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ORIGINAL ARTICLE

# Selective extraction of lactoferrin from acidic whey using CTAB/n-heptanol reverse micellar system

**Swapnali S. Pawar[1] · Regupathi Iyyaswami[1] · Prasanna D. Belur[1]**

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**Abstract** A reverse micellar system comprising CTAB/n-heptanol, developed for extracting lactoferrin (LF) from a synthetic solution of LF, was investigated for the selective extraction of LF from synthetic whey protein solution, which was prepared by mixing the pure whey proteins. The process conditions obtained during the process was further extended to extract the LF from real acidic whey. The selective extraction of LF was improved by studying the effect of NaCl concentration (additive) and aqueous phase pH on the partitioning of LF into the micellar phase. The highest extraction of LF (98.7%) from acidic whey to micellar phase was achieved at the aqueous phase pH of 10.3 and NaCl concentration of 1.1 M. The LF was back extracted to the aqueous stripping phase with 94% extraction efficiency and 100% purity. The recycling capacity of the organic phase after the back extraction of LF was analyzed to make the process more economical.

**Keywords** Lactoferrin · Whey · Reverse micellar extraction

## Introduction

Whey is a by-product of cheese manufacturing industry and produced in enormous quantity. It is considered to be a potent pollutant because of its very high biological oxygen demand (BOD). However, it is a rich source of proteins like α-lactalbumin (α-LA:1.5 g/l), β-lactoglobulin (β-LG:3–4 g/l), bovine serum albumin (BSA:0.3–0.6 g/l) and immunoglobulins (Ig:0.6–0.9 g/l). The proteins like lactoperoxidase (LPO:0.001–0.003 g/l) and LF(0.003–0.1 g/l) are also present in the whey at comparatively less concentration (Du et al. 2013). Based on the milk processing methods, whey is categorized as acidic and sweet (rennet) whey. The analysis of acidic and sweet whey show the similarity in total whey protein concentration, sugar and fat concentration, and total solid content. However, higher concentrations of calcium, phosphorus and lactic acid were reported for acidic whey (Smithers 2015). The acid whey was underutilized and often discarded as an effluent due to their higher acidity. Even though the whey proteins concentrate is utilized for several applications, the demand for individual pure whey proteins by pharmaceutical and food industries is increasing day by day. Among the whey proteins, LF has many health benefits and has high biomedical value. The global demand for LF has increased in recent years due to its increasing applications in the medicinal and food industry. It is also used as an important component of infant health formulation (Satue-Gracia et al. 2000) and used as food additive in meat and poultry industries to inhibit bacterial growth and extend the shelf life of the meat (Naidu 2002). The dairy industries explicitly add the LF to the cheese to prevent the spoilage and extend the shelf life by controlling the growth of *Pseudomonas* (Quintieri et al. 2013). LF inhibits the growth of yeast *Dekkera bruxellesis* and hence used to prevent the deterioration and to improve the quality of the wine (Duran and Kahve 2017). It is a potential additive for cancer treatment, autoimmune disorders, and antibiotic or antimicrobial therapy where drugs alone have failed to reduce the risks (Marshall 2004).

LF occurs in other biological fluids like saliva, seminal plasma and tears in very less concentration compared to milk

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and whey (Pawar et al. 2017). Even though the raw material is easily available and cheap, the cost of the pure LF in the open market is high due to the difficulties associated with the purification process, which has multiple process steps. The conventional purification methods including the chromatographic method failed to achieve the higher yield with desired purity. Chromatographic separation using cryogel column (Carvalho et al. 2014), gel filtration chromatography (Al-Mashikhi and Nakai 1987), semi-batch foaming process (Saleh and Hossain 2001), and ultrafiltration coupled with cation exchange membranes (Lu et al. 2007) are reported in the literature to extract and purify LF. Electrodialysis with an ultrafiltration membrane (Ndiaye et al. 2010) is an alternative to chromatography but the poor selectivity and fouling are the associated issues. The lower LF separation and purity was observed in the electrically enhanced membrane filtration due to the poor selectivity of the membrane for LF and migration of other whey proteins along with LF (Brisson et al. 2007). The limitations and drawbacks of the conventional and chromatographic processes facilitate the development of a cost-effective and environmentally benign method for purification of LF.

The non-conventional liquid–liquid extraction methods viz, aqueous two-phase extraction and reverse micellar extraction have a great potential to isolate the desired proteins with higher yields, purities, and lower process cost because of its unique characteristics like higher extraction capacity, better selectivity, and integration of recovery and purification. Anjana et al. (2010) carried out LF extraction from whey using the cationic reverse micelles formed by mixed surfactants. After encapsulating LF into cationic micellar phase, column chromatography was used to extract LF from the cationic micellar phase instead of the usual back extraction into the stripping phase. Alvarez-Guerra and Irabien (2012) performed LF extraction from its synthetic solution using imidazolium based hydrophobic ionic liquids. Nevertheless, only 20% of extraction efficiency was reported. Ionic liquid based three-phase partitioning (ILTPP) was used by Alvarez-Guerra and Irabien (2014) for LF extraction from bovine whey and 74 to 99% LF recovery has been observed at the interface of the system. Partitioning of commercially available LF was studied in an aqueous two-phase system formed by PEG4000 (10% w/w)—sodium citrate (14% w/w) and a 1000-fold increase of LF concentration in the salt-rich bottom phase was reported (Da Costa et al. 2015).

