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Full Length Article

Effect of Microencapsulator Speed on Composite Microcapsule Diameter Tailored to Fish Larvae Mouth Openings

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Abstract

This research aimed to determine the effect of stirring speed on the size and structural characteristics of microcapsule particles and to evaluate the morphological and physical characteristics of composite microcapsules using different combinations of matrix materials. The technique used in this research was modified suspension cross-linking. The results showed that stirring speed significantly influenced the size and structural characteristics of microcapsules. Higher stirring speed (1300 rpm) correlated with smaller sizes, increased particle density and thicker matrix layer. Combinations of matrix materials (composites) exhibited similar morphological characteristics but differed in their physical properties. Notably, experiments using alginate and gelatin as composite materials showed best results compared to other combinations. Interestingly, the use of cost-effective ingredients such as tapioca-egg yielded the most favorable results as larvae feed. The microcapsules produced through modifications in manufacturing technique resulted in droplet sizes tailored to the mouth openings of fish larvae.

Keywords: Feed technology; Microcapsules; Suspension cross-linking; Fish larvae

Introduction

Aquaculture is one of the Indonesia most developed industries, particularly in the fisheries sector. The increase in aquaculture production in 2020 reached 18.44 million tons. The target for 2021 increases to 19.47 million tonnes (Ministry of Maritime Affairs and Fisheries 2020). Increased cultivation productivity is in line with the development of technology used in maintenance systems and management. Currently, various technologies are developing very rapidly, starting from technical techniques to the feed provided to make cultivation activities more efficient. Several studies showed that feed absorbs the largest costs in cultivation activities (de Verdal *et al.* 2018; Adeleke *et al.* 2020; Naseem *et al.* 2021). However, in cultivation, some of the feed is wasted and damaged by the environment before it enters the fish's digestion. This is a challenge in the future regarding efforts to utilize feed optimally. The feed used in cultivation must be adjusted to the size of the fish's mouth opening (To'bungan 2019). The level of life for fish larvae has been very low until now. The availability of food that matches the larva's mouth opening is one factor that influences the level of larval life. (Sukardi *et al.* 2014).

Microcapsules are packaging technology for active ingredients in the form of solid, liquid or gas with small particle sizes. This technology has been developed in various fields such as health, pharmacy, animal husbandry and fisheries, generally in the form of feed. Microcapsule feed technology has several advantages, including maintaining nutritional stability. Microcapsules have a polymer layer that protects the active ingredients from the environment. In simple terms, microcapsules have two different parts, namely the outer part or capsule and the core part which can be a crystal material, serrated absorbent, emulsion, solid suspension or other suspension (Poshadri and Kuna 2010). Meanwhile, the protective part is made of material that has high viscosity properties. The type of core and matrix base material can influence the characteristics of microcapsules, both shape and size of microcapsules because each material has its own characteristics (Ye *et al.* 2018). However, previous studies have shown that the manufacturing techniques can also influence the characteristics of microcapsules (Calderón-Oliver and Ponce-Alquicira 2022). This experiment will focus on the speed of stirring in droplet formation and different types of matrix materials using the suspension cross-linking technique to obtain the best characteristics of the microcapsules.

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Various techniques for making microcapsules have been widely used to obtain ideal products, such as spray drying, complex coacervation, encapsulation in cyclodextrins, spray chilling, extrusion, freeze drying (Calderón-Oliver and Ponce-Alquicira 2022) and suspension cross-linking (Arshady 1990). Each encapsulation technique has its own advantages and disadvantages. Generally, each technique used considers the type of material used. The suspension cross-linking technique is simple, practical, and effective, and can be used with several readily available materials. In previous research, the suspension cross-linking method was successfully used to make microcapsules made from tubifex sp. and spirulina sp. Which had shown promising results (Sukardi *et al.* 2018, 2019). The advantage of the cross-link method is that it can be used on materials that are mutually soluble, insoluble, liquid, or solid and can be used to produce microparticles and nanoparticle sizes (Jayanudin *et al.* 2018). Cross-linking capsule formation is a process that can modify the structure and molecular properties of microcapsules. Cross-linking functions to harden the wall material after capsule formation. The purpose of capsule hardening is to make the microcapsules more stable during drying as well as to impart some special properties of the wall material, such as modifying the physical state (Leclercq *et al.* 2009). However, in developing, this technique is necessary to make several modifications both technically and to the basic materials used as the matrix.

