processes

MDPI

Article

Recovery of Protein from Dairy Milk Waste Product Using Alcohol-Salt Liquid Biphasic Flotation

Pei En Tham [1], Yan Jer Ng [1], Revathy Sankaran [2], Kuan Shiong Khoo [1], Kit Wayne Chew [3,*], Yee Jiun Yap [3], Masnindah Malahubban [4], Fitri Abdul Aziz Zakry [4] and Pau Loke Show [1,*]

1 Department of Chemical and Environmental Engineering, Faculty of Science and Engineering, University of Nottingham Malaysia, Jalan Broga, Semenyih 43500, Selangor Darul Ehsan, Malaysia; peien0405@gmail.com (P.E.T.); yanjer98@hotmail.com (Y.J.N.); kuanshiong.khoo@hotmail.com (K.S.K.)

2 Institute of Biological Sciences, Faculty of Science, University of Malaya, Jalan Universiti, Kuala Lumpur 50603, Malaysia; revathysankaran@ymail.com

3 School of Mathematical Sciences, Faculty of Science and Engineering, University of Nottingham Malaysia, Jalan Broga, Semenyih 43500, Selangor Darul Ehsan, Malaysia; YeeJiun.Yap@nottingham.edu.my

4 Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Sarawak Campus, Bintulu 97008, Sarawak, Malaysia; masnindah@upm.edu.my (M.M.); zakryfitri@upm.edu.my (F.A.A.Z.)

* Correspondence: KitWayne.Chew@nottingham.edu.my or kitwayne.chew@gmail.com (K.W.C.); PauLoke.Show@nottingham.edu.my or showpauloke@gmail.com (P.L.S.); Tel.: +603-8924-8605 (P.L.S.)

Received: 25 October 2019; Accepted: 19 November 2019; Published: 21 November 2019

Abstract: Expired dairy products are often disposed of due to the potential health hazard they pose to living organisms. Lack of methods to recover valuable components from them are also a reason for manufactures to dispose of the expired dairy products. Milk encompasses several different components with their own functional properties that can be applied in production of food and non-food technical products. This study aims to investigate the novel approach of using liquid biphasic flotation (LBF) method for protein extraction from expired milk products and obtaining the optimal operating conditions for protein extraction. The optimized conditions were found at 80% concentration ethanol as top phase, 150 g/L dipotassium hydrogen phosphate along with 10% (*w*/*v*) milk as bottom phase, and a flotation time of 7.5 min. The protein recovery yield and separation efficiency after optimization were 94.97% and 86.289%, respectively. The experiment has been scaled up by 40 times to ensure it can be commercialized, and the protein recovery yield and separation efficiency were found to be 78.92% and 85.62%, respectively. This novel approach gives a chance for expired milk products to be changed from waste to raw materials which is beneficial for the environment and the economy.

Keywords: milk; protein; liquid biphasic flotation; dairy waste; recovery

1. Introduction

A large quantity of dairy waste is produced per annum in every country. Taking UK as an example, a total of 330,000 tons of milk waste is produced annually with approximately 90% of the total waste produced from homes. This is equivalent to 490 million pints nationwide or 18.5 pints per household. Milk should be kept at the right temperature to prevent it from spoiling before the expiry date. However, the typical household UK fridge operates at a temperature that is 2 °C warmer than the recommended storage temperature of milk, which is between 0 and 5 °C [1]. This amount of milk waste creates an environmental problem as it creates greenhouse gas emissions equivalent to approximately 20,000 cars annually [2].

Milk contains approximately 87.4% of water and 12.6% of milk solids. Fats makes up 3.7% of the 12.6% milk solids while the remaining 8.9% is 3.4% of proteins, 4.8% lactose, and 0.7% minerals.

Of the proteins in milk, 80% is Casein and the remaining 20% is Whey protein [3]. Casein is chiefly phosphate-conjugated and mainly consists of calcium phosphate-micelle complex. Whey protein is a collection of a globular proteins with a high level of α-helix structure and the acidic-basic and hydrophobic-hydrophilic amino acids are distributed in a fairly balanced form. Whey proteins have substantial levels of secondary, tertiary, and quaternary structure. They are heat-labile stabilizing their protein structure through intermolecular disulfide linkage [4]. The proteins in milk are considered to be complete as they contain all types of essential amino acids in amounts that match the amino acid requirements. They are used as a standard reference for proteins to compare with other food proteins due to their high quality. Branched-chain amino acids contents such as valine, isoleucine, and leucine in milk are also higher than many other foods [4].

Since the conventional technique for extracting bioactive compounds need longer extraction time yet cost-consuming with complex scale-up, the liquid biphasic flotation (LBF) method was proposed [5]. LBF system is an integration of the adsorptive bubbles floatation system, where the biphasic system is supported with air bubbling to transport the biomolecules from one phase to another. The surface-active compound of biomolecules present will be absorbed onto the surface of ascending gas bubble and be brought from the bottom phase to the top organic phase [6]. LBF is formed by combining an immiscible polymer and a salt solution. Addition of salt to water will cause segregation of ions into their preferred water structuring [7]. Aqueous biphasic systems will occur when certain solutes cause an aqueous solution to fully separate into two aqueous phases. The basic aqueous two-phase system (ATPS) phasing strategy is based on the separation of proteins into one phase with the contaminants being present in the other phase. The smaller biomolecules will be present mostly at the bottom phase which also can be known as the salt-rich phase. Whereas proteins will be brought up to the top phase [8]. Polarity is believed to play a role in the separation; molecules with lower polarity will be partitioned to the top phase, while molecules with higher polarity will be partitioned to the bottom phase. This aqueous liquid–liquid two-phase system is more widely used in the extractive separation of labile biomolecules such as proteins. This system operates under mild conditions due to the low interfacial tension between the two phases, achieving small droplet size, large interfacial areas, and efficient mixing under very gentle stirring and rapid partition.

LBF is a well-known method for the separation, concentration, and purification of biological material, particularly for protein, enzyme, and DNA [9]. Extraction using LBF is based on the separation of biomolecules between the two aqueous phases [10]. Much work has been done by using LBF to exploit and study the behavior of the aqueous rich phase and driving forces which will affect the partitioning of biomolecules in the separation process. These systems were based on aqueous mixtures of two incompatible polymers, such as polyethylene glycol (PEG), dextran, and/or maltodextrin [11]. Since then, many immiscible aqueous systems were found by using hydrophilic solutions. However, other types of LBF, with components of different phase, had been focused on to increase the mass transfer rates and the selectivity of certain biomolecules. Ionic liquids [12], inorganic salts, and carbohydrates are three examples of solutes used in LBF [13]. These molecules were applied in the separation or purification of a wide range of compounds, including proteins, enzymes, antibiotics, organic acids, and many other bio- or synthetic molecules [13].

LBF is a very promising method, and it indicates a great potential for a wider usage in partitioning, concentrating, and purification of labile biology products from natural sources or fermentation broths, as well as in enzyme technology during industrial or laboratory production of enzymes. LBF is an integration of the principles of ATPS but with additional bubbling action to enhance the separation of biomolecules. This integration will utilize the adsorptive gas bubble separation technique in which the biomolecules with surface-active sites in the bottom aqueous phase are selectively adsorbed onto the surface of the ascending gas bubbles which are then collected in the immiscible top aqueous phase. With this, water soluble biomolecules can be separated from their crude aqueous extracts [9].

A detailed study was made with aims to obtain optimal operating conditions for the extraction of protein from expired milk using alcohol/salt LBF. The effect of milk concentration, type of salt,

type of alcohol, concentration of salt solution, concentration of alcohol solution, pH, flotation time along with a scaled-up LBF system were studied. Up to current date, no study has been made on recovery of protein from expired milk using alcohol/salt LBF. Partitioning of the milk protein into the alcohol phase through LBF using low-cost and recyclable phase forming components would lead to a cost-efficient protein recovery process. Additionally, alcohol-salt LBF has a low viscosity, easier constituent for recovery and short settling time. As such, this approach would enable the production of milk protein to be economically feasible and sustainable. This study has led to a novel discovery of liquid biphasic flotation application for protein extraction from milk waste with economic processes that will be beneficial at the industrial scale.

