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The effect of inoculum size and incubation temperature on cell growth, acid production and curd formation during milk fermentation by *Lactobacillus plantarum* Dad 13

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Abstract

The aims of this study were to study the effect of inoculum concentration and incubation temperature on the growth of *L. plantarum* Dad 13, acidity, and formation of curd on milk fermentation. *Lactobacillus plantarum* Dad 13 is an indigenous probiotic candidate culture which was isolated from Dadih, a traditional fermented buffalo milk from West Sumatera, Indonesia. Each of skim-milk (10% w/v) was inoculated with 1, 3, 5, and 10% of 20 h starter culture respectively and incubated at 37°C for 24 h. To study the effect of fermentation temperature, milk was fermented at temperatures of 30, 34, 37, and 40°C for 24 h respectively, using chosen inoculum concentration. The results show that increase inoculum concentration from 1 to 5% increased the viable cells and decreased pH at the end of fermentation significantly. *Lactobacillus plantarum* Dad 13 grew well at 30, 34, and 37°C, but they grew very slowly at 40°C. The higher the incubation temperature up to 37° the higher the acid production. Further increased in temperature to 40°C resulted in the decrease in acid production drastically. Increased incubation temperature of milk fermentation up to 37°C reduced the time for curd formation. No curd was performed at fermentation temperature of 40°C. The optimum temperature for milk fermentation by *L. plantarum* Dad 13 was at 37°C for 8 h.

Introduction

Fermented dairy products have long been important components of human nutritional diet. Traditional fermented milk are produced as a result of the activities of natural microflora present in the food or added from the surrounding. Over the period, microorganisms involved in fermented milk have been isolated and characterized and used as a starter culture. Lactic acid bacteria (LAB) are widely used as starter cultures in milk fermentation. Yogurt represents a major commercial fermented milks around the world which is produced from the two cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. However other fermented milk products may require a different starter culture either mixed cultures or single culture. For example Dahi, a commercial important fermented milk in India, is produced by mixed starter cultures of S. *thermophilus, Lactococcus lactis* biovar *diacetylactis* and *Lactococcus lactis* subsp *cremoris,* meanwhile, Bulgarian buttermilk is made from a single culture of *L. delbrueckii* subsp. *bulgaricus* (Tamime and Robinson, 2000). The main bacteria involved in the production of Caucasian yogurt, so-called as “Caspian Sea yogurt” in Japan, were *Lactococcus* lactis ssp. *cremoris* and *Acetobacter orientalis* (Ishida *et al*., 2005). This fermented milk was traditionally produced in the Caucasus region in the western part of Eurasia. Dadih, a traditional fermented buffalo milk which popular among people in West Sumatra, Indonesia is made by pouring fresh raw unheated buffalo milk into a bamboo tube covered with a banana leaf. Spontaneously fermentation was carried at room temperature for two days. The indigenous lactic acid bacteria involved in dadih fermentation could be derived from the bamboo tubes, buffalo milk or banana leaves. Fermented milks are also popular as vehichles of probiotic by supplementation of starter culture with probiotic organisms to increase their health benefits (Panesar, 2011). The probiotic bacteria used in commercial product are mainly members of genera of *Lactobacillus* and *Bifidobacterium* (Hope and Larsen, 2009).

Lactic acid bacteria are widespread in nature. Indonesian fermented foods are mostly produced traditionally by spontaneous fermentation. The genus of *Lactobacillus* plays the most important role in Indonesian fermented foods, followed by *Pediococcus, Streptococcus, Leuconostoc, Enterococcus,* and *Weissella*, and *L. plantarum* has

