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Effect of different extraction processes on the recovery of ghee residue proteins

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ABSTRACT

Ghee residue (GR) is the by-product of ghee industry, possessing huge nutritional potential that still lacks in its valorization. This investigation was aimed to recover proteins from GR, by processing with different treatments such as boiling water (30 min), washing with 50% alcohol, isoelectric precipitation, boiling in sodium tri polyphosphate concentration (STPP) solution (1, 2, 3% strength, 30 min) and employing ultrafiltration membrane process. Extraction of proteins from the processed ghee residue (PGR) was observed to be of lower proteins purity. Commonly used acid precipitation method also failed to precipitate proteins from GR, but ultrafiltration resulted in efficient concentration of these proteins. Decrease in concentration of ghee residue and increase in dissolving time, significantly increased solubility of GR. Similarly, addition of sodium tri polyphosphate concentration (STPP) and dissolving time also significantly improved GR solubility. Ghee residue solution was concentrated up to 5.2 ultrafiltration (UF) folds, after that membrane flux approached to zero. Further, ultrafiltration of GR solution resulted in 97% recovery and 70% purity of GR proteins. Findings of this study could significantly increase the profit of organized dairy plants and significantly reduce the waste disposal problem. About 1,00,000 tonnes of proteins can be recovered annually from GR only; that can satisfy yearly protein requirement of about 45,66,000 healthy persons and might help the nation in combating severe problem of protein energy malnutrition.

Key words: Ghee residue proteins, Isoelectric precipitation, Recovery, Sodium tri polyphosphate, Ultrafiltration

Milk production in India has reached 146.3 million tonnes during 2014–15 and the estimated production for the current year is 154 million tonnes (Srivastava 2016). About 30–35% of the total Indian milk production is converted into ghee (Gnadhi *et al.* 2013). Ghee is produced by several methods such as by desi method, creamery method (Rikhari *et al.* 2012), pre-stratification method, its clarification (Tyagi *et al.* 2006) and by its mechanization also as reported by Dodeja and Agrawala (2005). Therefore, about 50.82 (~33%) million tonnes milk will used to produce ghee in this year. Assuming 5% fat in mixed milk, total 2.54 million ghee production can be expected, which will also produce 0.254 million tonnes GR (one tenth of total ghee production) as per earlier reports (Dairy India 2007) in the current year. The solid not fat (SNF) present in cream or butter settles down during clarification of ghee in the form of small particles known as GR. GR has been reported as one of the largest by-product of dairy industry (Janghu *et al.* 2014), which is left when molten ghee is drained off after ghee preparation from cream, *makkhan* or butter. It has smooth to granular texture with glossy exterior due to the presence of excessive free fat (Verma and Raju 2008). It has great potential as a natural antioxidant and flavor enhancer compounds viz. free fatty acids (FFA), carbonyls and lactones (Galhotra and Wadhwa 1993). Owing to the presence of higher levels of reducing substances including free sulphydryls, GR possesses anti-oxidant properties (Santha and Narayanan 1979b). It contains variable amount of milk constituents such milk fat, proteins, lactose, minerals and moisture. The amount of moisture, fat, protein, lactose and ash contents of GR was in the range of 8–30, 32–70, 12–39, 8–30, 2–4 and 1–8%, respectively as reported by Santha and Narayanan (1978a); Relwani (1978) and Grewal (1979). Moreover, the colour, quality and quantity of GR constituents vary based upon the method of manufacture and the temperature used during ghee clarification. Thus, GR is a rich source of lipids and proteins, but also contains a considerable amount of minerals. The yield of GR varies considerably depending on the starting material. The average yield of GR is maximum in direct creamery method (12%) followed by about 3.7% yield in creamery butter and desi butter method. During household production of ghee, GR is consumed either by spreading over *chapattis* or mixing with cooked rice from decades. Thus, GR can be valorized for its nutritive value as a human dietary supplement.

