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The Effect of Different Freeze Dried Formulations on The Viability of Lactic Acid Bacteria In Lactose-Free Yogurt Production

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ABSTRACT

Lactose-free yogurt was produced by fermentation of coconut cake, by-product of coconut processing with mixtures of lactic acid bacteria**.** The purpose of this study was to determine the effect of different carrier in freeze dried formulations on the viability of lactic acid bacteria in fermented coconut cake for the production of lactose-free yogurt. A total of 200 kg yogurt was produced in 250 L bioreactor with a heater system for in-situ pasteurization and agitation that was set to 150 rpm. A mixture of *Streptococcus thermopilus, Lactobacillus plantarum and Lactobacillus bulgaricus* was inoculated to the pasteurized coconut solution to initiate fermentation. Fermentation data was analysed for CFU/ml, pH and titratable lactic acid concentration for every 4 hours sampling. After incubation of 48 hours, the yogurt was harvested and product was formulated with solid carriers for encapsulation during freeze drying process. Yogurt solution was mixed with carriers which were 7% w/w sucrose, 10% w/w maltodextrin and 0.24%w/w guar gum. The carriers were tested for its individual effect, combination of two and three carriers on the cultures' viability after freeze drying. The colony forming unit, cfu/ml of the product was analysed before and after freeze drying. Initially, the cultured solution contained 5.25 – 6.70 x 10^8 cfu/ml. The most recovered cfu/ml was analysed from the combination of sucrose and guar gum with 1.03 x 10^8, however the highest % of viability after rehydration was analysed from combination of the three carriers which was 17.42%, as the lowest was seen from the combination of maltodextrin and guar gum only, without the sucrose which were 2.41 and 3.56%, respectively. The moisture content of all products formulation was in range of 1.43 to 2.00% indicated that moisture was almost completely removed from the product during drying process**.** This work shows that freeze drying with sucrose, maltodextrin and guar gum as encapsulants enhance the viability of the freeze dried lactose-free yogurt.

INTRODUCTION

The popularity of probitic food products has becoming a rapid growing interest to consumers lately. Probiotic products with live microbial strain earn its attention due to its health-promoting benefits that highly publicity by the manufacturers. Almost all of the probiotic products offer a diary base alternative and least option for the lactose-intolerance niche. The demand in probiotic consumption is proportionally increased with the expanding trend of vegetarianism and diet in health and wellness promoting foods (Martins *et al*., 2013).

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Hence, the increase of number of vegetarians emphasizes the significance of developing the non-diary probiotic options (He and Hekmat, 2015). The lactose-free yogurt was developed from coconut cake, the by-product of virgin coconut oil processing. The development of producing value added product is envisaged to provide a non-diary yogurt alternative with health beneficial functionalities.

The maintenance of the viability of the probiotic microbes and probiotic characteristics in commercial starter cultures and during the products storage are important to preserve its health benefits (Rathnayaka *et al*., 2013). Dehydration process has been adopted to increase the shelf life of probiotic products which is freeze drying. Freeze drying is an alternative method for longer preservation of bioactive materials which involve dehydration process that lead to little shrinkage and produce easily soluble products (Chen *et al*., 2006). Also known as lyophilization, this method is usually used to preserve starter cultures of lactic acid bacteria in diary and food fermentations (Lodato *et al*., 1999). However, as reported by Abadias *et al*., (2001) not all of the strain could survive during freeze drying and the quantitative viability rates was as low as 0.1%. As been mentioned by Conrad *et al*., (2000) the major cause of the loss of cell viability in freeze drying are probably ice crystal formation, high osmolarity due to high concentration of internal solutes with membrane damage, macromolecule denaturation and the removed water which affect the properties of many hydrophilic macromolecules in cells.

