Improvement of Polymer Grade L-Lactic Acid Production Using Lactobacillus rhamnosus SCJ9 from Low-Grade Cassava Chips by Simultaneous Saccharification and Fermentation

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Keywords: Lactobacillus sp, low-grade cassava chip, RSM, L-lactic acid fermentation

Abstract:

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Abstract: The present study aims to examine the process for L-lactic acid production from low-grade cassava chips (LGC) using a two-step fermentation approach (TSF) and simultaneous saccharification and fermentation (SSF) by proficient, newly isolated *Lactobacillus rhamnosus* strain SCJ9. The optimized medium composition revealed by response surface methodology for TSF was 166 g/L LGC hydrolysate and 20 g/L yeast extract (YE), while other medium components were fixed (g/L) as follows: tween80 (2.0), $(NH_4)_2HPO_4$ (2.0), $CH_3COONa{\cdot}3H_2O$ (6.0), $(NH_4)_2HC_6H_5O_7$ (2.0), $MgSO_4{\cdot}7H_2O$ (0.5), and $MnSO_4{\cdot}H_2O$ (0.3). Based on the optimization conditions, the maximum experimental L-lactic acid of 134.6 g/L was achieved at 60 h fermentation time with a production efficiency of 89.73%, 0.95 g/g yield and 2.24 g/L/h productivity. In contrast, L-lactic acid production by SSF under optimized concentrations of thermostable-α-amylase (AA) and glucoamylase (GA) gave maximum L-lactic acid of 125.79 g/L at only 36 h fermentation time which calculated to the production efficiency, yield and productivity of 83.86%, 0.93 g/g and 3.49 g/L/h, respectively. The L-lactic acid production obtained from SSF was significantly improved when compared to TSF based on lower enzyme loading usage, shorter hydrolysis time and increase in production efficiency and productivity. Furthermore, there were no significant differences in the production by SSF between experiments conducted in laboratory bottle and 10-L fermenter. The results indicated the success of up-scaling for L-lactic acid production by SSF which could be developed for a further pilot-scale production of L-lactic acid.

Keywords: L-lactic acid fermentation; RSM; low-grade cassava chip; *Lactobacillus* sp

1. Introduction

Lactic acid is a natural organic acid with multifunctional in different ranges of application such as beverages, flavorings and food preservatives, animal feed, drugs, and leather and textile

industries [1,2]. The most recent and attractive applications of lactic acid are found in the manufacturing of biodegradable agro-based products as well as poly-lactic acid (PLA), which is more environment eco-friendly products and alternatives to petroleum-based plastics [3–5]. Since optically pure D- and L-lactic acid is the precursor in PLA production, the demand for both forms of lactic acid is increasing continuously. In 2013, the universal market demands for lactic acid and PLA were estimated to be 714.2 and 360.8 kilo tons and it is predicted to reach 1960 and 1205 kilo tons, respectively, by 2020 [6]. Usually, L-lactic acid constitutes 92 to 99% of total lactic acid in various commercial PLA polymers. In addition, a high percentage of optically pure L-lactic acid provides a good-quality PLA polymer with high crystallinity and melting point [6]. Lactic acid can be commercially produced either by chemically synthesis or microbial fermentation systems. Since the chemically synthesis from petroleum substrates produces a racemic mixture of the two isomeric forms, the optically pure D- or L-lactic acid production via microbial fermentation system is now commonly occupied. Additionally, approximately 90% of industrial lactic acid production worldwide is carried out by microorganisms [7]. Another significant advantage over chemical synthesis is that microbial fermentation can utilize cheap, abundant and renewable raw materials such as starch, cellulosic materials, whey and other carbohydrate-rich materials [8,9]. Among raw materials used for fermentation, starch has the potential to be used as a substrate for many reasons. Pretreatment with cellulosic material has been found to cause several technical problems such as inhibition of the enzyme involved in cellulose hydrolysis by intermediate products. On the contrary, various biomass materials in the form of starch such as corn, cassava, potato, barley, and wheat have been used as carbon sources for the production of lactic acid [10–12]. In addition, starch-containing wastes have also been reportedly used as the main substrate for lactic acid fermentation [13,14].

The cassava is a tropical plant capable of growing on poor soils or soils with depleted organic matter which typically causes low yields and productivity in other crops. Additionally, it has the lowest prices (~32 to 63 USD/ton) among the starch-based materials used as substrate [15]. According to its availability and low cost in many tropical regions, cassava has found worldwide use as an important source of starch as well as raw material for various industrial applications which include food, animal feed, pharmaceuticals, modified starch, beverages and alcoholic fermentation. Furthermore, the cassava-based residues can be renewed into different value products via microbial fermentation to maximize its effective utilization as a vital bioresource [16]. The world trade in cassava starch has long been dominated by Thailand since 1967, which supplied 67% of the global market in 2016 [17,18]. Cassava roots are processed into cassava chips by a chopping machine and dried by either sun-drying or in a drying machine until the final moisture content declines below 14% [19]. Most cassava chips are directly used as raw substrate for starch-producing factories, feed mills, ethanol industries and export. The recent statistic indicates that chip-form cassava is ranked among the most (up to approximately 60% in 2018) exported of the cassava products in Thailand [20]. However, the exported cassava chips are required to meet quality control standards, particularly the cassava chips intended for the feed industry—i.e., starch content at least 70% (European Unionpolarimetric method) or 75% by weight (nitrogen-free extract method), maximum moisture, fiber and sand are limited and not higher than 13, 4 and 2%, respectively, no mold or irregular odor [21]. If the cassava chip that does not match these standards, they are treated as low-grade cassava chips (LGC) and will be used in non-food- and feed-related industries.

In this study, we intended to develop an efficient and economic L-lactic acid fermentation process using a newly isolated *Lactobacillus rhamnosus* with rewards over other lactic acid-producing bacteria regarding the highly efficient production of optically pure L-lactic acid as well as homofermentative metabolism. This report mainly described the optimization of the culture conditions and medium components using the statistical experimental design response based on the employing of LGC as the primary substrate. The fermentation strategies of two-step fermentation (TSF) and simultaneous saccharification and fermentation (SSF) were comparatively investigated.

2. Materials and Methods

2.1. Chemicals

LGC was obtained from the local cassava production farm in Kamphaeng Phet Province, Thailand. The LGC proximate analysis was carried out for dry matter, moisture, protein, fat, fiber content, ash and nitrogen-free extract, following the AOAC method [22]. The commercial thermostable-α-amylase (AA) with the activity of 5.28×10^3 U/mL was purchased from DuPont Company (Wilmington, DE, USA). Glucoamylase (GA) with an activity of 5.44×10^4 U/mL was purchased from Sunson Industry Group Co., Ltd. (Beijing, China). Bacterial cultivation medium de Man, Rogosa and Sharpe (MRS) was purchased from HiMedia Laboratories (Mumbai, India). Other chemicals used in these experiments were of an analytical grade and procured domestically.

