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Liquid Biphasic Flotation System (LBFS) for Separation of Protein from *Azolla pinnata*

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ABSTRACT

The demand for plant-based protein has surged as consumers nowadays are highly conscious about their daily intake; thus, this has driven extensive research on plant-based protein. Industries are now focusing on minimal operational costs while being environmentally friendly. This project specifically targets the extraction of protein from *Azolla pinnata* using a liquid biphasic flotation system (LBFS). LBFS is a promising extraction method incorporating microbubbles to enhance protein separation efficiency in liquid-liquid extraction. The LBFS processing parameters are the type of solvent (ethanol and 2-propanol), solvent concentration (75-100%), salt concentration (200-500 g/L), biomass load (100-400 mg), and flotation time (5-15 min). The study's findings revealed that 2-propanol, with its polarity, yielded the highest protein recovery and separation efficiency. Increasing the solvent concentration led to a higher yield of extracted protein due to the greater number of hydroxyl groups per unit volume. Higher salt concentrations aid the separation process by enhancing hydrophobic contact between the protein and water. A higher biomass load resulted in greater protein recovery, while a longer flotation duration improved protein extraction due to lower particle surface tension. However, vigorous flotation conditions could lead to protein degradation. Based on the study's optimisation, the LBFS conditions that yielded the best results were 80% 2-propanol solvent, 400 g/L salt concentration, 300 mg biomass load, and a flotation time of 10 min. These conditions resulted in a protein yield recovery of 78.19% ±4.31 and a protein separation efficiency of

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79.39% ±2.80. This method provides a high protein yield recovery and separation efficiency for the extraction of biomolecules.

Keywords: Azolla pinnata, liquid biphasic flotation system, liquid-liquid extraction, plant-based protein, protein separation.

INTRODUCTION

Azolla pinnata, a free-floating fern from the *Azollaceae* family, is a rich source of protein that contains practically all essential amino acids, along with numerous important minerals (Cherryl et al., 2014). Figure 1 shows the image of an *Azolla pinnata* plant. It is commonly used as a feed ingredient in the poultry industry due to its high protein content of 25%–30% and its ease of cultivation (Swain et al., 2022). Plant-derived proteins are currently undergoing extensive research, as consumers nowadays are highly health-conscious about their daily intake. The growing global demand for plant-based protein is driven by the increasing number of people who are more mindful of their health in the post-COVID-19 pandemic. According to the World Health Organization (WHO), cardiovascular disease accounts for 31% of deaths worldwide. To reduce the number of deaths caused by this disease, an alternative protein source is necessary, and plant-based protein is an optimal choice, as it contains lower levels of saturated fatty acids compared to animal-based protein (Zhubi-Bakija et al., 2021). Moreover, *Azolla pinnata* contains up to 18 amino acids, of which glutamic acid makes up to 12.6% and glutamic acid is known to reduce the risk of cardiovascular diseases by lowering blood pressure, thus reducing the risk factors (Refaey et al., 2023).

Plant-based protein sources that are readily available in the market include soybean, quinoa, chickpea, rice, beans, and chia seeds (Darmalinggam & Kaliannan, 2020). In Malaysia, up to 90% of soybeans are imported from various countries to obtain plant-based protein (AgFlow, 2023). The abundant protein content found in *Azolla pinnata* presents a promising alternative source of protein for various industries, including the food and pharmaceutical sectors. It can be utilised to develop vaccines and produce plant-based, protein-based foods. Malaysia, being a tropical country with ample resources of *Azolla pinnata*, has

Figure 1. Shows the leaves of an *Azolla pinnata* plant

the potential to produce plant-based protein domestically instead of relying on imports, thus contributing to the country's economic development. Besides that, *Azolla pinnata* is able to grow in nitrogen-depleted areas, and it is also an effective nitrogen fixer due to the symbiont Anabaena azollae, which is present within the leaf cavities of the *Azolla pinnata* (Kaur et al., 2018).

