EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 7
156-162
Year: 2018

TEST VIABILITY MICROENCAPSULATION *LACTOBACILLUS ACIDOPHILUS* USING ALGINATE-CHITOSAN POLYMER IN SIMULATED GASTRIC FLUID

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Article Received on 11/05/2018 **Article Revised on 01/06/2018** **Article Accepted on 21/06/2018**

ABSTRACT
Objective: *Lactobacillus acidophilus* is one of the strains of lactic acid bacteria that has been widely used as a probiotic. The ability of *Lactobacillus acidophilus* to grow in the digestive system can suppress the growth of enteric pathogenic bacteria and improve the balance of microflora in the digestive system so that it can be utilized to maintain a healthy body. *Lactobacillus acidophilus* bacteria exhibit short stationary phases and follow rapid cell viability loss, although stored at freezing temperatures. This study aims to determine the viability of bacterial microenkaosulasi to simulated gastric fluid (SGF). **Methods:** The microencapsulation of bacteria was performed by using a chitosan-alginate polymer, the method used was the extrusion method. Bacterial microencapsulation viability tests were performed by incubating microencapsulation in the simulated gastric fluid. **Result:** The microencapsulated *Lactobacillus acidophilus* ATCC 4356 cell population amounted to $3.9x10^7$ colonies/ml of suspension, microencapsulated using sodium alginate-Chitosan polymer 2:0.1%; 2:0.2%; 2:0.3% with a cell density of $1.4x10^7$; $1.6x10^7$; $3.0x10^7$ colonies/g can still be preserved *Lactobacillus acidophilus* cells incubated in SGF pH 1.5 for 120 minutes. **Conclusion:** Polymer sodium alginate-chitosan can maintain the cell number of the cell (Free cell) were still able to retain *Lactobacillus acidophilus* cells incubated in SGF pH 1.5 for 120 minutes.

KEYWORDS: Lactobacillus Acidophilus, Lactic Acid Bacteria, Probiotic.

INTRODUCTION
Lactobacillus acidophilus is one of the strains of lactic acid bacteria that has been widely used as a probiotic. The ability of *Lactobacillus acidophilus* to grow in the digestive system can suppress the growth of enteric pathogenic bacteria and improve the balance of microflora in the digestive system so that it can be utilized to maintain a healthy body.[1,2] The *Lactobacillus acidophilus* bacterium exhibits a short stationary phase and is followed by rapid cell loss, although stored at freezing temperatures. The short lifetime of this probiotic makes the problem of how to maintain the viability of this probiotic in order to provide a functional effect. One way to maintain viability is by microencapsulation.

One way to prevent damage and decrease the amount of lactic acid bacteria and probiotics is to perform the encapsulation process. Encapsulation is applied to probiotics in order to protect probiotics alive from extreme conditions due to drying, storage and digestive tract fluid. The encapsulation process has been widely used in the chemical, pharmaceutical and food industries in order to protect the active compounds from environmental conditions (oxygen, water, acid, interaction with other materials), which may affect stability during processing, to provide controlled release or to alter physical properties, reducing stiffness during storage or transportation.[3] In addition, probiotics that circulate in the market in liquid form, are less efficient in terms of stability during storage and in packing and the possibilities for other bacteria to grow larger than in powder form.[4]

Several bacterial microencapsulation studies have been conducted, 4% *Lactobacillus casei*has not been able to maintain bacterial viability and decrease cell by 99.96%, with a cell number of $4.5x10^3$ colonies/gram from the initial cell count of $7.41x10^4$ Colonies/g before incubation in gastric acid simulation fluid.[5] Matrix chitosan for a microcapsulation viability test of *the Lactobacillus casei* with concentrations of 2%, 2.5% and 3% with a density of $1.8025x10^5$ colony/g; $1,5225x10^4$ colony/g; <30 colonies/g, but has not been able to sustain probiotic bacterial cells incubated in a simulated gastric fluid.[6] Meanwhile, according to[7] the optimum condition of microcapsule at 0.62% (b/v) alginate concentration of a chitosan combination of 1.75% (w/v) and glutaraldehyde 4.63% (v/v) slow release preparation.

