The potency of cassava starch (var. kristal merah and var. revita) for bio-succinic acid production using indigenous lactic acid bacteria (Leuconostoc sp)

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Abstract. Cassava of local strain, a plentiful agricultural product in Indonesia was evaluated for biosuccinic acid production via saccharification and fermentation using indigenous lactic acid bacteria (Leuconostoc sp). Cassava starch is an ideal biosuccinic acid feedstock due to its low price and high sugar content. Conversion of cassava starch into succinic acid is an effort to obtain added-value of the local product. Succinic acid has been well-known as an important platform chemical and being top 12 building block in the chemical industries. In the current study, saccharification of starch was carried out using available glucoamylase enzymes produced by Aspergillus awamori KT 11 (Glutech) in the temperature 60°C and 200 rpm. Results revealed the best yield of total reducing sugar accumulation (62.3 g L⁻¹) was obtained when the ratio of starch solution of var. kristal merah (25% w/v) and glucoamylase was kept to 3:1. HPLC analysis of sugar also showed three different sugar were produced during saccharification: glucose, maltose, and maltotriose. Fermentation of the liquid sugar as a carbon source in various media showed that Leuconostoc sp could produce highest succinic acid in medium SPM, with concentration 3.4 g L⁻¹ in 72 h for var. kristal merah and 3.6 g L⁻¹ in 48 h for var. revita. The other valuable organic acids, such as lactic acid, citric acid, formic acid, and acetic acid were also formed during fermentation process, with concentration less than 3 g L⁻¹. The results suggested that cassava starch of 2 local strains (var. kristal merah and var. revita) has the potential to be an alternative substrate for efficient and economic valuable organic acids production such as succinic acid.

1. Introduction

Succinic acid, known as butanedioic acid, is one of the most important platform compounds that can be used as a precursor for industrial chemicals, such as 1, 4-butanediol, tetrahydrofuran, maleic anhydride, g-butyrolactone, adipic acid, and polymers through esterification process [1-3]. For sustainability purpose in this era of petroleum shortages, the production of succinic acid via the microbial conversion of renewable feedstock has attracted great interest. The production of succinic acid can be performed by
fermentation using anaerobic or facultative anaerobic microorganisms. Succinic acid is a common intermediate in the metabolic pathway of the microorganisms [3]. The wild-type rumen bacterial strains such as *Actinobacillus succinogenes* and *Basfia succiniciproducens* are two of the most promising strains for industrial application [4]. However, the utilization of anaerobic microorganisms needs extra treatment to create a suitable condition for growing. Among succinic acid-producing strains, Lactic Acid Bacteria (LAB) has great potential because they can use different carbon sources to produce various valuable organic acids such as succinic acid in both aerobic and anaerobic condition. In the present study, *Leuconostoc sp*, was selected after an extensive screening assay of hundreds of LAB carried out in our laboratory (unpublished data).

The other important aspect in the production of bio-based chemicals such as biosuccinic acid is that low-cost raw materials with high supply should be used as fermentation feedstocks. Some researcher has reported the utilization of low-cost carbon sources such as sweet potato granular [5], spent sulphite liquor (SSL) [4], fresh cassava root [6], citrus peel [7], cane molasses, hydrolysate of carbon fibre, and wood hydrolysate [1] for fermentation to produce succinic acid. In the present study, a hydrolysate of cassava starch from 2 different local strains was used as a low-cost carbon source for fermentation by selected LAB, *Leuconostoc sp*. Enzymatic hydrolysis using glucoamylase was performed as saccharification method to obtain liquid sugar (glucose). The liquid was then used as carbon source in the fermentation process by *Leuconostoc sp*. In this study, we also determined the most suitable mineral medium for the microbe of organic acids production. Utilization of low-cost starchy materials could be another effective approach for reducing petroleum-based chemicals and also the cost of succinic acid production.

### 2. Methods

#### 2.1. Chemicals

MRS agar and MRS broth were purchased from Wako Co., Ltd. (Osaka, Japan). Starch from 2 strains of cassava (kristal merah and revita) was obtained from Laboratory Molecular Genetics and Biosynthesis Pathway Modification of Plant, Research Centre for Biotechnology, Indonesian Institute of Sciences (LIPI).

