Utilization of various encapsulation methods in proteolytic bacteria to maintain biomass cells during storage

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Abstract. Bacterial isolates used are proteolytic bacteria. Bacterial cell encapsulation aimed to protect cells from some external environmental factors that can interfere with the metabolism of the cell, so as to maintain the viability of bacteria, extending the shelf life and facilitate the distribution and utilization. In this study, the encapsulation of the bacteria was carried out by nanoencapsulation method using a spray drier and electrospun nanofibers. Encapsulation in nanosize has advantages such as immobilization efficiency because of the increased surface area and volume, providing protection due to the increased flexibility of the encapsulation material. Nanoencapsulation the proteolytic bacteria was using the coating material in the form of skim milk and maltodextrin. Electrospun nanofiber for proteolytic bacteria was using polyvinyl alcohol (PVA) and skim milk as a coating material. The results of the viability of proteolytic nanoencapsulation were higher when encapsulated using skim milk at a concentration of 15% was 44.07%. Results of encapsulation of bacteria using electrospun nanofiber method showed three parameters that affected the process of electrospun nanofiber: the coating material parameters (concentration, viscosity and mixing ratio), the process parameters (voltage, flow rate, and the distance between the collector and needle), and environmental parameters (relative humidity and temperature).

1. Introduction
Artificial civet coffee is intended to improve the quality of civet coffee. Artificial civet coffee is processed by fermentation using bacteria isolated from the digestive system of the civet. Artificial civet coffee quality improvement characterized by the decreased levels of caffeine, oxalic acid and an increase in lactic acid and butyric acid [1] [2]. In early development, civet coffee is fermented using proteolytic bacterial isolates in liquid dosage forms, the problem in the form of a liquid preparation was that isolates should be rejuvenated every time they are used, reducing the practicality and increasing the possibility of bacterial contamination. Then isolate bacterial fermentation developed in the form of dried starter but a significant decrease in bacterial cell viability.

Based on the problems faced by the fermentation of bacterial isolates, therefore, encapsulation bacteria were proposed. Encapsulation is the process of wrapping the core material, in this case, the bacteria encapsulation aims to maintain viability, protecting the bacteria from damage due to unfavorable environmental conditions and facilitate the use and distribution [3]. Viability is the ability of bacteria or bacterial cell viability to grow normally under optimal conditions.

Selection of encapsulation technique conducted with respect to methods of encapsulation, encapsulation materials, and encapsulation material concentration. Nanoencapsulation techniques with
Spray-dyer and electrospun nanofibers were chosen as the encapsulation techniques of proteolytic bacteria. Encapsulation of bacteria by utilizing nanotechnology has been done by researchers to maintain the viability of bacteria, such as encapsulation with electrospun nanofiber in the bacteria *Lactobacillus acidophilus* to maintain the viability of these bacteria up to 78.6% to 90% [4]. Encapsulation of *Lactic acid* bacteria using alginate generate increased viability during storage [5]. In some studies, many materials have been developed as materials for nanofiber using electrospinning methods, such as chitosan, alginate, collagen, and PVA.

Electrospun nanofiber encapsulation method is a method of forming the encapsulation using electrospinning that will produce fine nano-sized line (fiber) of the coating solution used. One ingredient that has been used as a material for the formation of electrospun nanofiber is PVA (Polyvinyl Alcohol) because it has hydrophilicity and compatibility characteristics, non-toxic, high water levels, strong mechanical properties, and chemical stability better than other synthetic polymers.

The purpose of this study was to investigate the effects of nanoencapsulation and electrospun nanofiber as proteolytic bacteria encapsulation method and the use of different types of carrier material in the encapsulation of bacteria on the viability of bacterial cells.

2. Materials and Methods

2.1. Bacterial Strain

Proteolytic bacteria (*Bacillus aerophilous*) used in this study were derived from civet feces the results of isolation in previous studies.

2.2. Culture Preparation

A total of 1-2 culture dose on a slant medium was taken and used for cell propagation. The media for proteolysis was skim milk and nutrient broth. The cultures were incubated at 37 °C for 24 h at a speed of 100 rpm under static microaerobic conditions. Then 10 ml culture was inoculated into a liquid medium by incubating for 24 h and at the same shaker speed. After 18 h, the bacteria were harvested by means of centrifugation at 3500 rpm for 15 min to separate the biomass and the supernatant.

