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Efficient production of lactic acid from cellulose and xylan in sugarcane bagasse by newly isolated *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* through simultaneous saccharification and co-fermentation process

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ABSTRACT

Sugarcane bagasse is one of the promising lignocellulosic feedstocks for bio-based chemicals production. However, to date, most research focuses mainly on the cellulose conversion process, while hemicellulose remains largely underutilized. The conversion of glucose and xylose derived from lignocellulosic biomass can be a promising strategy to improve utilization efficiencies of resources, energy, and water, and at the same time reduce wastes generated from the process. Here, attempts were made to convert cellulose and xylan in sugarcane bagasse (SB) into lactic acid (LA) through a pre-hydrolysis and simultaneous saccharification and co-fermentation (SScF) process using newly isolated *Lactiplantibacillus plantarum* TSKKU P-8 and *Levilactobacillus brevis* CHKKU N-6. The process yielded 91.9 g/L of LA, with a volumetric productivity of 0.85 g/(L·h). This was equivalent to 137.8 ± 3.4 g-LA, a yield on substrate (pretreated SB) of 0.86 g/g, and a productivity of 1.28 g/h, based on a final volume of 1.5 L. On the other hand, pre-hydrolysis and simultaneous saccharification and fermentation (SSF) process using *La. plantarum* TSKKU P-8 as a monoculture gave 86.7 ± 0.2 g/L of LA and a volumetric productivity of 0.8 g/(L·h), which were equivalent to 104.8 ± 0.3 g-LA, a yield on substrate of 0.65 g/g, and a productivity of 0.97 g/h, based on a final volume of 1.2 L. Mass balance calculated based on mass of raw SB entering the process showed that the SScF process improved the product yield by 32% as compared with SSF process, resulting in 14% improvement in medium-based economic yield.

1. Introduction

Lactic acid (LA) is one of industrially important organic acids, owing to its versatile applications in the food industry (as food acidulant, flavor enhancer, food preservative, antioxidant), chemical industry (as descaling agent, pH regulator, green solvent, cleaning agent, precursor for polylactic acid), cosmetic industry (as moisturizer, lightning agent, anti-acne agents, humectants), medicine and pharmaceutical industry (as dialysis solution, mineral preparation, immune stimulant), etc. [1,2]. Demand for LA has

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been increasing in the past decades. In 2013, the global market for LA was 714 kt [3] and this rose to 1220 kt in 2016. Recently, it was projected that this would increase to 1960 kt in 2025, with an annual growth of 16.2% [4]. Due to this vigorously increasing demand, development of cost-effective and efficient process for LA production has been extensively investigated and increasing attention has been paid to the use of fermentation process as it offers several advantages over LA synthesis through a chemical route, e.g., high LA yield, and requirement of less energy and lower temperature [2].

Around 90% of LA available in the market is produced by fermentation of sugars. Lactic acid bacteria (LAB) are the most widely used LA producers due to their ability to produce LA at high yield during a short fermentation time, using simple sugars, e.g., glucose, fructose, lactose, as the carbon source [2]. However, due to the high cost of refined sugars, e.g., 904 USD/ton for food grade glucose [5], the use of these leads to high production cost [2,6], and thus the selling price [4]. Furthermore, the use of sugars in non-food industry could compromise the food security [2]. To alleviate these problems, non-food and low-cost biomasses, e.g., starchy and lignocellulosic biomasses, have been investigated as alternative LA feedstocks. Among others, sugarcane bagasse (SB) holds a high potential, owing to its high cellulose content and availability. This biomass is readily available in many countries, including Thailand, which generates around 15 million tons of SB annually [7]. Due to its high cellulose content (*ca.* 25–47%, w/w) [8–10], saccharification of SB yields high glucose concentration that could be a good carbon source for LA fermentation. Fermentative production of LA from SB is conventionally carried out using separate hydrolysis and fermentation (SHF) process, through which the biomass is pretreated, hydrolyzed using dilute acid or enzymes, and the resulting sugars are fermented into LA. This sequential process, however, is known to have some serious limitations, e.g., risks of feedback and substrate inhibitions and prolonged period of biomass saccharification, that affect LA yield and productivity [11].

Problems related to the use of SHF process could be mitigated using simultaneous saccharification and fermentation (SSF) process, particularly the productivity and production cost [11,12]. SSF process is conducted using a single bioreactor, in which biomass saccharification and fermentation of the resulting sugars occur simultaneously. SSF has been shown to offer several advantages, e.g., reduced capital and operating costs, preventing accumulation of sugars in the system, and avoiding feedback and substrate inhibitions. It has also been shown to give high hydrolysis and product yields during a short processing time [2,3]. Recently, Maslova et al. [13] showed that SSF process enhanced LA titer, yield, and productivity by over 17% compared with SHF process. These improvements were also reported by Berlowska et al. [14] using sugar beet pulp as the substrate. However, a literature survey revealed that most SSF processes utilize cellulose as the fermentation substrate and hemicellulose, which is also present in biomass at considerable proportions (19–33%) [10], is usually discarded. Since hemicellulose comprises xylan and other carbohydrates that can also be converted into LA, the use of cellulose as a single substrate could lead to the loss of economic value of these sugars. In this regard, simultaneous saccharification and co-fermentation (SScF) process, in which two or more sugars are co-utilized [3], can be used. Co-utilization of sugars during LA fermentation can be achieved using either a monoculture or mixed cultures. However, co-cultivation of two LAB strains has been demonstrated to give high LA titer and productivity. For instance, *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* (formerly known as *Lactobacillus plantarum* and *Lactobacillus brevis*, respectively) were co-cultivated to ferment corn stover, yielding 31.2 $\pm$ 0.3 g/L of LA, with the overall yield of 0.78 g/g-substrate [15]. In another study, *Bacillus coagulans* LA-15-2 and *Lacticaseibacillus rhamnosus* (formerly known as *Lactobacillus rhamnosus*) LA-04-1 were co-cultivated to produce LA from cassava bagasse under SScF process, yielding as high as 112.5 g/L of LA and a yield of 0.88 g/g-substrate (starch, cellulose, hemicellulose) [16]. However, despite holding a high potential for LA production, the use of SScF process has been scarcely reported in the literature. Furthermore, to the best of our knowledge, there has been only a handful of works investigating LA production from SB through SScF process. Therefore, the present study was designed to improve efficiency of SScF process, aiming mainly to convert cellulose and xylan in SB into LA. Potential glucose- and xylose-utilizing LAB were isolated and characterized. Conditions for enzymatic saccharification of alkaline-pretreated SB were statistically optimized and subsequently used in the SSF and SScF processes. Based on the results, mass balance was calculated, and medium-based economic analysis was conducted to assess the feasibility of the SScF process.

