

The Effect of Extrusion Voltage and Flowrate to the Viability and Survivability of Probiotic *L. casei* Encapsulated in Alginate-Chitosan

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ABSTRACT--- Chitosan-coated *L. casei* containing alginate capsules (shortened as *L. casei* capsules) were prepared by extruding *L. casei* containing alginate solution at different extrusion voltage and and flow rate followed by coating the wet capsules in chitosan solution. This study aimed to determine the effect of extrusion voltage and sodium alginate liquid flow rate on the viability of *L. casei* bacteria in the encapsulation process. The encapsulation process in this study was carried out by the extrusion method using sodium alginate of 1% (w/v) and chitosan of 0.2% (w/v). The resulted beads were immersed in a simulated gastric fluid (SGF) (NaCl 0.2%; HCl 0.5 M with a pH of 1.5) for 1, 60, and 120 min at 37 °C. The number of *L. casei* cells before encapsulation was 12.3 log CFU. After encapsulation, the maximum viability of *L. Casei* obtained by voltage variations of 0 kV and flow rate 5 mL/min were 12.26 log CFU. After testing the beads in SGF for 1 min, the results obtained indicate that viability of *L.casei* in the sodium alginate - chitosan beads with an extrusion voltage of 0 kV and 5 mL/min was 11.8 log CFU/g. The result indicated that encapsulated *L. casei* in the sodium alginate - chitosan beads with a voltage of 0 kV and 5 mL/min was the highest survivability level of 97.38 %. The conclusions of the study were The higher extrusion voltage can kill more *L. casei* while the higher extrusion flow rate can protect more *L. casei*.

Keywords--- Encapsulation, probiotic, *L.casei*, na-alginate, chitosan, extrusion

1. INTRODUCTION

The term probiotic defined as living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition [1]. Their health effects are due to the nutritional and therapeutic benefits, especially protection against harmful microorganisms and the improvement of the immune system [2]. Modulation of the endogenous flora and osmotic changed in the gastrointestinal tract are prebiotics mechanisms of action. The world consumption of probiotic foods has grown greatly in recent times. Probiotic bacteria were mainly incorporated into dairy products such as ice cream, frozen desserts products, cheese, fermented milk, milk powder, and yogurt [3-5]. However, with an increase in consumer vegetarianism throughout the developed countries, there was an increasing demand for vegetarian probiotic products (non-dairy probiotic products) [3]. To show their health benefits, the probiotic bacteria must survive during gastrointestinal transition [4].

Encapsulation of probiotics in hydrocolloid beads, was an appropriate procedure to protect the bacterial cells from the damages caused by the external environment in food products and during gastrointestinal transition. Several studies have reported the probiotic microencapsulation by using alginate and chitosan to provide protection to Bifidobacteria and Lactobacilli [7-10]. Among the encapsulating materials, alginate was the most commonly used polymer for encapsulating viable cells [7]. It consist of L-gulucoronic acid and D-mannuronic acid. Alginate can form beads with calcium chloride to encapsulate probiotic bacteria that was susceptible to acidic environments but alginate beads was porous, so that acidic fluid can enter the inside of alginate beads and killed probiotics[8]. Polycations, such as chitosan or polyamine acids can reduce the porosity of the alginate beads [6]. Chitosan is a natural, non-toxic and inexpensive linear polysaccharide with positive charge at low pH, which has been used for coating the alginate beads. Low concentration chitosan solution (2 - 4 g/m³) has been applied for shell-making on alginate and gelatin capsules [8,11,12]. Extrusion is one of the ancient and usual method to produce hydrocolloid capsules using a simple and low cost procedure that makes minimal injuries to probiotic cells while maintaining comparatively higher viability [11]. The hydrocolloid solution is mixed with microorganisms and mixture is delivered through an extruder (syringe) in a droplet form into hardening solution (calcium chloride) that consists of multivalent cations. After dripping, the cells are immediately entrapped by the polymers leading to three-dimensional lattices that cross links with calcium ions [8]. Mostly, alginate and calcium chloride concentration ranges from 0.5% to 4% and 0.05–1.5 M, respectively. The beads