Recently, the authors studied the extraction of LF from its synthetic solution using cationic reverse micelles formed by 50 mM CTAB in n-heptanol at pH 10 with the addition of 1 M NaCl and obtained 100% entrapment of protein in micellar phase. The 98% of LF was back-extracted into the aqueous stripping phase at a pH of 6 in the presence of additives (7% n-propanol or n-butanol) and electrolyte (1.3 M KCl) (Pawar et al. 2017). The suitability of the reported system is studied by extending the obtained process conditions for the selective extraction of LF from model whey proteins solution as well as acidic bovine whey. The process condition and parameters were further tuned for the better extraction of LF with higher purity. In order to make the extraction process cost-effective, the organic phase obtained after back extraction of LF was collected and used in subsequent extraction cycles.

## Materials and methods

### Materials

Cetyltrimethylammonium bromide (CTAB) of 99% purity, α-LA, β-LG, LF of more than 85% purity and LPO (> 150U/mg) were obtained from Sigma-Aldrich. Molecular grade BSA having more than 98% was procured from Hi-media, India. Acetonitrile and Trifluoroacetic acid of HPLC grade solvents and Folin-Ciocalteu reagent (FCR) were purchased from Merck, India. Other organic solvents n-heptanol and n-butanol were procured from Loba Chemie, India. Inorganic salts like potassium chloride (KCl), sodium chloride (NaCl) were taken from Spectrum Chemicals, India.

### Reverse micellar extraction of lactoferrin

The reverse micellar extraction of LF was initially studied with the synthetic solution of whey proteins (α-LA, β-LG, LF, BSA, and LPO), which was prepared according to the protein concentrations reported in the literature (Du et al. 2013). Later, the extraction conditions obtained for the synthetic protein solution was extended to the crude whey. Whey was prepared by acidification (pH 4.2) of the pasteurized milk. Casein was precipitated during acidification and it was removed by centrifugation at 15,000 g for 40 min at 4 °C (Kubota 6930, Japan). The straw-colored whey was stored at 4 °C for future use. Total protein concentration in whey was measured by Lowry's Assay by taking absorbance at 660 nm. The physical and chemical properties of whey (Table 1) like Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD) and Total Solid Content (TSC) were estimated. The phenol–sulfuric acid assay was performed to measure carbohydrates present in the whey (Albalasmeh et al. 2013). Metal content in the whey was analyzed using Atomic Absorption Spectrophotometer (AAS) (GBC 932 plus, Australia) for magnesium, zinc, copper, and iron. Whereas, the sodium ($Na^+$) and potassium ($K^+$) content were measured by a flame photometer (Elico –CL 378, India).

n-Heptanol with CTAB was used as an organic phase by dissolving 50 $mMl^{-1}$ of CTAB to heptanol for the

**Table 1** Whey characterization

| Parameters | Present work | Literature Value | Reference |
|---|---|---|---|
| BOD (ppm) | 21,000 | > 35,000 | Smithers (2015) |
| COD (ppm) | 41,680 | ~ 80,000 | |
| Total protein (mg/ml) | 6.473 | 6.8 | Du et al. (2013) |
| Total Solids (%) | 3.0658 | 6.5 | |
| Total Carbohydrate (mg/ml) | 43.5 | 47 | |
| Density (gm/cm$^3$) | 1.01553 | 1.0654 | |
| *Minerals* | | | |
| Sodium (ppm) | 97.5 | 500 | |
| Potassium (ppm) | 257.3 | 1500 | |
| Calcium (ppm) | 189.1 | 600 | |
| Magnesium (ppm) | 72 | 100 | |
| Zinc (ppm) | 2.26 | 1.5 | |
| Iron (ppm) | 0.529 | 0.6 | |

formation of water in oil emulsion. NaCl concentration was maintained at 1 M and feed phase pH was adjusted using NaOH. 20 ml of the reverse micellar system was prepared with a phase volume ratio of one (organic: aqueous phase) for all the experiments. Forward extraction was carried out by mixing both the phases for 25–30 min using magnetic stirrer at 800 rpm and room temperature. Further to separate organic and aqueous phase, the reaction mixture was centrifuged at 5000 g for 20 min (Remi C-24 plus, India). The organic phase was further used for back extraction.

Back extraction was carried out by introducing an equal volume of fresh aqueous stripping phase containing 1.3 M KCl as an additive and 7% V/V n-butanol as co-solvents to the organic phase obtained from forward extraction. The phases were mixed at 800 rpm for 1 h using magnetic stirrer and centrifuged at 5000 g for 30 min (Pawar et al. 2017). After the phase separation, the phases are subjected to LF analysis. Folin-Lowry's assay was used to measure the LF concentration in the aqueous and organic phases after forward and back extraction by obtaining the absorbance at the wavelength of 660 nm using UV/Vis spectrophotometer (UV3000$^+$, Lab India). The extraction efficiency (%) (Eqs. 1 and 2) and Yield (%) (Eqs. 3 and 4) of LF during forward and back extraction and the purity of LF (Eq. 5) after back extraction were calculated. The overall efficiency was calculated using Eq. (6).