2. Materials and methods

2.1. Isolation and identification of LAB

Potential LAB capable of producing LA from glucose and xylose were isolated from Thai traditional fermented fish (Plasom) and fermented pork (Nham). Isolations were performed by suspending 1 g (wet basis) of the samples in 10 mL of 0.85% (w/v) NaCl solution, 10-fold serial dilution of the samples, and cultivation on De Man, Rogosa, and Sharpe (MRS) agar containing 1% (w/v) calcium carbonate (KEMAUS, Cas. No. 471-34-1) at 37 °C for 72 h. MRS agar comprised 10 g/L peptone (HiMedia laboratories, Cas. No. 68990-09-0), 5 g/L yeast extract (HiMedia laboratories, Cas. No. 8013-01-2), 1 g/L Tween 80 (Ajax Finechem, Cas. No. 9005-65-6), 5 g/L sodium acetate (Loba Chemie, Cas. No. 127-09-3), 1.84 g/L citric acid (RCI Labscan, Cas. No. 5949-29-1), 0.1 g/L $MgSO_4$ (RCI Labscan, Cas. No. 10034-99-8), 0.05 g/L $MnSO_4$ (Loba Chemie, Cas. No. 10034-96-5), 2 g/L K_2HPO_4 (QRëC, Cas. No. 7758-11-4), 20 g/L of glucose (Ajax Finechem, Cas. No. 50-99-7) or xylose (TCI Chemicals, Cas. No. 58-86-6), and 12 g/L agar (HiMedia Laboratories, Cas. No. 9002-18-0). The cultivation was repeated until single colonies were obtained. One potential glucose-utilizing LAB and another one utilizing xylose were selected based on the diameter of clear zone produced during the incubation. Identification of the isolates were conducted at the Thailand Bioresource Research Center (TBRC), Pathum Thani, Thailand, using 16S rDNA sequence analysis. According to the TBRC report, two universal primers were used, i.e., 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) or 800R (5′-TAC CAG GGT ATC TAA TCC-3′) and 518F (5′-CCA GCA GCC GCG GTA ATA CG-3′) or 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) [17]. The identification was carried out using the BLASTN program against 16S rDNA sequence database of validly published prokaryotes.

After the identifications, the two LAB were tested for their ability to ferment glucose and xylose into LA. This was performed using

MRS medium containing 20 g/L of glucose, or 20 g/L of xylose, as the carbon source. Sterile media were inoculated with each LAB at 0.5 unit of optical density at 600 nm (OD_{600}), and incubated at 37 °C, initial pH 6.5, 150 rpm, for 72 h. Samples were taken to determine cell growth (in terms of OD_{600}), sugar utilization, and products formation. Another experiment was conducted using MRS medium containing *ca.* 60 g/L of glucose and 20 g/L of xylose as the mixed carbon sources (the concentrations of glucose and xylose were adjusted to simulate SB hydrolysate obtained after enzymatic saccharification (see Section 2.5)). The fermentation was carried out under the same conditions as above, but for an incubation time of 84 h.

2.2. Inoculum preparation and microbial culture preservation

Inocula of glucose-utilizing and xylose-utilizing LAB were prepared by transferring one full loop of the bacteria growing on MRS agar into 500-mL Erlenmeyer flasks containing 100 mL of MRS broth containing appropriate carbon sources. The cultures were then incubated at 37 °C, 150 rpm, for 12–15 h before use. Stock cultures of both LAB were prepared by growing the microorganisms in MRS broth containing appropriate carbon source at 37 °C for 12 h. Then, the cultures were centrifuged at 10,000 rpm for 4 min, and the cells were washed twice with sterile distilled water. The cell pellets were then resuspended in MRS broth containing 20% (v/v) pure glycerol and stored at −20 °C.

2.3. Sugarcane bagasse and enzymes

SB was provided by Mitr Phu Viang Sugar Co. Ltd., Nong Ruea, Khon Kaen, Thailand. It was sun-dried for 4–5 days, followed by drying in a hot-air oven at 80 °C for 1–2 days. Dry SB was milled and sieved to obtain particles of 1–3.35 mm and stored in air-tight plastic containers at 25–30 °C until use. Compositions of SB are given in Table 1.

Commercial cellulase (Cellic® CTec3) was purchased from Novozyme, Denmark. It had cellulase activity of 240 filter paper unit (FPU) per mL. Commercial xylanase (iKnowZyme XL), with a xylanase activity of 380 U/mL, was purchased from Reach Biotechnology Co. Ltd., Bangkok, Thailand. Both enzymes were stored in screw-capped plastic bottles at 4 °C until use.

2.4. Pretreatment of sugarcane bagasse

SB was pretreated by alkaline-thermal methods following the method of Vásquez et al. [18]. SB was suspended in 4% (w/v) NaOH solution at a ratio 1 g to 20 mL, and heated in an autoclave at 121 °C for 30 min. After cooling to room temperature, the suspension was filtered through muslin cloth, and the solids were washed thoroughly with tap water to remove residual NaOH. The solids were then dried at 60 °C for 1–2 days to reduce its moisture content to *ca.* 10% (w/w) and stored in air-tight plastic containers at 25–30 °C until use.

2.5. Enzymatic hydrolysis of sugarcane bagasse

Conditions for enzymatic hydrolysis of SB were optimized using response surface methodology (RSM) with Box-Behnken design (BBD). Three factors were investigated, i.e., SB concentration (80–160 g/L), cellulase loading (20–80 FPU/g-SB), and xylanase loading (50–150 U/g-SB). Reducing sugars (glucose and xylose) production was used as the response. The design matrix comprising 17 experimental runs are shown in Table 2. The results obtained from each experimental run were used to develop a mathematical model to describe the relationship between the response and the variables, and to optimize the hydrolysis conditions. Analysis of variance (ANOVA) was used to identify the statistically significant terms of the model at a confidence level of 95% [19]. The experiment was carried out by suspending the designated amount of pretreated SB in 100 mL of sodium-citrate buffer (pH 4.8). After pre-warming the suspension at 50 °C for 5 min, filter sterilized (through 0.2-μm membrane) cellulase and xylanase were added at the designated loadings. The suspensions were then incubated at 50 °C for 24 h. Samples were taken at the beginning and the end of the incubation. The samples were centrifuged at 10,000 rpm for 10 min, and the supernatants were collected for the determination of reducing sugar concentration. A confirmation experiment was conducted under the predicted conditions in triplicate. The optimum conditions obtained in this experiment were subsequently used in the SSF and SScF processes for LA production.

2.6. LA production from alkaline-pretreated SB under SSF and SScF processes

For SSF process, sterile alkaline-pretreated SB (160 g) was suspended in 1 L of sterile distilled water contained in a 2-L bioreactor (Bioneer-neo-2L, B.E. Marubishi, Thailand). Then, filter sterilized cellulase and xylanase were added at the optimum loadings,

Table 1
Compositions of sugarcane bagasse before and after pretreatments.

