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Investigate the Conditions of Oil Extraction by Enzyme-Assisted Aqueous Extraction Method

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Cashew nut shell liquid (CNSL) is a liquid with a dark brown color in the soft honeycomb structure of the cashew nut shell, which is the by-product of the processing industry of the cashew nut. In this study, the enzyme-assisted aqueous extraction (EAAE) method was used to extract CNSL. The effect of several parameters such as water/material ratio, enzyme/material ratio, time and temperature for enzyme incubation on the oil yield was examined. The results indicated that the suitable conditions to extract CNSL were 6 mL water/g cashew nut shell (CNS),15 mL enzyme/g CNS for 2 h at 50 °C. Extracting oil was compared with commercial oil by polyphenol content and antioxidant capacity. The suitable conditions for oil release were found with the water-CNS ratio of 6 mL/g, and enzyme/CNS of 15 µL/g in 2 h of enzyme incubation at 50 °C. The polyphenol oil content of EAAE was 130.90 ± 3.86 µg/mg of GAE and the IC_{50} value was 734 µg/mL. The EAAE method increased CNSL extraction efficiency and the content of polyphenol compounds compared to commercial oil extracted by traditional methods.

1. Introduction

Vietnam is the world's largest cashew kernel exporter with a total value of 3.4B USD and brings a significant source of foreign currency to the country. Cashew nut shell (CNS) accounts for approximately 25 % of the cashew nut weight, so the amount of cashew shells discharged in industrial processing is very large. This amount of cashew nut shell is an abundant source of raw materials for the production of CNSL to supply the growing global demand (Zafeer and Bhat, 2023). The main content of CNSL is polyphenols, which have been widely studied and applied in many industries such as plastics, adhesives, drugs, fertilizers, pesticides, and potential green fuels (Pandiyan et al., 2020). Vietnam is one of the three largest CNSL-producing countries in the world, so it is necessary to have studies to improve the efficiency of CNSL extraction to improve economic efficiency.

Water extraction is a traditional method for oil extraction in that the extraction solvent is water. The method's efficiency is limited because the water assumes an extended time to break down the cellulose wall of the oil-containing material (Idris and Sulaiman, 2017). To overcome this limitation, oil extraction by the enzyme-assisted aqueous extraction (EAAE) method uses the enzyme and water combination to weaken the cell wall bonds of the material's oil-containing for easier and faster oil release (Nguyen and Ngoc, 2022). EAAE is an extraction method that has outstanding advantages over traditional methods. Molecules of lipids are amphoteric, just the part of water-soluble diffuses into water, the remaining components float to the surface to form an emulsion. The oil is further demulsified using enzymes that melt or change the temperature of the emulsion (Wu et al., 2018). Using enzymes allows the separation of the desirable components without altering their properties and positively affects the organoleptic and taste properties of the final product (Kriisa et al., 2022). EAAE can simultaneously extract the oil and protein based on the water's insolubility property in oil as separate and recover the desired compounds without changing their properties (Tirgarian et al., 2019). This method is considered more economical and safer than the organic solvent process, which is suitable for small and flexible plants (France-Oliveira et al., 2021). The extraction method in this study could provide an effective CNSL extraction solution that is more economical and environmentally friendly than previous methods.

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2. Materials and methods

2.1 Materials

The cashew nut shell was purchased at Minh Long cashew nut processing factory in Xuan Loc district, Dong Nai province, then ground by a grinder at a traditional medicine store in Ho Chi Minh City. Commercial CNSL was supplied by Quang Minh Company, Binh Phuoc province, Vietnam. The viscozyme L enzyme provided by Novozyme (Denmark) was derived from the *Aspergillus aculeatus* strain. The viscozyme L was an enzyme complex consisting of arabinase, cellulase, beta-glucanase, hemicellulase, and xylanase.