According to[8] microencapsulation of *Lactobacillus acidophilus* using carrageenan polymer has not been able to provide protection against *Lactobacillus acidophilus* where bacterial viability at pH 2 and pH 7 of 10^6 CFU/mg to 10^3CFU/mg and 10^2CFU/mg and still not fulfill the value minimum WHO probiotics.

Based on the above reasons, it is necessary to conduct research by making microencapsulated *Lactobacillus acidophilus* using a combination of sodium alginate polymer and Chitosan. Alginate is a commonly used ingredient in probiotic encapsulation. Bead alginate has been tested to increase probiotic survival. Alginate can also be used and safe for food. The alginate used is the form of salt, sodium alginate and crosslinking with calcium ions to form a controlled reaction, ie gel form.[9] Chitosan has a positively charged amines group that can form a semipermeable membrane around a negatively charged polymer, in addition chitosan is a natural, biocompatible, biodegradable, non-toxic polymer, and has the ability to form films.[10]

MATERIALS AND METHOD

Materials: The microorganisms used in this study were *Lactobacillus acidophilus* ATCC 4356. The chemicals used were De Man Rogosa Sharpe agar (MRSA) and De Man Rogosa Sharpe Broth (MRSB), chitosan, alginate, inulin, $CaCl_2$, NaCl physiological 0.9%, sterile distilled water, glycerol, SGF (0.08 M HCl in 0.2% NaCl with pH 1.598 without pepsin), and Gram staining reagents (crystal violet, lugol, 96% alcohol and safranin).

Instrument: Petri dish, ose needle, beaker glass, volumetric flask, erlenmeyer, stirrer, spatula, volume pipette, vaporizer, glassware, micropipette, glasses, oven, bunsen, autoclave (Vision), thermometer, Vision, Incubator (Fisher Scientic), Cooler (LG), Dropper Pipes, Aluminum foil, Vortex (Thermo), Colony Counters, Balance Sheet, Microscope, Hot Plate (Favor), Magnetic Stirrer (Thermo), Laminar Air Flow (LAF) (Isocide), pH meters (Hanna), syringe (Terumo) 23x1¼G, filter paper, centrifuge (Thermo), Scanning Electron Microscopy (SEM).

Verification of Test Microbes: The rejuvenated *Lactobacillus acidophilus* was taken 1 ose and then scraped on the glass surface of the object that has been spilled with 0.9% NaCl, then fixed with hot Bunsen to form a stain on the glass object. Preparations dripped with violet crystal 1 drop, then stand for 1 minute, washed with aquadest until the color fades and then dried. The preparation was re-dripped with 1 drop of lugol solution, allowed to stand for 1 minute and then washed with dried water and dried. The preparations are then re-dripped with 1 drop of alcohol, washed with aquadest and dried. The preparation is re-infused with 1 drop of safranin solution, let stand for 1 minute then washed with dried and dried. Afterwards the preparations were observed using a microscope with 100x magnification.[11]

Production of Bacterial Suspension

A total of 1 ose *L. acidophilus* extracted from the stock culture was inoculated in 10 ml MRS broth, then incubated at 37º C for 24 h in the incubator shaker. The culture was transferred into 100 ml MRS broth, then incubated again at 37º C for 24 h in the incubator shaker. Cells were harvested by centrifugation 4400 RPM for 10 min at 4ºC, then washing twice, the supernatant was discarded and the cell deposits suspended with 0.9% sterile NaCl solution then centrifuged, the supernatant was discarded. The cell precipitate was resuspended with 0.9% NaCl sterile and obtained *L. acidophilus* cell suspension.[12] Then performed cell population calculations on the suspension of *L. acidophilus*.

