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Microencapsulation of probiotic lactic acid bacteria using freeze-drying with isolated whey protein and trehalose as coating material

[Mikroenkapsulasi bakteri asam laktat probiotik menggunakan pengeringan beku dengan protein whey isolat dan trehalosa sebagai material pelapis]

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ABSTRACT

Microencapsulation could be employed to coat bacteria with protective compounds to enhance their viability. The freeze-drying method uses low temperatures, thereby reducing heat damage. Bifidobacterium breve was used as a probiotic along with Streptococcus thermophilus and Lactobacillus bulgaricus, two common yogurt cultures. Yogurt, a nutrient-rich milk product, has the potential to be an effective probiotic carrier. This research aimed to examine how the freeze-drying process with varying ratios of coating materials affects the viability of the bacteria combination B. breve, S. thermophilus, and L. bulgaricus under acidic and bile salt conditions, as well as the microencapsulation efficiency and particle size. The treatments tested different ratios of whey protein isolate to trehalose as a coating (1:1, 1.5:1, 2:1, 2.5:1, 3:1). The 1:1 ratio yielded the best results, with lactic acid bacteria counts of 6.60 log colony/mL at pH 2.0, 6.84 log colony/mL at pH 3.0, 7.39 log colony/mL at pH 4.0, 7.47 log colony/mL at pH 5.0, 7.70 log colony/mL at pH 6.0, and 7.05 log colony/mL in a bile salt environment. This ratio demonstrated 107.96% microencapsulation efficiency and a particle size of 9.66 µm.

Keywords: bacteria, Bifidobacterium breve, microencapsulation, probiotic lactic acid

ABSTRAK

Mikroenkapsulasi dapat digunakan untuk melapisi bakteri dengan senyawa pelindung guna meningkatkan kelangsungan hidupnya. Metode pengeringan beku menggunakan suhu rendah, sehingga mengurangi kerusakan akibat panas. Bifidobacterium breve digunakan sebagai probiotik bersama dengan Streptococcus thermophilus dan Lactobacillus bulgaricus, dua kultur yogurt yang umum. Yogurt, produk susu yang kaya nutrisi, memiliki potensi sebagai pembawa probiotik yang efektif. Penelitian ini bertujuan untuk mengkaji bagaimana proses pengeringan beku dengan rasio bahan pelapis yang berbeda memengaruhi kelangsungan hidup kombinasi bakteri B. breve, S. thermophilus, dan L. bulgaricus dalam kondisi asam dan garam empedu, serta efisiensi mikroenkapsulasi dan ukuran partikel. Perlakuan menguji berbagai rasio isolat protein whey terhadap trehalosa sebagai pelapis (1:1, 1,5:1, 2:1, 2,5:1, 3:1). Rasio 1:1 memberikan hasil terbaik, dengan jumlah bakteri asam laktat sebesar 6,60 log koloni/mL pada pH 2,0, 6,84 log koloni/mL pada pH 3,0, 7,39 log koloni/mL pada pH 4,0, 7,47 log koloni/mL pada pH 5,0, 7,70 log koloni/mL pada pH 6,0, dan 7,05 log koloni/mL dalam lingkungan garam empedu. Rasio ini menunjukkan efisiensi mikroenkapsulasi sebesar 107,96% dan ukuran partikel sebesar 9,66 µm.Kata kunci: bakteri, *Bifidobacterium breve,* mikroenkapsulasi, probiotik asam laktat

Introduction

Bifidobacterium is a bacteria found naturally in the human digestive tract, especially in infants who fed with breastmilk than formula-fed infants (Men et al., 2020; Tušar et al., 2014). The growth of Bifidobacterium spp. is promoted by the presence of Human Milk Oligosaccharides (HMO) found in breast milk. *B. breve* is one of the bacteria found in breast milk and can utilize this substrate well (Cukrowska et al., 2020).

Lactic acid bacteria (LAB) found in breastmilk, such as *Bifidobacterium* and *Lactobacillus* can minimize the risk of infection in infants, by producing anti-microbial compounds and increasing the intestine defense system (Tušar et al., 2014). Probiotics generally come from a group of LAB that can survive and form colonies in the human intestine, which benefits humans (Yaoet al., 2020; Sutandi et al., 2022).