2. Materials and Methods

2.1. Materials

Food grade alcohols of ethanol, 1-propanol, 2-propanol (R&M Chemicals, Selangor, Malaysia) were used as the extraction solvents of proteins. Salts for the bottom phase that are utilized in this study were ammonium sulphate [$(NH_4)_2SO_4$], di-potassium hydrogen phosphate (K_2HPO_4), sodium sulphate (Na_2SO_4), di-sodium hydrogen phosphate (Na_2HPO_4) and magnesium sulphate ($MgSO_4$) purchased from R&M Chemicals (Selangor, Malaysia). Bradford reagent was used to quantify protein concentration in the two solutions (top phase and bottom phase) after the flotation. Bradford reagent is also purchased from R&M Chemicals (Selangor, Malaysia).

2.2. Apparatus

Liquid biphasic flotation unit of 50 mL volume capacity was used as the separation system, and it was obtained from Donewell Resources (Puchong, Selangor, Malaysia). A 50 mL glass tube was connected from the bottom to a gas compressor. The bottom of the glass tube was drilled and fitted with a rubber tube to be connected to the gas compressor. A sintered glass disk (Grade 4 (G4) porosity) was fitted at the bottom of the glass tube so that air bubbles will be produced when compressed air is passed through it. The flowrate of air supplied to the LBF system is controlled by using a flowmeter (model: RMA-26-SSV) with a range of 50 to 200 cc/min (Dwyer, Michigan, IN, USA). The air compressor is powered by plugging it into a wall socket. Figure 1 shows the schematic diagram of the LBF system.

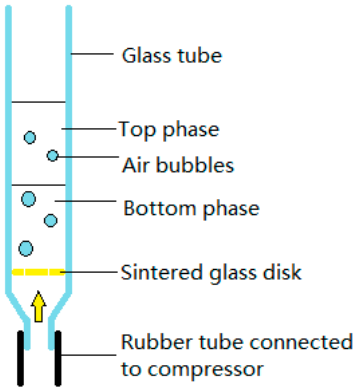

Figure 1. Figure illustrating schematic diagram of LBF system.

2.3. Preparation of Milk Samples

Expired milk was supplied by local producers (Dutch Lady) and was stored under room temperature. The concentration of proteins in the milk was tested to be 13.64 mg/mL. The milk was stored into a refrigerator at a temperature of 2 °C to reduce the effects of bacteria activity.

2.4. Protein Assay

The protein is determined by using Bradford Reagent. The dilution has been prepared with 10× dilution with adding 2 mL reagent with 0.2 mL of either top or bottom solution and was incubated for 10 min before the reading was tested using UV-Vis spectrophotometer at a reading wavelength of 595 nm. The absorbance of the protein concentration was based on the calibration between BSA concentration and OD_{595}.

2.5. Protein Extraction Using LBF

A mixture of salt solution and expired milk was mixed and top up to 15 mL to be used as the bottom phase of the experiment. 15 mL of pure alcohol was used as the top phase of the experiment. After pouring the two solutions into the LBF system, the mixture was allowed to settle for 30 s so that two layers of liquid can be formed inside the system. The flowmeter was set to 25 cc/min to allow compressed air to flow into the system. Air was passed through the system for 10 min before closing the flowmeter and allowing the system to settle for 5 min again. The top and bottom layer was pipetted out from the glass tube and tested for their respective protein concentrations.

2.6. Optimization of LBF Operating Parameters

The operating parameters of LBF such as type of salt/alcohol, concentration of salt/alcohol/milk, pH of the bottom phase, and the flotation time were investigated by one factor at a time (OFAT) approach to maximize protein extraction and recovery. The optimization started with the initial operating conditions which is 100% of 2-propanol, 20 g/L salt solution, 15% (*w*/*v*) milk solution, flotation time of 10 min and the initial pH of the solution. The initial volume for both top phase and bottom phase was kept at 15 mL each, and the experiment was carried out at room temperature. Table 1 shows the parameters and variables tested for this experiment.

Table 1. Parameters and variables.

No.	Condition	Initial Setting	Variables	Unit	Justification
1.	Type of salt	-	Ammonium sulphate, Magnesium sulphate, Sodium sulphate, Dipotassium hydrogen phosphate, Disodium hydrogen phosphate	-	The salt that would result in the best yield was chosen from the five salts used
2.	Type of alcohol	2-Propanol	Ethanol, 1-Propanol, 2-Propanol	-	After the selection of salt was completed, the type of alcohol that would result in the best yield was determined
3.	Concentration of salt	20	150, 200, 250, 300, 350	g/L	The percentage of salt was set according the Separation and Purification Technology Recovery of lipase derived from Burkholderia cenocepacia ST8 using sustainable aqueous two-phase flotation composed of recycling hydrophilic organic solvent and inorganic salt.
4.	Concentration of alcohol	100	60, 70, 80, 90, 100	%	A total of 15 mL of top phase solution is added into the system and the concentration of alcohol adjusted by using deionized water.
5.	Concentration of milk	15	5, 10, 15, 20, 25	% (*w*/*v*)	After the few parameters are stable, alteration of milk concentration begins.
6.	pH	9.15	6.5, 7, 7.5, 8, 9.15	-	Condition of milk has been altered to set the right pH for the whole system.
7.	Operation time	10	5, 7.5, 10, 12.5, 15	min	Initial setting without alcohol; after 10-min alcohol is added because no two-phase forming.

2.7. Calculations of Recovery Yield and Separation Efficiency

Recovery yield (R) of proteins in the hydrophilic organic solvent phase was measured using the following equation:

$$R = \frac{C_T V_T}{M} \times 100\%$$

where,

R is the recovery yield

C_T is the concentration of proteins in the hydrophilic organic solvent phase

V_T is the volume of the hydrophilic organic solvent phase

M is the total mass of proteins in the initial milk used

The separation efficiency from the milk to the top phase after LBF is calculated by the following equation:

$$E = \frac{C_T V_T}{C_T V_T + C_B V_B} \times 100\%$$

where,

E is the separation efficiency

C_T is the concentration of proteins in the hydrophilic organic solvent phase

V_T is the volume of the hydrophilic organic solvent phase

C_B is the concentration of proteins at the bottom phase

V_B is the volume of the bottom phase

3. Results and Discussion

3.1. Effects of Different Types of Inorganic Salt in Protein Recovery Using LBF

The type of salts used for LBF is a key for protein extraction in this system as different salts induce different interactions with the protein, causing the separation efficiency of the proteins to alter. This is because salt solutions at the bottom phase are responsible of manipulating the surface tension of water thus changing the hydrophobic interactions between proteins and water at bottom phase [14]. As a result, when the protein solubility is reduced, proteins will start to migrate to the top phase when aided by flotation. It is reported that the Gibbs free energy of hydration of salt was the key to the formation of a biphasic system between salt and alcohol solution [15].

This experiment was conducted by firstly determining the most suitable type of salt to be used to obtain the best results and then changing the type of alcohol used. The types of salts used include ammonium sulphate, dipotassium hydrogen phosphate, disodium hydrogen phosphate, sodium sulphate, and magnesium sulphate. The results are illustrated in Figure 2. Dipotassium hydrogen phosphate and disodium hydrogen phosphate show a higher efficiency followed by ammonium sulphate and sodium sulphate in the descending order of 85.18%, 68.99%, 58.85%, and 53.81%, respectively. Magnesium sulphate showed the lowest separation efficiency which is 50.10%. For disodium hydrogen phosphate, the bottom phase forms salt crystals after flotation has been completed. This is due to the volume of the bottom phase being reduced; thus, the salt is unable to be fully dissolved in the remaining solution. Higher maintenance cost of the system is required if disodium hydrogen phosphate were to be used as a medium to create the biphasic conditions of this system.