Calculation of Suspension Cell Population *L. Acidophilus:* Cell population calculation on *L. acidophilus* suspension was done by dilution to 109 colonies / ml. Dilution was carried out by taking as much as 1 ml of suspension of *L. acidophilus* using a micropipette then inserted into a sterile reaction tube containing 9 ml of sterile 0.9% NaCl then vortex. After that the dilution of *L. acidophilus* dilution was then taken as much as 100 µl using a micropipette and then inserted into a sterile petri dish which contained MRS for sterile after it was done spraying the spread method by using sterile L stems until the MRS surface to dry. Furthermore, the petri dish is inserted into the incubator for incubation for 48 hours at 37ºC. The growing colony was then calculated using a colony counter. The calculation of cell count is done by the formula of Pradikaningrum.[6]

$$\text{Number of cells/ml} = \frac{\text{number of cells}}{\text{volume spread in petri dish x dilution factor}}$$

Making Microencapsulated *L. acidophilus* Using Sodium Alginate-Chitosan: Preparation of *L. acidophilus* microcapsule formula using sodium alginate-chitosan each with a concentration of 2: 0.1%, 2: 0.2% and 2: 0.3%. Initially made an alginate mixture with bacterial suspension, then microencapsulated process and then formed microcapsules coated with Chitosan.

Table. 3.1. The microencapsulation formula of *L. acidophilus* uses sodiumalginate-chitosan

Material	Formula I	Formula II	Formula III
Alginate	2%	2%	2%
Chitosan	0,1%	0,2%	0,3%
Acetic acid	1%	1%	1%
Inulin	2%	2%	2%
$CaCl_2$	0,1 M	0,1 M	0,1 M
Suspense *L. acidophilus*	50 ml	50 ml	50 ml

Preparation of $CaCl_2$ 0.1 M solution: A total of 14.7 g of $CaCl_2$ was weighed and then dissolved in 1000 ml of distilled water in a measuring flask, stirred until

homogeneous. The solution was sterilized by autoclave at 121ºC for 15 minutes.

Making Sodium Alginate Solution Contains Bacterial Suspension: Preparation of sodium alginate solution was first prepared a solution of alginate with a concentration of 2% (w/v) with aquadest solvent (v/v). Added 2% inulin to the last while stirring until homogeneous and sufficient to 50 ml, then sterilized by autoclave at 121ºC for 15 minutes. *L. acidophilus* suspension was added 50 mL.

Making Chitosan Solution: The preparation of Chitosan solution was made in three concentrations, such as Chitosan concentration 0.1%, 0.2% and 0.3%. Each chitosan was dissolved in 1% acetic acid solution, then the solution was sterilized by autoclave at 121° C for 15 minutes.

Microencapsulation Making: Furthermore sterile alginate solution containing *L. acidophilus* suspension was introduced into the syringe and then dropped into a 0.1 M $CaCl_2$ solution, let stand for one hour until a solid microencapsulation was formed, then the formed microcapsules were transferred into sterile distilled water and stirred slowly using shaker for nsatu hours to remove $CaCl_2$ residue, then strain.

Alginate microcapsules that have been formed later coated with Chitosan. Insert the alginate beads into each 0.1%, 0.2% and 0.3% Chitosan solution, stirred at a rate of 600 RPM for 15 min until the surface of the coated microcapsules. Then filtered with filter paper and microcapsules rinsed with sterile distilled water twice.

Morphological Analysis of Microencapsulated *L. Acidophilus:* As much as 1 g microencapsulation *L. acidophilus* polymer, sodium alginate-chitosan was analyzed morphology and bead diameter using Scanning Electron Microscopy (SEM).

Calculation of encapsulated *L. Acidophilus:* A 1 g microencapsulation of *L. acidophilus* was added with 9 ml phosphate buffer (pH 6.8) then distirer for 2 hours for each formula. After that, dilution with 0.9% NaCl was sterilized, 100 µL taken and then transferred to MRS agar medium and then incubated at 37ºC for 72 hours.[13] The bacterial density of each 1 g formed was calculated by the formula.

$$\text{Number of cells / g} = \frac{\text{Number of colonies}}{\text{volume distributed in petri dish x dilution factor}}$$