#### 2.2. Microorganisms

The lactic acid bacteria (LAB) strain used in this study was *Leuconostoc mesenteroides* (obtained from InaCC with code LAB100). The fungus was cultivated at 25 °C on MRS agar medium for 3 days in a disposable plastic Petri dish and maintained at 4 °C prior to use. The roadmap oh this research was shown in Figure 1.

#### 2.3 Enzymatic hydrolysis of starch by crude glucoamylase produced by Aspergillus awamori KT 11

Experiments using various combination enzyme and substrate (E/S) was performed in 100-mL Erlenmeyer flask to identify the most suitable combination E/S for liquid sugar production. Gelatinization of starch (25% w/v) at 100 °C for 10 minutes at a stirring water bath was conducted prior to reaction with glucoamylase. The hydrolysis was conducted for 24, 48, and 72 h at 60 °C and agitation 200 rpm. The composition reaction for enzyme and substrate as shown in Table 1.

| Volume glucoamylase (mL) | Starch (gram) | Aquades (mL) | E/S  |
|--------------------------|---------------|-------------|------|
| 10                       | 5             | 10          | 1/1  |
| 5                        | 5             | 15          | 1/3  |
| 4                        | 5             | 16          | 1/5  |
| 2                        | 5             | 18          | 1/9  |
Table 2. Four various of culture medium for fermentation *Leuconostoc sp*

| Component                        | MRS (gram/L) | Medium 2* (gram/L) | Medium 3** (gram/L) | Medium 4*** (gram/L) |
|----------------------------------|--------------|--------------------|---------------------|----------------------|
| D (+) Glucose                    | 20           | -                  | -                   | -                    |
| Enzymatic digest of Casein       | 10           | -                  | -                   | -                    |
| Peptone                          | -            | 4                  | -                   | 4                    |
| Tripton                          | -            | -                  | 1.1                 | -                    |
| Beef/Meat Extract                | 10           | -                  | -                   | -                    |
| Yeast Extract                    | 4            | 3.1                | 0.5                 | 4                    |
| Tween 80                         | 1 ml         | 0.2 ml             | -                   | 1 ml                 |
| Disodium Phosphate (Na2HPO4)     | -            | -                  | -                   | 2                    |
| Potassium Phosphate (K2HPO4)     | 2            | 4                  | 0.3                 | 2                    |
| Magnesium Sulphate (MgSO4.7H2O)  | 2            | -                  | -                   | 0.1                  |
| Manganese Sulphate (MnSO4.5H2O)  | 0.04         | 0.04               | -                   | 0.05                 |
| Tri-Sodium Citrate               | -            | 0.01               | -                   | -                    |
| NaCl                             | -            | -                  | 0.1                 | -                    |
| (NH4)2SO4                        | -            | -                  | 0.1                 | -                    |
| CaCl.H2O                         | -            | -                  | 0.02                | -                    |
| MgCl2.5H2O                       | -            | -                  | 0.02                | -                    |
| Na2CO3                           | -            | -                  | 0.1                 | -                    |
| (NH4)2HPO4                       | -            | -                  | 0.55                | -                    |
| pH                               | 6.5          | 6.5                | 6.5                 | 6.5                  |

References: *[8]***[3]***[9]

2.4 Reducing sugar and glucose content analysis
Reducing sugar was analyzed using DNS (dinitrosalicylic acid) method by Miller [10]. 500 µL sugar solution (dilution factor = 2000) and 750 µL DNS were mixed and incubated at 100 °C for 15 minutes. The mixed solution then was precooled in the ice water bath for 10 minutes prior to absorbance measurement by spectrophotometer at wavelength 540 nm.

2.5 Selection of culture medium for fermentation by *Leuconostoc sp*
To obtain a suitable medium for fermentation of liquid sugar by *Leuconostoc sp*, 4 type media were selected. The content of each medium was shown in Table 1. The strain was inoculated for preculture in each medium (@ 5 mL) in a capped tube to minimize air circulation. The inoculated medium was incubated for 24 hours with agitation 150 rpm and temperature 37 °C. Ten percent of preculture was then inoculated into a fresh medium containing 20% of sugar liquid of cassava. Fermentation was conducted for 24, 48, 72, 120, and 168 h.

2.6 HPLC analysis of glucose and succinic acid content
The concentration of succinic acid, glucose, and other organic acids was analyzed by an HPLC system. Isocratic system using H2SO4 5 mM (flow rate 0.6 µL) as mobile phase and Aminex column as stationary phase were used in the analysis. The temperature of the column was set at 60 °C with the injection volume was 10 µL. RID was used as detector for analysis of these samples.