During freeze drying and storage, addition of protective agents may enhance the stability of probiotic microorganisms (Zayed & Roos, 2004). To reduce the loss in viability of probiotic cultures due to freeze drying, cryoprotectants such as skim milk are commonly used (Carvalho *et al*., 2002). In a way to protect the viability of probiotics during dehydration, a variety of protectants such as skim milk, whey protein, glucose, maltodextrin and trehalose have been added to the drying media before freeze drying (Martín *et al*., 2015). Rathnayaka *et al*., (2013) used UHT milk base and combine with sucrose, sorbitol and trehalose to study the effect of the additives on viability and probiotic properties of a probiotic microbial mixture containing Lactobacillus *rhamnosus* and *L. plantarum*. Zayed and Roos (2004) studied the influence of trehalose and moisture content on the survival of *L. salivarius* and found that trehalose and sucrose with skim milk were the most effective combination as freeze drying additive. Addition of sucrose or galactose as lyoprotectants significantly improved the viabilities of lactic acid bacteria and yeast for freeze-dried kefir (Chen *et al*., 2006). Chen and Mustapha (2012) used sucrose and trehalose as cryoprotectants for microencapsulation of α-galactosidase producing probiotics, *Lactobacillus acidophilus* LA2 incorporated into the soy bar matrix prior to freeze drying. Further, other than disaccharides (e.g sucrose and trehalose), polymers (e.g gelatin, maltodextrin and xanthan gum) has been used on freeze drying of *L.casei* ssp. *rhamnosus, Bifidobacterium longum, Lactococcus lactis* ssp. *lactis* and *Streptococcus thermophilus* and resulted as gelatin improved the stability most of the lactic acid bacteria (Champange *et al*., 1996). Both *Lactobacillus rhamnosus* and *L. paracasei* showed better stabilized viability when used glycerin and mannitol as protectants before freeze drying and increase the possibility of using prebiotics as cryoprotectans of freeze-dried probiotics (Savini *et al*., 2010). Hence, the influence of the additives on the survival rates of probitic bacteria and verification of the suitable combination that provides an effective agent for freeze drying is reasonable for technologically and economically assessment and verification (Jalali *et al*., 2012).

However, for the production of lactose-free yogurt, the limitation is to utilize only the non-diary base additive. The application of protective additives were only restricted to lactose free lyoprotectants such as sucrose, maltodextrin and guar gum. Hence, the overall purpose of this study was to determine the effect of different carrier in freeze dried formulations on the viability of lactic acid bacteria in fermented coconut cake for the production of lactose-free yogurt. In addition, this study was also carried out to determine the feasibility of adopting freeze drying method for the production of non diary base yogurt powder.

MATERIALS AND METHODS

Substrate:

Coconut cake was obtained as by-product from the processing of virgin coconut oil (VCO). The VCO plant is located at Pasir Gudang, Johor and every batch of coconut cake was supplied by the authorized company (Wawasan Tebrau Sdn Bhd.).

Preparation of Substrate:

A total of 200 kg coconut cake solution (20% wt) was mixed with distilled water in 250 L bioreactor. This custom fabricated bioreactor is a jacketed bioreactor with working capacity of 200 L and equipped with heater, agitation system and thermocouple both for product and jacketed temperature detection. Pasteurization was done in-situ where the temperature was heated up to 90^0C for prolonged to 30 min with agitation was set to 150 rpm. The pasteurized coconut cake was left to cool until temperature of 37^0C before cultures of lactic acid bacteria been inoculated for yogurt production.

Fermentation of coconut cake:

The temperature of the pasteurized coconut cake solution was in range of 37 to 40^{0}C before inoculation to prevent cultures denaturation. A mixture of 48 hour *Streptococcus thermopilus, Lactobacillus plantarum and Lactobacillus bulgaricus* was inoculated to the pasteurized coconut solution to initiate fermentation. Fermentation took place for 48 hours, at 37±2^{0}C and 150 rpm. Sampling was done in every 4 hours to determine its cultures' growth and pH. Temperature and titratable acidity was also done for every sampling point as correlation of cell growth.

Cultures' growth analysis:

Method for cultures' growth was adopted from Sieuwerts *et al*. (2008). A total of 100 µL of fermented sample was mixed with 900 µL ringers solution and series of dilution was done until 10^{-6}. The diluted samples were vortexed to ensure its homogenization before 30 µL of sample for each series of dilution was transferred onto MRS agar and incubated for 36 to 48 hours at 37^{0}C. The growth of cultures was calculated by using the following equation.

$$\text{Colony forming unit} = \frac{\text{Number of cells on plate x Dilution factor}}{\text{Volume of sample}}$$

The growth of these cultures was expressed as colony forming unit, CFU/ml.