$$\text{Forward Extraction Efficiency}\,(\%) = \left[\frac{\text{LF conc. in the organic phase}\,(\text{mg/ml})}{\text{LF conc. in aqueous feed phase}\,(\text{mg/ml})}\right] \times 100 \quad (1)$$

$$\text{Back Extraction Efficiency}\,(\%) = \left[\frac{\text{LF conc. in the stripping phase}\,(\text{mg/ml})}{\text{LF conc. in the organic phase}\,(\text{mg/ml})}\right] \times 100 \quad (2)$$

$$\text{Forward Extraction Yield}\,(\%) = \left[\frac{([\text{LF conc. in org phase}]\,\text{mg/ml}) \times (\text{Vol of org phase}\,(\text{ml}))}{([\text{LF conc. in the feed phase}]\,\text{mg/ml}) \times (\text{Vol of feed phase}\,(\text{ml}))}\right] \times 100 \quad (3)$$

$$\text{Back extraction Yield}\,(\%) = \left[\frac{([\text{LF conc. in stripping phase}]\,\text{mg/ml}) \times (\text{Vol of stripping phase}\,(\text{ml}))}{([\text{LF conc. in org phase}]\,\text{mg/ml}) \times (\text{Vol of org phase}\,(\text{ml}))}\right] \times 100 \quad (4)$$

$$\text{Purity of back extracted LF}\,(\%) = \left[\frac{\text{LF conc. in the stripping phase}\,(\text{mg/ml})}{\text{Total protein conc. in the stripping phase}\,(\text{mg/ml})}\right] \times 100 \quad (5)$$

$$\text{Overall Efficiency}\,\% = \left[\frac{\text{Conc. of Back Extracted LF}\,(\text{mg/ml})}{\text{Initial LF conc. in Whey}\,(\text{mg/ml})}\right] \times 100 \quad (6)$$

The statistical analysis of the experimental data was performed using the statistical software 'Minitab 18.0'. All the experiments were conducted in triplicate and the means were reported. The analysis of variance (ANOVA) and Tukey's test method were used to compare the means. The significance of means was measured at $P < 0.05$.

### HPLC analysis

HPLC (Shimadzu, LC-20AD, Japan) was performed using C18 (Shim-pack Solar, Shimadzu, Japan) column with size 250 × 4.6 mm l.D. Water and acetonitrile with 0.1% trifluoroacetic acid were used as mobile phase. Binary gradient mode was chosen with 0.5 ml/min flow rate and the column temperature was maintained at 25 °C. The binary gradient mode was maintained at 10% solvent B for 0.01 to 2 min and 90% solvent B was maintained till 15 min and till 17 min solvent B concentration was 0%. The runtime was 20 min. Absorbance was measured at 254 nm using a UV detector. The column was prior equilibrated with mobile phases for 30 min for sample injection. The samples of standard LF, synthetic whey protein solution and acidic whey were analyzed for the LF concentration using the chromatogram obtained during the analysis. The LF extracted in the organic phase during forward extraction and in the stripping phase during back extraction was also analyzed.

### Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

In order to assess the purity of extracted LF during forward and back extraction, SDS-PAGE analysis was performed using 12% resolving gel and 4% stacking gel. Whey, commercially available pure LF, the micellar phase containing LF (obtained during forward extraction) and stripping phase containing LF (obtained during back extraction) were loaded in gel and compared with wide range protein marker. The 10 µl volume of each sample was loaded. Electrophoresis was conducted at 75 V, for 3 h. Further, the gel was stained using staining solution prepared by dissolving 0.05% (W/V) coomassie brilliant blue R-250 in a mixture of glacial acetic acid, methanol and distilled water in the proportion 1:4:5 for 90 min and destained with the same solution without coomassie brilliant R-250 blue for overnight.

## Results and discussion

Whey is a complex mixture of proteins, carbohydrates, and traces of minerals. The analysis of the whey prepared in the present work is analyzed (Table 1), since the properties and composition of whey, which affect the selective extraction of LF, are dependent on the preparation methodology and the source of milk (Fee and Chand 2006). The physicochemical properties of whey were compared with the values reported in the literature (Du et al. 2013; Smithers 2015). The obtained concentrations of protein and carbohydrates were within the reported range. However, the lower concentration of metal ions (sodium, potassium, calcium, magnesium) was observed when compared to the literature (Table 1). The variation in the concentration of metals and other impurities in the whey may significantly influence the selective extraction of LF. The selective solubilization of LF from the synthetic whey protein solution, which was prepared by mixing the pure whey proteins, namely, α-LA, β-LG, LF, BSA, and LPO, into the organic phase was carried out at the reported optimized condition (50 mM CTAB in n-heptanol at pH 10 with the addition of 1 M NaCl) (Pawar et al. 2017). Further, the experiments were also conducted for the acidic bovine whey, which was prepared in the laboratory. Relatively less transfer of LF (88%—synthetic whey protein solution and 84.66%—acidic bovine whey) to RM was observed at this condition when compared with the 100% extraction of LF from synthetic solution. Hence the experiments are performed to improve the extraction efficiency by studying the effect of important process variables.

### Effect of aqueous phase pH

The variation in the whey protein properties can be exploited to selectively purify a specific protein from whey. The change in surface charge density of protein surface or any solute in response to the changed pH (Krishna et al. 2002) is considered to be one of the important characteristics for the selective extraction of the target protein. Specifically, the electrostatic interaction between the solute and the charged surfactant head group is the major driving forces during the reverse micellar extraction of the biomolecule.

