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