Fermentation data analysis:

A set of data for pH, temperature and titratable acidity was collected at every sampling point (every 4 hours of fermentation). pH data was analyzed by using pH meter and temperature of each sample was determined by using digital thermometer. Titratable acidity was done to calculate production of lactic acid throughout fermentation (GEA Niro Method No. A 19a, 2006). NaOH solution with molarity of 0.5 M was placed in burette and was titrated into 10 ml sample with addition of phenolphthalein as indicator. The titration was completed upon changes of sample's color to pinkish solution. The titratable acidity was calculated as following equation:

$$\text{Titratable acidity} = \frac{\text{Volume of NaOH for titration x Molarity of NaOH (0.5 M) x 90.08 g/mol x 100\%}}{\text{Volume of sample (ml) x 1000}}$$

Molecular weight of Lactic Acid = 90.08 g/mol

Freeze Drying Formulations:

Lactose –free yogurt was the produced by fermentation process of coconut cake. To improve shelf life and stability of the yogurt, freeze dry was applied to remove moisture in the product and at the same time preserved its cultures viability. Different solid carriers were added into the product to determine its effect and thus select the most suitable lyoprotectants for freeze drying of lactose-free yogurt. Three categories of carriers were chosen to determine its effect toward freeze dry recovery and cell survival. Sucrose was used as polysaccharides while Maltodextrin (Glucidex, DE 12) and 3% of guar gum was used as dextrin-based and gum based, respectively to differentiate the effect of each lyoprotectants. 7% of sucrose, 10 % of Maltodextrin and 8.33% of guar gum (3% stock) were added to determine the effect of individual additive, combination of two and three additives to viability of lactic acid bacteria after freeze drying process.

Freeze drying of lactose-free yogurt:

Each formulation was prepared in 100 ml solution. The mixtures were kept in gradually increased surrounding temperature prior to freeze dryer. In this study, a laboratory freeze dryer (Alpha 1,4- LD Plus Martin Christ) was used and the parameters were set at temperature of -55^{0}C and vacuum condition. The freeze drying process took place about 5 to 7 days, depended on the dryness of the sample. The duration for the freeze drying process to complete took longer days because of the sample was placed in jars and less surface area contacted with the low temperature conditions. Therefore, to reduce the time of freeze drying, tray system freeze drying can be used to shorten time for drying yogurt. This kind of system is usually applied in industrial freeze drying process in way to increase rate of drying and yield of a process.

Freeze dried samples' analysis:

After freeze drying process completed, the samples were analyzed for its moisture content and cells' viability. The powder's moisture content was analyzed using moisture analyzer. The cells were rehydrated using deionized water to determine its CFU/ml after freeze drying.

RESULTS AND DISCUSSION

Fermentation of coconut cake for production of lactose-free yogurt:

As for production of lactose-free yogurt, a non dairy based substrate, coconut cake was used in the fermentation. Coconut cake is a by-product of virgin coconut oil processing. It is mainly contains of fat (36-45%), carbohydrate (16-32%), fiber (12-22%), protein (8-15%), ash (3-9%) and moisture (3-5%). Coconut cake is a cheap, ready substrate and consists of significant nutritional values potentially to be use as growth media for lactic acid bacteria (LAB). Lactic acid bacteria are often implicated as probiotics which can provide a beneficial effect on digestive and immune system of the human body. Thus, coconut cake, a non-dairy substrate fermented with LAB has high prospective to be developed as functional foods or ingredients which could be beneficially used in wellness industries.

Innoculation of *Streptococcus thermopilus*, *Lactobacillus plantarum* and *Lactobacillus bulgaricus* started the fermentation of coconut cake substrate. A mixture of lactic acid bacteria was used as each LAB function differently in production of yogurt. *Streptococcus thermopilus* and *Lactobacillus bulgaricus* are the most common LAB cultures used in yoghurt manufacture in association and synegistically, produce volatile metabolites that determine the flavour of yoghurt (Widyastuti *et al*., 2014). In this study, the potentialities of the coconut cake as growth media for LAB was tested. Figure 1 shows the changes of growth of the microbes which is expressed as colony forming unit, CFU/ml and pH for every sampling point of fermentation of coconut cake.