Pawar et al. (2017) reported that pH 10 is the suitable pH for highest solubilization of LF during the forward extraction of LF from the synthetic solution of pure LF. In the whey, proteins like α-LA, β-LG, and BSA are present in high concentration compared to LF. Hence, there is a possible interference of such proteins during the entrapment of LF into RM (Fee and Chand 2006). Also, the weak molecular interaction between surfactant head group (positively charged) and LF (negatively charged) lead to the lesser extraction of LF at pH 10 (Li et al. 2007; Zhao et al. 2010). Hence, the pH of the aqueous phase was slightly adjusted within the range of 10 to 11 to improve the extraction efficiency. The maximum solubilization of 96.33 and 97.46% of LF into the micellar phase has been observed at pH 10.3 for synthetic whey protein solution and acidic bovine whey, respectively. The increase in pH of the aqueous phase resulted in partial precipitation of major proteins like α-LA, β-LG, and BSA. The precipitation is the result of high protein-surfactant ratio that decreases the hydrophobicity of the system and tends to aggregate proteins instead of solubilizing into micellar

phase. Similar precipitation of solute was observed during micellar extraction of Penicillin-G extraction by Mohd-Setapar et al. (2009). Consequently, the loss of total protein (6.8 to 2.13 mg/ml) in the aqueous phase has been observed. Also, the increased pH led to conformational changes of α-LA (Dhanapati et al. 1997) and irreversible transformation of β-LG (Tanford et al. 1959). The increase in pH reduces the LF extraction efficiency up to 62% for synthetic whey protein solution as well as acidic bovine whey (Fig. 1a). As the pH of the solution gradually increases, the concentration of basic ions ($OH^-$) in the aqueous solution found to increase. Further, the basic ions tend to interact with a positive head group of CTAB; but simultaneously, the LF bears negative charge beyond its isoelectric point of 9.4 (Steijns and van Hooijdonk 2000) also interact with polar head group of CTAB and tend to increases the basic ion concentration in the aqueous phase and resulted in the repulsion of LF molecule. Thus, the solubilization of LF in RM beyond the equilibrium of electrostatic interaction was limited (Pires and Cabral 1996). Similarly, water content (8.1) was found to be high at pH 10.3 and subsequent fall (4.3) was observed as extraction efficiency decreases at pH 11.

## Effect of ionic strength

The electrostatic repulsion between the surfactant head groups in the RM may be modified by varying the ionic strength through dissolving the electrolyte salts. The absence of ions in the system results in the accumulation of surfactant and/or the surfactant and protein complex which leads to the precipitation of the protein with surfactant molecule and hinders the phase. The addition of electrolyte salts at an optimum concentration may improve the solubilization of solute into the organic phase. Further, the addition of electrolyte helps to reduce the interfacial tension of the solution and thereby improved the formation of RM through the inverse emulsion. The addition of 1 M NaCl improved the solubility of LF (99%) to the RM phase during the extraction of LF from the aqueous solution of pure LF (Pawar et al. 2017), which reveals that the ionic strength has a significant effect over the solubilization of the LF to the RM phase. Hence, the effect of ionic strength on the LF solubilization from the synthetic whey protein solution and acidic bovine whey to micellar phase was studied (Fig. 1b). The maximum amount of LF solubilization to RM phase from synthetic whey protein solution and acidic bovine whey was observed as 98.04% and