As for the protein recovery yield, dipotassium hydrogen phosphate exhibits a higher protein recovery yield than all other salts, with a recovery yield of 29.997%. The lowest recovery yield of all salt tested was ammonium sulphate, which has a recovery yield of 7.74%. The other salts, which are disodium hydrogen phosphate, magnesium sulphate, and sodium sulphate, each has a recovery yield of 20.33%, 16.04%, and 10.81% respectively. The recovery yield for dipotassium hydrogen phosphate is significantly higher than that of disodium hydrogen phosphate. Taking into consideration the fact that when disodium hydrogen phosphate is used, the bottom phase will form salt crystals after flotation,

and the two results obtained, dipotassium hydrogen phosphate was chosen to be the inorganic salt used in the following tests.

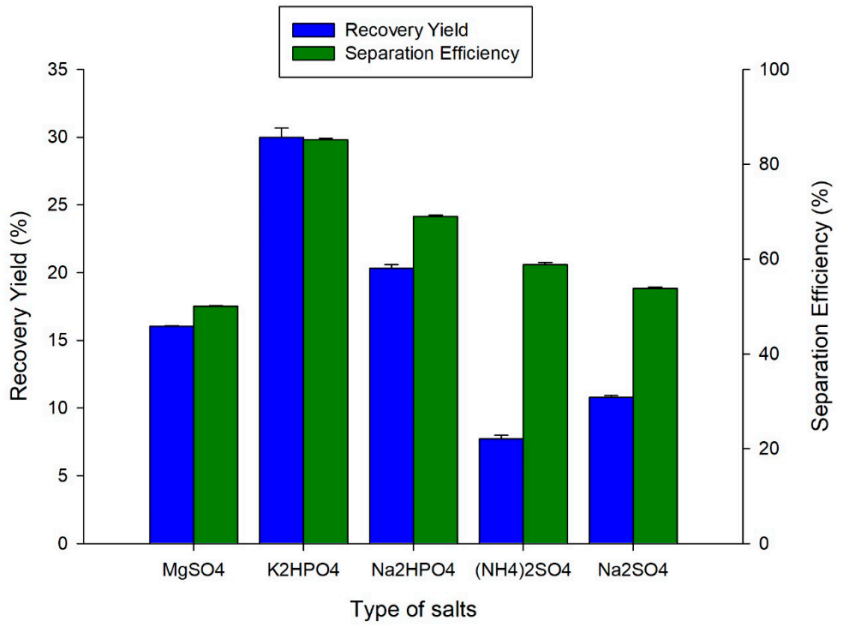

Figure 2. Figure showing the effect of different types of salts on the protein recovery yield and separation efficiency using LBF system.

3.2. *Effect of Different Types of Alcohols for Protein Recovery*

The type of alcohol used for LBF plays an important role in the system as different types of alcohol have different levels of interactions with the proteins, which will determine how much protein can the system extract. The type of alcohol used also affects the formation of biphasic system with salt solution. Many proteins are found to be non-compatible with the alcohol-rich top phase in the LBF process [16]. Some alcohols such as methanol will form triphasic solutions rather than biphasic solutions when mixed with salt solution. The selection of the type of alcohol used is very crucial as it will affect the overall performance of the system. In this study, ethanol, 1-propanol and 2-propanol of 100% were selected to form a biphasic system with dipotassium hydrogen phosphate at a concentration of 20 g/L.

All three alcohols were found to successfully form a biphasic system with the dipotassium hydrogen phosphate solution and the protein recovery yield for ethanol, 1-propanol and 2-propanol found to be 42.00%, 13.23%, and 23.35%, respectively. Based on Figure 3, ethanol has outperformed both 1-propanol and 2-propanol in terms of recovery yield, being almost two times the yield of 2-propanol and almost three times the yield of 1-propanol. In terms of separation efficiency, ethanol also outperforms both 1-propanol and 2-propanol. The separation efficiency of ethanol, 1-propanol, and 2-propanol is found to be 92.20%, 87.54%, and 90.56%, respectively. Alcohols usually contain a carbon chain and a functional group (-OH); the difference between ethanol and the other two alcohols is that ethanol has a shorter carbon chain, resulting in it having more ethanol molecules at the same volume. This can be proven by dividing the density of the respective alcohol with its molar mass. For example, the density of ethanol is 0.789 g/cm^3 at 20 °C [17] and ethanol has a molar mass of 46.07 g/mol [18], this will result in ethanol a volume of 0.01715 mol/cm^3. This is higher than 1-propanol and 2-propanol as they have volumes of 0.01336 mol/cm^3 and 0.01306 mol/cm^3, respectively. The density of 1-propanol and 2-propanol is 0.803 g/cm^3 [19] and 0.785 g/cm^3 [20], respectively at 20 °C while their molar mass is 60.096 g/mol [21] and 60.1 g/mol [22], respectively. This difference will result in the protein molecules being able to interact more with the alcohol molecules and not settle back to the bottom phase after flotation. Additionally, the high recovery yield and separation efficiency in ethanol could be because of high polarity of the alcohol compared to the other two alcohols used. High

hydroxyl group in ethanol could allow more protein to be accumulated at the top phase thus, giving high recovery yield [23]. Due to the above reasons, ethanol is chosen to carry out the following tests.

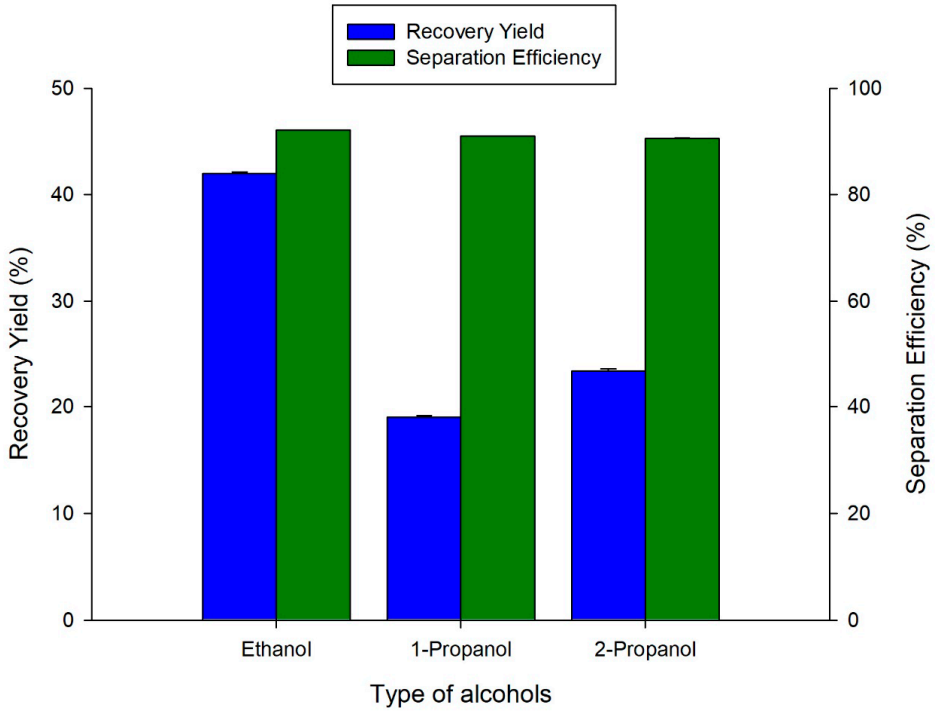

Figure 3. Effect of various types of alcohols on protein recovery yield and separation efficiency using LBF system.