Several widely used protein extraction processes include alkaline extraction-isoelectric precipitation, salt extraction-dialysis, membrane extraction, ultrasonic, and chromatography (Stone et al., 2015). The challenges encountered in the conventional extraction process are achieving high yield efficiency and managing the associated costs. Recent studies have demonstrated the promising nature of liquid-liquid extraction methods in achieving high yields at a cost-effective rate. This includes a novel technology known as the liquid biphasic flotation system (LBFS), which offers several advantages in solving complex processes, reducing time consumption, and minimising energy input during the extraction process. LBFS is a developing and rapid extraction process characterised by high-yield efficiency and cost-effectiveness (Saw et al., 2020). Its purification and separation capabilities are excellent due to low interfacial tension, which prevents the denaturation of biological activities (Asenjo & Andrews, 2012). Previous studies have reported successful protein extraction using liquid biphasic systems from *Persicaria tenulla* leaf (Saw et al., 2020), microalgae (Sankaran et al., 2018), and *Chlorella vulgaris* (Koyande et al., 2019). However, no documentation exists regarding using the same extraction process for separating proteins from *Azolla pinnata*. Therefore, this study aims to optimise selected processing factors of the liquid biphasic flotation system (LBFS) to recover protein from *Azolla pinnata*.

MATERIALS AND METHODS

Materials

Ammonium sulphate ($(NH_4)_2SO_4$), 2-propanol (C_3H_8O), ethanol (C_2H_6O), Bradford reagent and a disposable 2.5 mL cuvette were purchased from R&M Chemicals (Selangor, Malaysia).

Sample Preparation

The matured *Azolla pinnata* plant in the cultivation tank was harvested. The plant is then dried in an oven at 40 °C overnight because the protein found in *Azolla pinnata* denatures at higher temperatures, and studies have shown that *Azolla pinnata* protein hydrolysates (AFPHs) are still present at a maximum temperature of 70 °C (Qoms et al., 2024). The dried plant is then ground and sieved to obtain uniform particle size. An airtight container was used to prevent air and moisture from clumping the powder, which can also cause chemical reactions that can cause degradation.

Liquid Biphasic Flotation System

The experiment began with the preparation of the chemical; for this research, ammonium sulphate and 2-propanol were used. These solvents were chosen due to the ability of the solvent to form separation phases, which aids in the extraction process based on the compounds' polarity (Cumplido et al., 2018). The initial state of the experiment was conducted with the preparation of 250 g/L of ammonium sulphate, which was dissolved with 20 mL of distilled water, 10 mL of 100% 2-propanol, and 300 mg of *Azolla pinnata* powder. This formulation was based on our preliminary experiments.

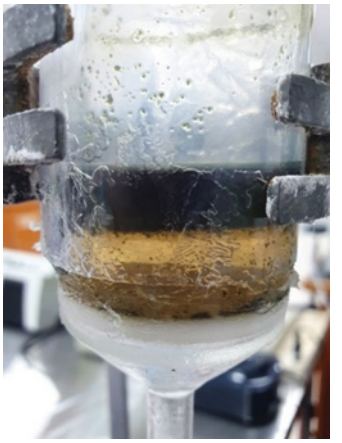

Figure 2. The separation layer of the solvent and salt solution of the liquid biphasic flotation system

First, the dissolved salt solution was poured into the sintered glass tube, followed by the solvent. Then, the Azolla pinnata powder was added last to the sintered glass tube. The sintered glass tube aids the mixture and separation of the solution as air passes through the sintered disk with G4 porosity ranging from 5 to 15 µm. After the mixture had settled, the air pump was turned on for 10 minutes for flotation to occur. Once the air pump was turned off, the separation layer could be seen instantaneously, as shown in Figure 2. The top layer and bottom layer would then be pipetted into separate 15 mL centrifuge tubes, and the volumes would be recorded. The separated layers were analysed further for the protein content.

Protein Assay

The concentration of protein obtained was determined through the Bradford reagent method (Saw et al., 2020). The Bradford method is a rapid, simple and refined approach that precisely measures the protein concentration (Kielkopf et al., 2020). Within a cuvette, 0.25 mL of the extracted protein sample was mixed with 2.5 mL of the Bradford reagent. The mixture was then stirred. After 10 min of homogenisation, the absorbance reading was recorded using the UV-Vis spectrophotometer set at 595 nm. The obtained absorbance reading was then converted to a protein concentration using a standard calibration curve established using a standard protein, specifically bovine serum albumin (BSA). This curve determined the relationship between the absorbance reading and the protein concentration. The findings were reported using a mean value calculated from measurements replicated four times (Saw et al., 2020).