size ranges from 2 to 3 mm in diameter which mostly depended upon the distance between the syringe and hardening solution, polymer type, viscosity, concentration, extrusion voltage, extrusion flow rate, and diameter of the extruder [12]. The aim of this study was to determine the effect of extrusion voltage and sodium alginate flow rate on the viability of *Lactobacillus casei* (*L. casei*) in the encapsulation process.

2. EXPERIMENTAL SECTION

Equipments

Ose needles, measuring flask (Pyrex), petri dishes (Normax), beaker glass (Pyrex), Erlenmeyer, funnels, test tubes, spatulas, volume pipettes, micropipettes (Effendorf), stirring rods, pH meter (Horiba, Japan), filter paper Whatman no. 40, watch glass, autoclaves (Tomy SX- 300), shaker incubator (CERTOMAT® BS-1), incubator (Memmert 854 Schwabach), vortex (Thermo), refrigerators, aluminum foil, plastic wrap, analytical balance, glass preparations, microscopes, hot plates, magnetic stirrers, Scanning Electron Microscopy (SEM) (JEOL JSM-IT300), Laminar Air Flow (ETL), syringe (Therumo) 22 G (inner diameter 0.394 mm), syringe 60 mL.

Materials

The chemicals used were chitosan 0.2% (Sigma Aldrich PCode: 101729402), MRSA (de Man Rogosa Sharpe Agar) and MRSB (de Man Rogosa Sharpe Broth) (Merck, KGaA), Na-alginate 1% (HIMEDIA® REF MB-114-100G), NaCl 0.2% pH 1.5, CaCl₂ Solution 32 g / L, Na-Citrate 1%.

Procedure

Preparation of Sodium Alginate Solution Containing Bacterial Suspension

Preparation of sodium alginate solution was made from an alginate solution 40 mL with a concentration of 1% (w/v) with distilled water. At that point sterilized via autoclaving at 121°C for 15 min. After the water has been cooled to room temperature, then 10 mL *L. casei* suspension was included.

Preparation of Chitosan Solution

The chitosan solution was made with a concentration of chitosan 0.2% (w/v). Chitosan was dissolved in a 1% (v/v) acetic acid solution; then, the solution was sterilized by autoclaving at 121 °C for 15 min.

Surface morphology and bead size determination

The shape and surface characteristics were determined by scanning electron microscopy (SEM) using a gold sputter technique. The microcapsules were vacuum-dried, coated with gold-palladium, and observed microscopically. The size of the *L. casei* loaded microcapsules was measured with SEM.

Survival of *L. casei* loaded in microcapsules

To determine the viable counts of the encapsulated *L. casei*, 1 g of capsules were resuspended in 9 mL of sodium citrate (1 %) and stirred for 120 min using a magnetic stirrer. The complete release of bacteria from the microcapsules in 120 min was previously assured by comparing the released number of cells from the capsules. The colony-forming units (CFU/g) were determined by anaerobic plating on MRS agar plate and incubating at 37 °C for 48 h. The plating procedures were carried out in triplicates. Nonencapsulated *L. casei* was enumerated in the MRS agar as control.