2.3. Characterization of Bacterial Isolates

2.3.1. Growth Curve

2 - 3 cultures from the solid media were rejuvenated and inoculated in 50 ml of liquid media. The culture was incubated in 30 °C shaker at 100 rpm and the absorbance was measured using a 620 nm spectrophotometer to reach an optical density of 0.8 - 1.1 ml of culture was transferred to 99 ml of liquid media. The culture was then incubated in 30 °C shaker at 100 rpm for 54 hours. Every 6 hours starting from the 0 hours, the absorbance measurement was carried out using a 620 nm spectrophotometer.

2.3.2. Protease Enzyme Activity

Determination of the activity of protease enzymes was based on the modified Kunitz method [6]. Protease enzyme activity measurements were performed by measuring the levels of the crude enzyme using a 1% casein substrate. Measurement was done by taking samples every two hours for 24 h.

2.4. Preparation of Material Carrier for Nanoencapsulation

The carrier material for encapsulation was first dissolved to nano-size. Skim milk solution with a concentration of 10%, 15%, and 20% were stirred using a magnetic stirrer with a speed of 15000 rpm, a temperature of 50 °C for 30 min until homogeneous. Maltodextrin solutions with a concentration of 15%, 20%, and 25% were stirred using a magnetic stirrer with a speed of 15000 rpm for 5 minutes until homogeneous.
The concentrations of PVA used as an electrospun nanofiber encapsulation were 10%, 15%, and 20% while skim milk were 10%, 15%, and 20% respectively were stirred with a magnetic stirrer at a speed of 500 rpm at a temperature of 50 °C for 30 min. Furthermore, mixing of PVA and skim milk was continued using the Daihan WiseTis homogenizer HG-15D series at a speed of 2000 rpm for 5 min. The particle size for all encapsulated material was measured using a Particle Size Analyzer (PSA).

2.5. Preparation of Bacterial Nanoencapsulation
Bacterial biomass mixed with each skim milk and maltodextrin solution then was stirred using a shaker at a speed of 500 rpm for 5 min until the bacteria were mixed homogeneously in the solution. The homogeneous mixture was then dried with spray dryer at an inlet temperature of 125 °C and a flow rate of 4-6 mL/min, the pressure on the spray nozzle was 1 bar with a fixed volume of air dryer to form nanoencapsulation. The encapsulated dried culture was obtained and then tested for viability during drying.

2.6. Preparation of Bacterial Electrospun Nanofiber
Bacterial biomass was mixed with a mixture of PVA solution and skim milk then was stirred using a shaker at a speed of 500 rpm for 5 minutes until the bacteria were homogeneously mixed in solution. The process conditions for electrospinning were regulated; the voltage was 6 kV, the flow rate was varied by 35 ml/h, 55 ml/h and 70 ml/h, the distance between the needle and the collector was varied by 8 cm, 10 cm, and 12 cm.

The homogeneous mixture solution of 5 ml was injected into the syringe of the electrospinning (pump tool). The flow rate of the solution coming out of the pump was regulated, then a different electric charge (cathode and anode) was given to the tip of the needle and collector. The injected solution was subjected to electric voltage and form a beam towards the collector. Nanofiber will be formed due to solvent evaporation. The formed nanofiber was captured by the collector. Environmental influences were maintained at 25 °C and 50% relative air humidity.

2.7. Cell Viability of Nanoencapsulation and Electrospun Nanofiber Bacteria
Bacteria cell viability determination was based on the ratio of bacterial counts before and after the storage and expressed in percent. The viability of cells based on the number of proteolytic isolates was calculated using total plate count (TPC). A total of 0.1 g of each nanoencapsulation was diluted with 900 μL NaCl physiologically to obtain the first dilution (10⁻¹) and then serial dilution was conducted until 10⁻⁶. A total of 1 ml of dilution yield was planted by the spread plate method on CMC and skim milk agar, and then incubated at 37 °C for 48 h. The results of encapsulation were tested for bacterial viability by counting the number of bacterial colonies using total plate number (TPC).

3. Results and Discussion
3.1. Characterization of Bacterial Isolates
The characterization of isolates was done by measuring the growth curve and activity of the enzyme. Growth curve measurements were conducted to determine the optimal time of bacterial growth. Bacterial growth is said to be optimal if the number or cell biomass increases rapidly or reached the exponential phase [7].