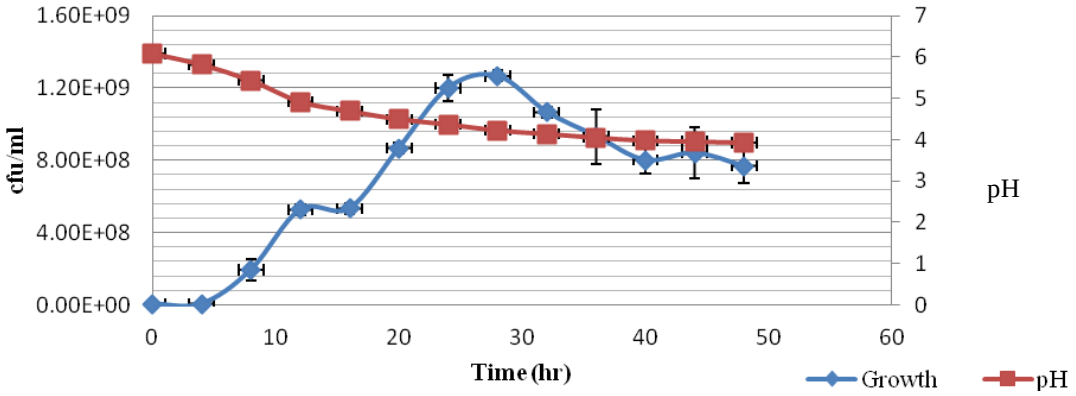

Fig. 1: Changes of growth rate and pH during 48 hours fermentation period

According to Figure 1, the growth of the LABs showed exponential growth. The growth of the microbes was gradually increased from the 4th hour of fermentation and kept increasing until the 28th hour. However, the growth slightly decreased from 32th hour of fermentation until the completion of the fermentation time at 48th hour. From Figure 1, the cfu/ml at 48 hours fermentation is 7.67 x 10^{8}. This data indicated that coconut cake substrate is a good growth media of the LAB mixtures in par with He and Hekmat (2015) report where soy, almond, and peanut milk samples successfully support the growth of *Lactobacillus rhamnosus* GR-1 with beyond 10^{6} cfu/ml. The pH data shows a decreasing of values at every sampling point and at 32th hour, the pH value was about to constant until fermentation was completed. The inversely data of CFU and pH showed the good correlation of growth and pH value whereby as growth was positive, pH value should decrease, as indicator of good growth. This data was also supported by the data of titratable acidity. Titratable acidity was determined to calculate the production of lactic acid in the fermented substrate throughout fermentation. This analysis also can be served as indicator of positive growth of the lactic acid bacteria as growth was correlated with the production of lactic acid. It is also important to determine the suitability of the substrate used for its acid production. As assumed by Horackova *et al*., (2015) dairy milk is suitable medium for acid production in comparison with soy milk as non-dairy based substrate for yogurt production. Figure 2 shows the data of titratable acidity and temperature throughout the fermentation process. As shows in Figure 2, the temperature of the fermented broth was in ranged of 32.9 to 36.0^{0}C and the titratable acidity was a positive linear during the fermentation period. More lactic acid were produced with increasing value of cfu/ml. Thus, from this set of data, good growth, decreasing pH and increasing lactic acid concentration showed coconut cake is a good growth medium for LAB.

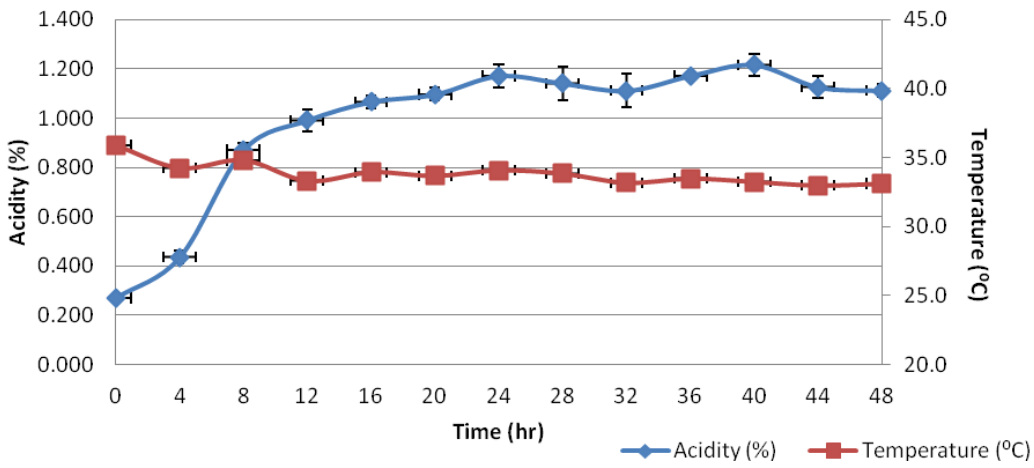

Fig. 2: Titratable acidity and temperature profiling during fermentation of coconut cake

