# Quantification of Lactic Acid as Secondary Metabolite of Lactic Acid Bacteria Isolated from Milk and Its Derivatived Products

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Keywords: Lactic Acid, Lactic Acid Bacteria, Total Acid Test, Secondary Metabolite.

Abstract: Lactic acid is a widely used secondary metabolite product. Titrated Total Acid (TTA) analysis is one of method to learn quantification process of lactic acid as a secondary metabolite of Lactic Acid Bacteria (LAB). The TTA test was carried out on 158 isolates from 11 samples with 2 replications using culture supernatant. The test was started with preparing growth media then rejuvenating the culture. NaOH 0.1 N, oxalic acid 0.1 N, and PP 1% indicator were prepared as reagents for analysis. As the result, the smallest TTA value is shown in the 1-Sa-L sample with a TTA value of 0.41% and the largest TTA value is shown in the KGL-5 sample with a TTA value of 1.56%.

## 1 INTRODUCTION

Lactic acid bacteria (LAB) is common used bacteria as starter to produce food product such as yoghurt, cheese, and sausage. LAB has food equivalent safety (food grade). LAB could reproduce in digestive tract, affect balance of digestive tract bacteria which provide a healthy effect, as probiotic. LAB is isolated from various sources and explored functional potential effect to enhance human health. Milk is rich of protein, fat, carbohydrate (mainly lactose), and vitamin and mineral (Park et al., 2007), and become ideal habiTTA for microorganism growth. Lactose, as main carbohydrate compound in milk, is fermented with microorganism, especially LAB group to produce lactic acid as major metabolite, creating milk with sour taste. Lactic acid is secondary metabolite product, could be produced in two ways which are using chemical synthesis and microbiological fermenTTAion.

Lactic acid production using microbiological fermenTTAion has some advantages, one of the advantage is the high purity (90 – 95 %) (Kotzamanidis et al., 2002) of optical L(+) lactic acid with high crystallinity and melting point. In the other hand, chemical synthesis of lactic acid produce mix result in D-L configuration. FermenTTAion in producing lactic acid has some disadvantages such as bacteria growth medium is not economical since it contains some expensive composition like yeast extract and peptone. FermenTTAion process produces primary and secondary metabolite. Primary metabolite is chemical compound produced and used by microbes to regenerate which are lactic acid and alcohol. Secondary metabolite is compound produced by microbes but not used for physiological activity like bacteriocin. Kefir fermenTTAion is done with LAB and yeast. LAB is used to produce lactic acid from glucose and triggered yeast's growth. In kefir making process, yeast has function to produce ethanol and flavor component as specific kefir taste. (Usmiati, 2007)

Whey contains high nutrition and has benefit for health such as controlling body metabolism, probiotic, animal antitumor, and antibacterial (Farnworth, 2003). Whey is defined as serum or the water portion of the milk that remains after separation of curd (cheese) and is the result of coagulation of milk protein with acids and proteolytic enzymes (Usmiati, 2007). One kilogram of cheese produced will produce as much as 8 to 10 liters of whey (Farnworth, 2003). Whey in Indonesia is generally underutilized, thus polluting the environment. Whey cheese has a Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) content which does not meet the permitted safe limits, namely 50,000 mg / L (BOD) and a COD level of 80,000 mg / L (Guimaraes et al., 2010). Environmental pollution can be reduced by utilizing whey as a basic ingredient



Afiati, F., Setiyoningrum, F., Priadi, G. and Qyasaty, V.
Quantification of Lactic Acid as Secondary Metabolite of Lactic Acid Bacteria Isolated from Milk and Its Derivatived Products.
DOI: 10.5220/0010546700003108
In *Proceedings of the 6th Food Ingredient Asia Conference (6th FiAC 2020) - Food Science, Nutrition and Health*, pages 132-136
ISBN: 978-989-758-540-1

in the manufacture of probiotic functional drinks such as yogurt or kefir. Whey kefir is the latest innovation in whey processing which is fermented by a number of microbes, namely lactic acid-producing bacteria (LAB), acetic acid-producing bacteria, and yeast.

Inhibition zone produced by the metabolite compound L. plantarum against Gram-negative and positive pathogenic bacteria is a result of the presence of primary metabolite such as lactic acid, ethanol, diacetyl, and carbon dioxide and secondary metabolites such as bacteriocins and hydrogen peroxide.

