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RESISTANCE OF *Lactobacillus sp.*ISOLATED FROM BOVINE COLOSTRUM THAT ENCAPSULATED ON VARIOUS FORMULA TOWARDS ACIDIC CONDITIONS AND BILE SALTS AS PROBIOTIC CANDIDATE

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Abstract

Research about characterization of probiotic Lactobacillus sp. origin from bovine colostrum in some types of formula encapsulation has been done. The research method was carried out experimentally using a factorial Completly Randomized Design with two factor: a x b, the value of a and b depend on the level of each treatment, which a was bacteria that encapsulated in some types of formula and b was characterization of probiotic test, with three times repetition. The data were analyzed using analysis of variance with a level of 95%, followed by Duncan's Multiple Range Test. The results showed that L. paracasei in alginate tapioca and L. curvatus in alginate skim had high viability during storage of 4 weeks with each of the bacterial population were 2,98 x 1010 CFU/ml and 2,25 x 1010 CFU/ml. Bacteria L. curvatus encapsulated in alginate skim had the highest resistant to the acidic environment of pH 2 and 4 with each of the bacterial population were 1,02 x 103 CFU/ml and 3,69 x 105 CFU/ml and resistant to bile salt concentration of 0.3% and 0 , 5% with each of the bacterial population were 3,5 x 1010 CFU/ml and 2,4 x 1010 CFU/ml. The supernatant of L. curvatus in alginate skim also had the highest antimicrobial activity against pathogenic bacteria Escherichia coli and Salmonella typhimurium with each inhibitory diameter was 20.83 mm and 12.67 mm. Encapsulation formula, especially the combination of alginate and skim milk, can protect the probiotic during processed, storage, and applied in digestive tract.

Key words: *encapsulation, Lactobacillus, probiotik iv.*

INTRODUCTION

Bovine colostrum is pre-milk fluid produced by cows in the first 24-48 hours after birth and several types of microorganisms that have been widely used as probiotics found on it (Brandano, et al., 2004; Lindner, et al., 2011). Probiotics are living microorganisms that are beneficial to humans, especially in maintaining health and preventing disease. They can live in digestive tract to control the balance of gut microbial and has characteristics such as non-pathogenic, resistant to stomach acid conditions, resistant to bile salt concentration in the intestine, producing organic acids, and also has antimicrobial properties against pathogens digestion (Salminen and von Wright, 2004). One of the probiotic bacteria found in bovine colostrum is *Lactobacillus sp.* (Anal and Singh, 2007). The bacteria have the ability to attach to host cells, remove or reduce pathogenic bacteria, produce acid, hydrogen peroxide and bacteriocins which able to inhibit the growth of pathogenic bacteria (Rizqihati, et al., 2009).

Due to environmental factors that are less conducive for probiotic bacteria to survive, viability of probiotic bacteria in product should reach 10^7-10^9 CFU/ g. Low pH of 1.5 to 2.0 for an empty stomach or pH of 4.5 to 5.0 for filled stomach and presence of bile salts in the small intestine which must pass by the bacteria would decrease the viability while in the digestive tract or even during storage. Bile salt concentration equivalent to physiological concentrations of bile salts in the duodenum is 0.5% (Puspawati, 2010). While all microbial that could live in 0.3% bile salt was resistant to bile salts in the small intestine (Wijayanto, 2009).

Encapsulation is a process of core material coating, in this research the probiotic bacteria. Specific encapsulation material used to maintain viability and protect probiotics bacteria from damage due to the environmental conditions (Rizqihati et al., 2009). The encapsulation

material that often used is alginate. Alginate used to protect or wrap core material from unfavorable environmental factors (Desmond, et al., 2002). The advantages of alginate use is easy to form gel matrix overlying bacteria, safe to consumed, easy to obtain, and can release the trapped cells. However, alginate also has the disadvantage that vulnerable in an acidic environment. Therefore, in order to optimize the alginate encapsulation process, combination of alginate with a variety of other polymer compounds needed. The addition of starch, skim milk, gelatin, and chitosan combined with alginate can provide better resistance on encapsulation (Chavarri, et al., 2013).

In this study, resilience of probiotic bacteria *Lactobacillus sp.*, which encapsulated with various encapsulation formula tested to acidic conditions and high bile salts to determine the best encapsulation material formula that can be select for encapsulation process on probiotic bacteria.