Yoghurt is a popular fermented milk product high in nutrients and sensory attributes (Santos etal., 2018). Milk products can be a good probiotic carrier as it forms a protective matrix around the probiotic and provides nutrients that allows probiotic growth (Koh et al., 2022; Wang et al., 2019).Yoghurt commonly uses *S. thermophilus* and *L. bulgaricus*, but yoghurts with just the two shows minimal probiotic effects. It is advisable to add another culture such as *L. acidophilus* (Kamara et al.,2016). However, interaction between starter bacteria may cause a difference in the characteristics of yoghurt resulted (Sutandi et al., 2022).

Commercial probiotic food products have shown a significant decline in total viable bacteria in simulated digestive systems rendering it ineffective (Yao et al., 2020). Bile salt can damage the cell membrane hence detrimental to bacteria (Urdaneta et al., 2017). In addition, the acidic condition in the stomach may also cause cell death (Sensoy et al., 2021).

Microencapsulation forms a wall around the probiotic cell that protects it from direct contact with food components and the digestive system, which then minimizes damage in the production and digestion process so it can reach the intestines (Koh et al., 2022; Yao et al., 2020). Microencapsulation can also prevent cell death during yoghurt fermentation (Li et al., 2019). Freeze-drying is a microencapsulation method that uses a low temperature thus minimizing damage caused by heat. Nevertheless, cell viability can still decline. Therefore, coating materials are needed to increase cell survivability (Jalali et al., 2012). WPI can be used as a culture media as it contains lactose and soluble protein (Aragón-Rojas et al., 2018). WPI forms a film that can protect probiotics. However, it is recommended to combine with polysaccharides to prevent the protein from dissolving. Trehalose is a sugar that can minimize cell rupture caused by ice crystals and decrease the solubility in WPI to strengthen the structure (Sun et al., 2021).

The experiment conducted involves probiotic microencapsulation through freeze-drying, using different ratios of WPI and trehalose as treatments (1:1, 1.5:1, 2:1, 2.5:1, 3:1). The objectives of this experiment were to develop probiotic microcapsules containing *Bifidobacterium breve*, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* for use in yogurt production; to assess the impact of different ratios of these bacterial cultures on the characteristics of the resulting yogurt; and to evaluate the effect of varying ratios of WPI and trehalose as coating materials on the yogurt produced. Microcapsules produced are then analyzed based on cell viability in acid and bile salt, microencapsulation efficiency, and particle size.

Materials and methods

Materials and equipment

Materials used in this research include *Bifidobacterium breve* ND3A, *Lactobacillus bulgaricus* FNCC0041, *Streptococcus thermophilus* FNCC 0040, demineralized water, WPI, and trehalose. Meanwhile, the equipment used were a freeze-dryer, freezer, incubator, laminar air flow, centrifuge, hotplate, microscope, micropipette, and colony counter.

Research method

Microencapsulation was done based on the method previously reported by Bora et al. (2018) with modifications. *B. breve, S. thermophilus*, and *L. bulgaricus* with a ratio of 1:2:1 is inoculated in MRS broth and incubated at 37°Cfor 72 hours. The ratio of 1:2:1 was based on experiments by Lang et al.(2022) with modifications, who used cultures of *B. animalis, S. thermophilus*, and *L. bulgaricus*. Besides that according to Vitheejongjaroen et al. (2021), the use of *S. thermophilus* with a higher concentration can speed up the fermentation time in digesting sugar in milk. The mixture was then centrifugated at 6000 rpm for 10 minutes. The supernatant was carefully separated from the pellet. The biomass obtained was then mixed with coating materials at a 1:10 ratio. Coating materials were pre-mixed beforehand with the ratio between WPI and trehalose as such, 1:1, 1.5:1,2:1, 2.5:1, and 3:1. Then, each of the mixtures was put in the freezer for

24 hours before freeze drying for 24 hours. Probiotics encapsulated were then analyzed on the viability in acid and bile salt, as well as microencapsulation efficiency and particle size.