3.3. Effect of Different Concentration of K_2HPO_4 Salt on the Recovery of Proteins

The concentration of salt used for the bottom phase is also optimized in this study. Varying salt concentrations have been used in the separation of proteins. When dipotassium hydrogen phosphate concentration is increased from 150 g/L to 350 g/L, the volume of top phase showed a decreasing trend while the volume of bottom phase showed an increasing trend. More proteins are retained in the lower phase.

From Figure 4, the highest protein recovery yield is exhibited by a salt concentration of 150 g/L, with a yield of 46.83%, while the lowest yield was obtained from a salt concentration of 350 g/L, with a yield of 37.14%. At salt concentration of 200 g/L, 250 g/L, and 300 g/L, the recovery yield of proteins is 45.01%, 39.36%, and 44.96% respectively. When increasing the salt concentration, the yield shows a decreasing trend due to more proteins being denatured when exposed to higher concentrations of salts. Thus, the lowest concentration of salt to form a biphasic solution should be obtained [24]. As for the separation efficiency when salt concentration is altered, the separation efficiency shows a decreasing trend when the salt concentration is increased. This is also due to the proteins in the milk being denatured by the salts when the solution is mixed together. Due to the above reasons, a salt concentration of 150 g/L was used for the following tests.

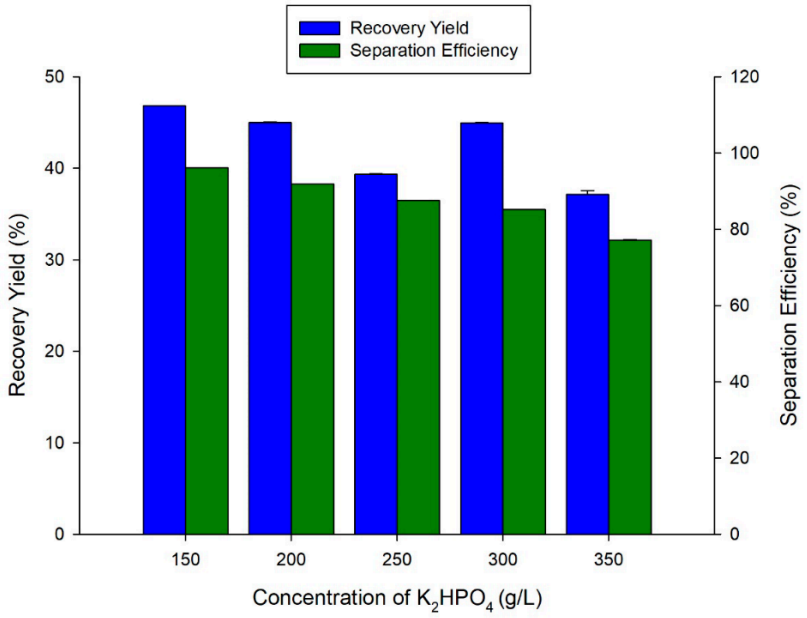

Figure 4. Figure illustrating effect of various K_2HPO_4 concentrations on protein recovery yield and separation efficiency using LBF system.

3.4. Effect of Different Concentrations of Ethanol on the Recovery of Proteins

The concentration of alcohol used will also affect the overall performance of the LBF system. Thus, the next parameter to be optimized is the concentration of alcohol. Various concentrations from 60% to 100% of ethanol were tested by using 15% (g/L) dipotassium hydrogen phosphate. As shown in Figure 5, 80% (*W*/*V*) shows the best recovery yield of 77.30%, followed by 70% of ethanol concentration which shows a yield of 54.50%. Concentrations of 60%, 90%, and 100% show 45.88%, 46.30%, and 41.48% yield, respectively. The protein recovery yield increases when the ethanol concentration increases, however when the alcohol concentration exceeds a certain point, the protein recovery yield starts to reduce. This is because the formation of the biphasic layers is weak when the concentration of alcohol is low. This result follows the trend in the previous study on protein recovery of wet microalgae using LBF where the highest protein recovery was obtained at 60% of 1-propanol, and the recovery yield decreased when the concentration of alcohol reduced below 40% [25]. These phenomena are due to the concentration of alcohol decreasing; more hydrophilic proteins can be dissolved into the top phase when the proteins are brought up by flotation air bubbles. However, when the concentration of alcohol decreases, the water at the top phase tends to migrate down to the bottom phase of the LBF system [16].

In terms of separation efficiency, ethanol solution with 80% concentration also has the highest separation efficiency, being at a value of 93.82%. The separation efficiency is followed by a pure ethanol solution which has a value of 90.59%. Values for 60%, 70%, and 90% are 69.16%, 54.17%, and 84.60%, respectively. The high value obtained at 80% of ethanol could be contributed due to cluster formation of ethanol. The number and size of the clusters strongly depend on the number of hydrogen bonds, and at higher concentration of ethanol, the cluster size is higher, which contributed to higher recovery of protein. Generally, ethanol has maximum viscosity of 75% to 80%, thus, this supports the high recovery of protein and separation efficiency. This indicates that at 80% of ethanol concentration, more proteins can be separated from the bottom phase which is the main point of this study. Therefore, 80% concentration ethanol solutions will be used in the following optimizations.

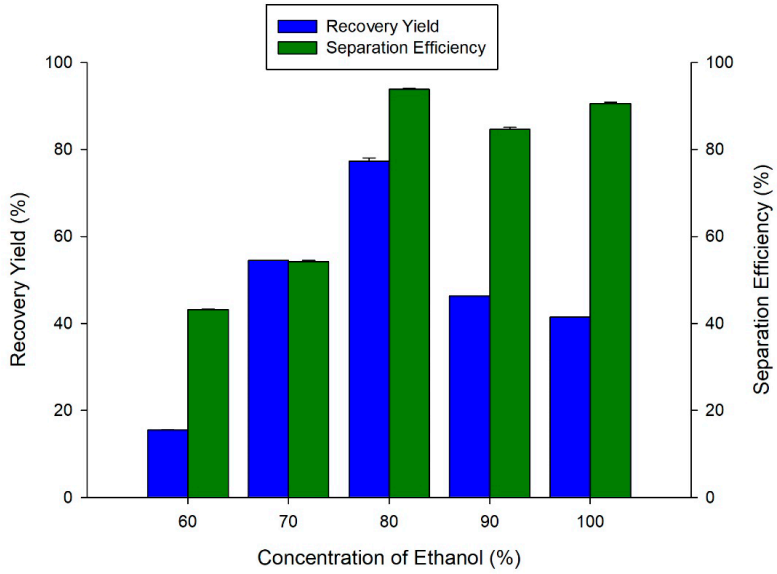

Figure 5. Figure showing effect of various concentrations of ethanol on protein recovery yield and separation efficiency using LBF system.

3.5. Effect of Various Concentrations of Milk

In this section, the effect of milk concentration of the bottom phase of the LBF system was studied. The concentration of milk may pose a potential effect on protein extraction by affecting the formation of the biphasic system, indicating that the concentration of milk used for extraction will have an impact on the yield of proteins recovered [26]. Milk with concentrations of 5% (*w*/*v*), 10% (*w*/*v*), 15% (*w*/*v*), 20% (*w*/*v*), and 25% (*w*/*v*) mixed along with dipotassium hydrogen phosphate solution with 150 g/L concentration were tested to investigate the effects of milk concentration on protein recovery. The highest protein yield obtained is at 10% (*w*/*v*), closely followed by 5% (*w*/*v*), which has the values of 93.96% and 93.75%, respectively. At 25% (*w*/*v*), the recovery yield of proteins is the lowest, having a value of 50.98%. At 15% (*w*/*v*) and 20% (*w*/*v*), it has values of 89.77% and 64.74%, respectively. According to Figure 6, it was observed that the concentration of milk has increased from 5% (*w*/*v*) to 10% (*w*/*v*), the recovery yield has only increased slightly; however, when the concentration is further increased, the yield starts to drop significantly, especially from 15% (*w*/*v*) to 20% (*w*/*v*), with a total drop of more than 25% protein yield. This is due to the fact that when a high concentration of milk is used, the salt solution mixture tends to form a liquid of which its high viscosity will result in the formation of flotation bubbles to be too difficult to control. By increasing the concentration of milk used, the performance of the LBF would be reduced as the level of impurities within the solution will also increase. The overall composition of the bottom phase will be altered as there is a lot of impurities in the milk [16,27]. In terms of separation efficiency, however, the highest value obtained is 94.02%, which is achieved by 25% (*w*/*v*). The separation efficiency gradually increases as the concentration is increased, starting from 5% (*w*/*v*), the separation efficiency was found to be 72.62%, 87.14%, 90.96%, and 92.59%, respectively. Due to the higher separation efficiency, 10% (*w*/*v*) of milk concentration was selected to carry on the following tests.

