

Production of high concentration of L-lactic acid from oil palm empty fruit bunch by thermophilic *B. coagulans* JI12

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Abstract

Thermophilic *Bacillus coagulans* JI12 was used to ferment hemicellulose hydrolysate obtained by acid hydrolysis of oil palm empty fruit bunch (EFB) at 50°C and pH6, producing 105.4 g/L of L-lactic acid with a productivity of 9.3 g/L/h by fed batch fermentation under unsterilized conditions. Simultaneous saccharification and fermentation (SSF) was performed at pH5.5 and 50°C to convert both hemicellulose hydrolysate and cellulose-lignin complex in the presence of Cellic Ctec2 cellulases using yeast extract (20 g/L) as the nitrogen source, giving 114.0 g/L of L-lactic acid with a productivity of 5.7 g/L/h. The SSF was also conducted by replacing yeast extract with equal amount of dry Bakers' yeast, achieving 120.0 g/L of L-lactic acid with a productivity of 4.3 g/L/h. To the best of our knowledge, these lactic acid titers and productivities are the highest ever reported from lignocellulose hydrolysates.

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1. Introduction

Lactic acid is an industrially important chemical, which was first discovered and isolated from sour milk in 1780 by a Swedish chemist Carl Wilhelm Scheele and its structure was elucidated by a German chemist Johannes Wislicenus in 1873. Traditionally, lactic acid is used in food industry for processing meats as preservative and for salad dressing, in the pharmaceutical industry as a pH regulator, in metal sequestration, in the detergent industry as a descaling agent and in the dairy industry for improving the performance of farm animals (<http://www.lactic-acid.com>). Lactic acid is a chemically attractive molecule with both hydroxyl and carboxylic acid functional groups, which makes it an ideal platform molecule for the production of many value-added chemicals including poly lactic acid (PLA), lactide (3, 6-dimethyl-1, 4-dioxane-2,5-dione), 1, 2-propanediol, 2, 3-pentanedione, acrylic acid and pyruvic acid [1].

The chemical synthesis of lactic acid from plant biomass containing cellulose and water uses lead as the catalyst giving a racemic mixture containing both D- and L-isomers with a total yield of 60 % [2, 3]. Lactic acid production by microbial fermentation is more commercially attractive as it provides a cleaner, greener and more economical alternate. In 1856, Louis Pasteur discovered *Lactobacillus* and its role in lactic acid production. In 1895, a German company Boehringer Ingelheim started the industrial production of lactic acid by fermentation [4]. The demand for lactic acid is expected to increase at a compound annual growth rate (CAGR) of 18.6% from 2015 to reach USD 3.82 billion by 2020. The demand for polylactic acid market is expected to grow at a CAGR of 20.9% from 2015 to reach USD 5.16 billion by 2020. Nature Works LLC (140,000 ton, USA), Purac (100,000 ton, Netherlands), Pyramid Bioplastics GubenGmbH (60,000 ton, Germany), Archer Daniels

Midland Company (USA) and Henan Jindan (China) are the major companies for lactic acid and PLA production (<http://www.marketsandmarkets.com/Market-Reports/polylacticacid-387.html>) [5, 6].

Traditionally, lactic acid is produced using glucose, starch (potato, cassava, wheat, rice and sorghum) and sucrose as the carbon sources, which not only increases the lactic acid production cost but also competes with global food and feed supplies [7]. Therefore, lactic acid production using inexpensive carbon sources is of a great commercial attraction. The great abundance of lignocellulose biomass on earth makes it an ideal feed stock for lactic acid production. In south East Asia, Indonesia and Malaysia are leading producers of palm oil. Production of every 1 kg of crude palm oil will generate approximately 4 kg of waste biomass and one third of it is empty fruit bunch (EFB). It has been reported that 22 million ton of EFB in Indonesia and 19 million ton of EFB in Malaysia are discarded annually (https://www.asiabiomass.jp/english/topics/1001_03.html). This large amount of EFB would be an ideal biomass resource for producing lactic acid, which has a huge market demand due to the rapid growth of poly lactic acid (PLA) industry [8-11]. EFB is composed of 34.3% glucan, 21.8% xylan, 21.5% lignin and 22.4% others [12]. Depolymerization of EFB to produce fermentable sugars for microbial fermentation is the prerequisite step. Depolymerization of EFB can be conducted by both enzymes and chemical hydrolyses. Enzymatic depolymerization produces clean sugars without any inhibitors due to mild conditions. On contrary, chemical hydrolysis is conducted at high temperatures ($>100^{\circ}\text{C}$) producing sugar degradation products such as furfural and hydroxyl methyl furfural along with the fermentable sugars. The concentration of fermentable sugars obtained after acid hydrolysis is high which is industrially attractive but the sugar degradation products formed are inhibitory to microbes. The hydrolysate contains predominantly hemicellulose (C5) sugars. The left over solid is cellulose-lignin complex, which needs to be further converted to