Component	Raw SB	Alkaline-pretreated SB	% Removal
Cellulose (%, w/w)	39.8 ± 0.9	66.8 ± 0.5	29.5 ± 1.8
Hemicellulose (%, w/w)	19.5 ± 0.7	13.9 ± 0.3	70.2 ± 0.7
Xylan (%, w/w)	16.9 ± 0.6	12.0 ± 0.4	70.2 ± 0.6
Lignin (%, w/w)	22.2 ± 2.1	7.3 ± 0.7	85.9 ± 1.3

Table 2
Box-Behnken design (BBD) matrix and results for optimizing saccharification of alkaline-pretreated sugarcane bagasse.

Run	Pretreated SB (g/L)	Cellulase (FPU/g)	Xylanase (U/g)	Reducing sugar (g/L)
1	120	50	100	74.7
2	120	50	100	73.0
3	80	50	50	54.3
4	120	50	100	75.0
5	160	50	50	84.2
6	120	80	50	84.5
7	120	20	150	75.3
8	160	50	150	105.0
9	160	20	100	92.3
10	80	50	150	59.2
11	120	50	100	75.5
12	120	20	50	73.8
13	160	80	100	94.6
14	120	80	150	108.3
15	80	20	100	59.5
16	120	50	100	74.9
17	80	80	100	60.1

followed by hydrolyzing the suspension at 50 °C, pH 5.0 ± 0.2, 150 rpm, for 12 h. Then, the temperature of the suspension was lowered to 37 °C, and pH was adjusted to 6.5 using 10 M NaOH. Inoculum of TSKKU P-8 (*ca.* 200 mL) was then added into the bioreactor to reach 1 OD_{600} unit, and the fermentation was carried out at 37 °C, pH 6.5 ± 0.2 (controlled using 10 M NaOH and 1 M HCl), 150 rpm, for 108 h. As for SScF process, the fermentation was carried out using the same method and conditions as in the SSF experiment, but when glucose concentration in the fermentation broth decreased below 10 g/L (at around 60 h), inoculum of CHKKU N-6 (*ca.* 200 mL) was added into the bioreactor to reach 1 OD_{600} unit, and the fermentation was allowed to proceed under the same conditions until 108 h. Samples were taken regularly to determine glucose, xylose, LA, acetic acid, and ethanol concentrations. Volume of samples, as well as NaOH and HCl solutions used for pH control, were recorded to estimate the final volume of the fermentation broth. The experiments were conducted in duplicate, and the means and standard deviation of the means are reported.

2.7. Medium-based economic analysis

Medium-based economics was calculated in terms of economic yield (*EY*, $-LA/$-nutrients), which is defined as monetary unit (USD) of LA produced per monetary unit (USD) spent in fermentation medium. It was calculated using Eq. (1), which is a modified version of an equation proposed by Bustos et al. [20], where *LA* is the mass of LA produced (kg), *C* is the cost of each nutrient and raw SB used in the fermentation (USD/kg), *N* is the mass of each nutrient used (kg), and *I* is the selling price of LA, which was 1.3 USD/kg [21]. The price of SB was 0.01 USD/kg [7], and prices of other nutrients were taken from www.sigmaaldrich.com, and are given in Supplementary Table S1.

$$EY = \frac{LA}{\sum (CN)} I \tag{1}$$

2.8. Analytical methods

Cell growth was measured in terms of OD_{600} using a UV-VIS spectrophotometer (UV mini-1240, Shimadzu, Japan). Cellulose, hemicellulose and lignin contents were analyzed following the method of Sluiter et al. [22]. Activity of cellulase was determined using the methods of Adney and Baker [23]. Xylanase activity was determined using the method of Bhalla et al. [24] with some modifications, i.e., 1% (w/v) xylan was prepared in sodium acetate-acetic acid buffer pH 5.0 and the incubation temperature was controlled at 50 °C. Reducing sugar concentration was determined using the DNS method [25], using glucose as the standard. Concentration of glucose, xylose, LA, acetic acid, and ethanol were determined using high performance liquid chromatography (Waters, model 2414), equipped with an Aminex HPX-87H column (Bio-Rad, USA), and a refractive index (RI) detector. Sulfuric acid (5 mM) was used as the mobile phase at a flow rate of 0.5 mL/min. The detector temperature and injection volume were 45 °C and 20 μL, respectively. Optical purity of LA was determined using a rapid assay kit (Product code: K-DLATE) purchased from Megazyme, Ireland.

Removal efficiency of hemicellulose and lignin, as well as cellulose degradation, after the pretreatment was calculated based on mass of the component in SB before and after pretreatment using Eq. (2) [26]. Here, *R* is the removal efficiency of hemicellulose or lignin, or cellulose degradation (%), *m* is the mass of cellulose, hemicellulose, or lignin in the pretreated SB (g), m_0 is the mass of cellulose, hemicellulose, or lignin in the untreated (raw) SB (g).

$$R = \left(1 - \frac{m}{m_0}\right) \times 100 \tag{2}$$

Theoretical LA production from pretreated SB was estimated using Eq. (3), assuming that glucose and xylose was used under Embden-Meyerhof-Parnas (EMP) and phosphoketolase (PK) pathways, which yielded 1 g-LA/g-glucose and 0.6 g-LA/g-xylose,

respectively. Here, LA_{Th} is the theoretical LA production (g), *Biomass* is the mass of the pretreated SB used in the process (g), *Cellulose* is the cellulose content in the biomass (%), 1.11 is the conversion factor for cellulose to glucose, 1 is the theoretical yield of LA on glucose (g/g), *Xylan* is the xylan content in the biomass (%), 1.14 is the conversion factor for xylan to xylose, and 0.6 is the theoretical yield of LA on xylose (g/g). Fermentation efficiency (η) was calculated using Eq. (4), where *LA* is the mass of LA produced (g).

$$LA_{Th} = (Biomass \times Cellulose \times 1.11 \times 1) + (Biomass \times Xylan \times 1.14 \times 0.6) \quad (3)$$

$$\eta = \frac{LA}{LA_{Th}} \times 100 \quad (4)$$

3. Results and discussion

3.1. Identification of the isolated LAB

Based on diameter of clear zone developed during an incubation (data not shown), one potential glucose-utilizing LAB (Isolate TSKKU P-8) and one xylose-utilizing LAB (Isolate CHKKU N-6) were selected for identification. The former was obtained from fermented fish, while the latter was isolated from fermented pork. The analysis of 16S rDNA sequences using the BLASTN program showed that TSKKU P-8 had 99.66% similarity to *Lactiplantibacillus plantarum* subsp. *plantarum*, and CHKKU N-6 had 99.73% similarity to *Levilactobacillus brevis*, and therefore these were named *La. plantarum* TSKKU P-8 and *Le. Brevis* CHKKU N-6, respectively. Hereafter, the former is referred to as TSKKU P-8 and the latter as CHKKU N-6. A rapid test for optical purity of LA produced by the two strains showed that both TSKKU P-8 and CHKKU N-6 produced both D-LA and L-LA.