3. Result and Discussion
3.1 Enzymatic hydrolysis of starch by crude glucoamylase produced by *Aspergillus awamori KT11*
In the present study, non-simultaneous (separation) saccharification fermentation was used for biosuccinic acid production. Starch, a polysaccharide material, was breakdown into more digestible sugar for microorganisms (glucose and/or oligosaccharide) using glucoamylase prior to use in the fermentation step. Several combinations of enzyme and substrate (E/S) treatment were selected for saccharification to obtain the most effective and efficient method for sugar production. Based on enzyme activity analysis, glucoamylase powder from Glutech has activity 16.9 U/mL when diluted in 9 mL aquadest (1:9). The enzyme has moderate thermophilic characteristics with the optimum reaction temperature at 60 °C. Initial high concentration of starch (25% w/v) was used to achieve high yield and
full utilization of substrate [2]. Among the combination treatment (Figure 1) the two highest total reducing sugar revealed at combination E/S (1/1) and (1/3), with the concentration 61.3 and 62.3 g L⁻¹, respectively at 48 hours. Under these conditions, a saccharification rate of approximately 25% was obtained. Many factors can cause this low conversion of starch into glucose. Unsuitable gelatinization process, such as temperature, and utilization of unpurified enzymes are two factors that cause low yield on enzymatic hydrolysis of starchy materials [5]. The reducing sugar concentration tends to decrease after 48 h, except for treatment E/S (1/9) which gradually increased up to 43.9 g L⁻¹ until 72 hours.

![Figure 1. Total reducing sugar of hydrolysate of cassava starch (var. Kristal merah)](image)

Based on HPLC analysis of sugar content in the hydrolysate, three different sugars were found as products: glucose, maltose, and maltotriose (Table 3). In all treatment, glucose revealed as a major product of the enzymatic hydrolysis of starch by glucoamylase. In this study, since glucoamylase, *Glutech* is an unpurified enzyme product, various products of sugar were produced. The glucose accumulation in hydrolysate (1/3) after 96 h reached 47.71 g L⁻¹ which was slightly higher compared with treatment (1/1), that used the highest enzyme loading (50%). The chromatogram of hydrolysate in various treatment can be seen in Figure 3.

Some study has reported the utilization of low-cost carbon sources for fermentation of succinic acid. Akhtar and Idris [11] evaluated for the production of succinic acid via simultaneous saccharification and fermentation (SSF) using an anaerobic microbe, *A. Succinogenes* ATCC 55618 and oil palm empty fruit bunch (EFB) as a carbon source. Dessie et al., [12] also reported the utilization of fruit and vegetable wastes for saccharification in the solid-state fermentation. Chen et al., [2] reported the production of succinic acid from cassava starch and raw cassava instead of glucose by *Escherichia coli* NZN111. However, the utilization of local cassava starch for succinic acid production by LAB has not been reported to date.

| Treatment | Incubation time (h) | Sugar content (g/L) | glucose | maltose | maltotriose |
|-----------|---------------------|---------------------|---------|---------|-------------|
| E/S (1:1) | 48 h                | 35.22               | 4.30    | 2.94    |
|           | 96 h                | 46.77               | 4.87    | 3.30    |
| E/S (1:3) | 48 h                | 43.59               | 3.44    | 1.83    |
|           | 96 h                | 47.71               | 4.30    | 2.94    |
| E/S (1:4) | 48 h                | 35.14               | 4.15    | 2.18    |
|           | 96 h                | 37.06               | 4.28    | 1.50    |
| E/S (1:9) | 48 h                | 23.01               | 5.99    | 3.01    |
|           | 96 h                | 32.02               | 2.87    | 1.45    |
Figure 2. HPLC Chromatogram of hydrolisate of cassava starch (var. kristal merah) in various treatment E/S: 1/1 (a); 1/3 (b); 1/4 (c); and 1/9 (d).