glucose by cellulases before it can be utilized by microbes to produce lactic acid [6]. We have isolated several thermophilic *Bacillus coagulans* strains including *B. coagulans* WCP10-4, *B. coagulans* JI12 and *B. coagulans* C-106 that can produce L-lactic acid from all lignocellulose sugars at 50°C under non-sterilized conditions [13-16]. In a simultaneous detoxification, saccharification and co-fermentation of EFB hydrolysate using *B. coagulans* JI12, lactic acid reached 80.6 g/L with a productivity of 3.4 g/L/h [17]. Yeast extract and peptone are routinely used nitrogen sources. Yeast extract has high nitrogen content in the form of proteins and free amino acids along with high content of vitamin B which is known to enhance lactic acid production (<http://www.solabia.com/solabia/content/NT0000440A.pdf>) [7]. However, yeast extract is expensive and can account up to 38 % of the raw material cost [18]. Therefore, a cheaper nitrogen source and higher lactic titer are needed to further reduce the lactic acid production cost. A lot of researchers have been reported to replace expensive yeast extract with inexpensive nitrogen sources such as agricultural wastes (flour of red lentils), gluten, corn steep liquor, Bakers' yeast and inorganic $(\text{NH}_4)_2\text{SO}_4$ for lactic acid production [19-22, 27].

Here we report the efficient conversion of EFB to L-lactic acid at high concentration (>100 g/L) by thermophilic *B. coagulans* JI12 using inexpensive dry Bakers' yeast to replace expensive yeast extract as the nitrogen source. To the best of our knowledge, the lactic acid titers and productivities achieved here are the highest ever reported from lignocellulose hydrolysates.

2. Materials and methods

2.1. Milling of EFB

EFB was obtained from an oil palm field in Malaysia. The intact raw EFB was first passed through a wood crusher (SHAPU, China) to get loose and fluffy long fiber for easier grinding in the successive step. The long EFB fiber was grinded to 8 mm, 4 mm and finally 2 mm particles using a RETSCH SM 100 cutting mill (Retsch, Düsseldorf, Germany). The final 2 mm particles were used for the acid-catalyzed hydrolysis to get fermentable sugars.

2.2. Acid-catalyzed hydrolysis of EFB

Acid-catalyzed hydrolysis of EFB was performed in a 20 L Parr reactor (Parr, Illinois, USA). EFB powder (2.0 kg) was mixed with tap water (10 L) containing H_2SO_4 (2 %, w/v) and H_3PO_4 (0.8%, w/v). The mixture was heated at 130°C for 36 min with stirring at 250 rpm. Afterwards, the mixture was cooled down to 50°C by circulating cold water and then collected into a vertical tank (Nalgene, USA). After reaching room temperature, the hydrolysate was separated from the cellulose-lignin complex by centrifugation (Beckman Coulter, USA) at 5000 rpm for 10 min. The hydrolysate was then concentrated at 50°C by evaporation to reach the desired sugar concentrations for lactic acid fermentation. The sugar concentrations were analyzed by HPLC after appropriate dilutions.

2.3. Detoxification and neutralization of EFB hydrolysate and cellulose-lignin complex

The concentrated hydrolysate was detoxified with charcoal to remove carbohydrate degradation products and lignin degradation products. For every 1 L of hydrolysate 20 g of activated charcoal was added and the mixture was incubated for 8 h at room temperature with stirring at 150 rpm. After that, the hydrolysate was neutralized by adding $\text{Ca}(\text{OH})_2$ to increase

the pH to 5.5-6.0. The charcoal and precipitated CaSO_4 were removed by centrifugation at 5000 rpm for 20 min (Beckman Coulter, USA). The hydrolysate was preserved at 4°C till further use. This detoxified hydrolysate was directly used as the carbon source for lactic acid fermentation without sterilization.

