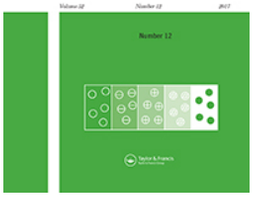
Taylor & Francis
Taylor & Francis Group

SEPARATION SCIENCE AND TECHNOLOGY

Separation Science and Technology



ISSN: 0149-6395 (Print) 1520-5754 (Online) Journal homepage: http://www.tandfonline.com/loi/lsst20

# Nonionic surfactants induced cloud point extraction of Polyhydroxyalkanoate from Cupriavidus necator

Sivananth Murugesan & Regupathi Iyyasamy

**To cite this article:** Sivananth Murugesan & Regupathi Iyyasamy (2017) Nonionic surfactants induced cloud point extraction of Polyhydroxyalkanoate from Cupriavidus necator, Separation Science and Technology, 52:12, 1929-1937, DOI: 10.1080/01496395.2017.1307227

**To link to this article:** http://dx.doi.org/10.1080/01496395.2017.1307227

Accepted author version posted online: 20 Mar 2017.
Published online: 20 Mar 2017.

SEPARATION SCIENCE AND TECHNOLOGY
2017, VOL. 52, NO. 12, 1929–1937
https://doi.org/10.1080/01496395.2017.1307227

Taylor & Francis
Taylor & Francis Group



# Nonionic surfactants induced cloud point extraction of Polyhydroxyalkanoate from *Cupriavidus necator*

Sivananth Murugesan and Regupathi Iyyasamy

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

**ABSTRACT**

Polyhydroxyalkanoate synthesized by Cupriavidus necator DSM 428 was purified from the crude fermentation broth as such by performing nonionic surfactants (Triton X100, Triton X114 & Tergitol 6) induced cloud point extraction. Polyhydroxyalkanoate was extracted into the micelle-rich bottom phase (coacervate phase), while most of the cellular impurities partitioned into the aqueous phase. Cloud point temperatures and the extraction efficiency of different cloud point systems were studied at different pH value and in the presence of additives. Maximum extraction of biopolymer was achieved (recovery of 84.4%) with a purity of 92.49% at 3 pH with the addition of 0.1 M ammonium chloride in the mixed surfactant system at a reduced cloud point temperature of 33°C.

**ARTICLE HISTORY**
Received 12 June 2016
Accepted 13 March 2017

**KEYWORDS**
Cloud point extraction; *Cupriavidus necator*; nonionic surfactant; polyhydroxyalkanoate (PHA)



## Introduction

Polyhydroxyalkanoates (PHA) are biopolyesters that possess physical properties similar to that of commercial plastics and are synthesized by a variety of microbes under limited nutrient conditions with surplus carbon source.[1] Over the current decade, there has been a growing market for PHA in the field of packaging,[2] medical and pharmaceutical,[3] and in the fuel industry.[4] Several strategies have been employed to synthesize different classes of PHA from complex to simple carbon sources, especially waste that could be utilized as carbon source[5] by various microbes ranging from wild to genetically modified strains.[6] Downstream processing of PHA is of current research interest owing to the difficulty associated with the separation of amorphous plastic from the fermentation broth. In general, purification of PHA is a crucial step as the process involves release of PHA into the medium during cell lysis and which is then processed further for analysis and usage. Cell disruption techniques like sonication, dissolved air flotation, gamma irradiation and sodium hypochlorite disruption are slow and require several cycles, while biological treatment involves large volumes of enzymes for cell disruption.[7] Although LLE techniques such as solvent extraction, super critical fluid extraction and aqueous two phase extraction have been experimented on PHA extraction, solvent extraction involving highly toxic and volatile solvents has been commercially employed for the purification of PHA. Utilization of hazardous solvents in bulk volumes leads to an increase in the overall purification cost and loss of nativity of the polymer due to the reduction in molar mass caused by the breakage of polymer granule.[6] Development of separation process to extract hydrophobic compounds from the complex mixtures such as fermentation broth has been of wide research interest as large scale operations involve higher operation and maintenance cost, while most of the process are difficult to scale-up to industrial level.[8] With the invention of surfactants and their varied types, aqueous two phase micellar separation has been found to be an effective extraction process for hydrophobic solutes. Continuous addition of surfactant to a solvent increases the monomeric surfactant concentration, and micelles are found to form beyond certain surfactant concentration. The lowest surfactant concentration required to form micelle is referred to as CMC.[9] With inclining temperature, the solubility of the micelles decreases and the single aqueous surfactant solution turn into two coexisting isotropic phases (noted as cloud point temperature), namely micelle-rich lower phase (coacervate phase) and an upper aqueous phase.[10] Most of the hydrophobic solutes get partitioned in the surfactant-rich bottom phase, while the hydrophilic solutes partition into the aqueous phase.[11] Cloud point extraction (CPE) has been extensively researched toward the extraction of metal agents like zinc, silver, cobalt, mercury, manganese, copper, chromium and lanthanides; organic pollutants like humic acid, fulvic acid, phenols and phenylamines; dyes like yellow dye, malachite green, crystal violet and proteins

**CONTACT** Regupathi Iyyaswami regupathi@nitk.ac.in Department of Chemical Engineering, National Institute of Technology Karnataka, Surathkal, Mangalore 575025, India.

including bacteriorhodpsin, cytochrome c and other hydrophobic membrane proteins from their respective feed mixtures.[12] Nonionic surfactants such as Triton X 100 and Triton X 114 have been widely used for the separation of compounds owing to their mild and non-denaturing nature toward the solutes; apart, ionic surfactants have also been reported in CPE process. In case of nonionic surfactants, the extraction of solute molecules from the feed involves stronger hydrophobic interaction with the surfactant tail group in a micelle. While electrostatic interaction exists between the solute and the charged ionic surfactant heads during CPE. Such interactions can be altered by varying the surfactant concentration, addition of co-surfactant, Ionic strength and pH value.[12–14] Being advantageous like biodegradable and eco-friendly, minimum amount of surfactant required to form micelles, hydrophobicity based extraction, higher efficiency on extraction, scale-up opportunities, the possibility of recycling the surfactant during continuous extraction process over other separation process and most importantly its ability to retain the nativity of the molecules during extraction, the present research article focuses on nonionic surfactants induced CPE of PHA in its native form from the biomass present in the fermentation broth.