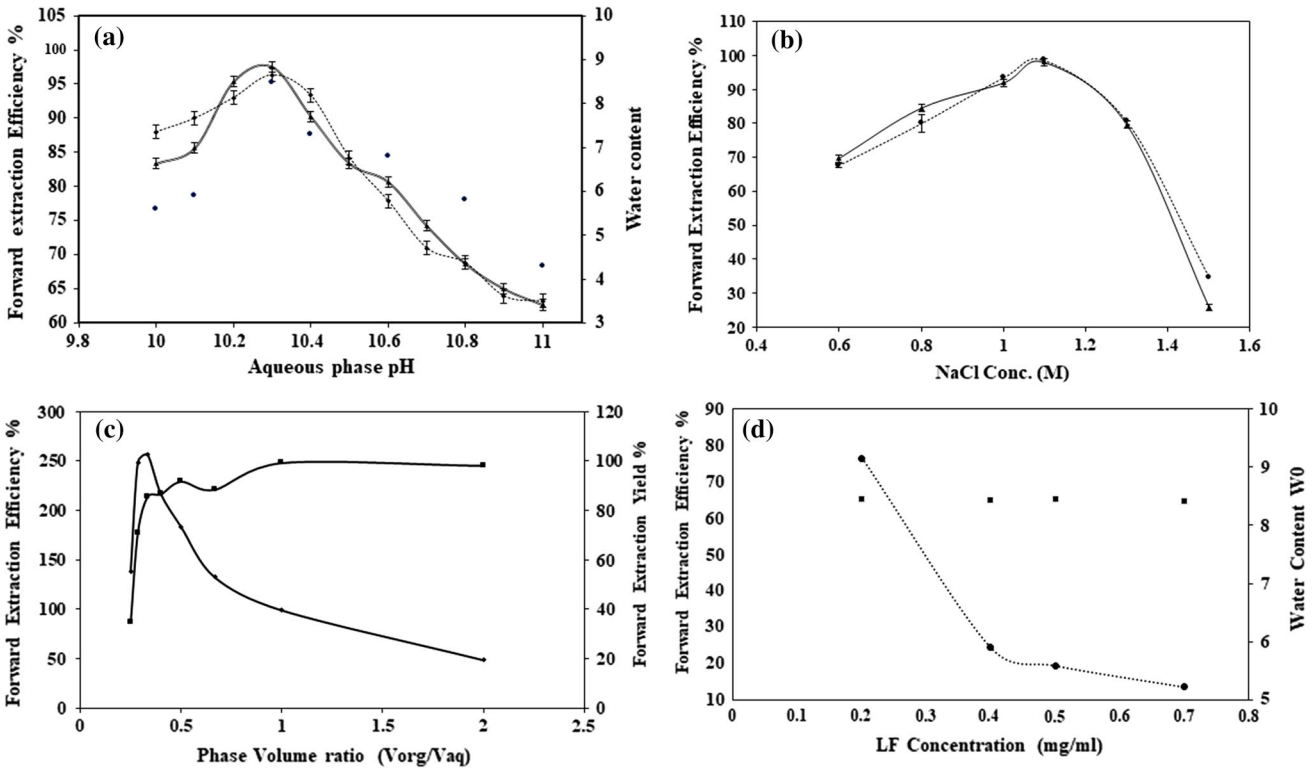


**Fig. 1** Effect of process vaiables on the forward extraction of LF. **a** Aqueous phase pH (filled diamond, synthetic whey protein solution; upward filled triangle, acidic bovine whey and filled circle, water content (acidic bovine whey)); **b** salt concentration (upward filled triangle, synthetic whey protein solution; filled circle, acidic bovine whey); **c** phase volume ratio ($V_{org}/V_{aq}$) (forward extraction efficiency (filled diamond) and yield (filled square)); **d** LF concentration in aqueous phase (forward extraction efficiency (filled circle) and water content (filled square))

98.7% at the NaCl concentration of 1.1 M. Whereas, a very sharp drop in LF transfer to RM i.e. 79.66% to 25.66% in the synthetic whey protein solution and 80.66% to 34.56% in case of acidic bovine whey was observed between 1.3 and 1.5 M of NaCl (Fig. 1b). When electrolytic strength is increased, ions tend to form an electrostatic layer and surrounds the micelles polar core that tend to reduce electrostatic attraction between the charged protein and the charged inner core of micelles. Along with this, the smaller ions ($Cl^-$) produce less screening effect and allow more protein to entrap in RM. The anions pronounce more effect on the extraction than the cations. During LF extraction, anions ($Cl^-$) were also transferred into the reversed micelles by electrostatic interaction (Krei and Hustedt 1992; Li et al. 2007). The decrease of LF transfer efficiency with the increase of salt concentration is the result of reduced Debye length due to the increased electrostatic interaction between ion and surfactant and reduced interaction between solute and surfactant (Tonova and Lazarova 2008).

The increased salt concentration in the system also alters the RM size and resulted in the exclusion of LF from RM and affects extraction efficiency (Nandini and Rastogi 2009). The direct effect of water content on RM size and extraction efficiency has been reported at different operating conditions (Pawar et al. 2017). The size of micelles was measured at optimized parameters for forward extraction of LF from a synthetic solution of whey proteins as well as acidic bovine whey. RM size was 138 μm for synthetic whey protein solution and acidic bovine whey. The size of RM was remarkably less for the empty RM in both cases (7.83 μm) with the water content of 7.6. As the water content of RM is increased the water-CTAB ratio is increased and allows access of more hydrogen bonding site on surfactant as well as water to solubilize protein in RM (Jeffrey and Saenger 1991).

### Effect of phase volume ratio on forward extraction ($V_{org}/V_{aq}$)

The phase volume ratio ($V_{org}/V_{aq}$) was studied with acidic bovine whey to improve the yield of LF in the RM phase. The experiments were conducted between the phase volume ratios of 0.25 to 2.0 and found that the extraction efficiency of LF increases from 138.47 to 256.31% with increasing phase volume ratio till 0.3. Further, the extraction efficiency was found to gradually decline with increasing volume ratio and reduced to 49.84% at the ratio of 2 (Fig. 1c). The highest yield of LF in the RM phase was observed at a volume ratio of 1. Even though the maximum LF concentration was achieved at the volume ratio of 0.3, the relatively lesser yield was observed due to the lower volume of RM phase. At lower phase volume ratio, the volume of the organic phase is not sufficient to accommodate all the LF from the aqueous phase. Further the RM also less stable due to the saturated concentration of LF in the RM phase and consequently resulted in the leakage of the aqueous phase from the organic phase (Bhavya et al. 2012). However, the LF concentration in the RM phase decreases with increasing volume ratio due to the decrease in overall surfactant concentration in the system (Zhao et al. 2010) and hence the RM was less stable with reduced interactive force with LF. The yield is not decreased at higher volume ratio since sufficient RM are available to accommodate all the LF at lower concentration (Fig. 1c).

### Effect of concentration of LF in whey

The maximum capacity of the RM may be realized by studying the LF concentration in the crude. The experiments are planned to increase the LF yield in the RM phase by increasing the LF concentration in the crude during the forward extraction. The effect of increasing LF concentration from 0.2 to 0.7 mg/ml was studied during the forward extraction. Highest LF recovery (76.5%) was obtained when LF concentration was maintained at 0.2 mg/ml in the whey (Fig. 1d). The drastic fall in the recovery of LF was observed as the concentration of LF was increased beyond 0.2 mg/ml. $W_0$ was found to be constant (8.4) for all the RM formed irrespective of LF concentrations (Fig. 1d). Hence, the size of the micelles is not changing with the increased load of LF concentration. The decreased solubility of LF to organic phase was observed at higher LF concentration due to the insufficient number of CTAB molecules and a lesser number of RMs in the organic phase. It was believed that the increased concentration of target protein in the feed phase may demand a higher concentration of surfactant to increase the extraction efficiency by providing an ample amount of RM (Mohd-Setapar et al. 2009).