2.4. Treatment of cellulose-lignin complex prior to use for fermentation

The collected cellulose-lignin complex was washed 3-5 times with tap water to remove the entrapped water-soluble substances. The washed cellulose-lignin complex was then dried in a hot air oven at 80°C for 48 h. This dried cellulose-lignin complex was powdered in a blender and used for simultaneous saccharification and fermentation (SSF) to produce lactic acid. The compositions of cellulose-lignin complex and the xylooligomers in the detoxified hydrolysate and the unused sugars from the leftover cellulose-lignin complex after fermentation were analyzed following the NREL protocols [23].

2.5. Lactic acid fermentation

Lactic acid fermentation was performed in 2 L fermenters (Sartorius, Germany). The broth for lactic acid fermentation was freshly prepared by mixing 0.5 L of hemicellulose hydrolysate, 20 g/L of yeast extract and 2 g/L of $(\text{NH}_4)_2\text{SO}_4$ followed by inoculation of seed culture, which was prepared in 200 mL mineral salts medium containing 5% of xylose and 1% of yeast extract in 500 mL conical flasks at 50°C and 200 rpm overnight. The fermentation was conducted without bubbling any inert gases into the system and medium was constantly agitated at 350 rpm. The pH was controlled at 6.0 by adding 35% (w/v) of $\text{Ca}(\text{OH})_2$. After 10 h, 100 ml of hydrolysate (20 g of fermentable sugars) was supplemented for fed-batch fermentation. In the case of SSF, after 10 h, stirring was stopped temporarily, 75 g of cellulose-lignin complex and Cellic Ctec2 (50 FPU Ctec₂/g of cellulose) cellulase (Novozymes, Bagsværd, Denmark) were added and the stirring was then resumed. The pH of

the medium was controlled at 5.5. Other conditions were the same as those used for the fermentation of hemicellulose hydrolysate. The SSF was also conducted by replacing yeast extract with equal amount of dry Bakers' yeast (Wenzhou Kaipu Biochemistry, China) while keeping all other conditions the same. All the results were the average of 2 parallel experiments.

2.6. Analytical methods

Xylose, glucose, arabinose, lactic acid, acetic acid and furfural were analyzed by HPLC (LC-10AT, refractive index detector SPD-10A, Shimadzu, Kyoto, Japan) equipped with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) at 50°C. The mobile phase was 12 mM H₂SO₄ at 0.65 mL/min.

3. Results and discussion

3.1. Pretreatment of EFB and detoxification of cellulose-lignin complex

After the acid-catalyzed hydrolysis of EFB, the brown hemicellulose hydrolysate (7 L) was separated from the cellulose-lignin complex (1.4 kg, dry weight) by simple filtration. The composition of the hydrolysate was shown in Table 1. The hydrolysate contained 0.283 kg of fermentable sugars, which were composed of primarily xylose (33.26 g/L) and arabinose (1.9 g/L). After detoxification with charcoal, no furfural and 5-hydroxymethyl furfural were detected by HPLC, indicating the complete removal of these inhibitors. The cellulose-lignin complex was washed with water to remove the toxic substances on the solid surface. The cellulose-lignin complex contained 40.9% of cellulose and small amount of xylose (2.7%) in addition to lignin. The total sugar yield based on the acid hydrolysate and cellulose-lignin

complex was 90.0%, which equals to 0.45 g sugars /g EFB. The analysis following NREL protocols indicated the presence of 4.5 g/L xylooligomers in the detoxified hydrolysate.

3.2. Lactic acid fermentation

The fed-batch fermentation of hemicellulose hydrolysate by *B. coagulans* JI12 was conducted at pH 6.0 and 50°C with an initial fermentable sugar concentration of 100.0 g/L using yeast extract as the nitrogen source (Fig 1). The strain could metabolize both pentose and hexose sugars simultaneously. After 8.5 h, more hemicellulose hydrolysate was supplemented to make the total fermentable sugars reach 120.0 g/L. All the sugars were consumed within 11.5 h, producing 105.4 g/L of L-lactic acid at a productivity of 9.3 g/L/h and a yield of 87.9%.