Bacteria viability in acid and bile salt

The total viable bacteria in acid and bile salt conditions were assessed using the pour plate method with MRS agar, following the modified protocols of Soesetyaningsih & Azizah (2020) andBurton (2014). MRS broth was first adjusted with HCl to achieve pH levels of 2.0, 3.0, 4.0, 5.0, and 6.0. Another batch of MRS broth was supplemented with 0.5% bile salt. For each treatment, 0.5 g of microencapsulated bacteria samples were incubated in 4.5 mL of the conditioned MRS broth for 30 minutes. Then, 1 mL of each conditioned sample was serially diluted with saline solution. The number of bacteria in the diluted samples was then counted using the total plate count method with MRS agar.

Microencapsulation efficiency

Microencapsulation efficiency was determined using a modified version of the methods described by Jouki et al. (2021) and Li et al. (2019). 1 mL sample of mixed culture intended for microencapsulation was incubated in MRS broth for 24 hours. Following incubation, 1 mL of the culture was diluted, and the total bacteria was measured with the total plate count method.

Microcapsule particle size

Microcapsule particle size was determined using the 'Olympus Start' app in reference to the research previously done by Siregar and Kristanti (2019). Microcapsules of each variation of ratio obtained are spread even on a microscope glass and then covered with the cover glass. It is then viewed under 1000× magnification.

Statistical analysis

This research uses ANOVA analysis with a significance level of 5% ($p<0.05$) calculated to determine whether the differences caused by varying ratios of coating materials were significant. Significantly different data from ANOVA analysis were tested with further tests which is Duncan's Test. Statistical analysis is done with IBM SPSS Statistics 25. The research was done with 5 different ratios of coating materials, 2 repetitions, and in duplo.

Result and discussion

Viability of bacteria in acid and bile salt conditions

Microcapsules obtained after freeze-drying are analyzed on their ability to withstand the acid environment. Based on the results (Table 1), different ratios of WPI and trehalose do not affect the total viable bacteria significantly ($p>0.05$). However, the microencapsulation process clearly increased the number of cells obtained compared the control treatment, which was not microencapsulated. In pH 2.0, the control shows no bacteria growth mean while the microencapsulated bacteria have grown more than 6 log colony/mL. Meanwhile, in pH 3.0, the control bacteria grew but not as much as the microencapsulated counter parts. This result shows similar results to the research done by Li et al. (2019) where encapsulated *B. breve* has a total of 9.45 log colony/mL bacteria whereas non-encapsulated *B. breve* only has a total of 6.17 log colony/mL, both being subjected to acid treatment for 2 hours.

Table 1. Total lactic bacteria viability of mixed culture at different acid pH and different ratios of WPI and trehalose

Formulation of WPI and trehalose (*B.b* : *S.t* : *L.b*)	pH 2.0 (log colony /mL)	pH 3.0 (log colony /mL)	pH 4.0 (log colony /mL)	pH 5.0 (log colony /mL)	pH 6.0 (log colony /mL)
Control	0,00	5,80	5,74	5,36	5,38
1:1:1	6,60±1,00[a]	6,84±0,42[a]	7,39±0,00[a]	7,47±0,05[a]	7,71±0,02[a]
2:1:1	6,89±1,06[a]	7,08±1,18[a]	7,35±0,94[a]	7,63±0,75[a]	7,30±0,92[a]
3:1:1	7,05±0,01[a]	7,34±0,48[a]	6,84±0,06[a]	7,13±0,05[a]	6,93±0,01[a]
1:2:1	7,10±0,05[a]	7,34±0,01[a]	7,48±0,20[a]	7,56±0,24[a]	7,33±0,77[a]
1:1:2	7,25±0,03[a]	7,53±0,50[a]	7,38±0,49[a]	7,53±0,23[a]	7,29±0,24[a]

Note: Different superscripts indicate the significant differences between the coating ratios used (Duncan's, $p < 0.05$).

All microencapsulated probiotics demonstrated LAB growth exceeding 6 log colony/mL from pH 2.0 to pH 6.0, suggesting that microencapsulation effectively protects probiotics inacidic conditions. The pH value in the stomach can vary for different conditions and individuals. It usually ranges from 1-3. WPI and trehalose construct a structure that may prevent direct contact between the bacteria and the acid (Wang et al., 2017). WPI may also go through denaturation and gelation in low pH conditions (Wagner et al., 2019). In low pH conditions, bacteria need more energy to retain the intracellular pH, and if that condition is not met, the bacteria will die (Rizal et al., 2016). The minimum requirement of total viable cells to give benefits to the body is 6 to 7 log colony/mL (Salihu et al., 2019).