2.2 Extract CNSL by EAAE method

The extraction process was performed according to the method of Phuong and Tuan (2016). The ground cashew nut shell with 4 g was dispersed into distilled water at the rate of 7 mL/g cashew nut shell in a 50 mL Falcon tube, then 40 µL of Viscozyme L enzyme was added and shaken well and incubated in a shaker at 55 °C at 100 rpm. After incubation, Falcon tubes were centrifuged for 30 min at 4,000 rpm. At the end of treatment, the CNSL was collected, and the drying process of the residual material was conducted under constant condition at 70 °C for 3 h. The raw material (m_{before}) and final cashew residue (m_{after}) was weighed to calculate the amount of oil. The amount of oil was calculated by Eq (1):

$$m_{oil}(g) = m_{before} - m_{after} \quad (1)$$

2.3 Investigate the conditions of CNSL extraction by enzyme-assisted aqueous extraction method

Expected factors affecting CNSL extraction by the EAAE method on cashew nut shells included water-CNS ratio (mL/g), enzyme-CNS ratio (µL/g), incubation temperature (°C), and incubation time (h). Experiments were performed with each factor in turn. The first was a change in the water-CNS ratio (5 mL/g, 6 mL/g, 7 mL/g, 8 mL/g, and 9 mL/g) with a fixed condition with enzyme: CSN 10 µL/g for 2 h at 50 °C. After choosing the appropriate ratio, the experiments continued to be conducted on the impact of enzyme-CNS rate on CNSL extraction. Enzyme-CNS ratios were 0 mL/g, 5 mL/g, 10 mL/g, 15 mL/g, and 20 mL/g under same time and temperature conditions. The experiment to investigate the incubation time was performed with the optimal ratios of the water-CNS and enzyme-CNS from the above experiments, the incubation temperature was 50 ºC and the incubation time changed with 1 h, 2 h, 3 h, 4 h, and 5 h. The experiment to evaluate the effect of temperature on oil extraction efficiency was performed with the best conditions from the above experiments and the extraction temperature changed from 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C. To evaluate the effectiveness of the EAAE method compared with the pressing method used in industry, the polyphenols and antioxidant capacity of these two types of CNSL were quantified.

Polyphenols content was measured by the Folin-Ciocalteu method. Experiments were taken with 0.5 mL and 2.5 mL of sample and Folin-Ciocalteu solution per test tube. 2 mL of Na2CO3 (7.5 % w/v) was added after 5 min and kept in the dark for 2 h to react, then measure the absorbance at 760 nm. Gallic acid was utilized as the standard solution. The total polyphenol content was represented as the gallic acid equivalent to 1 mg of CNSL (Lawag et al., 2023). Antioxidant capacity was determined using the DPPH method. CNSL was dissolved in methanol at different concentrations. 0.3 mL of CNSL at various concentrations was added to 0.3 mL of 0.2 mM DPPH. Shook well, and left in the darkness for 30 min. Ascorbic acid was utilized as the standard solution. The experiment results using the optical absorbance method at 517 nm wavelength.

2.4 Statistical analysis

The results of the experiments were analyzed and data was processed using one-way ANOVA and DUNCAN analysis ($p \leq 0.05$) through the SPSS software v.20. Graphical representation was performed using Origin 2021 software.

3. Results and discussion

3.1 Oil extraction conditions by EAAE method

The results of the water-CNS ratio, as shown in Figure 1, indicate that the yield of oil obtained at the rate of 5 mL/g was 0.75 g. The results of three treatments ranging from 6-8 mL/g increased with the oil volume of 0.78 g, 0.81 g, and 0.78 g. The oil yield decreased as the amount of water increased to 9 mL/g, resulting in a yield of 0.7 g. The yield of oil obtained in 3 treatments 6-8 mL/g had no significant difference ($p>0.05$). The suitable water-CNS ratio for oil extraction was 6 mL/g to save water and energy for the next steps. In the previous study by Phuong and Tuan (2016), the water-CNS ratio was 9 mL/g on the raw material which was cashew shell. The difference could be explained by the size and source of raw materials, as well as enzymes used for extracting, which were viscozyme cassava C and viscozyme L (Phuong and Tuan, 2016).