Microencapsulation *L. acidophilus* Viability Test On SGF: A 1 g microencapsulated *L. acidophilus* was dispersed with 10 ml sterile SGF (0.2% NaCl, 0.08 M HCl pH 1.5) and then incubated at 37ºC for 120 min. After incubation, microencapsulated *L. acidophilus* was washed with a sterile 0.9% NaCl solution. Then calculated *L. acidophilus* colonies with methods such as point 3.3.7.[14]

Water content analysis: On the measurement of water content according to[15], the cup is first dried with oven for 15 minutes and cooled in a denticator and then weighed. After that the microcapsule is rapidly weighed as much as 0.5 g. Furthermore, the microcapsule plate is inserted into the oven at 105ºC for 6 hours. The microcapsule plate is cooled in the denticator and weighed in weight. The cup and microcapsules are put back into the oven until a fixed weight (3 decimal) is obtained. Water content is calculated on a dry basis.

RESULTS AND DISCUSSION

Bacterial Verification of *Lactobacillus acidophilus* Test ATCC 4356: Observation of the colony's morphological characteristics.Verification of the test microbes was performed to look at the type of bacteria based on the shape and color of bacterial cells, and to prove that the growing colony is *L. acidophilus*. One of the verification processes undertaken to identify bacteria is by doing Gram staining. Gram staining is able to distinguish between two groups of bacteria, Gram positive and Gram negative bacteria. Purple bacteria are classified into Gram positive, whereas red bacteria are classified into Gram negative.[11] *L. acidophilus* is a Gram-positive bacterium that does not have cells with bacillus (stem) forms and is anaerobic facultative.[16] The result of observation of bacterial staining of *Lactobacillus acidophilus* with microscope in Figure 4.1 shows the blue-purple color indicates the bacteria as Gram positive bacteria. This is in accorandce with the literature that says that the bacterium *L. acidophilus* has a form of bacil and is a Gram positive bacteria.

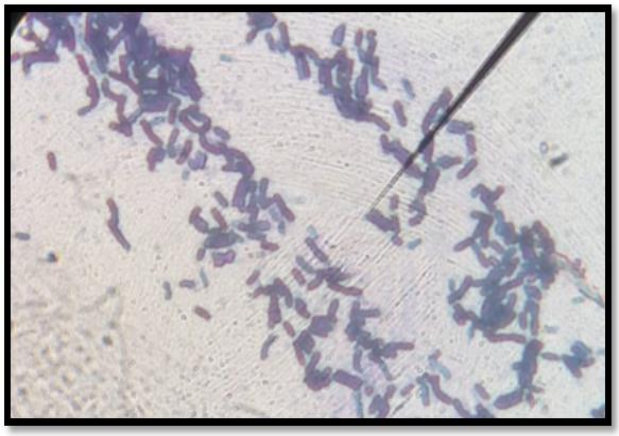


Figure. 4.1. Cell *Lactobacillus acidophilus* ATCC 4356 at 100x magnification.

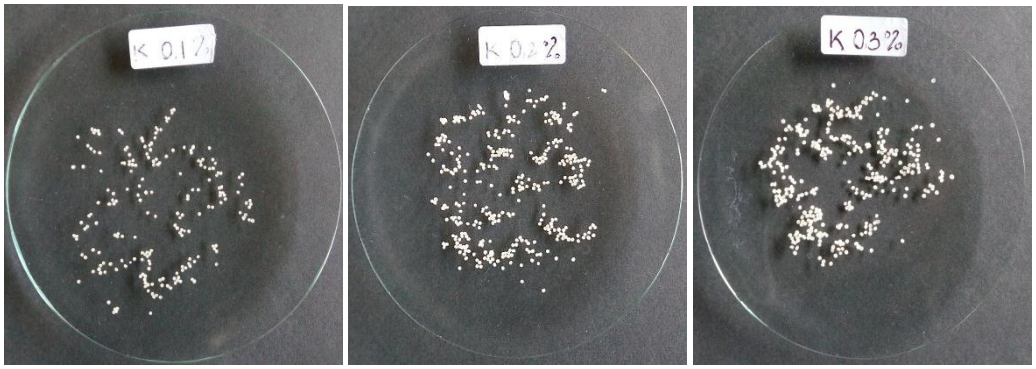
Ratio of Suspension Cell Population *Lactobacillus acidophilus:* Calculations were performed by dilution using a 0.9% NaCl sterile solution. Then the suspension pippeted out as much as 100 µl into a petri dish containing MRS for sterile. After incubation for 48 hours with temperature 37°C, the number of cells obtained as much as 3.9 x 107 colony / ml.

