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ORIGINAL ARTICLE

Double Emulsion Based Alginate/Chitosan Prepared by Ultrasound for Bioactive Encapsulation

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ABSTRACT – Water-in-oil-in-water (W/O/W) emulsion is an effective technology for encapsulating bioactivity but has poor stability. This study aims to evaluate the effect of ultrasonication on the preparation of double emulsions with varying alginate/chitosan ratios (1:1, 1:2, 2:1) and sonication times between 0 and 7 minutes. The results showed that a sonication time of 7 minutes resulted in the best storage stability (0.00–1.02%) and thermal stability (17.13–23.21%), a significant decrease in droplet size (2.08–2.87 μm), and higher emulsion activity index (EAI) (16.11–38.10 m^2/g) and emulsion stability index (ESI) (63.28–89.74 min) values. The alginate/chitosan ratio of 2:1 also gave the most optimal results with storage stability of 4.58%, thermal stability of 36.23%, droplet size of 2.08–4.14 μm, and EAI and ESI values of 16.12 m^2/g and 48.42 min. The ultrasonic-assisted alginate/chitosan double emulsion method effectively improved the stability and bioavailability of bioactive and presents significant potential for food and pharmaceutical applications.

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INTRODUCTION

Double emulsions are complex structures characterized by droplets of one liquid phase encapsulated in droplets of another liquid phase, all suspended in a continuous medium. The most common type is the water-in-oil-in-water (W1/O/W2) emulsion, where water droplets are contained in oil droplets, which are then dispersed in the water phase [1]. This technology has a variety of important applications, especially in the pharmaceutical, food, and cosmetic industries. For example, W/O/W emulsions are used to control the release of active substances or nutrients in the body, as well as to maintain the stability of sensitive materials. However, double emulsions are considered unstable, causing aggregation of oil droplets, internal water phase migration, and flocculation or phase separation during processing or storage, thus limiting its functional nutritional applications [2]. To overcome these obstacles, ultrasonication technology can be applied.

Ultrasonication is a process that utilizes ultrasonic waves to create cavitation in a liquid, which produces strong shear forces to form very fine and stable emulsion droplets [3]. Ultrasound is needed to influence the mechanical effect so that the particle size can be easily decreased [4]. The advantages of ultrasonic emulsification are lower cost and great availability [5]. This ultrasonication process can help reduce particle size significantly. The ultrasonic-assisted emulsification process will produce energy waves that can accelerate the entry of active ingredients and improve the permeability properties of the coating polymer layer. The factors that influence the sonication process are time, power, frequency, and temperature. Pratap-Singh et al. (2021) found that the optimal ultrasonication process time to reduce the particle size of samples was 10 minutes and was roughly constant regardless of the process parameters (frequency and power) [6]. In the study of Guzmán et al. (2020), the optimal ultrasonication time for emulsification of Passiflora edulis seed oil was 5.96 minutes, which significantly improved the physical properties of the emulsion [7]. However, excessive time can also cause degradation.

One of the applications of W/O/W emulsions is to encapsulate lipophilic and hydrophilic bioactive compounds commonly found in plant extracts [8]. Moringa oleifera Lam., often known as the Moringa tree, is a plant with high nutritional value and various health benefits. Parts of the plant, such as leaves, seeds, flowers, and pods, contain various essential nutrients, including vitamins, minerals, and antioxidants that are very beneficial for human health [9]. Although Moringa oleifera offers various benefits, its utilization can be constrained by several factors, such as the stability of bioactive compounds, low bioavailability, and unpleasant taste and aroma [10]. Therefore, a method is needed to increase the stability and effectiveness of Moringa oleifera utilization, one of which is encapsulation.

In the encapsulation process, the encapsulated substance is called the core material/active agent, while the