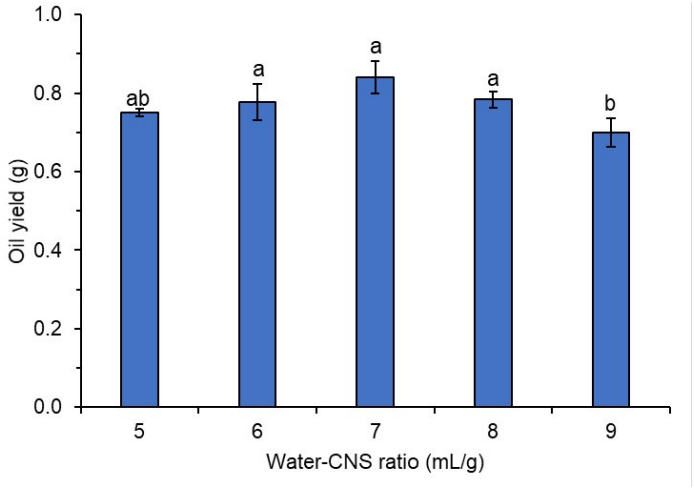

Figure 1: Effect of water-CNS ratio on the oil yield

The highest yield of CNSL obtained was 0.95 g at the enzyme-CNS ratio of 15 µL/g, which was statistically different from the other treatments (p-value <0.05) and the lowest CNSL yield obtained was 0.47 g when the enzyme was not added (Figure 2). When the amount of enzyme increased at 20 µL/g, the yield of CNSL obtained was 0.88 g lower than in the treatment of 15 µL/g, this result showed the amount of enzyme exceeds the optimal catalytic threshold for the process. This result showed that increasing the enzyme ratio beyond the optimal threshold did not increase extraction efficiency (Martins et al., 2014). The selected enzyme-CNS ratio for CNSL extraction was 15 µL/g.

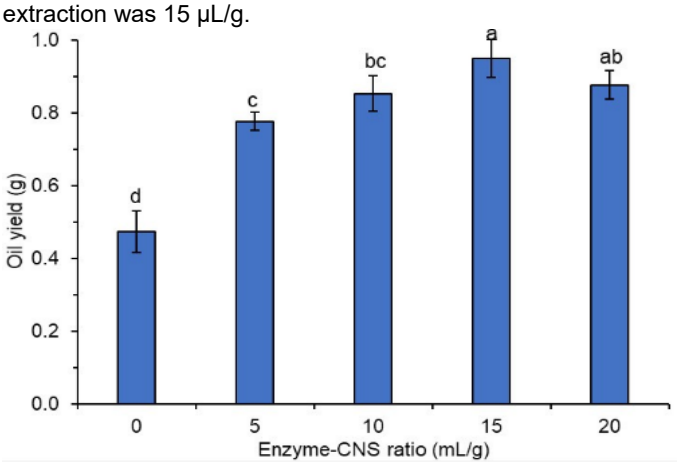

Figure 2: Effect of enzyme-CNS ratio on extracted CNSL by EAAE method

The yield of CNSL obtained in the treatment without enzyme addition (0.47 g) was lower than all the treatments with enzyme effect and only half of that was in the treatment with enzyme supplementation at the rate of 15 µL/g (0.95 g). This result highlighted the role of enzymes in breaking down plant cell walls to increase the efficiency of oil extraction from plants. The viscozyme L contained a mixture of carbohydrate enzymes that help to weaken the bonds in the cell wall of the cashew shell (Giovannoni et al., 2020). Glucanase could not hydrolyze cellulose but could act on the cell wall to release xyloglucan oligosaccharide which was a substrate for the hemicellulase group (arabinase, xylanase). Hemicellulase and cellulase cleaved glycoside bonds to form sugar molecules with smaller molecular weights such as glucose, arabinose, and xylose which helped the oil molecules in the honeycomb structure to escape easily (Ahmed and Claire, 2023).

Experiments with time intervals of 1 h, 2 h, 3 h, 4 h, and 5 h were conducted with initial conditions of 6 mL/g water-CNS ratio, 15 µL/g enzyme-CNS ratio, and 50 °C temperature. The results in Figure 3 showed that the incubation time had an effect on the extraction efficiency with the highest yield of CNSL obtained at 0.94 g and statistically significant at 2 h and then slightly decreased at 0.87 g when the incubation time increased to 3 hours. This proves that the long incubation time didn't increase the oil extraction efficiency but also rose the production cost. According to conventional extraction processes, when the extraction time was longer, the

enzyme had enough time to break more molecular bonds, so the yield of oil present in the solvent was higher. But the extraction time was too long, the enzyme could inactivate and the reaction rate to decrease. The decrease in oil yield in the long-term treatments could also be due to the oil being re-adsorbed on the residual solid. A long reaction time could lead to a risk of oil damage by hydrolysis or oxidation reactions (Huynh et al., 2013).