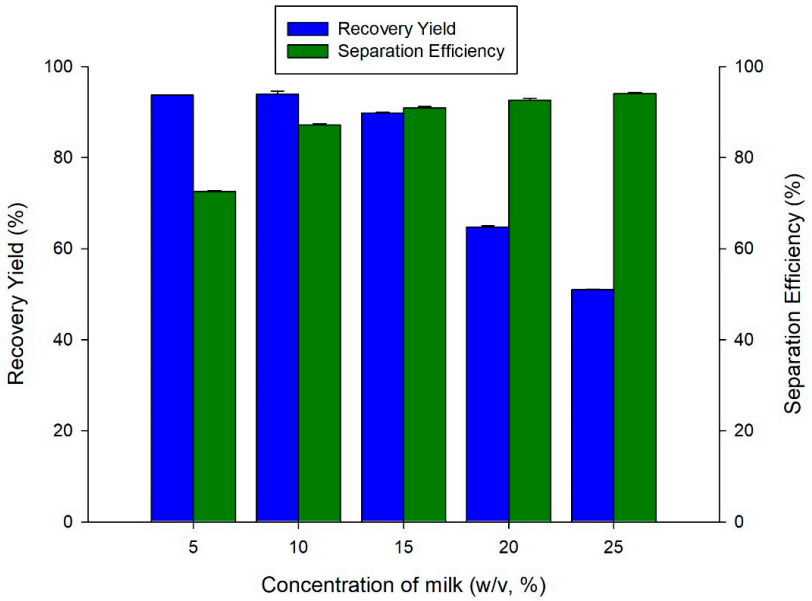

Figure 6. The effect of different concentrations of milk on the protein recovery yield and separation efficiency utilizing LBF system.

3.6. Effect of pH on the Recovery of Proteins

Impact of pH partitioning of proteins and enzymes to the phases in the LBF system depends on their isoelectric points. The pH of the system, however, affects the charge of target protein molecules and ionic composition, as well as introduces differential partitioning into the two phases. Most of the biomolecules, especially proteins and enzymes, are stable at neutral pH, which is a favorable condition to conduct the LBF partitioning. However, an increase in pH of the LBF from 7.0 to 9.0 reduced the protein recovery and activity recovered. Enzyme stability is slightly reduced in the acidic area except at the lowest pH and was dramatically lost at pH above 9.0. This dependence on pH for optimal protein recovery can be explained in terms of the charge in the protein. The protein in the LBF is predominantly casein. From the literature, isoelectric point of casein is 4.6, and since the pH of milk is 6.6, casein molecules are positively charged due to the protons provided by the milk medium. Given that the formula for pH is $pH = -log[H^+]$, where H^+ is concentration of hydrogen ions, a pH of 7 to 8 in the system (see Figure 7 corresponds to a negatively charged medium. With a positive charge, the casein molecules are thus hydrophobic, making them less soluble in water. Given that bubbles are used to push the particles up to the top phase and that the casein molecules are positively charged, the surface charge on the bubbles plays a vital role in the protein extraction efficiency. In particular, the charge of the casein and the surface charge of the bubble will be responsible for the adsorption of the protein molecule to the bubble surface. In order for the protein molecules to be attached to the bubble surface, the charge of the bubble surface must therefore be negative.

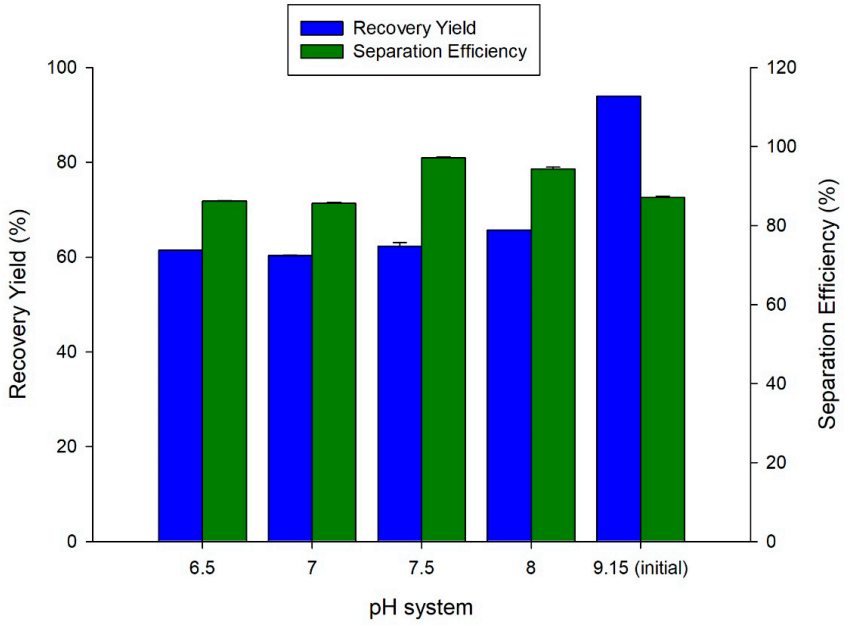

Figure 7. Figure showing the effect of pH system on protein recovery yield and separation efficiency.

To understand the mechanics behind the adsorption of the bubbles, we have developed a mathematical model with the use of partial differential equations (PDEs).

The region of interaction in our model is constructed using the same approach as the Lamm equations (Lamm O., 1929), which is to divide the volume of the container into sector-shaped cells. With reference to Figure 8 consider a region R in a sector-shaped cell within the chamber of the flotation system. Let M, M_{in}, and M_{out} be the mass of solute inside R, the mass flow into R, and the mass flow out of R, respectively. By the principle of conservation of mass,

$$\frac{\partial M}{\partial t} = \frac{\partial M_{in}}{\partial t} - \frac{\partial M_{out}}{\partial t} \tag{1}$$

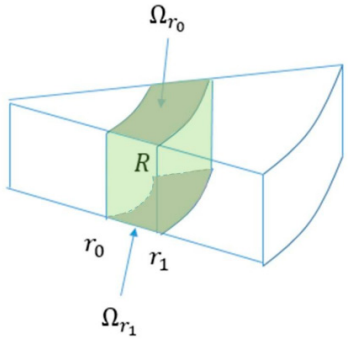

Figure 8. Region of collision of the protein particles against the bubbles, R.

Flux $\bar{J}$ is defined as the number of bubbles passing though an area A per unit time. Assuming no diffusion, i.e., only convection,

$$\bar{J} = \sigma \bar{s} \tag{2}$$

where σ and $\bar{s}$ are the density and velocity of the bubbles respectively.

Putting Equation (2) into (1) gives

$$\frac{\partial \sigma}{\partial t} = -\nabla \cdot \bar{J}. \tag{3}$$

The bubbles are assumed to move into the region R, resulting in a negative $\nabla \cdot \bar{j}$, and the negative sign is to make it positive.