Organized dairy plants, producing ghee do not utilize GR profitably except for the extraction of milk fat from it. Fresh GR has soft and smooth body that hardened during

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storage, which can be eliminated adopting suitable treatments. Before subjecting the residue to any-treatment, its lumps are broken and then pulverized by passing through 40 mesh sieve (Verma and Raju 2008). The treated GR is known as processed ghee residue (PGR) which had soft and smooth texture. PGR was blended with different other ingredients to produce edible preparations like chocolate, candy and burfi (Verma and De 1978, Janghu *et al.* 2014). Tamine (2009) reported that GR can also be employed during sweets making, bakery products and as a flavour enhancer. But, none of the above mentioned approaches of GR utilization have been adopted on a commercial scale. Thus, the valorization of the nutritional potential of GR, still remains untapped.

Ultrafiltration is a well-known pressure driven membrane process that has been used for the concentration and purification of milk proteins during standardization of cheese milk, production of high protein milk, curd, yoghurt and production of high protein, low lactose powders like dairy whiteners, milk and whey protein concentrates and isolates (Meena *et al.* 2015). During ultrafiltration, molecules which are higher in size such as proteins, fat and insoluble salts are commonly retained and concentrated while smaller size molecules like lactose, soluble salts, vitamins and water pass through the membrane in to permeate. This process is widely being used for the concentration and purification of animal, vegetable, plant and fish proteins from decades. Ultrafiltration is used in water, beverage, juice, wine, beer, alcohol, pharmaceutical and nutraceutical industries, but dairy industry has the largest applications of ultrafiltration process. This process is capable to fractionate, concentrate and purify proteins which is even not possible with available chemical methods. This process has many advantages like energy saving, process simplification, higher solids recovery, low product cost, better nutritional and functional properties, improved product quality, no phase change and reduction of pollution hazards.

Protein Energy Malnutrition (PEM) is a major cause of concern in our country. As per National Health Profile (2007), more than 85% children in India suffer from malnutrition (44% mild malnutrition, 38% moderate malnutrition and 4.6% severe malnutrition). On the other side, GR contains significant amount of proteins which can be made available for human nutrition after its isolation using efficient processes like ultrafiltration. Thus, total 1, 00, 000 tonnes of protein can be recovered from GR (39% protein content) yearly. As per ICMR (2005), the per annum protein requirement (recommended daily allowance of protein) for a 60 kg health man, involved in moderate work activity is 21.9 kg. Hence, the proteins recovered (100%) from ghee residue can satisfy the yearly protein requirement of about 45,66,000 persons.

Attempts have not been made to extract proteins from ghee residue till now. Therefore, this investigation was aimed to extract the milk proteins present in the ghee residue using ultrafiltration technique. Further, such valorization of GR could reduce the waste disposal problem, and can also give better economic return to dairy plants.

MATERIALS AND METHODS

Processing of GR: Fresh GR was procured from the experimental dairy of National Dairy Research Institute, Karnal and subjected to different treatments as discussed here.

Cooking the residue in boiling water: After achieving vigorous boiling of water, the bundle of procured GR in a muslin cloth was suspended and kept immersed in the boiling water for 30 min. At the end of the specified period, the bundle was removed and allowed to drain out as much water as possible. Later, it was manually squeezed to press-out remaining water.

Cooking of the GR in water and 1% sodium-bi-carbonate solution: GR was cooked using 1% strength of food grade of tri sodium polyphosphate ($Na_5O_{10}P_3$) that was procured from Johnson Chemical India Ltd., Mumbai. In this case, cooking medium was stirred continuously to avoid excessive foam formation and consequent overflow of the boiling solution.

Washing the GR with 50% alcohol: This was carried out by lixiviating the GR with alcohol. In a stainless steel vat, 3 ml of alcohol/gram of GR used was taken. The GR was thoroughly washed in alcohol added water by continuous manual string of the solution followed by leaving the solution undisturbed for 15 min. Then, it was filtered through a muslin cloth and treated GR was subsequently cooked like it's cooking in boiling water.