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encapsulating substance is called the carrier/wall material [11]. The wall material must form a cohesive film to the active agent, be inert to the active agent, and have coating properties such as strength, flexibility, impermeability, optical properties, and stability [12]. The wall material will affect the properties of the core material, including drying results, encapsulation efficiency, water content, dispersibility properties, etc. [13]. In this study, a combination of alginate and chitosan was used as the wall material. Alginate and chitosan were selected as encapsulating agents due to their characteristics, which fulfill the essential requirements for effective wall materials. Alginate, which comes from brown algae, can form a stable gel through interaction with calcium ions. It is inert to active agents and has mechanical strength, flexibility, and semi-permeable properties that protect active agents while controlling their release. Meanwhile, chitosan, which comes from the deacetylation of chitin, has adhesive properties, is biocompatible, can form a protective layer with high impermeability, and offers additional stability with its antimicrobial properties. Using a combination of alginate and chitosan (Alg/Ks) as an encapsulation material offers enhanced stability. Alginate is an anionic polymer with carboxylic groups ($-COO^-$), while chitosan is a cationic polymer with positively charged amino groups ($-NH_2$). When these two polymers are mixed in the appropriate ratio, electrostatic interactions occur, forming a polyelectrolyte complex. This complex forms a protective layer on the oil droplet, preventing coalescence through steric and electrostatic repulsion.

Chitosan, which remains stable at higher pH levels, helps prevent alginate from degrading in such conditions [14]. The combination of physical and morphological properties of Alg/Ks will specifically improve the performance of the encapsulation process and protect the biological activity function of the drug or bioactive compound carried. Alginate will quickly form a gel in the presence of metal cations [15]. The addition of chitosan to encapsulation provides dual benefits, namely increasing the nutrition and functional properties of the material. Research conducted by Prasetyaningrum et al. (2021) found that the combination of alginate and chitosan (Alg/Ks) will provide a synergistic effect to prevent the release of Citronella bioactive and protect against oxidation reactions [16]. In addition, Li et al. (2013) found that double emulsions of alginate and chitosan can encapsulate insulin and create a stable oral delivery system [17]. However, research on the effect of ultrasonication on Alg/Ks double emulsions is still limited. In this study, W1/O/W2 (water-in-oil-in-water) type double emulsion was used for encapsulation, where the water phase is dispersed in the oil phase, which is then dispersed in another water phase. The treatment of alginate/chitosan mixtures using the ultrasonic method also improves the chemical and physical properties of the material. Therefore, this study will be carried out with ultrasonication-assisted encapsulation of moringa oleifera in W/O/W double emulsion.

EXPERIMENTAL METHOD

Materials

Moringa oleifera extract was obtained from NutriOne, alginate (molar mass 216.12 g/mol with CAS Number 9005-38-3 SIGMA-Aldrich, USA), chitosan (75–85% degree of deacetylation with CAS Number 9012-76-4 SIGMA Aldrich, USA). Chemicals such as acetic acid, Tween 80, lecithin, and sodium dodecyl sulfate (SDS) were of commercial grade.

Preparation of W1/O Emulsion

The oil phase (O) was formed by adding lecithin (1% v/v) to soybean oil, and the mixture was homogenized for 30 minutes. The internal water phase (W1) phase was created by dissolving 2% w/v Moringa extract in distilled water and stirring for 30 min. The W1/O emulsion was prepared by mixing W1 and O in a 6:4 ratio, followed by homogenization for 3 minutes at a speed of 10,000 rpm.

Preparation of W1/O/W2 Emulsion

The external phase (W2) consisted of a mixture of Alg/Ks with 1:1, 1:2, and 2:1 ratios. Tween 80 (1%, v/v) was added as an emulsifier. To prepare the W1/O/W2 emulsion, the W1/O emulsion was added to the W2 phase and homogenized for 1 minute and 30 s. Double emulsions were prepared using 25% (w/w) W1/O and 75% (w/w) of the external aqueous phase (W2). These emulsions were then sonicated for 0, 2, 5, and 7 minutes (20 kHz, 5 s on, 4 s off, Biobase UCD-250).

Storage Stability

The samples were preserved at 4oC for 7 days. The creaming index (CI) was determined by assessing the total height (cm) of the emulsion (Ht) in conjunction with the height (cm) of the cream layer (Hs). The CI was calculated in Equation (1).

$$CI\ (\%) = \frac{Hs}{Ht} \times 100\% \quad (1)$$

Thermal Stability

10 mL of sample was placed in a beaker glass and heated using a hotplate stirrer at a temperature of 70°C for 30 minutes. The CI values of the thermally treated specimens were assessed as delineated in Equation (**Error! Reference source not found.**).

Microstructure and Particle Size

The W1/O/W2 emulsion was observed using an optical microscope (Olympus CX43, Japan). One drop of the emulsion was placed on a microscope slide and diluted with two drops of 0.5% Tween 80. The coverslip was dropped without forming air bubbles, and images were taken using ImageJ software using an optical microscope at 100× magnification. The size of droplets was measured in micrometers (μm) using the radius tool in ImageJ software.