Determination of Separation Efficiency and Recovery Yield

The percentage of protein that was successfully extracted in the solvent phase is referred to as the separation efficiency (E) of the top phase. The derived estimated efficiency before and after flotation of the process between the protein concentration of the bottom phase and the top phase was analysed using Equation 1.

$$E = \left(\frac{C_B - C_{Bi}}{C_{Bi}}\right) \times 100\% \quad [1]$$

where C_B stands for the concentration of protein in the bottom phase after flotation, and C_{Bi} stands for the concentration of protein in the bottom phase before flotation. The E value gives an indication of the amount of protein that has been effectively extracted from the alcohol-rich top phase (Saw et al., 2020).

The application of Equation 2 was used to determine the overall recovery yield (R) of protein. C_T resembles the protein concentration that is recovered in the top phase, whereas V_T volume is in the top phase. The recovery yield (R) was calculated by comparing the quantity that was acquired with the theoretical amount of protein contained in mg. This is done based on the protein concentration obtained from the top phase (Saw et al., 2020).

$$R(\%) = (C_T \, x \, V_T) / \, (Protein \; content \; based \; on \; proximate \; analysis) \times 100\% \quad [2]$$

Statistical Analysis

All measurements were performed in triplicate, and statistical analysis was conducted using Minitab version 18.1. A one-factor analysis of variance (ANOVA) was carried out using the Tukey LSD (least significant difference) method to compare the treatments. It is to assess any significant differences ($p<0.05$), and the results are displayed as mean ± standard error.

RESULT AND DISCUSSION

Proximate Analysis of *Azolla pinnata* Powder

The *Azolla pinnata* powder sample was subjected to proximate analysis, which yielded various data points, as shown in Table 1. The moisture content of the *Azolla pinnata* powder is 10.9%; this could be due to the nature of the plant, as it is grown in the water surrounding area; thus, moisture can still be retained in the cell wall of the plant, although the plant has been dried at 40 °C overnight. The fibrous properties of the *Azolla pinnata* plant, which have 27.5%, make it prone to the retention of moisture. The ash content of *Azolla pinnata* powder was 16.2%, followed by the total fat, 4.9%, protein, 26.1%, and carbohydrate, 41.9%, which contributes to 316 kcal of energy. The proximate analysis is

based on 100 g of *Azolla pinnata* powder. The data obtained are similar to those from the proximate analysis of *Azolla pinnata*, which was used as a feed supplement for poultry (Kumar et al., 2018).

Table 1
Proximate analysis of Azolla pinnata *powder*

Analysis	**Amount Per 100g of *Azolla pinnata* Powder**
Total Ash	16.2%
Moisture	10.9%
Total Fat	4.9%
Protein	26.1%
Carbohydrate	41.9%
Energy	316kcal
Crude Fiber	27.5%

The most important component that is focused on in the study is protein. The amount of protein available in the *Azolla pinnata* powder is 26.1% per 100 g of sample, which is slightly higher than the 22.25% reported by Kumar et al. (2018). The difference in protein content could be due to the different environments and cultivation practices (Hertzler et al., 2020). It may also be due to variations in the drying process, as the protein is sensitive to heat. The protein content of *Azolla pinnata* is still not as high as the protein content of soybean, which is reported to be 45 g of protein per 100 g of sample (Kakati et al., 2024). Nevertheless, the cultivation period of *Azolla pinnata* is significantly shorter, with only 14 days (Utomo et al., 2019), compared to soybean, which will take up to 70 to 80 days to be harvested (Idaryani et al., 2021). A shorter cultivation time leads to lower operating costs, making Azolla pinnata a promising alternative for high-protein-based products that can replace soybeans. To facilitate this, the subsequent sections will discuss the protein extraction of *Azolla pinnata*, particularly using the liquid biphasic flotation system (LBFS).