Encapsulation of *L. casei* Using Sodium Alginate-Chitosan Mixture

The *L. casei* probiotic encapsulation method chosen was the extrusion method and the ionic gelation method. The extrusion method was chosen because besides using a simple tool in the form of a syringe, this method was also chosen to avoid extreme temperatures and pressures and from unfavorable environments such as low temperatures in the freeze-drying method, which can result in reduced bacterial viability [13]. While the choice of ionic gelation method was due to a simple process, did not use organic solvents, and can be controlled easily. While the principle of particle formation in the ionic gelation method was the occurrence of ionic interactions between the divalent cation (Ca²⁺) and the carboxylic anion (COO⁻) of the alginate monomer. Cross-linking occurs because a calcium ion replaced two sodium ions in alginate. This crosslinking structure caused limited molecular motion and inhibited the development of polymers in a medium [14]. The extrusion technique of microencapsulation was used [15]. Alginate microcapsules were prepared as follows: sodium alginate was dissolved in distilled water (1%, w/v) and sterilized at 121°C for 15 min. After cooling the alginate mixture, the cell suspension (10 mL) was mixed with the alginate mixture (40 mL) homogeneously and injected through a syringe into sterilized 125 mL CaCl₂ (32 g/L) solution that was stirred continuously to form capsules. The capsules were allowed to harden for about 30 min in the CaCl₂ solution and then washed with the distilled water. The beads were dried under controlled air flow and temperature (4°C). Alginate–chitosan microcapsules were prepared as follows: the wet state of alginate coated beads was immersed in 50 mL of chitosan solution (0,2%, w/v) and stirred for 15 min using a magnetic stirrer. The beads were dried under controlled air flow and temperature (4°C) [16].

Viability and Survivability Testing of *L. casei* probiotic

Simulation gastric fluid (SGF) consisted of 0.2% Sodium chloride with a pH of 1.5 (adjusting the pH by adding 0.5 M hydrochloric acid). 1 g capsule (extrusion flow rate of 1, 3, and 5 mL/min with extruder voltage of 0, 10, and 20 kiloVolt (kV)) were immersed in 9 mL SGF, incubated for 1, 60, and 120 min at a temperature of 37° C. After that, it was filtered using Wattman 40 filter paper then immersed in 9 mL (1 g/100 mL) sterile sodium citrate solution with slow stirring at room temperature. Then sequential dilution was prepared to reach the number of cells that can be calculated by pouring a suspension technique that was spread on MRS Agar media. After that, it was incubated for 48 hours at 37 °C [17]. The total colony can be calculated by the Total Plate Count (TPC) method.

3. RESULTS AND DISCUSSION

Encapsulation probiotic *L. casei*

The result of making encapsulation with sodium alginate: chitosan (1%: 0.2%) with a flow rate of 5 mL/min and voltages of 0, 10, and 20 kV showed in figure 1.a.

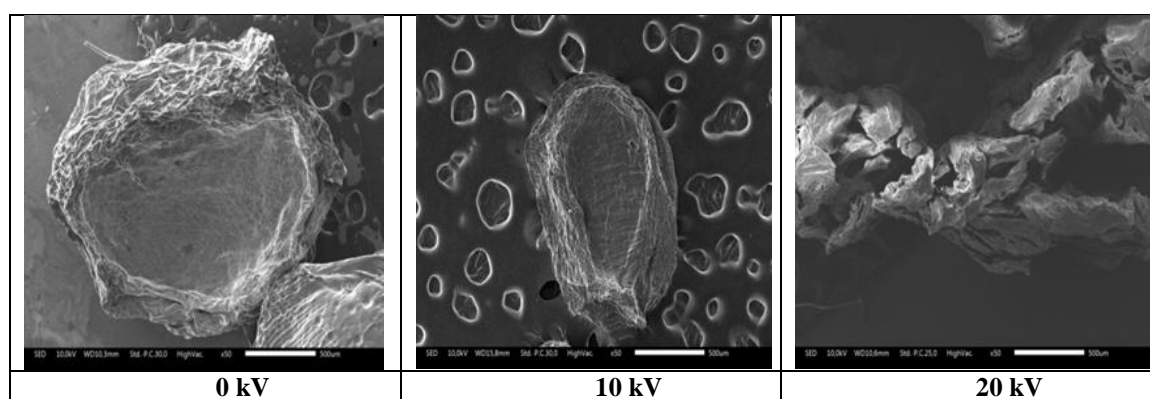


Figure 1. a. Encapsulated beads on various voltage variations.