The matrix or capsule is the most important part as it has the function of protecting the core material. The type of material used is an important factor determining the characteristics of microcapsules (Choudhury *et al.* 2021). Selecting the right matrices can increase the stability of nutritional value and increase feed efficiency for use by fish (Heck *et al.* 2020; Mohammed *et al.* 2020). Some of the basics for selecting materials used in making matrices are local and abundant availability and cost-effectiveness. Previous research revealed that several materials could be used as matrices, namely eggs, gelatin, alginate and tapioca flour (Sukardi *et al.* 2018). It is necessary to combine different materials, commonly referred to as a composite matrix, to achieve maximum results because each type of material has different characteristics (Shao *et al.* 2019). Composite microcapsules are microcapsules whose walls consist of two or more different materials. Composite matrices can improve the mechanical performance of microcapsules as protectors of the core material and optimize their efficiency values, but the amount of material used as a composite matrix must be correct (Zhou *et al.* 2018). Characteristics can be evaluated simply by several parameters such as morphological characteristics (Zhu *et al.* 2018) and physical properties (Santos *et al.* 2021). To determine the microcapsule particle size according to the fish's mouth opening, different stirring speeds were utilized in the present study to get desired sized microcapsule particles. Different composite matrices were also tested to obtain the most efficient ingredients through evaluation of their morphological and physical properties.

Materials and Methods

Time and location

This research was conducted experimentally and observationally, with many core materials and matrices being tested in this research. The research was conducted from August to September 2023 at the Feed Technology Laboratory, Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University.

Research procedure

In general, this research consists of two research stages, namely 1) measuring the speed of the microcapsule particles formed. 2) observing the morphological and physicochemical characteristics of the microcapsule matrix polymer. During the first stage, microcapsules were created through the suspension cross-linking technique with a modified mixer and hot plate (Sukardi *et al.* 2018). The material composition for testing the effect of different speeds on diameter size of microcapsules has been detailed in Table 1. The speeds used in this test were 500 rpm, 700 rpm, 900 rpm, 1100 rpm and 1300 rpm. During the second stage, a formulation was prepared to assess differences in polymer matrix materials using the same technique as in the first stage. However, the stirring speed was determined based on achieving the smallest particle size observed during the first stage, specifically at 1300 rpm. The formulation for testing the matrix materials is detailed in Table 1.

Then, after preparing the composition formulation of each ingredient, the process of making microcapsules is carried out, by following the modified procedure of Sukardi *et al.* (2018). Several stages were carried out in making microcapsules, namely droplet formation, suspension cross-linking and recovery. The matrix and inclusion material components were mixed in a speed encapsulator for 5 min to form droplets. The suspension cross-linking procedure was then employed by stirring and heating on a hot plate to reduce the water content, allowing the droplets to gradually harden. This heating and stirring process continued using an encapsulator machine until the cross-linking polymer occurred, lasting approximately 5 min until the temperature reached 70°C. The final stage in making microcapsules involved leaving the microcapsules undisturbed for about 5 min. The microcapsules in their wet form were collected using a dropper pipette, placed on a glass slide, covered with a cover glass, and observed under a microscope at 10× magnification to examine the microcapsules that had been formed. After observation, the microcapsules were dried at 60°C for 24 h to solidify their structure, enabling them to be ground into powder form.

Microcapsule diameter measurement

Microcapsules in wet form were dropped on a glass slide, covered with a cover glass, and diameter of the microcapsule was measured using microscope at 10X magnification. The measurement was conducted based on the scale visible through the microscope lens multiplied by the magnification used (μm). For each experiment, the diameter of 15 randomly selected microcapsules was measured.

Morphological characteristics of microcapsules

Observation of microcapsule morphology was conducted using a microscope with 10x magnification. The morphological characteristics assessed included shape, color, edges and size of microcapsules.

Physical observations of microcapsules

Buoyancy: The buoyancy test involved immersing dry microcapsules in water and measuring the time required for them to sink to the bottom. A longer sinking time indicates higher microcapsule quality.