Figure 1. Growth Curve and Enzyme Activity of Proteolytic Bacteria

The results of proteolytic activity showed that proteolytic bacteria produced proteases during their growth. Figure 1 shows that the bacteria had the highest quantitative proteolytic activity of 0.06 U/ml with an incubation time of 18 hours. If it is connected between the bacterial growth curve and the proteolytic activity test it can be seen that in the exponential growth phase, the bacteria produced high proteolytic activity which was achieved at intervals of incubation of 0-18 hours. This was due to the availability of large amounts of nutrients needed by bacterial cells to carry out cell metabolism, so that bacterial growth increased.

3.2. Particle Size of Solution for Nanoencapsulation

Measurement of particle distribution aimed to determine the size distribution of the resulting nanoencapsulation. Nanoparticle size and particle size stability are strongly influenced by the rotational speed and the length of time of the homogenization [8]. Tests were carried out using the Particle Size Analyzer (PSA). Skim milk solution was homogenized at 15000 rpm for 5 min had a distributed particle size of 383.7 nm (99.2%) to 4815 nm (0.8%). Overall, the average particle size of the skim milk solution was 316 nm. As for the particle size distribution of maltodextrin solutions ranged from 7.4 nm (92.3) to 3612 nm (7.7%). Overall the maltodextrin solution particle size was 380.4 nm. The particle size distributions of each solution are shown in Figure 2 and Figure 3.

Figure 2. Particle Size Distribution of skim milk Figure 3. Particle Size Distribution of maltodextrin

3.3. Nanoencapsulation of Bacteria

In the process of nano-encapsulating proteolytic bacteria using a spray dryer, the principle of this tool is to use high temperatures in the grinding process. One way to maintain bacterial viability and evenly distributed encapsulation size distribution is to adjust operating conditions such as inlet and outlet temperatures [9]. The best condition is at the inlet temperature of 120 °C and the outlet temperature is
70 °C. From the results of nanoencapsulation using a spray dryer, the number of proteolytic bacteria cells in skim milk and maltodextrin coating was decreased. The number of bacterial cells that lived after nanoencapsulation ranged from 11-13.6 x 10^8 cfu/ml. The viability of nanoencapsulation bacteria expressed in percent was 35.45% - 44.07%. The results of viability testing are presented in Table 1.

Cell viability was highest in 15% maltodextrin and 20% skim milk. The highest viability in maltodextrin reached 41.91% while the highest viability in skim milk reached 44.07%. The higher the concentration of coating materials, the encapsulation efficiency will increase [10]. It is evident that the addition of skim milk concentration resulted in higher viability.

| Material Carrier | Before encapsulation (cfu/g) | After encapsulation (cfu/g) | Viability (%) |
|------------------|-----------------------------|-----------------------------|--------------|
| Skim milk 10%    | 11.0 x 10^8                 | 3.9 x 10^8                  | 35.45        |
| Skim milk 15%    | 12.9 x 10^8                 | 5.0 x 10^8                  | 38.76        |
| Skim milk 20%    | 11.8 x 10^8                 | 5.2 x 10^8                  | 44.07        |
| Maltodextrin 15% | 13.6 x 10^8                 | 5.7 x 10^8                  | 41.91        |
| Maltodextrin 20% | 12.5 x 10^8                 | 4.8 x 10^8                  | 38.40        |
| Maltodextrin 25% | 12.7 x 10^8                 | 4.4 x 10^8                  | 34.65        |

To find the resistance of the bacterial cell viability during storage, a shelf-life prediction of bacterial isolates that have been encapsulated was conducted by counting the number of live bacteria before storage with the number of bacteria after storage. The result was that after storage for 2 weeks at 30 °C for the treatment of 20% skim milk, cell number was 7.0x10^5, while with maltodextrin 15% the number of cells was 1.6x10^6. There was a decrease in the number of cells that were not so significant.

The optimum temperature of the proteolytic bacteria is 37 °C, at the optimum temperature bacteria will easily adapt.

3.4. Encapsulation Bacteria by Electrospun Nanofibers

Application of electrospun nanofiber as cell encapsulation for proteolytic bacteria was to protect cells from some external environmental factors that can interfere with the metabolism of cells, controls the release of the cell, so as to maintain the viability of the bacteria during the storage process. The success of encapsulated bacteria with nanofiber using the electrospinning method has been demonstrated by Zussman [11]. Bacterial cells that have a diameter of a micrometer can be absorbed into the nanofiber because it is porous and semi-permeable.