The extruded electric voltage of 0 kV produces a capsule size of 1000 μm - 1,100 μm. The extruded 10 kV voltage produces a capsule size of 500 μm - 1000 μm. Extruded 20 kV voltage produces capsules of 100 μm - 200 μm, and the capsules tend to be damaged. Figure 1.a showed that the higher extrusion voltage resulted in the smaller size of the beads. This happened because of high voltage can create a high electrostatic force so that the liquid in the extruder was pulled to the bottom faster and even bursts. The result of making encapsulation with sodium alginate: chitosan (1%: 0.2%) with a voltage of 0 kV and flow rates of 1, 3, and 5 mL/min showed in figure 1.b.

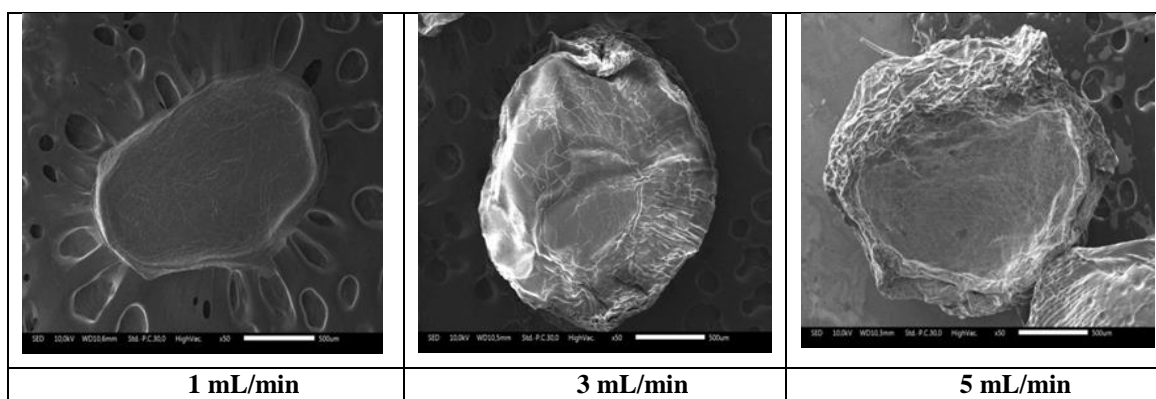


Figure 1. b. Encapsulated beads on various flow rate variations.

Figure 1.b showed that the higher flow rate resulted in the bigger size of the beads. The extrusion flow rate of 1 mL/min produces a capsule size of 600 μm - 800 μm. The extrusion flow rate of 3 mL/min produces a capsule size of 750 μm - 1000 μm. The extrusion flow rate of 5 mL/min produces a capsule size of 1,100 μm - 1,200 μm. A higher extrusion rate resulted in a higher amount of the encapsulating material forming a larger bead size than at a lower extrusion rate.

Test of viability and yield of probiotic *L. casei* encapsulation process in sodium citrate solution

The newly encapsulated particles (1 g), soaked in 9 mL (1 g/100 mL) sterile sodium citrate solution with slow stirring at room temperature. Then sequential dilution was prepared to reach the number of cells that can be calculated by pouring plates in MRS Agar.