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been found to be the most common species found in traditional Indonesian fermented food (Rahayu, 2003). Many lactic acid bacteria strains isolated from traditional Indonesian fermented food have met the basic requirement as probiotics and posses some functional properties (Nuraida, 2015). *Lactobacillus plantarum* Dad 13 which was isolated from dadih, a traditional fermented buffalo milk is a probiotic candidate. This microorganism was relatively stable at pH 3.0 for 3 h and could grow in MRSL containing 0.5% oxgall (Utami *et al*., 2009). *In vitro* study showed that this microorganism could inhibit the growth of pathogenic *E. coli* (Sumaryati *et al*., 2009). Feeding of *L. plantarum* Dad 13 and inulin increased the concentration SCFAs in rat fecal. (Utami *et al*., 2010). Fermentation of sesame milk with this bacteria increased the antioxidant activity due to the activity of β-glucosidase to hydrolyze sesaminol triglucoside into active aglycone sesaminol (Ulyatu *et al*., 2015). Single culture of *L. plantarum* Dad 13 could grow well in MRS-Lactose media and skim-milk, and produced acid and formed curd, and therefore can be used as a strater culture for milk fermentation (Kasmiati *et al*., 2002; Lestari *et al*., 2003).

Starter cultures play an important roles in fermented milk. The growth of starter culture is affected by many factors such as milk chemical composition, the amount of inoculum, temperature and time of incubations and the cooling time. Yoghurt fermentation is commonly prepared by adding *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and incubated at 40-45°C for 2.5-3 h or at ambient temperature for 18 hours or more until certain acid level (Tamime and Robinson, 2000). Chen *et al.* (2015) found that the optimum inoculum size of L. acidophilus and *L. casei* were all 7% on goat milk fermentation at 39°C. Meanwhile Shu *et al.* (2014) reported that the optimum inoculum concentration in goat milk was 3% with the incubation temperature at 43°C using *L. bulgaricus* and *S. thermophilus* as starter cultures. Fermentation of Caucasian yogurt or known as Caspian Sea yogurt was carried out at a lower temperature than typical yogurts, approximately 25 to 30°C and produced a lower acidity and greater viscosity (Kiryu *et al*., 2009). Inoculum size may has effect on pH, acidity, viable counts and flavor of fermented milk. Different microorganisms may have different optimum temperature for their growth and metabolite activities. In this study, milk fermentation using *L.plantarum* Dad 13 as a starter culture was carried out at various inoculum size and temperature incubation to evaluate its cell growth, acid production, pH reduction and curd formation.

Material and Methods

Microorganism

The strain *L. plantarum* Dad 13 was obtained from Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta. Stock culture was maintained at -45°C in the mixture of 1:1 of 20% (v/v) sterile glycerol and 5% (v/v) skim-milk. The culture was maintained on MRS (Oxoid) slope agar by subculturing aseptically at two week intervals and stored at 4°C until further use. For starter culture preparation the bacterial culture was inoculated into 80 ml of sterile MRS medium, and incubated at 37oC for 20 h.

Fermentation conditions

Skim-milk powder (Lactona) from local supermarket was reconstituted with water to prepare liquid skim-milk (10% w/v). Each of 80 ml of liquid skim-milk in 100 ml erlenmeyer flask was sterilized at 110°C for 10 min. The fermentation medium was used for milk fermentation using *L. plantarum* Dad 13.

For study the effect of inoculum size on milk fermentation, each of 10% w/v sterile skim-milk was inoculated with 20 h inoculum of *L. plantarum* Dad 13 at various concentrations ranging from 1% to 10% (v/v). Fermentation was carried out at 37oC for 24 h. At the initial fermentation time, 4, 8, 12, 18 and 24 h fermentations samples were taken and analysed the viable cells of lactic acid bacteria, titratable acidity, pH and development of curd. Selected inoculum size then was used to study the effect of temperature incubation on milk fermentation. Sterile skim-milk liquid (10% w/v) was inoculated with selected size of L. plantarum Dad 13 and incubated at 30, 34, 37 and 40°C for 24 h. At the initial fermentation time, 4, 8, 12, 18 and 24 h fermentations, samples were taken and analysed as in the experiment with inoculum size.

Enumeration of lactic acid bacteria

Population of lactic acid bacteria was determined by serially diluting sample in 0.85% NaCl solution and plating on MRS agar with 1% $CaCO_3$. After 48 h of incubation at 37°C, the colonies with the clear zone that appeared on the plates were counted and calculated as CFU/ml.