MATERIALS AND METHODS

The tools used in this study are centrifuge Hermle Z 300, syringe, filter, and vortex. Materials used are alginate acid (sodium salt) from Sigma, acetic acid, *Lactobacillus paracasei* and *Lactobacillus curvatus* isolated from cow colostrum, bile salt (Oxoid), phosphate buffer, 0.1 M $CaCl_2$, Nutrient Agar (NA), Nutrient Broth (NB), skim milk and tapioca.

Biomass Production (Puspawati, 2010)

Lactobacillus sp. biomass production obtained by sub-culture the isolate on NB for 24 h at 37°C. Sterilized NB inoculated by the 10% culture and then incubated at 37°C for 18-20 hours. Culture were centrifuged at a speed of 5000rpm for 10 minutes. Supernatant separated with the filtrate to obtained biomass.

Encapsulation with Alginate (Purwandhani, et al., 2007)

Bacterial biomass suspended in physiological NaCl at MacFarland turbidity three then counted by Total Plate Count (TPC) to determine the number of bacteria before encapsulated. Three ml cultured *Lactobacillus paracasei* and *Lactobacillus curvatus* re-suspended in 10 ml of sterilized NaCl then 60 ml of Sodium Alginate 3% (w/v) were added. After mixing dropped into 200 ml of $CaCl_2$ 0.1 while stirring with a magnetic stirrer then washed with sterilized 0.8% NaCl and dried.

Encapsulation with Skim Alginate (Purwandhani, et al., 2007)

Isolates of *Lactobacillus paracasei* and *Lactobacillus curvatus* subcultured in 2.5 ml NB incubated for 9 hours, then re-subcultured in consortium twice at 25 ml and 250 ml of skim milk and incubated for 9 hours. The inoculum of bacteria in skim milk centrifuged at 5000 rpm for 10 min at 4°C. Biomass obtained suspended in 100 ml distilled water then mixed with 100 ml of 3% alginate as carrier material. The mixture then put into a syringe and dropped into $CaCl_2.2H_2O$ 0.1 mol/l with 10 cm distance from syringe tip and the surface of CaCl2.$2H_2O$. Hold for an hour so that the grains of the encapsulation yields harden while kept on a shaker. The encapsulation pellets yields rinsed with sterilized 0.8% NaCl and sterilized distilled water, then dried.

Encapsulation with Tapioca Alginate (Wijayanti, 2010)

Sterilized sodium alginate as much as 1% (w / v) dissolved into 90 ml of distilled water followed by addition of 3% (w / v) tapioca powder. Ten percent biomass consortium aseptically inoculated into 90 ml of coating material formula. The mixture of biomass consortium and coating material dropped into 0.1 M $CaCl_2.2H_2O$ and then washed twice using sterilized distilled water.

Ca-alginate capsule that washed inoculated into NB and incubated for 24-30 h in a rotary shaker with speed 150 rpm at room temperature for secondary multiplication. Washed twice using sterilized distilled water and then dried.

Acid Tolerant Test (Lian, et al., 2003)

One gram encapsulated isolates of *Lactobacillus paracasei* and *Lactobacillus curvatus* grown at 9 ml NB with pH 2, pH 4, pH 6 and control. Vortexed and incubated at 37°C for 6 hours then centrifuge at 5000× g for 10 min at 4°C and washed twice with phosphate buffer. Pellet diluted in 10 ml of sterilized distilled water then poured into NA and incubated at 37°C for 48 hours then the number of colonies counted.

Bile Salt Tolerant Test (Lian, et al., 2003)

One gram encapsulated isolates inoculated into 9 ml NB that bile salt added (0.3% and 0.5%) and

control. Vortexed and incubated at 37 ° C for 6 hours, centrifuged at 5000 × g for 10 min at 4°C then washed twice with phosphate buffer. Pellet diluted in 10 ml of sterilized distilled water then poured into NA and incubated at 37°C for 48 hours then the number of colonies counted.

Data Analysis

Data analyzed using analysis of variance with the level of 95% and treatment effect will analyzed by Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSIONS

Probiotic candidate isolated from bovine colostrum

Two species of bacteria of *L. paracasei* and *L. curvatus* isolated from bovine colostrum on preliminary research. Both have proved as type of probiotic bacteria naturally found in the human digestive tract because their ability to survive in acidic stomach conditions and high concentration of bile in the intestine (Anal and Singh, 2007). Isolates of *L. paracasei* and *L. curvatus* encapsulated to maintain their probiotic characteristics while in the digestive tract.