Precipitation of GR proteins: Precipitation of GR solution was carried out with diluted hydrochloric acid (1:6), which was procured from sd- FINE CHEM Ltd., Mumbai. The diluted acid was added drop by drop with continuous stirring at 36±1°C. The pH of the solution was reduced up to 3.8. Digital pH meter (PHAN LAB INDIA, Labtek Engg. Pvt. Ltd., India) was used for pH measurement.

Concentration of GR proteins

Preparation of GR solution: The GR solution was prepared by suspending about 100 g of fresh GR in one liter of water. About 2% sodium tri poly phosphate on dry matter basis was added to GR solution. To obtain a good solution of dissolved GR proteins, its pH was adjusted in 7.0–7.5 range using 10% NaOH solution and mechanically stirred at 4,000 rpm till 4 h at 60–90°C. The mechanical stirrer of HARCO India was used in this study. Filtration of solution was carried out to separate insoluble materials form the solution using double folded muslin cloth at 35±2°C.

Clarification and cream separation of GR solution: The clarification and cream separation of GR solution was carried out in the laboratory cream separator (Chadha Electro Industries, Delhi) having capacity of 110 litres/h at 50±2°C.

Concentration of GR proteins by ultrafiltration: Millipore ultrafiltration unit (Pellicon, Millipore Corporation,

Billerica, MA) was used for concentration of GR protein. This plant was equipped with 30,000 MWCO, Polyethersulfone (PES) membrane having 0.1 m^2 membrane area. The GR solution were heated to 50±1°C and then passed through Millipore pre-filter in a Millipore pre-filtration assembly. The feed and retentate pipes were kept in the sample reservoir to concentrate protein in a loop while permeate was collected and measured separately. The inlet pressure was maintained below 15 psi throughout the process. The filtration process was continued until the sample was concentrated up to 5.2 times concentration factor (volume of original feed/volume of final feed) after that membrane flux was almost nil. UF retentate samples were stored in sample bottles at –20°C till further use. After each run, membrane cassette was washed with 0.1 N NaOH (approximately 7 litres) and finally rinsed with distilled water (approximately 5 litres). It was then removed from cassette holder and stored in 0.1 N NaOH at 4°C for further use.

Total solids (TS) and ash contents of all the samples were determined gravimetrically as per the method of BIS (2001a) while their crude protein content was determined using Macro Kjeldahl Method (IDF 20B: 1993). Crude protein content of the samples were multiplied with factor 6.38 to obtain their respective total protein content. Fat content of these samples was determined by mojonnier method using the procedure reported in BIS (2001a). Lactose content of the samples was determined by difference by subtracting protein, fat and ash from their concerned TS *i.e.* lactose (%) = TS (%) - (% protein-% fat-% ash).

Per cent recovery and per cent purity of GR proteins after ultrafiltration

Per cent recovery and per cent purity of GR proteins was calculated using the following formulas:

$$\% \text{ Recovery} = \frac{\text{Kg of proteins in retentate}}{\text{Kg of proteins in ghee residue}} \times 100$$

$$\% \text{ Protein purity} = \frac{\% \text{ proteins in retentate}}{\% \text{ total solids in retentate}} \times 100$$

RESULTS AND DISCUSSION

Chemical composition of GR: The proximate composition of fresh GR procured from Experimental Dairy Plant of NDRI Karnal, India, is presented in Table 1. The GR was observed to have on an average, 78.19±0.22% total solids, 35.99±0.39% fat, 19.97±0.27% proteins, 17.88±0.10% lactose and 3.81±0.31% ash. This proximate composition of GR was in good agreement with the previously reported values by Santha and Narayanan (1978a), Relwani (1978) and Grewal (1979).