Emulsion Activity Index/Emulsion Stability Index (EAI/ESI)

The emulsifying characteristics were evaluated by determining the EAI/ESI. An aliquot of 50 μL of the freshly prepared sample was promptly combined with 5 mL of 0.1% SDS, and the absorbance measurement at 500 nm was conducted utilizing an ultraviolet-visible (UV-vis) spectrophotometer (Shimadzu UV-1900i, Japan). The calculation formula is shown in Equation (2) and Equation (3):

$$\text{EAI } (m^2/g) = 2 \times 2.303 \times \frac{A0 \times N}{10000 C \theta} \quad (2)$$

$$\text{ESI } (min) = \frac{A0}{A0\text{-}A30} \times 30 \quad (3)$$

where A0 and A30 signify the absorbance measured at 0 min and 30 min, respectively, N refers to the dilution factor, θ represents the proportion of the oil phase, and the concentration of the emulsifier is expressed in grams per milliliter (g/mL).

RESULT AND DISCUSSION

Storage Stability

The creaming index (CI) is a parameter used to measure the stability of an emulsion. As illustrated in Figure 1, the CI was monitored during storage. Ultrasonication time significantly affects the CI for various alginate and chitosan (Alg/Ks) ratios. In the sample without sonication (Figure 1 (a)), the emulsion stability is still relatively low with a high CI, particularly at an Alg/Ks ratio of 2:1. At an ultrasonication time of 2 minutes (Figure 1 (b)), there is a significant decrease in CI for all ratios, indicating that ultrasonication helps improve emulsion stability. At an ultrasonication time of 5 minutes (Figure 1 (c)), it also shows an increase in stability for all ratios. At an ultrasonication time of 7 minutes (Figure 1 (d)), all ratios reach almost stable CI values. The Alg/Ks ratio of 1:2 remains the most unstable at all ultrasonication times, with the highest CI value. Overall, longer ultrasonication times improve emulsion stability.

After 7 days of storage, the double emulsion prepared without ultrasonic treatment showed obvious separation between the internal water in oil (W1/O) emulsion and the external water phase (W2), which led to high CI values. This indicated the poor stability of the double emulsion prepared in the sample without ultrasonic. Compared to the sample without ultrasound treatment, changes in CI values were not clearly observed after ultrasound treatment, indicating that this treatment enhanced stability. Ultrasonication can help achieve better homogeneity in emulsions through several mechanisms. First, ultrasonication can reduce the droplet size in the emulsion, thereby increasing emulsion stability and decreasing the creaming index, as smaller droplets are less prone to float and coalesce due to the reduced gravitational force [18]. Second, ultrasonication can create a more uniform droplet size distribution, which also contributes to emulsion stability [2]. Additionally, ultrasonication enhances the interaction between surfactant molecules and oil droplets, helping stabilize droplets and prevent coalescence, lowering the creaming index [19]. Moreover, the polysaccharide ratio affects emulsion stability. The sample with an Alg/Ks ratio of 2:1 remained relatively stable after storage, likely due to the compact coating structure formed by the Alg/Ks complex, which prevents droplet aggregation. Meanwhile, samples with an Alg/Ks ratio of 1:2 showed the lowest stability compared to other ratios. If the amount of chitosan is too high, the interaction between the positively charged amino groups ($-NH_3^+$) in excess chitosan with the negatively charged carboxylate groups ($-COO^-$) in alginate can cause the formation of inhomogeneous aggregates so that the particles become larger and are not well distributed in the emulsion. The

imbalance of the electrostatic charges of the two polymers can trigger system instability, which causes flocculation and coalescence of particles. This is in accordance with research by Sapei et al. (2022), where increasing the concentration of chitosan will reduce the interaction between chitosan and oil due to stronger molecular interactions between chitosan molecules due to high electrostatic forces [20].