When SSF of whole EFB hydrolysates was conducted, hemicellulose hydrolysate (100 g/L) was mixed with cellulose-lignin complex containing 20.0 g/L of cellulose and 1.3 g/L of xylan as the carbon sources. When yeast extract (20 g/L) was used as the nitrogen source, 113.9 g/L of lactic acid was produced within 20 h at a productivity of 5.1 g/L/h and a yield of 94.9% (0.47 g lactic acid/g EFB)(Fig 2). When the yeast extract was replaced with equal amount of dry Bakers' yeast, 120 g/L of lactic acid was produced within 28 h at a productivity of 4.3 g/L/h and a yield of 99.9 % (0.49 g lactic acid/g EFB) (Fig 3). Analysis of the leftover cellulose-lignin complex after fermentations did not show any leftover sugars indicating the complete depolymerisation of cellulose by cellulases and subsequent utilization by *B. coagulans* JI12. Our group has reported 80.6 g/L lactic acid from EFB [17], here we increased it to 120 g/L, which is, to the best of our knowledge, the highest lactic acid titer from lignocellulose hydrolysate ever reported in literature.

It is noticed that for the SSF, the lactic acid yield was almost near to its theoretical yield of 100% in the case of using dry Bakers' yeast as the nitrogen source, which might be

attributed to the sugars contained in the dry Bakers' yeast [24]. In the case of using Baker's yeast to replace yeast extract, almost the same lactic acid titer and yield were achieved but the productivity was slightly lower in the former case. This might be due to the gradual release of the nitrogen substances from the yeast cells affecting the productivity. Therefore, the replacement of yeast extract with cheaper dry Bakers' yeast as the nitrogen source for lactic acid production is feasible in terms of the almost same lactic acid titer, yield and only slightly lower productivity. As the fermentation was conducted at 50°C, at which the dry Bakers' yeast cells could not grow anymore while began to autolyze to release nutrients, making the fermentation conducted smoothly without the concern of sugars being consumed by the yeast cells, which are usually most alive at 30°C. Yeast extract is produced from Bakers' yeast or brewer's yeast through autolysis at a solid recovery yield of 42-62% [24]. The yeast extract has been reported to account for 38% of the total medium cost for lactic acid production [17, 24-26]. It has been estimated that the use of dry yeast cells to replace yeast extract would reduce the cost of nitrogen source from 38% to 23% of the total medium cost [27]. The combined use of lignocellulose sugars and dry yeast cells and thermophilic *Bacillus coagulans* strains is expected to be a commercially attractive way to produce L-lactic acid. Effort will be put to increase the acid tolerance of the thermophilic strains to make the fermentation conduct at free pH without the need of neutralization during the fermentation to further reduce the lactic acid production cost.

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Table 1. Compositions of EFB hydrolysate obtained from the Parr reactor (The standard errors were <5%)

No	Component	Concentration (g/L)
1	Glucose	5.2
2	Xylose	33.3
3	Arabinose	1.9
4	Acetic acid	3.8
5	Furfural	3.9

FIG. 1 A summary of value-added chemicals that can be produced from lactic acid (Corma et al., 2007)

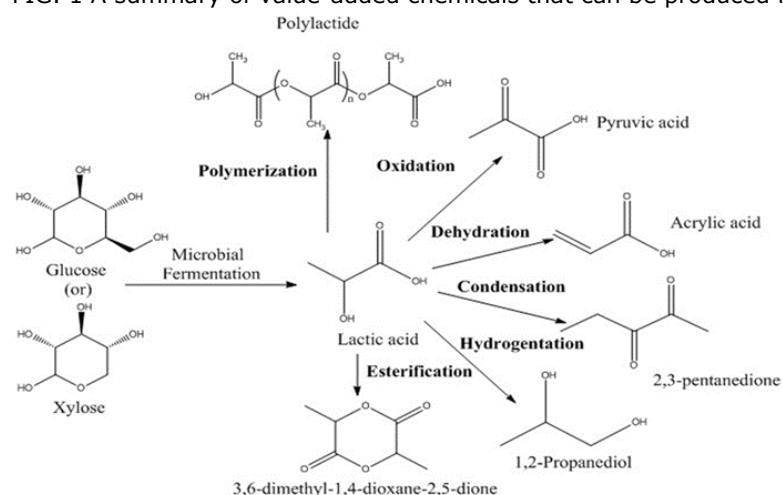


FIG.2 Time courses of L-lactic acid production (O) from EFB hemicellulose hydrolysate by *Bacillus coagulans* JI12. Fermentation conditions: 20 g/L of yeast extract, pH 6.0, 50 °C, 35% Ca(OH)₂ as the neutralizing agent. Xylose (□), glucose (X), arabinose (Δ). The final xylose concentration was 13.9 g/L which is close to the theoretical value of 14.31 g/L. The average standard error was ± 5.21.