Water content

Water content was measured by weighing the material both before and after processing it into dry microcapsules. The formula used was as follows (Srifiana *et al.* 2014):

$$\text{Water content} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100\%$$

Yield value

The yield value was calculated by comparing the weight of the microcapsules obtained with the total weight of the inclusion and matrix materials used. The formula used was as follows (Xiao *et al.* 2007):

$$\text{Yield} = \frac{\text{Wt} - \text{Wo}}{\text{Wo}} \times 100\%$$

Where: Wt = initial weight (g)
Wo = final weigth (g)

Water absorption capacity

Analysis of the water absorption capacity of microcapsules was done using the method described by Xiao *et al.* (2007) with slight modification. Briefly, 0.2 g of dry microcapsules was soaked in 45 mL of water at room temperature for 1 h. The microcapsules that absorbed water were filtered to remove unabsorbed water and then weighed. The absorbed water per gram of dry microcapsules was calculated using the following equation:

$$\text{DA} = \left(\frac{\text{M}}{\text{M}_0} - 1\right) \times 100\%$$

Where M is the weight of the microcapsules that absorbed water and M0 is the dry microcapsules.

Organoleptic testing

Organoleptic testing was conducted to observe the stability of microcapsules during storage period. Tests were conducted daily for one week, evaluating the color, odor and texture of microcapsules.

***In vivo* experimental design**

Some composite microcapsule feeds were tested as the primary feed for nilem larvae over a 30-day period in a Recirculating Aquaculture System (34.5 cm deep x 38.2 cm width x 58 cm length) with a water volume maintained at 50 L and an initial abundance of 20 larvae per treatment. The feeds tested included P4 (alginate-gelatin), P5 (alginate-egg), P6 (tapioca-egg), and a control (fish meal), selected based on the cost-effectiveness of their materials. Parameters measured included specific growth rate (SGR), percentage weight gain (PWG), and survival rate (SR) (Mani *et al.* 2021).

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Data analysis

The research data obtained was analyzed descriptively based on calculations and observations of each parameter. Data analysis is displayed in the form of figures and tables. Then, the results obtained were discussed and compared with several previous references to obtain conclusions.

Results

The first part of this research involved varying the speed of droplet formation to observe its effect on microcapsule size. Measurements were taken before and after each observation to assess any size changes. The measurements of microcapsule diameters have been presented in Table 2.

The results showed that different speeds yield varying average diameters of microcapsules. When considering microcapsule diameter based on size range, speed significantly influenced both diameter size and the density

Table 1: Formulations with different matrix materials and speed test

Ingredient	Treatment (%)						
	Speed test	T1	T2	T3	T4	T5	T6
Material of Matrix	94.2	56.0	56.0	56.0	56.0	56.0	56.0
Spirulina	2.0	-	-	-	-	-	-
Fish meal	-	14.0	14.0	14.0	14.0	14.0	14.0
Fish oil	0.2	1.0	1.0	1.0	1.0	1.0	1.0
Tween	-	1.0	1.0	1.0	1.0	1.0	1.0
Water	12.8	28.0	28.0	28.0	28.0	28.0	28.0

Materials of matrix information: Speed test= Egg, T1= Gelatine-egg, T2= Alginat-tapioca, T3= Gelatine-tapioca, T4= Alginate-gelatin, T5= Alginate-egg, T6= Tapioca-egg

Table 2: The size of the microcapsule diameter with reference to stirring speed

Treatment	Speed (rpm)	Time (min)	Diameter Size (µm)	
			Average (n=45)	Range
P1	500	5	90±3.0	42-210
P2	700	5	82±6.0	50-160
P3	900	5	74±8.0	30-100
P4	1100	5	66±8.0	30-97
P5	1300	5	72±6.0	30-97

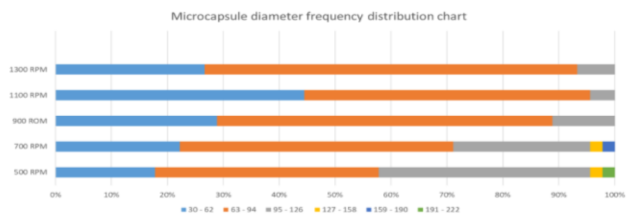

Fig. 1: Frequency distribution of microcapsules

and thickness of the microcapsule polymer matrix layer. At lower speeds (500 rpm), the size range was 50–210 µm with a central cavity and a thin polymer layer. Conversely, higher speeds (1300 rpm) resulted in a smaller size range of 30–90 µm with higher inclusion density and a thicker polymer layer. These measurements highlighted the influence of speed on the size and structural characteristics of the produced microcapsules. Furthermore, the distribution frequency of microcapsule sizes indicated a predominantly uniform distribution, as depicted by the orange line in Fig. 1. These findings collectively demonstrated that increasing the speed during droplet formation leads to smaller microcapsule particles.