### Back extraction

Selectively extracted LF to the RM phase has to be back extracted to a fresh aqueous stripping phase. The process condition should be maintained in such a way that the interactive forces between the RM and LF should be destroyed to release the LF from the RM. The repulsive force between protein and a polar head group of surfactant is mainly responsible to release the protein from RM (Krishna et al. 2002; Nandini and Rastogi 2009). Such repulsive force may be created by modifying the ionic strength of the system through the addition of an appropriate concentration of ions and change the pH of the aqueous stripping phase. The back extraction of LF from the RM phase of forward extraction with pure synthetic

solution was studied by (Pawar et al. 2017) and reported that the pH 6 with the addition of a small amount of co-solvent (7% n-propanol or n-butanol) and electrolyte (1.3 to 1.5 M KCl) is the optimum condition to back-extract the LF. The Chaotropic (water repelling) ions like KCl was found to be more effective to rupture RM (Gaikaiwari et al. 2012). The back-extraction efficiency was studied by extending the findings of Pawar et al. (2017) and 93.42% and 94.2% of LF was back extracted from the micellar phase to the stripping phase at 1.5 M KCl concentration for the synthetic whey protein solution and acidic bovine whey, respectively (Fig. 2a). The increased KCl concentration (> 1.7 M) in stripping phase resulted in precipitation of protein at the interface that ultimately reduces the LF extraction efficiency for both synthetic as well as acidic bovine whey to 54.5% and 44.9%, respectively (Fig. 2a). Along with potassium ion, the co-solvent n-butanol also acts as a chaotropic agent that helps to weaken the hydrogen bonding network between a water molecule and also reduces the stability of the native state of proteins by weakening the hydrophobic effect (Bhaganna et al. 2010). The effect of n-butanol as co-solvent at different concentration was studied but failed to increase the extraction efficiency significantly other than 7%, which was reported earlier for the pure LF extraction studies (Pawar et al. 2017).

The effect of volume ratio ($V_{org}/V_{aq}$) on the back-extraction efficiency and yield of LF was studied for acidic bovine whey. It was observed that the back-extraction efficiency was increased from 9.4 to 97.77% as the volume ratio increases from 0.25 to 1 and it was found to decrease to 67.27% as volume ratio increased to 2. However, the yield was found to increase from 37.98 to 97.77% until the volume ratio of 1 and gradually decreased thereafter to 33.63% by increasing the volume ratio to 2 (Fig. 2b). The higher volume of stripping phase at lower volume ratio tends to increase the ionic strength and weaken the hydrogen bonding between the protein and water, which results in the release of LF from the RM. The yield and extraction efficiency found to decrease at the higher volume ratio 2 due to the relatively lesser volume of stripping phase which may not able to accommodate all the LF released from the RM phase. However, the less extraction efficiency was noticed at extreme volume ratios of 0.25 and 2, due to the denaturation and precipitation of LF.

## Purity analysis of LF

The area of the chromatogram peak corresponding to LF for different samples were compared with the standard graph developed at different concentration of LF. The LF peak elution was obtained at 9.038 min (Fig. 3a) for the standard LF. The synthetic whey protein solution prepared by dissolving the pure proteins like α-LA, β-LG, BSA and LPO with LF corresponding to the whey composition was subjected for the HPLC and the obtained chromatogram is presented as (Fig. 3d). With similar chromatographic conditions, forward as well as back extracted LF sample was analyzed and peaks were observed at 9.017 min (Fig. 3b) and 8.851 min (Fig. 3c), respectively. In the case of RM phase sample obtained from the forward extraction, the additional peaks are obtained for the solvent and surfactant other than the proteins peaks (Fig. 3b). Those peaks are confirmed by comparing with the chromatogram obtained for the empty RM without LF. Interestingly, the LF alone selectively get extracted from the whey as well as the synthetic solution of whey proteins by leaving all the other proteins and impurities during the forward extraction, which was confirmed by the chromatogram with a single peak for LF at 9.017 min along with the peak of solvent