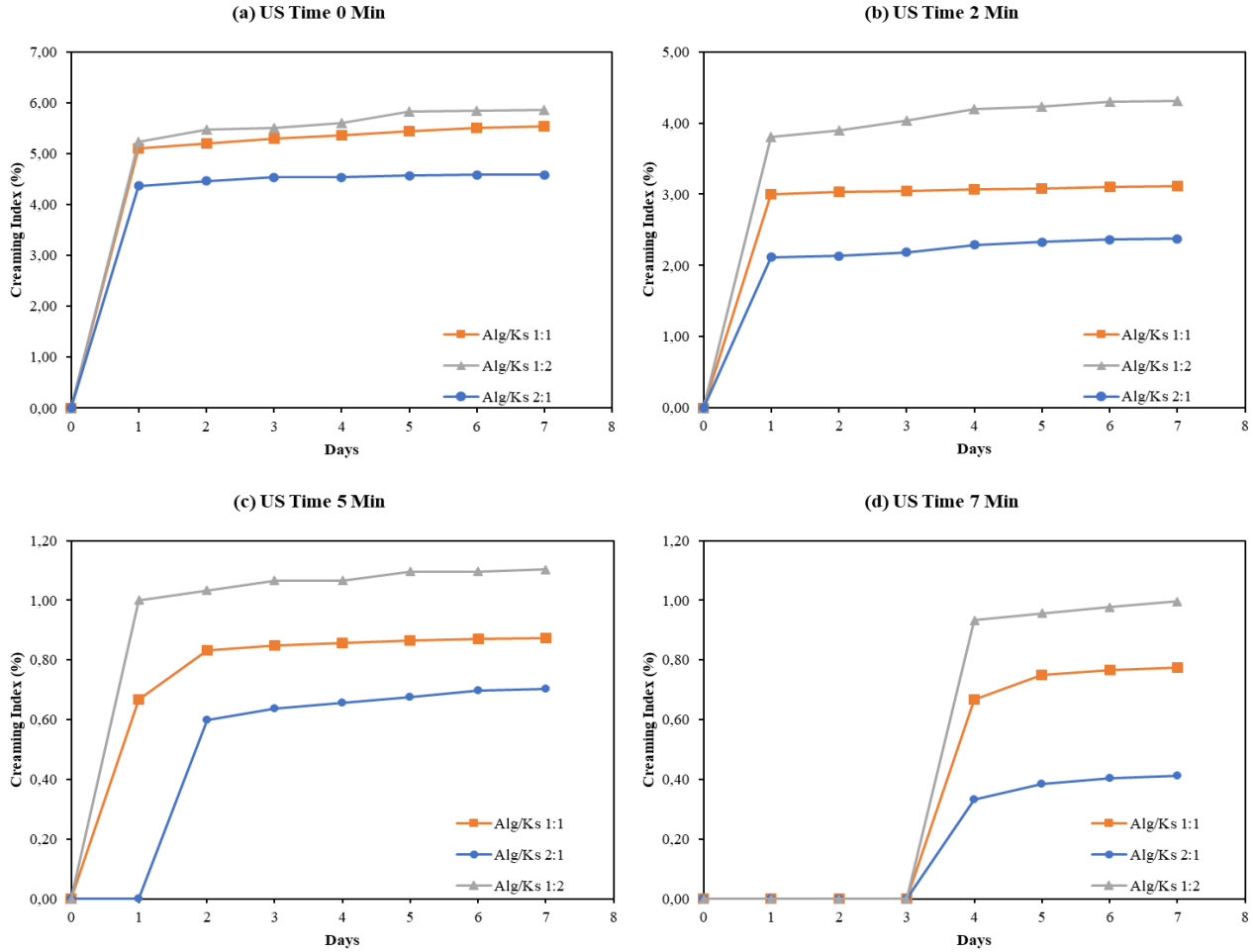

Figure 1. Storage stability of W/O/W emulsion under different ultrasound (US) time and Alg/Ks ratio