The morphological characteristics of microcapsules were then examined to determine the optimal combination of matrix materials, with fish meal used as the inclusion. The characteristics observed included shape, color, edges and size. The morphological characteristics of microcapsules have been shown in Table 3 and Fig. 2.

Based on observation of morphology of microcapsules, the shape, color, and edges were the same but varied in their sizes. The shape obtained from each experiment has granules with irregular surfaces, appeared brown in color with jagged edges. Buoyancy tests indicated mostly similar values across experiments. However, P6 (tapioca and eggs) showed very low buoyancy (6–56s).

Table 3: Microcapsule morphological characteristics

Treatment	Inclusion	Shape	Color	Edge	Size (µm)
P1	Fish flour	Granular, irregular	Brown, white	Jagged	30-150
P2	Fish flour	Granular, irregular	Brown, white	Jagged	60-200
P3	Fish flour	Granular, irregular	Brown, white	Jagged	50-290
P4	Fish flour	Granular, irregular	Brown, white	Jagged	50-140
P5	Fish flour	Granular, irregular	Brown, white	Jagged	50-250
P6	Fish flour	Granular, irregular	Brown, white	Jagged	50-250

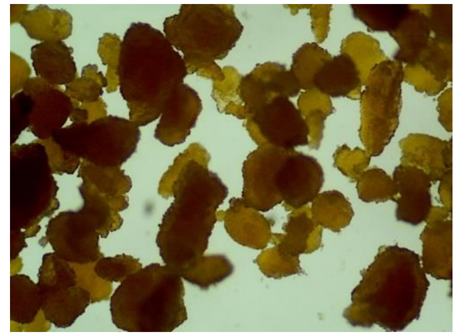

Fig. 2: Fish meal microcapsules

Furthermore, the highest yield value (14.28%) was obtained in experiment P4 (alginate and gelatin). The water contents remained relatively consistent, while organoleptic testing showed the same results, such as soft texture, brown color and fishy smell.

The experimental results showed that feeding microcapsules had a positive effect on fish larvae. This is evident from the higher values observed in the PWG and SGR parameters for all treatments compared to the control. Particularly, the tapioca-egg treatment showed the best across all parameters.

Discussion

Aquaculture has recently developed very rapidly, employing various cultivation technology systems, such as recirculation (Badiola *et al.* 2018), biofloc (Khanjani and Sharifinia 2020), and mina rice to super intensive cultivation systems (Suantika *et al.* 2020; Palupi *et al.* 2023). All these technologies in aquaculture aim to increase production scale and efficiency. The advancements in feed technology, such as microcapsule feeds, are gaining attention globally because of their flexibility and efficiency (Sánchez-Osorno *et al.* 2023). Our study focused on two main aspects of microcapsule formation in aquaculture, including 1) determination of impact of stirring speed on particle size during microcapsules production so that the results of this study can be applied to fish seed cultivation based on the mouth opening. 2) evaluation of various types of matrix materials to achieve a stable capsule wall to maintain the bioactive core. Although, these materials were chosen based on their availability and cost-effectiveness. In this case we evaluated simply by looking at the morphology and physical appearance of the microcapsules, with a focus on how the

Table 4: Physical characteristics of microcapsules

Treatment	DSA (%)	DA (s)	Yield (%)	Water content (%)	Organoleptic		
					Texture	Color	Smell
P1	0,06	1142	3,79	6,97	Soft	Brown	Fishy
P2	0,17	1023	5,14	4,95	Soft	Brown	Fishy
P3	0,25	1142	5,06	3,86	Soft	Brown	Fishy
P4	0,03	1165	14,28	5,65	Soft	Brown	Fishy
P5	0,02	1114	3,47	3,25	Soft	Brown	Fishy
P6	0,79	948	3,27	3,78	Soft	Brown	Fishy

