RESEARCH ARTICLE

Optimization of protein extraction from *Chlorella Vulgaris* via novel sugaring-out assisted liquid biphasic electric flotation system

Apurav Krishna Koyande¹ | Kit Wayne Chew² | Jun-Wei Lim³ | Sze Ying Lee⁴ |
Man Kee Lam⁵ | Pau-Loke Show¹

¹Department of Chemical Engineering,
University of Nottingham Malaysia, Selangor
Darul Ehsan, Malaysia

²School of Mathematical Sciences, University
of Nottingham Malaysia, Selangor Darul
Ehsan, Malaysia

³Department of Fundamental & Applied
Sciences, Universiti Teknologi PETRONAS,
Perak, Malaysia

⁴Department of Chemical Engineering,
Universiti Tunku Abdul Rahman, Selangor
Darul Ehsan, Malaysia

⁵Department of Chemical Engineering,
Universiti Teknologi PETRONAS, Perak,
Malaysia

Correspondence

Dr. Pau-Loke Show, Department of Chemical Engineering, University of Nottingham Malaysia, 43500 Selangor Darul Ehsan, Malaysia.

Email: showpauloke@gmail.com

Abstract

Microalgae biomass has been consumed as animal feed, fish feed and in human diet due to its high nutritional value. In this experiment, microalgae specie of *Chlorella Vulgaris* FSP-E was utilized for protein extraction via simple sugaring-out assisted liquid biphasic electric flotation system. The external electric force provided to the two-phase system assists in disruption of rigid microalgae cell wall and releases the contents of microalgae cell. This experiment manipulates various parameters to optimize the set-up. The liquid biphasic electric flotation set-up is compared with a control liquid biphasic flotation experiment without the electric field supply. The optimized separation efficiency of the liquid biphasic electric flotation system was $73.999 \pm 0.739\%$ and protein recovery of $69.665 \pm 0.862\%$ compared with liquid biphasic flotation, the separation efficiency was $61.584 \pm 0.360\%$ and protein recovery was $48.779 \pm 0.480\%$. The separation efficiency and protein recovery for $5 \times$ time scaled-up system was observed at $52.871 \pm 1.236\%$ and $73.294 \pm 0.701\%$. The integration of simultaneous cell-disruption and protein extraction ensures high yield of protein from microalgae. This integrated method for protein extraction from microalgae demonstrated its potential and further research can lead this technology to commercialization.

KEYWORDS

glucose, liquid biphasic electric flotation system, protein recovery, separation efficiency, sugaring-out

1 | INTRODUCTION

Microalgae are photosynthetic unicellular organisms which are capable of rapid growth and survival at harsh conditions [1]. Compared to vegetable and other source of proteins, microalgae biomass contains high amount of protein content. The microalgae species of *Spirulina* and *Chlorella* are noted to contain more than 70% of their dry matter as proteins [2].

Due to such high protein content of microalgae, their biomass has attracted attention by various industries such as poultry, aquaculture, animal feed, etc. The World Health Organization (WHO) also recommends consumption of microalgae biomass of *Chlorella* species in human diet due to its complete essential amino acids profile [3]. Protein extraction from microalgae is currently carried out via expensive separation processes such as crystallization, column chromatography, ultrafiltration, membrane separation, and precipitation. These conventional processes are time consuming, complicated due to several steps, require high maintenance cost, and are

Abbreviations: LBEF, liquid biphasic electric flotation; LBF, liquid biphasic flotation; SE, separation efficiency.

difficult to scale-up. On the other hand, liquid biphasic flotation (LBF) is a novel upcoming technology which is capable of replacing the conventional technologies [4]. LBF system is a combination of solvent sublation (SS) and aqueous two phase system (ATPS). In the LBF system, the air bubbles provided at the bottom of the set-up adsorb the bio-active components of the bottom phase. They are transported to the top phase by the rising gas bubbles. This novel method has been utilized at experimental scale for extraction of various bio-active components such as betacyanins [5], lipase [6], bacteriocin [7], protein [8,9], etc. The major advantages of utilizing LBF process are that it is a simple, economical technique with high SE and concentration co-efficient. It is also cost-effective compared to other conventional techniques due to its recyclable nature [10].

The concept of LBF system is similar to the aqueous two phase flotation (ATPF) system which depends on the salting-out effect for the separation of bio-active components. However, in this experiment, a novel of sugaring-out method is explored. This method is specifically designed to counter the difficulties faced in the salting-out experiment. In the sugaring-out effect, the bio-active component are reported to preserve their properties and structure due to the lower influence of sugar solution on the target products [11]. The introduction of sugar instead of salt solution also ensures that there is no pH change in the system and equipment are not damaged due to corrosion [12]. In sugaring-out system, the top organic phase consists of ACN instead of conventional alcohols. ACN is capable of rapid two-phase formation while providing stable and friendly environment for target bio-molecules. Studies have been conducted with different organic solvents, however, ACN has been most promising of all as it is miscible with water at all concentrations and thus potentially extract wide range of biomolecules [13]. Additionally, ACN is less harmful compared to other solvents used in traditional liquid-liquid extraction systems, therefore it is considered more favourable.