## Materials and methods

Surfactants (TX100, TX114 and TMN6), Polymers (PEG 4000, 6000 & 8000) and Standard PHBV (12%) were purchased from Sigma Aldrich, India. Sodium sulfate, sodium chloride, ammonium sulfate and ammonium chloride were purchased from CDH, India. HPLC grade acetonitrile, HPLC grade TFA and concentrated sulfuric acid ($H_2SO_4$) (98%) were purchased from Merck, India. Deionized water was used during the protocols, and the experiments were conducted at room temperature unless and otherwise stated. *Cupriavidus necator* DSM 428 procured from MTCC, IMTECH Chandigarh, India was used in the submerged batch fermentative production of PHA under limited nutrient condition. Ammonium sulfate as limited nitrogen source and abundant crude glycerol as carbon source were used in the medium. About 85% of PHA accumulation in biomass was calculated by performing crotonic acid assay as described in literature.[15] Fermentation broth after incubation was used as such for the CPE protocol. LABINDIA analytical UV 3000 + UV/Vis spectrophotometer and Shimadzu HPLC LC 20 A series were used for the UV spectral analysis, and Shimadzu LC-MS 2020 was used for chromatography and Mass spectrometry analysis. Effect of individual surfactant concentration on the extraction of PHA from the fermentation broth was studied by considering nonionic surfactants TX100, TX114 and TMN6 at varying concentrations of 1–10 wt %. Equal volumes of broth and desired concentration of surfactant (wt %) were added to individual dry pre-weighed tubes. The tubes were subjected to temperature change in a temperature controlled water bath to form two phases. The phase formation was examined visually for the onset of cloud point and the clear separation of two distinct phases. Later, the tubes were withdrawn and were allowed to cool down to room temperature. Pellets were obtained by centrifuging the mixture at 5000 rpm for 10 minutes and dried in a hot air oven for 1 hour at 100°C. The tubes were allowed to cool down to room temperature, and the post-weight of the tubes was recorded. The difference between post-weight and pre-weight of the tubes containing pellet is noted down as cell dry mass (CDM). Further, the obtained pellets were subjected to chloroform solubilization, and required amount of the chloroform dissolved sample was used to estimate the PHA content by performing crotonic acid assay.[15] The absorbance recorded at 235 nm in the UV/Vis spectrophotometer was used to calculate the PHA amount present in the sample using the calibration curve developed at different concentrations of standard PHBV. The purity % (Eq. (1)) and recovery % (Eq. (2)) of PHA were estimated.

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

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

Effect of surfactant mixtures -TX100 + TX114, TX114 + TMN6, TX100 + TMN6 on the cloud point extraction of PHA from the biomass was studied by considering different wt % of surfactant mixtures (1%, 5%, and 7%). The Mixed surfactant system which gave maximum purity % was considered to study the effect of fermentation broth pH value (2–9), and the broth pH in the presence of mixed surfactant system which gave maximum purity of PHA was considered for further purification studies. Effect of additives was studied by considering polymer- PEG 4000, 6000 and 8000 with the concentrations of 0.1, 0.5 and 1 wt %, while effect of electrolytes was studied by considering salts from the Hofmeister series like ammonium sulfate, ammonium chloride, sodium sulfate and sodium chloride with a concentration range of 0.1–1 M.

### *Chromatographic analysis of cloud point extracted PHA*

Chromatographic analysis was performed in HPLC (Shimadzu LC 20 A) unit fitted with Rezex ROA organic acid H+ (8%) column from Phenomenex, USA. About 20 µl of the methanolysis PHA samples from individual CPE optimization step which gave

maximum purity of PHA was injected to the column separately. About 0.014 N $H_2SO_4$ was used as the mobile phase, while column oven temperature of 30°C and PDA temperature of 40°C were maintained. Chromatograms obtained at 235 nm were compared to analyze the retention time of the peaks and their respective peak intensities.

LC-MS analysis of standard PHBV solubilized in chloroform was performed by running 20 µl of sample in a RP-column capcell pak C18 MG II type fitted in Shimadzu HPLC LC-MS 2020. Column oven was maintained at 30°C, while mobile phase consisting of HPLC grade acetonitrile and water at 50:50 (vol: vol) was used in an isocratic mode at a flow rate of 1 mL/min. Similarly, bottom micelle phase of the cloud point system that gave highest purity of PHA during the CPE as such and the chloroform derived micelle phase of the same system were analyzed at the above said operational conditions. Chromatogram at 235 nm was recorded along with the peak-specific mass scan obtained from ESI-MS. Nitrogen was used as nebulizing gas maintained at a flow rate of 1.5 L/min, and the same was used as drying gas maintained at 15 L/min. MS unit heat block temperature was maintained at 200°C, while the ion interface temperature was maintained at 350°C. Chromatograms and the negative ion m/z peaks obtained were compared.