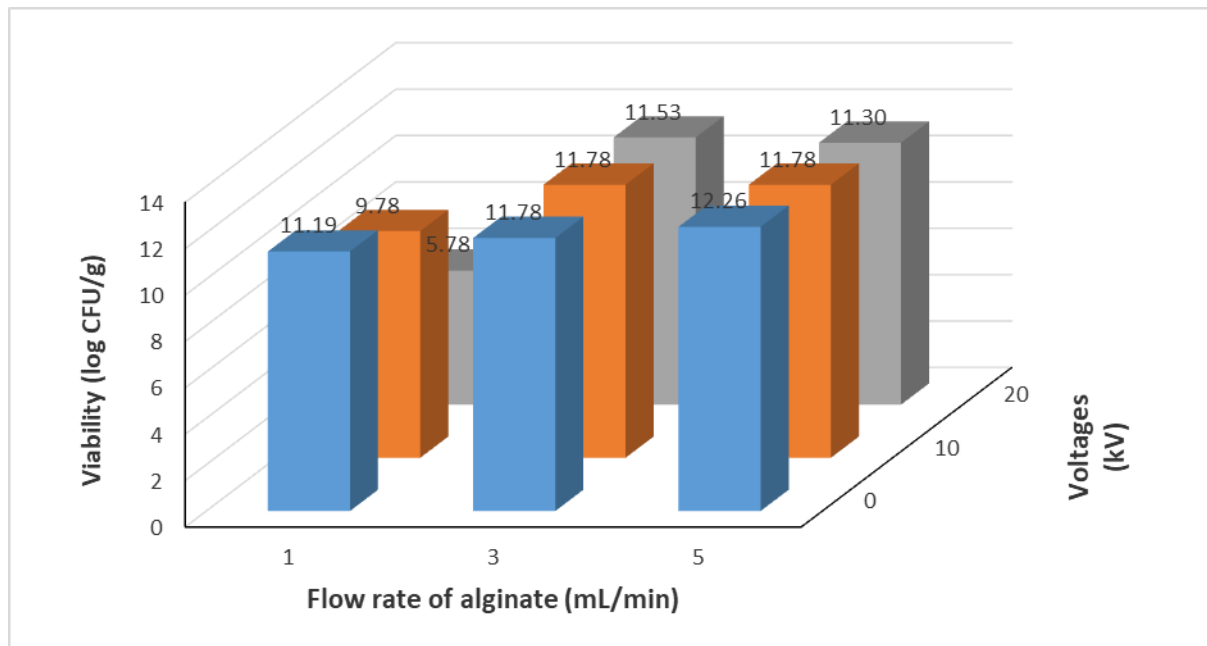


Figure 2. Viability of *L. casei* on various voltage variations and flow rate of extrusion.

The number of *L. casei* cells before encapsulation was 12.3 log CFU (2×10^{12} CFU). After encapsulation, the maximum viability of *L. casei* was 12.26 log CFU (1.8×10^{12} CFU) (Figure 2) was obtained for the variation of 5 mL/min flow rate and the 0 kV extrusion voltages. The higher voltage can decrease probiotics viability while the greater flow rate of alginate can protect probiotics viability. While the level of yield was shown in Figure 3 below.