Thermal Stability

Heat treatment is a common technique used in food processing because it can kill microorganisms in food and increase its safety [21]. Therefore, the emulsion must show good thermal stability. Figure 2 illustrates the thermal stability after heating at 70°C for 30 minutes. The CI of the emulsion without ultrasound treatment was the largest (29.67–36.23%). Emulsion stability can be damaged by heating. This may be due to extensive layer damage caused by heating so that the droplets merge and become non-uniform in size [22]. This phenomenon is similar to the study of Geng et al. (2024), where the heated emulsion showed layer separation [23]. The emulsion subjected to ultrasound treatment showed no separation after heating, and the CI value did not change significantly compared to the non-ultrasonic treatment. Furthermore, it was observed that the Alg/Ks polysaccharide ratio influenced thermal stability due to the interfacial viscoelasticity formed by the polysaccharide complex. However, the emulsion with an Alg/Ks ratio of 1:2 exhibited aggregation after heating, with a higher CI value, which was associated with the larger initial particle size and looser interfacial structure. Emulsions with an Alg/Ks ratio of 2:1 showed better thermal stability compared to other ratios due to more balanced polyelectrolyte interactions and a more organized polymer network structure. This ratio allows the formation of stronger polyelectrolyte complexes, creating a denser hydrogel matrix that is resistant to temperature changes. This ratio also produces a viscosity that is high enough to slow down the movement of water and oil molecules during heating, thereby reducing the possibility of phase separation or coalescence.

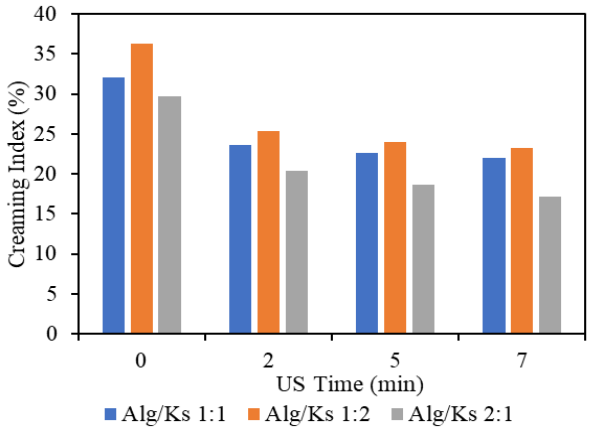

Figure 2. Thermal stability of W/O/W emulsion under different ultrasound (US) time and Alg/Ks ratio

Microstructure and Droplet Size

An optical microscope was used to understand the microstructure of the W/O/W emulsion. Table 1 and Figure 3 show that ultrasonication can reduce particle size, thereby improving emulsion stability. Initially, as the sonication time increases, the mean droplet diameter decreases. Smaller and more uniform droplet sizes make the emulsion more stable, as smaller droplets tend to undergo sedimentation or coalescence more slowly, which enhances the emulsion stability index [24]. This is because the ultrasonic waves break the liquid into smaller droplets, leading to a reduction in droplet size. The energy input during sonication, which is affected by both time and power, plays a crucial role in determining droplet size distribution. Higher energy levels can create more turbulence and shear forces, leading to smaller droplet sizes. This phenomenon is similar to the study of Zhou et al. (2022), where ultrasonic assistance had a positive impact on the particle size and distribution of the emulsion [25].

At the same ultrasound time, the droplet size of the W/O/W emulsion was affected by the proportion of polysaccharides. The Alg/Ks ratio exhibits a more complex behavior in regulating particle size. Emulsion with an Alg/Ks ratio of 2:1 has a higher number of carboxylic groups ($-COO^-$) of alginate compared to amino groups ($-NH_2$) of chitosan, which causes the polyelectrolyte complex to form stably without causing excessive aggregation. A sufficient alginate layer can cover the droplet well, while a lower amount of chitosan prevents excessive interaction that can cause flocculation and increase particle size. Higher amounts of chitosan in the Alg/Ks blend result in larger particles and lower encapsulation efficiency. This is due to the higher affinity of amino groups in chitosan for mannuronic (M) residues in alginate, which can cause particle aggregation [26]. Ultrasonic treatment could decrease the viscosity of polysaccharide molecules in the aqueous phase during emulsification. These changes could accelerate the adsorption of polysaccharide molecules at the oil/water interface, reducing the interfacial tension and thereby enhancing the stability of emulsions via steric repulsion [27].

Table 1. The droplet size of W/O/W emulsion under different ultrasound (US) times and Alg/Ks ratio

US Time	Alg/Ks	Droplet Size (μm)
0 min	1:1	4.78 ± 0.0087
	1:2	5.09 ± 0.0053
	2:1	4.14 ± 0.0071
2 min	1:1	4.40 ± 0.0029
	1:2	4.59 ± 0.0084
	2:1	3.86 ± 0.0112
5 min	1:1	3.12 ± 0.0036
	1:2	3.96 ± 0.0078
	2:1	2.32 ± 0.0065
7 min	1:1	2.52 ± 0.0039
	1:2	2.87 ± 0.0043
	2:1	2.08 ± 0.0035