Type of Solvent

Figure 3 displays the LBFS yield recovery and separation efficiency of two types of solvents, ethanol and 2-propanol, for extracting protein from *Azolla pinnata*. A solvent is an essential chemical in this study since it extracts soluble compounds during the LBFS process and creates the separation later with an aqueous salt solution. The finding indicates that 2-propanol has a higher recovery yield of 78.19% ±3.74 of protein and separation efficiency of 79.39% ±2.16 compared to ethanol, with 42.66% ±4.65 recovery yield and 76.53% ±2.84 separation efficiency, respectively.

This might be due to a better solubility characteristic offered by 2-propanol, particularly for extracting protein from *Azolla pinnata* (Jouyban et al., 2018). The polarity of solvents

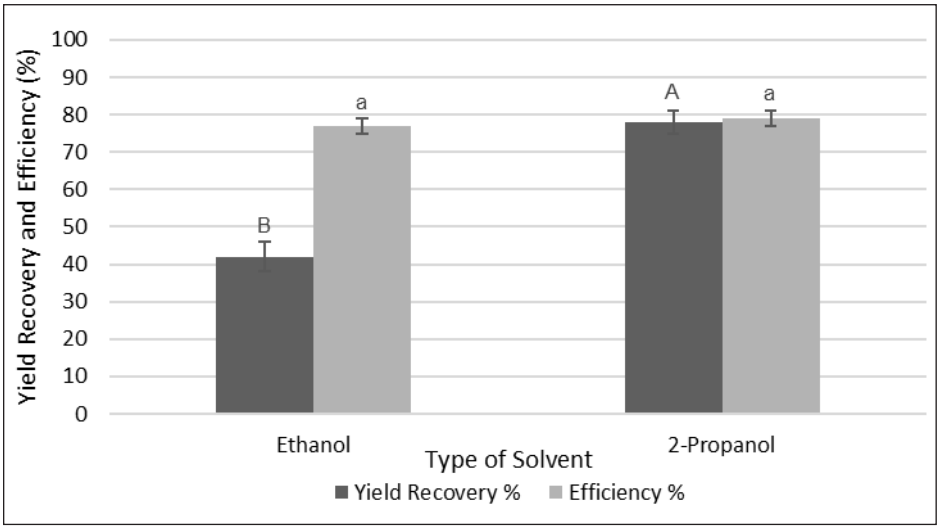

Figure 3. Effect of the type of alcohol on the yield recovery and separation efficiency of protein from *Azolla pinnata* during the LBFS process. (The alphabetical indication of capital and lower letters states the yield recovery and efficiency indicate the significantly different means of $p \leq 0.05$)

could also affect the extraction of the compounds, including protein (Susanto et al., 2018). A solvent is an important element in the LBFS for the formation of the separation layer to aid the extraction process of the solvent-soluble compounds. Besides that, 2-propanol has a higher hydrophobicity than ethanol, which is less soluble in water and provides better protein precipitation, allowing for better protein recovery (Chow et al., 2023). Hence, 2-propanol has been chosen as the solvent for the LBFS for the subsequent extraction processes in this study. The study was performed using 250 g/L of ammonium sulphate, and 100% propanol was chosen for the following parameters.

Concentration of Solvent

As shown in Figure 4, the effect of solvent concentration on the yield recovery and separation efficiency of the protein from Azolla pinnata during the LBFS process is illustrated. The solvent concentration (2-propanol) ranged from 75% to 100% with a 5% variation. Distilled water was used to lower the concentration of the solvent. Based on our preliminary findings, the solvent with a concentration of less than 75% was unable to form a visible two-phase layer, which might be due to high dilution that reduces the solvent's solubility capability. A low concentration of alcohol can result in weak ionic and van der Waals interactions, which could not be strong enough to attract biomolecules to the upper phase and vice versa (Aron et al., 2022)