Integrating,

$$\frac{\partial}{\partial t}\int_{h_0}^{h_1} \sigma(h,t)A(\Omega_h)dh = -\int_{h_0}^{h_1} \frac{\partial}{\partial h}[j(h,t)A(\Omega_h)]dh \tag{4}$$

where $\sigma(h,t)$ and $j(h,t)$ are density and magnitude of flux of bubbles respectively, $A(\Omega_h)$ is the area in the top and bottom surfaces of the region R (see Figure 1), and cylindrical coordinates are used: $r = \sqrt{x^2+y^2}$, $\phi = \tan^{-1}\frac{y}{x}$, $h = z$.

From Sminov and Berry (Smirnov et al., 2015), the velocity of the bubble is given by

$$s = \frac{2dg{r_b}^2}{9\nu} \tag{5}$$

where g is the free fall acceleration, d is the density difference for liquid and air inside bubbles or the liquid density, r_b is the radius of bubble, ν is the liquid viscosity.

From previous work (Lin YK, 2015), the number of bubbles that can be adsorbed to the surface of a bubble, N_p, is given by $N_p = \pi\left(\frac{r_b}{r_p}\right)^2$ where r_p is the radius of the particle.

For maximum adsorption, the number of particles in the region R must be $\pi\left(\frac{r_b}{r_p}\right)^2$ times the number of bubbles in the same region R, and assuming a very small R, all bubbles that go into the R will collide with all particles that go into R.

From Equation (4), substituting $A(\Omega_r) = h\phi r$ and rearranging gives

$$\int_{h_o}^{h_1} h\phi r\frac{\partial \sigma}{\partial t} + \frac{\partial \sigma}{\partial r}(h\phi r j)dh = 0 \tag{6}$$

From Equation (4),

$$\int_{z_0}^{z_1} \frac{\partial}{\partial t}[\sigma(z,t)A(\Omega_z)] + \frac{\partial}{\partial z}[j(z,t)A(\Omega_z)]dz = 0. \tag{7}$$

Putting $Area = r\phi(r_1 - r_0)$ into Equation (7) and integrated w.r.t. z gives

$$r\phi(r_1 - r_0)\frac{\partial}{\partial t}\sigma(z,t) + r\phi(r_1 - r_0)\frac{\partial}{\partial z}j(z,t) = 0$$

Rearranging, we have

$$\frac{\partial}{\partial t}\sigma(z,t) + \frac{\partial}{\partial z}j(z,t) = 0 \tag{8}$$

Putting $j(z,t) = \sigma s$ into Equation (8) yields

$$\frac{\partial}{\partial t}\sigma(z,t) + \frac{\partial}{\partial z}(\sigma s) = 0$$

$$\frac{\partial}{\partial t}\sigma(z,t) + s\frac{\partial}{\partial z}\sigma(z,t) + \sigma(z,t)\frac{\partial}{\partial z}s = 0 \tag{9}$$

Given that $s = \frac{2dg{r_b}^2}{9\nu}$, we can take $d \approx \rho$. We know that

$$P = \rho g h \tag{10}$$

where P is pressure of fluid, which is equal to the pressure in the bubble, ρ is density of fluid, g is acceleration due to gravity, and h is height. From Sminov and Berry,

$$P = P_{ex} + \frac{2\alpha}{r_b} \tag{11}$$

where P_{ex} is exteral pressure acting on liquid, α is the surface tension and is a constant, and r_b is the radius of the bubble. Combining Equations (10) and (11) gives:

$$r_b = \frac{2\alpha}{\rho g h - P_{ex}} \tag{12}$$

where P_{ex} is the pressure of air in the room (most likely atmospheric pressure, 1 atm).

Putting Equation (12) into Equation (6) gives:

$$s = \frac{2\rho g\left(\frac{2\alpha}{\rho g h - P_{ex}}\right)^2}{9\nu} = \frac{8\rho g}{9\nu}\left(\frac{\alpha}{\rho g z - P_{ex}}\right)^2 = f(z) \tag{13}$$

where, $h = z$.

Putting (13) into (9) gives:

$$\frac{\partial}{\partial t}\sigma(z,t) + \frac{8\rho g}{9\nu}\left(\frac{\alpha}{\rho g z - P_{ex}}\right)^2 \frac{\partial}{\partial z}\sigma(z,t) - \frac{16(\rho g)^2\alpha^2}{9\nu(\rho g z - P_{ex})}\sigma(z,t) = 0 \tag{14}$$

$$\frac{\partial}{\partial t}\sigma(z,t) + f(z)\frac{\partial}{\partial z}\sigma(z,t) + \sigma(z,t)\frac{d}{dz}f(z) = 0 \tag{15}$$

$$f(z)\sigma(z,t)_z + \sigma(z,t)_t + F(z)\sigma(z,t) = 0 \tag{16}$$

where, $F(z) \equiv \frac{d}{dz}f(z)$.

To solve Equation (16), Laplace transform is applied to Equation (16), giving

$$f(z)\overline{\sigma}_z(z,s) + s\overline{\sigma}(z,s) - \sigma(z,0) + F(z)\overline{\sigma}(z,s) = 0$$

$$f(z)\overline{\sigma}_z(z,s) + [s + F(z)]\overline{\sigma}(z,s) - \sigma(z,0) = 0 \tag{17}$$

Since $\sigma(z,0) = 0$, Equation (17) becomes

$$f(z)\overline{\sigma}_z(z,s) + [s + F(z)]\overline{\sigma}(z,s) = 0 \tag{18}$$

To solve Equation (18), we divide Equation (18) by $f(z)$ to give

$$\overline{\sigma}_z(z,s) + \frac{s + F(z)}{f(z)}\overline{\sigma}(z,s) = 0 \tag{19}$$

Multiplying Equation (19) with integrating factor $e^{\int \frac{s+F(z)}{f(z)}dz}$ yields

$$\frac{d}{dz}\left[e^{q(z,s)}\overline{\sigma}(z,s)\right] = 0 \tag{20}$$

where,

$$q(z,s) = \frac{9s\nu}{8\rho g\alpha^2}\left(\frac{\rho^2 g^2 z^3}{3} - \rho g z^2 P_{ex} + (P_{ex})^2 z\right) + ln\left|\frac{8\rho g\alpha^2}{9\nu(\rho g z - P_{ex})^2}\right| \tag{21}$$

Integrating Equation (20) gives

$$\overline{\sigma}_z(z,s) = Ce^{Q(z,s)} \tag{22}$$

where C is a constant, and $Q(z,s) = q(z,s)^{-1}$. Transforming Equation (22) from the s domain back to the t domain yields

$$L^{-1}\{\overline{\sigma}_z(z,s)\} = \sigma_z(z,t) = \delta\left[t - \frac{9\nu}{8\rho g a^2}\left(\rho g z^2 P_{ex} - (P_{ex})^2 z - \frac{\rho^2 g^2 z^3}{3}\right) - ln\left|\frac{8\rho g a^2}{9\nu(\rho g z - P_{ex})^2}\right|\right] \quad (23)$$

where L is Laplace transform.

From Equation (23), for the bubbles to hit all protein molecules, a sufficient amount of time is required. In particular, the Dirac delta function in Equation (23) indicates that for maximum protein extraction, $t \sim O\left(z^2\right)$. Thus, with a uniform distribution of bubble holes at the bottom of the chamber, the time it takes to collect all molecules increases as the square of the height of the chamber. This explains the increasing amount of time required for higher protein collection in Figure 9, which shows both the theoretical and experimental data. It can be seen from this figure that the experimental data is in agreement with the theoretical data for flotation time less than 8 min. The reduction in yield after 8 min can be attributed to the lack of protein in the system after prolonged extraction.