Microencapsulated *L. acidophilus* Using Alginate Polymers Chitosan: The encapsulated probiotic method of *L. acidophilus* selected is the extrusion method and ionic gelation method. The extrusion method was chosen because it uses a simple tool, done by using spray drying method[17], and from unfavorable environment. Drying, which can lead to reduced viability of the bacteria[18], and also greater shape compared to the emulsion method.[19] The selection of ionic gelation method because of its simple process, does not use organic solvent, and can access easily.[20] In the first extrusion technique hydrocolloid solution, then probiotics and used to take into the solution. While the principle of particle formation in the ionic gelation method is the ionic interaction between the carboxylic anion (COO^-) of the alginate monomer and the divalent cation (Ca^+). Crosslinking occurs by way of calcium ions for two sodium ions in alginate. This crosslinking structure is a restricted molecular movement and hinders media development in the media.[14] The microencapsulation is then performed by organoleptic examination, the result of *L. acidophilus* microencapsulation using sodium alginate-chitosan polymer can be seen in table 4.1. and Figure 4.1.

Table. 4.1 Results of microcapsulated organoleptic examination of *L. acidophilus* uses sodium alginate-chitosan polymer.

Concentration		Organoleptic		
Alginate	**Chitosan**	**Form**	**Color**	**Odor**
2%	0,1%	Spheric	Dark white	Odorless
2%	0,2%	Spheric	Dark white	Odorless
2%	0,3%	Spheric	Dark white	Odorless

Based on Table 4.1 and Figure 4.2 it is seen that the organoleptis of the microencapsulated *L. acidophilus* produced in all three concentrations has a whole round shape. While the colors resulting from all three concentrations are cloudy white. And the three microencapsulated formulations of *L. acidophilus* are odorless.



(a) (b) (c)

Figure 4.2 Microencapsulated *Lactobacillus acidophilus* sodium alginate-Chitosan.

Description: (a) Microencapsulated *L.acidopilus* sodium alginate-chitosan 2-0,1%,
(b) Microencapsulated *L.acidopilus* sodium alginate-chitosan 2-0,2%,
(c) Microencapsulated *L.acidopilus* sodium alginate-chitosan 2-0.3%.

Table. 4.2 Results of water content analysis and *L. actobacillus* microencapsulated weight uses a sodium alginate-chitosan polymer.

Concentration		Water content	Microcapsule weight	
Alginate	**Chitosan**		**A**	**B**
2%	0,1%	9,0 %	10,022 gr	10,031 gr
2%	0,2%	9,3 %	10,016 gr	10,033 gr
2%	0,3%	9,8 %	10, 019 gr	10,044 gr

Description: (A) Before chitosan is added
(B) After chitosan is added

Based on table 4.2 can be seen that the water content of the three formulas large enough where the amount of water ranges from 9.0 to 9.8%.[21] reported a moisture probiotic encapsulation of 7-12%.[22] Reported that the Bifidobacteria microencapsulated water content of some sinbiotics by spray drying method ranges from 6-10%, where the use of spray drying will result in a reduction in the moisture content of the material. While the addition of microcapsules weight from before and after coated chitosan on the variation of concentration yields different weight. And it can be concluded that there is a layer of chitosan that protects microencapsulation and maintains the viability of probiotics.

Miroencapsulation Analysis of *L. acidophilus*

Based on the image from the SEM (Scanning Electron Microscopy) analysis it is known that the particle size of the sodium alginate-chitosan microcapsule with the extrusion method varies, can be seen from different diameters of some particles. Where the diameter for each microcapsule is 0,976mm to 1.091mm.