## Results and discussion

### Effect of individual surfactants

Extraction studies were performed by considering nonionic surfactants TX100, TX114 and TMN6 due to their mild and non-denaturing characteristics on biomolecules.[16] Cloud point shift on addition of fermentation broth to the surfactant solutions was noted, and it was observed that the cloud point temperature was found to decrease with increasing concentrations of TX100 and TMN6, while it increased with increasing concentration of TX114 as shown in Fig. 1a. Similar results have been reported for increasing surfactant concentrations and cloud point temperatures.[17] On addition of surfactant to the fermentation broth, surfactant monomers solubilize the lipid bilayer of the cell wall and forms micelles, thereby leading to cell disruption and cell leakage.[18] Hydrophobic tail of the surfactants interacts with hydrophobic solutes such as PHA, hydrophobic proteins and lipid molecules, while the hydrophilic head groups remain solubilized in the surrounding water and interact with hydrophilic protein molecules suspended in the aqueous environment. As a result of stronger hydrophobic interaction between micelle and PHA molecules in the medium, a stable micelle-PHA complex is formed, as explained by necklace bead model[19] and this complex settles down as micelle-rich bottom phase (coacervate phase).

Purity and recovery % of PHA were found to increase in the order of TX100 < TMN6 < TX114 as shown in Fig. 1b. As Hydrophile Lipophile Balance number (HLB) of the surfactant solution (HLB value of TX114-12.4, TMN6-13.1 & TX100-13.5) increases with increasing surfactant concentrations, purity and recovery of PHA declined as a result of decrease in hydrophobicity of the surfactant system.[20] At increased surfactant concentrations, the excess surfactant micelles present in the solution encapsulate the proteins and other biomolecules and settle down along with the micelle-PHA complex, ultimately reducing the purity and recovery of PHA.

### Effect of mixed surfactants

Mixed surfactant systems were studied to in order to foresee their effect on cloud point temperature and extraction efficiency. As a result of mixing two different

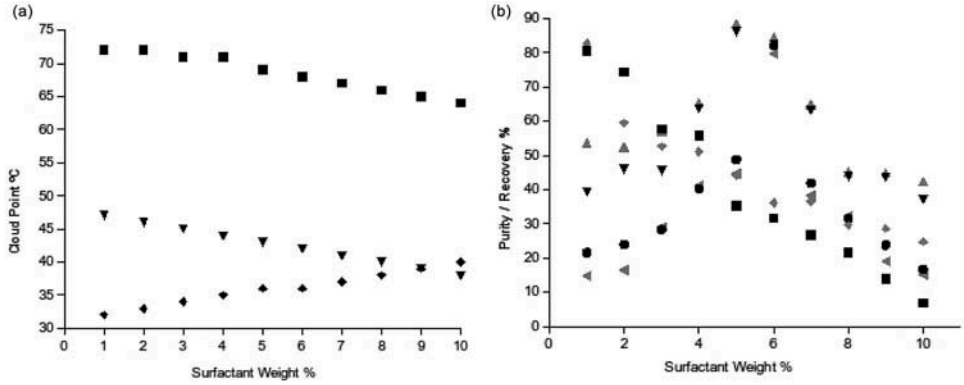



Figure 1. (a) Cloud point temperature variation of different nonionic surfactants and their varying concentrations. ■ – TX100; ♦ – TX114; ▼ – TMN6 and (b) purity and Recovery % of different nonionic surfactants with varying concentrations. Purity : ■ – TX100; ▼ – TX114; ● – TMN6; Recovery : ♦ – TX100; ▲ – TX114; ◄ – TMN6.

surfactants at varying concentrations results in variation of HLB value of the micelle system which in turn affects the cloud point temperature and the extraction efficiency,[21] It was observed that the cloud point temperature of mixtures containing TX114, that is, TX114 +TMN6 and TX114+TX100 had lower cloud points than TX100+TMN6 mixture *(data not represented)*. Extraction efficiency was found to decline with increasing HLB value of the respective mixed micelle systems in the following order TMN6+TX114>TX100 +TX114>TMN6+TX100. Higher concentrations of TMN6 and TX100 present in the mixture lead to increase in the HLB value. Thus, increasing the extraction of hydrophilic solutes and reducing the purity and recovery of PHA extracted from the fermentation broth.[20]

As seen in Fig. 2, purity % was found to increase with increasing concentrations of TX114 in the surfactant mixture TX114 + TMN6, while the yield % decreased owing to elevating hydrophilicity exerted by increase in TMN6 concentration in the mixture. TX114 + TMN6 mixture with a total weight % of 5 (TX114 –4.5%+ TMN6 –0.5%) was found to give maximum purity of 74.13%, while the cloud point was noted to be at 36°C, yield % was about 16.18. Similar effects reported in literature confirming that the increasing concentration of TX114 in the surfactant mixture of TX114 and Sodium Dodecyl Sulfate had a positive influence on the extraction efficiency of chromium.[14]

### Effect of fermentation broth pH

Effect of pH was studied to increase PHA recovery with higher purity. On varying the broth pH value, the net positive or negative charge of the cellular proteins attained based on their pI and their interaction with water molecules and micelles affects the extraction efficiency. Fermentation broth pH value was varied between 2 and 9 for the extraction, and the results obtained are graphically represented in Fig. 3. Even on varying the broth pH value, cloud point temperature of the systems remained same at 36°C as that of mixed surfactant system - 5 wt % of TX114+TMN6. As observed in the figure, the purity and recovery % was found to increase until pH value of 3 and further increase in pH value reduces the purity and recovery %. At strongly acidic pH value of 3, most of the proteins attain a net positive charge and are attracted to the aqueous phase thus resulting in a maximum purity % of 79.38. Further increase in broth pH value leads to variation in the surface charge of the proteins, which might have led to protein-protein interaction and aggregation over the coacervate phase, thus reducing the extraction efficiency.