The LBF system with sugaring-out effect is solely not efficient in extraction of protein from microalgae. The microalgae cells are surrounded by a rigid cell wall which ensures that the valuable bio-active compounds present in the cytoplasm and other organelles are preserved [14]. Hence, to efficiently extract proteins from microalgae, a pretreatment for disrupting the cell wall is necessary. It is essential to select the suitable cell disruption technique to be coupled with optimum extraction method to optimize the protein recovery from the microalgae while avoiding the contamination of the target components. There are many reports regarding increase in protein recovery due to pretreatment of microalgae with various cell disruption techniques such as pulsed electric field [15], ultra-sonication [16], and high voltage electrical discharges [17]. The disruption methods need to be chosen as such that the downstream processing of the extracted

PRACTICAL APPLICATION

The affordable protein production from microalgae is a bottleneck that needs to be resolved to manipulate the unicellular micro-organisms to its full potential. Complicated downstream processing of the harvested microalgae biomass with numerous time-consuming, expensive operating procedures are few hurdles that prevent commercialization of microalgae derived proteins. These obstacles can be tackled by adopting a novel liquid biphasic electric flotation system. This integrated system disrupts the rigid cell wall, releases bio-active components in the surrounding environment, and simultaneously extracts the proteins to the top phase. Due to its rapid two-phase formation, the required bio-active components can be extracted from the cell debris easily. The positive results of the scale-up model hints towards a potential high capacity prototype and possibly industrial commercialization. Hence, this simple, fast, stable, and scalable integrated protein extraction system should be explored for further applications.

components should not be affected by it. This experiment utilizes electrolysis as a cell disruption method for microalgae. In this method, electric field is passed through the system to initiate movement of negatively charged microalgae components to the anode and rise up to the top solvent phase via gas bubbles [10].

In the current experiment, the cell-disruption method of electrolysis was integrated with LBF system to extract proteins from *Chlorella Vulgaris* FSP-E biomass. The main aim of this study is to optimize the operating parameters of the integrated liquid biphasic electric flotation (LBEF) system. The parameters manipulated include the microalgae biomass loading, voltage of the DC electric supply, concentration and type of sugar, concentration of ACN, air flowrate, and duration of flotation system. The system was compared with control LBF system and scalable nature of the system was also investigated.

2 | MATERIALS AND METHODS

2.1 | Materials

The chemicals required for this experiment, namely, glucose, sucrose, maltose, fructose, xylose, ACN, and Bradford's reagent were purchased from R&M Chemicals, Malaysia. The bovine serum albumin (BSA) protein required for protein assay was also purchased from R&M Chemicals (Selangor,

Malaysia). All the purchased chemicals were of analytical grade.

2.2 | LBF equipment set-up

The apparatus for LBF System of 500 mL was obtained from Donewell Resources of Puchong, Malaysia. The LBF set-up consists of a glass cylinder with inner diameter of 7 cm, height of 15 cm, and volume of 500 mL. At the bottom of the unit, a hole was drilled to connect 50 mL centrifugal tube. The air bubble filter required for flotation in the column is installed at the bottom of the cylinder. It is a sintered disk of G4 porosity with pore size of 10 μm . Compressed air was supplied through the bottom of the unit with the help of an air compressor. The flowrate of compressed air was controlled with a flowmeter (model: RMA-26-SSV, Dwyer, USA) of range between 50–200 cc/min.

2.3 | Microalgae strain and medium selection

Chlorella Vulgaris FSP-E algal strain was obtained from National Cheng Kung University of Taiwan. This specific strain was selected due to its high protein percentage (58%) as dry matter [18]. The *C. Vulgaris* FSP-E microalgae was pre-cultured using BG-11 medium for 7 days followed by batch culture for 14 days. The microalgae biomass was harvested after stagnant growth rate. The BG-11 medium consists of NaNO_3 as 1.5 g/L, K_2HPO_4 as 0.03 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ as 0.075 g/L, $\text{C}_6\text{H}_8\text{O}_7$ as 0.006 g/L, Na_2CO_3 as 2 g/L, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ as 3.6 g/L, $\text{FeH}_4\text{N}_5\text{O}_{12}$ as 0.6 g/L, EDTA as 0.1 g/L, H_3BO_3 as 2.86 g/L, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ as 1.81 g/L, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ as 0.222 g/L, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ as 0.39 g/L, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ as 0.079 g/L, and $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ as 0.049 g/L. To prevent contamination, the initial pre-culture and the following batch-culture was carried out in the laminar flow chamber.

2.4 | Microalgae biomass cultivation

The cultivation of microalgae was carried out in laboratory using a small-scale photo bioreactor consisting of glass vessels with 1 L working volume. The photo bioreactor was covered with artificial lights with illumination of 200 $\mu\text{mol}/\text{m}^2/\text{s}$. The temperature was fixed at around $25 \pm 1^\circ\text{C}$. The culture medium was constantly aerated at 400 mL/min with mixture of CO_2 and air. The mixture was agitated at 450 rpm and the cultivation was carried out for 14 days. The biomass was obtained from the broth using a centrifugation at 6500 rpm for 5 min using a centrifuge (Eppendorf, 5430). The supernatant was discarded and the remaining paste was freeze dried for 36 h. The dried biomass was stored at -20°C .

