Wheat Germ Protein Extraction Via Subcritical Water For Water Treatment Process

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The attractive characteristics of subcritical water (SubCW) lie in its ability to extract and hydrolyze bioactive compounds from natural matrices. These properties allow water to act as an efficient solvent with a short extraction time. This paper aims to study the effect of SubCW temperature on wheat germ protein extraction for application in the coagulation of the water treatment process. Concerning coagulation performance, a mass-to-water ratio and extraction time are other factors studied, besides the SubCW temperature. Wheat germ (WG) is an excellent source of plant-based protein that is suitable as a biocoagulant for substituting the widely used chemical coagulants. Experiments were conducted in a batch reactor at a SubCW temperature between 100 and 170°C with a solid-to-water ratio (s/w) of 0.5:25–2:25 and an extraction time of 5–30 min. The extracts obtained after the SubCW process contained a distinctive amount of protein, which was then used as a coagulant extract solution in the coagulation process. The highest total protein yield was 22.93 g/100 g-WG, obtained at 160°C, which corresponds to protein extraction of 82.8%. The lowest turbidity, 48.9 NTU, was achieved at 120°C in SubCW extracts, which resulted in a 98.8% turbidity reduction. From the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), it was observed that proteins with a molecular weight less than 117 kDa exhibit superior coagulation activity. Consequently, wheat germ protein was efficiently extracted by SubCW and can be used as a promising bio-coagulant alternative in waste treatment facilities.

Keywords: Coagulation; Protein; Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Subcritical Water; Wheat Germ

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1. Introduction

Extraction is essential in scientific studies related to the isolation and analysis of bioactive compounds from environmental and biological systems. It has been an important unit operation in the agricultural commodities, food, chemicals, biotechnological, environmental, and pharmaceutical industries. The use of substantial amounts of organic solvent in both conventional and modern techniques for extraction raises issues of safety, environmental concerns, and cost effectiveness. Conventional extraction methods like alkaline treatment result in high production costs as the product yield depends mainly on the alkaline solution [1]. For instance, in the study done by [1], three different biomass brewers' spent grain (BSG), pasture grass (PG), and cyanobacteria (Arthrospira platensis; AP) were suspended in 0.1 M NaOH and distilled water to achieve a pH of more than 11. The mixture is then stirred for 2 hours at 40°C before being centrifuged for 20 minutes. In contrast, the use of Soxhlet extraction, maceration, and hydrodistillation leads to time-consuming, poor selectivity, costly, and decomposition risk of thermolabile compounds, as some organic solvents may harm the environment as well as human wellness [1]. Also, the analytes with different

physical and chemical characteristics need to be considered to suit the extraction process. Recently, the extraction of bioactive compounds using critical fluids has widely attracted researchers due to its environmentally friendly characteristics. Subcritical water (SubCW) is a cheap and user-friendly green technology as it utilizes water as an extraction solvent. It has the ability to replace ethanol, methanol, and acetone because water is readily available and non-toxic [2]. The wide applications of SubCW were mainly to produce pharmaceutical extracts from various types of medicinal plants [3] and herbs such as Rosmarinus officinalis (rosemary), Matricaria recutita (German chamomile), Cassia angustifolia (senna), Valeriana officinalis (valerian), Scutellaria baicalensis (Baikal skullcap), Schisandra chinensis (Wuweizu), Zingiber officinale (ginger), Astragalus Membranaceus, etc. The extracts produced by the plants include volatile oil, tannins, polyphenols, anthraquinone, and lactone, which were the active components that gave positive effects against cardiovascular diseases, neurodegeneration, cancer, and skin aging, mainly due to their antioxidant, anti-inflammatory, or anti-proliferative activities, as reported in different human trials [4].

Previous studies had shown that SubCW successfully extracted protein from various plants, by-products, or even waste from the food industry. A study conducted by [5] successfully extracted $84.9 \pm 13.2\%$ of the total protein from the green seaweed Ulva sp. The result was achieved at a process temperature of 180°C and 10.5 bar pressure during 40 min of extraction time with an 8%w/w solid load. This is a much higher protein recovery level as compared to other methods reported for protein extraction from seaweeds. The present study explores the extraction of selected proteins from wheat germs, which have an active component that promotes coagulation in water treatment via SubCW. Wheat germ is an excellent source of plant-based protein that is suitable as a biocoagulant for substituting the widely used chemical coagulants for water treatment purposes. They could be extracted from sustainable and low-cost plant-derived waste from agriculture and byproducts of the food and crop industries. This led to a reduction in food waste [6]. Previous works had applied reverse micelles, and a combination of both reverse micelles for ultrasoundassisted extraction (UAE) only recorded 37% and 57% of protein extraction from wheat germ, respectively [7]. Deep Eutectic Solvent (DES) has already been used as a co-solvent in extraction techniques such as UAE [8], and the advantages of DES are its low cost, environmental friendliness, and ease of synthesis [9, 10]. Although DES has a similar advantage to SubCW, DES has a high viscosity, which does not occur in SubCW. Thus, this paper aims to study the effect of SubCW on wheat germ protein extraction for the application in coagulation of the water treatment process.