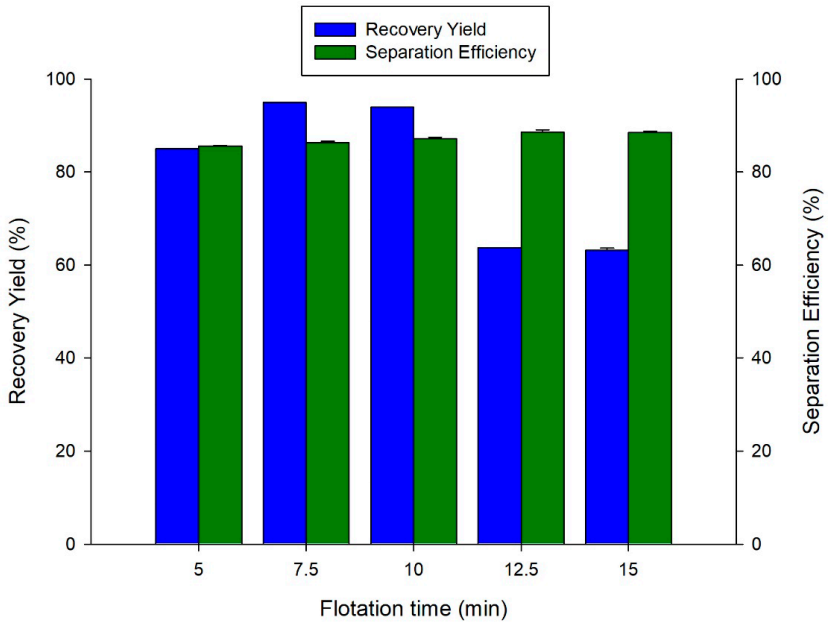

Figure 9. Figure illustrating the effect of flotation time on protein recovery yield and separation efficiency.

Besides allowing for a better understanding of the mechanism behind the system, this model also serves to provide some insights on design of efficient flotation systems at industrial scale. In particular, since it has been mathematically shown that an increase in height of the chamber drastically increases the flotation time, future chambers of flotation should preferably be as low as industrially viable.

The effect of pH has been studied by altering the pH of the bottom phase by using 1M hydrochloric acid. The initial pH of the bottom phase is 9.15, the tested pH is 6.5, 7.0, 7.5, and 8.0. The acid is added drop by drop until the bottom phase reached the desired pH with the aid of a pH meter. The highest recovery yield of proteins is obtained by the solution to which hydrochloric acid has not been added, which is the solution with a pH of 9.15. The recovery yield of the solution with this pH is at 93.96%, surpassing the second highest, which is a pH of 8.0 with a value of 65.71%, by more than 28%. The other three pH, being 6.5, 7.0, and 7.5, each has a recovery yield of 61.45%, 60.37%, and 62.33%, respectively. The big difference in yield is caused by the hydrochloric acid used to alter the pH having denatured the proteins, thus greatly reducing the yield of the LBF [24]. As the acid was added drop by drop, each time a drop of acid hits the surface of the bottom phase, the extreme pH of the acid will denature some of the proteins at the bottom phase before being diluted by the rest of the solution at this phase. This results in the big difference in the recovery yield when comparing between

a bottom phase of pH 9.15 and 8.0; this is also why the difference in recovery yield between pH 8 and pH 6.5 does not show a big difference when compared with 9.15 and 8.0. The separation efficiency increases when the pH is changed from neutral to 6.5, which is from 85.59% to 86.21%. At a pH of 7.5, the separation efficiency is the highest which is at 97.18%. The separation efficiency of pH 8.0 and 9.15 is 94.33% and 87.14%, respectively. Due to the above reasons, the following test was conducted without altering the pH system.

3.7. Effect of Flotation Time on the Recovery of Proteins

The duration of the flotation process being conducted is very important as it could cause a major impact on the area of air–water interface per unit volume of aqueous solution in time [27]. Flotation times of 5, 7.5, 10, 12.5, and 15 min are tested for this study. The highest recovery yield of proteins is obtained when the system is run for 7.5 min, having a yield of 94.97%, closely followed by a flotation time of 10 min having a yield of 93.96%. With a flotation time of 5, 12 and 15 min, the yield is 85.03%, 63.76%, and 63.24%, respectively. The yield of proteins obtained shows an increasing trend when the flotation time is increased; however, it starts to decrease when the flotation time exceeds 10 min. This is because molecules other than proteins is being blown to the top phase, causing the overall concentration of the proteins at the top phase to be reduced. This is proven as the volume of the bottom phase shows a decreasing trend when the flotation time is increased, decreasing from 4 mL for 5 min flotation time to 2.5 mL for 15 min flotation time. In terms of separation efficiency, the values show a general increasing trend, but the increase in efficiency is only by a small amount, starting from 5 min flotation time, the separation efficiency is 85.58%, 86.29%, 87.20%, 88.64%, and 88.46%, respectively. Due to the reasons stated above, a flotation time of 7.5 min is taken to be optimum for this study. In general, the recovery of protein from the beginning of the experiment is low, however; it increases as the optimization is carried out. The low in recovery may be due to high contamination of bacteria that could affect the recovery of protein.

3.8. Effect of Scaling up LBF for Industrial Application Purposes

For industrial reasons, this experiment was tested again under large scale conditions. The experiment was scaled up by 40 times (from 30 mL to 1.2 L), and the results show that this experiment is suitable to be scaled up, with a recovery yield of more than 70% and a separation efficiency of more than 80%. This indicates that LBF is suitable to be commercialized as a method to separate proteins from expired milk wastes.

In this study, several parameters and their impacts on the LBF system have been studied by using the one-factor-at-a-time approach. Given that there might be a possibility that there will be interaction effects between several parameters, further studies on interaction factors such as milk concentration and flotation time could have a high chance of further optimizing this process. There is also a need to further improve this method so that protein recovery rate may be increased. An air compressor that can achieve higher and more accurate flowrates can be used in future LBF experiments. Furthermore, different types of gases can be used in replacement of atmospheric air for flotation. Different types of gases may help to bring up proteins or have other interactions with proteins that can improve the protein recovery yield. Thus, the effects of different types of gases such as pure oxygen or pure nitrogen can be tested to improve the recovery yield of the protein. Besides, the liquid used as top phase can be changed to different materials as using large amount of alcohol in industrial scale is a safety hazard. Alternative materials such as other organic solvents can be considered as an alternative to alcohol. Another aspect that is worth mentioning is the brand of milk used. As each brand of milk has a different formula for the milk they produce, changing the brand of milk used may also improve the protein recovery yield as there might be some components in milk from other brands that help in protein separation. Moreover, milk of different expiry dates can also be tested as well, as the level of microorganisms inside the milk may differ as time progresses. Further studies can be carried out by

comparing milk of different expiry dates and some milk that is close to their expiry date so that the effects are clear.

4. Conclusions

The parameters for protein extraction from expired dairy products were optimized in this study. The effects of the type of inorganic salt used, the type of alcohol used, the concentration of salt used, the concentration of alcohol used, the concentration of raw material (milk) used, pH of the bottom phase, and the flotation time of the LBF system were discussed. The optimum conditions for protein extraction from dairy wastes tested in this study were found to be 150 g/L dipotassium hydrogen phosphate, 80% of ethanol, 10% (*w*/*v*) milk, a pH system of 9.15 (initial pH), and a flotation time of 7.5 min. The final protein recovery yield and separation efficiency after optimization were 94.97% and 86.29%, respectively. A scaling up of the LBF system was also performed at a factor of 40 times, and the protein recovery yield and separation efficiency for this test were 78.92% and 85.62% respectively. This study showed that proteins can be extracted from dairy waste effectively. The advantages of this novel approach include providing a use for expired milk products, reducing wastes being thrown away and benefiting the environment, and turning waste that once needed money to be disposed of into a raw material that can provide profit. Additionally, the utilization of high concentration of alcohol and salt will help inhibit further contamination by bacteria as they cannot survive in high alcohol and salt concentration environments. The concern with environmental impact due to high salt and alcohol concentration can be avoided by studying the recycling ability of the phase components. Studies have shown that there is a great potential to reuse recycling phase components in the subsequent extraction of LBF. Future studies on the interaction of parameters and methods for recycling the top and bottom phases after separation will provide a great opportunity for future industries to apply this method as a waste treatment process.