Measurement of pH and titratable acidity

The pH of the culture media was directly determined using a pH-meter (Eutech 510) at room temperature. Acid production was determined as titratable acidity using Sodium hydroxide titration with phenolptalein 1% as an indicator (AOAC, 1999).

Results and Discussion

The effect of inoculum size on the growth of lactic acid bacteria, production of acid and formation of curd during milk fermentation

Different inoculum size affected the initial population of bacteria in the fermentation medium. Initial population of lactic acid bacteria in skim-milk media with inoculum size of 1, 3, 5, and 10% (v/v) were 2.39 x 10^7 cfu/ml, 7.93 x 10^7 cfu/ml, 1.21 x 10^{8c}fu/ml and 2.81 x 10^8 cfu/ml respectively. It can be seen in Figure 1, that the growth rate of *L. plantarum* Dad 13 was very slowly in the case of 1% (v/v) inoculum. On the other hand, addition of 3, 5 and 10% inoculums resulted in the significantly increase population of lactic acid bacteria during milk fermentation. Higher concentration of inoculum can reduce the lag phase. After 12 h fermentation time, the population of lactic acid bacteria with inoculum size of 1%, 3%, 5%, and 10% (v/v) were 1.7 x 10^8 cfu/ml, 5.78 x 10^8 cfu/ml, 9.25 x 10^8, and 1.58 x 10^9 cfu/ml respectively.

Table 1 shows the effect of inoculum size on the production of acid, pH value and curd formation during milk fermentation by *L. plantarum* Dad 13 at various inoculum size. The bigger the inoculum size the higher the acid production. During milk fermentation, lactic acid bacteria utilized lactose in milk as a carbon source for its growth and metabolic activity. Inoculum size had an effect on the rate of acid production. The greater the inoculum size, the faster the acid production rate. Lowest acid production rate was found in fermentation with 1% (v/v) inoculum. At the end of fermentation time the concentration of titratable acidity was only 0.46%. Higher inoculum size of 3%, 5%, and 10% tend to be faster acid production. Fermented milk with inoculum size of 3%, 5% and 10% (v/v) had titratable acidity of 0.67; 0.90; and 1.20%. The result was in agreement with the result reported by Shu *et al.* (2014) for the production of yoghurt using S. thermophilus and *L. delbrueckii* subsp *bulgaricus* at various concentration of inoculum. Panesar *et al.* (2010) reported that lactose utilization and acid production increased with the increase in inoculum size up to 2% (v/v) and no improvement of acid production at higher inoculum size. The low acid production at 1%(v/v) inoculum size could be due to the low density of strater culture.

Production of acid during milk fermentation caused the decrease of pH. Higher inoculum size gave lower initial pH due to some acid production during starter preparation. After 24 h fermentation the pH of fermented milk with inoculum size of 3%, 5%, and 10% drop the pH to 4.41; 3.94, 4.05 respectively. On the other hand, milk fermentation using 1% (v/v) inoculum only reduced the pH to 5.27.

Table 1. Titratable acidity, pH and curd formation during milk fermentation at 37°C using various concentration of inoculum

Inoculum size (%)	Fermentation time (h)	Titratable acidity (%)	pH	Physical characteristic
1	0	0.22±0.02	6.34±0.16	Liquid
	4	0.22±0.01	6.22±0.08	Liquid
	8	0.27±0.01	5.95±0.17	Liquid
	12	0.32 ±0.02	5.70±0.25	Liquid
	24	0.46±0.03	5.27±0.23	Liquid
3	0	0.21±0.02	6.19±0.12	Liquid
	4	0.27±0.00	5.80±0.07	Liquid
	8	0.41±0.06	5.25±0.07	Liquid
	12	0.47±0.13	4.99±0.01	Curd
	18	0.57±0.05	4.74±0.02	Curd, syneresis
	24	0.67±0.01	4.41±0.15	Curd, syneresis
5	0	0.25±0.01	5.81±0.09	Liquid
	4	0.33±0.02	5.55±0.00	Liquid
	8	0.51±0.01	4.85±0.01	Curd
	12	0.64±0.01	4.38±0.01	Curd, syneresis
	18	0.75±0.01	3.99±0.00	Curd, syneresis
	24	0.90±0.01	3.94±0.04	Curd, syneresis
10	0	0.33±0.01	5.64±0.01	Liquid
	4	0.46±0.01	5.15±0.03	Liquid
	8	0.78±0.01	4.45±0.00	Curd, syneresis
	12	0.91±0.02	4.27±0.02	Curd, syneresis
	18	1.13±0.01	4.04±0.04	Curd, syneresis
	24	1.20±0.01	4.05±0.04	Curd, syneresis