Where: DSA = water absorption capacity, DA = buoyancy, KA = water content

Table 5: Effect of microcapsule feed on larvae

Composite	Parameter		
	PWG	SGR	SR
Control	4.00±1.73	2.34±0.43	73.33±16.07
P4	12.00±3.60	3.66±0.43	46.66±24.66
P5	16.33±2.30	4.11±0.19	61.66±16.07
P6	34.00±16.46	5.05±0.63	78.33±22.54

Where: PWG = percent weight gain, SGR = specific growth rate, SR = survival rate

Table 6: Characteristics of the polymers used in the research

Polymer	Characteristic	References
Alginate	Hydrophilic, highly biodegradable, clear and transparent after gelatinization	(Comaposada *et al.* 2015)
Tapioca flour	It has a fairly wide temperature range, quite high swelling ability, quite strong and transparent gel characteristics	(Herawati 2012)
Egg	Low adhesion, has a low temperature range, low viscosity	(Wulandari and Arief 2022)
Gelatin	Biodegradable, non-toxic, easy to cross-link	(Wati *et al.* 2022)

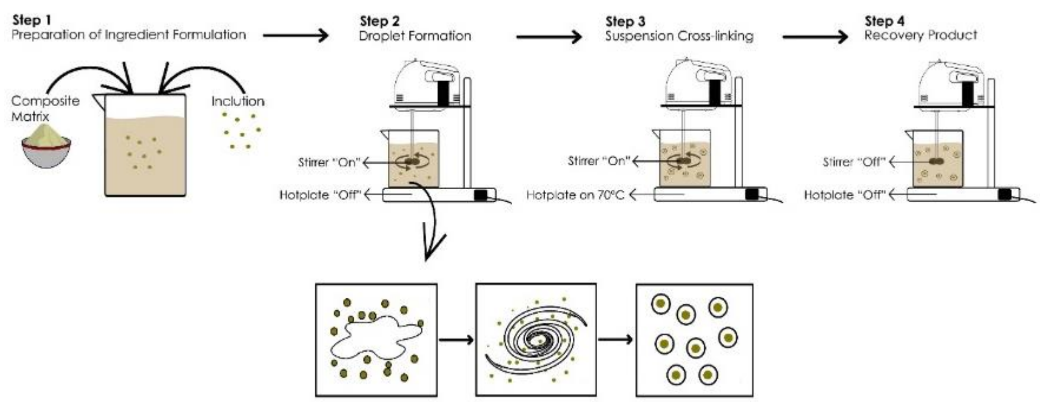

Fig. 3: Microencapsulation scheme using suspension cross-linking technique

polymer matrix characteristics influence shape and size (Choudhury *et al.* 2021), as mentioned in Tables 5, 6.

Various methods and techniques for making microcapsules have been developed in several countries, including Indonesia, such as chemical, physico-chemical and physico-mechanical methods (Giro-paloma *et al.* 2016). According to Giro-paloma *et al.* (2016), the encapsulation method in this research is classified as a chemical method with a modified suspension cross-linking technique to simplify the encapsulation technique and be efficient. Even though, it is considered very simple and has several disadvantages, such as it produces particle sizes in a wide range. Thus, this method was used in this research experiments to utilize different stirring speeds to obtain the desired droplet or microcapsule particle size (Fig. 3). Then, the techniques in microencapsulation methodology are shown as a comparison as well as to see several methodologies and techniques in microencapsulation which are presented in Table 7.

The scheme above illustrates the stages and process of microcapsule production using the suspension cross-linking technique. Modifications in this research were carried out in step 2, namely droplet formation. This stage is important in this technique where microcapsule granules are formed. The stirring process using a stirrer will change the formation of the polymer structure. Polysaccharide materials such as tapioca and gelatin, having gel-like properties, will form granular or droplet formations with the addition of oil, which in this research, fish oil was used so that cross-linking between molecules occurs (Arshady 1990; Sukardi *et al.*

Table 7: Methodology and techniques in microencapsulation (Giro-paloma *et al.* 2016)