Freeze drying product analysis:

After the 48 hour fermentation, fermented coconut cake was harvested and subjected to freeze drying for product preservation. Different formulations of lyoprotectants had been used to investigate the effect of each additive. The selected additive chosen in this study were based on three different categories, which were, disaccharides-based (sucrose), starch-based (maltodextrin) and gum-based (guar gum). The formulations were as shown in Table 1. To obtain the effects of each additive, three of the first treatment was added with the individual additives, next three treatments with combination of two additives and a combination of three lyoprotectants. For the purpose of lyophilization, the fermented broth needed to be added with encapsulants not only to have better recovery but the main objective was to ensure the viability of the lactic acid bacteria and hence, preserved its functionality as lactose-free yogurt. As shown in Table 1, all of the moisture content of freeze dried powder for each formulation was in ranged of 1.43 to 2.00%. This data indicated that an amount of moisture had been removed from the yogurt. Zayed and Roos, (2004) convinced that certain amount of water must remain in the dehydrated state of *Lactobacillus salivarius* freeze-dried powder for a satisfactory survival rate.

Table 1: Different freeze dried formulation and the moisture content of the freeze dried yogurt powder

Sample	Additives			Moisture content (%)
	Sucrose, S(%)	Maltodextrin, M(%)	Guar gum, GG(%)	
1	7			1.43 ± 0.32
2		10		1.54 ± 0.02
3			8.33	2.33 ± 0.18
4	7	10		2.00 ± 0.05
5	7		8.33	1.49 ± 0.06
6	7	10	8.33	1.86 ± 0.27
7		10	8.33	1.73 ± 0.13

Effect of different freeze dried formulations on lactic acid bacteria viability:

The ability of each freeze dried additive to ensure the survival of the mixture of lactic acid bacteria was investigated in this study. As shown in Figure 3, all of the cultures maintained its viability after freeze drying. Initially, the cultured solution contained 5.25 – 6.70 x 10^8CFU/ml. The highest CFU/ml was obtained from the combination of sucrose & guar gum and followed by the combination of all additives with 1.03 x 10^8 and 9.35 x 10^7 CFU/ml, respectively. Both of this treatment had the highest initial CFU/ml.

Table 2 shows the log of reduction and % of viability after rehydration for each of the formulations. The highest % of viability after rehydration was obtained from the combination of three additives; sucrose, maltodextrin and guar gum with less than 1 log of reduction followed by the combination of sucrose and guar gum. Among all of the formulations, the lowest % of viability after rehydration was seen from the combination of maltodextrin and guar gum. This result indicated that addition of sucrose was significance in way to protect and reduce the log of reduction and thus increases the survival rates of the LAB after freeze drying process. Many studies reported that sucrose had been determined to serve as good lyoprotectant which improve the tolerance of microbes in comparison to other cryoprotectants during freeze drying (Saarela *et al*., 2004; Siaterlis *et al*., 2008). Chen *et al*., (2006) mentioned in his report that sucrose provide the best protection and improve the survival rate for the microorganisms in kefir during freeze drying. This is also in line with Zayed and Ross, (2004) where the combination of sucrose with trehalose was the most efficient protective media with highest

survival rate of *Lactobacillus salivarius subsp. salivarius* and improved its stability during 7 weeks of storage compared to when used alone. In Heckley and Quay (1983) report, it is mentioned that loss of viability of freeze dried bacteria when exposed to air that associated with the free radical production was inhibited by sugars, for instance sucrose.