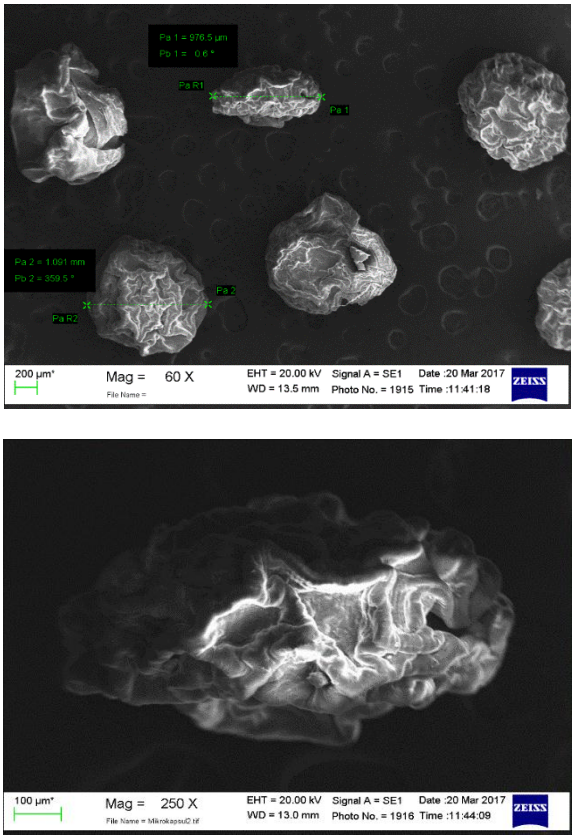


Figure. 4.3. Results of SEM analysis (Scanning Electron Microscopy) on 60x and 250x magnification.

The size of the diameter of the encapsulation affects the matrix's ability to protect the bacteria inside it. The larger size of encapsulation (2-4 mm) with the extrusion technique in the Muthukumarasamy, et al study can better protect *Lactobacillus reuteri* bacteria compared to the 20-1000 µm encapsulation size.[23] The size of the various microcapsules is influenced by many factors, including: polymer concentration, the distance between the syringe and the microcapsule forming solution, the pressure difference during the formation of microcapsules through a syringe, and the size of the syringe diameter used in the process of extraction.[24]

Calculation of Encapsulated *L. acidophilus*

The population calculation of encapsulated *L. acidophilus* is performed to determine the success of bacteria in microcapsules, so that it can be used as a comparison to the next stage test such as viability for simulation of acid liquid of gastric and bile salt.

One way to increase the viability of probiotic bacteria is in the process of encapsulation. The encapsulation method used is an extraction method to avoid extreme temperatures during encapsulation processes that can reduce the amount and viability of the bacteria. The result of *L. acidophilus* ATCC 4356 bacterial suspension was then mixed with coating material, namely sodium alginate-chitosan with a ratio of 1:1. The biomass mixed with coating is then homogenized prior to the encapsulation by the extrusion method.

Table. 4.3 Results of calculations of *L. acidophilus* encapsulated in sodium alginate and chitosan.

Concentration Sodium alginate - chitosan	Free cell *L. acidophilus* (colonies/ml)	Encapsulated *L. acidophilus* (colonies/ml)
2 : 0,1%	3,9 x 10^7	1,6 x 10^7
2 : 0,2%	3,9 x 10^7	1,9 x 10^7
2 : 0,3%	3,9 x 10^7	3,8 x 10^7

The initial cell count before the encapsulation process was 3.9 x 10^7 colonies/ml for each concentration. To determine the number of bacterial cell populations after the encapsulation process, the microcapsules that have formed are resuspended to be able to be confined. After calculation, we get the number of encapsulated bacteria that are consecutive of alginate-chitosan 2:0,1%; 2:0.2% and 2:0.3% are 1.4x10^7 colonies/grams; 1.9x10^7 colonies/grams and 3.8x10^7 colonies/grams. The number of bacterial calculations is shown in Table 4.2. From these results can be seen the number of bacterial cells encapsulated sodium alginate-chitosan 2:0.3% more than the number of encapsulated bacterial cells using sodium alginate-chitosan 2:0.1% and 2:0.2%. This is because the efficiency of encapsulation increases significantly with increasing concentrations of biopolymers.[23] According to[25], lactic acid bacteria suffer from pressure caused by environmental changes so that the number of *L. acidophilus* cells decreases viability. According to[23], factors that may cause a decrease in the number of encapsulated *L. acidophilus* cells are cell loss in $CaCl_2$ solution and and loss of viability in microencapsulation.

