Formulation and Storage Stability of Nanoencapsulated
Proteus penneri and Bacillus aerophilus

Y I P Misto¹, R D M C Sitorus¹, I D Permatasari¹, E Noor¹*, T C Sunarti¹

¹ Department of Agroindustrial Technology, Bogor Agricultural University, Bogor, Indonesia
*Email: erlizanoor@yahoo.com

Abstract. Proteus penneri and Bacillus aerophilus are bacterial that can be used as dry starter in the fermentation of artificial civet coffee. The use of dried starter will facilitate the handling, distribution and transportation of culture. The aims of this research were to evaluate the suitable nanoencapsulation coating materials of Proteus penneri and Bacillus aerophilus to increase biomass cells during drying and storage. Nanoencapsulated starter using carrier materials maltodextrin, sodium alginate and skim milk with spray drying method. The Nanoencapsulated Proteus penneri and Bacillus aerophilus were tested for stability during storage for up to 14 days at different temperatures (4 and 37 °C). Proteus penneri isolates had higher viability when encapsulated using maltodextrin with a concentration of 30%. Bacillus aerophilus isolates had higher viability when encapsulated using maltodextrin at 15% concentration. Viability of Bacillus aerophilus and Proteus penneri in dry starter with filler coffee pulp powder and coffee bean powder was less than after nanoencapsulation. The storage stability studies demonstrated the storage time and temperature affecting the survival of cells of dried starter cultures.

1. Introduction

Cellulolytic bacteria Proteus penneri and proteolytic bacteria Bacillus aerophilus were isolated from civet feces [1]. The isolates were used for fermentation in vitro of artificial civet coffee. Fermentation of coffee beans using these isolates in liquid starter has been performed either singly or in combination [2]. The use of the liquid starter in the fermentation process of civet coffee has limitations, such as inefficient in terms of stability and packaging. Besides, the possibility to grow other bacteria is bigger than in powder form. One method to extend the isolates storage is by drying. In addition, drying aims to facilitate the distribution and ease of maintenance.

The field of microbiology, encapsulation of various bacterial cultures has become a common practice to increase shelf life and provide safe handling. Encapsulation is a technology that has been widely developed to be applied in the food industry as an effort to protect bacterial cells. Encapsulation generates particles with micrometer diameters up to nanometers [3]. Nanoencapsulation is one way of maintaining the stability of a compound through a coating process in the form of nanoparticles. The nanoencapsulated product has diameters between 10 to 1,000 nm [4]. Compared to micron-sized carriers, nanocarriers provide more surface area; thus increasing their solubility and improving bioavailability [5].

There are several techniques such as spray drying, freeze drying, fluidized bed drying for the production of encapsulation dried starter cultures [6]. In general, freeze drying leads to high survival rates, but due to the high costs of the freeze-drying process, and the long drying times, alternative drying techniques
for starter cultures are becoming more and more important. The alternative drying processes that are mostly investigated for the drying of starter cultures are spray drying and vacuum drying [6]. Dry powder from spray drying containing microorganisms is a suitable form for storage and application purposes in the fermentation of artificial civet coffee. The dryer used for spray drying is the reduction in viability that occurs during processing and storage of the powder. The survival rate of the culture during spray drying and subsequent storage depends upon a number of factors, including the species and strain of culture, the drying conditions, the inoculum, and medium used, preadaptation of the culture to acquire resistance to processing conditions and the use of protective agents [7].

The objective of this study were to investigate the effect of bacterial encapsulation of *Proteus penneri* and *Bacillus aerophilus* on bacterial resistance after drying and viability after two weeks storage at low temperature (4 °C) and room temperature (30 °C).

2. Materials and Methods

2.1. Bacterial Strain
The bacteria used in this study is isolate derived from fresh civet feces *Proteus penneri* and *Bacillus aerophilus* which is isolated in previous study [1]. The isolates were obtained from the Laboratory of Animal Biotechnology PAU, IPB.

2.2. Preparation of Culture
Bacterial cell mass propagation of *Proteus penneri* was conducted in the carboxymethyl cellulose (CMC) medium and *Bacillus aerophilus* in Skim milk medium. The strain incubated at 37 °C for 24 h under microaerobic-static conditions and then used as an inoculum.