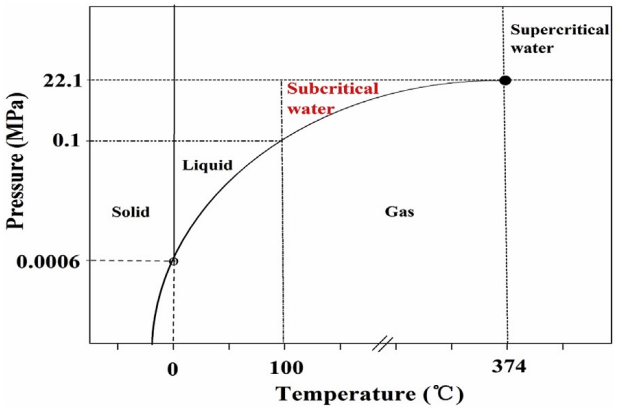

Fig. 1. The phase diagram of water under different temperature and pressure

2. Theory and formula

At room temperature and atmospheric pressure, water has a high polarity and dielectric constant due to its strong hydrogen bonding structure. Hence, water could not extract non-polar or organic compounds. However, under high temperature and pressure, the strong hydrogen bond breaks, changing its properties. Fig. 1 shows the phase diagram of water in different states under different temperatures and pressures [11].

The SubCW condition occurs at a temperature between 100 and 374°C and a pressure between 0.101-22.064 MPa [12]. At higher temperatures, the viscosity and surface tension of water decreased steadily, but its diffusivity increased. The supplied energy can disrupt the interactions between solute-matrix (adhesive) and solute-solute (cohesive) by lowering the activation energy required for the desorption process [11]. Thus improving solubility and effective mass transfer for the elution of strongly bound compounds to cell walls [2]. In the meantime, the extraction process is assisted by the higher pressure that forces the water to penetrate into the matrix (pores), which this condition could not achieve under normal pressure [11]. Besides, the pressure is high enough to maintain the water in a liquid state, and under subcritical conditions, the water has distinctive properties such as a high ionic product (H^+ allows water to behave like an acid and $OH-$ allows water to behave as a base) and a low dielectric constant (water behaves like an organic solvent) [13, 14].

The greater ionic product resulted in the promotion of acid- and base-catalyzed reactions without using acid or

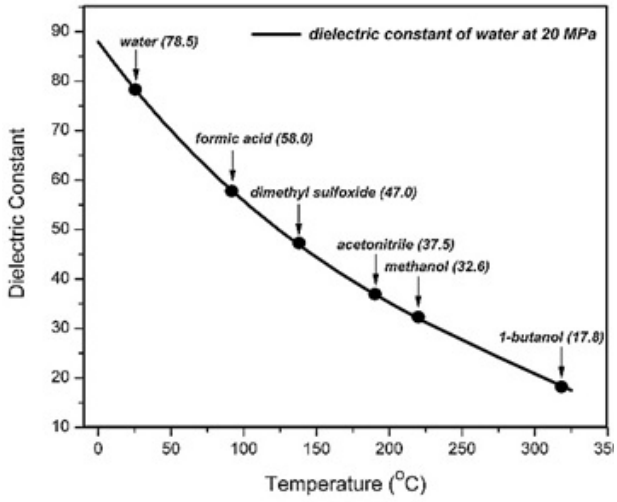

Fig. 2. The tunable polarity of subcritical water due to changes of dielectric constant result from temperature and pressure adjustment.

alkali that contribute to the production of waste in hydrolysis reactions [15]. Meanwhile, the extractive efficiency characteristics of SubCW lie in its dielectric constant. As the temperature rises to 250°C, the dielectric constant of water drops from 80 (at room temperature) to 25, which falls between those of ethanol ($\varepsilon = 24$) and methanol ($\varepsilon = 33$) (as organic solvents at 25°C). Fig. 2 shows the tunable polarity of subcritical water due to changes in the dielectric constant resulting from temperature and pressure adjustments.

Under such circumstances, some properties of water are comparable to those of organic solvents that can dissolve various medium and low-polarity compounds. Thus, polar, medium-polar, low-polar, and non-polar compounds can be separated, respectively. A main advantage of SubCW for natural products is that water is nontoxic; hence, it is more suitable for the extraction of vegetables, fruits, and herbs, where the extracts can be safely consumed by humans or animals. In addition, in the SubCW, the liquid waste generated does not need waste disposal if no organic modifiers are used [16]. Due to several beneficial characteristics, SubCW technology has been used in a broad range of applications, including waste reuse, recycling, and treatment [14].

3. Experimental setup

The natural raw wheat germ was purchased from a local company (LOHAS, Malaysia). The nutritional facts of wheat germ are shown in Table 1.

The wheat germ was milled and sieved into powder

Table 1. Nutritional facts of raw wheat germ.