Both TSKKU P-8 and CHKKU N-6 were tested for their ability to grow on glucose and xylose. TSKKU P-8 grew well on glucose and produced LA. Low growth was observed when it was grown on xylose, though small amounts of LA and acetic acid were detected in the medium. This was possibly due to the conversion of residual glucose carried over from the inoculum. These results suggested that TSKKU P-8 preferred glucose to xylose for its metabolism. As a facultative heterofermentative LAB, *La. plantarum* can produce 2 mol-LA/mol-glucose through EMP pathway [3,27], and so TSKKU P-8 was considered a potential strain for high-level LA production from glucose derived from SB. On the other hand, CHKKU N-6 grew well on both glucose and xylose. When grown on glucose, it produced LA, acetic acid, and ethanol. However, no ethanol was detected when it was grown on xylose. *Le. brevis* is recognized as obligate heterofermentative LAB that use PK pathway to metabolize hexoses and pentoses. When glucose is used as the carbon source, LA, acetic acid, and ethanol are produced, whereas only LA and acetic acid are produced when xylose is the carbon source [3]. These results implied that CHKKU N-6 could be used to simultaneously ferment both glucose and xylose derived from SB to produce LA. However, LA yield would be low as only 1 mol-LA would be obtained from 1 mol-sugar [2].

TSKKU P-8 and CHKKU N-6 were further tested for their ability to utilize mixed carbon sources (glucose and xylose). TSKKU P-8 completely consumed glucose, while xylose was partially consumed (Table 3). This hierarchical utilization of sugars was due to carbon catabolite repression (CCR) that is observed in most LAB, including *La. plantarum*. In the presence of preferred sugars, e.g., glucose, the utilization of the less favorable ones, e.g., xylose and arabinose, is repressed [28]. This is due to the regulation of genes and operons involving in the catabolism of the less favorable carbon sources by catabolite control protein A (CcpA) at the transcriptional level [29]. The detection of LA and acetic acid at 43.4 and 3.3 g/L, respectively, suggested that TSKKU P-8 primarily used EMP pathway to produce LA, and when glucose was depleted, PK pathway was used, producing acetic acid as the co-product [3]. On the other hand, CHKKU N-6 did not completely consume glucose and it seemed to simultaneously utilize glucose and xylose to produce LA, acetic acid, and ethanol. *Le. brevis* has been reported to lack CCR [30], and its ability to simultaneously convert C-5 and C-6 sugars into LA has previously been reported [31]. However, despite possessing the ability to co-utilize glucose and xylose, it should be noted that the use of *Le. brevis* would give low LA yield as mentioned above (1 mol-LA/mol-sugar). For this reason, rather than using CHKKU N-6 as a single LA producer, sequential inoculation of glucose-utilizing LAB (TSKKU P-8) followed by xylose-utilizing one (CHKKU N-6) would be more effective in converting mixed sugars into LA. In this way, maximum glucose conversion to LA and utilization of xylose could be achieved [32]. A theoretical LA yield would be 3 mol-LA/mol-sugar (2 mol-LA/mol-glucose and 1 mol-LA/mol-xylose), which is higher than the use of either LAB individually.

3.2. Pretreatment of sugarcane bagasse

SB was pretreated using NaOH solution at high temperature, aiming to remove lignin from the biomass, as well as to partially destroy the biomass structure to facilitate subsequent fermentations. Alkaline pretreatment using NaOH is one of the most effective methods for delignification of lignocellulosic biomass. It causes the biomass to swell, as well as causing solvation and saponification of

Table 3
Growth, sugars consumption, and metabolites production by *La. plantarum* TSKKU P-8 and *Le. brevis* CHKKU N-6 grown on mixed sugars.

LAB	Glucose consumed (%)	Xylose consumed (%)	Lactic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)	OD_{600}
TSKKU P-8	100	27.6	43.4	3.3	0.0	9.98
CHKKU N-6	76.4	25.1	25.4	9.3	8.3	5.03

Note: OD_{600} represents growth of the LAB and was calculated as the difference between the final and initial OD_{600} values.

intermolecular ester bonds that cross-link xylan and lignin. These result in removal of the cross-links, disruption of lignin structure, increasing internal surface area, and decreasing degree of polymerization and crystallinity [33]. SB used in this study contained 39.8% cellulose, 19.5% hemicellulose, and 22.2% lignin, which complied well with the ranges reported in the literature [8,9]. Noted that hemicellulose content reported here included only xylan and arabinan contents in the biomass, and the real hemicellulose content could be slightly higher. Xylan and arabinan were used to represent hemicellulose fraction since these are the predominant hemicellulosic carbohydrates in SB [9]. In the present study, NaOH pretreatment reduced the lignin content from 22.2 ± 2.1% to 7.3 ± 0.7%, equivalent to the removal efficiency of 85.9 ± 1.3% (Table 1). Previously, Siqueira et al. [34] reported that lignin removal of at least 60% was sufficient to achieve cellulose hydrolysis of over 80%. This suggested that the pretreatment used in the present study was very effective and was adequate to support subsequent enzymatic hydrolysis of cellulose. However, NaOH pretreatment also partially solubilized carbohydrates in the biomass [33,35]. Hemicellulose, particularly xylan, is known to be alkali-soluble compound [9]. Alkalis can break the hydrogen bonds linking xylan-xylan and xylan-cellulose, as well as covalent bonds between xylan and lignin, leading to xylan solubilization [36]. As seen in Table 1, xylan was solubilized during the pretreatment, resulting in hemicellulose content being reduced from 19.5 ± 0.7% to 13.9 ± 0.3%, with a removal efficiency of 70.2 ± 0.7%. This effect was considered not favorable in this study as this reduced the substrate for LA fermentation. Nevertheless, the removal of lignin and hemicellulose increased cellulose content in the biomass to 66.8 ± 0.5%, despite 29.5 ± 1.8% of cellulose being solubilized during the process. After the pretreatment, dry weight of the biomass was reduced by 58%.