2.2. Isolation, Identification and Inoculum Preparation

The bacterial strain SCJ9, proficient in producing L-lactic acid, was obtained from raw sugarcane juice collected from the domestic sugar factory in north Thailand. The isolation method for the bacterial strain was carried out following our previous report [12]. Briefly, 1 mL of sample was transferred to 10 mL of MRS broth and incubated at 37 °C for 24 h. Then, 1 mL of the mother culture broth was then spread on MRS agar supplemented with 125 ppm of bromocresol purple as indicator, then incubated anaerobically at 37 °C for 24–48 h. The single colony surrounded with the yellow clear zone assumed as a lactic acid producing strain was selected and purified by streaking on MRS agar. All selected isolates were grown in 10 mL of MRS broth at 37 °C for 24 h to investigate the capability for lactic acid production. The optically pure L- and D-lactic acids were analyzed using Megazyme L-/D-Lactic acid kit method (Megazyme International Ireland, Bray, Co. Wicklow, Ireland).

The identification of the isolate SCJ9 was performed based on Bergey's Manual methods [23]. In case of molecular identification, the sequencing study of the 16S rRNA gene was performed using the method described by Kanpiengjai et al. [24]. In briefly, genomic DNA of the isolate SCJ9 was extracted from 5 mL of bacterial culture broth cultivated overnight at 37 °C using the standard protocol as described by Sambrook and Russell [25]. The 16S rDNA fragments were amplified using bacterial genomic DNA as a template with bacterial-specific primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′). The PCR amplification reactions were carried out in a DNA thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were then purified with the Qiagen PCR Purification kit (Qiagen, Chatsworth, CA, USA) and, finally, the DNA sequence was sent to 1st BASE Laboratory Company (Singapore) for their sequencing service. Further, the 16S rDNA sequence retrieved from NCBI database and multiple sequence alignment were performed using BioEdit 7.0 software tool (Ibis Therapeutics, Carlsbad, CA, USA). The phylogenetic tree was constructed based on the neighbor-joining methods by MEGA version 4.0 [26]. The newly isolated strain SCJ9 was maintained in MRS broth containing 15% (*v*/*v*) glycerol and stored at −80 °C. To prepare seed inoculum, a single colony of the isolate SCJ9 was inoculated into 10 mL of the MRS broth and further incubated at 37 °C under the static condition for 12–18 h to OD_{600} of 0.6–0.8.

2.3. Effect of Enzyme Concentration on LGC Hydrolysis

To find out the best condition for starch hydrolysis and glucose release, the effect of AA and GA dosages on concentrations of glucose released from LGC were investigated. The LGC hydrolysis reaction was prepared using 47.2 g of LGC, equivalent to 40.0 g of starch, adjusted volume to 200 mL with citrate phosphate buffer (50 mM, pH 4.5) in 250-mL laboratory plastic container and then sterilized at 121 °C for 15 min. Gelatinized LGC was initially hydrolyzed by AA dosages of 86, 172, and 344 U/g dry weight (DW) at 85 °C in a water bath with stirring every 15 min. Samples were taken at 1, 2, 3, and 4 h to determine the reducing sugars. Under these conditions, GA dosages were also varied through 40, 80, 160, and 320 U/g DW. The mixtures were incubated at 55 °C on a 100-rpm incubator shaker.

Samples were taken after 0, 6, 12, and 18 h of saccharification, followed by centrifuging at 13,000× *g* for 10 min to remove precipitates.

2.4. Statistical Medium Optimization of Two-Step Fermentation (TSF) Strategy

2.4.1. Preparation of Low-Grade Cassava Chip Hydrolysate

Based on the optimal levels of AA and GA for LGC hydrolysis, 86 U of AA/g DW was applied into 472 g of LGC (corresponding 400 g of starch) in 2000 mL of citrate phosphate buffer (50 mM, pH 4.5) and maintained at 85 °C for 3 h followed by GA addition at a dose of 80 U/g DW and further hydrolysis at 55 °C, pH 4.5 for 12 h. The reducing sugar obtained after the saccharification process was approximately 184 g/L. The hydrolysate was centrifuge at 6000 rpm for 20 min to eliminate insoluble particles and concentrated using the EYELA series N-1000 rotary evaporator (Tokyo Rika-kikai, Co. Ltd., Tokyo, Japan) prior to use as the carbon source for medium optimization for L-lactic acid production.

2.4.2. Plackett–Burman Design (PBD)

LGC hydrolysate was used as the sole carbon source in the L-lactic acid production medium using the TSF process. Inoculum size of 5% (*v*/*v*) was transferred to 250-mL narrow-necked bottle containing 200 mL of the production medium and incubated at 37 °C. During the fermentation, the pH of the culture was manually adjusted to 6.0–7.0 with 3 h intervals using 10 M NaOH (Labscan, Bangkok, Thailand) and or 5 M HCl (Labscan, Bangkok, Thailand). The essential medium nutritional factors influenced on L-lactic acid production were screened using PBD. The eight independent variables including LGC hydrolysate, YE, tween80, $(NH_4)_2HPO_4$, $CH_3COONa{\cdot}3H_2O$, $(NH_4)_2HC_6H_5O_7$, $MgSO_4{\cdot}7H_2O$, and $MnSO_4{\cdot}H_2O$ were investigated to identify the significant factors for the L-lactic acid production. In this study, a 15-run PBD was used to evaluate the eight factors. Each independent variable was evaluated at two levels: +1 for the high level and −1 for the low level. The actual lactic acid concentration obtained from each treatment was determined in triplicates for the mean value calculation and analyzed by statistical software package Design-Expert 8.0 (Stat-Ease Inc., Minneapolis, MN, USA). The nutritional factors influenced on L-lactic acid production were statistically identified through the significance of *p*-value and selectively considered for further optimization.

2.4.3. Central Composite Design

The central composite design (CCD) was employed for finding the optimal concentration of the significant influencing factors for maximum productivity. In this study, the two-factor analysis at five levels of the CCD model was applied in optimization of two selected factors obtained from PBD, LGC hydrolysate (g/L) and YE (g/L). The statistical design matrix was set with five different levels of each variable including the points (−1, +1), axial point (−α, +α) and center point (0), as presented in Table 1. The experimental L-lactic acid data were analyzed using multiple regression analysis. A second-order polynomial equation and the data were fitted using the quadratic model. The regression coefficients and the model significance were then analyzed by Design-Expert 8.0 software. A second-order polynomial model is described as follows:

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{1} \sum_{j=i+1}^{2} \beta_{ij} X_i X_j \quad (1)$$

where Y is the dependent response (L-lactic acid concentration, g/L), X_i and X_j are the independent factors (LGC hydrolysate and YE) and β_0, β_i and β_{ij} are the model coefficients that were obtained using the linear least squares method. The predicted levels of significant medium formulae were validated as the optimal media for L-lactic acid production. The 5% (*v*/*v*) of inoculums was transferred to the optimal medium and incubated at 37 °C under controlled culture pH of 6.0 during fermentation. Samples were taken periodically with 12 h intervals for 84 h to determine L-lactic acid and total carbohydrate.

Table 1. Central composite design (CCD) experimental range and levels of the independent variables effect on L-lactic acid production by *L. rhamnosus* SCJ9 using two-step fermentation (TSF).