Probiotic encapsulation

Encapsulation of probiotics is the process of wrapping probiotic bacterial cells using protective materials. Protective material used in this study is alginate, skim, and tapioca. Alginate used as a protective material because the ability to wrap core material, in order to protect from unfavorable factors (Desmond, et al., 2002). The use of alginate combined with another polymer compound such skim milk and tapioca. Skim milk was a good source of nutrients for microbes, especially sugars and proteins (Triana, et al., 2006). Tapioca also a good source of nutrients for microbes due to high carbohydrate content (Vidhyalakshmi, et al., 2009). The formulations used as encapsulation material in this study is alginate, alginate skim, and alginate tapioca that will form gel encapsulation containing bacterial biomass (Figure 1).

Gambar 1. The encapsulan gels of *Lactobacillus curvatus* and *Lactobacillus paracasei* in alginate formula, skim alginate and tapioca alginate

Before encapsulation process *L. paracasei* biomass as much as 1.55 x 10^{10} CFU / ml and *L. curvatus* as much as 1.21 x 10^{10} CFU / ml. Bacterial biomass produced mixed into the formula protective material. Mixture dropped into $CaCl_2$ solution to form gel encapsulation. Gel encapsulation formed by the reaction of sodium alginate with calcium chloride ($CaCl_2$) which causing gelatinization of calcium alginate gel-matrix occured. Encapsulan tested for acidic conditions and various bile salt levels resistance.

Encapsulated Probiotic Tolerance towards Acidic Environment

Test done to determine the encapsulated probiotic tolerance towards environment pH of 2, 4, and 6. Based on the analysis of variance, it known that there was an interaction between pH and the probiotic bacteria inside the encapsulation formula. Test continued with DMRT at 5% significance level and the results shown at Table 1.

Table 1. DMRT at 5% of encapsulated probiotic tolerance towards acidic environment
Description: The mean treatment followed by the same capital letter (vertical direction) and lowercase letters the same

pH	Bacteria (CFU/ml)					
	e_1	e_2	e_3	e_4	e_5	e_6
p_1	5,05 x 10^2 C b	4,33 x 10^2 c C B	6,12 x 10^2 c C ab	1,81 x 10^2 c C c	1,02 x 10^2 c a	1,08 x 10^2 c d
p_2	7,53 x 10^4 B C	2,84 x 10^5 b B ab	1,86 x 10^5 b b	2,17 x 10^5 b ab	3,69 x 10^5 b a	2,08 x 10^5 b b
p_3	4,03 x 10^8 A A	3,93 x 10^8 a A A	4,57 x 10^8 a a	3,26 x 10^8 a A	3,71 x 10^8 a a	3,86 x 10^8 a A a

(horizontal direction) are not significantly different according to DMRT 0.05

Table 1 showed that higher pH value gave higher number of probiotics colonies bacteria in the encapsulation material. Probiotic bacteria *L. paracasei* and *L. curvatus* that already encapsulated by the formula were optimally grew at pH 6. It is because *Lactobacillus* bacteria are optimally grew at the pH 5.5 to 6.2. Some can survive at low pH of 3.2, high pH of 9.6 and several of them only grows in a narrow pH range (4.0 to 4.5) (Wijayanto, et al., 2009).

Probiotic bacteria *L. curvatus* in skim alginate formula has high resistance towards acidic environment which shown by lower number of decreased bacterial colonies than another encapsulation formula, such as 5.556 cycles log CFU / ml. *Lactobacillus curvatus* in tapioca alginate formulas have highest colonies decreased among other encapsulated formula, namely 6.605 cycles log CFU / ml. The number of bacteria colonies declined because of low pH values (pH 2 and 4) which destruct the bacterial cell. Denaturing effect of enzymes that exist on the surface of cells, damage lipoposaccharide, outer membrane and cytoplasmic pH through increased membrane permeability causing bacteria cell destruction (Puspawati, 2010). Skim addition as protective material able to increased *L. curvatus* tolerance towards acidic environments than other materials. Skim milk composed by variety of complex materials such as lactose, casein, citrate and phosphate which able to act as buffer so that could protect the bacteria from acid and bile salts exposure (Puspawati, 2010).

Decreased of total bacteria was occured after 5 hours incubation in acidic media (Puspawati, 2010). Addition of skim milk, lactose and maltodextrin as protective material could increased tolerance of *L.brevis* A17 towards low pH (each declined of 5.37 log CFU/ml; 4.78 log CFU/ml; 5.13 log CFU/ml). While addition of all kinds of protective material on *L. rhamnosus* R21 could increased the tolerance towards low pH with the amount of decreased of 3.95 log CFU/ml in sucrose; 3.21 log CFU/ml in skim milk; 5.09 log CFU/ml on lactose; and 1.38 log CFU/ml in maltodextrin (Puspawati, 2010).