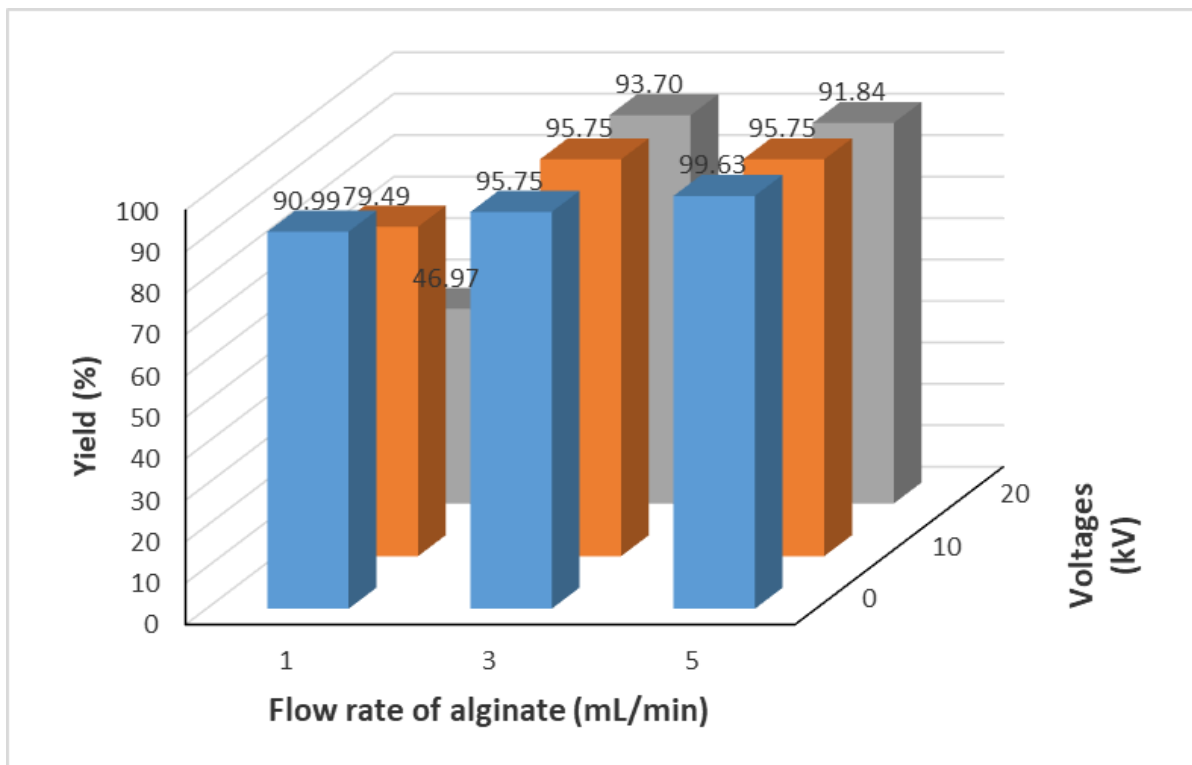


Figure 3. *L. casei* yield on various voltage variations and flow rate of extrusion.

Figure 3 above showed that the highest *L. casei* yield of 99.63 % was obtained for the variation of 5 mL/min flow rate and the 0 kV extrusion voltage. While on the high voltage, the yield of *L. casei* decreased. This result showed that *L. casei* could not withstand high voltages.

Viability and survivability test of *L. casei* in simulation gastric fluid (not encapsulated cells)

The results of the *L. casei* probiotic Total Plate Count (TPC) test in broth liquid had a viability of 2×10^{11} CFU/mL. *L. casei* preparations, which had a viability of 2×10^{11} CFU/mL, were immersed in a simulated gastric fluid solution containing 0.2% NaCl solution pH 1.5 at various variations of immersion time, i.e., 1, 60, and 120 min. The result of the calculation of the Total Plate Count (TPC) of the probiotic *L. casei* showed the viability of 0 CFU/mL for 1 min. This result meant that *L. casei*, which was not encapsulated, will experience direct death once immersed in simulation gastric fluid pH 1.5, or in other words, the survivability was 0%.

Test the viability and survivability of *L. casei* in simulation gastric fluid (encapsulated cells)

A 0.5 mm-sized bead shape produced by *L. casei* probiotic, which was encapsulated with the alginate-chitosan matrix through an extrusion method. Then the beads are immersed in a simulation gastric fluid containing 0.2% NaCl solution with a pH of 1.5 and varying incubation time from 1 min, 60 min and 120 min. When the beads were immersed in the liquid, they shrink in size. After the beads were immersed, they were removed and dried. Then the beads were immersed in sodium citrate pH 8. As a result of immersion in the sodium citrate solution, the beads swell, so the probiotics trapped in the beads can come out. Then the probiotic viability was tested using the TPC method. The test results were shown in Figure 4, 5, and 6 below.