3.3. Enzymatic saccharification of sugarcane bagasse

Conditions for enzymatic saccharification of the pretreated SB were optimized for use in the subsequent fermentations. Table 2 shows that solids concentration, cellulase loading, and xylanase loading affected reducing sugar production from the biomass. Either linear (Eq. (5)) or quadratic equations (Eq. (6)) could fit the results well with R^2 of 0.8142 and 0.9428, respectively. However, inspection of the coefficients of the interaction and quadratic terms in Eq. (6) showed that these were very small and not significant (data not shown). Therefore, the linear equation was considered adequate to predict the response in the present study. Using Eq. (5), analysis of variance (ANOVA) showed that all the factors in the range tested had significant effects on reducing sugar production (Table 4). This was because increasing SB concentration increased substrates (cellulose and xylan) availability in the system, resulting in higher reducing sugar production as seen in Fig. 1A and B. As for the effects of enzymes loadings, adding more cellulase and xylanase to the reaction mixture promoted biomass saccharification, leading to increased reducing sugar production (Fig. 1A through 1C). Although Cellic® CTec3 is known to contain cellulases, as well as hemicellulase and other accessory enzymes [37,38], the accessory enzymes might not be sufficient to support efficient xylan degradation, and so xylanase could be added to improve xylan hydrolysis [38,39]. Our results indicated that high SB concentration, as well as cellulase and xylanase at high loadings, were necessary to achieve high reducing sugar production, which agreed well with a previous report by Plaza et al. [40]. However, SB concentration cannot be overly increased, as too high of a biomass concentration would cause high-solid effects that impedes efficient mixing. Solid loadings in a range 12%–15% has been reported to represent the upper limit for efficient mixing [41], and in the present study, 160 g/L of SB was the highest concentration that still gave a mixable suspension. Beyond 160 g/L, no free liquid was observed in the system, making it very difficult to mix the suspension. Likewise, high enzyme loading has been reported to be necessary for effective saccharification process. This is due partly to the loss of hydrolytic activity caused by nonproductive interaction between the enzymes and lignin in the biomass [42]. However, using high enzyme loadings does not always lead to high sugar production. Pallapolu et al. [43] demonstrated that increasing cellulase loading beyond a certain point resulted in only slight increase in cellulose digestibility and for some biomasses, no positive effects were observed. This could be because enzymes were present in excess of the substrate. Using too high of an enzyme loading would also lead to increased operating costs, making the process not economically feasible [42]. Therefore, it was necessary to optimize SB concentration and enzymes loadings to achieve efficient saccharification while maintaining low operating costs.

$$RS = 1.88 + 0.45SB + 0.19Cellulase + 0.13\text{Xylanse} \quad (5)$$

$$RS = 44.39 + 0.81SB - 0.87Cellulase - 0.70Xylanase + 0.0004(SB \times Cellulase) + 0.002(SB \times Xylanase) + 0.004(Cellulase \times Xylanase) - 0.002SB^2 + 0.007Cellulase^2 + 0.002Xylanse^2 \quad (6)$$

where *RS* is the reducing sugar production (g/L), *SB* is the concentration of alkaline-pretreated sugarcane bagasse (g/L), *Cellulase* is the cellulase loading (FPU/g), and *Xylanase* is the xylanase loading (U/g).

Table 4
Analysis of variance of results obtained from the RSM-BBD experiments.

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F
Model	3148.97	3	1049.66	18.99	<0.0001
A-SB	2555.49	1	2555.49	46.23	<0.0001
B-Cellulase	269.23	1	269.23	4.87	0.0459
C-Xylanase	324.24	1	324.24	5.87	0.0308
Residual	718.61	13	55.28		
Lack of Fit	714.73	9	79.41	81.93	0.0004
Pure Error	3.88	4	0.97		
Cor Total	3867.57	16			

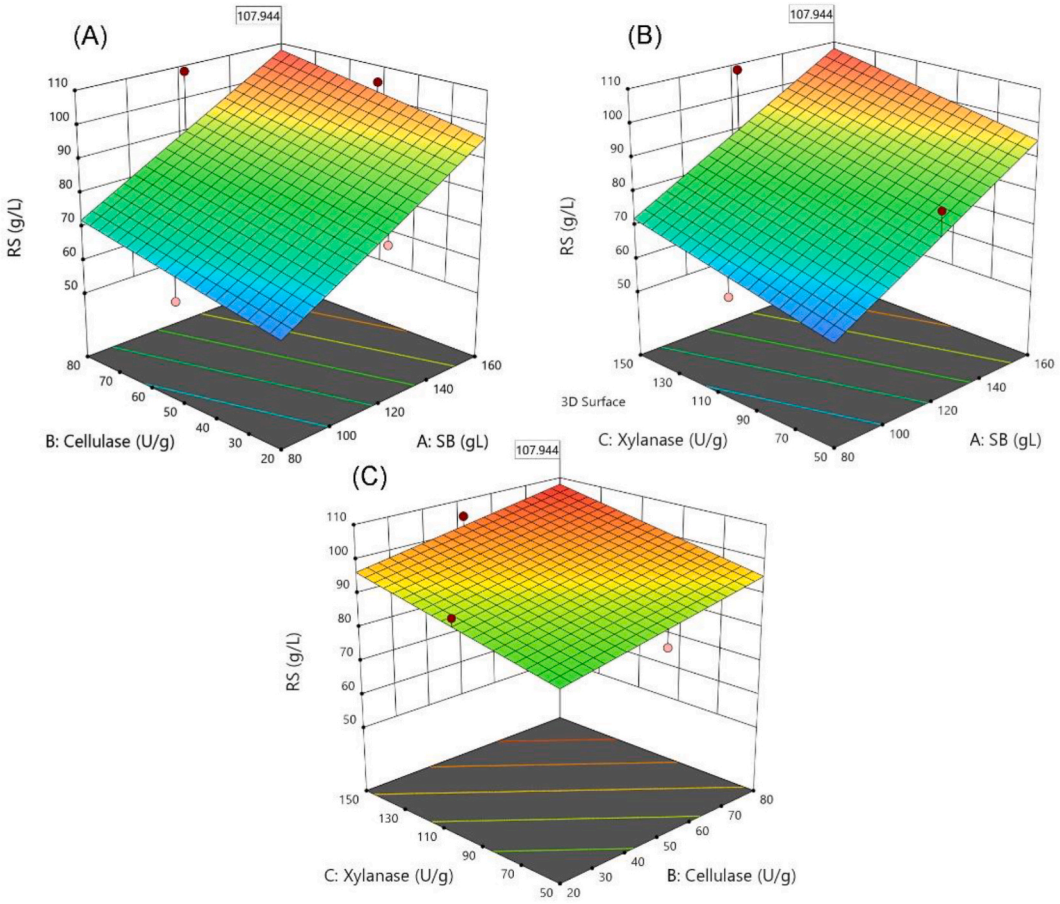

Fig. 1. Interaction effects between alkaline-pretreated sugarcane bagasse (SB) concentration and cellulase loading (A), SB concentration and xylanase loading (B), and cellulase and xylanase loadings (C) on reducing sugar production. RS is reducing sugar production.