3.2. Fermentation of starch hydrolysate by Leuconostoc sp for succinic acid production

Figure 3 Microbial growth during fermentation of Leuconostoc sp; a) kristal merah; b) revita
After completion of enzymatic hydrolysis of starch on the optimum incubation time (48 hours), the hydrolyzed products were added into fermentation medium for further process. Culture medium is known to play an important role in maintaining cellular metabolism and enzyme activities during fermentation [3]. In the present study, we selected three types of mineral medium which specific for lactic acid bacteria, and also MRS medium as a positive control for bacterial growth. Medium 2 [8] has been reported as a suitable medium for *Lactobacillus salivarius* L29 and allows the attainment of high levels of cell mass and lactic acid production. Medium 3 [3] has been used as the growing medium for *Enterococcus flavescens* on succinic acid production. Medium 4 supported with sweet potatoes as carbon sources [9] has been reported as the developed medium for *Lactobacilli*. Twenty percent of sugar liquid from the previous study was added into each medium and inoculated with 10% bacterial inoculum (24 h). As shown in Figure 3, all medium was a suitable medium for *Leuconostoc* to grow. When using hydrolysate of var. kristal merah and var. revita, the growth rate in all three media was higher compared with MRS medium. The optimum growth rate was achieved at 24 h for medium 4 and start to enter the stationary phase after 24 h.

![Figure 4. Total succinic acid production during fermentation by *Leuconostoc* sp: a.) var. kristal merah, and b.) var. Revita](image)

Among four fermentation medium tested, the highest production of 3.4 g L\(^{-1}\) succinic acid was obtained in 72 h using medium 3 (M3) for var. kristal merah and 3.6 g L\(^{-1}\) succinic acid in 48 h using medium 2 (M2) for var. revita (Figure 4). There was no increase in amount of succinic acid produced on subsequent increase in incubation time. The content of mineral medium M3 is more complex than M2, with only contain minimum media such as yeast extract and peptone as protein sources, potassium phosphate and manganese sulfate as mineral sources [5] [8].

Metabolically engineered *E. coli* NZN111, constructed by disruption of ldhA and pflB encoding lactate dehydrogenase (LDH) and pyruvate-formatelyase (PFL), has been reported as a potential succinate producer with formation 106.17 g L\(^{-1}\) of succinic acid when the liquefied crude cassava powder was used directly in SSF, 106.17 g L\(^{-1}\) \[2\]. *Actinobacillus succinogenes* also has been reported on succinic acid production with maximum concentration, (33.4 g L\(^{-1}\)), yield (30.47 g g\(^{-1}\) substrate) and productivity 0.69 g L\(^{-1}\) h\(^{-1}\) when using pretreated empty fruit bunch sample as a carbon source [11].
Succinic acid production by *Leuconostoc sp* (only 3.6 g L\(^{-1}\)) is lower than 2 recent studies above. Many anaerobic and facultative anaerobic microorganisms such as *Leuconostoc sp*. ferment carbohydrates to a mixture of acids, e.g., formate, acetate, lactate, and succinate as end products. Phosphoenolpyruvate (PEP) is one of the central intermediates during the mixed acid fermentation. It is either converted into pyruvate resulting acetate, formate, ethanol, and lactate or it is converted into oxaloacetate resulting succinate and propionate as end products via the reversible arm of tricarboxylic acid (TCA) cycle depend on environmental factors [3]. To boost succinic acid production by *Leuconostoc sp*, the effect of various environmental factors should be conducted. Beside succinic acid, the microbe also produced organic acids such as citric acid, formic acid, and acetic acid (less than 3 g L\(^{-1}\)). Lactic acid was produced in the highest concentration, up to 10.62 g L\(^{-1}\).

This is first study regarding succinic acid production by *Leuconostoc sp*. Many studies reported anaerobic microorganisms such as succinic acid production. *Actinobacillus succinogenes*, *Mannheimia succiniciproducens* (Lee et al., 2003), *Anaerobiospirillum succiniciproducens* (Meynial-Salles, et al. 2008) and *Corynebacterium crenatum* (Chen et al. 2013). Facultative microorganisms can produce organic acids such as succinic acid under aerobic and anaerobic condition.

4. Conclusion

Fermentation of the liquid sugar as a carbon source in various media showed that *Leuconostoc sp* could produce highest succinic acid in medium SPM, with concentration 3.4 g L\(^{-1}\) in 72 h for var. kristal merah and 3.6 g L\(^{-1}\) g in 48 h for var. revita. The other valuable organic acids, such as lactic acid, citric acid, formic acid, and acetic acid were also formed during fermentation process, with concentration less than 3 g L\(^{-1}\). The results suggested that cassava starch of 2 local strains (var. kristal merah and var. revita) has the potential to be an alternative substrate for efficient and economic valuable organic acids production such as succinic acid.

5. References

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