Milk fermentation is usually stopped when the pH reaches 4.6 with certain acid concentration depends on the characteristic of the product needed (Tamime and Robinson, 2000). Higher inoculum size reduced the time to reach pH 4.6. Milk fermentation with inoculum size of 3%, 5%, and 10% reached pH 4.6 between 18 to 24 h, 8 to 12 h and 8 h fermentation time respectively. On the other hand, in the case of 1% inoculum, pH 4.6 could not be reached even for 24 h fermentation. These corresponded with the time of curd formation. No curd was formed in the milk fermentation with 1% (v/v) inoculum due to low acid production. In this case, the population of lactic acid bacteria was very low therefore the metabolic activity was also low resulted in the low acid production. Lee and Lucey (2004) reported that a more interconnected structure was found in yogurt gel inoculated with 3 or 4% inoculum, compared to the one made with 0.5% inoculum. Curd formations were occurred at fermentation time of 12 h, 8 h, and 8 h for milk fermentation with 3%, 5%, and 10% (v/v) inoculum size respectively. Based on the cell growth, production of acid and pH level of fermented milk,

Table 2. Titratable acidity, pH and curd formation during milk fermentation by *L. plantarum* Dad 13 at various temperatures

Temperature (°C)	Fermentation time (h)	Titratable acidity (%)	pH	Physical characteristic
30	0	0.24±0.03	5.97±0.09	Liquid
	4	0.30±0.01	5.78±0.08	Liquid
	8	0.39±0.02	5.29±0.01	Liquid
	12	0.49 ±0.01	5.00±0.06	Liquid
	18	0.62±0.04	4.72±0.05	Curd, syneresis
	24	0.71±0.01	4.56±0.08	Curd, syneresis
34	0	0.23±0.00	6.03±0.02	Liquid
	4	0.28±0.00	5.60±0.12	Liquid
	8	0.46±0.01	4.97±0.01	Liquid
	12	0.59±0.01	4.55±0.02	Curd, syneresis
	18	0.70±0.01	4.30±0.02	Curd, syneresis
	24	0.84±0.00	4.09±0.01	Curd, syneresis
37	0	0.25±0.01	5.81±0.09	Liquid
	4	0.33±0.02	5.55±0.00	Liquid
	8	0.51±0.01	4.85±0.01	Curd
	12	0.64±0.01	4.38±0.01	Curd, syneresis
	18	0.75±0.01	3.99±0.00	Curd, syneresis
	24	0.90±0.01	3.94±0.04	Curd, syneresis
40	0	0.24±0.03	5.98±0.11	Liquid
	4	0.29±0.00	5.79±0.00	Liquid
	8	0.33±0.04	5.57±0.12	Liquid
	12	0.38±0.04	5.40±0.12	Liquid
	18	0.40±0.01	5.33±0.12	Liquid
	24	0.44±0.02	5.20±0.02	Liquid

inoculum size of 5% was chosen for further study.

The effect of incubation temperature on the growth of lactic acid bacteria, production of acid and formation of curd during milk fermentation

To study the optimum temperature for milk fermentation using *L. plantarum* Dad 13, skim-milk (10% w/v) was inoculated with 5% (v/v) inoculum and incubated at various temperatures (30 to 40°C). *Lactobacillus plantarum* Dad 13 grew well and had similar growth pattern when milk fermentation was carried out at 30, 34, and 37°C, and after 24 h fermentation the viable cells were 1.5 x10^9, 1,28 x 10^9, and 1,23 x 10^9 cfu/ml respectively (Figure 2). Conversely, *L. plantarum* Dad 13 could not grow well at 40°C, and no increase in viable cell after 4 h fermentation. At the end of fermentation time the viable cell only reached 2.5 x 10^8 cfu/ml or only increased 0.20 log cycles.

