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Analysis of organic acids produced by lactic acid bacteria

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Abstract. Organic acids can be useful as starting materials in multiple applications including for food supplements, bio-based material, and biodegradable polymers. The use of microorganisms for organic acids production has been promising to replace petroleum-based processes because of their abilities to utilize inexpensive and renewable feedstocks. In order to obtain potential bacteria for organic acids production, a total of 4 selected lactic acid bacteria (LAB) isolates were investigated. The production of organic acids was carried out for 5 days of incubation using a liquid fermentation process. The organic acids analyzed were acetate, citrate, formate, lactate, and succinate. The analysis of these compounds was performed using High – Performance Liquid Chromatography (HPLC). Results revealed the growth of 4 LAB isolates increased and reached about 10^7 CFU/ml during 5 days of fermentation. Lactate and acetate were found as the dominant products of organic acid fermentation. The highest amount of acetate, lactate, and formate was achieved at 44.81 g/L, 17.11 g/L, and 7.90 g/L respectively by BAL 100 isolate whereas the highest content of succinate was 20.83 g/L and produced by BAL 690 isolate. The experiments indicated that BAL 100 isolate could be the most potential for organic acid production and BAL 690 isolate is considered as promising isolate for succinic acid production.

1. Introduction

Organic acids are widely applied in many industries because of its versatile use as monomer and starting materials for food supplements, bio-based material, and biodegradable polymers [1] [2]. Those can be useful as chelating agents because of providing both protons and organic anions and become a key group among building block chemicals [3].

Organic acids can be produced by microbial processes. Most of them are natural product or intermediates in major metabolite pathways of microbes. The use of bio-based process for organic acids production can replace petroleum-based [4]. Due to the increase of crude oil price and the reduction of fossil fuel resources, organic acids production from petrochemicals are becoming costly [5]. It leads some manufacturers to find new methods which can utilize inexpensive and renewable feedstocks.

The Lactic Acid Bacteria (LAB) are popular bacteria in producing organic acids such as lactate, acetate, formate, succinate, and citrate. The LAB is Gram-positive microorganisms that produce lactic acid as a major fermentation product. It is called homofermentative if lactic acid is yielded as single fermentation products and the other is called heterofermentative if ethanol and CO₂ are mainly produced as by-products [6].



Sugar is the preferred substrates of the heterofermentative LAB. However, the homofermentative LAB can ferment hexose and produce lactic acid by the reaction of glycolysis and lactate dehydrogenase. Catabolism of LAB plays an important role to determine the fermentation products formed. At limiting catabolism, mixed acid fermentation with formate, lactic, ethanol and acetate as the products is generated. Whereas catabolism with high glycolytic flux, homofermentative metabolism with lactic acid as the sole product is produced. Under slow growth condition and low glycolytic flux rates, the homofermentative LAB change to mixed acid fermentation [7].

Development of microbial processes for organic acids production by employing LAB has been studied by many researchers. Lactate and succinate are currently produced at commercial scale; further research are required to scale up the other organic acids from laboratory to commercial scale processes [1]. Succinate, acetate, and formate can be produced by LAB through citrate metabolism. There are at least two different mechanisms of citrate utilization in the LAB. First, citrate conversion to succinate by reducing oxaloacetate using the enzymes malate dehydrogenase, fumarate reductase, and fumarase [8]. Second, citrate conversion to acetate and formate via pyruvate [9].

The analysis of organic acids in fermentative product is necessary in order to find potential Lactic Acid Bacteria. This research focuses on characterizing the selected LAB isolates and obtaining their potentiality for organic acids production. The organic acids can be determined and quantified by various methods, one of them is usually performed by high-performance liquid chromatography (HPLC). In a combination of RP-C18 columns, 5mM sulfuric acid as eluent and UV for detection are used for the separation of these compounds [10].

2. Materials and Methods

2.1. Media

The medium used for stock cultures was de Man, Rogosa and Sharpe (MRS) agar containing (g/liter): enzymatic digest of casein 10.0, meat extract 10.0, yeast extract 4.0, D(+)glucose 20.0, dipotassium hydrogen phosphate 2.0, tween 80 1.0, di-ammonium hydrogen citrate 2.0, sodium acetate 5.0, magnesium sulfate heptahydrate 0.2, manganese sulfate monohydrate 0.04 and agar 14.0. The liquid medium used for inoculums and fermentations was MRS broth.