Parameter	Nutritional Facts (%)
Moisture	12.6 ± 0.19
Ash	3 ± 0.18
Crude fat	8.99 ± 0.32
Crude protein	27.69 ± 0.44
Crude fiber	1.5 ± 0.18
Carbohydrate	46.07
Food energy value (kcal/100 g)	375.95

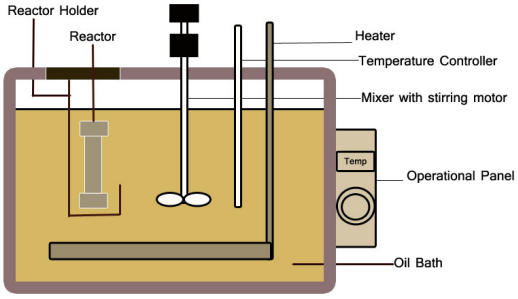

Fig. 3. The schematic diagram of the batch mode of SubCW apparatus (Oil Bath).

form through a 0.44-mm sieve. The fraction with a particle size less than 0.4 mm was used in this study. It was then mixed with water at a fixed solid-to-water (s/w) ratio of 1.25 before being loaded into a standard stainless steel batch reactor (Swagelok & Co.) with a 35-ml capacity, approximately 16 mm in diameter, and 150 mm in length, with Swagelok caps at both ends of the reactor. Argon gas is used to release trapped air from the reactor before it is tightly closed. The sample-filled tube was immersed in the oil bath at a specific temperature and time. The experiment was conducted in batch subCW mode.

The schematic diagram of the batch mode of the SubCW apparatus is shown in Fig. 3. The oil bath (Thomas Kagaku Co. Ltd.) was installed with a stirrer and temperature-control unit in order for the reaction to be performed at a temperature variation of 100°C 180°C and pressures of 0.101–1 MPa. The pressure within the reaction tube was estimated from the steam table under subcritical conditions. The time required to elevate to the operating temperature was around 30 minutes.

The parameters being investigated were temperature ($100 - 180$°C) with a 10 -minute extraction time and a 1:25 solids to water ratio. Then the reactor tube was quenched

in a cooling water basin to room temperature to terminate the reaction. It took about 1-5 minutes for the reactor to reach room temperature before the reaction products were transferred to a $50-$ mL centrifuge tube and centrifuged (KUBOTA 2420, Japan) at 4000rpm for 10 minutes. There are two layers formed resulting from the centrifugation: the aqueous phase and the solid phase.The supernatant was withdrawn and separated for total protein analysis before being applied in the jar test as a coagulant solution. Total protein content was determined using the Bradford assay [17]. A 0.25-ml sample was mixed with 2.5 ml of Bradford reagent. After 20 min, the sample was transferred into a cuvette, and by using a UV spectrophotometer (UV-160A, Shimadzu, Japan), the absorbance was measured at 595 nm. The protein yield (%) from the SubCW process is calculated as Eq. (1):

$$\frac{\text{Total Protein in SubCW extract } (\text{g}/100\text{ g WG})}{\text{Protein in Raw Wheat germ } (\text{g}/100\text{ g WG})} \times 100\% \tag{1}$$

A coagulation process was conducted via jar test. 15 g of kaolin was added to 1 L of tap water, and the suspensions were stirred for 2 hours at 120rpm for a uniform dispersion of kaolin particles in the water. It was then left without mixing for 24 hours for complete particle hydration. A fixed 20 mL of SubCW extracts at different temperatures ($100°C, 120°C, 140°C$, $160°C$, and $180°C$) were added, respectively, in a beaker that contained 100 mL of synthetic turbid water.

The suspensions were mixed rapidly at 100 rpm for 2 min, followed by a slow mixing at 30 rpm for 20 min. The agitation was switched off, and the suspension was allowed to form flocs within 1 hour of sedimentation. All the experiments were performed at the acidic pH (pH 4) of the synthetic turbid water. The supernatant was taken out for turbidity measurements. The coagulation performance is assessed using the formula for percentage tubidity reduction Eq. (2):

$$\%\text{ Turbidity Reduction } = \frac{T_i - T_f}{T_i} \times 100 \tag{2}$$

Where T_i is the initial turbidity of the sample before coagulation and T_f is the final turbidity after coagulation. Meanwhile, the molecular weight of the SubCW extracts was analyzed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide gel) on a vertical electrophoresis chamber (AE-6500 Dual Mini Slab) (Ato Corporation, China). The electrophoresis was conducted at a voltage of 75 volts for 15 minutes, increased to 120 volts, and ran for another 1 hour and 10 minutes. After electrophoresis, the gel was stained with Coomasie Brilliant Blue-R-250 0.01% (w/v) for 30 minutes and subsequently destained for another 30 minutes. After the destaining process was completed, the gel was stored in distilled water and transferred to a densitometer for image scanning and analysis. A statistical analysis in this study involved the use of SPSS software (version 20, USA). All data were expressed as mean $\pm$ standard deviation values. The correlation between factors of temperature, solid-to-water ratio, extraction time, and protein yield was analyzed using Pearson's correlation analysis in the SPSS software, with correlation coefficients at a significance level of 0.01 [18, 19].

4. Results and discussion

Fig. 4 shows the appearance of wheat germ extracts at different SubCW temperatures. The wheat germ, after underwent the SubCW process, generated products with a liquid phase and a water-insoluble solid phase. The liquid phase is considered in this study as an extract that contains a significant amount of protein, which can be used as a coagulant in the water treatment. At $100°C$, the liquid phase (extracts) appears clear until it reaches $130\ °C$. Then the liquid appearance starts to become turbid at $140\ °C$, which shows the denaturation of proteins. The denaturation of protein phenomena can be seen in Fig. 4, as the liquid phase in the extracts turns from clear to opaque. The change in color is due to the increased amount of hydrolysis and decomposition of products, which gradually resulted from denaturation at high temperatures [14]. A common consequence of denaturation is loss of biological activity (e.g., loss of the catalytic ability of an enzyme [20], which will affect the coagulation performance as denaturing could affect protein functional properties [1].