Using Eq. (5), it could be calculated that the maximum reducing sugar production would be 107.9 g/L, under the optimum pretreated SB concentration of 160 g/L, and cellulase and xylanase loadings of 80 FPU/g, and 150 U/g, respectively. A confirmation experiment conducted under these conditions for 24 h yielded 91.9 ± 0.8 g/L of reducing sugars, 88% of which was glucose, and the rest was xylose. This reducing sugar production was within the 95% Prediction Intervals of 88.7–127.2 g/L, indicating that the predicted conditions were valid. Nevertheless, analysis of samples taken at 12 h of the saccharification process showed that the hydrolysate contained 89.8 ± 1.8 g/L of reducing sugar (82% glucose and 18% xylose). This suggested that the reaction time could be shortened to 12 h while a reasonably high reducing sugars production could still be attained. Therefore, pre-hydrolyzing SB for 12 h was considered adequate for use preceding SSF and SScF processes. This could shorten the processing time, thus reducing the operating costs and enhancing process productivity.

3.4. LA production from alkaline-pretreated SB under SSF and SScF processes

Fig. 2A shows LA production during SSF of alkaline-pretreated SB. Pre-hydrolyzing the biomass under the optimum conditions for 12 h led to the production of 84.9 ± 2.5 g/L of glucose, and 20.6 ± 0.6 g/L of xylose. Inoculation of TSKKU P-8 increased the total volume of the fermentation broth, which slightly diluted the sugars concentrations. Upon the inoculation, the bacterium consumed glucose rapidly and produced LA at a high rate, suggesting that glucose available in the system supported rapid growth of the bacterium. The rate of LA production decreased after 60 h, corresponding to the low concentration of glucose in the broth. The final LA concentration was 86.7 ± 0.2 g/L, and volumetric LA productivity was 0.8 g/(L·h). Further inspection of the results revealed that glucose in the fermentation broth was depleted at 84 h, while the concentration of xylose only decreased slightly from 19.0 ± 0.4 g/L at 12 h (after the inoculation) to 15.3 ± 0.7 g/L at the end of the process. Possibly, this might be due to facilitated diffusion of xylose into the cells in the presence of glucose [30,44]. However, since *La. plantarum* is CCR positive, xylose was considered contributing negligibly to bacterial growth and LA production. Small amount of acetic acid was detected at 84 h and its concentration increased subtly toward the end of the process. This indicated that TSKKU P-8 shifted its fermentation pathway from homofermentative to heterofermentative to utilize xylose as the carbon source, which produced acetic acid as the co-product of xylose fermentation.

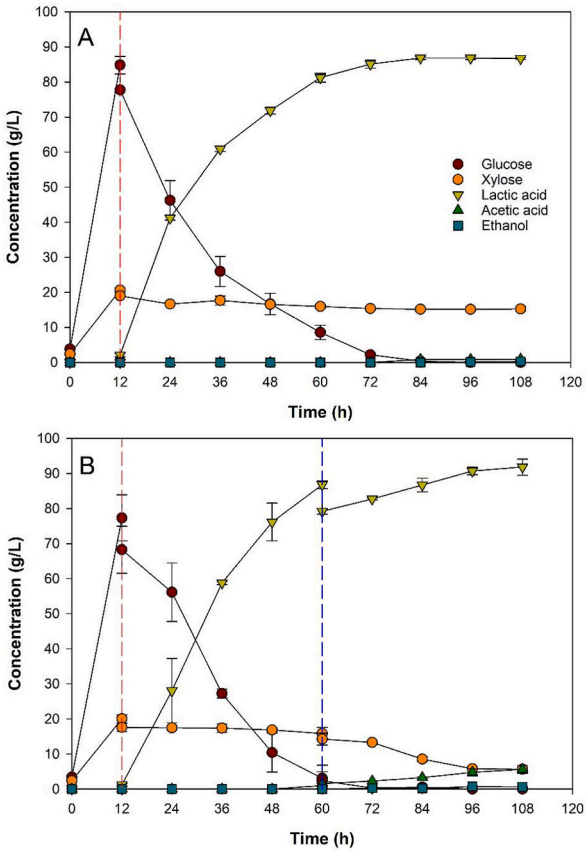

Fig. 2. Profiles of sugars and metabolites during SSF (A) and SScF (B) of alkaline-pretreated sugarcane bagasse. The red dash line represents the end of the pre-hydrolysis period and the time at which *La. plantarum* TSKKU P-8 was added into the bioreactor. The blue dash line represents the time at which *Le. brevis* CHKKU N-6 was added into the bioreactor. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Interestingly, ethanol was also detected, but at a very low level. It could be possible that there was small amount of glucose remaining in the fermentation broth or cellulase in the system continued to hydrolyze the biomass, though at a low rate, releasing glucose into the broth and the bacterium utilized this through the phosphoketolase pathway, forming ethanol as the co-product of glucose fermentation [45]. It is important to note that the concentrations of glucose and xylose shown in the figure are measurable concentrations, i.e., the sugars remaining after the consumption by the bacterium under SSF process. These measurable concentrations should not be used to calculate LA yield as these do not reflect the 'real' amount of sugar consumed during the process [12]. Alternatively, fermentation efficiency (η) could be calculated and used to demonstrate the process efficiency as this reflects the ability of the process to convert the substrate into product. Since the final volume of the SSF system was 1.2 L, the mass of LA produced in this process was 104.1 g. Using

Table 5
Lactic acid production from alkaline-pretreated sugarcane bagasse under SSF and SScF processes.

Parameter	Process	
	SSF	SScF
Feedstock (g)[a]	160	160
Final volume[b] (L)	1.2	1.5
LA (g)	104.8 ± 0.3	137.8 ± 3.4
Acetic acid (g)	1.1 ± 0.2	8.4 ± 1.7
Ethanol (g)	0.3 ± 0.2	0.9 ± 0.1
Fermentation efficiency (%)	79.1	104.6
LA yield on feedstock (g/g)	0.65	0.86
LA productivity (g/h)	0.97	1.28

[a] Cellulose and xylan in the feedstock were 106.9 and 19.1 g, equivalent to 118.7 g-glucose and 21.8 g-xylose, respectively.

[b] Final volume was estimated accounting for the volume of samples taken, and acid and alkaline solutions used for pH control during the process (initial volume was 1 L).

Table 6
Lactic acid production from lignocellulosic biomass under different processes.