Lactobacillus plantarum Dad 13 has different optimum growth temperature from yoghurt starter of *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus.* Yoghurt starter cultures are belong to thermophile bacteria and the temperature growth was 45°C. Meanwhile *L. plantarum* Dad 13 can grow well at 30-37°C but not at 40°C. These could be due to the fact that *L. plantarum* Dad 13 is mesophile lactic

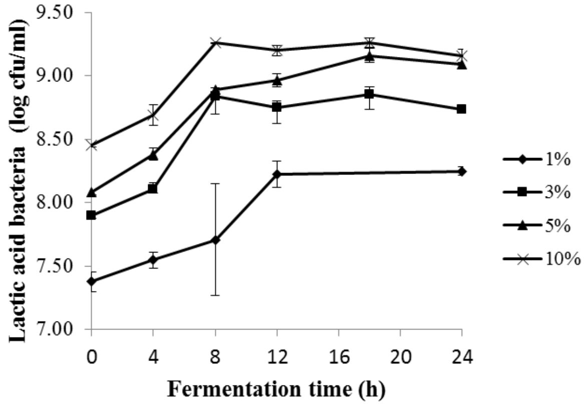

Figure 1. The growth of *L. plantarum* Dad 13 during milk fermentation at 37°C using various concentration of inoculum

acid bacteria which has optimum temperature for growth 20-35°C. *Lactobacillus plantarum* Dad 13 was isolated from dadih, a traditional fermented buffalo milk which was incubated in bamboo tubes at room temperature. Fermentation of Caspian Sea Yoghurt which the main bacteria involved were *Lactococcus lactis* ssp. *cremoris* and *Acetobacter orientalis* was carried out at lower temperature, 25-30°C (Ishida *et al*., 2005). Microorganisms involved in fermented dairy product have different growth temperature ranges, therefore the temperature and time incubation (Vedamuthu, 2006). *Lactobacillus delbruecki*i subsp *bulgaricus* and *S. thermophilus* have optimal temperature for growth at 45 and 40°C respectively, with similar minimum and maximum growth temperature of 22°C and 52°C respectively. *Lactococcus lactis* subsp *cremoris* has minimum, optimum, and maximum temperature for growth at 8°C, 22°C, and 37°C respectively (Panesar, 2011). By knowing the ranges of growth temperature, we could design the fermentation process, especially temperature and time.

There is a closed-relationship between the growth of starter culture and the acid production pattern during fermentation. Increase incubation temperature from 30°C to 37°C would increase the rate of acid production (Table 2). Further higher temperature to 40°C decreased the rate of acid production. The acid production increased with the increase in temperature up to 37°C, and markedly decrease in acid production was found at 40°C.

Panesar *et al.* (2010) found that the maximum lactic acid production from whey by *L. casei* was observed at 37°C, however, an insignificant decrease in lactic acid production was found at 40°C. Shiphrah *et al.* (2013) also reported that incubation temperature at 37°C produced higher amount of lactic acid compared to that of at other temperatures.

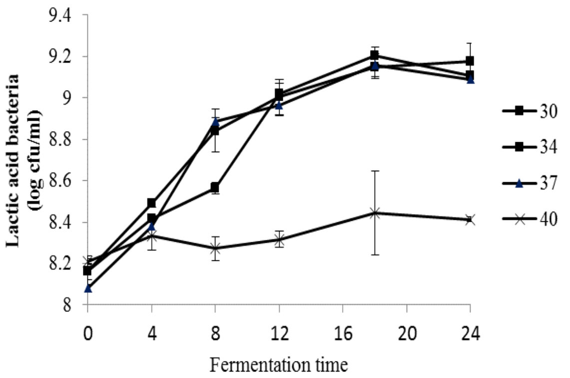

Figure 2. The growth of *L. plantarum* Dad 13 during milk fermentation at various temperatures