The total protein extracted at different SubCW temperature extracts is shown in Fig. 5.

As shown in Fig. 5, at $100°C$ and $120°C$ extracts, the total protein was low at around 19 g/100 g-WG. However, when the SubCW temperature increased above $130°C$, the total protein in the extracts increased to a maximum of $22.93\ \text{g}/100\ \text{g} - \text{WG}$ at $160°C$. The figure indicates the positive correlation between increasing temperature and the yield of protein from wheat germ. The highest protein yield occurred at 10 minutes of extraction time and a 1:25 solid-to-water ratio, corresponding to 82.8% of the protein extracted from the crude wheat germ protein composition of 27.69 g/100 g-WG in the proximate analysis. The excellent correlation between protein yield and SubCW temperature results could be due to the enhanced solubility of proteins at high temperatures [21]. At $160°C$, the dielectric constant (ε) of water drops to 45 , which lies be-

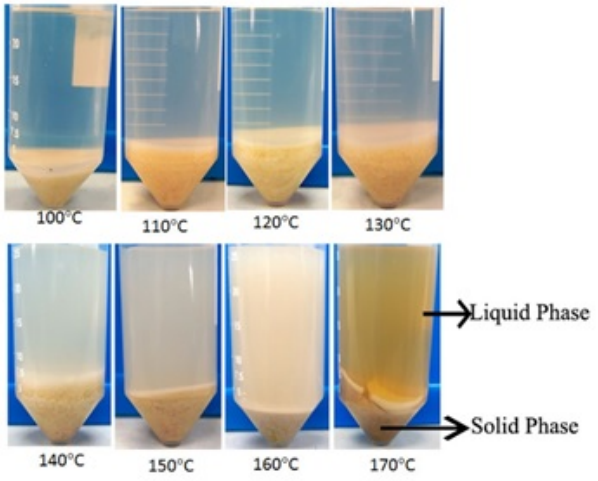

Fig. 4. Extracts from SubCW treatment of wheat germ at different temperatures after centrifuged.

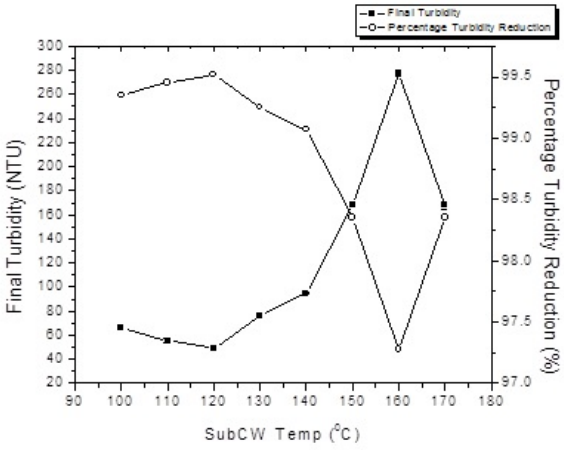

Fig. 6. Final turbidity of Kaolin after coagulation ■ and percentage turbidity reduction (o) as the function of SubCW temperature.

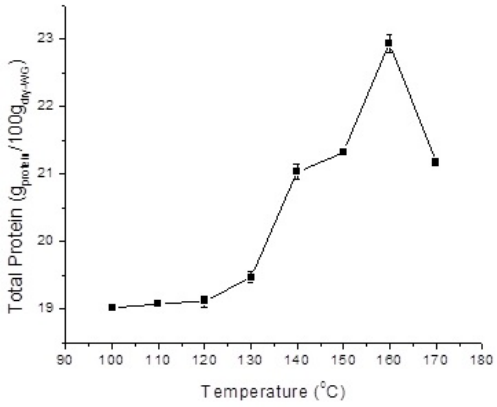

Fig. 5. The total protein obtained in the SubCW extracts, expressed as g protein/100 g-WG

tween those of dimethyl sulfoxide ($\varepsilon = 47$) and acetonitrile ($\varepsilon = 37.5$). This condition allows the properties of water to act like an organic solvent, which can dissolve various mediums such as proteins [11]. In addition to that, at high temperatures, the extraction efficiency improved since the solvent viscosity and surface tension decreased. This could allow the heat energy to accelerate desorption of the target compound (protein) from their complexes and allow better pore filling and good contact between the solvent and matrix active site. The sequential steps for the extraction process in SubCW are as follows: (1) entry of fluid; (2) desorption of solutes from the matrix active site; (3) solute diffusion through organic materials (wheat germ); (4) solute diffusion through static fluid in porous material; (5) solute diffusion through the stagnant fluid layer; and (6) solute eluted by the flowing bulk of fluid [22].

However, the protein yield was reduced to 21 g/100 g-WG at 170 °C, and at this temperature, the extract produced was brown in colour with a burning smell [23]. This may be due to the degradation of some of the constituents at higher temperatures. The degradation occurs due to the increase in hydronium and hydroxide ion concentrations that allow water to act as an acid or base catalyst, facilitating the hydrolysis of proteins into smaller peptides and low-molecular-mass products such as amino acids [13] and organic acids [14]. Besides the degradation of temperature-sensitive compounds, elevated temperatures could involve high investment costs and could also promote the generation of unwanted reactions such as caramelization, Maillard reactions, and toxic compounds. This is because water is very reactive, which could result in oxidation or catalyze the hydrolysis of some compounds. For example, substantial amounts of sugars can generate Maillard reactions and caramelization with the formation of new compounds [8, 24].