2.5 | Determination of microalgae concentration

The microalgae concentration determination for cell growth in the culture was carried out daily by using optical density of the obtained sample. The culture broth was diluted ten times and mixed well in the cuvette. The mixture was analyzed in UV-Vis Spectrophotometer (UV-1800, Shimadzu) at wavelength of 680.8 nm.

2.6 | LBEF integration

The LBF set-up mentioned in Section 2.2 is integrated to equip two carbon electrodes (diameter 10 mm). These electrodes are attached to an insulated polystyrene ring which rests on top of the LBF cylinder. The electrodes are placed 2 cm apart. The electric current was provided by a GW GPS-3030 single DC (0–30 V/3 A) power supply to two carbon electrodes which acted like anode and cathode, respectively. The two lead electrodes were purchased from BTSCIENCE SDN BHD, Selangor, Malaysia.

The dried microalgae biomass utilized in this experiment was first weighed to desired amount. It was then dissolved in the prepared sugar solution of desired concentration. The solution was mixed well and then added to the LBEF unit. This serves as the bottom phase. The ACN solution of desired concentration was added to form the top phase of the biphasic system. Compressed air of desired flowrate was introduced to the system. The two electrodes were placed in the LBF unit and DC power supply of fixed voltage was provided. This initiated the experiment. The experiment was conducted for pre-determined duration. At the end of the experiment, the two phase solution was transferred to a measuring cylinder to note down the volume of top and bottom phase. The experimental set-up for the experiment is shown in Figure 1. A 10 mL sample was collected from top and bottom phase for analysis and the experiment was carried out in triplicate.

2.7 | Protein assay

The protein content in each sample was determined by Bradford's method using a BSA standard solution. The collected bottom and top phase samples of 0.2 mL were mixed with 2.5 mL of Bradford reagent. The mixture was analyzed in UV-Vis Spectrophotometer (UV-1800, Shimadzu) at 595 nm wavelength after 10 min. The obtained results were compared with standard calibration curve of BSA protein.

2.8 | Operating parameters and optimization of LBEF system

The protein extraction via LBF and electrolysis was carried out simultaneously. The method implemented here is sugaring-out instead of the conventional salting-out method.

FIGURE 1 The set up for liquid biphasic electric flotation system

The introduction of electric current promotes cell disruption and cell contents are released effectively including the proteins. The continuous flotation will transfer these proteins from bottom phase to the top phase by two phase partitioning and can be extracted easily. The method employed for optimization of protein extraction is One Variable At a Time (OVAT). The parameters manipulated in this study include microalgae biomass loading, voltage of DC current provided, position of electrodes, concentration and type of sugar, concentration of ACN, air flowrate, and flotation time of the experiment. The initial working parameters were fixed at working volume of aqueous solution of 150 mL, volume of ACN at 150 mL. The biomass amount was chosen as 0.1 g and the sugar concentration of 150 g/L. The flotation system was operated with air flowrate of 75 cc/min for 10 min. The air was filtered using a 0.2 μ m filter. The DC electric supply to the electrodes was 5 V with the tip of electrodes in the bottom phase. After manipulating all the variables, a comparison study between LBF and LBEF was conducted.

2.9 | Determination of separation efficiency and protein recovery (yield)

The results were analyzed using separation efficiency and protein recovery/yield to optimize each parameter. The separation efficiency (SE) is defined as yield in terms of concentration in the top phase. It is calculated by

$$SE = \left[1 - \frac{C_P}{C_{Pi}} \right] \times 100\%$$

where SE is separation efficiency, C_P is protein concentration at bottom phase at end of the experiment and C_{Pi} is protein concentration of the initial crude microalgae.

The protein recovery or yield is calculated by

$$Y_P = \frac{P_T}{P_i} \times 100\%$$

where Y_P is protein recovery, P_T is total protein recovered in top phase, and P_i is initial protein content in the crude microalgae feedstock.

The overall protein in the crude feedstock was determined by following equation:

$$P_i = M_i \times Nea \times NTP$$

where M_i is microalgae biomass loading in grams, Nea is percentage of total nitrogen in *C. vulgaris* via elemental analysis, and NTP corresponds to the total nitrogen to protein conversion factor. The values for Nea and NTP were calculated as 7.81% and 6.35, respectively [19,20].

2.10 | Statistical analysis

The data presented was average value of triplicates of each parameter. The values are depicted as mean $\pm$ SD. The triplication of each parameter was conducted to validate the data obtained. Further, a one-way ANOVA was conducted on the data obtained via Microsoft Excel to assess the sum of differences and confirm that $p \leq 0.05$.

3 | RESULTS AND DISCUSSION FOR OPTIMIZATION OF LBEF SYSTEM

3.1 | Influence of microalgae biomass loading on the LBEF system

The crude microalgae biomass loading was investigated first to determine the biomass loading for determining the critical loading level of the microalgae in the LBEF system. It is noted that, biomass loading potentially affects two phase formation thus affecting the total protein yield [21]. Previous reports also suggest that crude biomass loading of greater than 5% w/w result in decrease in protein separation efficiency [22]. To evaluate the biomass loading on the system, all the other parameters were fixed at a constant and the microalgae biomass content was tested between 0.05 and 0.5 g. This range is between the critical threshold quantities of 5% w/w necessary for optimum two-phase formation. Figure 2 illustrates the separation efficiency and protein recovery of varying biomass loading.