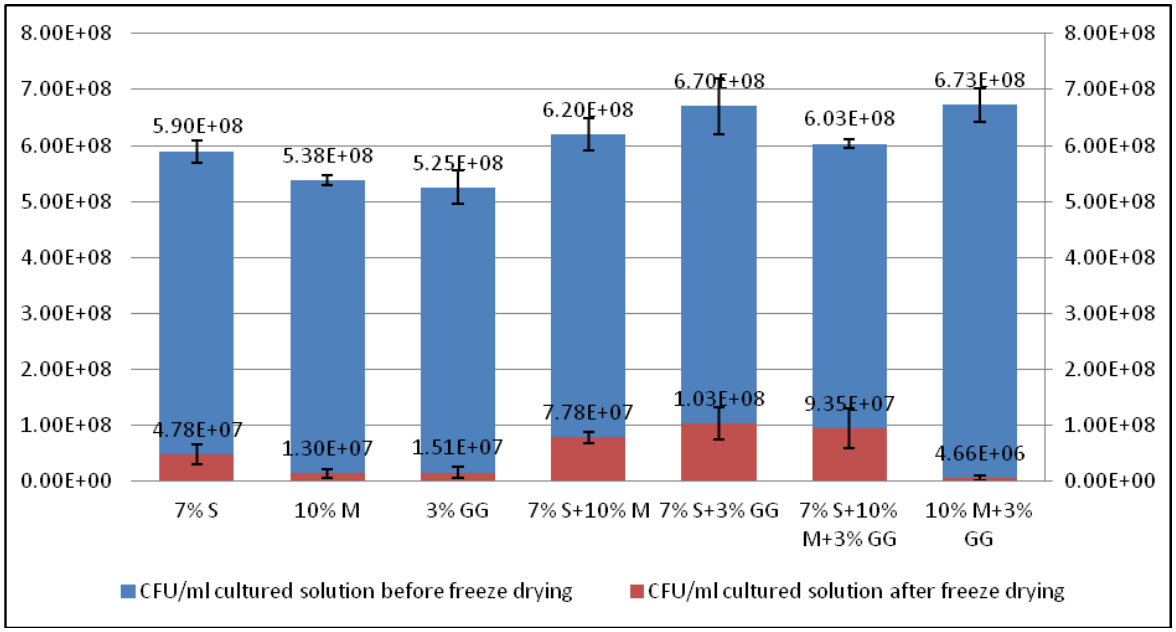

Fig. 3: Changes CFU/ml before and after freeze drying for different formulation

From Figure 3, the cfu/ml data showed variation for each formulation before freeze drying. Thus, for comparison, the reduction of log for each of the treatment was calculated as well as the percentage of the survival after rehydration. These were the equations used for both calculations.

Log of reduction = Log_{10} (A/B)

% of viability after rehydration = (A-B) x 100

Where; A = CFU/ml cultured solution before freeze drying

B = CFU/ml cultured solution after freeze drying

Table 2: Reduction of log and viability of lactic acid bacteria after freeze dry for each formulation

	Drying medium						
	Suc	Malto	GG	Suc +Malto	Suc + GG	Suc + Malto + GG	Malto +GG
Log of reduction	+	+	+	+	++	++	-
% of viability after rehydration	8.10	2.42	2.88	12.55	15.37	15.51	0.69

Key: ++ less than 1 log reduction; + less than 2 log reduction; - more than 2 log reduction

On the other hand, the effect of maltodextrin and guar gum were not really significance, especially when it was used alone. This result might be due to increase quantity of powder obtained with given cell concentrate by large amount of added maltodextrin (Champange *et al*., 1996). However, the addition of maltodextrin as lyoprotectants improved the survival of lactic acid bacteria in the yogurt especially when in combination with other protectants. As reported by Sohail *et al*. (2013), the alginate microspheres as combined with maltodextrin protected *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* from fragmentation during freeze drying and hence improved the survival rates of the bacteria.

Same result showed by addition of guar gum. The addition of guar gum was initially to avoid phase separation and keep the homogenization of the fermented solution. Thus, the potentialities of guar gum can be explored more in future as additive of freeze drying to high fat content substrate, such as coconut cake. This finding was as the same as reported by Champange *et al*., (1996) where addition of xanthan gum did not significantly improve the viability of the *L.casei* ssp. *rhamnosus, Bifidobacterium longum, Lactococcus lactis* ssp. *lactis* and *Streptococcus thermophilus*.

Conclusion:

The fermentation of coconut cake for production of lactose-free yogurt was successfully done in 250L bioreactor. The results from this study indicated the potentiality and feasibility to produce lactose-free yogurt powder by freeze drying. The effect of different freeze dried encapsulants was investigated in this study. Addition of sucrose as lyoprotectants is important as % of viability of lactic acid bacteria improved compared to other individual additives, maltodextrin and guar gum. The survival rate of the lactic acid bacteria was improved with combinations of the encapsulants except the combination without sucrose. The most effective combination was from the three additives where the less than one reduction of log and highest % of viability after freeze drying for the production of lactose-free yogurt freeze dried powder.

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