Variable Code	Factors	Unit	Range and Levels				
			$-\alpha$	−1	0	+1	$+\alpha$
A	LGC hydrolysate	g/L	55.85	80	140	200	224.85
B	YE	g/L	5.86	10	20	30	34.14

2.5. Optimization of Enzyme Dosages for the Simultaneous Saccharification and Fermentation Process

To find valuable conditions for L-lactic acid production by simultaneous saccharification and fermentation (SSF), CCD was used to analyze the optimal dosages of AA and GA and their interaction. The statistical design for two variables was created for five different levels of AA and GA dosages, as seen in Table 2. Thirteen treatments including five center points were constructed by the design. In the experiment, L-lactic acid data were analyzed by multiple regression analysis. All treatments obtained from the design were used for the production of L-lactic acid by SSF strategy. Briefly, 177 g/L of LGC with the equivalent amount of an optimal level of 166 g/L of LGC hydrolysate were used as a sole carbon source. The initial pH of the production culture medium was adjusted to 6.0. Initially, the gelatinized LGC in the production medium after autoclaving was liquefied with AA at 85 °C for 3 h. After cooling down to 37 °C, the inoculums size of 5% (*v*/*v*) and GA were simultaneously added in a 250-mL laboratory bottle containing a 200-mL optimal medium. The incubation was carried out at 37 °C in a shaker incubator at 100 rpm. During the fermentation stage, the pH of the culture was adjusted in 3-h intervals to 6.0–7.0. Finally, the experimental L-lactic acid values obtained at 60 h of fermentation time were analyzed by the least square regression and the validation of the predicted values was performed to ensure quadratic model design.

Table 2. Central composite design experimental values of the independent variables effecting on L-lactic acid production by *L. rhamnosus* SCJ9 using SSF.

Variable Code	Factors	Unit	Range and Levels				
			$-\alpha$	−1	0	+1	$+\alpha$
X	AA	U/g DW	1.72	19.60	62.78	105.96	123.84
Y	GA	U/g DW	0.80	12.46	40.60	68.74	80.40

2.6. Large Scale of L-Lactic Acid Production by SSF Strategy in a 10-L Fermenter

The experiment in a 10-L, large-scale, fermented production of L-lactic acid (BEMT Expert of Bioengineering model, Marubishi Ltd., Tokyo, Japan) was performed using the working volume of 8000 mL. The medium was prepared by mixing 1416 g of LGC and volume was adjusted to 8000 mL with an aqueous solution containing the fermenting supplements and then sterilized at 121 °C for 15 min. The optimum dosage of AA (66.6 U/g DW) was added into the production medium for liquefaction and then incubated at 85 °C with a stirring speed of 100 rpm for 3 h. After cooling down to 37 °C, the inoculum size of 5% (*v*/*v*) and the optimal GA dosage (54.4 U/g DW) were simultaneously added to the production medium. During fermentation, the stirrer speed was set at 100 rpm, the temperature was controlled at 37 °C and the pH was controlled automatically at pH 6.0. Tested samples were withdrawn at different time durations (0, 6, 12, 18, 24, 36, 48, 60, and 72 h) for the analysis of L-lactic acid, total carbohydrate and viable cell counts.

2.7. Analytical Method

The total carbohydrate concentration was measured using the method of Phenol-sulfuric acid, as described by Dubois et al. [27]. L-lactic acid concentration was determined using HPLC with a Rezex™ ROA-Organic Acid H+ (8%) column (300 × 7.8 mm) (Phenomenex, Torrance, CA, USA)

with mobile phase as 0.005 N H_2SO_4 at a flow rate of 0.50 mL/min. The temperature of column was maintained at 40 °C and the separated sample was detected using a 210 nm UV detector. The statistical experiments analyses by one-way analysis of variance (ANOVA) were performed by Design-Expert software version 8.0 (Stat-Ease Inc., Minneapolis, MN, USA). Differences between mean values were analyzed using the least significant difference (LSD) method, the analysis was performed by Statistix software, version 8.0 (Statistix Analytical Software, Tallahassee, FL, USA) with significance defined as *p*-values less than 0.05.

3. Results and Discussion

3.1. Identification of L-Lactic Acid Producing Bacterium

Among ten lactic acid producing bacteria isolated from the fresh sugarcane juice, the proficient lactic acid producing bacterial isolate SCJ9 was selected owing to the fastest and biggest yellow clear zone formed on MRS agar containing 125 ppm of bromocresol purple after cultivation at 37 °C for 24 h. It was Gram-positive, rod-shaped, non-spore-forming bacterium and produced highly optically pure L-lactic acid, around 98.7%, with a maximum lactic acid concentration of 8.4 from 10.0 g/L glucose used as the sole carbon source after incubation at 37 °C for 24 h. These observed characteristics hold huge promise as highly optically pure D- or L-lactic acid is needed to produce the lactic acid monomer required for the industrial synthesis of PLA [6]. The molecular identification revealed that the 1490 bp of the 16S rRNA gene sequence of the strain showed the highest similarity to *L. rhamnosus* JCM 1136 (accession no. BALT01000058) with a pairwise similarity of 99.86%. The 16S rRNA gene sequence of the strain SCJ9 has been deposited in the NCBI GenBank database under the accession number MT941265 and a phylogenetic tree constructed from the 16S rDNA sequence analysis is presented in Figure 1. *L. rhamnosus* has been extensively studied for various applications particularly the use as probiotic against diarrhea and arthritis [28]. This bacterial species was commonly found to remarkable tolerance when exposing to the harsh acidic condition which is a typical condition in the stomach and digestive tract [29,30] and it has also been used in lactic acid production [10,31]. Regarding the fast-growing and highly optically pure L-lactic acid-producing capabilities, not only the application in L-lactic acid production, a newly isolated *L. rhamnosus* SCJ9 may provide the suitable characteristics for the use as a probiotic in the food and feed industries.

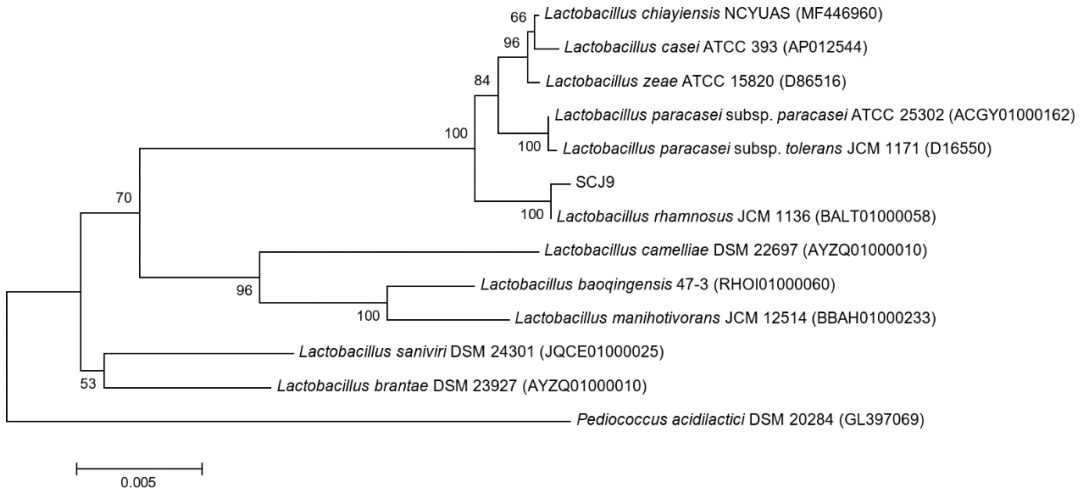

Figure 1. Phylogenetic tree demonstrating the relationships of the 16S rRNA gene sequences of the isolate SCJ9 and related sequences obtained from NCBI. Scale bar, 0.005 substitutions per nucleotide position.