FIGURE 2 Effect of biomass loading in LBEF system with 150 mL of 100% ACN, 150 mL of glucose solution with concentration of 150 g/L, air supply of 75 cc/min with flotation time of 10 min and DC supply of 5 V with tip of the electrode in the bottom phase

From Figure 2, it can be noted that the separation efficiency of LBEF system steadily decreases from 0.05 to 0.3 g. However, in case of protein recovery, there is steep decline from 0.05 to 0.1 g. The protein recovery remains in the same range until 0.3 g biomass loading and after 0.3 g there is a sharp decline in the yield. This is mainly attributed to the increase in viscosity of the bottom phase corresponding to higher biomass loading. The flotation efficiency of the system decreases due to increase in the viscosity of the bottom phase. Therefore, yield is reduced considerably. The highest SE and Y_p of $55.452 \pm 0.815\%$ and $30.208 \pm 1.276\%$ was noted for biomass loading of 0.05 g, respectively. The biomass loading of 0.1 g and 0.2 g have similar separation efficiencies at $51.277 \pm 0.167\%$ and $51.072 \pm 0.625\%$, respectively. However, the corresponding protein recovery is lower at $18.796 \pm 0.278\%$ and $18.453 \pm 1.042\%$, respectively. The decrease in protein recovery is noted due to the increase in impurities and contaminants in the aqueous system at higher biomass loading [23]. In addition to that, due to presence of higher biomass in the bottom phase, there is more

precipitation on the ACN/sugar interface which hinders the efficient air supply. This results in lower protein recovery as the biomass loading is increased. Therefore, from this study, biomass loading of 0.05 g was selected as a constant for further variable manipulation.

3.2 | Influence of electric current (voltage) and position of electrode on LBEF system

The DC electric current supply for this experiment was manipulated by varying voltage supply to the electrodes from 5–30 V. The results obtained from this set-up are depicted in Figure 3A. As the voltage supply is increased from 5 to 15 V, the SE and Y_p increases. Further increase in voltage supply (20–30V) illustrates a decreasing trend in the total yield. A similar trend was observed by Leong et al. in extraction of betacyanins from peel of red-purple pitaya via LBEF system [5]. According to Leong et al., it is possible that the mass transfer in the experiment has reached an equilibrium stage and addition voltage supply is unnecessary for the system [5]. The highest SE and Y_p of $55.165 \pm 1.253\%$ and $43.209 \pm 1.586\%$ were obtained at DC supply of 15 V, respectively. Although the SE at 5 V ($55.452 \pm 0.815\%$) is similar to that of 15 V, the corresponding Y_p is low ($30.208 \pm 1.277\%$).

The supply of electricity in the LBEF system causes electroporation of the microalgae cell membrane. This enhances the extraction of proteins from microalgae biomass. Increase in the permeability of cell membrane certainly increases the amount of proteins released from the cell structure to the external solution [24]. The application of voltage or electric field to the system is a non-thermal process. The electric field ranging from 5–30V passes through the cell structure and initiate membrane rupture as the cell membrane are dipole in nature [25]. The mass transfer energy of the system increases due to the addition of electric field. When the electric voltage is supplied to the system, the ions in the liquids move from anode or cathode depending on the charge of the cells. The microalgae cell carry a negative charge on the cell membrane and thus move towards anode when electric

FIGURE 3 Effect of (A) voltage supply (V) and (B) electrode position in LBEF system

FIGURE 4 Effect of (A) glucose concentration (g/L) and (B) type of sugar on LBEF system

field is introduced. This produces oxygen and hydrogen gas at the electrodes. The proteins bind to these bubbles and rise to the top ACN-rich phase. The emission of hydrogen and oxygen bubbles result in higher turbulence in the two phase system. Statistical analysis of this study suggested that the data obtained is significant ($p < 0.05$) and 15 V of DC electric field supply is enough to induce higher separation efficiency and protein recovery. Therefore, 15 V of DC supply will be utilized in the next parameters.

After operating the LBEF system at 15 V, the position of the tip of the electrodes was analyzed. The tip of the electrode was positioned at the bottom, top, and middle phase. The results for this set-up are depicted in Figure 3B. The highest separation efficiency and protein recovery was observed when the tip of the electrode was at the bottom phase. When the tip of the electrode was at the bottom phase, the SE was $55.165 \pm 1.253\%$ and Y_p was $43.209 \pm 1.587\%$. The lowest SE and Y_p of $49.886 \pm 0.041\%$ and $3.114 \pm 0.080\%$ was observed when the tip was placed at the middle or at the interface of the two phase system, respectively.

As observed from the Figure 3B, it is evident that electric field supply to the bottom phase results in higher protein extraction efficiency. As the microalgae biomass is present in the bottom phase, the supply of electric field to bottom phase releases the protein and the subsequent flotation carries the proteins to the top phase. However, when the electric field is supplied to middle and top phase, the protein extraction efficiency is reduced due to the absence of microalgae biomass initially. As the flotation progresses and proteins are extracted in top phase, the electric field is effective. However, it can't compete with electrodes tip in bottom phase. Therefore, in the further optimization experiments, the tip of the electrodes is immersed in the bottom phase.