Fig. 6 illustrates the turbidity of kaolin as a function of SubCW temperature. The higher level of protein solubility in the water resulted in better coagulation performance [25]. The highest coagulation performance expressed by the lowest turbidity (48.9 NTU) is achieved at 120°C with a 20-ml coagulant solution and synthetic wastewater operating at a pH of 4.

In acidic conditions, amino groups in the protein are protonated. Since colloidal particles in kaolin are negatively charged, the protonated amino acid is attracted to negatively charged particles. This charge will promote co-

agulation as these particles bind together and grow in size to form a floc. As a result, larger particles or floc become heavy and settle to the bottom, leaving the supernatant as clear water. Hence, the turbidity value of the supernatant becomes lower, resulting from the effective coagulation process. At higher temperatures of 130°C, 140°C, 150°C, and 160°C, the turbidity keeps increasing to 76.2 NTU, 94.8 NTU, 168 NTU, and 278 NTU, respectively. This shows lower coagulation performance resulting from protein denaturation. The effect of mass loading and reaction time in SubCW on the efficiency of coagulation is shown in Figs. 7 and 8, respectively.

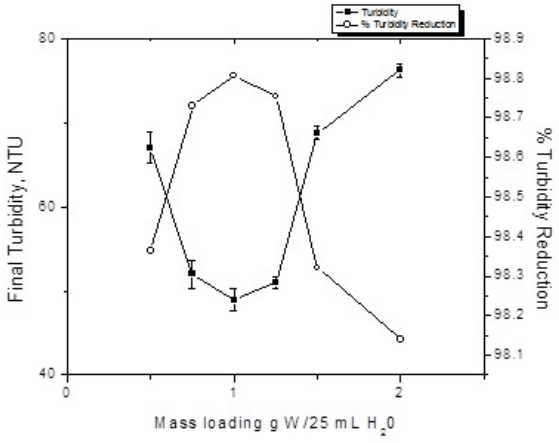

Fig. 7. Final turbidity of Kaolin after coagulation ■ and percentage turbidity reduction (o) at various mass loading (constant reaction time 10 min)

The highest coagulation efficiency was obtained at 1:25 s/w ratio (Fig. 7) and a reaction time of 10 min (Fig. 8), corresponding to the lowest turbidity (48.9 NTU) and highest percentage of turbidity reduction (98.8%). At this optimum condition, the amount of WG put in the reactor allows more protein to be extracted [13] to be used as a coagulant in the coagulation of kaolin suspension. The high s/w resulted in low coagulation performance (high turbidity), which recorded the highest turbidity (80 NTU) due to the excess of WG in the reactor. The excess might prevent the good contact of the protein or peptide with the water [13], resulting in poor coagulation performance. Meanwhile, prolonged reaction time causes the denatured of protein and coagulation efficiency is low under this condition due to the loss of the catalytic ability of the enzyme [26].

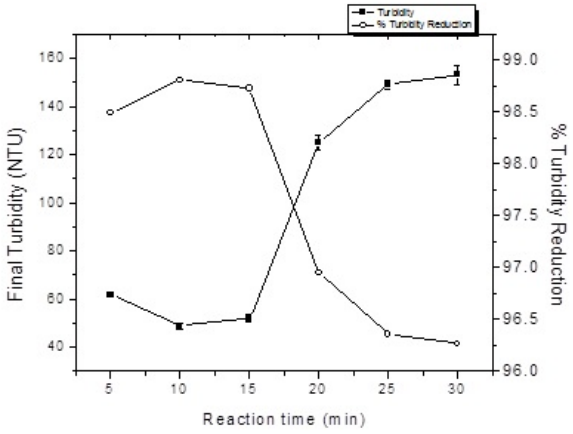

Fig. 8. Final turbidity of Kaolin after coagulation ■ and percentage turbidity reduction (o) at various reaction time (constant mass loading 1:2.5).

4.1. The correlation between SubCW temperature on protein yield and mass loading and reaction time on the final turbidity

The correlation between SubCW temperature, mass loading, and reaction times on protein yield and final turbidity was performed using Pearson's correlation analysis. In the current study, there is a positive correlation and a strong relationship (r = 0.865) between temperature and protein yield. Meanwhile, the relationship between mass loading and final turbidity showed a positive correlation with a moderate relationship (r = 0.524), whereas there is a strong and positive correlation (r = 0.897) between reaction time and final turbidity. It is clearly shown that the extraction temperature of SubCW, extraction time, and mass loading play an important role in the extraction of target compounds such as proteins. This study is in accordance with the study done by [27, 28].

4.2. Molecular weight of SubCW extracts

SDS-PAGE analysis was conducted using 12% gel in order to obtain information on the relation between the molecular weight of extracts at different temperatures and their contribution to the performance of turbidity removal at different protein fractions. Fig. 9 shows various bands and sizes of proteins, and some similar distinct protein bands can be observed across all the extracts at temperatures ranging from 100°C to 160°C, except for 180°C.