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Logarithmic phase is 15-24 hours on incubation period. Metabolite compounds from L. plantarum can inhibit Gram-positive and negative pathogenic bacteria which are often found in foodstuffs of animal origin, especially meat and milk, so that it has a good enough potential to be studied in more depth regarding its use directly in foodstuffs of animal origin. The process of producing bio preservation materials for food from the metabolism of L. plantarum has the potential to be developed and applied to foodstuffs of animal origin, especially milk and meat.

# 2 MATERIALS AND METHODS

## 2.1 Preparation of Growth Media and LAB Culture Growth

The growth media for Lactic Acid Bacteria (LAB) are MRS-agar and MRS-broth. Sterile Lactobacillus MRS Broth (HIMEDIA, M369) was used. Pre-culture stage was needed to refresh isolates which used to be stored at -80 °C before use, then isolates were incubated for 24 hours at 37 °C, characterized by a white sediment and the growing medium becomes cloudier. After that, isolates were homogenized and 5 µL was regrown in sterile MRS Broth media to be incubated for 18 hours (overnight) at 37 °C

## 2.2 Total Titrated Acid Test (TTA)

One of the reagents in the Titrated Total Acid (TTA) test is 0.1 N NaOH. Volume of NaOH needed showing the acid present in isolates because NaOH would neutralize the acid and was indicated by a change in color to pink.

The amount of NaOH and distilled water used in order to get the desired normality follows the following formula:

$$N\ NaOH = gram/MR \times 1000/(v\ (mL)) \quad (1)$$

## 2.3 Preparation of 0.1 N Oxalic Acid

Preparation of 0.1 N Oxalic Acid solution aims to standardize and determine the normality of NaOH precisely. Weighed 0.9 gram of powdered oxalic acid and dissolved in 100 mL of distilled water then stirred until the solids and solution were completely mixed to form a 0.1 N oxalic acid solution. The calculation of the amount of powdered oxalic acid with a relative molecular mass value of 90 (Mr = 90) and the distilled water used in order to obtain the desired normality follows the following formula:

$$N\ oxalic\ acid = gram/MR \times 1000/(v\ (mL)) \quad (2)$$

## 2.4 Standardization of NaOH

NaOH standardization is carried out to determine the normality value of NaOH in more detail. NaOH standardization is carried out by using the titration principle. The 0.1 N oxalic acid solution was put into a 100 mL erlenmeyer, while the NaOH solution was put into a burette. The 0.1 N oxalic acid solution was then titrated with NaOH solution until it turned pink. Titration is carried out three times so that the results can be more accurate. At each titration, the volume of NaOH used was recorded. The volume of NaOH obtained is then averaged so that the final volume will be used in the calculation. The standardized value of NaOH concentration is 0.1135 N. Calculation of the NaOH concentration follows the following formula:

$$N\ NaOH = (Volume\ oxalic\ acid \times N\ oxalic\ acid)/(Volume\ NaOH) \quad (3)$$

## 2.5 Preparation of PP (Phenolphtalein) Indicator 1%

The PP indicator is used as a color indicator in the titration process both in the total acid test and when standardizing NaOH. The PP indicator serves to determine the exact equivalence point in a solution marked by a change in color. 0.5 grams PP in powder form is weighed using a digital scale, then dissolved with 96% ethanol 50 mL in a beaker glass until well blended. The finished PP indicator solution is then put

in a dark glass bottle to keep the concentration unchanged.

TTA test was carried out on 80 samples with twice analysis each sample. Repetition is purposed to obtain more accurate results. If the standard deviation between repetitions does not exceed 10%, the results obtained can be used as final data. The TTA test was carried out to determine the acid content found in LAB isolates. The measurement of the total titrated acid (TTA) is the determination of the total acid concentration. Total Titrated Acid (TTA) relates to the measurement of the total acid contained. The TTA value includes the measurement of total dissociated and undissociated acid. The results of TTA measurement are used to determine the amount of organic acids in fruits and vegetables.

TTA test is carried out only on the supernatant part of the culture so that before the TTA test is carried out, the sample is centrifuged first. The sample to be used is put into a microtube for centrifuge. The centrifuge was carried out at a speed of 9000 rpm for 2 minutes.

TTA test basically uses the titration principle. 1 ml sample is pipetted into a 100 mL erlenmeyer, then diluted up to 10 times by adding 9 mL of sterile distilled water. To show the equivalence point in the solution, the PP indicator is added so that the solution will turn pink when it reaches the equivalence point. When it reaches the equivalence point, the titration is stopped and the initial volume and final volume of NaOH are recorded which will be used in the calculation to obtain the final data.