3.3 | Effect of sugar concentration and type of sugar on LBEF study

In the conventional salting-out two phase systems, the type of salt and its corresponding concentration plays a major role.

These two parameters are vital for effective two-phase formation and thus affect the efficiency of the system. Similarly, in sugaring-out effect, optimization of type of sugar and its concentration are necessary. In this section, the sugar concentration for glucose is optimized. The ACN molecules interact with water molecules via hydrogen bonding and dipole-dipole interactions to form a 3-D cluster (N-H-O complexes) [26]. The formation of two-phase in sugaring-out system is predicted due to the substitution of hydrogen bonds between ACN-water by the added sugar molecules [11]. Therefore, ACN molecules are separated and form two-phase system. According to Wang et al., increase in sugar concentration should enhance and strengthen the two-phase formation [27]. Higher sugar concentration also ensures higher ACN volume in the top phase for recycling [27]. Figure 4A illustrates the results of this experiment with glucose concentration ranging from 100 to 300 g/L.

As previously suggested by the literature study, the results of the experiment follow similar trend. As the glucose concentration is increased from 100 to 300 g/L, there is steady increase in separation efficiency. The SE rose from $50.14 \pm 0.082\%$ at 100 g/L to $69.592 \pm 1.522\%$ at 300 g/L. The protein recovery for glucose concentration of 100 g/L is very low ($3.360 \pm 0.159\%$) due to the weak two-phase formation. At 100 g/L, there are less sugar molecules to replace the ACN-water complex. This results in weaker two-phase formation and introduction of turbulence due to flotation eventually collapses the two-phase formed. However, there is a steady increase in protein recovery from 150 g/L ($43.209 \pm 1.587\%$) to 300 g/L ($59.456 \pm 2.029\%$). As the sugar concentration of bottom phase increases, there are lesser hydrogen bonds available for the released proteins to attach, they rise to the top phase and can be extracted. The increase in volume of ACN with higher glucose concentration also enhances extraction of non-polar proteins as ACN is hydrophobic (non-polar) compared to water [28]. Statistical analysis of this study suggested that the data obtained is significant ($p < 0.05$) and 300 g/L of sugar concentration was selected for optimization of further parameters.

As the sugar concentration was determined, the corresponding type of sugar was experimented. For this study, five types of sugars were selected namely glucose, sucrose, fructose, maltose, and xylose. Their SE and Y_p are illustrated in Figure 4B. Based on the results from Figure 4B, it is evident that sucrose, maltose, and xylose had lowest Y_p of $48.174 \pm 0.580\%$, $44.477 \pm 1.614\%$, and $37.923 \pm 1.328\%$, respectively. The highest Y_p was reported by glucose at $59.456 \pm 2.029\%$, followed by fructose at $58.023 \pm 1.836\%$. The SE was highest for fructose at $72.624 \pm 1.197\%$ followed by $69.592 \pm 1.522\%$ for glucose. The lowest SE was observed by maltose followed by xylose and sucrose at $65.298 \pm 1.009\%$, $66.140 \pm 0.725\%$, and $67.609 \pm 0.362\%$, respectively.

Previous reports suggest that two-phase formation is largely influenced by placement of hydroxyl group on the 4th carbon of the circular ring of the sugars. For monosaccharide sugars like glucose, fructose, and xylose, glucose has higher protein recovery compared to fructose and xylose. Fructose is a ketose with five chain carbon ring and xylose is an aldose on a five chain carbon ring whereas glucose has six chain ring which increases the availability of number of hydrogen bonds [29]. The low protein recovery for sucrose and maltose can be explained by their molecular structure. Both these sugars are disaccharides. Sucrose contains one glucose and one fructose molecule compared to two glucose molecules of maltose. They each can form eight hydrogen bonds whereas glucose and xylose can form only five and four bonds, respectively [28]. However, the difference in number of moles of sucrose (0.876 moles) and maltose (0.876 moles) for the same mass of glucose (1.665 moles) at 300 g/L limits the efficiency of two phase formation [29]. Statistical analysis of this study via one-way ANOVA indicated that the data obtained is significant ($p < 0.05$) and hence, glucose was selected for further optimization.

3.4 | Effect of concentration of ACN on LBEF system

In this section, the effect of concentration of ACN on the LBEF system was explored. The ACN concentration was manipulated from 70–100%. Figure 5 illustrates the results obtained from various concentration of ACN. The ACN was diluted to 70, 80, 90, and 100% and the experiment was conducted with the corresponding concentration as top phase of the LBEF system. According to Figure 5, it is clearly evident that ACN concentration of 100% exhibits the highest SE and Y_p of $69.592 \pm 1.522\%$ and $59.456 \pm 2.029\%$, respectively. As the concentration of ACN was reduced, the protein separation efficiency and protein recovery observed a decreasing trend. The lowest SE ($49.998 \pm 0.092\%$) and Y_p ($3.330 \pm 0.178\%$) was obtained at 70% ACN concentration. At such low concentration, the two phase formation was weak

FIGURE 5 Effect of ACN concentration (%) on LBEF system

and couldn't sustain it throughout the experiment. As the concentration of ACN is reduced, the ACN and water molecules bind firmly due to the hydrogen bonds between them. As water molecules are higher in number at such low ACN concentration, the glucose will form hydrogen bonds with these water molecules and result in weaker two phase formation [9]. Therefore, in further optimization, ACN with 100% concentration was employed.