Extraction of proteins from GR

Effect of processing of GR by cooking in boiling water (*T-I*)*:* It was observed that when the bundle of GR was suspended into boiling water, then water penetrated inside the core of GR so the same becomes soft and greasy. Subsequently, due to water penetration, moisture content of processed GR was observed to be higher compared to unprocessed GR. During its boiling in water, slight reduction in protein content but higher reduction in fat, lactose and ash content was observed. The chemical composition of processed GR is presented in Table 1.

Effect of processing of GR by cooking in 1% sodium-bi-carbonate solution (*T-II*)*:* Cooking of GR in 1% solution of sodium-bi-carbonate, considerably improved the texture but, similar alteration in moisture content was at par with moisture content obtained after cooking of GR in water (Table 1). The extent of reduction in fat and lactose content of GR cooked in both cooking mediums remained almost same, but higher reduction in proteins content was observed during cooking in soda solution. Higher retention of ash percentage was observed in GR cooked in soda solution than the unprocessed GR as shown in Table 1.

Effect of alcohol washing on quantity of cooked GR during its processing (*T-III*)*:* As compared to unwashed GR, higher quantity of moisture was retained in GR when washed with alcohol prior to it's processing in boiling water, as presented in Table 2. The fat content of cooked GR was considerably reduced due to its washing with 50% alcohol solution (i.e. higher fat loss). Proteins content of processed residue was not adversely affected with alcohol washing. Lactose content of processed GR that was earlier washed with alcohol solution; was lesser than other two treatments. Percentage of ash in the processed GR (treated with alcohol) was slightly lower than untreated processed GR (Table 1). Similar results were also reported by Prahlad (1954).

Protein content (on dry matter basis) of control and processed ghee residues (T-I, TII, T-III) were 25.29, 34.40, 24.33 and 37.87%, respectively. Thus, these approaches failed to enhance the protein purity to a marked level, hence discontinued.

Acid precipitation of GR proteins: GR proteins were tried to separate by acid precipitation method using diluted hydrochloric acid (1:6). At 36°C, pH of solution was first reduced to 4.6, and then reduced to 3.8. These pH adjusted

Table 1. Chemical composition of processed GR*

Constituent	GR (unprocessed)	GR (processed)		
		T-I	T-II	T-III
Total solids (%)	78.96± 0.019	36.65± 0.34	32.34± 0.34	33.95± 0.05
Fat (%)	35.99± 0.39	18.05± 0.165	17.86± 0.195	16.09± 0.08
Lactose (%)	17.88± 0.10	3.43± 0.21	3.58± 0.06	2.18± 0.02
Ash (%)	3.81± 0.31	2.56± 0.31	4.06± 0.04	2.96± 0.04
Protein (%) on dry matter basis	25.29	34.40	24.33	37.87

*Mean±SE (n=3)

solutions were stored for 12 h but precipitation of casein was not appeared. It was observed that caseins in GR cannot be precipitated by acidification at 36°C which might be attributed to its severe denaturation during ghee clarification between110–118°C that might have altered its precipitation behaviour at its isoelectric point (pH-4.6) and even below that (pH-3.8). Therefore, GR proteins were further concentrated in an ultrafiltration plant.

Solution preparation for ultrafiltration

Effect of concentration of GR solution and dissolving time on GR solubility: Trials were conducted to get primary information for the preparation of high quality GR solution in order to concentrate GR proteins. Solution was made using 1:1.75 GR to water ratio, without maintaining pH at 35±1°C temperature followed by 1 h continuous mixing. It was observed that after one hour dissolving time, particles not only completely settled but also, resulted in higher amount of insoluble material during filtration. Two fold clarification of the solution was not able to remove the slime completely. Using one part of GR and two parts of water, GR solution was dissolved till 4 h by maintaining its pH and temperature to 7 at 60±1°C, which reduced the amount of insoluble materials obtained at 35±1°C (without pH adjustment), but five times more separation was required for the removal of slime. On the basis of these results, it was revealed that effective filtration could be performed on higher values of solution concentration, dissolving time, pH and temperature.