2.3. Preparation of bacterial nanoencapsulation
The nanoencapsulation process is carried out using a homogenizer. Encapsulation materials in the form of sodium alginate and maltodextrin are homogenized using homogenizer. Before the encapsulation process, measurements of the encapsulating material particles using the Particle Size Analyzer (PSA) were performed. Cellulolytic bacterial biomass *Proteus penneri* was made by using CMC media and *Bacillus aerophilus* in skim milk media. In a sterile medium inoculated bacterial culture of 10% and then incubated at 37 °C for 18 hours. The culture was then harvested and centrifuged at 4000 rpm for 15 minutes. The supernatant was removed to obtain wet biomass. The addition of encapsulant material to bacteria *Proteus penneri* was using maltodextrin with concentration of 15%, 20% and 30% and sodium alginate concentration 0.50%, 0.75% and 1%. *Bacillus aerophilus* was using maltodextrin with concentration of 15%, 20% and 25% and skim milk concentration 10%, 15% and 20%. Wet biomass is then mixed with a protective material according to a predetermined concentration using a magnetic stirrer at 500 rpm for 5 min. The homogeneous mixture is then dried with a spray dryer at an inlet temperature of 125 °C and a flow rate of 4-6 mL / min, a pressure on a 1 bar atomizer nozzle with a fixed air dryer volume to form a nanocapsule. The dried cultures obtained were then tested viability during drying.

2.4. Dried Starter
Dry pulp and coffee beans (13% moisture content) were ground and sieved (60 mesh). The inoculum that has been cultured in a liquid medium is combined with a comparison of *Bacillus aerophilus* : *Proteus penneri* 1 : 1 (cell/ml). Then added a filler for every 10 ml of microbe in accordance with the treatment of 10 g of coffee pulp flour and 10 g of coffee beans flour. Starter was dried by vacuum drying at 45 °C for 2 hours up to ± 10% water content.
2.5. Storage of Nanocapsul and Dried Starter

Bacterial nanocapsule of Provent penneri and Bacillus aerophilus were packed into polyethylene plastic and stored at low temperature (4°C) and room temperature (30°C) for two weeks of viability testing.

2.6. Cell viability

Bacteria cell viability testing was calculated based on the ratio of bacterial counts before and after storage and expressed in percent. A total of 0.1 g of each nanocapsule was diluted with 900 µL NaCl physiologically (0.85%) to obtain the first dilution (10^-1). Then conducted serial dilution until the 8th dilution. A total of 1 ml of dilution yield was planted by spread plate method on CMC and skim milk agar, and then incubated at 37°C for 48 hours. The colony was calculated, then calculated viability.

3. Result

3.1. Nanocapsulation of Bacteria

Preparation of bacterial nanoencapsulation by dissolving the carrier materials maltodextrin, sodium alginate and skim milk in aquades then homogenized by using homogenizer at 15,000 rpm for 5 minutes to obtain a nano-sized coating material. Then the bacterial cells that have been incubated for 24 hours were mixed with the coating material using a magnetic stirrer device at 500 rpm for 5 minutes and dinanoencapsulated with a spray dryer at 125 °C. According to [4], a nano-sized material has a size range from 10 to 1000 nm. Test results using the Particle Size Analyzer (PSA) tool based on the volume distribution showed maltodextrin particle size between 1.66-5.55 nm, sodium alginate size between 9.27-48.38 nm, and skim milk size between 316.0-383.7 nm.

3.2. Viability of Nanoencapsulated Bacteria

One way to increase the viability of cellulolytic and proteolytic bacteria are nanoencapsulation process. The method used is spray drying method. Nano-sized coating particles can protect bacteria from unfavorable environmental conditions such as heat and chemicals.

| Coating material | Mean count before nanoencapsulation (cfu/ml) | Mean count after nanoencapsulation (cfu/g) | Count of bacteria at storage (cfu/g) |
|------------------|---------------------------------------------|------------------------------------------|-----------------------------------|
| Maltodextrin 30% | 1.72 x 10^8                                  | 1.57 x 10^8                             | 1.54 x 10^8 7 x 10^7              |
| Maltodextrin 20% | 1.72 x 10^8                                  | 1.38 x 10^8                             | 8.5 x 10^7 7.4 x 10^7              |
| Maltodextrin 15% | 1.72 x 10^8                                  | 1.25 x 10^8                             | 7.3 x 10^7 6.1 x 10^7              |
| Sodium alginate 1% | 1.72 x 10^8                              | 1.525 x 10^8                            | 1.8 x 10^7 1.72 x 10^7             |
| Sodium alginate 0.75% | 1.72 x 10^8                          | 1.22 x 10^8                             | 1.2 x 10^7 1.18 x 10^7             |
| Sodium alginate 0.5% | 1.72 x 10^8                           | 1.34 x 10^8                             | 1.26 x 10^7 1.01 x 10^7             |
Figure 1. Graph of *Proteus penneri* viability after drying process