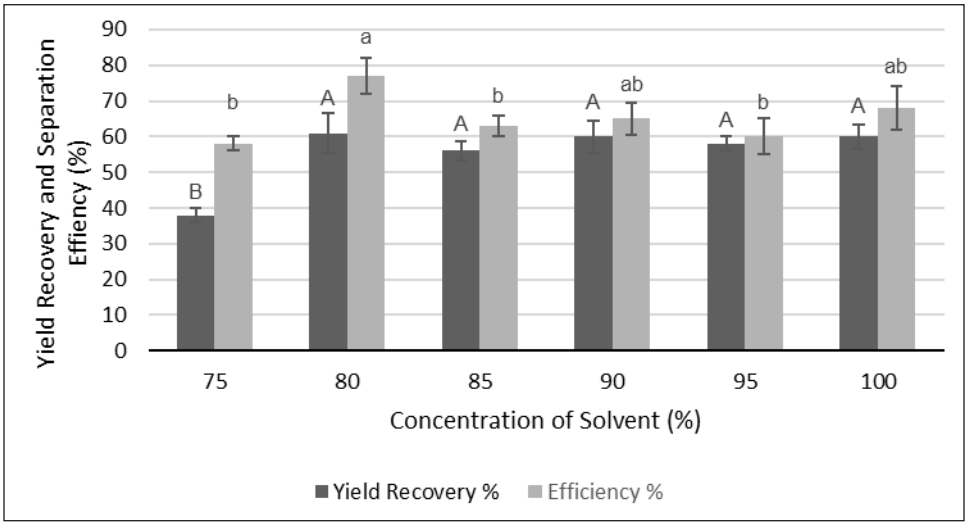

Figure 4. Effect of concentration of solvent on the yield recovery and separation efficiency of protein from *Azolla pinnata* during the LBFS process. (The alphabetical indication of capital and lower letters states the yield recovery, and efficiency indicates the significantly different means of $p \leq 0.05$)

Based on Figure 4, the yield recovery and protein separation efficiency are relatively constant throughout the entire range of 2-propanol concentrations except at 75%. It can be seen that 75% of concentration has the lowest recovery yield; this could be due to the polarity of the solvent; when it is too diluted, the polarity reduces, which also decreases the hydrophobicity, thus reducing the solubility as protein with polar amino acids tend to be more soluble in polar solvent (Madeira et al., 2024). Higher solvent concentration contains a greater number of hydroxyl groups per unit volume; hence, it has the potential to attract a greater number of biomolecules (Aron et al., 2022). The concentration of solvent at 80% resulted in the highest yield recovery of 60.76% ±5.57 and protein separation efficiency of 76.19% ±5.18. This might be due to the availability of the biomolecules in the sample, as a higher solvent concentration of more than 80% did not result in any further increment. Hence, an 80% solvent concentration is chosen for further optimisation in this study.

Concentration of Salt

Figure 5 depicts the influence of salt concentration on LBFS yield recovery and protein separation efficiency from *Azolla pinnata*. The concentration of salt is another factor that should be focused on in the liquid biphasic flotation system, as it affects the salting-out effect to separate water-miscible organic solvents in the liquid biphasic flotation system. The study on the effect of salt concentration was initiated with a constant concentration of solvent, which was set to 80%, and the amount of *Azolla pinnata* powder sample that was used was 300mg for each concentration. Based on Figure 5, as the concentration of salt