To optimize various processing conditions, further experiments were conducted as presented in Table 2. Concentration of GR solution was varied by adding different part of water (from 2.5–40 parts) to one part of GR. Its pH, temperature and dissolving time were varied in the range of 7.0–7.5, 60–90°C and 8–20 h, respectively. Both decrease in GR concentration and increase in dissolving time resulted in considerable decrease in slime formation i.e. achieved better solubility of GR. The statistical analysis (ANOVA) showed that the GR concentration, dissolving time and their interaction had highly significant (P<0.01) effect on the GR solubility (Table 3). As GR concentration increased, slime formation increased. It was also observed that as the amount of water in GR to water ratio was increased, it resulted in higher fat separation efficiency of GR solution during centrifugation and decrease in formation of slime on separator discs. At minimum GR solution concentration (i.e. one part of GR and 40 parts of water), the slime formation was least (29.6 g/100 g of GR) which was not economically desirable due to extensive dilution of the solution. Therefore, higher GR solution concentration (i.e. one part of GR and ten parts of water) was selected for further studies. It was observed that above this concentration the formation of slime was relatively more.

Table 2. Effect of concentration of GR solution and dissolving time on GR solubility*

GR solution (Part of GR: parts of water)	Dissolving time (h)	No. of separation required	Slime formation (per 100 g of GR)	Fat (%)
1:2.5	8	4	96.28±0.1	1.1±0.01
1:5	8	4	93.33±0.4	0.9±0.02
1:7.5	8	4	91.42±0.2	0.9±0.08
1:10	10	3	76.01±0.5	0.7±0.02
1:12	10	3	70.01±0.7	0.7±0.06
1:14	10	3	66.13±0.6	0.6±0.07
1:16	12	3	51.87±0.2	0.5±0.09
1:20	12	2	46.88±0.9	0.4±0.03
1:24	12	2	43.22±0.5	0.4±0.06
1:26	20	2	40.39±0.6	0.4±0.07
1:30	20	1	38.28±0.4	0.3±0.04
1:40	20	1	29.6±0.6	0.2±0.03

*Mean±SE (n=3)

Table 3. ANOVA for effect of concentration of GR solution and dissolving time on GR solubility

Source	df	SS	MSS	F-value
Corrected model	11	18569.177	1688.102	1731.021**
Conc. of GR solution (A)	8	442.346	55.293	0.994**
Time (B)	3	68.639	17.020	6.446 **
A × B	24	34.239	0.975	0.912**
Error	46	23.405		
Total	93	155622.052		

**Significant (P<0.01)

Effect of concentration of sodium tri polyphashate (STPP) and dissolving time on GR solubility: To determine the effect of added concentration of STPP and dissolving time on GR solubility, three different concentration (i.e. 1, 2 and 3%) and time duration (i.e. 4, 8 and 12 h) were studied. During this study, the concentration of GR (i.e. one part of GR and ten parts of water) was kept constant while, pH and temperature were fixed in the range of 7.0–7.5 and 60–90°C , respectively (Table 4).With increase in concentration of STPP and dissolving time, solubility of GR increased.

Table 4. Effect of concentration of sodium tri polyphosphate (STPP) and dissolving time on the GR solubility*

Conc. of STPP (%)	Dissolving time (h)	No. of separation required	Slime formation (per 100 g of GR)	Fat (%)
1	12	2	52.06±0.3	0.5±0.04
	8	3	66.09±0.6	0.5±0.03
	4	3	69.76±0.4	0.6±0.04
2	12	2	37.03±0.01	0.3±0.06
	8	2	45.54±0.08	0.4±0.01
	4	2	50.09±0.07	0.4±0.02
3	12	2	35.3±0.05	0.3±0.04
	8	2	41.98±0.06	0.4±0.06
	4	2	47.02±0.04	0.4±0.01

*Mean±SE (n=3)

As mentioned above, GR concentration with minimum dissolving time (4 h) and 1, 2 and 3% STPP concentration the formation of slime was 69.76, 50.09 and 47.02 g, respectively (Table 4). Whereas, the observed slime formation was 52.06, 37.03 and 35.3 g at maximum dissolving time (12 h) and different STPP concentration (1, 2 and 3%). The effect of STPP concentration and dissolving time on GR solubility is given in Fig. 1.