Encapsulated Probiotic Tolerance towards Various Bile Salt Concentrations

Test conducted to determine the probiotic bacteria tolerance in an environment that has a high concentration of bile salts (0.3% and 0.5%). Analysis of variance results showed that there were no interaction between concentration of bile salts towards encapsulated probiotic bacteria *L. paracasei* and *L. curvatus*. Various concentration of bile salts did not gave significant effect towards probiotic bacteria colonies number, so that DMRT was not carried out.

The results showed that the encapsulation formula has a high resistance to 0.3% and 0.5% bile salts after 6 hours incubation. Probiotic bacteria colonies in the encapsulation material shown approximately 10^{10} CFU / ml. *Lacto-bacillus sp.*could tolerates high concentrations of bile salts because the bacteria own bile salt hydrolase (BSH) enzyme. The enzyme was able to change physico-chemical abilities possessed bile salts so that not harmed the lactic acid bacteria (Puspawati, 2010). Bacteria tolerance towards bile salts thought to be caused by the role of polysaccharides as constituent of the cell wall

of gram-positive bacteria (Surono, 2000). In addition, the presence of lipid components in the membrane of gram-positive bacteria were also an important part to maintained the structure of membrane because fatty acids has role in lowered cell leakage caused by bile salts (Kimoto, et al., 2002).

Based on the results of encapsulated probiotic bacteria tolerance towards bile salt concentration of 0.3% and 0.5% (Table 2), *L. curvatus* in skim alginate formula has the highest average colony. Although the results did not have significantly different with other encapsulation materials, but it can concluded that the combination of skim and alginate was good formula in protecting bacteria against high concentration of bile salts. Skim milk contains complex nutrients that can act as buffer that protects the bacteria from high bile salts environmental conditions (Puspawati, 2010).

Table 2. Average colony of encapsulated *Lactobacillus paracasei* and *Lactobacillus curvatus* towards various bile salt concentration

Probiotic	Formula	Bile Salt	CFU/ml	Average
L. paracasei	Alginate	0.3%	3.50×10^{10}	2.97×10^{10}
			1.99×10^{10}	
			3.42×10^{10}	
		0.5%	3.74×10^{10}	3.10×10^{10}
			3.59×10^{10}	
			1.98×10^{10}	
	Skim-Alginate	0.3%	2.75×10^{10}	3.11×10^{10}
			3.30×10^{10}	
			3.16×10^{10}	
		0.5%	2.90×10^{10}	3.99×10^{10}
			3.18×10^{10}	
			3.35×10^{10}	
	Tapioca-Alginate	0.3%	4.36×10^{10}	3.11×10^{10}
			3.90×10^{10}	
			1.07×10^{10}	
		0.5%	3.60×10^{10}	3.14×10^{10}
			4.40×10^{10}	
			3.97×10^{10}	
L. curvatus	Alginate	0.3%	1.40×10^{10}	2.73×10^{10}
			3.89×10^{10}	
			2.90×10^{10}	
		0.5%	3.78×10^{10}	3.10×10^{10}
			3.30×10^{10}	
			3.84×10^{10}	
	Skim-Alginate	0.3%	2.55×10^{10}	4.42×10^{10}
			5.14×10^{10}	
			5.57×10^{10}	
		0.5%	4.68×10^{10}	4.43×10^{10}
			5.21×10^{10}	
			3.40×10^{10}	
	Tapioca-Alginate	0.3%	4.04×10^{10}	3.80×10^{10}
			3.57×10^{10}	
			3.80×10^{10}	
		0.5%	4.18×10^{10}	3.12×10^{10}
			0.85×10^{10}	
			4.34×10^{10}	

CONCLUSIONS AND SUGGESTIONS

Conclusions

Lactobacillus paracasei and *Lactobacillus curvatus* colostrum origin that encapsulated in various formulas could tolerates acidic conditions and high levels of bile salts. Skim-alginate encapsulation formula was the best coating material that could maintained viability of bacteria towards acidic conditions and high levels of bile salts

Suggestions

To produce encapsulan gels that have high tolerance towards low pH and bile salts advisable to combine alginate with other polymer compounds such as skim, tapioca and others. The presence of second coating will protect bacteria better from unfavorable environmental conditions.

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