2.2. Microorganisms

The isolates used were obtained from Culture Collection of Biocatalyst and Fermentation Laboratory, Research Center for Biotechnology, LIPI. A total of 4 selected LAB isolates were used in this work. Those were given labels as LAB 100, LAB 120, LAB 690, and LAB 761. The strains were stocked on MRS agar plate.

2.3. Inoculum Preparation

A single colony from each LAB isolate on MRS agar plate was selected and inoculated in 15 ml MRS broth medium. The inoculums were aerobically incubated at 37 °C and shaken at 150 rpm for 24 hours.

2.4. Fermentation Process

The fermentation process was carried out in a screw cap test tube containing 5 ml of fermentative medium and inoculated with 5% of the inoculum. The temperature of fermentation was set at 37 °C and constant agitation of 150 rpm for 5 days.

2.5. Sample Preparation

All the experiments were carried out in triplicates. The LAB culture in MRS media was prepared as the samples. The sampling process was performed by withdrawing each test tube periodically at an interval of 24h for 5 days of fermentation. Each sample was centrifuged at 10.000 rpm, temperature at

4°C for 20 minutes. The supernatant was then used for HPLC analysis. A 400 µl of supernatant was filtered through syringeless filter Whatman pore size 0.45 µm.

2.6. Analytical Methods

The cell number of each LAB isolate was measured using spectrophotometer based on the optical density at λ 600 nm and converted into Colony Forming Unit using Total Plate Count method. Samples were diluted in serial dilution of 10^{-1} to 10^{-6} and poured them into 15 ml MRS agar plate. After 3 days of incubation, the colony number was enumerated. Correlation value was measured by comparing the colony number and absorbance.

The products of organic acids were analyzed using Shimadzu HPLC System equipped with Aminex column compartment, autosampler and refractive index detector (RID). A volume of 10 µl was injected with a run time of 40 minutes for each sample. The mobile phase used was 5.0 mM/L H_2SO_4 in ultrapure water (HPLC grade) at a flow rate of 0,6 mL per minute. Standard solutions were injected to obtain the retention time for each compound.

3. Results and Discussion

3.1. Lactic Acid Bacteria Growth

The growth of LAB in MRS broth medium was observed from 0 to 120 hour of the experiment (5 days of fermentation). The growth increased, starting at about $10^2 - 10^3$ CFU/ml after inoculation and exceeded 10^7 CFU/ml at the end of fermentation. The exponential phase was reached at about 0 to 24h whereas, at above 24h, the growth of LAB was on the stationary phase (Figure 1). Due to the reduction of nutrients in the medium, the LAB's growth remains steady. In the stage of stationery, the LAB started to produced secondary metabolites, such as organic acids.

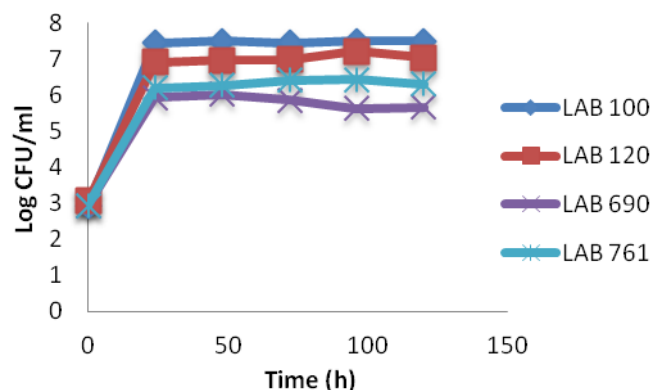


Figure 1. The growth of Lactic Acid Bacteria in MRS broth medium for 5 days of fermentation

3.2. Organic Acids Production

A total of 4 selected LAB isolates have been investigated their ability in producing 5 compounds of organic acids: acetate, citrate, formate, lactate, and succinate. The main activity of the LAB is to produce the end product lactic acid by metabolizing the sugar. In this experiment, results showed that acetate was present in large amounts in all the fermentation broths compared to the other organic acids. The highest amount of acetate was reached at 44.81 g/L whereas that of lactate was only 17.11 g/L. A small amount of lactate in fermentation broth might be caused by the presence of oxygen. The fermentation process was not completely carried out in anaerobic condition. The limited oxygen was still present in liquid medium. It could trigger the LAB to produce acetate with high content instead of lactate.