3.5 | Effect of air flowrate and flotation time on the LBEF system

The air flowrate for LBEF experiment plays an important role in optimizing the SE and protein recovery of protein from microalgae. In this part of the experiment, the air flowrate for flotation system was manipulated from 50 to 200 cc/min. The results are shown in Figure 6A. The SE for 50, 75, 100, 125, and 200 cc/min of air flowrate were $70.187 \pm 1.262\%$, $69.592 \pm 1.522\%$, $68.494 \pm 1.330\%$, $67.845 \pm 0.903\%$, and $68.147 \pm 0.883\%$, respectively. All these values range from 65–70%, however, at air flowrate of 150 cc/min, the SE is the highest at $72.810 \pm 0.576\%$. In case of protein recovery, similar trend is followed for air flow rates of 50, 75, 100, 125, and 200 cc/min with values ranging from 57–60%. Although, at 150 cc/min, the Y_p is highest at $66.465 \pm 0.710\%$.

The increase in air flowrate from 50 to 150 cc/min illustrates an escalation in protein recovery. However, with further increase in the air flowrate, the protein recovery decreases. As the air flowrate increases, more air bubbles are introduced to the bottom phase. This increases the efficiency of bio-active protein molecules to rise above to the top phase. The turbulence caused by increasing air flowrate also ensures higher protein adsorption on the air bubbles and thus higher Y_p [30]. However, after crossing 150 cc/min, the Y_p decreases from $66.465 \pm 0.710\%$ to $57.529 \pm 1.178\%$. This steep decrease in Y_p can be explained by increase in turbulence caused at higher air flowrate. At 200 cc/min, the time of contact between

FIGURE 6 Effect of (A) air flowrate (cc/min) and (B) flotation time on LBEF system

the protein and air bubbles is very low and thus inefficient adsorption is observed. The intense turbulence due to high air flowrate is also responsible for re-dissolution of the bio-active proteins in the sugar solution [31]. This doesn't promote mass transfer and thus reduces the protein recovery of the whole system. Statistical analysis of this study suggested that the data obtained is significant ($p < 0.05$) and hence, 150 cc/min of air flowrate was utilized for further optimization of this experiment.

The flotation time for the chosen air flowrate was examined in this part of the experiment. The flotation time tested was 5, 10, 15, 20, and 30 min, respectively. It is evident from Figure 6B that protein separation efficiency and protein recovery increases progressively with increase in flotation duration. However, after 15 minutes, prolonged flotation reduces the SE and Y_p , respectively. The SE for 5, 10, 15, 20, and 30 min were $67.580 \pm 1.522\%$, $72.810 \pm 0.576\%$, $73.999 \pm 0.739\%$, $72.870 \pm 1.973\%$, and $67.005 \pm 0.552\%$, respectively. The corresponding Y_p values were $66.499 \pm 1.573\%$, $66.465 \pm 0.710\%$, $69.665 \pm 0.862\%$, $65.635 \pm 2.499\%$, and $60.407 \pm 0.662\%$, respectively. The separation efficiency and protein recovery increases linearly with increase in flotation time and this phenomenon is observed due to higher contact duration between proteins and air bubbles introduced in the system [10]. Although, as the flotation time is prolonged, an opposite trend is observed. This might be because the system is in equilibrium at 15 min and any increase in flotation duration doesn't provide higher efficiency. Statistical analysis of this study suggested that the data obtained is significant ($p < 0.05$). Therefore, flotation duration of 15 min was selected as the last step for optimization of this experiment.

3.6 | Comparison between LBEF and LBF and scale-up approach

As all the parameters were optimized, a comparison study was conducted between the newly optimized LBEF system with the control LBF set-up. This was mainly conducted to

TABLE 1 Comparison study of LBF & LBEF system and large-scale approach

	Separation efficiency (SE%)	Protein recovery (Y_p %)
Type of system		
LBF	$61.584 \pm 0.360\%$	$48.779 \pm 0.480\%$
LBEF	$73.998 \pm 0.739\%$	$69.665 \pm 0.862\%$
Integrated LBEF system		
Small-scale	$73.998 \pm 0.739\%$	$69.665 \pm 0.862\%$
Large-scale	$52.871 \pm 1.236\%$	$73.293 \pm 0.701\%$

investigate the change in protein recovery and SE induced by the electrolysis pretreatment of the microalgae cells. The results are illustrated in Table 1. In the LBF set-up, the electric voltage supply was not provided. In this case, there was no external cell disruption technique involved and thus resulted in lower separation efficiency and protein recovery. The lack of external electrolysis effect of electric field in the microalgae rich bottom phase limits the cell wall disruption, therefore the amount of protein released from microalgae cells is low, which is reflected in Table 1.