Temperature of incubation is one of the important factors that affects the metabolic activity of enzymes. Enzymes are most active at optimum temperature, and enzymatic reaction proceeds at maximum rate. The optimal temperature for lactic acid bacteria growth varies with genera and also species from 20 to 45°C. An appreciable increase in titratable acidity and decrease in pH were noted in milk inoculated with *L. plantarum* Dad 13 at 30-37°C. At the end of 24 h fermentation, titratable acidity increased to 0.71%, 0.84 %, and 0.90% at incubation temperatures of 30°C, 34°C and 37°C, meanwhile the pH declined to 4.56, 4.09 and 3.94 respectively. On the other hand very little changes in titratable acidity and pH was found in milk fermentation at 40°C. From the above obervations, a temperature of 37°C was considered optimal for milk fermentation by *L. plantarum* Dad 13.

During fermentation, lactic acid bacteria degraded lactose to glucose and galactose and then to acid, especially lactic acid, and made the pH decrease. Incubation temperature affected the reduction of pH value. The initial pH was 5.97-6.03, and during fermentation the pH decreased. At the end of fermentation time, the pH value of fermented milk that incubated at 30, 34, 37, and 40°C were 4.56; 4.09; 3.94; and 5.20 respectively. The decrease in pH value corresponds to the increase of acid production. The result also showed that although the growth rate of lactic acid bacteria at 30°, 34°, and 37°C were quite similar but exhibited different acid production rate and pH value. Temperature of 37°C could be an optimal temperature for fermentation of milk at 37°C by *L. plantarum* Dad 13 which showed fastest acid production and pH reduction. Study carried out by De Brabandere and De Baerdemaeker (1999) also found that incubation temperatures that were lower than the optimal temperature caused slower pH reduction.

The main function of starter is to generate lactic acid by the fermentation of lactose. The rate of acid development depends on starter used, and the fermentation temperature. As acid accumulates during fermentation of milk sugar, the pH gradually decreases. When the pH drops to the isoelectric points of casein, the colloidal dispersion of casein micelles collapses, and the acid casein precipitates forming the curd (Vedamuthu, 2006). Thus, the acid generated not only contributes to a pleasantly acid flavor to the product, but also transforms the liquid milk into a semi solid curd. The curd formation during fermentation of milk at various fermentation temperatures is presented in Table 2.

The higher the fermentation temperatures up to 37°C the shorter the time for curd formation. No curd formation was occurred at fermentation temperature of 40°C since the growth of lactic acid bacteria was very limited, so acid production. The acidification process results in the formation of three-dimesional network consisting of cluster and casein chains (Lee and Lucey, 2010). Thus low acid production resulted in low three dimensional network formation, and no curd formation. Fermentation of milk at 37°C for 8 h produced curd with the pH values approximately 4.85 with tittratable acidity around 0.51%. Prolong the fermentation time resulted in reduction of pH and appearing of some syneresis. It could be that curd formation of milk fermentation at 30°C and 34°C occurred between 12 – 18 h and 8 – 12 h respectively. It seems that longer fermentation period will resulted in the reduction of the curd and separation of whey and soluble component, thus rapid and proper cooling at desired acid level is needed.

Conclusion

Inoculum size of *L. plantarum* Dad 13 and fermentation temperature had significant effects on viable counts, pH, acidity and development of curd on milk fermentation. The optimum inoculum size in milk fermentation by *L. plantarum* Dad 13 was 5% (v/v). *Lactobacillus plantarum* Dad 13 can grow well at temperature of 30, 34, and 37°C, but the growth at 40°C was very slowly. No curd was performed at fermentation temperature of 40°C. The higher the incubation temperature up to 37° the higher the acid production. Further increased in temperature to 40°C resulted in the decrease in acid production drastically. Increased incubation temperature of milk fermentation reduced the time for curd formation. The optimum incubation temperature for milk fermentation using *L. plantarum* Dad 13 was 37°C. Increase temperature from 30 to 37°C increase the acid production and reduce the time for curd formation. Single culture of *L. plantarum* Dad 13 can be used as a strater culture for milk fermentation with mild acidity.

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