3.2. *Effect of Enzyme Concentration on LGC Hydrolysis Efficacy*

The resulting proximate composition indicated that LGC consisted of 12.03 ± 0.04, 1.23 ± 0.04, 3.26 ± 0.03, 1.74 ± 0.03, 2.04 ± 0.01 and 79.24 ± 0.01% of moisture, crude fat, crude fiber, crude protein, ash, and nitrogen-free extract, respectively. The LGC contained mainly starch up to 84.75 ± 1.90% of its dry weight. In this study, a total of 200 g/L of starch-existing LGC was hydrolyzed at 85 °C with different dosages of AA in the first 3 h followed by the saccharification step where all hydrolysis was carried out at 55 °C until steady state for production of reducing sugars, as shown in Figure 2. It was found that gelatinized LGC was gradually liquefied with AA and reached a steady phase within the first 3 h. Reducing sugar concentrations increased corresponding to the reduction of the starch slurry viscosity as well as the increase of AA dosages. Under previously described conditions, 59.35 ± 0.98, 72.09 ± 1.27, and 81.93 ± 1.60 g/L of reducing sugars were obtained at 3 h of incubation when AA was applied 86, 172, and 344 U/g DW of AA, respectively (Figure 2A–C). GA shows the critical role for glucose released. Reducing sugar increased rapidly within 6 h when added GA in each liquefied LGC and further slightly increased after that until 15 h of incubation. The maximal concentration of reducing sugar of 195.43 ± 3.43 g/L was achieved when applied the maximum dosages of AA and GA (344 and 320 U/g DW). Considering, based on LGC hydrolysis efficiency, the highest efficiency with approximately 184.05 ± 4.37 g/L was obtained when applied 86 and 80 U/g DW of AA and GA, respectively. In contrast, the higher dosages of both enzymes were not increased the starch hydrolysis efficiency significantly. AA is an important endoamylase for partially gelatinized starch hydrolysis, and the rapid loss in viscosity of the starch slurry was observed due to random cleaving the α-1,4 glycosidic bonds in the inner regions of the starch molecule into shorter oligosaccharides of varying lengths [32]. However, Souza [33] reported that the AA activity could be inhibited by its products, an α-configuration and α-limit dextrins, that are a mixture of maltose, maltotriose, and of 6-8 glucose units branched oligosaccharides which consist of both α-1,4 and α-1,6 glycosidic bonds.

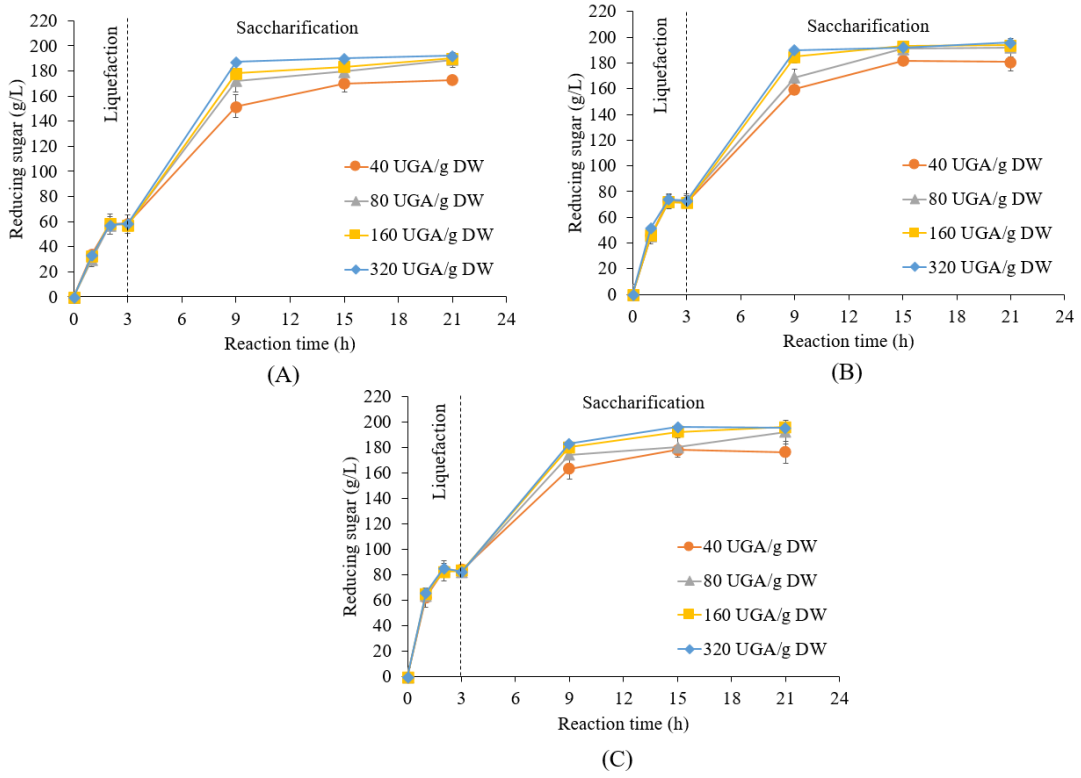

Figure 2. Effect of AA and GA dosages on glucose released from 200 g/L starch existing in LGC. Different dosages of AA were varied from 86 UAA/g DW (**A**), 172 UAA/g DW (**B**), and 344 U AA/g DW (**C**).

3.3. Statistical Optimization of Medium Composition for Lactic Acid Production by TSF Strategy

The PBD was used to find out the significant medium compositions for L-lactic acid production by the strain SCJ9 among eight medium components based on the modified MRS medium [34] including LGC hydrolysate, YE, tween80, $(NH_4)_2HPO_4$, $CH_3COONa{\cdot}3H_2O$, $(NH_4)_2HC_6H_5O_7$, $MgSO_4{\cdot}7H_2O$ and $MnSO_4{\cdot}H_2O$. The 15 runs, and their responsive results of L-lactic acid achieved in the design matrix, are presented in Table 3. Analysis of the data by the linear regression to fit with the first-order model found that the model was highly significant at p-value < 0.0001 with R^2 and adjusted R^2 values of 0.9972 and 0.9909, respectively. The ANOVA presented in Table S1 revealed that two variables, LGC hydrolysate (A) and YE (B), exhibited the most significance (p-value < 0.0001) in enhancing L-lactic acid production. We also found a significant curvature from the interaction of AB at p-value of 0.0002, which indicates both LGC hydrolysate (A) and YE (B) strongly enhanced the production of L-lactic acid. The CCD was further employed for finding the optimal concentrations of these two variables for maximum L-lactic acid production. The design matrix of the variables in actual values was presented in Table 4 with the response results. Fitting the experimental lactic acids with the least square linear regression models, the simulated second order equation was obtained as follows:

$$\text{L-lactic acid (g/L)} = 120.43 + 29.11A + 10.16B + 5.30AB - 14.92A^2 - 10.61B^2 \quad (2)$$

where A and B were LGC hydrolysate and YE, respectively.