Substrate	Process	LA producer	Substrate loading (%)	Working volume (L)	LA (g/L)	LA yield (g/g-sugar)	LA yield (g/g-substrate[a])	LA productivity (g/(L·h))	Reference
Spent coffee ground	SHF	*L. parabuchneri*	–	0.05	4.6	–	0.101	0.14[b]	[54]
SB	SHF	*R. oryzae* F-814	–	–	12.1	–	0.121	0.303	[13]
Cassava biomass	SHF	*L. fermentum* S1a	–	–	22.74	0.81	–	0.47[b]	[6]
SB	SHF	*L. plantarum* 23 immobilized on poly-(vinyl alcohol)	–	0.2	42.4	0.98	–	7.0	[52]
Corn cob	SHF	*B. coagulans* IPE22	–	3	53.51	0.92	–	2.97	[55]
Wheat straw	SHF	Thermotolerant *Bacillus sonorenesis*	–	0.1	55.9	0.97	–	0.77	[56]
Eucalyptus sawdust	Fed-batch	*Carnobacterium* sp.	–	1.5	29.6	0.50	–	0.31	[57]
SB	Continuous	*L. plantarum* 23 immobilized on poly-(vinyl alcohol)	–	0.2	41.2	1.0	–	10.3	[52]
SB	SSF	*R. oryzae* F-814	–	–	14.2	–	0.142	0.355	[13]
Mixed lignocellulosic biomass	SSF	*L. rhamnosus* ATCC 7469	16	2	61.74	0.97	–	1.4[b]	[58]
SB	SSF	*L. pentosus*	30	1	72.75	0.61	0.244	1.01	[59]
Oil palm empty fruit bunch	SSF	Thermophilic *Bacillus coagulans* JI12	–	0.5	114	–	0.47	5.7	[51]
Corn stover	Fed-batch SSF	*L. pentosus* FL0421	8	1.5	92.3	–	0.66	1.92	[60]
Aging paddy rice with hull	SLSF[c]	*L. rhamnosus* DUT1908	20	2	107.8	–	0.89	3.4	[61]
SB	SScF	*L. pentosus*	–	0.725[b]	65	0.93	–	1.01	[62]
SB	SScF	*L. brevis* MTCC 4460	1	–	–	–	0.52	–	[63]
SB	SScF	*Pediococcus acidilactici* XH1		0.1	57.0	–	0.58	0.50	[64]
Cassava bagasse	SScF	*B. coagulans* LA-15-2 and *L. rhamnosus* LA-04-1	–	–	112.5	–	0.88	2.74	[16]
Wheat straw	SScF	Genetically engineered *P. acidilactici*	30	–	130.8	–	–	1.82	[50]
SB	SSF	*La. plantarum* and *Le. brevis*	16	1.2	86.7	–	0.65	0.8 (0.97[d])	This study
SB	SScF	*La. plantarum* and *Le. brevis*	16	1.5	91.9	–	0.86	0.85 (1.28[d])	This study

[a] Substrate used in the fermentation process.
[b] Calculated based on results reported in the original publication.
[c] SLSF: simultaneous liquefaction, saccharification and fermentation.
[d] Productivity in the unit of g/h.

Eq. (3), a theoretical LA production (LA_{Th}) was 131.7 g. Therefore, the fermentation efficiency of the process was 79.1%. LA yield on substrate (pretreated SB) and productivity were 0.65 g/g and 0.97 g/h, respectively (Table 5). Overall, the results obtained using SSF process were satisfactory. However, as could be seen in Fig. 2A, a considerable amount of xylose remained unutilized. Considering that xylose could be used by xylose-utilizing LAB to increase LA yield on the substrate and improve process economic feasibility, SScF process employing CHKKU N-6 as the second LA producer was carried out.

Using SScF process, pre-hydrolysis for 12 h under the optimum conditions yielded 77.3 ± 6.6 g/L of glucose and 20.0 ± 1.1 g/L of xylose (Fig. 2B). After lowering the temperature to 37 °C and inoculation of TSKKU P-8 at 12 h, rapid consumption of glucose was observed, corresponding to the rise of LA concentration to 86.8 ± 1.1 g/L at 60 h, with a volumetric productivity of 1.45 g/(L·h). At 60 h, 1.0 ± 0.0 g/L of acetic acid was detected. Similar results have been reported by Liu et al. [46] that LA production from glucose by *Lactobacillus amylophilus* through EMP pathway could yield other acids, e.g., acetate and tartrate, and ethanol, as the co-products. It was also noticeable that the concentration of xylose decreased slightly during 12–60 h, possibly due to the facilitated diffusion as discussed above. When glucose was at low level (at 60 h), CHKKU N-6 was inoculated into the bioreactor to convert xylose into LA. Inoculation of CHKKU N-6 at this time was to avoid glucose conversion through the PK pathway, which results in lower LA yield. From Fig. 2B, it can be seen that xylose concentration decreased steadily from 14.3 ± 1.7 g/L to 5.6 ± 0.5 g/L, and LA concentration increased steadily toward the end of the process. At 108 h, 91.9 ± 2.3 g/L of LA was obtained, with 5.6 ± 1.1 g/L of acetic acid and 0.6 ± 0.0 g/L of ethanol being produced as the co-products. Considering that no measurable glucose was present in the system after 60 h, the production of LA after the inoculation of CHKKU N-6 was considered due mainly to xylose conversion. At the end of the process, the final volume of the system was 1.5 L, and so the total mass of LA produced was 137.8 g. Noted that although a similar combination use of *La. plantarum* and *Le. brevis* has been reported earlier, different substrate was used (corn stover) and much lower LA production was attained [15]. Interestingly, the total mass of LA produced under SScF process was higher than the LA_{Th} of 131.7 g, resulting in a fermentation efficiency of 104.6%. This strongly suggested that the use of two LAB strains was highly efficient in converting glucose and xylose into LA, and that additional LA could be produced from other sugars that were not quantified during the process. Since arabinan was also detected in the alkaline-pretreated SB, but at a low level of 1.9 ± 0.1%, and *La. plantarum* has been reported to be able to produce LA from arabinose, mannose, and galactose, while *Le. brevis* could produce LA from arabinose and galactose [47,48], it was possible that the bacteria utilized arabinose and other sugars during the fermentation. Based on the results, LA yield on the substrate and productivity attained under SScF process were 0.86 g/g and 1.28 g/h, respectively (Table 5).