The number of bacteria from free cells used in the encapsulation process will greatly affect the amount of bacteria to be absorbed into the polymer. The higher the number of bacterial cells to be used, the higher the amount of bacteria being absorbed into the polymer. So the viability of the bacteria after the encapsulation process will be maintained in accorandce with WHO standards 10^6-10^7cfu/gram (log).[26]

During the encapsulation process many things can affect the decrease of bacterial viability. The amount of bacteria absorbed into the sodium alginate-chitosan matrix is not optimum, this can be seen from the decrease in the number of cell population after the encapsulation process. Bacterial suspension may be left in the container or syringe during the microcapsule formation process. So not all bacteria from the beginning of the encapsulation process are absorbed into the polymer.

Viability of Microencapsulated *L. acidophilus* in SGF

The three microcapsule formulas containing *L. acidophilus* were tested for resistance to SGF with pH 1.5, then incubated in a shaker incubator at 37°C for 120 min. The results of the viability test are shown in Table 4.3.

Table 4.4. Microencapsulated resistance of *L. acidophilus* in simulated stomach waters.

Sodium Alginate concentration-Chitosan	Before Simulation of Gastric Fluid (colonies/g)	After Simulation of Gastric Fluid (colonies/g)
2 : 0,1%	$1,6 \times 10^7$	$1,4 \times 10^7$
2 : 0,2%	$1,9 \times 10^7$	$1,6 \times 10^7$
2 : 0,3%	$3,8 \times 10^7$	$3,0 \times 10^7$

Table 4.4. Shows the number of colonies that are absorbed in microencapsulated after incubation in a simulated gastric fluid. It turned out that after incubation of sodium alginate-chitosan was still able to maintain the survival of *L. acidophilus*, but the number of the surviving cell population experienced a slight decrease, but still fulfilled the viability standard by WHO of 10^7 CFU it passed stomach acid.

The decrease in the amount of bacteria occurring in the simulated gastric fluid due to the very acidic pH of the stomach will affect the strength of the sodium alginate-chitosan polymer as the *L. acidophilus* bacterial encapsulation matrix. According to[27], the encapsulated material can be released by several ceremonies such as breaking the encapsulating wall, dissolving the encapsulated material, and diffuse through the encapsulating material.

The calcium alginate membrane readily degrades rapidly at low pH and loses its stability if there are chelating compounds such as phosphate, lactate and citrate.[28] The degradation of calcium alginate can cause cells to exit into the environment. The concentration of chitosan in the coating of sodium alginate beads determines the ability of viability, the authors assume the higher concentration of chitosan then the ability of polymers in protecting *L. acidophilus* bacterial cells will increase in the stimulation of acidic stomach acid.

CONCLUTION

L. acidophilus can be encapsulated with sodium alginate-chitosan by extrusion method resulting in a round, intact white, odorless white microcapsule. While the bead size 976,5 mm to 1,091 mm. Polymer sodium alginate-chitosan can maintain the cell number of the cell (Free cell) of $3.9x10^7$colonies/ml to $1.6x10^7$; $1.9x10^7$ and $3.8x10^7$ colonies/ml for formulas I, II and III. Microencapsulated *L.accidophilus* using sodium alginate-chitosan polymer 2:0.1%; 2:0.2%; 2:0.3% with a cell density of $1.4x10^7$; $1.6x10^7$; $3.0x10^7$ colonies/g were still able to retain *L. acidophilus* cells incubated in SGF pH 1.5 for 120 minutes simulation.

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