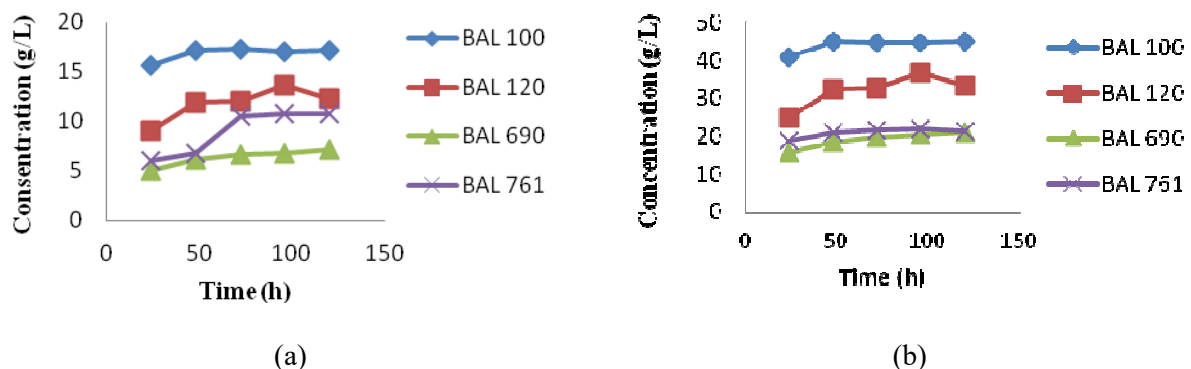


Figure 2. The content of lactate (a) and acetate (b) produced by the Lactic Acid Bacteria

The BAL 100 isolate had the highest amount of lactate and acetate, followed by BAL 120, BAL 690 and BAL 761 (Figure 2). Lactate and acetate were dominant products in this work. It has been reported by Paillart et al. (2016) that the LAB was able to produce acetic and lactic acid as the dominant products in food fermentation [11].

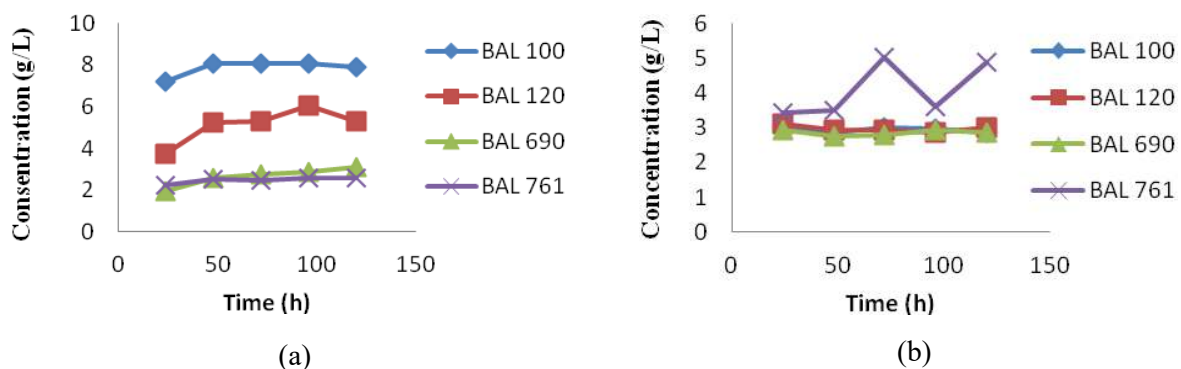


Figure 3. The content of formate (a) and citrate (b) produced by the Lactic Acid Bacteria

Beside lactate, formate and citrate were also formed in the fermentation broths. The results showed formate and citrate that produced by four isolates were detected in small amounts. A fluctuate graph of citrate was shown in Fig 3 as citrate is an intermediate of the tricarboxylic acid (TCA) cycle and it is necessary to provide precursor for its cycle [12]. The limited number of LAB are able to ferment organic acids including citrate [13].