A summary of the ANOVA for the response surface quadratic model was presented in Table S2. The highly significant model at p-value < 0.0001 with R^2 value > 0.95 which was in agreement with the adjusted R^2 value (adjusted R^2 > 0.90) indicated the excellent fit of the model. This result moreover predicated that LGC hydrolysate and YE were the most significant variable for L-lactic acid production by *L. rhamnosus* SCJ9 with p-value < 0.0001. Significant effects of the two variables interaction were also observed correspondingly to the convex shape of the 3D contour response surface plot (Figure 3). The quadratic model predicted the maximum level of L-lactic acid at 130.24 g/L after 72 h of fermentation under the optimized medium containing 166.0 g/L LGC hydrolysate and 20.0 g/L YE as a carbon source and an organic nitrogen source, respectively. In comparison, other medium components were fixed following the result of PBD that consisted of 2.0 g/L tween80, 2.0 g/L $(NH_4)_2HPO_4$, 6.0 g/L $CH_3COONa{\cdot}3H_2O$, 2.0 g/L $(NH_4)_2HC_6H_5O_7$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 0.3 g/L $MnSO_4{\cdot}H_2O$.

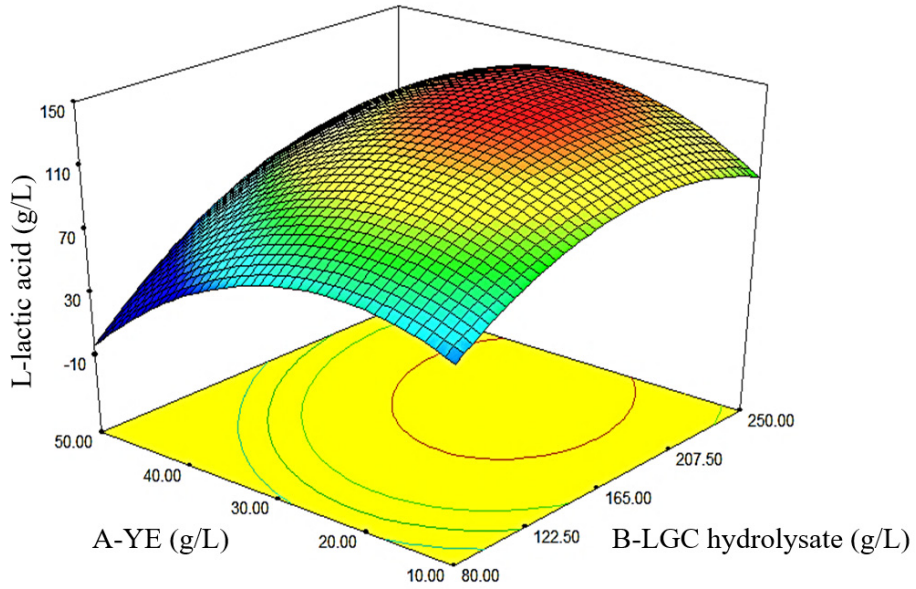

Figure 3. Response surface and contour plots of the combined effects of LGC hydrolysate and YE concentrations on the L-lactic acid production by *L. rhamnosus* SCJ9 at 37 °C.

Table 3. Experimental design matrix of Plackett–Burman for screening the significant medium compositions of L-lactic acid production by *L. rhamnosus* SCJ9 using TSF.

Run	A: LGC Hydrolysate (g/L)	B: YE (g/L)	C: Tween80 (g/L)	D:$(NH_4)_2HPO_4$ (g/L)	E: $CH_3COONa{\cdot}3H_2O$ (g/L)	F: $(NH_4)_2H$ $C_6H_5O_7$(g/L)	G: $MgSO_4{\cdot}7H_2O$ (g/L)	H: $MnSO_4{\cdot}H_2O$ (g/L)	L-Lactic Acid (g/L)
1	80	10	1	2	6	2	0.1	0.1	73.4
2	40	10	2	0.5	6	2	0.5	0.1	37.5
3	80	2	2	2	2	2	0.5	0.5	37.1
4	40	10	1	2	6	0.5	0.5	0.5	36.1
5	40	2	2	0.5	6	2	0.1	0.5	33.2
6	40	2	1	2	2	2	0.5	0.1	37.7
7	80	2	1	0.5	6	0.5	0.5	0.5	35.1
8	80	10	1	0.5	2	2	0.1	0.5	68.9
9	80	10	2	0.5	2	0.5	0.5	0.1	74.1
10	40	10	2	2	2	0.5	0.1	0.5	35.0
11	80	2	2	2	6	0.5	0.1	0.1	35.6
12	40	2	1	0.5	2	0.5	0.1	0.1	27.5
13	60	6	1	1.25	4	1.25	0.3	0.3	55.5
14	60	6	1	1.25	4	1.25	0.3	0.3	56.3
15	60	6	1	1.25	4	1.25	0.3	0.3	55.4

Table 4. Experimental designs and the results of the CCD for L-lactic acid production by *L. rhamnosus* SCJ9 using TSF.

Run	A-LGC Hydrolysate (g/L)	B-YE (g/L)	L-Lactic Acid (g/L)	
			Experimental Values	Predicted Values
1	80(−1)	10(−1)	65.8	60.9
2	200(+1)	10(−1)	110.6	108.5
3	80(−1)	30(+1)	70.2	70.6
4	200(+1)	30(+1)	136.2	139.4
5	55.15(−α)	20(0)	46.6	49.4
6	224.85(+α)	20(0)	133.0	131.7
7	140(0)	5.86(−α)	80.3	84.8
8	140(0)	34.14(+α)	116.6	113.5
9	140(0)	20(0)	123.0	120.4
10	140(0)	20(0)	117.8	120.4
11	140(0)	20(0)	121.5	120.4
12	140(0)	20(0)	118.3	120.4
13	140(0)	20(0)	121.6	120.4

The L-lactic acid production model was validated under an optimized medium within 72 h of fermentation and the result is presented in Figure 4. *L. rhamnosus* SCJ9 proliferated and entered the log phase within 6 h and reached the stationary phase after 6 h. Initial total sugar concentration with approximately 166 g/L decreased rapidly within the first 48 h and reached a steady phase after 72 h. Considering that, for the L-lactic acid production, the experimental value achieved at 72 h of fermentation was 124.02 ± 4.35 g/L or 95% validation when compared to 130.24 g/L of the predicted value, it could be concluded that the optimization of medium composition using the statistical experimental design was successful.