Figure 2. Graph of *Proteus penneri* viability on some combination of encapsulation materials after being stored at low temperature (4 °C)

Figure 3. Graph of *Proteus penneri* viability on some combination of encapsulation materials after being stored at room temperature (30 °C)

Table 2. Data Count of Colonies of *Bacillus aerophilus* after nanoencapsulation

| Coating material | Mean count before nanoencapsulation (cfu/ml) | Mean count after nanoencapsulation (cfu/g) | Count of bacteria at storage (cfu/g) |
|------------------|----------------------------------------------|-------------------------------------------|-------------------------------------|
|                  |                                              | 4°C                                       | 30°C                                |
| Maltodextrin 25% | 1.92 x 10^7                                  | 7 x 10^6                                  | 1.6 x 10^6                          | 1.1 x 10^6                        |
| Maltodextrin 20% | 1.98 x 10^7                                  | 8.9 x 10^6                                | 2.3 x 10^6                          | 1.35 x 10^6                       |
| Maltodextrin 15% | 1.87 x 10^7                                  | 1.55 x 10^6                               | 1.5 x 10^6                          | 1.0 x 10^6                        |
| Skim Milk 20%    | 1.53 x 10^7                                  | 6.6 x 10^6                                | 7.3 x 10^5                          | 3.9 x 10^5                        |
| Skim Milk 15%    | 1.49 x 10^7                                  | 6.7 x 10^6                                | 6.2 x 10^5                          | 4.6 x 10^5                        |
| Skim Milk 10%    | 1.28 x 10^7                                  | 4.9 x 10^6                                | 3.6 x 10^5                          | 1.6 x 10^5                        |

Figure 4. Graph of *Bacillus aerophilus* viability after drying process
Based on Table 1. after storage for two weeks, the highest *Proteus penneri* viability for the maltodextrin coating agent was present at a concentration of 30% with a storage temperature of 4°C, viability of 90.80%. While the lowest is at concentration of 15% with a storage temperature of 30°C, which is viability of 72.30%. Highest *Proteus penneri* viability for sodium alginate coatings is present at 1% concentrations of 88.20% with 4°C storage temperature, while the lowest is at 0.75% concentration of 70.56% with storage temperature 30°C. Based on Table 2. highest viability *Bacillus aerophilus* for maltodextrin coating material was present at 15% concentration with 4°C storage temperature, ie via 27.27% viability. While the lowest is in the concentration of 20% with a storage temperature of 30°C, namely viability of 15.17%. The highest viability of *Bacillus aerophilus* for skim milk coatings was at a concentration of 20%, ie 11.06% with 4°C storage temperature. While the lowest is at concentration of 10% that is equal to 3.27% with storage temperature 30°C. According to [8], the proportion of bacterial counts and coating agents used should be considered so as to provide optimum protection for each bacterial cell. Higher coating concentrations are able to provide protection to each bacterial cell during the nano encapsulation process to heat generated by the spray dryer.

**Table 3.** Data Count of Colonies of *Bacillus aerophilus* after vacuum drying

| Filler material | Mean count before drying (cfu/ml) | Mean count after drying (cfu/g) | Count of bacteria at storage (cfu/g) |
|-----------------|----------------------------------|-------------------------------|----------------------------------|
| Coffee Beans    | $5.7 \times 10^6$                | $2.5 \times 10^8$              | $1.6 \times 10^7$ $1.2 \times 10^7$ |
| Pulp Coffee     | $5.7 \times 10^6$                | $2.3 \times 10^8$              | $1.1 \times 10^7$ $6.4 \times 10^6$ |

**Table 4.** Data Count of Colonies of *Proteus penneri* after vacuum drying

| Filler material | Mean count before drying (cfu/ml) | Mean count after drying (cfu/g) | Count of bacteria at storage (cfu/g) |
|-----------------|----------------------------------|-------------------------------|----------------------------------|
| Coffee Beans    | $6.7 \times 10^6$                | $2.8 \times 10^8$              | $1.9 \times 10^7$ $1.2 \times 10^7$ |
| Pulp Coffee     | $6.7 \times 10^6$                | $2.3 \times 10^8$              | $2.9 \times 10^7$ $1.4 \times 10^7$ |
Dry starter viability was determined by comparing colonies before and after drying. The results showed that the dry starter of bacteria isolated from civet feces after spray drying and vacuum drying had decreased in the count of cells but still had high viability. The protective effect of the protectant is probably due to their stabilization effect on cell membrane constituents, resulting in improved viability [9]. It is known that during the drying process, water removal can lead to destabilization of the structural integrity of cellular components, causing a loss of cellular viability and activity [10,11,12].