### Effect of additives

The presence of additives has a significant role in the reduction or increment of cloud point temperature of the micellar system and has an effect on the extraction efficiency by modulating the intermolecular forces between the solute and the micelles.[12] In order to increase the extraction efficiency further and to reduce the cloud point temperature of the mixed micelle systems, additives including polymers (PEG of varying molecular weight – 4000, 6000, 8000) and electrolytes from the hofmeister series (sodium sulfate, sodium chloride, ammonium sulfate,

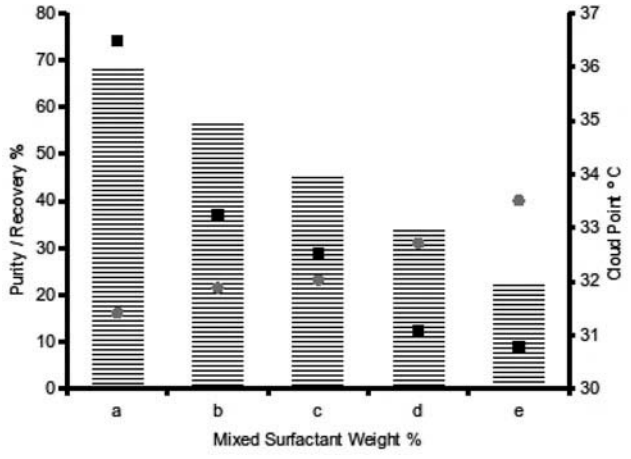



**Figure 2.** Purity/Recovery % and cloud point temperatures of Mixed surfactant mixture (TX114+TMN6) at 5 weight %; a - 4.5% + 0.5%, b – 3.75% + 1.25%, c – 2.5% + 2.5%, d – 1.25% + 3.75%, e – 0.5% + 4.5%. Horizontal line bars represent cloud point temperature °C, ■ – Purity and ● – Recovery.

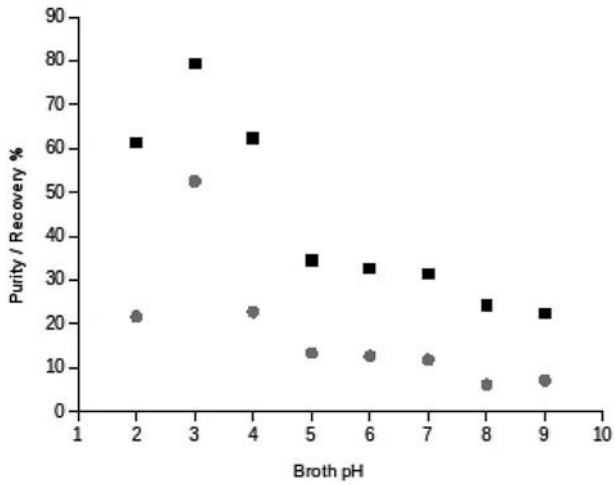



**Figure 3.** Effect of broth pH on Purity and Recovery % of PHA for TX114+TMN6 mixed micelle system. ■ – Purity and ● – Recovery.

ammonium chloride) were considered. Figure 4a depicts the effect on cloud point temperature on addition of 0.1, 0.5 and 1 wt % of different PEG molecules. PEG molecules form polymer–surfactant micelle aggregates in the presence of micelles as explained by necklace bead model[19] and results in reduction of surfactant concentration required to form micelles which is usually lower than that of CMC, known as critical aggregation concentration (CAC).[19] As a result of PEG-surfactant interaction, cloud point temperature decreases with increasing molecular weight and their increasing concentrations.[22]

Purity of PHA extracted into the micelle-rich phase was found to increase, while PHA recovery decreased with increasing molecular weight and concentrations of PEG. As PEG molecules are strongly hydrophilic compared to surfactants, they get solubilized in the aqueous layer and aid in the separation of proteins, while surfactants solubilize most of the hydrophobic solutes in the micellar phase.[23] As observed in Fig. 4b, purity and recovery % was found to increase with the addition of PEG molecules until 0.5 wt % and with further increase in concentration purity and recovery of PHA decreased, as a result of increasing hydrophobicity exerted by the PEG–surfactant micelle system.-[24,25] However, the maximum extraction efficiency was found at lower molecular weight PEG (i.e., PEG 4000) and increasing molecular weight resulted in lower purity and recovery of PHA as shown in Fig. 4b.

Four different electrolytes in the concentration range of 0.1–1 M were considered to study their effect on cloud point temperature and extraction efficiency. The ethylene oxide unit of the surfactant tails gets hydrated or dehydrated depending on the anions present in the salt, which leads to surfactant's structural deformation in a micelle and thereby increasing or decreasing the cloud point of the system.[26–28] From Fig. 5a, it is concluded that the addition of sulfate salts increased the cloud point temperature, while chloride salts decreased the cloud point temperature of the system. However, increasing concentration of sulfate declines the cloud point temperature, while increasing concentration of chloride salts resulted in increment of cloud point temperature as a result of ionic charge and hydrophobicity imparted on the micelles.[29]

Larger ionic radius of ammonium compared to that of sodium results in the reduction of cloud point temperature due to the screening of hydrogen bonds between water and the adjacent micelles. Further the exposure of hydrophobic sites of micelles leads to micelle–micelle interaction and micelle coalescence, which accelerate the formation of micellar-rich lower phase.[30,31] The presence of electrolytes in the medium imparts electrostatic interaction or repulsion with charged biomolecules, while the interaction between nonionic surfactant micelles and PHA is purely hydrophobic. Increasing salt concentrations leads to protein aggregation and precipitation that affects PHA extraction. From Fig. 5b, it is inferred that addition of 0.1 M ammonium chloride impose a maximum purity of 92.49% and recovery of 84.4%. In comparison to ammonium sulfate, ammonium chloride has a mild effect on the biomolecules and most of the cellular proteins are retained in the aqueous phase, thereby increase the purity and recovery of PHA in the micelle phase, while the presence of ammonium and sulfate salts and its increasing Ionic strength leads to stronger interaction and precipitate the proteins, which settle down over the coacervate phase and hence declining the overall extraction efficiency.