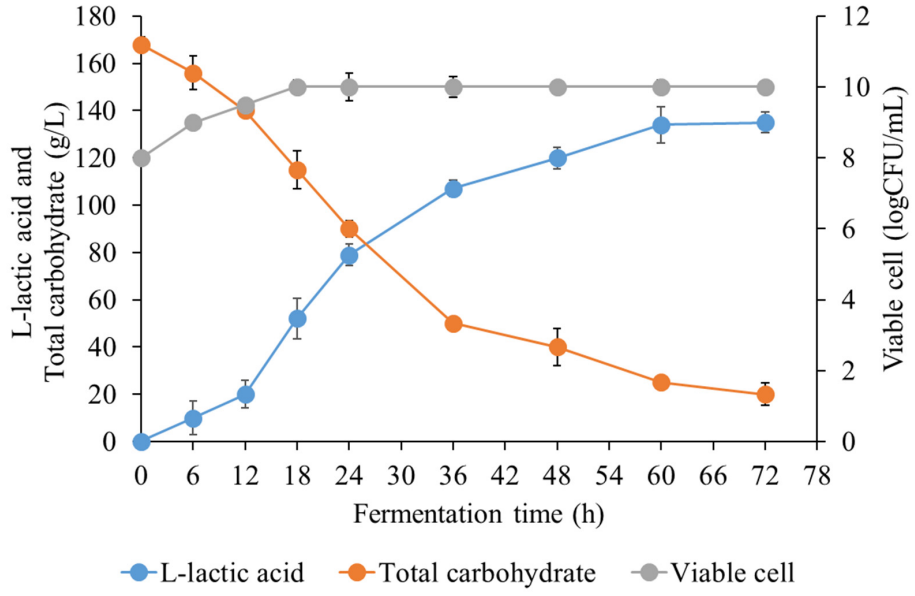

Figure 4. Profiles of viable cells, total carbohydrate consumption, and L-lactic acid during lactic acid production by *L. rhamnosus* SCJ9 under the optimized medium at 37 °C using TSF strategy.

Regarding the production of lactic acid by general lactic acid bacteria, there are many factors that influence productivity. Osmotic stress due to high initial sugar concentration resulted in decreased lactic acid production through the inhibition of microbial growth [35]. The yield of lactic acid production by *L. rhamnosus* CASL was also lowered due to initial glucose concentration (>200 g/L) [7]. The ratio of carbon and nitrogen sources (C/N ratio) is also another factor that mainly affected the enhancement

of lactic acid production [36]. Similarly, Hetényi et al. [37] also found that lactic acid productivity increased with a reduction of the C/N ratio from 37:1 to 19:1, while more decrease in the ratios did not promote the production of lactic acid by *Lactobacillus* sp. MKT-LC878. The production of L-lactic acid by *L. rhamnosus* SCJ9 presented from this experiment may acquire the drawback effect as described previously leads to the highest L-lactic acid was limited as only 166 g/L even at the maximum initial total sugar. Thus, finding the strategy to reduce the effect of initial total sugar might be positive to the lactic acid yield. Simultaneous saccharification and fermentation is one strategy that has been reported that providing the higher product yield via the overcome substrate inhibition effect leads to the reduction of initial sugar at the initial step of fermentation [38].

3.4. Process Optimization of Enzyme Dosages for SSF of L-Lactic Acid Production

Prior to the SSF, the starch degrading enzymes AA and GA were investigated for finding the enzyme dose performing the most effective saccharification activity using CCD experimental design under mixing conditions with the optimal medium obtained from the previous experiment. The CCD experimental design matrix generated a total of 13 experimental dosages of AA and GA used for lactic acid production (Table 5). After 60 h of the fermentation, the L-lactic acid values were analyzed by ANOVA and fitted with the second-order quadratic model; the simulated second-order polynomial equation based on actual values was obtained as follows:

$$\text{L-lactic acid (g/L)} = 130.19 + 2.86X + 8.98Y - 1.67XY - 11.29X^2 - 8.99Y^2$$

where X and Y were AA dosage (U/g DW) and GA dosage (U/g DW), respectively.

Table 5. Experimental designs and the results of the CCD for L-lactic acid production by *L. rhamnosus* SCJ9 using SSF.

Run	X-AA (U/g DW)	Y-GA (U/g DW)	L-Lactic Acid (g/L)	
			Experimental Values	Predicted Values
1	19.60(−1)	12.46(−1)	95.5	96.40
2	105.96(+1)	12.46(−1)	100.1	105.46
3	19.60(−1)	68.74(+1)	117.2	117.69
4	105.96(+1)	68.74(+1)	115.2	120.07
5	1.72(−α)	40.60(0)	103.3	103.57
6	123.84(+α)	40.60(0)	117.7	111.65
7	62.78(0)	0.80(−α)	102.7	99.51
8	62.78(0)	80.40(+α)	127.5	124.90
9	62.78(0)	40.60(0)	127.3	130.19
10	62.78(0)	40.60(0)	128.5	130.19
11	62.78(0)	40.60(0)	134.3	130.19
12	62.78(0)	40.60(0)	128.4	130.19
13	62.78(0)	40.60(0)	132.5	130.19

The ANOVA results revealed that the response surface methodology model displayed the *p*-value lower than 0.05 and lack of fit of the model was not significant indicating that the quadratic model was highly significant and could be applied to predict the optimum enzyme dosages correctly. Furthermore, R^2 value of the model at 0.9328 was also indicated high accuracy of the model. GA was found to be the most significant factor enhancing lactic acid production by SSF strategy at $p < 0.05$ while AA was not significantly. However, lower and higher dosages of these variables declined the lactic acid production significantly ($p < 0.05$) (Table S3). The lower dosages of AA and GA resulted in more effective reduction of the starchy media and low amounts of glucose production John et al. [39] and Wang et al. [7] and reported that the high use concentration of starchy materials made a medium slurry that negatively influenced the mixing of seed inoculum and enzymes. Hence, liquefaction with optimal AA dosage overcame the viscosity problem and led to the rapid bioconversion of starch to

dextrin. On the other hand, the high rate of glucose release was received from higher enzyme dosages. Much more glucose concentration can inhibit the ability of lactic acid production of LAB.

Although TSF for lactic acid production has been widely studied, the fermentation strategy requires time consuming caused by longer saccharification step and inhibition of bacterial growth during high concentration of hydrolysate at the initial fermentation stage might be a risk [38]. Single-step lactic acid production is preferable, since the saccharification can be simultaneously performed with lactic acid fermentation. During SSF, the starch degrading enzymes are simultaneously added to the starchy substrate along with the bacterial inoculums. Thus, the fermentation is faster than that occurring in TSF process. The interaction of AA and GA could be observed from both an ANOVA table (Table S3) and the 3D contour plot (Figure 5) as it presents in the convex shape, indicating the maximum level of L-lactic acid. The predicted optimal dosages of AA and GA were 66.65 and 54.42 U/g DW that provided the maximal L-lactic acid production of 132.52 g/L within 60 h of fermentation time at 37 °C. At the predicted point, the experimental lactic acid value was 127.0 ± 2.0 g/L which is 95.8% validation. Based on this achieved lactic acid value, the production efficiency, yield and productivity were 84.64 ± 1.69%, 0.86 ± 0.03 g/g and 2.12 ± 0.04 g/L/h, respectively. All presented values were comparable with those obtained from TSF, thus success in SSF, the production efficiency, yield and productivity from both strategies had close values (Table 6). However, considering the time course of L-lactic acid production by SSF strategy under the optimized enzyme dosages (Figure 6), it was found that total carbohydrate consumption and L-lactic acid production reached a stationary phase since only 36 h of fermentation time with the maximum L-lactic acid production of 125.79 ± 3.67 g/L, which was calculated to the production efficiency of 83.86 ± 2.45%, yield of 0.93 ± 0.04 g/g and productivity of 3.49 ± 0.10 g/L/h. In contrast, L-lactic acid production obtained from TSF strategy was only 107.30 ± 3.50 g/L, which was calculated to be 71.54 ± 2.33% production efficiency, 0.92 ± 0.06 g/g yield and 2.98 ± 0.10 g/L/h productivity (Table 6). The fermentation time of the SSF strategy was shorter than the TSF strategy for approximately 24 h, thus it provided the higher productivity. Moreover, the SSF strategy also provided higher lactic acid value and production efficiency at 36 h of fermentation. The results indicated and confirmed that SSF strategy appeared to be more suitable than the TSF strategy for producing L-lactic acid at an up-scale production and was chosen for further study.