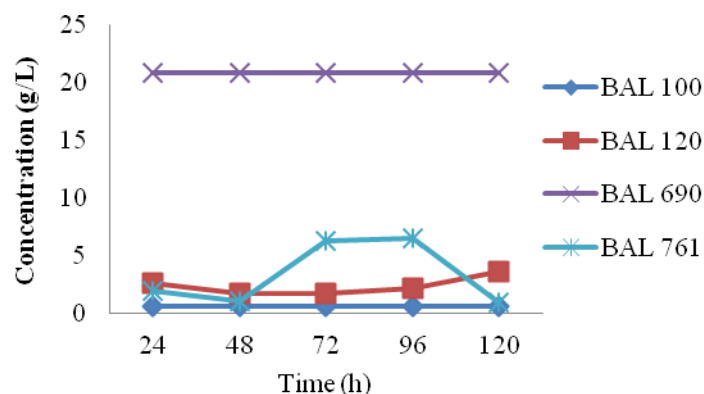


Figure 4. The content of succinate produced by Lactic Acid Bacteria

The largest content of succinate was reached by BAL 690 isolate at 20.83 g/L as depicted in Fig. 4. However, the ability of other isolates to produce succinate was low. The isolate of BAL 761 showed a fluctuate graph, and it was able to produce succinate in small amounts. Song and Lee (2006) has reported that succinate is one of the fermentation end-product of anaerobic metabolism and an intermediate of TCA cycle in aerobic condition [14]. A fluctuate graph might be caused by the use of succinate either in aerobic or anaerobic condition.

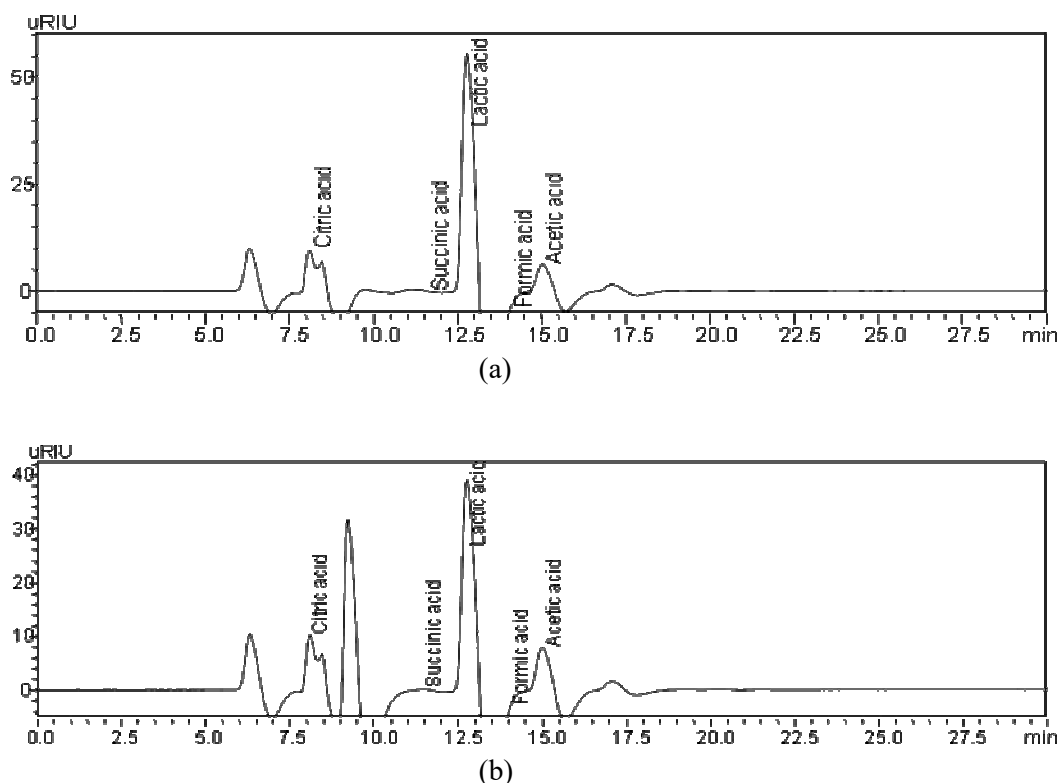


Figure 5. HPLC chromatograms of organic acids from fermentation medium for isolate: BAL 100 (a) and BAL 120 (b)