At 100°C, multiple bands can be observed with protein subunit MW ranging from 18 kDa to 63 kDa. As the temperature rises to 120 °C and 140°C, the image of SDS-PAGE reveals more bands as compared to the 100°C temperature. This is because at higher temperatures, more protein

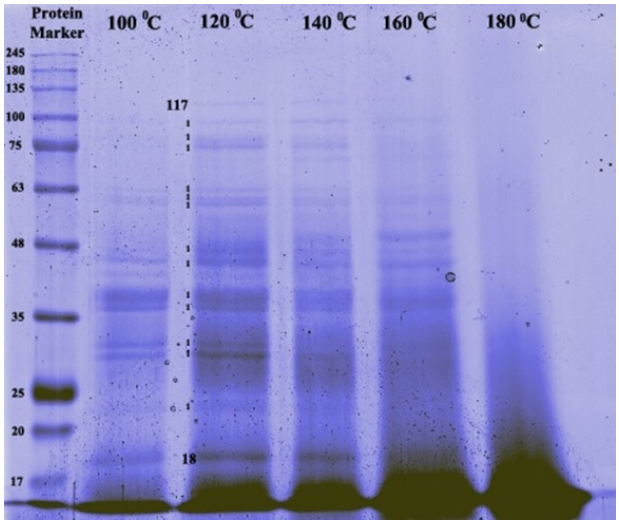

Fig. 9. SDS-PAGE Image of Protein Marker (Lane 1) and Wheat Germ Extracts at different temperature: 100°C (Lane 2), 120°C (Lane 3), 140°C (Lane 4) 160°C (Lane 5) and 180°C (Lane 6).

can be extracted from the wheat germ, resulting in multiple distinct bands in the SDS-PAGE gel lane [29]. There is clear evidence of similarity in protein bands, with the MW subunit ranging between 18 kDa and 117 kDa at both 120°C and 140°C extracts, but the denser and darker distinct bands, which indicate the presence of a larger number of soluble proteins, can be observed at 120°C. With the presence of a larger number of soluble proteins, the extracts at 120°C exhibited the highest coagulation activity, with turbidity removal of 98%, indicating that the higher level of protein solubility in the extracts resulted in better coagulation performance [25].

As the temperature rises to 160°C, the bands start to decrease, and the MW range is lower as compared to the MW range at 120°C and 140°C. There are only faint bands with MW ranging from 35 kDa to 100 kDa on the sds-page lane that can be observed at 160°C, indicating the relatively small amount of protein presented in the extracts, which resulted in lower coagulation performance with only 72% turbidity removal. Meanwhile, at 180 °C, only a smearing image and no bands can be observed. This may occur at high temperatures because of the degradation of proteins into smaller peptides or amino acids with a molecular weight below the protein standard, which is 11 kDa.

The smearing image may also be due to the generation of other products derived from the degradation, such as organic acids. A poor coagulation performance was obtained when extracts at 160 °C and 180°C were used as coagulants. This shows that the coagulation performance of this study works well with proteins with high molecular weight, as the coagulation performance decreased when the molecular weight was low and when there was little or no protein present in the extracts. This study is comparable to the studies done by [30, 31], where their active coagulant component extracted from Hibiscus is a protein with a MW ranging between 11–82 and 183 kDa, respectively. In the study done by [30], three hibiscus species were assessed, i.e., okra, sabdariffa, and kenaf. Although multiple proteins with varying MW sizes between 11 and 82 kDa are presented in the extracts, the purified samples contained a single coagulant protein band around 39 kDa. The purified samples of okra, sabdariffa, and kenaf protein achieved 98, 94, and 90% turbidity removal. Meanwhile, the combination of 100–500 mg/L of Hibiscus (with a molecular weight of 183 kDa) with 4000 mg/L of alum results in 60% turbidity removal [31]. According to [16], a polymer with a molecular weight of 100 kDa is defined as having a high MW.

A study conducted by [32] shows that the high molecular weight of microbial flocculant (120 kDa) that is obtained from Proteus mirabilis shows flocculating efficiencies (93.1%). This study obtained a coagulation optimum condition at 120°C that contained protein with a MW of 117 kDa in the extracts. Hence, the mechanism of coagulation by active coagulant from wheat germ is suggested to be bridging, as bridging is more effective at higher molecular weights. As depicted in Fig. 9, in the range of 18–117 kDa, the darkest protein band is visualized at a molecular weight of 30 kDa, which indicates a highly abundant protein in the sample. Hence, proteins with a molecular weight of 30 kDa are likely to be responsible for the coagulation property in this study. This is in accordance with the previous findings from other studies performed by [29–31]. The study conducted by [33] utilized Moringa oleifera lectin with a molecular weight of 26.5 kDa, and the obtained molecular weight is quite similar to the study conducted by [29], which reported proteins with a molecular weight less than 36 kDa isolated from MO seeds show superior coagulation activity.