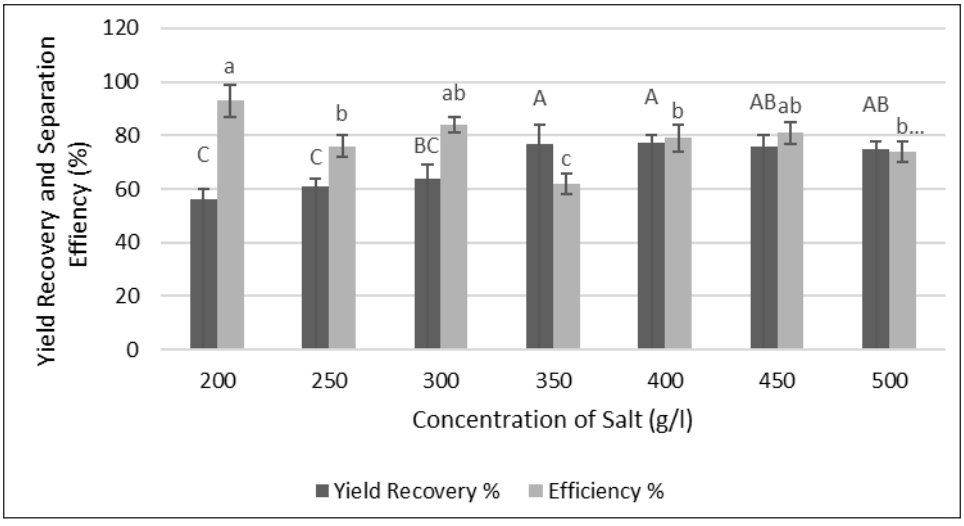

Figure 5. Effect of concentration of salt on the yield recovery and separation efficiency of protein from *Azolla pinnata* during the LBFS process. (The alphabetical indication of capital and lower letters states the yield recovery, and efficiency indicates the significantly different means of $p \leq 0.05$)

increased, so did the yield recovery of protein. The two-phase separation layer formed by this liquid-liquid separation process is also known as salt-induced phase separation (Majors, 2009). Because salt is present in the solution, the surface tension of the water will be altered, consequently increasing the hydrophobic contact between the protein and the water. The nature of the targeted protein will determine whether the protein remains in the aqueous phase after this alteration or moves into the solvent phase (Saw et al., 2020). Raj et al. (2023) studied the deconstruction of microalgae biomass and reported that when the phase molecules interact beyond a critical concentration, the attraction between the molecules and the phase components determines the selective extraction of specific biomolecular compounds. Although the recovery rate for concentrations of salt above 350 g/L to 500 g/L was relatively similar, 400 g/L of salt concentration showed the highest protein recovery of 78.19% ±2.50 with a separation efficiency of 79.39% ±5.73.

In Figure 5, the separation efficiency between different salt concentrations did not follow a consistent trend. At a 300 g/L salt concentration, the efficiency dropped drastically due to the high amount of undissolved salt in the solution, resulting in a salt precipitate that affected the extraction process. Besides, our preliminary findings also found that salt concentration below 200 g/L did not manage to produce the separation layer, as the polarity of the solution was too low to produce the separation layer (Asenjo & Andrews, 2012).

The salting-out effect can help us establish the upper boundary of salt concentration for the LBFS since different proteins salt out at different salt concentrations (Saw et al., 2020). A study conducted on the purification of amylase from sweet potato slurry through

a liquid biphasic system reported that a better purification factor can be achieved due to a better partition coefficient when the salt concentration is higher in the system (Yusree et al., 2022). Furthermore, another study on the extraction of protein from *Moringa oleifera* further describes that a higher concentration of salt is able to yield better protein extraction; however, excessively high salt concentration can also denature the protein, thus reducing the protein recovery (Illingworth et al., 2022). This study chose 400 g/L of salt solution as the optimised salt concentration for the LBFS of protein from *Azolla pinnata*.

Load of Biomass

The effect of biomass load on the LBFS yield recovery and separation efficiency of protein from *Azolla pinnata* is displayed in Figure 6. The load of *Azolla pinnata* powder used for the liquid biphasic flotation system can also influence the protein recovery. The sample that was experimented with in this study ranged from 200 mg to 350 mg. Due to the unique partition behaviour of the target protein, raising the concentration of protein sources can substantially affect the performance of phase separation (Chew et al., 2019). For this study, the concentration of solvent and salt was kept constant, whereby the concentration of solvent was 80%, and the concentration of salt was 400 g/L with a flotation time of 10 min.