# 3 RESULTS

The following are the results of the TTA analysis for each sample:

Table 1: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| K. Kam 1 | $0.996 \pm 0.04^{B}$ |
| K. Kam 2 | $1.468 \pm 0.09^{C}$ |
| K. Kam 3 | $1.124 \pm 0.04^{B}$ |
| K. Kam 4 | $0.562 \pm 0^{A}$ |
| K. Kam 5 | $1.660 \pm 0.07^{D}$ |
| K. Kam 6 | $0.562 \pm 0.07^{A}$ |

Table 2: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| W.K. Sapi 1 | $1.532 \pm 0^{C}$ |
| W.K Sapi 2 | $1.545 \pm 0.02^{C}$ |
| W.K Sapi 3 | $1.162 \pm 0.05^{A}$ |
| W.K Sapi 4 | $1.328 \pm 0.04^{B}$ |
| W.K Sapi 5 | $1.187 \pm 0.02^{A}$ |
| W.K Sapi 6 | $1.315 \pm 0.09^{B}$ |

Table 3: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| KGG - 1 | $0.664 \pm 0^{C}$ |
| KGG - 2 | $0.6 \pm 0.02^{AB}$ |
| KGG - 3 | $0.626 \pm 0.05^{BC}$ |
| KGG - 4 | $0.613 \pm 0^{ABC}$ |
| KGG - 5 | $0.562 \pm 0^{A}$ |
| KGG - 6 | $1.622 \pm 0.02^{D}$ |
| KGG - 7 | $1.609 \pm 0.04^{D}$ |
| KGG - 8 | $0.626 \pm 0.02^{BC}$ |

Table 4: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| KGP - 1 | $0.562 \pm 0.04^{A}$ |
| KGP - 2 | $0.6 \pm 0.05^{A}$ |
| KGP - 3 | $1.2 \pm 0.07^{BC}$ |
| KGP - 4 | $0.626 \pm 0.02^{A}$ |
| KGP - 5 | $1.302 \pm 0.07^{C}$ |
| KGP - 6 | $1.111 \pm 0.09^{B}$ |
| KGP - 7 | $1.124 \pm 0^{B}$ |
| KGP - 8 | $1.175 \pm 0.07^{BC}$ |
| KGP - 9 | $0.702 \pm 0.09^{A}$ |

Table 5: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| KGB - 1 | 0.702 ± 0.02A |
| KGB - 2 | 0.753 ± 0.02AB |
| KGB - 3 | 0.728 ± 0.02AB |
| KGB - 4 | 0.766 ± 0.04AB |
| KGB - 5 | 1.251 ± 0.07F |
| KGB - 6 | 1.149 ± 0EF |
| KGB - 7 | 0.958 ± 0.09CD |
| KGB - 8 | 1.047 ± 0DE |
| KGB - 9 | 0.856 ± 0.09BC |

Table 6: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| KGL - 1 | 1.468 ± 0.02A |
| KGL - 2 | 1.073 ± 0B |
| KGL - 3 | 1.085 ± 0.05B |
| KGL - 4 | 0.741 ± 0.04A |
| KGL - 5 | 1.558 ± 0.07A |
| KGL- 6 | 1.047 ± 0.04B |
| KGL- 7 | 0.729 ± 0.04A |
| KGL- 8 | 1.366 ± 0.02C |
| KGL- 9 | 1.302 ± 0.07C |

Table 7: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| SS - 1 | 1.353 ± 0.04E |
| SS - 2 | 1.047 ± 0D |
| SS - 3 | 0.843 ± 0.04C |
| SS - 4 | 0.536 ± 0A |
| SS - 5 | 0.779 ± 0.02B |

Table 8: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| 1-Sa-A | 0.511 ± 0.04A |
| 1-Sa-B | 0.677 ± 0.09B |
| 1-Sa-E | 0.485 ± 0.04A |
| 1-Sa-F | 0.447 ± 0.02A |
| 1-Sa-G | 0.421 ± 0.02A |
| 1-Sa-H | 0.498 ± 0.09A |
| 1-Sa-I | 0.421 ± 0.05A |
| 1-Sa-J | 0.498 ± 0.02A |
| 1-Sa-K | 0.409 ± 0.04A |
| 1-Sa-L | 0.409 ± 0.04A |