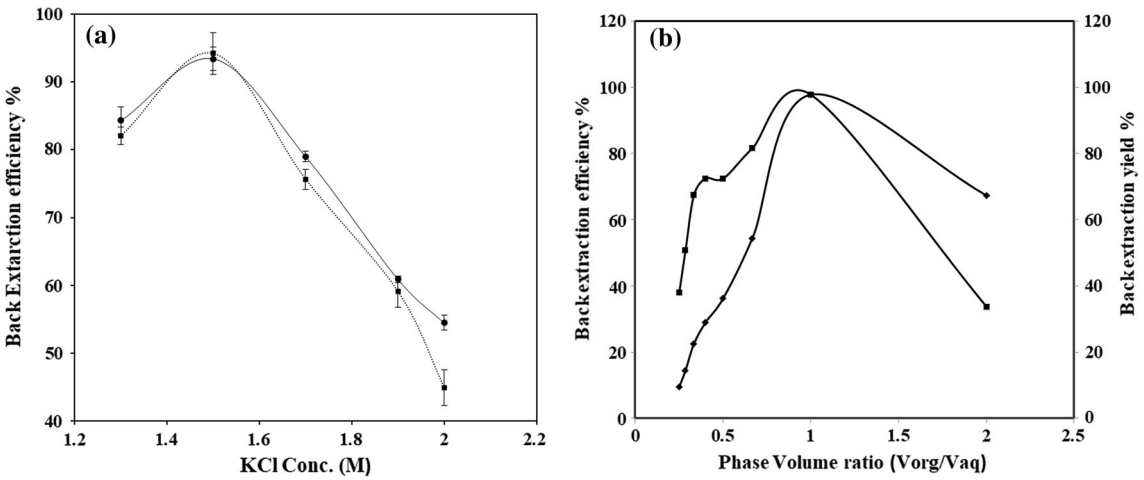


**Fig. 2** Effect of process vaiables on the back extraction of LF. **a** Salt concentration (acidic bovine whey (filled square) and synthetic whey protein solution (filled circle)); **b** phase volume ratio ($V_{org}/V_{aq}$) (back extraction efficiency (filled diamond) and yield (filled square))

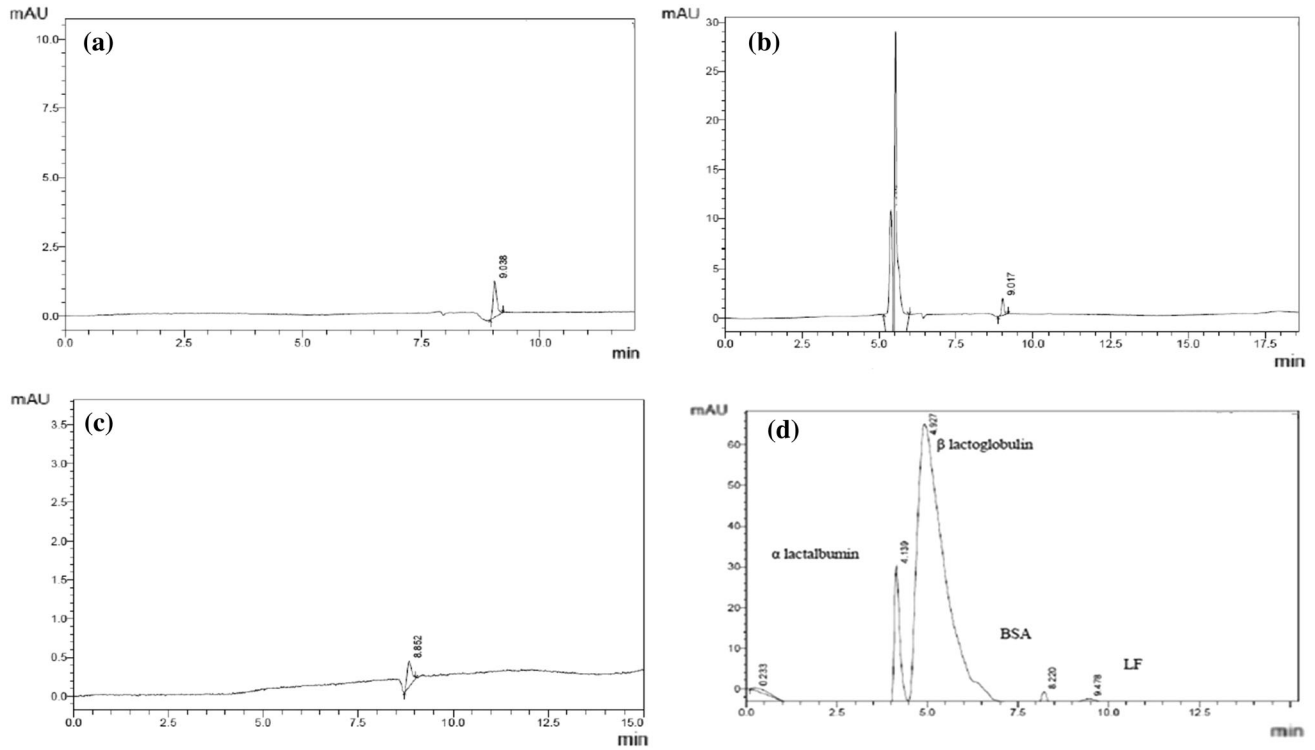


**Fig. 3** HPLC chromatogram of **a** pure LF; **b** forward extracted LF; **c** back extracted LF; **d** whey proteins present in the synthetic whey protein solution

and surfactant. However, a slight shift in the LF peak elution time was observed for the RM phase and stripping phase when compared with the standard LF elution time due to the interference of organic components and ions in the samples.

The SDS-PAGE image consisting of protein marker (Lane 5), whey (Lane 4), Pure LF (Lane 3), organic phase of forward extracted LF (Lane 2) and stripping phase of back extracted LF (Lane 1) was obtained (Fig. 4). The RM phase of forward extraction was loaded in Lane (2) but due to hindrance caused by the solvent of the organic phase, the protein band is not visible in the respective lane (2). It was observed from the Fig. 4 that the appearance of a single band of stripping phase (Lane 1) corresponds to protein band in lane (3, 4 and 5) and absence of other protein bands which were visible in lane 4 confirms the presence of LF alone in the stripping phase. The obtained results are in concurrence with the RP-HPLC results and confirm the selective extraction of LF.