The integrated LBEF system optimized in this experiment was conducted at a larger scale. The initial experiment was conducted with a working volume of 300 mL (150 mL of sugar solution and 150 mL of ACN). In the large-scale set-up, the experiment was conducted at five times the initial volume. The results are demonstrated in Table 1. The separation efficiency reduced, however, the protein recovery was a bit higher than the small-scale experiment. Therefore, this experiment can be verified with positive results from large-scale system.

4 | CONCLUDING REMARKS

This study investigates the effects of various factors for protein extraction using an integrated method for increasing the overall efficiency of the LBF system. The results suggest that the separation efficiency and protein recovery of LBEF

system increases significantly when compared with simple LBF system. Moreover, utilizing sugaring-out effect eliminates the corrosion and equipment damage caused by conventional salts in the biphasic system. The final result after optimization of all the parameters was obtained with 0.05 g of microalgae biomass with 15 V of DC current supply with tip of the electrode at the bottom phase. The sugar concentration of glucose was 300 g/L and ACN concentration was 100%. The air flowrate of the flotation system was 150 cc/min with flotation time of 15 min resulted in optimum values for SE and protein recovery. The optimized separation efficiency of the LBEF system was $73.999 \pm 0.739\%$ and protein recovery of $69.665 \pm 0.862\%$ compared to LBF, the separation efficiency was $61.584 \pm 0.360\%$ and protein recovery was $48.779 \pm 0.480\%$. The optimized parameters were utilized in a $5 \times$ time scaled-up LBEF system. The separation efficiency and protein recovery were noted at $52.871 \pm 1.236\%$ and $73.294 \pm 0.701\%$, respectively. The major drawback of this study was the smaller magnitude of the voltage supply and utilization of high-voltage pulsed electricity is necessary to effectively study the cell-disruption of microalgae. Furthermore, future studies can be conducted with different optimization approach that will take into consideration the interaction between the selected parameters. This novel, simple method is fast and reliable and can be a breakthrough in solving the tailbacks of conventional extraction technologies.

NOMENCLATURE

°C	degree celcius
μm	micrometer
μmol/m ² /s	micromoles per square meter per second
ACN	acetonitrile
cc/min	cubic centimeter per minute
DC	direct current
g	grams
g/L	grams per liter
L	liter
LBEF	liquid biphasic electric system
LBF	liquid biphasic system
mins	minutes
mL	milliliter
nm	nanometer
OD	optical density
OVAT	one variable at a time
rpm	revolutions per minute
SE	separation efficiency
UV-Vis	ultraviolet-visible
V	voltage
WHO	World Health Organization
Yp	protein recovery/yield

ACKNOWLEDGMENTS

This work was supported by the Fundamental Research Grant Scheme, Malaysia [FRGS/1/2019/UNIM].

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

REFERENCES

- dos Santos, R. R., Moreira, D. M., Kunigami, C. N., Aranda, D. A. G. et al., Comparison between several methods of total lipid extraction from *Chlorella vulgaris* biomass. *Ultrason. Sonochem.* 2015, 22, 95–99.
- Koyande, A. K., Chew, K. W., Rambabu, K., Tao, Y. et al., Microalgae: a potential alternative to health supplementation for humans. *Food Sci. Hum. Wellness* 2019, 8, 16–24.
- Chronakis, I. S., Madsen, M., proteins, A., in: Phillips, G. O., Williams, P. A. (Eds.), *Handbook of Food Proteins*, Woodhead Publishing Series in Food Sciences, Technology and Nutrition, 2011, pp. 353–394.
- Sankaran, R., Show, P. L., Lee, S. Y., Yap, Y. J. et al., Integration process of fermentation and liquid biphasic flotation for lipase separation from *Burkholderia cepacia*. *Bioresour. Technol.* 2018, 250, 306–316.
- Leong, H. Y., Ooi, C. W., Law, C. L., JULKIFLE, A. L. et al., Integration process for betacyanins extraction from peel and flesh of *Hylocereus polyrhizus* using liquid biphasic electric flotation system and antioxidant activity evaluation. *Sep. Purif. Technol.* 2019, 209, 193–201.
- Show, P. L., Ooi, C. W., Anuar, M. S., Ariff, A. et al., Recovery of lipase derived from *Burkholderia cenocepacia* ST8 using sustainable aqueous two-phase flotation composed of recycling hydrophilic organic solvent and inorganic salt. *Sep. Purif. Technol.* 2013, 110, 112–118.
- Sidek, N. L. M., Tan, J. S., Abbasiliasi, S., Wong, F. W. F. et al., Aqueous two-phase flotation for primary recovery of bacteriocin-like inhibitory substance (BLIS) from *Pediococcus acidilactici* Kp10. *J. Chromatogr. B* 2016, 1027, 81–87.
- Phong, W. N., Show, P. L., Teh, W. H., Teh, T. X. et al., Proteins recovery from wet microalgae using liquid biphasic flotation (LBF). *Bioresour. Technol.* 2017, 244, 1329–1336.
- Sankaran, R., Manickam, S., Yap, Y. J., Ling, T. C. et al., Extraction of proteins from microalgae using integrated method of sugaring-out assisted liquid biphasic flotation (LBF) and ultrasound. *Ultrason. Sonochem.* 2018, 48, 231–239.
- Sankaran, R., Show, P. L., Cheng, Y. S., Tao, Y. et al., Integration process for protein extraction from microalgae using liquid biphasic electric flotation (LBEF) system. *Mol. Biotechnol.* 2018, 60, 749–761.
- Dhamole, P. B., Mahajan, P., Feng, H., Sugaring out: a new method for removal of acetonitrile from preparative RP-HPLC eluent for protein purification. *Process Biochem.* 2010, 45, 1672–1676.
- Leinonen, H., Stress corrosion cracking and life prediction evaluation of austenitic stainless steels in calcium chloride solution. *CORROSION* 1996, 52, 337–346.