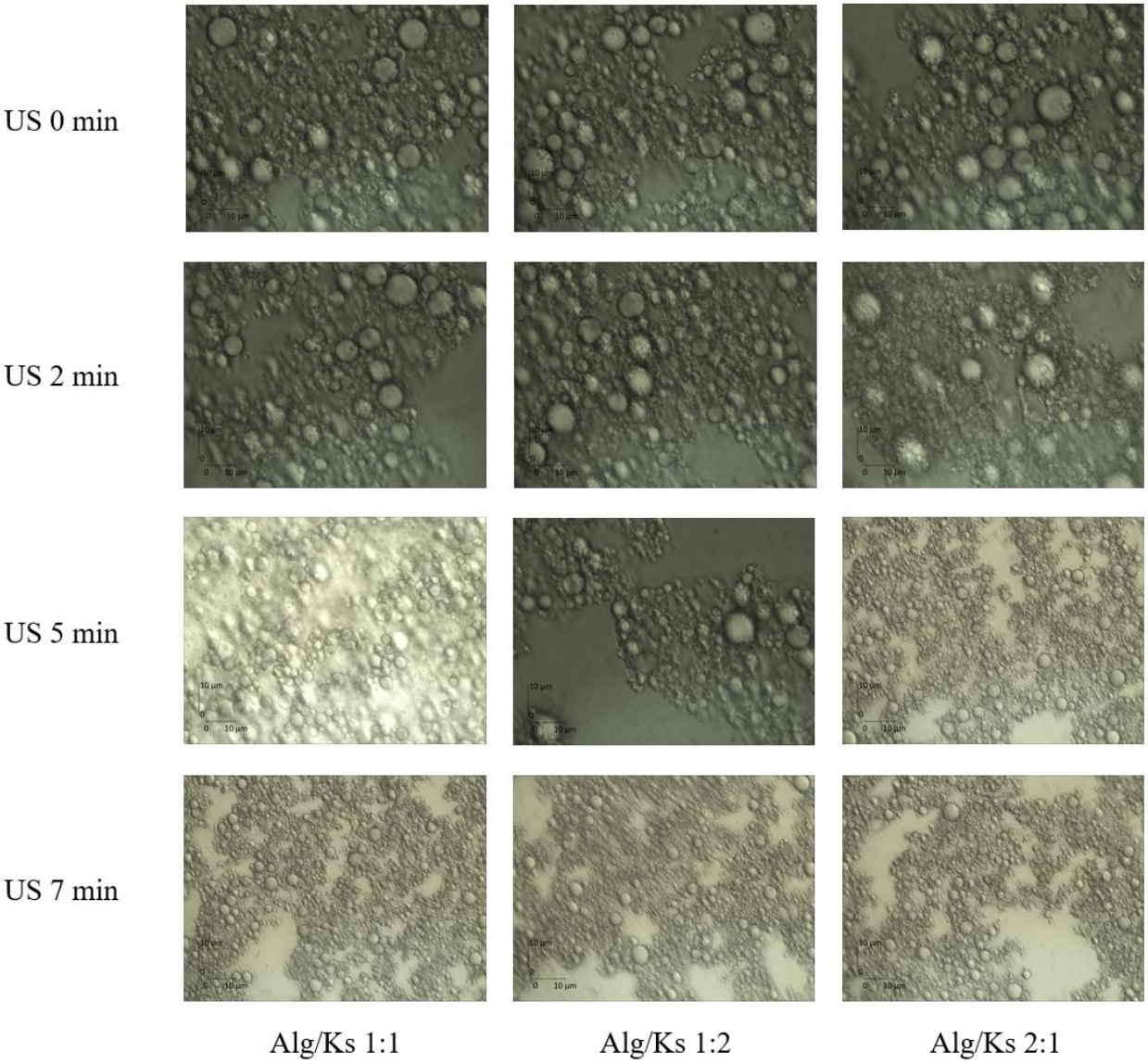

Figure 3. Microstructure of W/O/W emulsion under different ultrasound (US) times and Alg/Ks ratio

Emulsion Activity Index/Emulsion Stability Index (EAI/ESI)