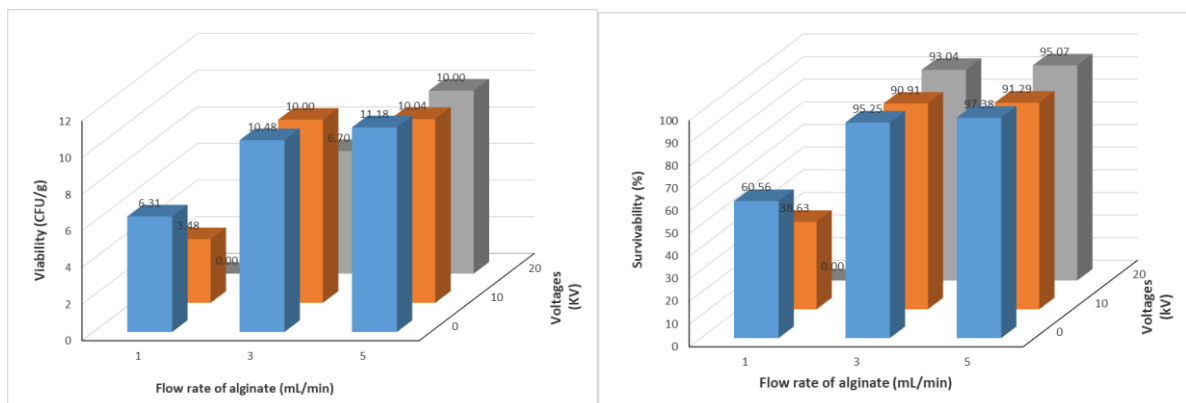


Figure 4. Viability (left) and survivability (right) of encapsulated *L. casei* in SGF immersion pH 1,5 for 1 min.

The viability of *L. casei* from beads immersed in SGF pH 1.5 for 1 min did not show a significant decrease except for the encapsulated beads with an extrusion flow rate of 1 mL/min. This happened because the beads formed by the encapsulation process with a flow rate of 1 mL/min produced small beads so that SGF easily killed *L. casei* in the beads (as shown in Figure 1.b.). Figure 4 above showed that the highest *L. casei* viability of 11.8 log CFU/g (1.5×10^{11} CFU/g) (survivability of 97.38 %) was obtained for the variation of the 0 kV voltage and 5 ml/minute flow rate. Whereas at the voltage of 20 kV and 1 mL/min flow rate, the viability value was 0 CFU/g (survivability of 0%).

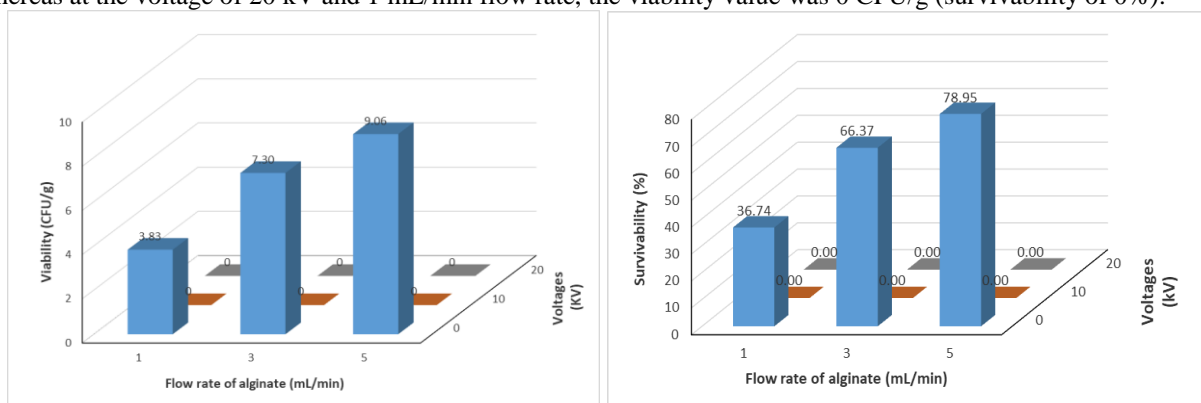


Figure 5. Viability (left) and survivability (right) of encapsulated *L. casei* in SGF immersion pH 1,5 for 60 min.