3.5. Potential of SScF process for LA production from SB

Bioconversion of sugars, e.g., glucose and xylose, derived from lignocellulosic biomass into LA has extensively been investigated. Several biomass pretreatments and fermentation strategies have been developed to enhance the fermentation yield, which is regarded as one of the key parameters to drive down the minimum product selling price (MPSP) of LA [49]. However, only a handful of previous reports showed promising results that could be extended to larger scale implementation, and only few of these were from SB (Table 6). From the table, it can be seen that LA titer varies depending on several factors, including type of feedstock, strain of microorganism,

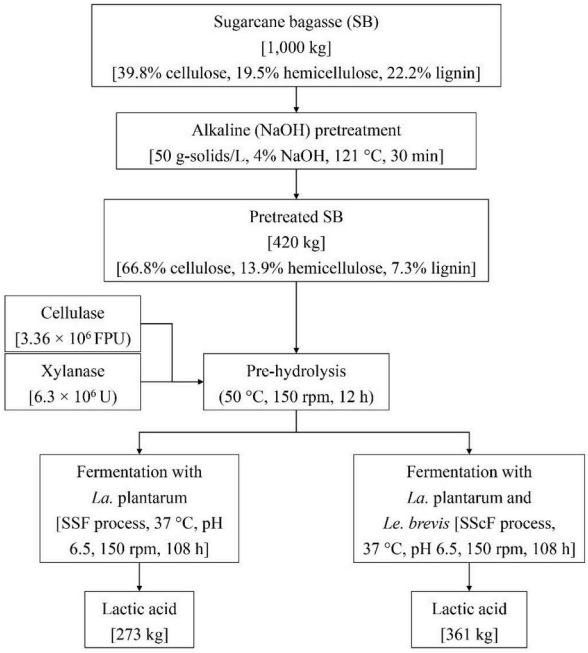

Fig. 3. Mass balance of LA production from SB through SSF and SScF process.

and fermentation process and conditions. Highest LA production was reported in a study using genetically engineered *Pediococcus acidilactici* under high-solid SScF process [50], while the use of wild type thermophilic LAB gave 114 g-LA/L [51]. Using SB as the substrate, LA titer ranged from 12.1 to 72.75 g/L. Interestingly, as high as 10.3 g/(L·h) of LA productivity has been reported using a continuous fermentation of SB hydrolysate, though LA titer is relatively low (41.2 g/L) [52]. In the present study, 91.9 g/L was attained through SScF process, with a yield of 0.86 g-LA/g-substrate (Table 6). These are much higher than the results obtained using the same combination of LAB (*La. plantarum* and *Le. brevis*) to ferment corn stover [15], and are in the upper range of the values reported in the literature [53]. The high LA production, being close to the 100 g-LA/L suggested for industrial LA production [11], and the high LA productivity, suggested that the SScF process used in this study is highly feasible for LA production. Furthermore, with no additional nutrients, e.g., nitrogen source and minerals, being used in the process, the economic feasibility of the process could be improved.

To assess the economic feasibility of the SScF process, mass balance was calculated (Fig. 3) and used to determine the medium-based economic yield (*EY*). Based on the results that dry weight loss after alkaline pretreatment was 58%, 420 kg of pretreated SB would be obtained from a ton of raw SB. The use of the pretreated SB in the pre-hydrolysis step would require 3.36×10^6 FPU of cellulase and 6.3×10^6 U of xylanase. The use of SSF process would yield 273 kg-LA, while 361 kg-LA would be attained under SScF process. These are equivalent to LA yield on raw SB of 0.27 and 0.36 kg/kg, respectively. Here, the use of SScF process improved LA yield on raw SB by 32%, confirming the potential of this process for LA production. Based on the mass balance, *EY* of the SSF process was 28.38 $-LA/$-nutrients, and this was enhanced to 31.77 $-LA/$-nutrients using SScF process. These are higher than the value 0.22 $-LA/$-nutrients estimated from a glucose-based medium [65], and are higher than those estimated using other low-cost feedstocks. For instance, Ma et al. [21] reported an *EY* of 0.466 $-LA/$-nutrients using food waste and spent mushroom substance as feedstock in a fermentation by *Enterococcus mundtii* CGMCC 22,227. In another study, Sharma et al. [65] achieved an *EY* of 1.25 $-LA/$-nutrients using whey permeate-based medium in a fermentation by *L. plantarum* CRA52, while *EY* of 10.47 $-LA/$-nutrients was attained in a study of Bustos et al. [20] using corn steep liquor as the sole nutrient to grow *L. coryniformis* ssp. *torquens* CECT 4129T. The high *EY* attained in the present study was due primarily to the use of SB, which could be acquired at a very low cost (0.01 USD/kg), and the efficient conversion of SB into LA. These results further confirmed the feasibility of the SScF process for LA production.

Despite the satisfactory results, it was considered that LA yield could be further enhanced through several approaches. For instance, acetate production ability of the LAB could be halted to channel sugars to LA synthesis pathway. Phosphotransacetylase (*pta*) gene in *La. plantarum* [66] and *Le. brevis* [67] could be disrupted to hinder the conversion of acetyl-CoA into acetyl-phosphate that is subsequently converted into acetic acid. This was shown to reduce acetic acid production and increase LA production in *Clostridium thermocellum* [68,69]. Inactivation of pyruvate dehydrogenase (*pdh*) and alcohol dehydrogenase (*adh*) genes could also be investigated to reduce ethanol production, as this was shown to be effective in improving LA production in *Saccharomyces cerevisiae* expressing bovine lactate dehydrogenase gene [70]. Another possible approach is to use effective delignification agent and optimization of the pretreatment process to selectively remove lignin from the biomass, while avoiding carbohydrates solubilization and increasing the fermentability of the pretreated biomass. In this regard, deep eutectic solvent (DES) may be worth investigation as its compositions (hydrogen bond doner and hydrogen bond acceptor) can be adjusted to change its property, e.g., polarity, to suit the desired purpose [71]. Pretreatment using DES also consumes less energy as compared with a conventional alkaline pretreatment. Besides, DES is a green solvent that could be prepared at low cost. It can also be reused, which could help reduce the operating cost [72].

4. Conclusions

The present study investigated the use of SB as a feedstock for LA production. SB was efficiently pretreated using NaOH solution, reducing considerably lignin content in the biomass that facilitated subsequent fermentations. The use of pretreated SB under SSF process employing *La. plantarum* TSKKU P-8 as a single LA producers gave 104.8 g-LA, equivalent to a yield of 0.65 g-LA/g-substrate, and a productivity of 0.97 g/h. LA production was enhanced through the use of SScF process using *La. plantarum* TSKKU P-8 and *Le. brevis* CHKKU N-6. As high as 137.8 g-LA was attained, with a yield and productivity of 0.86 g/g-substrate and 1.28 g/h, respectively. LA yield and productivity attained in the present study were in the upper range of those reported in the literature, suggesting that the SScF process developed in this study is highly feasible for LA production from SB. Further investigation could focus on enhancing LA synthesis by disrupting co-products (acetic acid and ethanol) formation, as well as finding a novel solvent for effective lignin removal at low cost.

Author contribution statement

Chularat Haokok: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Siriporn Lunprom: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Alissara Reungsang: Contributed reagents, materials, analysis tools or data.

Apilak Salakkam: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Additional information

Supplementary content related to this article has been published online at [URL].

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17935.

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