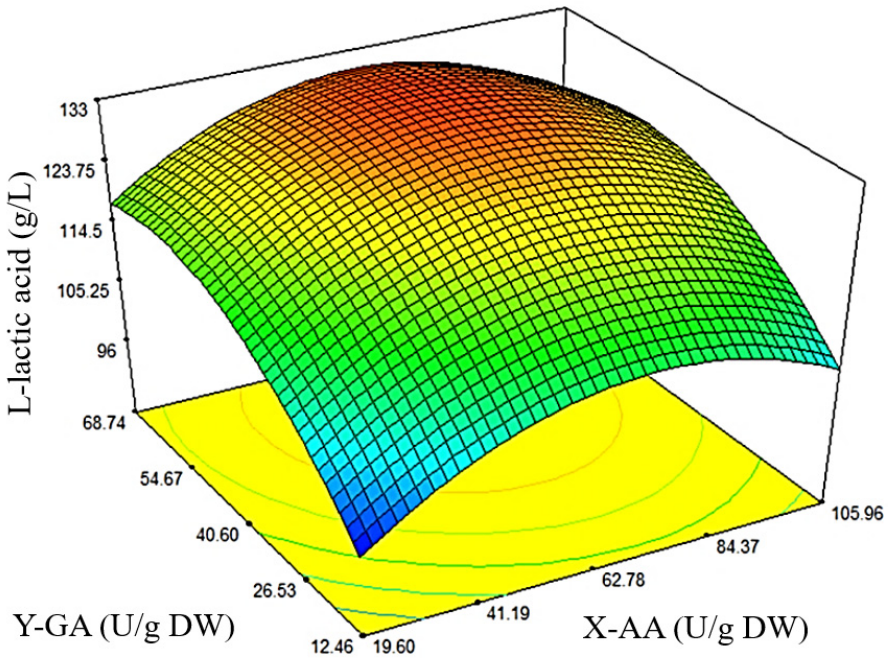

Figure 5. Response surface plot and contour showing the interaction between AA and GA dosages on L-lactic acid production by *L. rhamnosus* SCJ9 at 37 °C using SSF strategy.

Table 6. Comparison of L-lactic acid, yield, production efficiency and productivity of *L. rhamnosus* SCJ9 cultivated in the optimized medium using TSF and SSF fermentation strategies.

Strategies	Fermentation Time (h)	L-Lactic Acid (g/L)	Yield * (g/g)	Production Efficiency (%)	Productivity (g/L/h)
TSF	36	107.30 ± 3.50 ^{b}	0.92 ± 0.06 ^{a}	71.54 ± 2.33 ^{b}	2.98 ± 0.10 ^{a}
TSF	60	134.60 ± 0.20 ^{a}	0.95 ± 0.05 ^{a}	89.73 ± 0.13 ^{a}	2.24 ± 0.00 ^{b}
SSF	36	125.79 ± 3.67 ^{a}	0.93 ± 0.04 ^{a}	83.86 ± 2.45 ^{a}	3.49 ± 0.10 ^{a}
SSF	60	127.00 ± 2.00 ^{a}	0.96 ± 0.03 ^{a}	84.64 ± 1.69 ^{a}	2.12 ± 0.04 ^{b}

* Lactic acid yield (g/g) = Lactic acid formation (g)/substrate consumption (g). Different superscript small letter (a-b) within the same column indicate a statistically significant difference (p-value < 0.05) by Tukey's multiple range tests.

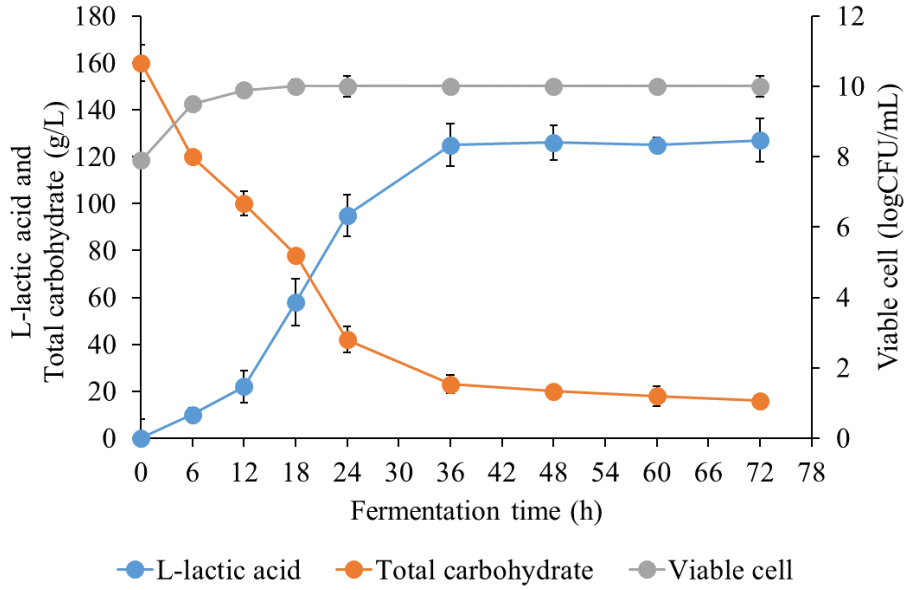

Figure 6. Time course of viable cells, total carbohydrate consumption, and L-lactic acid during lactic acid production by *L. rhamnosus* SCJ9 at 37 °C in the optimized medium using SSF strategy.