13. Valente, I. M., Gonçalves, L. M., Rodrigues, J. A., Another glimpse over the salting-out assisted liquid-liquid extraction in acetonitrile/water mixtures. *J. Chromatogr. A* 2013, *1308*, 58–62.
14. Günerken, E., D'Hondt, E., Eppink, M. H. M., Garcia-Gonzalez, L. et al., Cell disruption for microalgae biorefineries. *Biotechnol. Adv.* 2015, *33*, 243–260.
15. Zbinden, M. D. A., Sturm, B. S. M., Nord, R. D., Carey, W. J. et al., Pulsed electric field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae. *Biotechnol. Bioeng.* 2013, *110*, 1605–1615.
16. Mubarak, M., Shaija, A., Suchithra, T. V., Ultrasonication: an effective pre-treatment method for extracting lipid from *Salvinia molesta* for biodiesel production. *Resour. Technol.* 2016, *2*, 126–132.
17. Roselló-Soto, E., Barba, F. J., Parniakov, O., Galanakis, C. M. et al., High voltage electrical discharges, pulsed electric field, and ultrasound assisted extraction of protein and phenolic compounds from olive kernel. *Food Bioprocess Technol.* 2015, *8*, 885–894.
18. Becker, E. W., Micro-algae as a source of protein. *Biotechnol. Adv.* 2007, *25*, 207–210.
19. Lourenço, S. O., Barbarino, E., Lavín, P. L., Marquez, U. M. L. et al., Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors. *Eur. J. Phycol.* 2004, *39*, 17–32.
20. Safi, C., Charton, M., Pignolet, O., Silvestre, F. et al., Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors. *J. Appl. Phycol.* 2013, *25*, 523–529.
21. Chew, P. L., Annuar, M. S. M., Show, P. L., Ling, T. C., Extractive bioconversion of poly- ϵ -caprolactone by *Burkholderia cepacia* lipase in an aqueous two-phase system. *Biochem. Eng. J.* 2015, *101*, 9–17.
22. Selvakumar, P., Ling, T. C., Walker, S., Lyddiatt, A., A practical implementation and exploitation of ATPS for intensive processing of biological feedstock: a novel approach for heavily biological feedstock loaded ATPS. *Sep. Purif. Technol.* 2010, *75*, 323–331.
23. Show, P. L., Tan, C. P., Anuar, M. S., Ariff, A. et al., Direct recovery of lipase derived from *Burkholderia cepacia* in recycling aqueous two-phase flotation. *Sep. Purif. Technol.* 2011, *80*, 577–584.
24. Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M. et al., Techniques for extraction of bioactive compounds from plant materials: a review. *J. Food Eng.* 2013, *117*, 426–436.
25. Chemat, F., Rombaut, N., Meullemiestre, A., Turk, M. et al., Review of green food processing techniques. Preservation, transformation, and extraction. *Innov. Food Sci. Emerg. Technol.* 2017, *41*, 357–377.
26. Szydlowski, J., Szykuła, M., Isotope effect on miscibility of acetonitrile and water. *Fluid Phase Equilib.* 1999, *154*, 79–87.
27. Wang, B., Ezejias, T., Feng, H., Blaschek, H., Sugaring-out: a novel phase separation and extraction system. *Chem. Eng. Sci.* 2008, *63*, 2595–2600.
28. Dhamole, P. B., Chavan, S., Patil, R. G., Feng, H. et al., Extraction of p-coumaric acid from agricultural residues and separation using 'sugaring out'. *Korean J. Chem. Eng.* 2016, *33*, 1860–1864.
29. de Brito Cardoso, G., Mourão, T., Pereira, F. M., Freire, M. G. et al., Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin. *Sep. Purif. Technol.* 2013, *104*, 106–113.
30. Pakhale, S. V., Vetal, M. D., Rathod, V. K., Separation of bromelain by aqueous two phase flotation. *Sep. Sci. Technol.* 2013, *48*, 984–989.
31. Bi, P., Dong, H., Dong, J., The recent progress of solvent sublation. *J. Chromatogr. A* 2010, *1217*, 2716–2725.

How to cite this article: Koyande AK, Chew KW, Lim J-W, Lee SY, Lam MK, Show P-L. Optimization of protein extraction from *Chlorella Vulgaris* via novel sugaring-out assisted liquid biphasic electric flotation system. *Eng Life Sci.* 2019;19:968–977. <https://doi.org/10.1002/elsc.201900068>