Encapsulation with nanofiber provides advantages such as immobilization efficiency because of the increased surface area to volume ratio and strong protection for increased flexibility by encapsulation materials. Probiotic bacterial cells are encased by a nanofiber matrix, which is initially dispersed in a polymer solution and then cones and forms spheres in a nanofiber. The results of the viability test showed that probiotic bacteria that can survive reached 90% on day 21 of storage [4]. The factor that affects viability during storage is water activity and oxygen content which is reduced during the encapsulation process with nanofiber.

The encapsulation process by utilizing nanofiber electrospun was conducted using electrospinning devices. The principle of an electrospinning device is by pushing a polymer solution that is given a high voltage using a syringe pump to release a drop of solution at the end of the spinneret (in the form of a needle). The electrically induced polymer solution will move towards the electrode with the opposite charge while accompanied by the polymer solvent evaporation process so that only the dry polymer fiber is left behind [12]. According to Haider et al. [13], electrospinning devices utilizing
electrostatic power on polymer solutions will produce fine and dry fibers in very small sizes up to
nanometers (electrospun nanofibers). According to Li and Wang [14], the parameters that influence
the formation of nanofiber with electrospinning techniques are the parameters of the solution
(concentration, molecular weight (Mw), viscosity, surface tension, and conductivity), process
parameters (voltage, flow rate, collector, and distance between the collector and the syringe tip), and
environmental parameters (temperature and humidity). Electrospinning devices used in proteolytic
bacterial encapsulation are shown in Figure 4.

![Electrospinning devices used in proteolytic bacterial encapsulation](image)

**Figure 4.** Electrospinning devices used in the proteolytic bacterial encapsulation

The determination of the type of material, concentration, the ratio of encapsulant materials was carried
out to determine the formulation of the formation of nanofiber encapsulations for bacteria with
 optimum conditions on electrospinning. Making a PVA (Polyvinyl Alcohol) coating solution with
variations in concentrations of 10%, 15% and 20% and skim milk with a concentration of 10%. Each
solution was stirred using a magnetic stirrer at a speed of 400 - 450 rpm, a temperature of 50 °C for 30
min until the solution was completely homogeneous. Mixing ratios of PVA and skim milk were
60%:40%, 70%:30%, and 80%:20%, then the best mixing ratio is chosen to be processed using
electrospinning. Mixing of PVA and skim milk was by stirring using a magnetic stirrer at a speed of
500 rpm, a temperature of 50 °C for 30 min until the solution is completely homogeneous. Furthermore, mixing was continued using the Daihan WiseTis homogenizer HG-15D series (in 2016)
at a speed of 15000 rpm for 5 min. The solution of the nanofiber formation material (coating material)
was in the solution particle size determined using a particle size analyzer (PSA). Viscosity and pH
were also measured in the solution to be able to adjust to the electrospinning process conditions.

The conditions of the process of electrospinning used were the flow rate of the exit solution arranged
using an automatic pump in a continuous and stable manner starting from 15 ml/hour, 20 ml/hour, and
25 ml/hour. The voltage applied was varied from 5kV and 6kV. Different electrical voltages were
charged at the end of the nozzle or needle and collector. The solution of the material that contacted the
electric voltage will form a jet towards the collector. The emitted form will harden to form nanofiber
due to solvent evaporation. The formed nanofiber was captured by the collector. The distance between
the tip of the nozzle or needle and the collector ranged from 8 and 10 cm. Environmental influences
were maintained at 25 °C and 50% relative air humidity.

4. Conclusion
Encapsulation of proteolytic bacteria with nanoencapsulation and electrospun nanofiber technique has
been shown to maintain the viability of bacterial cells during storage. In nanoencapsulation
techniques, it was found that the results of bacterial viability encapsulated coating material using 20%
skim milk was 44.07%, the result was higher than maltodextrin coating material. After estimating the
shelf life for two weeks, it was shown that the number of cells did not decrease significantly, from
7.0x10^5 to 5.2x10^5. In the encapsulation technique with electrospun nanofiber, there are three
parameters that affect the process of electrospun nanofiber, i.e. the coating material parameters
(concentration, viscosity and mixing ratio), the process parameters (voltage, flow rate, and the distance between the collector and needle) and environmental parameters (relative humidity and temperature). Viability of bacteria can be maintained during storage by encapsulation with electrospun nanofiber due to the bacteria trapped in the shaft of nanometer-sized fibers.

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