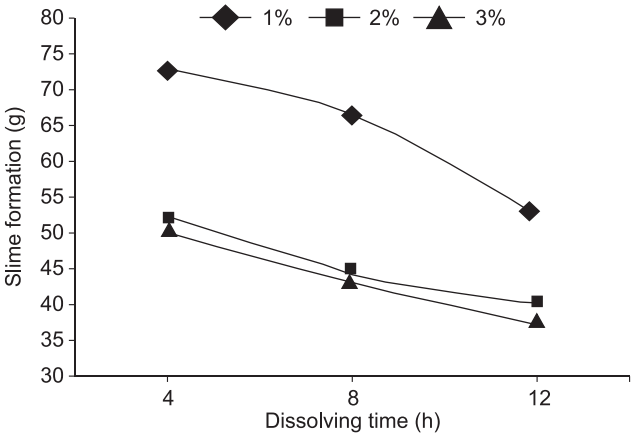

Fig. 1. Effect of added STPP concentration and dissolving time on the GR solubility.

Due to increase in STPP concentrations, dissolving time and their interaction, GR solubility increased significantly (P<0.01) as presented in Table 5. Thus, at 2 and 3% concentration of STPP and different dissolving times, almost equal amount of slime formation obtained which was lesser than the slime formed at 1% concentration of STPP. Moreover, least slime was formed with at highest dissolving time and higher concentration of STPP. Therefore, 2% STPP concentration was selected for further studies.

Effect of cream separation on chemical composition of GR solution: Amount of slime formed was directly related to the separation efficiency of GR solution. With increase in GR solubility, decrease in slime formation and increase in separation efficiency was observed. The chemical composition of GR solution before and after fat separation is presented in Table 6 indicating reduction in all constituents after separation of fat from dissolved GR solution (Table 7) that might be attributed to the composition

Table 5. ANOVA for effect of concentration of STPP and dissolving time on the GR solubility

Source	df	SS	MSS	F-ratio	CD
Conc. of STPP (A)	2	1272.98	1113.34	2003.34**	0.42
Time (B)	2	123.97	19876.05	3871.88**	1.48
A × B	4	75.29	27890.15	453.98**	1.27
Error	11	16.09	43.12		
Total	19	1488.16			

**Significant (P<0.01)

Table 6. Chemical composition of GR solution before and after cream separation*

Constituent	GR solution		UF retentate (5.2 folds)
	Before cream separation	After cream separation	
Total solids (%)	6.59±0.02	4.01±0.081	12.52±0.03
Fat (%)	2.85±0.06	0.65±0.08	3.30±0.02
Protein (%)	1.72±0.48	1.59±0.01	8.77±0.03
Lactose (%)	1.49±0.06	1.32±0.09	0.25±0.02
Ash (%)	0.49±0.02	0.45±0.02	0.20±0.01

*Mean±SE (n=3)

of slime as some milk solids were also lost in it.

Studies on recycling of slime: Solubility of slime was determined using three different slime concentrations and dissolving time durations as shown in Table 8. With the increase in dissolving time and decrease in concentration of slime solution, the formation of slime was reduced meaning enhancement of solubility. It was observed that concentration of slime solution, dissolving time and their interaction had significant (P<0.01) effect on slime formation (Table 9).

From Table 7, it was seen that the slime solution which had 1:2.5 ratio of slime to water with three hours dissolving time was better with respect to process economics, so selected for further studies.