Microencapsulated probiotics were also tested on their ability to survive against bile salt conditions (0.5% w/v). Based on the results provided in Table 2, different ratios of coating ratio do not affect the total viable cells obtained significantly ($p>0.05$).

Table 2. Bacteria viability of mixed culture at bile salt (pH 2) and different ratios of WPI and trehalose

Formulation of WPI and trehalose (*B.b* : *S.t* : *L.b*)	Total LAB in pH 2 (log colony/mL)
Control	4.09
1:1:1	7,06±0.70[a]
2:1:1	7,19±1.00[a]
3:1:1	6,81±0.19[a]
1:2:1	7,54±0.07[a]
1:1:2	7,82±0.65[a]

Note: Different superscripts indicate the significant differences between the coating ratios used (Duncan's, $p < 0.05$).

Aside from protecting bacteria from acid, microencapsulation can also increase the bacteria viability in bile salt conditions. The microcapsules obtained from all coating ratios showed higher total LAB in pH 2.0 compared to the control treatment. Total viable cells have also exceeded the minimum requirement of 6 log colony/mL.

Microencapsulation can increase the viability of bacteria exposed to bile salt (Li et al., 2019). Carbohydrates, in this case monosaccharides and oligosaccharides, can prevent cell damage due to bile salt (Salihu et al., 2019). With its ability to form film and gel, WPI can increase the viability of probiotics encapsulated. However, due to its high solubility, WPI may dissolve and hinder its potential as a protective agent. Trehalose and other polysaccharides added can reduce its solubility thus strengthening the structure (Sun et al., 2021).

Microencapsulation efficiency

Microencapsulation efficiency is calculated by comparing the total bacteria obtained before and after the microencapsulation process. As shown in Figure 1, the coating ratio used affects the microencapsulation efficiency significantly ($p<0.05$).

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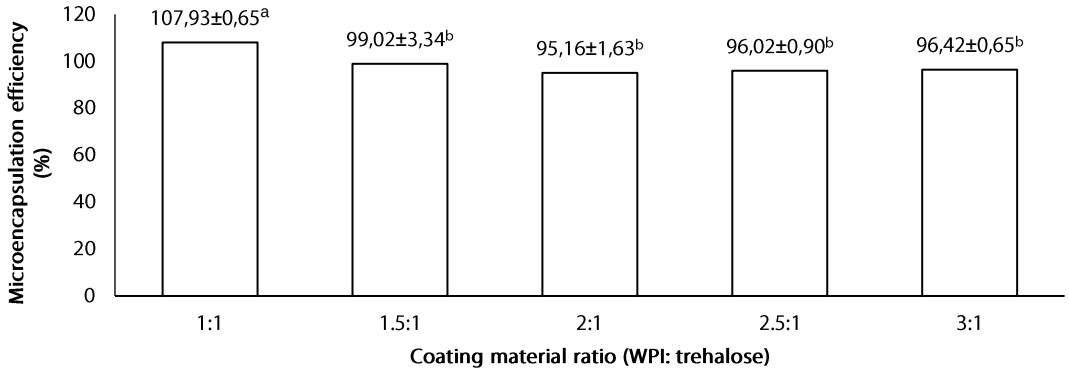

Figure 1. Microencapsulation efficiency of mixed culture at different ratios of WPI and trehalose. Different superscripts indicate the significant differences between the coating ratios used (Duncan's, $p < 0.05$).

Microcapsules produced with WPI and trehalose with a ratio of 1:1 have the highest efficiency among all treatments. Low temperature used in freeze drying and the addition of coating materials could protect cells therefore increasing bacteria survivability as well as microencapsulation efficiency. WPI forms a protective layer to prevent direct contact with digestive liquids. Meanwhile, trehalose minimizes the damage inflicted by the freezing process and strengthens the structure formed by whey (Salihu et al., 2019, Sun et al., 2021).