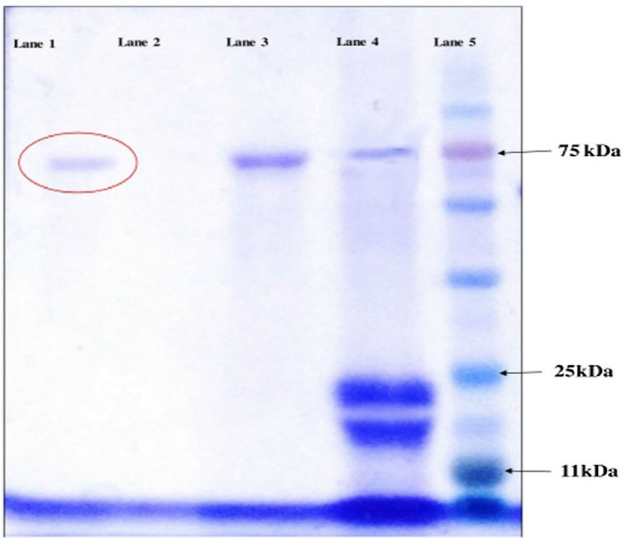


**Fig. 4** SDS PAGE profile of Back extracted LF (Lane 1), Forward extracted LF (Lane 2) and Pure LF (Lane 3), Whey (Lane 4) and Protein Marker (Lane 5) at optimised condition

## Recycling of RM phase for LF extraction

The studies were conducted to recycle the micellar phase obtained after the stripping operation for the extraction of LF from the fresh acidic bovine whey in order to make the process cost-effective and sustainable by reducing the disposal of spent organic phase. The RM phase was reused for a number of cycles and their extraction capacity and efficiency were analyzed (Fig. 5). The highest forward extraction efficiency of 97.5% was obtained in the first cycle and found to gradually decrease to 53% in the fourth cycle. Similarly, the back-extraction efficiency of LF also remarkably decreased from 95.43% in the 1st stage to

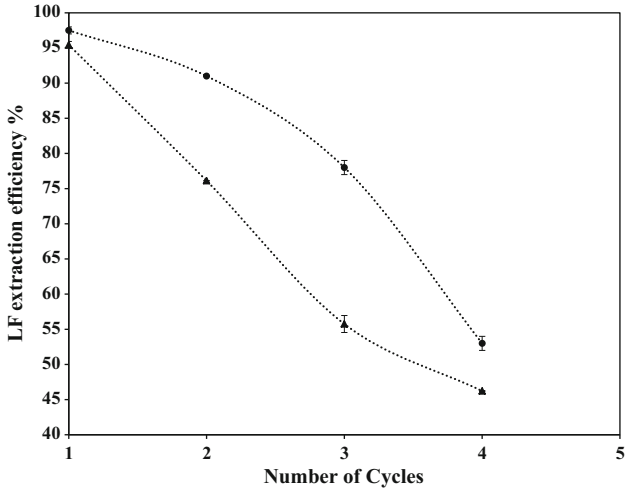


**Fig. 5** Recycling of organic phase for LF (filled circle) forward and (upward filled triangle) back extraction

46.22% in 4th stage (Fig. 5). The decreased extraction efficiency over the number of cycles could be due to loss of CTAB during extraction of LF (Nandini and Rastogi 2009).

The decrease in extraction efficiency after each cycle was analyzed by studying the RM size and water content of the RM phases. The RM with LF after the forward extraction was found to be stable till the fourth cycle with a similar RM size of 128 µm. The number of RM in the organic phase may decrease after each cycle due to the loss of CTAB. Even though significant increase in $W_0$ was observed between the empty RM (7.432) to RM which contains LF, the water content ($W_0$) of the RM phase found to be similar (9.018) after each cycle due to the simultaneous reduction in the number of surfactant molecules and resultant RMs. The difference in water content and size of empty and LF containing RM was the result of increased molecular interaction between chloride ions, protein molecule and a positively charged head group of surfactant present in organic phase due to the increase in the thickness of electric double layer for RMs (Fathi et al. 2012). Further the RM size and water content may vary if the ionic strength of the system varies due to the presence of $Na^+$ and $K^+$ ions. The ions $K^+$ from the stripping phase and $Na^+$ from the fresh whey may accumulate into the RM phase due to the recycling operation. $Na^+$ and $K^+$ concentrations were measured once the organic phase was obtained after back extraction at each cycle. The concentration of $Na^+$ was found to be almost constant (4 ppm) till the fourth cycle, however a slight increase in $K^+$ ion from 0.5 ppm for the first cycle to 3.5 ppm after the 4th cycle was observed. The instability of the RM after the fourth cycle may be due to the combined effect of loss of surfactant and increased concentration of $K^+$ ion. A detailed study is required to improve the extraction efficiency by adding additional solvent and surfactant to make up the organic phase.

## Conclusion

LF was extracted from model whey protein solution as well as acidic bovine whey with cationic RM system. 98.7% LF was solubilized into RM at the salt aqueous phase pH 10.3 with the addition of 1.1 M NaCl. Further LF was back extracted in a pure form into the stripping phase containing 1.5 M KCl and 7% n-butanol as a co-solvent at pH 6. In the present study, 94.2% of LF was recovered from bovine acidic whey without the interference of other whey proteins. The purity of the back extracted LF was confirmed with SDS PAGE as well as RP-HPLC analysis. The obtained result proves the suitability of the CTB/n-heptanol micellar system for the selective extraction of LF from complex biological sources like whey. The recycling of RM phase shows the feasibility of the process in industrial scale. The developed method avoids the pre-treatment steps of whey and makes it cost effective.

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