## *Chromatographic analysis of cloud point extracted PHA*

Results obtained from crotonic acid assay were verified by performing chromatographic analysis of PHA samples with maximum purity obtained during individual

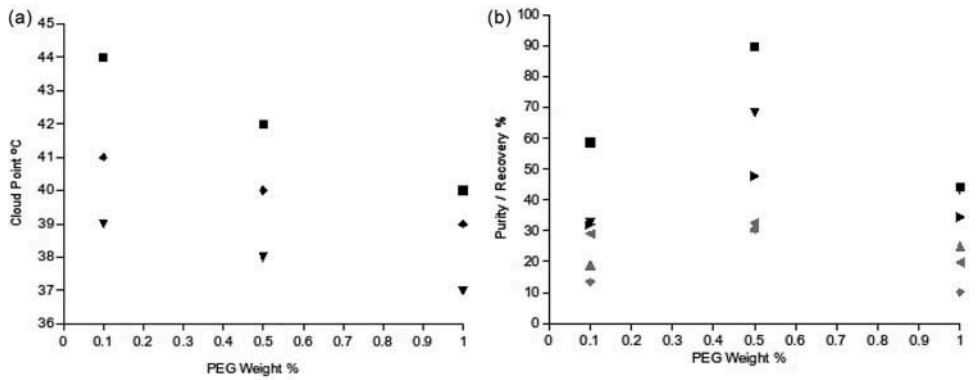



**Figure 4.** (a) Cloud point temperature variation on the addition of PEG (different molecular weights and concentrations) with TX114 +TMN6 mixed micelle system. ■ – PEG 4000, ♦ – PEG 6000, ▼ – PEG 8000 and (b) purity and Recovery % on the addition of on the addition of PEG (different molecular weights and concentrations) with TX114+TMN6 mixed micelle system . Purity : ■ – PEG 4000; ▼ – PEG 6000; ► – PEG 8000; Recovery : ♦ – PEG 4000; ▲ – PEG 6000; ◄ – PEG 8000.

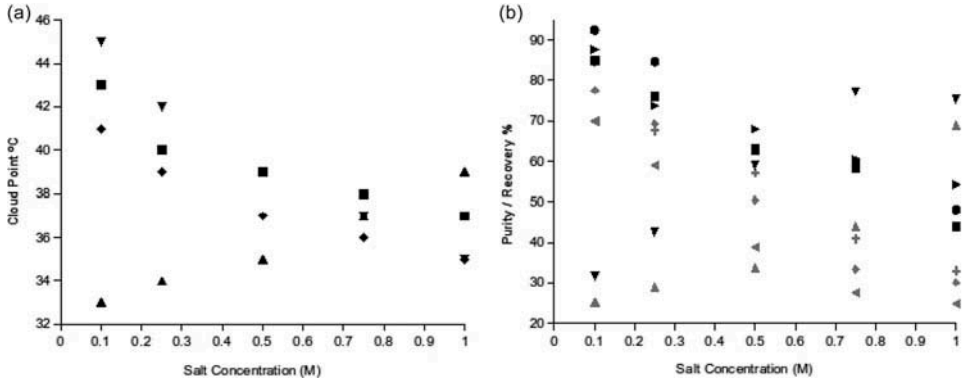



**Figure 5.** (a) Cloud point temperatures on the addition of salts at different concentrations with TX114+TMN6 mixed micelle system. ■ – sodium sulfate, ♦ – sodium chloride, ▼ – ammonium sulfate, ▲ – ammonium chloride and (b) purity and recovery % on the addition of salts at different concentrations with TX114+TMN6 mixed micelle system. Purity: ■ – sodium sulfate; ▼ – sodium chloride; ► – ammonium sulfate; ● – ammonium chloride; Recovery: ♦ – sodium sulfate; ▲ – sodium chloride; ◄ – ammonium sulfate; + – ammonium chloride.



extraction optimization steps. Samples were subjected to methanolysis[32], and the same was injected to the column maintained at the above said column conditions. It is inferred from Fig. 6a that the peak intensity of PHA samples obtained during CPE by individual surfactants increased in the order of TX100 < TMN6 < TX114, which is in agreement with the purity % of PHA obtained via crotonic acid assay.

Figure 6b depicts the effect of other process variables like mixed surfactants and their concentration, broth pH and additives (PEG and salt) and their concentration on the PHA purity. It was found that the peak intensity increased in the order TX114+TMN6 (4.5:0.5 – wt %:wt %) < pH 3 < PEG 4000 (0.5 wt %) < ammonium chloride (0.1 M). Maximum peak intensity acquired corresponds to the highest purity and recovery of PHA obtained at a cloud point temperature of 33°C for the mixed surfactant system with a total mixed surfactant concentration of 5 wt % (4.5% TX114 and 0.5% TMN6) at pH 3 and with the addition of 0.1 M ammonium chloride as additive.

To confirm the presence of PHA and analyze the effect of surfactant and chloroform on the retention of polymer nativity, the LC-MS analysis of samples was performed in LC-MS (Shimadzu LC-MS 2020) fitted with capcell pak C18 MG II column. Figure 7 indicates the retention time of standard PHBV peak, PHA from micellar phase of CPE and chloroform solubilized PHA from micellar phase of CPE. It can be observed from the figure that the standard PHBV had a retention time of ~4.1 minute, while PHA obtained through CPE had a retention time of about ~4.9 minutes. Further, the chloroform derivatized PHA from cloud point extracted micellar phase had a retention time of ~4.2 minutes. The variation in retention time of PHA from CPE and chloroform derivatized PHA reveals the fact that chloroform results in structural deformation of the polymer which is why, chloroform derivatized PHA is assumed to have low-molecular weight that elutes out at a reduced time interval than that of native PHA extracted by cloud point extraction.