Table 9: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| 2-Sa-A | 0.562 ± 0.07A |
| 2-Sa-B | 0.715 ± 0B |
| 2-Sa-C | 1.073 ± 0.07C |
| 2-Sa-D | 0.434 ± 0.04A |

Table 10: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| 2-JR-A | 0.485 ± 0A |
| 2-JR-B | 0.498 ± 0.02A |
| 2-JR-C | 0.524 ± 0.02AB |
| 2-JR-D | 0.511 ± 0.04AB |
| 2-JR-E | 0.562 ± 0.04BC |
| 2-JR-F | 0.536 ± 0.04ABC |
| 2-JR-G | 0.587 ± 0C |

Table 11: Total Titrated Acid (TTA) value from milk and its derivated products.

| Isolates | TTA (%) |
|---|---|
| 1-PE-A 1 | 0.69 ± 0.04C |
| 1-PE-C2 | 0.664 ± 0BC |
| 1-PE-D3 | 0.447 ± 0.05A |
| 1-PE-E4 | 0.562 ± 0.07AB |
| 1-PE-G5 | 0.549 ± 0.02AB |
| 1-PE-I 6 | 0.932 ± 0.05D |

According to Kusumawati et.al (2008), lactic acid bacteria produce compounds that are antimicrobial such as organic acids, hydrogen peroxide and protein compounds (bacteriocin). Lactobacilli species which produce sufficiently large amounts of hydrogen peroxide can inhibit growth and kill pathogenic microbes. The accumulation of these compounds in cells occurs because lactobacilli does not produce the enzyme catalase.

Lactic acid bacteria will convert carbohydrates into lactic acid under anaerobic conditions and this process can be divided into three stages. In the early stages, starch from carbohydrate sources will be hydrolyzed into maltose by α and β amylase, then this maltose molecule will be broken down into glucose by maltase and in the final stage lactic acid bacteria will convert glucose into lactic acid and other materials such as acetic acid and alcohol.

Secondary metabolites are compounds that are synthesized by microbes but are not a basic physiological requirement. One of the secondary metabolites that can function as antibacterial is bacteriocin (a protein compound that exhibits antibacterial activity and is able to prevent the growth of disease-causing bacteria). Bacteriocin produced in the decay or stationary phase, which is the phase when the substrate starts to run out, will stimulate the formation of enzymes that play a role in the formation of secondary metabolites.

Lactic acid bacteria (LAB) are a group of bacteria that have been widely used as a starter to produce food ingredients such as yogurt, cheese and sausages. Based on a long history of safety in the consumption of food produced using LAB, this class of bacteria is recognized as having food-grade safety. LAB isolates were characterized morphologically and physiologically based on Gram characteristics, gas production from glucose and catalase production. Only Gram positive and catalase negative isolates were further identified. Catalase testing is done by dropping one drop of LAB culture over 30% H2O2 solution. Positive catalase is characterized by the formation of foam (bubbles / foam). Gram staining was carried out to see cell morphology and

characteristics of Gram. Gas formation from glucose is carried out using hot loops and gas formation is characterized by the formation of froth. Lactic acid bacteria also produce other metabolites that function as anti-microbes such as acetic acid, hydrogen peroxide, and bacteriocins. The increase in lactic acid from secondary metabolites of lactic acid bacteria is caused by the increasing number of cell biomass that ferments the substrate into lactic acid and energy. According to Yusmarini and Efendi (2004), the sugar contained in the media is used by lactic acid bacteria as a carbon source to produce lactic acid and energy through the glycolysis process.

According to Farnworth (2003), fermentation of milk into kefir produces metabolites that are beneficial to health, namely exopolysaccharides and bioactive peptides. Both of these compounds will stimulate the immune system. The polysaccharides formed in kefir also act as anti-tumor agents. Antibacterial components are also produced during kefir fermentation such as organic acids (lactic and acetic acids), carbon dioxide, hydrogen peroxide, ethanol, diacetyl, and peptides (bacteriocins) which are not only useful for inhibiting the growth of pathogenic bacteria and spoilage bacteria during food processing and storage, but can also be used for the prevention of some digestive disorders and infections. The composition and taste of kefir will differ significantly depending on several factors. The main factor that can distinguish is the source of milk used whether it comes from cow's milk or goat's milk. Other factors that play a role are the fat content of the milk used, the composition of the kefir seeds and starter used, and the accompanying technological process. Traditionally kefir is produced by adding kefir seeds to some milk.

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