5. Conclusions

The study shows the ability of SubCW as a green solvent in the extraction of wheat germ protein to be utilized as a green coagulant in the coagulation process. The highest protein that can be extracted from the crude wheat germ is 82.8%. In the absence of hazardous chemicals and a short extraction time, the extracts from SubCW show significant efficiency in reducing the kaolin turbidity up to 98.8%. This study obtained a coagulation optimum condition at 120°C

that contained protein with a MW of 117 kDa in the extracts. Hence, the mechanism of coagulation by active coagulant from wheat germ is suggested to be bridging, as bridging is more effective at higher molecular weights. This indicates the wheat germ protein proved to be an important source of peptides from agro-food products with significant biocoagulant activities. Moreover, SubCW is an environmentally friendly method that can be used for the valorization of natural products in water treatment facilities. Such observations lead to the conclusion that subsequent protein exploitation from other natural products could be performed in order to replace the chemical coagulants, and the results have demonstrated the potential development of a large-scale SubCW process.

References

[1] L. Du, P. J. Arauzo, M. F. Meza Zavala, Z. Cao, M. P. Olszewski, and A. Kruse, (2020) *"Towards the properties of different biomass-derived proteins via various extraction methods"* **Molecules** ***25***(3): 488. DOI: 10.3390/molecules25030488.

[2] S. Ibrahim, R. Santos, and S. Bowra, (2018) *"Optimization of subcritical water mediated extraction of apple pomace polyphenolics and their antioxidant activity"* **Journal of Chromatography & Separation Techniques** ***9***(05):

[3] X. Liang, Q. Fan, et al., (2013) *"Application of subcritical water extraction in pharmaceutical industry"* **Journal of Materials Science and Chemical Engineering** ***1***(05): 1.

[4] A. M. Silva, A. S. Luís, M. M. Moreira, R. Ferraz, T. Brezo-Borjan, J. Švarc-Gajić, P. C. Costa, C. Delerue-Matos, and F. Rodrigues, (2022) *"Influence of temperature on the subcritical water extraction of Actinidia arguta leaves: A screening of pro-healthy compounds"* **Sustainable Chemistry and Pharmacy** ***25***: 100593. DOI: 10.1016/j.scp.2021.100593.

[5] M. Polikovsky, A. Gillis, E. Steinbruch, A. Robin, M. Epstein, A. Kribus, and A. Golberg, (2020) *"Biorefinery for the co-production of protein, hydrochar and additional co-products from a green seaweed Ulva sp. with subcritical water hydrolysis"* **Energy Conversion and Management** ***225***: 113380. DOI: 10.1016/j.enconman.2020.113380.

[6] M. N. Nasrabadi, A. S. Doost, and R. Mezzenga, (2021) *"Modification approaches of plant-based proteins to improve their techno-functionality and use in food products"* **Food Hydrocolloids** ***118***: 106789. DOI: 10.1016/j.foodhyd.2021.106789.

[7] K.-X. Zhu, X.-H. Sun, and H.-M. Zhou, (2009) *"Optimization of ultrasound-assisted extraction of defatted wheat germ proteins by reverse micelles"* **Journal of Cereal Science** ***50***(2): 266–271. DOI: 10.1016/j.jcs.2009.06.006.

[8] C. Ferreira, M. M. Moreira, C. Delerue-Matos, and M. Sarraguça, (2023) *"Subcritical Water Extraction to Valorize Grape Biomass—A Step Closer to Circular Economy"* **Molecules** ***28***(22): 7538. DOI: 10.3390/molecules28227538.

[9] S. Areeya, E. J. Panakkal, M. Sriariyanun, T. Kangsadan, A. Tawai, S. Amornraksa, U. W. Hartley, and P. Yasurin, (2023) *"A review on chemical pretreatment of lignocellulosic biomass for the production of bioproducts: mechanisms, challenges and applications"* **Applied Science and Engineering Progress** ***16***(3): 6767–6767. DOI: 10.14416/j.asep.2023.02.008.

[10] D. Jose, N. Kitiborwornkul, M. Sriariyanun, and K. Keerthi, (2022) *"A review on chemical pretreatment methods of lignocellulosic biomass: Recent advances and progress"* **Applied Science and Engineering Progress** ***15***(4): 6210–6210. DOI: 10.14416/j.asep.2022.08.001.

[11] J. Zhang, C. Wen, H. Zhang, Y. Duan, and H. Ma, (2020) *"Recent advances in the extraction of bioactive compounds with subcritical water: A review"* **Trends in Food Science & Technology** ***95***: 183–195. DOI: 10.1016/j.tifs.2019.11.018.

[12] N. L. Rahmah, S. M. M. Kamal, A. Sulaiman, F. S. Taip, S. I. Siajam, et al., (2022) *"Optimization Of Phenolic Compounds And Antioxidant Extraction From Piper Betle Linn. Leaves Using Pressurized Hot Water"* **Journal of Applied Science and Engineering** ***26***(2): 175–184. DOI: 10.6180/jase.202302_26(2).0003.

[13] N. H. Zainan, M. A. M. Sapardi, B. C. H. Ho, S. I. Siajam, S. M. M. Kamal, M. K. Danquah, and R. Harun, (2022) *"Correction to: Kinetic and thermodynamic characterization of amino acids generation via subcritical water reaction of microalgae Nannochloropsis sp. biomass"* **Biomass Conversion and Biorefinery**: 1–1. DOI: 10.1007/s13399-021-01908-w.