Table 7. Effect of concentration of solution and dissolving time on slime formation

Parts of slime: parts of water	Dissolving time (h)	No of separation required	Slime formation (per 100 g of original slime)
1:1.7	1	3	20.26±0.5
1:2.5	3	2	11.25±0.2
1:5	5	1	10.9±0.04

Table 8. ANOVA for effect of concentration of solution and dissolving time on slime formation

Source	df	SS	MSS	F-ratio	C.D
Conc. of slime solution (A)	2	1194.594	597.297	1380.255**	0.143
Time (B)	2	2396.731	296.731	1652.089**	0.987
A × B	4	1597.833	197.834	1884.151**	0.821
Error	7	0.345	0.058		
Total	15	5189.504			

**Significant (P<0.01)

Concentration of GR proteins by ultrafiltration: The GR proteins were concentrated according to the method as described in material and methods section using Millipore ultrafiltration unit.The gross chemical composition of 5.2 folds UF retentate (Table 6). Milk fat, protein, lactose, ash

and total solids were concentrated from 0.65 to 3.30%, 1.59% to 8.77%, 1.32% to 0.25%, 0.45% to 0.20% and total solids from 4.01% to 12.52% in separated GR solution and UF retentate, respectively. Protein content of cow and buffalo skim milk has been increased employing ultrafiltration in our laboratory earlier by Solanki and Gupta (2009), Khatkar *et al.* (2014) and Meena *et al.* (2015). Thus the calculated protein purity as given by [(8.77/12.52)×100] was 70% while the recovery of proteins was about 97%. Published information related to concentration of GR protein by ultrafiltration are not available in the literature, hence, direct comparison cannot be made, but efficient concentration of proteins by ultrafiltration technique is well established fact from decades in dairy industry.The optimized process for the extraction of GR proteins employing ultrafiltration process has been depicted in Fig. 2.

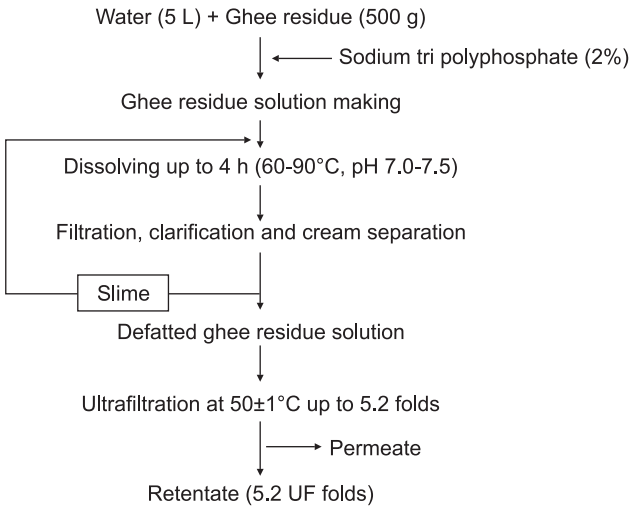

Fig. 2. Optimized process for the extraction of protein from ghee residue.

Being a by-product of ghee industry, GR is a rich source of milk fat and proteins, but still used in cattle feed after recovery of milk fat. The precipitation of GR proteins is not possible by acid precipitation method, but the same can efficiently concentrated by ultrafiltration process. Decreased concentration of GR solution and increased dissolving time had highly significant ($P<0.01$) effect on GR solubility. The increase in STPP concentration and dissolving time also significantly ($P<0.01$) enhanced GR solubility. Further concentration of GR solution beyond 5.2 UF folds was restricted by retentate viscosity that resulted in limited membrane flux. The observed recovery of proteins by ultrafiltration was 97.16% with 70% protein purity. The findings of this study might help organized dairy sector by improving their profit. It might also reduce the problem of protein malnutrition by making huge quantity of GR proteins available for human consumption as GR had 0.75 protein efficiency ratio (PER), 92.65 digestibility coefficient and 65.05% biological value (BV).

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