Author Contributions: Conceptualization, K.W.C.; data curation, P.E.T., Y.J.N., and K.W.C.; formal analysis, P.E.T. and Y.J.N.; funding acquisition, P.L.S.; methodology, R.S. and K.W.C.; supervision, K.W.C. and P.L.S.; visualization, K.S.K.; writing—original draft, P.E.T. and Y.J.N.; writing—review and editing, R.S., K.S.K., Y.J.Y., M.M., F.A.A.Z., and P.L.S.

Funding: This research was supported financially by SATU Joint Research Scheme (ST014-2018, ST022-2019), Geran Penyelidikan Universiti Malaya (UMRG Programme)—SUS (Sustainability Science) (RP025B-18SUS) and Fundamental Research Grant Scheme (Malaysia FRGS/1/2019/STG05/UNIM/02/2).

Acknowledgments: This study is supported by the Fundamental Research Grant Scheme (Malaysia, FRGS/1/2019/STG05/UNIM/02/2).

Conflicts of Interest: The authors declare that they have no conflicts of interests.

References

1. Milk Waste Produced. Available online: http://www.wrap.org.uk/content/wrap-comes-winning-formula-tackle-milk-waste (accessed on 9 July 2019).
2. Wasted Milk Is a Drain on Resources. Available online: https://www.ed.ac.uk/news/all-news/140512-climate (accessed on 9 July 2019).
3. American Association of Cereal Chemist. *Properties of Milk and Its Components*; American Association of Cereal Chemists: St. Paul, MN, USA, 1997; Volume 1, pp. 1–10.
4. Davoodi, S.H.; Shahbazi, R.; Esmaeili, S.; Sohrabvandi, S.; Mortazavian, A.; Jazayeri, S.; Taslimi, A. Health-Related Aspects of Milk Proteins. *Iran. J. Pharm. Res.* **2016**, *15*, 573–591. [PubMed]
5. Reen, C.S.; Wayne, C.K.; Loke, S.P.; Manickam, S.; Chuan, L.T.; Yang, T.A.O. Isolation of protein from Chlorella sorokiniana CY1 using liquid biphasic fl otation assisted with sonication through sugaring-out effect. *J. Oceanol. Limnol.* **2019**, *37*, 898–908.
6. Wayne, C.K.; Reen, S.; Krishnamoorthy, R.; Tao, Y.; Chu, D. Liquid biphasic flotation for the purification of C-phycocyanin from Spirulina platensis microalga. *Bioresour. Technol.* **2019**, *288*, 121519.
7. Bian, H.; Li, J.; Zhang, Q.; Chen, H.; Zhuang, W.; Gao, Y.Q.; Zheng, J. Ion Segregation in Aqueous Solutions. *J. Phys. Chem. B* **2012**, *116*, 14426–14432. [CrossRef] [PubMed]

8. Yuzugullu, Y.; Duman, Y.A. Aqueous Two-Phase (PEG4000/Na2SO4) Extraction and Characterization of an Acid Invertase from Potato Tuber(Solanum tuberosum). *Prep. Biochem. Biotechnol.* **2015**, *45*, 696–711. [CrossRef] [PubMed]
9. Show, P.L.; Ooi, C.W.; Anuar, M.S.; Ariff, A.; Yusof, Y.A.; Chen, S.K.; Annuar, M.S.; Ling, T.C. Recovery of lipase derived from Burkholderia cenocepacia ST8 using sustainable aqueous two-phase flotation composed of recycling hydrophilic organic solvent and inorganic slat. *Sep. Purif. Technol.* **2013**, *110*, 112–118. [CrossRef]
10. Lee, S.Y.; Khoiroh, I.; Ling, T.C.; Show, P.L. Aqueous Two-Phase Flotation for the Recovery of Biomolecules Aqueous Two-Phase Flotation for the Recovery of Biomolecules. *Sep. Purif. Rev.* **2015**, *45*, 81–92. [CrossRef]
11. Albertsson, P. Partition of cell particles and macromolecules in polymer two-phase systems. *Adv. Protein Chem.* **1970**, *24*, 309–341. [PubMed]
12. Ying, S.; Loke, P.; Chuan, T.; Chang, J. Single-step disruption and protein recovery from Chlorella vulgaris using ultradonication and ionic liquid buffer aqueous solutions as extractive solvents. *Biochem. Eng. J.* **2017**, *124*, 26–35.
13. Asenjo, J.A.; Andrews, B.A. Aqueous two-phase systems for protein separation. Phase separation and applications. *J. Chromatogr. A* **2012**, *1238*, 1–10. [CrossRef] [PubMed]
14. Wingfield, P.T. Protein precipitation using ammonium sulfate. *Curr. Protoc. Protein Sci.* **1998**, *13*, A.3F.1–A.3F.8.
15. Lu, Y.; Yu, M.; Tan, Z.; Yan, Y. Phase Equilibria and Salt Effect on the Aqueous Two-Phase System of Polyoxyethylene Cetyl Ether and Sulfate Salt at Three Temperatures. *J. Chem. Eng. Data* **2016**, *61*, 2135–2143. [CrossRef]
16. Sankaran, R.; Show, P.L.; Cheng, Y.S.; Tao, Y.; Ao, X.; Nguyen, T.D.; Van Quyen, D. Integration Process for Protein Extraction from Microalgae Using Liquid Biphasic Electric Flotation (LBEF) System. *Mol. Biotechnol.* **2018**, *60*, 749–761. [CrossRef] [PubMed]
17. Properties of Ethanol. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Ethanol#section=Density (accessed on 12 July 2019).
18. Properties of Ethanol. Available online: https://pubchem.ncbi.nlm.gov/compound/Ethanol#section=Computed-Properties (accessed on 12 July 2019).
19. Properties of 1-Propanol. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/1031#section=Density (accessed on 17 July 2019).
20. Properties of Isopropyl. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Isopropyl-alcohol#section=Density (accessed on 17 July 2019).
21. Properties of 1-Propanol. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/1031#section=Computed-Properties (accessed on 17 July 2019).
22. Properties of 2-Propanol. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Isopropyl-alcohol#section=Computed-Properties (accessed on 12 July 2019).
23. Mao, Y.; Sheng, X.; Pan, X. The effects of NaCl concentration and PH on the stability of hyperthermophilic protein Ssh10b. *BMC Biochem.* **2007**, *8*, 1–8. [CrossRef] [PubMed]
24. Phong, W.N.; Show, P.L.; Teh, W.H.; Teh, T.X.; Lim, H.M.; binti Nazri, N.S.; Tan, C.H.; Chang, J.S.; Ling, T.C. Proteins recovery from wet microalgae using liquid biphasic flotation. *Bioresour. Technol.* **2017**, *244*, 1329–1336. [CrossRef] [PubMed]
25. Ling, P.; Suffian, M.; Annuar, M.; Loke, P.; Chuan, T. Extractive bioconversion of poly-caprolactone by Burkholderia cepacia lipase in an aqueous two-phase system. *Biochem. Eng. J.* **2015**, *101*, 9–17.
26. Ooi, C.W.; Tey, B.T.; Hii, S.L.; Ariff, A.; Wu, H.S.; Lan, J.C.W.; Juang, R.S.; Kamal, S.M.M.; Ling, T.C. Direct purification of Burkholderia Pseudomallei lipase from fermentation broth using aqueous two-phase systems. *Biotechnol. Bioprocess. Eng.* **2009**, *101*, 811–818. [CrossRef]
27. Khoo, K.S.; Chew, K.W.; Ooi, C.W.; Ong, H.C.; Ling, T.C.; Show, P.L. Extraction of natural astaxanthin from Haematococcus pluvialis using liquid biphasic flotation system. *Bioresour. Technol.* **2019**, *290*, 121794. [CrossRef] [PubMed]