Based on Figure 6, generally, the increment of the *Azolla pinnata* powder sample load increased the efficiency of the separation as well as the yield recovery of the protein. The highest yield recovery protein was obtained at 300 mg sample load with 78.19% ±8.17, with a protein separation efficiency of 79.39% ±5.61. This indicates that 300 mg of *Azolla*

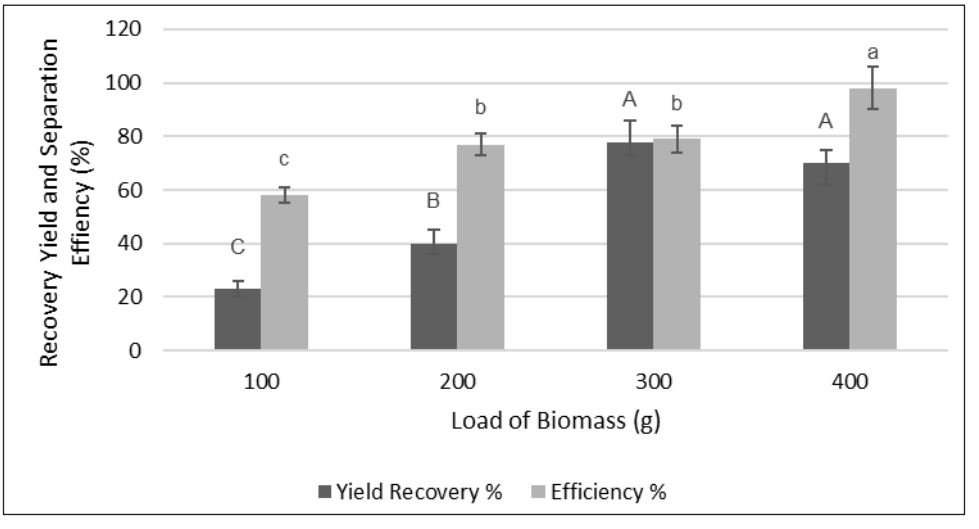

Figure 6. Effect of a load of biomass on the yield recovery and separation efficiency of protein from *Azolla pinnata* during the LBFS process. The alphabetical indication of capital and lower letters states the yield recovery, and efficiency indicates the significant difference ($p \leq 0.05$)

pinnata powder is the optimum amount needed to achieve the highest yield recovery and be as efficient as the rest of the sample sizes in this study. It can also be seen that as a load of biomass increases, the yield recovery of protein decreases; a similar trend was also noticed by a study of extraction protein from *Persiscaria tenulla,* and it was deduced that due to the high amount of biomass was present in the system, the increase of contaminant and impurities increases in the system as well which therefore reduces the performance of the LBFS separation (Saw et al., 2020). A study by Idowu et al. (2024) on protein extraction from *P. palmata* found that protein yield varies depending on the biomass-to-solvent ratio, which directly affects extraction efficiency. Therefore, optimising this ratio is essential to achieving a high protein recovery yield.

The data obtained from this study opposed the study conducted on the protein extraction from *Persicaria tenulla* leaf, which found that at 100 mg to 400 mg, it shows a downward trend of protein recovery (Saw et al., 2020). As for *Azolla pinnata* powder, it indicates an upward trend in protein recovery as the biomass load increases from 100 mg to 300 mg; however, once the load exceeds 300 mg, the protein recovery rate decreases. The difference in findings might be due to the availability of the biomolecules contained in the sample, whereby *Azolla pinnata* powder contains a higher amount of biomolecules, which portrays higher protein yield recovery (Saw et al., 2020). Moreover, Awad et al. (2021) stated that increasing the surface area and contact between the biomass and solvent enhances the yield and concentration of the active compound in the extract. However, an excessive biomass load can reduce protein recovery efficiency. Since 300 mg of *Azolla pinnata* powder sample load produces the highest yield of protein, it was chosen as the optimised condition for the LBFS extraction of protein from *Azolla pinnata*.

Flotation Time

Figure 7 displays the effect of floating time variation on the LBFS yield recovery and protein separation efficiency from *Azolla pinnata*. Flotation time is the period during which microbubbles are introduced into the liquid biphasic flotation system. The microbubbles aid the LBFS in separating the important compounds, which in this case are the proteins. The microbubbles reduce the surface tension of the particles during the process, which helps prevent the deterioration of the protein, as it is the main composition aimed at being extracted. The duration of the flotation process affects the final product, affecting the area of the air-water interface generated per unit volume of aqueous solution over time (Saw et al., 2020).