Negative ion peaks obtained during mass spectral analysis of peaks corresponding to chromatographic

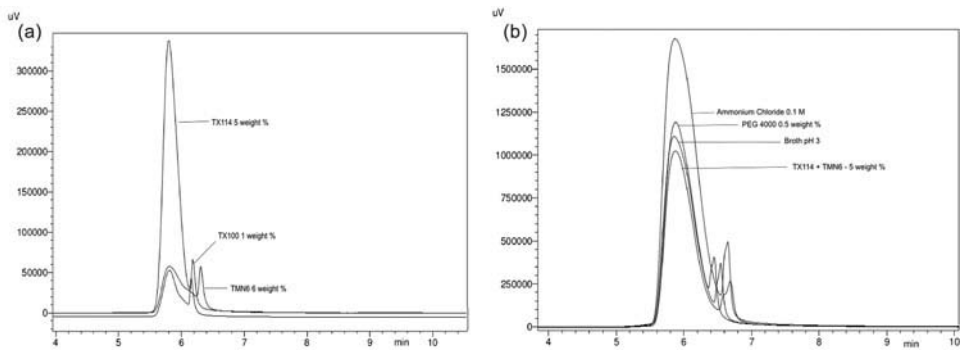



**Figure 6.** (a) Chromatographic peaks obtained at 235 nm for methanolysis samples of individual surfactants -TX114 (5%), TMN 6 (6%), TX100 (1%) and (b) chromatographic peaks obtained at 235 nm for methanolysis samples of mixed surfactants, broth pH and additives – PEG and Electrolyte.

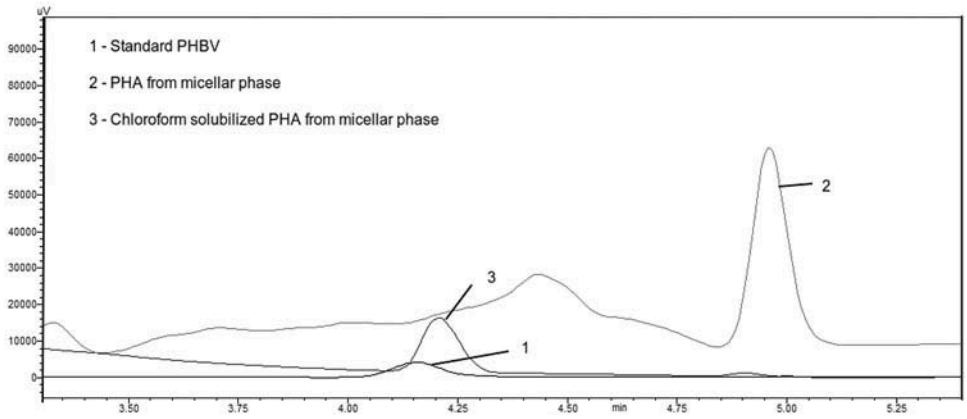



**Figure 7.** Chromatographic analysis of standard PHBV, PHA from micellar phase and chloroform solubilized PHA from micellar phase at UV-235 nm.



peaks with retention time as explained above were compared. MS detection results in random polymer fragmentation and is observed as ion peaks with respect to the molecule generated. Comparing the negative ion peaks obtained for PHA from CPE and chloroform derivatized PHA from CPE as shown in Fig. 8, it was found that a few peaks are absent in the case of negative spectrum of chloroform derivatized PHA, while most of the peaks have a low molecular m/z compared to that of native PHA from CPE.

### *Comparison of the extraction processes*

Conventional extraction processes were performed to compare the purity of PHA obtained with that of the current developed CPE process. It was observed that chloroform extraction of PHA[33] resulted in a purity of 88.76%, while sodium hypochlorite treatment[34] resulted in a purity of 90.47%, while ultrasonication of crude broth at 4 kHz for 5 minutes resulted in a purity of 78.37%. Although, comparatively higher purity was obtained for chloroform extraction, sodium hypochlorite treatment and ultrasonication processes were lower than the overall purity obtained via developed CPE process. CPE derived purity % was higher, while recovery % was comparatively lower than that of fixed temperature surfactant based extraction as reported by Yang et al.[35] Considering the facts that most of the nonionic surfactants are biodegradable, are very mild and do not possess environmental threat and reusage of surfactant during CPE process, CPE induced by nonionic surfactants is a sustainable method compared to solvent extraction methods (using hazardous solvents) which cannot be focused as application oriented separation process, such as pharmaceutical and medical applications.