Cell death that occurs because the drying process can be reduced by the addition of coating material or filler material into the cell suspension to be dried. The results of dry starter viability showed that both isolates had good resistance after being a dry starter. The ability to survive can be caused by the availability of food reserves on dry starters. The carriers provide a protective effect on the cells because they create a thick wall matrix encapsulating the cells inside the microparticle and can react with and stabilize cellular structures during drying [13]. The use of pulp coffee as filler material is the utilization of the by-products of coffee processing. Besides that, the added pulp and coffee beans have high carbohydrate, protein and fiber content.

Viability of dry starter with storage at 4 °C is the highest compared to storage method at 30 °C. At 4 °C, the cell metabolism takes place at a minimum or stops altogether, as a result of which the water content dried starter remains low, its cell viability remains stable. The temperature condition of 30 °C is a disadvantage for the stability of dry starter life during storage.

Based on the results showed that the count of cells of Bacillus aerophilus and Proteus penneri bacteria in dry starter with filler coffee pulp powder and coffee bean powder was less than after nanoencapsulation. This will affect the results of the coffee fermentation process. The less the count of bacterial cells used, the lower the fermentation results.

4. Conclusion

Proteus penneri isolates had higher viability when encapsulated using maltodextrin with a concentration of 30%. Bacillus aerophilus isolates had higher viability when encapsulated using maltodextrin at 15% concentration. Viability of Bacillus aerophilus and Proteus penneri in dry starter with filler coffee pulp powder and coffee bean powder was less than after nanoencapsulation.

5. Acknowledgement

This research was supported by Ministry of Research, Technology and Higher Education of the Republic of Indonesia through the Postgraduate Research Grant (No. 1546/IT3.11/PN/2018).

6. References

[1] Dewi SL 2012 Isolation of xylanolitic and cellulolytic bacteria from civet feces. (Bogor,ID: IPB)
[2] Rohman H 2013 Production of enzymatic coffee using proteolytic bacteria and combination of cellulolytic and xylanolytic bacteria from civet. (Bogor,ID: IPB)
[3] Zuidan NJ, Nedovic VA 2010 Encapsulation Technologies for Food Ingredients and Food Processing. New York (US): Springer
[4] Konan Y N, Gurny R and Allémann E 2002 Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles Int J Pharma 233:239–252.
[5] Kwak H 2014 Nano- and Microencapsulation for Foods. Oxford (UK): John Wiley & Sons
[6] Holzapfel W 2014 Advances in Fermented Foods and Beverages. Oxford (UK): Woodhead Publishing
[7] Desmond CC, Stanton GFK, Collins and Ross 2002 Improved survival of Lactobacillus paracasei NFBC 338 in spray dried powders containing gum acacia *J of Appl Microbiol* 93:1003-1012.

[8] Triana E, Yulianto E, Nurhidayat N 2006 Viability of encapsulated Lactobacillus sp. Mar 8 *Biodiversitas*. 7(2):114-117.

[9] Lapsiri W, Bhandari B, Wanchaitanawong P 2012 Viability of Lactobacillus plantarum TISTR 2075 in different protectants during spray drying and storage *Drying Technology* 30: 1407–1412.

[10] Ananta E, Volkert M, Knorr D 2005 Cellular injuries and storage stability of spray-dried Lactobacillus rhamnosus GG. *International Dairy Journal* 15:399–409.

[11] Yadav AK, Chaudhari AB, Kothari RM 2009 Enhanced viability of Bacillus coagulans after spray drying with calcium lactate, storage and re-hydration. *Indian Journal of Chemical Technology*, 16:519–522

[12] Golowczyc MA, Silva J, Teixeira P, De Antoni GL, Abraham AG 2011 Cellular injuries of spray-dried Lactobacillus spp. isolated from kefir and their impact on probiotic properties. *International Journal of Food Microbiology* 144:556–560.

[13] Fu N, Chen XD 2011 Towards a maximal cell survival in convective thermal drying processes. *Food Research International* 45: 1127–1149