The HPLC method was able to quantify different types of organic acids (acetate, citrate, succinate, formate, and lactate) that exist in the liquid medium of fermentation. The separation for each compound of the organic acids peaks was clear. In general, the use of HPLC is an accurate and convenient analytical technique for quantification and identification of organic compounds including organic acids [15].

Table 1. The content of organic acids products

Isolate	Organic Acids (g/L)				
	Acetate	Citrate	Formate	Lactate	Succinate
BAL 100	44.81	2.87	7.90	17.11	0.65
BAL 120	33.11	2.99	5.31	12.28	3.56
BAL 690	20.75	2.85	3.11	7.20	20.83
BAL 761	21.10	4.90	2.59	10.80	6.51

The content of organic acids produced by four LAB isolates was summarized in Table 1. The potential isolates were obtained based on their abilities to produce organic acids in high amounts.

4. Conclusions

This research concluded that Lactic Acid Bacteria (LAB) were able to produce various compounds of organic acids, in particular, lactate and acetate as dominant products during the fermentation. The highest amount of acetate, lactate, and formate was produced by BAL 100 isolate whereas the highest value of succinate was produced by BAL 690 isolate. The potential isolates for organic acids production were found through this research.

5. Acknowledgment

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6. References

- [1] Pleissner D, Dietz D, van Duuren J B J H, Wittmann C, Yang X, Lin CSK and Venus J 2017 *Adv. Biochem. Eng. Biotechnol.* 1-38
- [2] Liaud N, Ginies C, Navarro D, Fabre N, Crapart S, Gimbert I H, Levasseur A, Raouche S and Sigoillot J C 2014 *Fungal Biology and Biotechnology* 1 1
- [3] Posso E J S, de Prager M S and Rojas C A C 2017 *Acta Agron.* **66** (2) pp 241-247
- [4] Sauer M, Porro D, Mattanovich D and Branduardi P 2007 *Elsevier Ltd, Cell Press* pp 100-108
- [5] Cao Y, Zhang R, Sun C, Cheng T, Liu Y and Xian M 2013 *Biomed Research International.*
- [6] Madigan M T, Martinko J M, Stahl D A and Clark D P 2012 *Brock Biologi of Microorganisms 13th Edition* (San Francisco: Benjamin Cummings) p 375
- [7] Zaunmuller T, Eichert M, Richter H and Uden G 2006 *Appl. Microbiol. Biotechnol.* **72** 421-429
- [8] Cselovszky J, Wolf G and Hammes W P 1992 *Appl. Microbiol. Biotechnol.* **37** 94-97
- [9] Lindgren SE, Axelsson LT and McFeeters R 1990 *FEMS Microbiol. Lett.* **66** 209-214
- [10] Coelho E M, Padilha C V S, Miskinis G A, de Sa A G B, Pereira G E, de Avezedo L C and Lima M S 2017 *Journal of Food Composition and Analysis*
- [11] Paillart M J M, van der Vossen J M B M, Lommen E, Levin E, Otma E C, Snels J C M A and Woltering E J 2016 *Acta Hort.* pp 289-296
- [12] Hugenholtz J 1993 *FEMS Microbiology Reviews* **12** 165 – 178
- [13] Neal-McKinney J M, Lu X, Duong T, Larson C L, and Call D R 2012 *PLoS ONE* **7** (9)
- [14] Song H and Lee SY 2006 *Enzyme and Microbial Technology* **39** pp 352-361
- [15] Zaky A S, Pensupa N, Eiroa A A, Tucker G A and Du C 2017 *Journal of Food Composition and Analysis* **56** 25-33