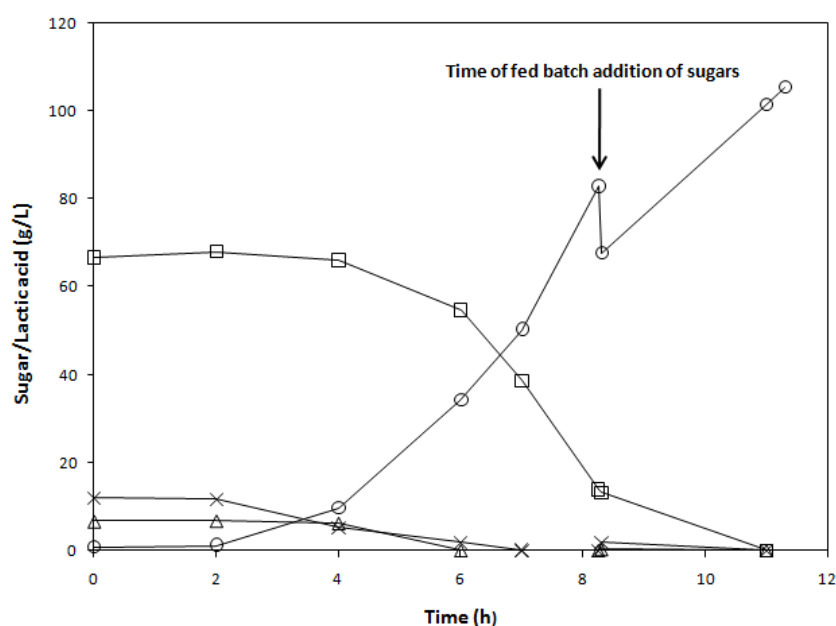


FIG.3 Time courses of L-lactic acid production (O) in SSF by *Bacillus coagulans* JI12. Fermentation conditions: 20 g/L of yeast extract, pH 5.5, 50 °C, 35% Ca(OH)₂ as the neutralizing agent. Xylose (□), glucose (X), arabinose (Δ). The average standard error was ± 5.89.

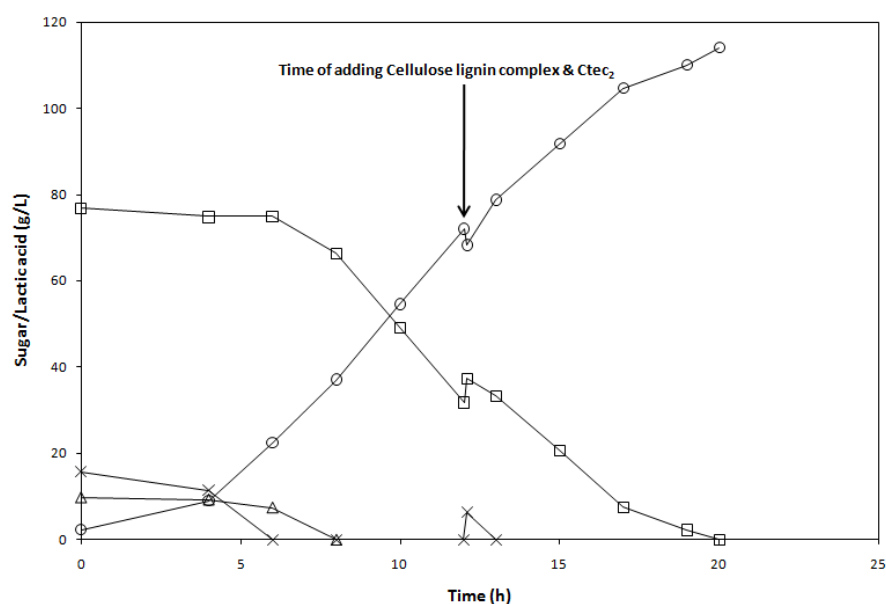


FIG. 4 Time courses of L-lactic acid production (O) in SSF of LA using *Bacillus coagulans* JI12. Fermentation conditions: 20 g/L dry Bakers' yeast, pH 5.5, 50 °C, 35% Ca(OH)₂ as the neutralizing agent. Xylose (□), glucose (X), arabinose (Δ). The average standard error was ± 3.37 .