[14] W. Abdelmoez and H. Yoshida, (2013) *"Production of amino and organic acids from protein using sub-critical water technology"* **International Journal of Chemical Reactor Engineering** ***11***(1): 369–384. DOI: 10.1515/ijcre-2013-0017.

[15] H. D. D. Ziero, L. C. Ampese, W. G. Sganzerla, P. C. Torres-Mayanga, M. T. Timko, S. I. Mussatto, and T. Forster-Carneiro, (2022) *"Subcritical water hydrolysis of poultry feathers for amino acids production"* **The Journal of Supercritical Fluids *181***: 105492. DOI: 10.1016/j.supflu.2021.105492.

[16] Y. Cheng, F. Xue, S. Yu, S. Du, and Y. Yang, (2021) *"Subcritical water extraction of natural products"* **Molecules *26***(13): 4004.

[17] F. He, (2011) *"Bradford protein assay"* **Bio-protocol**: e45–e45.

[18] M.-J. Ko, M.-R. Kwon, and M.-S. Chung, (2020) *"Pilot-scale subcritical-water extraction of nodakenin and decursin from Angelica gigas Nakai"* **Food Science and Biotechnology *29***: 631–639.

[19] A. A. Suleiman, U. A. Abdullahi, A. Suleiman, S. A. Suleiman, and H. U. Abubakar, (2022) *"Correlation and regression model for physicochemical quality of groundwater in the Jaen District of Kano State, Nigeria"* **Journal of Statistical Modeling & Analytics (JOSMA) *4***(1):

[20] V. V. Acharya and P. Chaudhuri, (2021) *"Modalities of protein denaturation and nature of denaturants"* **International Journal of Pharmaceutical Sciences Review and Research *69***(2): 19–24.

[21] G. Náthia-Neves and E. Alonso, (2024) *"Optimization of the subcritical water treatment from sunflower by-product for producing protein and sugar extracts"* **Biomass Conversion and Biorefinery *14***(2): 1637–1650.

[22] A. H. Asl and M. Khajenoori, (2013) *"Subcritical water extraction"* **Mass Transfer-Advances in sustainable energy and environment oriented numerical modeling**: 459–487.

[23] B. Díaz-Reinoso, S. Rivas, J. Rivas, and H. Domínguez, (2023) *"Subcritical water extraction of essential oils and plant oils"* **Sustainable Chemistry and Pharmacy *36***: 101332.

[24] S. Jokić, T. Gagić, Ž. Knez, D. Šubarić, and M. Škerget, (2018) *"Separation of active compounds from food by-product (cocoa shell) using subcritical water extraction"* **Molecules *23***(6): 1408. DOI: 10.3390/molecules23061408.

[25] T. Okuda, A. U. Baes, W. Nishijima, and M. Okada, (2001) *"Coagulation mechanism of salt solution-extracted active component in Moringa oleifera seeds"* **Water research *35***(3): 830–834. DOI: 10.1016/S0043-1354(00)00296-7.

[26] V. V. Acharya and P. Chaudhuri, (2021) *"Modalities of protein denaturation and nature of denaturants"* **International Journal of Pharmaceutical Sciences Review and Research *69***(2): 19–24.

[27] S. Awaluddin, S. Thiruvenkadam, S. Izhar, Y. Hiroyuki, M. K. Danquah, R. Harun, et al., (2016) *"Subcritical water technology for enhanced extraction of biochemical compounds from Chlorella vulgaris"* **BioMed research international *2016***: DOI: 10.1155/2016/5816974.

[28] J. Lu, X. Feng, Y. Han, and C. Xue, (2014) *"Optimization of subcritical fluid extraction of carotenoids and chlorophyll a from Laminaria japonica Aresch by response surface methodology"* **Journal of the Science of Food and Agriculture *94***(1): 139–145. DOI: 10.1002/jsfa.6224.

[29] M. Choudhary and S. Neogi, (2017) *"A natural coagulant protein from Moringa oleifera: isolation, characterization, and potential use for water treatment"* **Materials Research Express *4***(10): 105502. DOI: 10.1088/2053-1591/aa8b8c.

[30] A. N. Jones and J. Bridgeman, (2019) *"Identifying Molecular Mass of Coagulant Protein from Edible Hibiscus Seeds Using SDS-PAGE Analysis"* **Journal of Environmental Engineering *145***(11): 04019077. DOI: 10.1061/(ASCE)EE.1943-7870.0001595.

[31] N. A. Awang and H. A. Aziz, (2012) *"Hibiscus rosa-sinensis leaf extract as coagulant aid in leachate treatment"* **Applied Water Science *2***: 293–298. DOI: 10.1007/s13201-012-0049-y.

[32] Z. Zhang, S. Xia, J. Zhao, and J. Zhang, (2010) *"Characterization and flocculation mechanism of high-efficiency microbial flocculant TJ-F1 from Proteus mirabilis"* **Colloids and Surfaces B: Biointerfaces *75***(1): 247–251. DOI: 10.1016/j.colsurfb.2009.08.038.

[33] A. F. Santos, L. A. Luz, A. C. Argolo, J. A. Teixeira, P. M. Paiva, and L. C. Coelho, (2009) *"Isolation of a seed coagulant Moringa oleifera lectin"* **Process biochemistry *44***(4): 504–508. DOI: 10.1016/j.procbio.2009.01.002.