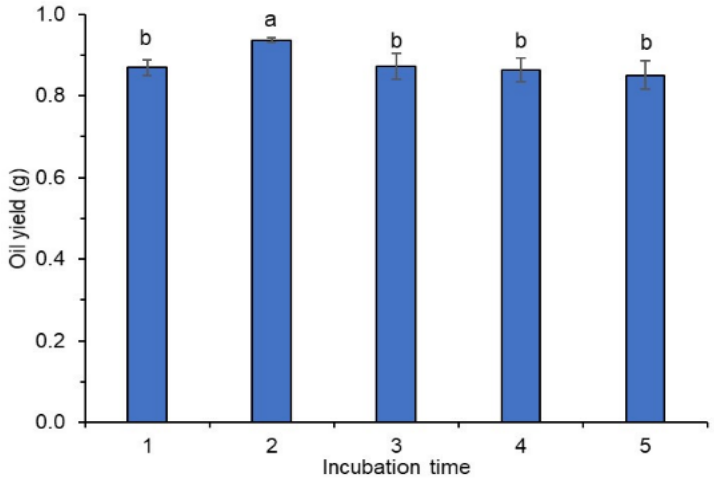

Figure 3: Effect of incubation time on CNSL yield

Investigate the effect of temperature on oil extraction at temperature conditions of 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C with the conditions of water-CNS 6 mL/g, enzyme-CNS 15 µL/g, and incubation time 2 h (Figure 4).

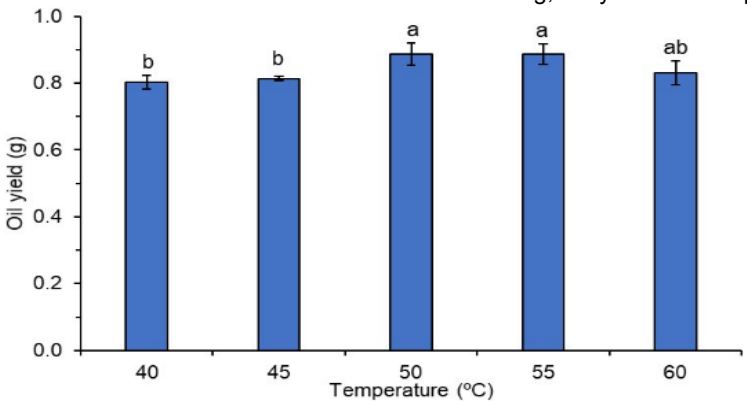

Figure 4: Effect of temperature *on oil yield*

The results showed that the mass of oil obtained was similar at two temperatures of 40 °C (0.80 g) and 45 °C with 0.81 g and increased to 0.89 g of oil at both 50 °C and 55 °C. The appropriate temperature for extracting CNSL using the Viscozyme L was 50 °C This result was consistent with the temperature evaluated as optimal for hemicellulase activity obtained from *Aspergillus aculeatus* (Ahmed and Claire, 2023). Temperature was one of the important factors for any oil extraction method, enzymes were temperature sensitive and operated over a narrow temperature range. If the temperature was too high compared to the optimal operating temperature, the enzyme was denatured, and darkening of the oil can occur, inactivation of proteins, and degradation of biologically active substances. The appropriate temperature for extraction depended on the enzyme used, the raw materials, and the target product (Mwaurah et al., 2020).