## Conclusions

Industrially, chloroform extraction is employed for the separation of PHA from biomass, which not only deteriorates the PHA nativity but also demerits the use of such extracted PHA in biological applications. CPE is globally endowed for its both solid-phase and liquid-phase

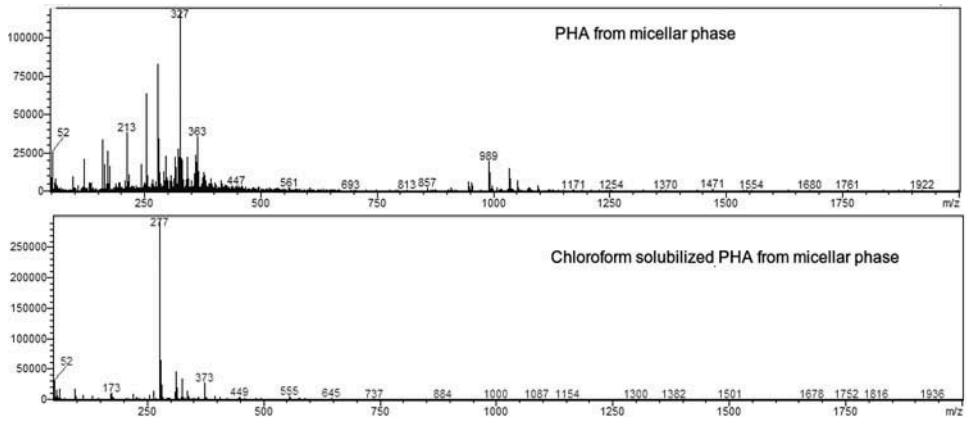



**Figure 8.** Comparison of Mass spectral data of PHA from micellar phase and chloroform solubilized PHA from micellar phase.

extraction and as a potent alternative of conventional extraction methods with its standalone advantages.[36] Though nonionic surfactants have been widely documented for its application toward CPE of membrane proteins and other biomolecules, there are no previous articles reported in the research domain to separate PHA from fermentation broth by performing CPE. The present article demonstrates the potential of nonionic surfactant induced CPE of PHA and obtained a recovery of 84.4% with 92.49% purity. Thus, nonionic surfactant induced CPE of PHA will reduce the overall operating cost in a more sustainable way by handling large volume, single step extraction process at a minimum time and at low cloud point temperatures. Chromatographic analysis reassures that the nativity of PHA molecule is retained to a larger extent and thereby, the molecules physiochemical and mechanical properties remain unaltered. Thus, nonionic surfactant induced cloud point extraction of PHA from *Cupriavidus necator* is first of its kind research which involves extraction of PHA in its native form and also involves removal of endotoxins in the most sustainable manner. The optimistic results obtained from the batch experiments of cloud point extraction can be further extended with insight toward continuous extraction involving recycling of surfactant which aids in further reduction in overall cost of the process.

## Abbreviations

| | |
|---|---|
| CDM | Cell Dry Mass |
| CMC | Critical Micelle Concentration |
| CPE | Cloud Point Extraction |
| ESI-MS | Electrospray Ionization Mass Spectrometry |
| HPLC | High Performance Liquid Chromatography |
| LC-MS | Liquid Chromatography Coupled Mass Spectrometry |
| LLE | Liquid -Liquid Extraction |
| m/z | mass-to-charge ratio |
| PEG | Polyethylene Glycol |
| pI | Isoelectric Point |
| PHA | Polyhydroxyalkanoate |
| PHBV | Poly (3-Hydroxybutyrate–co-3 Hydroxyvalerate) |
| rpm | Rotation Per Minute |
| TFA | Trifluoroacetic Acid |
| TMN6 | Terigtol 6 |
| TX100 | Triton X 100 |
| TX114 | Triton X 114 |
| Wt% | Weight% |

## ORCID

Regupathi Iyyasamy http://orcid.org/0000-0002-3379-0278

## References

[1] Akaraonye, E.; Keshavarz, T.; Roy, I. (2010) Production of polyhydroxyalkanoates: the future green materials of choice. *Journal of Chemical Technology and Biotechnology*, 85 (6): 732.
[2] Peelman, N.; Ragaert, P.; De Meulenaer, B.; Adons, D.; Peeters, R.; Cardon, L.; Van Impe, F.; Devlieghere, F. (2013) Application of bioplastics for food packaging. *Trends in Food Science & Technology*, 32 (2): 128.
[3] Chen, G.Q.; Wang, Y. (2013) Medical applications of biopolyesters polyhydroxyalkanoates. *Chinese Journal of Polymer Science*, 31 (5): 719.
[4] Snell, K.D.; Peoples, O.P. (2009) PHA bioplastic: a value-added coproduct for biomass biorefineries. *Biofuels, Bioproducts and Biorefining*, 3 (4): 456.
[5] Cruz, M.V.; Freitas, F.; Paiva, A.; Mano, F.; Dionísio, M.; Ramos, A.M.; Reis, M.A. (2016) Valorization of fatty acids-containing wastes and byproducts into short-and medium-chain length polyhydroxyalkanoates. *New Biotechnology*, 33 (1): 206.
[6] Koller, M.; Salerno, A.; Dias, M.; Reiterer, A.; Braunegg, G. (2010) Modern biotechnological polymer synthesis: a review. *Food Technology and Biotechnology*, 48 (3): 255.
[7] Kunasundari, B.; Sudesh, K. (2011) Isolation and recovery of microbial polyhydroxyalkanoates. *Express Polymer Letters*, 5 (7): 620.
[8] Angelova, B.; Schmauder, H.P. (1999) Lipophilic compounds in biotechnology—interactions with cells and technological problems. *Journal of Biotechnology*, 67 (1): 13.
[9] Blankschtein, D.; Thurston, G.M.; Benedek, G.B. (1986) Phenomenological theory of equilibrium thermodynamic properties and phase separation of micellar solutions. *Journal of Chemical Physical*, 85 (12): 7268.
[10] Corti, M.; Minero, C.; Degiorgio, V. (1984) Cloud point transition in nonionic micellar solutions. *The Journal of Physical Chemistry-US*, 88 (2): 309.
[11] Hinze, W.L.; Pramauro, E. (1993) A critical review of surfactant-mediated phase separations (cloud-point extractions): theory and applications. *CRC Critical Reviews Analysis Chemical*, 24 (2): 133.
[12] Mukherjee, P.; Padhan, S.K.; Dash, S.; Patel, S.; Mishra, B.K. (2011) Clouding behaviour in surfactant systems. *Advances in Colloid and Interface*, 162 (1): 59.
[13] Frankewich, R.P.; Hinze, W.L. (1994) Evaluation and optimization of the factors affecting nonionic surfactant-mediated phase separations. *Analytical Chemistry*, 66 (7): 944.
[14] Paleologos, E.K.; Giokas, D.L.; Karayannis, M.I. (2005) Micelle-mediated separation and cloud-point extraction. *TrAC Trends in Analytical Chemistry*, 24 (5): 426.
[15] Law, J.H.; Slepecky, R.A. (1961) Assay of poly-β-hydroxybutyric acid. *Journal of Bacteriology*, 82 (1): 33.
[16] Hirofumi, T.A.N.I.; Kamidate, T.; Watanabe, H. (1998) Aqueous micellar two-phase systems for protein separation. *Analysis Sciences*, 14 (5): 875.
[17] Gu, T.; Galera-Gomez, P.A. (1995) Clouding of Triton X-114: the effect of added electrolytes on the cloud point of Triton X-114 in the presence of ionic surfactants. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 104 (2–3): 307.