3.5. Scale-Up of L-Lactic Acid Production by SSF Strategy in a 10-L Fermenter

To investigate the feasibility of lactic acid production by SSF strategy from optimized medium containing 177 g/L of LGC and 20 g/L of YE as the carbon source and nitrogen source, respectively, the inoculum size of 5% (*v*/*v*) of *L. rhamnosus* SCJ9 and GA dosage of 80 U/g DW were simultaneously transferred into the production medium (with final volume at 8000 mL) after liquefaction with AA dosage of 86 U/g DW. The time course of total carbohydrate consumption, viable cell count and lactic acid produced by *L. rhamnosus* SCJ9 after cultivation at 37 °C, 100 rpm, and controlled pH at 6.0 during fermentation are presented in Figure 7. It was found that *L. rhamnosus* SCJ9 quickly consumed sugar hydrolyzed from LGC to produce lactic acid, along with cell growth. The bacterium rapidly grew within the first 12 h of fermentation and entered to stationary phase until the end of fermentation. Initial total carbohydrate (168.72 ± 4.67 g/L) expeditiously decreased within 30 h of fermentation and then slightly reduced until 36 h. After 36 h of fermentation, the remaining total carbohydrate with 20.95 ± 1.23 g/L was obtained, corresponding to the highest L-lactic acid production of 130.4 ± 1.9 g/L with the production efficiency of 86.92 ± 1.24%, yield of 0.88 ± 0.03 g/g and productivity of 3.62 ± 0.05 g/L/h. The result represented that lactic acid production, yield, production efficiency, and productivity obtained in 10-L fermenter was not significantly different from the result in the laboratory bottle scale at 36 and 60 h of fermentation, as showed in Table 6. Some publications are focusing on an investigation of the feasibility of lactic acid production from starch by SSF strategy in up-scaling production. For example, Wang et al. [7] investigated the production of lactic acid in SSF strategy from 275 g/L cassava powder by *L. rhamnosus* CASL in a 5-L fermenter with a working

volume of 2000 mL under automatic pH control at 5.6–6.0, 42 °C and static condition. They successfully obtained a high lactic acid concentration of 175.4 g/L with a yield and productivity of 0.71 g/g and 1.80 g/L/h, respectively. Nakano et al. [40] also reported on the lactic acid production from broken rice by SSF strategy using *L. delbrueckii* in 5-L fermenter with 2500 mL of medium at 40 °C, 150 rpm, and pH controlled at 6.0, this bacterial strain produced the maximum lactic acid value of 79.0 g/L, with a productivity of 3.58 g/L/h and yield of 0.81 g/g. Compared with the previously published results, our study showed a higher yield (0.88 ± 0.03 g/g) and productivity (3.62 ± 0.05 g/L/h) of lactic acid.

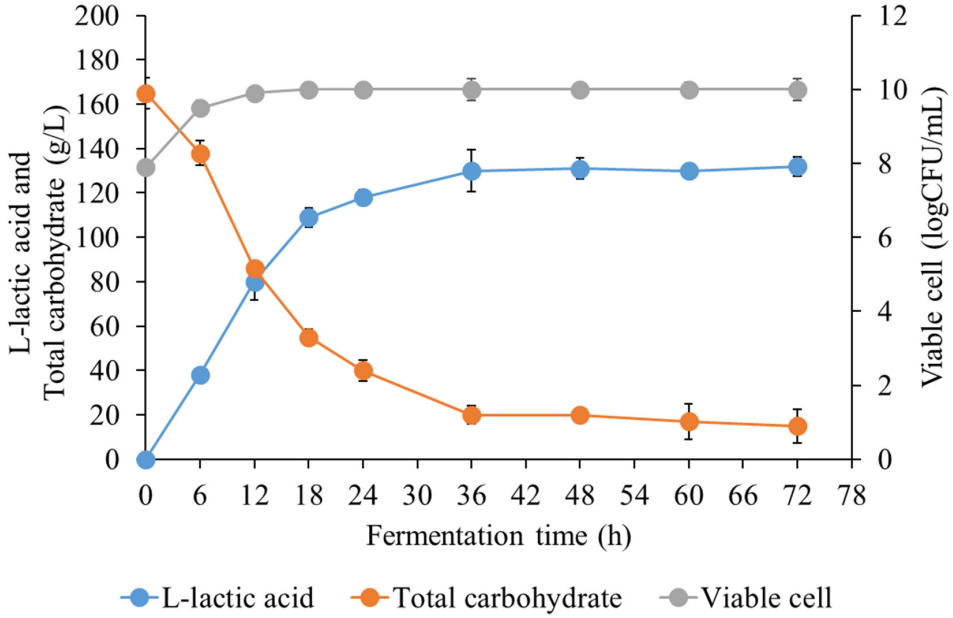

Figure 7. Profiles of viable cells, total carbohydrate consumption, and L-lactic acid during lactic acid production by *L. rhamnosus* SCJ9 at 37 °C in the optimized medium using SSF strategy in a 10-L fermenter.

The results from this study indicate the potential application of *L. rhamnosus* SCJ9 in bioconversion of low-grade cassava chips into an optically pure L-lactic acid which is required for the synthesis of biodegradable plastic polylactate. Besides LGC, other starch-related substrates or wastes have also been tried to use as the substrate for the cultivation of our newly isolated *L. rhamnosus* SCJ9 and the high potentiality was also observed. Cassava bagasse, which is a fibrous material by-product of the cassava-processing industry, contains approximately 30–50% starch on a dry weight basis [41] and can also serve as substrate for *L. rhamnosus* SCJ9. In addition, we also revealed the excellent viability of *L. rhamnosus* SCJ9 during fermentation even at the low pH of 3.8–4.0, which reached high viable cell numbers up to 10 log CFU/mL from 12 h of fermentation time for SSF, both during culture in flask and in a 10-L bioreactor. Regarding the fact that *L. rhamnosus* has been successfully developed and approved for being used as probiotic against diarrhea and arthritis [27], a by-product as a viable biomass cell is possible to be used as a probiotic especially in food and feed industries. However, the characterization and approval process of *L. rhamnosus* SCJ9 are essential and must be further investigated. Even though the results from this research mostly described the successful in L-lactic acid production from a low cost substrate as LGC, there are some limitations, such as a high-cost nutritional components, like yeast extract (YE), were included in the production medium. It is recognized worldwide that YE is a cost-effective medium component for industrial production of various biotechnological fermentative products [42]. Therefore, the finding of alternative substrate for either replacing or reducing the use of YE is necessary. Furthermore, the evaluation of L-lactic acid production cost from SSF strategy must be considered for industrial competitiveness and the techno-economic feasibility via economic model has to be further investigated.

4. Conclusions

The present study indicates the potential application of the newly isolated *L. rhamnosus* SCJ9 for optically pure L-lactic acid production using low-grade cassava chips. The improvement of L-lactic acid production was successfully achieved via the statistical optimization of nutritional components. The productivity was also more improved by using the response surface methodology to minimize the enzyme usage and hydrolysis time, whereas the production efficiency and productivity were increased. The success of up-scaling in a 10-L fermenter also confirmed that *L. rhamnosus* SCJ9 could be implemented for the larger-scale production of the polymer-grade L-lactic acid required in PLA synthesis. Furthermore, further investigations on the probiotic properties of *L. rhamnosus* SCJ9 biomass as the value-added by-product is in progress.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9717/8/9/1143/s1, Table S1: ANOVA analysis of the first order model for response variables in PBD for L-lactic acid production by *L. rhamnosus* SCJ9 using TSF. Table S2: ANOVA for response surface quadratic model of the CCD for L-lactic acid production by *L. rhamnosus* SCJ9 using TSF. Table S3: ANOVA for response surface quadratic model of L-lactic acid production by *L. rhamnosus* SCJ9 using SSF.

Author Contributions: Conceptualization, K.U., N.P., A.K., and C.K.; methodology, N.P., A.K., and C.K.; formal analysis, N.P., and A.K.; investigation, K.U., N.P., A.K., R.K.G., D.K., and C.K.; writing-original draft preparation, K.U., N.P., and C.K.; writing-review and editing, A.K., and C.K.; visualization, K.U.; supervision, C.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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