As illustrated in Figure 4, the EAI and ESI values were enhanced by ultrasonic treatment. This is primarily due to the polysaccharide structure being opened by ultrasound, which reduces interfacial tension and improves emulsifying properties [28]. Ultrasound treatment induces cavitation, which produces strong mechanical forces

that can break the glycosidic bonds of polysaccharides. This breakdown increases the surface area of the polysaccharides, allowing better interaction with the oil and water phases, thereby increasing the emulsification capacity [29]. Additionally, the emulsion with an Alg/Ks ratio of 2:1 exhibited superior emulsifying stability, likely due to the increased viscosity of the continuous phase and complex. In this context, the more organized arrangement of polysaccharide molecules enhances molecular interaction forces. The EAI and ESI values increased with a higher proportion of Alg in the Alg/Ks complex. This improvement in emulsifying properties is attributed to increased viscosity, electrostatic repulsion, and steric hindrance [30]. However, an excessively high concentration of Alg can also reduce emulsion stability due to larger particle sizes. In this study, the best emulsification was achieved when the Alg/Ks ratio was 2:1.

Controlling the stability of double emulsions is far more challenging than single emulsions. This is due to the osmotic pressure difference between the two aqueous phases and the requirement for at least two emulsifiers with different hydrophilic-lipophilic balance (HLB) values to stabilize the distinct oil-water interfaces [31]. Double emulsions are thermodynamically unstable systems, making them prone to destabilization by physical and chemical factors. The stability of different emulsions can be influenced by the percentage of emulsifiers used in the primary W/O emulsion [32]. Various other phenomena are associated with the instability of double emulsions, such as coalescence, sedimentation, creaming, flocculation, etc.

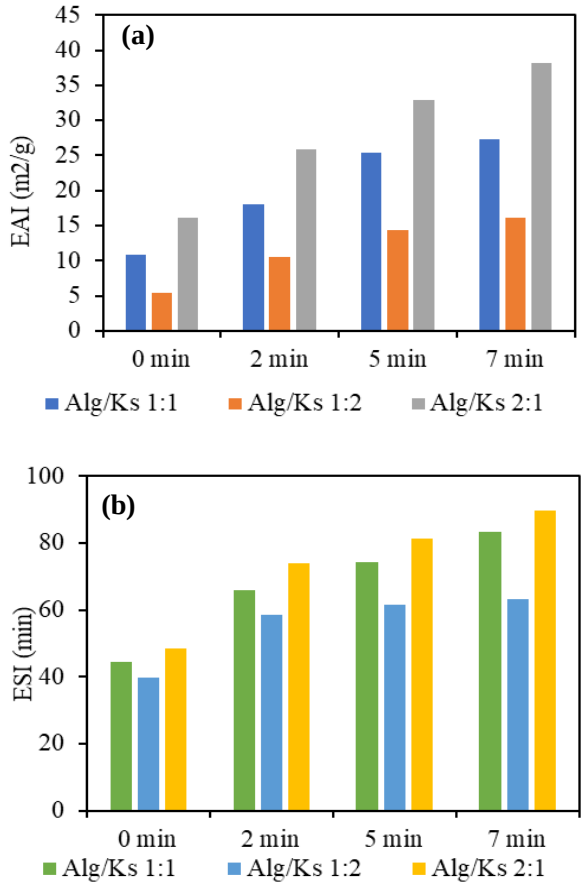

Figure 4. (a) EAI and (b) ESI of W/O/W emulsion under different ultrasound times and Alg/Ks ratios

CONCLUSION

Ultrasonic treatment had a positive effect on the encapsulation of moringa leaves in Alg/Ks double emulsion. The ultrasonication process has significant potential to improve the stability of the double emulsion. This is indicated by the low creaming index and the decrease in droplet size, which directly contribute to the improvement of the stability of the emulsion system. Thus, the ultrasound technique is a promising method to be used in the manufacture of functional food and pharmaceutical products that rely on the stability of double emulsions as a delivery system for active ingredients. Meanwhile, the water-in-oil-in-water (W/O/W) emulsion prepared with an alginate and chitosan (Alg/Ks) ratio of 2:1 had stronger environmental stability and higher bioaccessibility of bioactive substances than ratios of 1:1 and 1:2, which may be due to the higher affinity of amino groups in chitosan to mannuronate residues of alginate. In conclusion, the best physicochemical

properties of the W/O/W emulsion were obtained under the conditions of ultrasonic treatment for 7 minutes, and the Alg/Ks ratio was 2:1.

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