WPI and trehalose can also provide nutrients for the bacteria. *S. thermophilus* utilizes protein in whey to produce amino acid and other amines that promotes bacterial growth. Trehalose, as a sugar consisting of two glucose molecules, can be used by bacteria to produce ATP (Salihu et al., 2019).

Particle size

Coating materials ratio variation affects particle size significantly. Analysis results are displayed in Figure 2. Microcapsule particle sizes obtained from this research range from 6,59 to 9,66 µm. The standard criteria for microcapsule sizes is 1-1000 µm. Microcapsules were obtained in the research previously done by Wang et al. (2019) using WPI sizes 8,45 µm. The irregular form of microcapsules produced as a result of the gel layer formed by WPI or the homogenization process was not enough (Bora et al., 2018; Wang et al., 2019). Additionally, the drying process may have caused an uneven sublimation and consequently resulted in irregular microcapsules (Sun et al., 202).

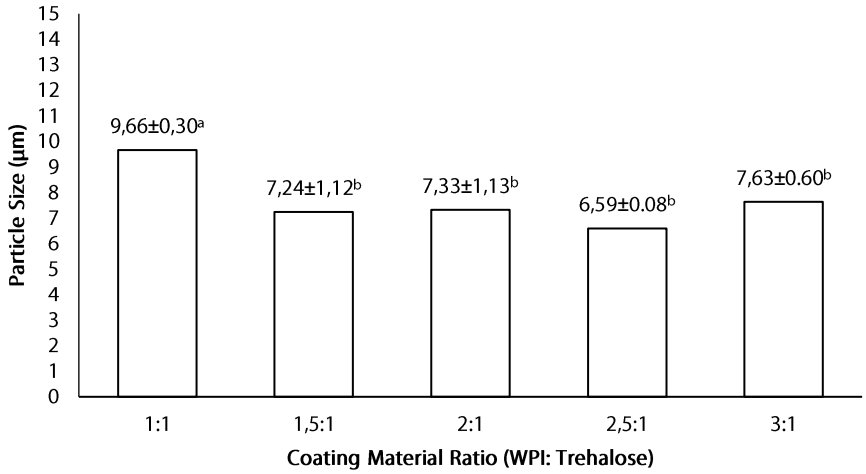

Figure 2. Particle size of mixed culture at different ratios of WPI and trehalose. Different superscripts indicate the significant differences between the coating ratios used (Duncan's, $p < 0.05$).

Determination of best treatment

Freeze-drying with WPI and trehalose as a coating can enhance bacterial viability in acidic and bile salt environments. The best ratio between WPI and trehalose was chosen based on its microcapsule efficiency and the highest particle size. Whereas, microcapsules with a dressing ratio of 1: 1 had total LAB (6.60±1.00) log colony/mL at pH 2.0, (6.84±0.42) log colony/mL at pH 3.0, (7.39±0.00) log colony/mL at pH 4.0, (7.47±0.05) log colony/mL at pH 5.0,(7.71±0.02) log colony/mL at pH 6.0, and (7.06±0.70) log colony/mL in bile salts which meet the minimum total LAB to be probiotic. The microcapsule efficiency was (107.96±0.65)% and the particle size produced was (9.66±0.30)µm which was higher than other ratios. The best ratio between the culture ratio of *Bifidobacterium breve*, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* of yoghurt was chosen based on its highest total LAB, highest total titratable acid, and the lowest pH value. Whereas the yogurt formulation with the ratio1:2:1 has the highest total LAB of (8.99±0.01) log colony/mL, the highest total titratable acid of (0.82±0.03)%, and the lowest pH value of 4.15±0.01 than other ratio.

Conclusion

Bacteria microencapsulated using WPI and trehalose as coating materials have shown higher viability in acid and bile salt conditions compared with the control treatment. The ratio of WPI to trehalose influences both microencapsulation efficiency and particle size. The microcapsules produced with WPI and trehalose with the ratio of 1:1 resulted in 6.60 log colony/mL at pH 2.0 and 7.06 log colony/mL in saline bile salt that meets the minimum total LAB to be probiotic. The microencapsulation resulted in 107.96% efficiency and 9.66µm particle size.

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