3.2 Evaluation of the CNSL properties

Using gallic acid as the standard, the polyphenol standard curve equation had the form y = 0.0121x+ 0.0181 with R^2=0.9981. The total polyphenol content of the extracted CNSL was 130.90 ± 3.86 (µg GAE/mg oil) higher than the commercial oil sample with 80.27 ± 3.62 (µg GAE/ mg of oil) (Figure 5). Polyphenol concentrations in some previous studies with water and ethanol solvents were 180.15 ± 9.47 and 125.6 ± 8.4 mg GAE/g oil. The CNSL was extracted by shaking and incubation with ethanol as a solvent with a polyphenol concentration of 243 mg

GAE/g oil (Sruthi et al., 2023). Pressed-extracted CNSL had higher impurity levels, higher viscosity, lower thermal oxidation stability, and lower failure temperature than cold solvent-extracted CNSL (Huynh et al., 2013).

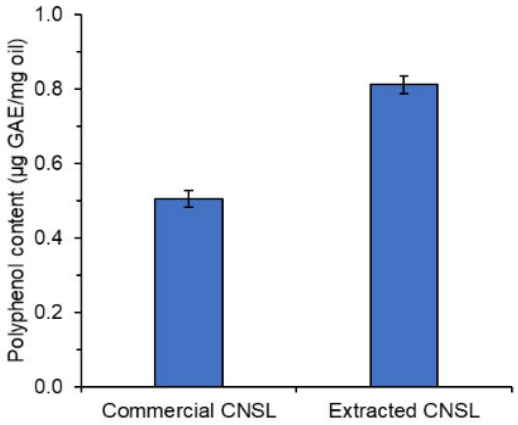

Figure 5: Polyphenol content in two types of CNSL

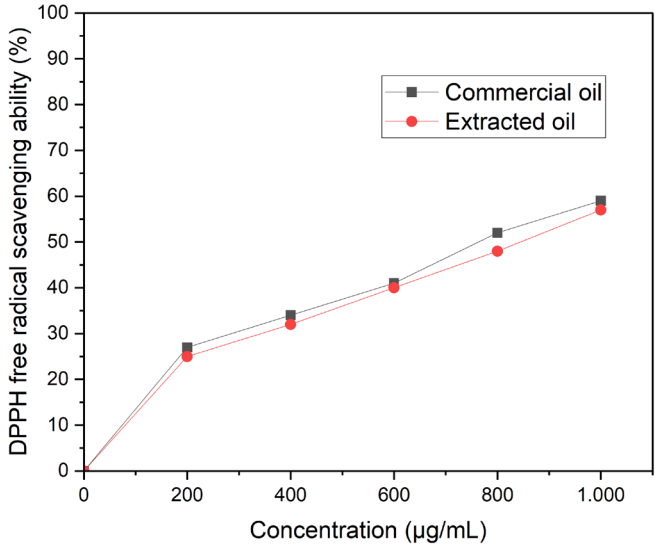

Figure 6: DPPH free radical scavenging ability of two types of CNSL

The antioxidant capacity of a substance was assessed based on the IC_{50} value. IC_{50} was the antioxidant content required to scavenge 50 % of the free radicals in the DPPH solution. The lower the IC_{50} value, the higher the antioxidant activity, and vice versa (Sruthi et al., 2023). The DPPH free radical scavenging ability of the commercial oil and the extracted oil was shown in Figure 6 showing that there was no significant difference in IC_{50} value between the commercial oil and extracted oil with 734 µg/mL and 781.78 µg/mL. CNSL was a hindered phenols mixture with a replacement of the long alkyl group at meta-position. The antioxidant capacity was correlated with the electron-donor nature and the substituent steric effect. The effect of electron-donating could enhance the oxygen's electron density of the phenol, which resulted in the high radical-trapping speed. Steric effect prevented the coupling of phenoxy radicals and raised the number of peroxy radicals trapped (Rodrigues et al, 2006). Allyl substituents captured peroxyl and alkyl radicals and increased antioxidant action (Morais et al., 2017).

4. Conclusions

The experiment to investigate the extraction conditions of cashew nut shell oil showed that the conditions were suitable to improve the extraction efficiency of CNSL by the EAAE method with the ratio of water-CNS 6 mL/g, the ratio of enzymes–CNS 15 µL/g for 2 h at 50 °C. Evaluating and comparing with commercial CNSL, the total

polyphenol content of the extracted oil sample was 130.90 ± 3.6 (μg GAE/mg oil) higher than that of the commercial CNSL sample with a content of 80.27 ± 3.62 (μg GAE/mg oil).

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