Methods	Technic	Advantages	Weakness
Chemistry	Suspension polymerization	Good heat control against reactions	Some monomers are soluble in water, particle size 2-4000 uL
	Dispersion polymerization	Micron-sized monodisperse polymer particles in one batch process	Not reported
	Emultion polymerization	High MW polymers quickly	Purifies polymers from surfactants
	In-situ polymerization	The coating is more uniform	Particle size 1-2000 uL
	Interfacial polymerization	Versatile, good properties in size, degradability, mechanical resistance	MPCN size 2-2000 uL
Physico-chemistry	Coacervation	Two versatile methods	Grouping
	Sol-gel encapsulation	Organic material with high thermal conductivity	Difficult to improve
	Supercritical CO2-assisted	Effective, cost-effective and environmentally friendly method	Not much use
Physical-mechanical	Spray drying	Easy to use, versatile, low cost	High temperature
	Electrostatic encapsulation	Round shape, micro size	Only for sizes less than 50 uL
	One-step method	Easy to upgrade,	Not reported
		Self-stabilization	Not reported

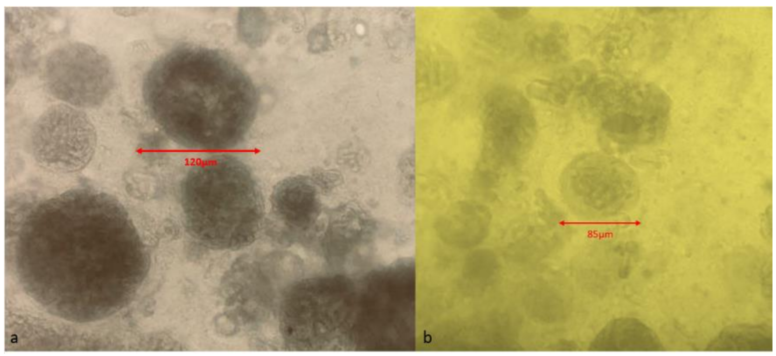

Fig. 4: Microcapsule particles. a= 500 rpm, b= 1300 rpm

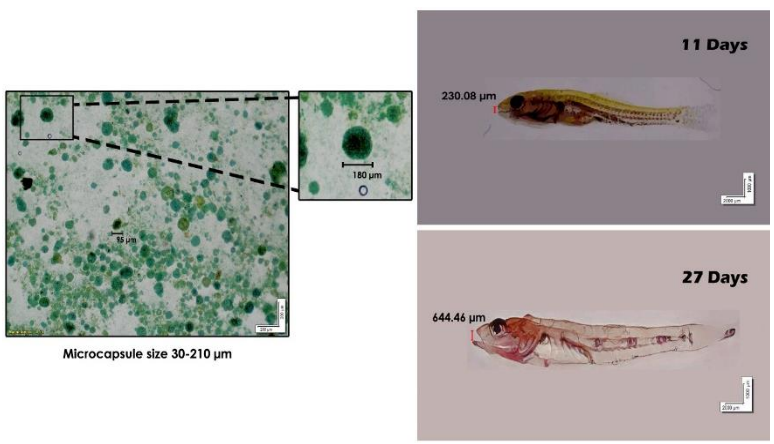

Fig. 5: Illustration of the relationship between fish mouth opening and microcapsule particle size

2018). In this process, molecules with a higher mass are trapped, which in this case are the core material or inclusions, so that they form particles covered by polymer as a matrix. The results of this research showed that the stirring speed influences the particle size of the microcapsules. Importantly, the process of stirring and heating in this technique results in slow evaporation so that the droplets formed are more stable.

Regarding the effect of stirring speed on the size of particles produced in microcapsules, it is thought that it affects the size of the particles. The results of this research showed that experiments on making microcapsules made from egg polymer and spirulina inclusions showed varying sizes. Experiments at low speed (500 rpm) showed a size range of 40–210 µm. then experiments at high speed showed results in a particle size range of 30–90 µm. When compared from low to high speeds, it showed that there is a difference in the size of the microcapsule particles. Stirring at low speed produces a larger particle size compared to the particle size at high stirring speed. This can be seen from the range of particle sizes in Table 4. According to (Jegat and Taverdet 2000; Lemos *et al.* 2017), the speed of stirring in the formation of microcapsule droplets has an influence on the size of the particles formed. However, they have documented that increasing the speed with a slightly higher rotation did not have a real effect on particle size. This shows the similarities in the experiments carried out in this study, i.e. increasing the speed slightly (200 rpm) did not show significant results on the particle size of the microcapsules. However, it can be seen in the results that the stirring speed had an influence on the particle size of the microcapsules. In addition, high speed has an effect on the density of inclusion particles and the thickness of the polymer layer (Jegat and Taverdet 2000). This was also found in the experiments in this study. The higher the stirring speed, the higher the density of inclusion particles and the thickness of the matrix layer (Fig. 4). It can be observed in the experiment that at a speed of 500 rpm there is a cavity in the center of the inclusion and the matrix layer has a thin layer. On the other hand, the 1300 rpm experiment showed a thicker and clearer matrix layer covered in inclusion particles.