[18] Lai, Y.S.; De Francesco, F.; Aguinaga, A.; Parameswaran, P.; Rittmann, B.E. (2016) Improving lipid recovery from Scenedesmus wet biomass by surfactant-assisted disruption. *Green Chemistry*, 18 (5): 1319.
[19] Hansson, P.; Lindman, B. (1996) Surfactant-polymer interactions. *Current Opinion in Colloid and Interface*, 1 (5): 604.
[20] Egan, R.W. (1976) Hydrophile-lipophile balance and critical micelle concentration as key factors influencing surfactant disruption of mitochondrial membranes. *The Journal of Biological Chemistry*, 251 (14): 4442.
[21] Kunieda, H.; Shinoda, K. (1985) Evaluation of the hydrophile-lipophile balance (HLB) of nonionic surfactants. I. Multisurfactant systems. *Journal of Colloid and Interface Science*, 107 (1): 107.
[22] Naqvi, A.Z.; Khatoon, S. (2011) Phase separation phenomenon in non-ionic surfactant TX-114 micellar solutions: effect of added surfactants and polymers. *Journal of Solution Chemistry*, 40 (4): 643.
[23] Sivars, U.; Tjerneld, F. (2000) Mechanisms of phase behaviour and protein partitioning in detergent/polymer aqueous two-phase systems for purification of integral membrane proteins. *BBA-Genetics Subjects*, 1474 (2): 133.
[24] Saitoh, T.; Hirofumi, T.A.N.I.; Kamidate, T.; Kamataki, T.; Watanabe, H. (1994) Polymer-induced phase separation in aqueous micellar solutions of alkylglucosides for protein extraction. *Analytical Sciences*, 10 (2): 299.
[25] Tani, H.; Saitoh, T.; Kamidate, T.; Kamataki, T.; Watanabe, H. (1997) Polymer-induced phase separation in aqueous micellar solutions of octyl-β-D-thioglucoside for extraction of membrane proteins. *Biotechnology and Bioengineering*, 56 (3): 311.
[26] Fricke, B. (1993) Phase separation of nonionic detergents by salt addition and its application to membrane proteins. *Analytical Biochemistry*, 212 (1): 154.
[27] Wang, Y.; Kimura, K.; Huang, Q.; Dubin, P.L.; Jaeger, W. (1999) Effects of salt on polyelectrolyte – micelle coacervation. *Macromolecules*, 32 (21): 7128.
[28] Lopes, A.M.; Santos-Ebinuma, V.C.; Pessoa Júnior, A.; Rangel-Yagui, C.O. (2014) Influence of salts on the coexistence curve and protein partitioning in nonionic aqueous two-phase micellar systems. *Brazilian Journal of Chemical Engineering*, 31 (4): 1057.
[29] Leontidis, E. (2002) Hofmeister anion effects on surfactant self-assembly and the formation of mesoporous solids. *Current Opinion in Colloid and Interface*, 7 (1): 81.
[30] Israelachvili, J.N. (2011) *Intermolecular and Surface Forces*; Academic Press, Amsterdam, the Netherlands.
[31] Parikh, J.; Rathore, J.; Bhatt, D.; Desai, M. (2013) Clouding behavior and thermodynamic study of nonionic surfactants in presence of additives. *Journal of Dispersion Science and Technology*, 34 (10): 1392.
[32] Lo, C.W.; Wu, H.S.; Wei, Y.H. (2009) Optimizing acidic methanolysis of poly (3-hydroxyalkanoates) in gas chromatography analysis. *Asia-Pacific Journal of Chemical Engineering*, 4 (4): 487.
[33] Ramsay, J.A.; Berger, E.; Voyer, R.; Chavarie, C.; Ramsay, B.A. (1994) Extraction of poly-3-hydroxybutyrate using chlorinated solvents. *Biotechnology Technical*, 8 (8): 589.
[34] Heinrich, D.; Madkour, M.H.; Al-Ghamdi, M.A.; Shabbaj, I.I.; Steinbüchel, A. (2012) Large scale extraction of poly (3-hydroxybutyrate) from Ralstonia eutropha H16 using sodium hypochlorite. *AMB Express*, 2 (1): 59.
[35] Yang, Y.H.; Brigham, C.; Willis, L.; Rha, C.; Sinskey, A. (2011) Improved detergent-based recovery of polyhydroxyalkanoates (PHAs). *Biotechnology Letters*, 33 (5): 937.
[36] Moradi, M.; Yamini, Y. (2012) Surfactant roles in modern sample preparation techniques: a review. *Journal of Separation Science*, 35 (18): 2319.