The study was initiated with the concentration of solvent and salt being kept constant at 80% solvent concentration and 400 g/L salt concentration, with a 300 mg sample load, as optimised in the previous sections. The flotation system was compared with flotation times of 5 to 15 minutes. Based on Figure 7, a longer flotation time resulted in a higher

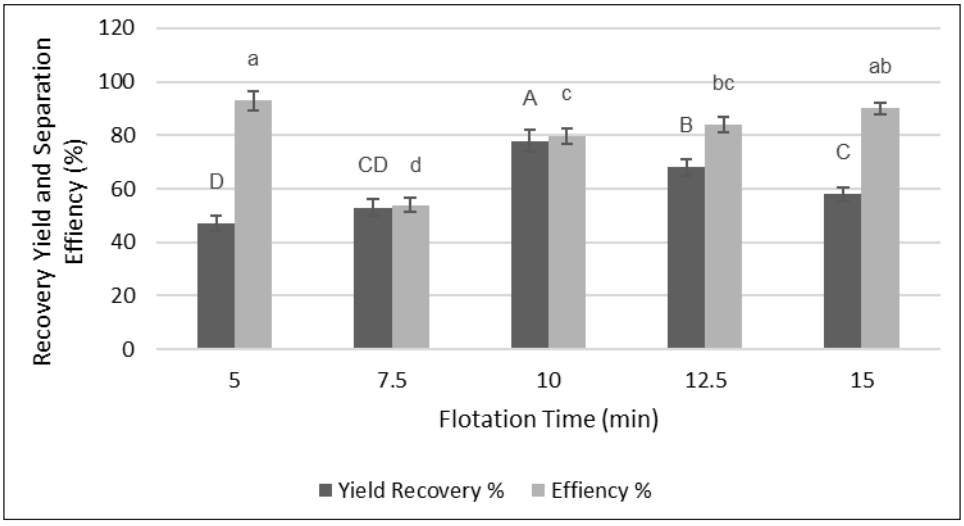

Figure 7. Effect of flotation time on the yield recovery and separation efficiency of protein from *Azolla pinnata* during the LBFS process. (The alphabetical indication of capital and lower letters states the yield recovery, and efficiency indicates the significantly different means of $p \leq 0.05$)

protein recovery yield and efficiency, with the optimised condition obtained after 10 min of flotation time (78.19% ±4.31 yield recovery and 79.39% ±2.80 separation efficiency). A decrease in yield and efficiency was observed when the flotation time exceeded 10 min, likely due to protein denaturation caused by prolonged exposure to vigorous flotation conditions. Besides, from an operational point of view, the process should be performed as quickly as possible to reduce operating costs. All in all, 10 min would be the optimum choice, as it yields the highest protein recovery and protein separation efficiency.

A study by Koyande et al. (2019) on protein extraction from *Chlorella vulgaris* suggests that a high biomass load can reduce the efficiency of flotation systems. This occurs because the increased biomass load raises the viscosity of the mixture, making it more difficult for microbubbles to form. As a result, both yield recovery and separation efficiency are impaired. Another study on the recovery of protein from dairy milk waste products states that the increase in flotation time exceeding 10 minutes reduces the protein recovery yield. This is because molecules that are not proteins are being blown to the top phase, causing the total concentration of proteins at the top phase to decrease (Tham et al., 2019).

CONCLUSION

This study evaluated the potential of a liquid biphasic flotation system as a liquid-liquid extraction method to extract protein from *Azolla pinnata* powder. According to the results of this research, it is possible to achieve a high rate of protein recovery and separation

efficiency by employing the LBFS method. The parameters used for this system have to be optimised to achieve the highest protein recovery and separation efficiency. The optimised parameter can produce a separation layer as the polarity of the solvent and salt can produce the top and bottom layers to separate the protein. The optimised value is 80% concentration of 2-propanol, 400 g/L concentration of ammonium sulphate, 300 mg of *Azolla pinnata* powder and 10 min of flotation time, which results in 78.19% ±4.31 protein yield recovery and 79.39% ±2.80 protein separation efficiency. Upscaling the process can further improve protein recovery and separation efficiency. Thus, the liquid biphasic flotation system is a promising technology for extracting plant-based protein, which can benefit both the food industry and the pharmaceutical industry.

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