Based on observations of the morphological characteristics of the microcapsule particles, the same shape and edge characteristics were seen in each experiment. The exact reason for the results obtained has not been found. According to Carneiro *et al.* (2013), the shape and surface structure of microcapsule particles can be influenced by the polymer matrix material since they have different basic properties. However, other references also state that there are many other factors that can influence the shape characteristics of microcapsules such as the technique used, pH conditions and temperature (Carneiro *et al.* 2013; Li *et al.* 2019). Based on several references, morphologically, different types of shapes and edges should be obtained. The basic reason behind these results may be the similarity in the type of inclusion and the preparation process when emulsifying the matrix material. In the matrix material emulsion process we tried to equalize the viscosity level by adding water which is thought to provide the same characteristics. According to (Sah 1997; Xiao *et al.* 2007), the preparation and emulsion process can provide characteristic morphological properties to microcapsules.

Based on the physical characteristics observed, the experimental results showed varied results across most of the measured parameters. These results indicated that the matrix materials posed diverse influences on physical characteristics. Particularly, experiment P4 exhibited distinct characteristics compared to other experiments. It demonstrated the highest yield percentage, followed by water content, although buoyancy was not notably high. The yield and buoyancy values observed in this study align with previous research findings where yield was 15.58% and water content was <10% (Mardiana and Fatmawati 2014). According to several studies, these characteristics can be influenced by various factors, including different types of emulsifiers (Chatterjee and Judeh 2017). We hypothesize that the physical characteristics of microcapsules may influence their viability and stability during storage.

In aquaculture, microcapsules provide a new breakthrough to maintain viability and stability of the nutritional composition of feed. Microcapsules can be applied in several cultivated biota such as fish (Sukardi *et al.* 2018), crustaceans (Jaroensaensuai *et al.* 2022), molluscs (Luzardo-Alvarez *et al.* 2010). Microcapsules can be modified in several parts such as the type of inclusion, polymer matrix and size suitability to the cultivated biota. This is usually associated with mouth opening in fish, especially in fried fish. In fish maintenance management, the fry phase is important because the fish are still susceptible to disease attacks and require adequate nutrition for growth. Microcapsule feed is very appropriate to be applied in this phase of maintenance because microcapsules can be modified by adding supplements, probiotics and other bioactives which are protected using a polymer matrix so that it is more efficient in utilizing feed nutrients with particle sizes adjusted to the mouth opening. In the illustration in the picture above, we try to relate the size of the microcapsule product from the experiments carried out in this research. The results of the mixing speed experiment on the size of the microcapsules showed differences which may relate with the mouth opening of the fish seeds. One of the basic principles regarding microcapsule products that can be applied to fish with small mouth openings is generally larvae. Based on the illustrative image, this experiment resulted in a microcapsule size of 30–210 µm, where this range corresponds to the mouth opening of nilem fish seeds (*Osteochilus vittatus*) which have a mouth opening of 230–644 µm at the age of 11–27 days. This indicates that the microcapsule product can be applied to larval nilems.

Conclusion

The present study on microcapsules revealed that stirring speed significantly influences their size and morphological characteristics. High stirring speeds decrease the size of microcapsules, increase the density of included particles, and thicken the polymer matrix layer. The combination of composite matrix materials yielded similar morphological characteristics but varied in the physical properties observed, with the best results obtained using alginate and gelatin. Interestingly, the use of cost-effective ingredients like tapioca-egg showed the best performance as larvae feed. The effect of speed modifications in producing microcapsule sizes is associated with the mouth opening size of fish larvae.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Data Availability

Not applicable.

Ethics Approval

Not applicable.

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