Figure 5 above showed that the highest *L. casei* viability of 9.06 log CFU/g (1.15×10^9 CFU/g) (survivability of 78.95 %) was obtained for the variation of the 0 kV voltage and 5 mL/min flow rate. Whereas at the voltages of 10, and 20 kV

and 1, 3, and 5 mL/min flow rates, the viability value was 0 CFU/g (survivability of 0%). This happened because the beads formed from the extrusion process with a voltage of 10, and 20 kV tended to be small and damaged (as shown in Figure 1.a.) so that SGF easily made contact with *L. casei* in these beads that they were killed so easily.

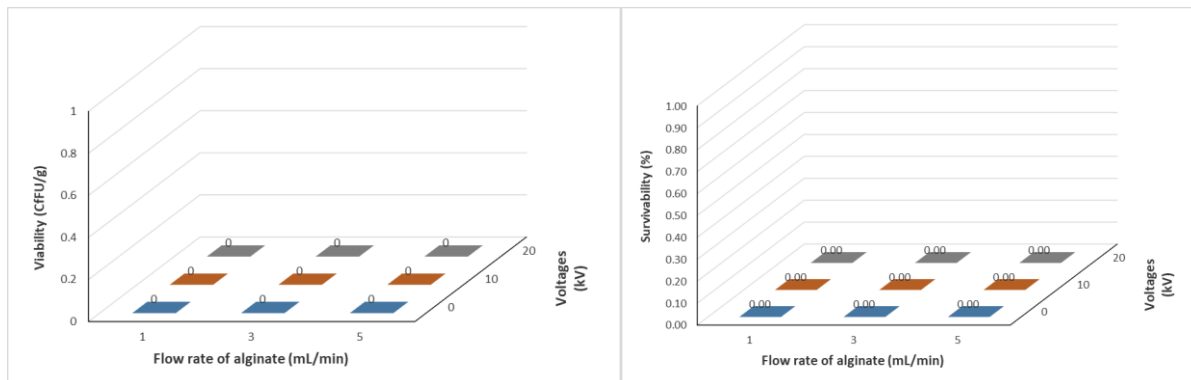


Figure 6. Viability (left) and survivability (right) of encapsulated *L. casei* in SGF immersion pH 1,5 for 120 min.

Figure 6 above showed that the viability of *L. casei* was 0 CFU/g (survivability of 0%) for all experiment variations when beads were immersed for 120 min in simulation gastric fluid pH 1.5. This happened because for 120 min the chitosan that filled the pores dissolved completely in SGF so that SGF can enter the alginate pores which resulted in SGF came into contact with *L. casei* and killed it [18-20].

4. CONCLUSION

The number of *L. casei* cells before encapsulation was 12.3 log CFU. After encapsulation, the maximum viability of *L. casei* obtained by voltage variations of 0 kV and flow rate 5 mL/min were 12.26 log CFU. Encapsulated *L. casei* immersed in SGF pH 1.5 for 1 minute resulted in the highest *L. casei* viability of 11.8 log CFU/g (survivability of 97.38 %) obtained for 0 kV voltage and 5 mL/min flow rate. Whereas at the voltage of 20 kV and extrusion flow rate of 1 mL/min, the viability value was 0 CFU/g (survivability of 0%). Encapsulated *L. casei* immersed in SGF pH 1.5 for 60 min resulted in the highest *L. casei* viability of 9.06 log CFU/g (survivability of 78.95 %) obtained for 0 kV voltage and extrusion flow rate of 5 mL/min. Whereas at the voltages of 10 and 20 kV and extrusion flow rates of 1, 3, and 5 mL/min, the viability value was 0 CFU/g (survivability of 0%). On the encapsulation process, the higher extrusion voltage can kill more *L. casei* while the higher extrusion flow rate can protect more *L. casei*.

5. REFERENCES

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