



Article

Simultaneous Bioconversion of Gelatinized Starchy Waste from the Rice Noodle Manufacturing Process to Lactic Acid and Maltose-Forming α -Amylase by *Lactobacillus plantarum* S21, Using a Low-Cost Medium

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Abstract: A direct bioconversion of gelatinized starchy waste (GSW) to lactic acid by amylolytic lactic acid bacterium *Lactobacillus plantarum* S21 was investigated. Corn steep liquor (CSL) was selected as the most suitable low-cost nitrogen source for replacing yeast extract, beef extract, and peptone in De Man, Rogosa and Sharpe (MRS) medium. Plackett–Burman design results indicated that GSW and CSL were the two most nutrients that significantly influence lactic acid production, among eight medium components, including GSW, CSL, K_2HPO_4 , CH_3COONa , $(NH_4)_2HC_6H_5O_7$, $MgSO_4$, $MnSO_4$, and Tween 80. A new low-cost medium containing only GSW (134.4 g/L) and CSL (187.7 g/L) was achieved as omitting other six components from the optimized medium had no effect on lactic acid yield. Batch fermentation at 37 °C both in 1 L and 10 L jar fermenters showed non-significantly different productivity. A by-product, maltose-forming α -amylase, was successfully achieved up to 96% recovery yield using an ultrafiltration unit equipped with a 50 kDa cut-off membrane. Crude lactic acid exhibited the additional benefit of antimicrobial activity against food and feed pathogens *Salmonella enterica* serovar Typhimurium TISTR 292, *Vibrio cholerae* TH-001, and also *E. coli* ATCC 25922. This study presents a promising bioprocess for the simultaneous production of lactic acid, and a value-added food enzyme, using only two industrial wastes, GSW and CSL, as the medium components.

Keywords: lactic acid; amylolytic lactic acid bacteria; gelatinized starchy waste; *Lactobacillus plantarum*; corn steep liquor; antimicrobial activity

1. Introduction

Lactic acid (2-hydroxypropanoic acid) is an important organic acid that is widely used in various industries, particularly food and feed, pharmaceutical, and chemical industries. Owing to the advantageous functions of lactic acid such as improving protein digestion, and inhibiting the growth of Gram-negative microflora in the gastrointestinal tract [1–3], lactic acid and calcium lactate are presently listed in the European Union (EU) Register of Feed Additives, as technological

additives (functional group: preservatives) for use with feed for all animal species and categories without restriction, and are subject to re-evaluation [4]. Lactic acid can be commercially produced by either chemical synthesis or biotechnological fermentation; however, approximately 90% is produced by microbial fermentation [5]. In case of fermentation, lactic acid bacteria (LAB) consume sugar, particularly glucose, as a carbon source and metabolize it to lactic acid [6]. Various kinds of raw materials containing metabolizable sugars such as sucrose, molasses, and other biomasses can be also used as substrates for lactic acid fermentation, but the important considerations for substrate selection in industrial lactic acid production are economic reasons and substrate availability [7]. Wastes from agricultural industries, such as starchy wastes, lignocellulosic biomass, and food wastes have been extensively investigated in recent years, as alternative and economic raw materials to reduce the cost of lactic acid production [5,8–11]. Besides, utilizing these agricultural wastes is also an effective strategy in environmental waste management [12]. Gelatinized starchy waste (GSW) is a type of starchy waste that is discharged daily from noodle manufacturing processes in high quantities. It has been used to substitute expensive carbon sources for lactic acid fermentation [13]. In addition to the substrate or carbon source, another important economic factor of lactic acid production is the high cost of nitrogen sources like peptone and yeast extract [14]. Yousuf et al. [15] reported on the effect of total solid content and pretreatment on lactic acid production by a mixed culture using food waste as substrate; a similar purpose of utilizing food waste for lactic acid production was also reported by Tang et al. [16]. Corn steep liquor (CSL) is a low-cost alternative medium component for fermentation. It is a by-product of wet corn milling that has been reported as a rich source of proteins, and it contains vitamins, minerals, and carbohydrates [9,17]. CSL has been successfully used as an alternative for expensive nitrogen sources in many fermentation processes [8,9,18,19].

Lactobacillus is one of the most common genera of LAB used in diverse food and agricultural applications such as starter culture [20], probiotics [21], and food preservatives [22] and also food processing [23]. In addition, *Lactobacillus* spp. are also utilized as the key microbe for industrial lactic acid production [5,24]. *Lactobacillus plantarum* S21, an amylolytic lactic acid bacterium isolated from Thai indigenous food products from north Thailand, is selected as a highly efficient LAB strain that is capable of direct lactic acid production from starchy substrates, due to its ability for extracellular amylase production for starch hydrolysis, to release sugars for bacterial growth and lactic acid production [25]. The amylase produced by *L. plantarum* S21 is well-characterized, and it is identified as a maltose-forming α -amylase, with the capability to generate maltose from soluble starch. Regarding the enzyme, it is produced by a generally recognized as a safe (GRAS) microbe. The maltose-forming α -amylase from *L. plantarum* S21 is expected to be used in the production of food grade maltose directly from starchy substrate [26]. This enzyme was attractively stable in a low pH condition, and it always remained in the culture broth, after the growth of *L. plantarum* S21 in liquid medium using starch as a substrate [13]. In order to apply the rare type of amylolytic lactic acid bacterial strain as *L. plantarum* S21, and also regarding a huge quantity of daily discharged GSW from a noodle manufacturing plant, the conceptual idea for the bioconversion process for the simultaneous production of lactic acid and a value-added by-product as maltose-forming α -amylase using GSW as a substrate, was initiated.

This paper describes the selection and optimization of nutritional components for the bioconversion of GSW to lactic acid by the selected amylolytic lactic acid bacterium *L. plantarum* S21, using a statistical experimental design. A strategic model process for starchy waste treatment and its utilization by the simultaneous production of lactic acid, and maltose-forming α -amylase, as a value-added by-product, is also demonstrated. In addition, the simple process of the product separation of lactic acid and maltose, forming α -amylases is also described.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

Lactobacillus plantarum S21, an amyolytic lactic acid bacterium isolated from Thai indigenous food products [25], was maintained in de Man, Rogosa and Sharpe (MRS) broth with 15% (v/v) glycerol at $-80\text{ }^{\circ}\text{C}$. It was cultured in MRS broth and streaked, to obtain a single colony, prior to utilizing it for the study. *Salmonella enterica* serovar Typhimurium TISTR 292 was maintained in Salmonella–Shigella (SS) agar. The *Vibrio cholerae* used in this experiment was a clinical strain that was kindly provided by the School of Allied Health Sciences, University of Phayao. *Escherichia coli* ATCC 25922 was also used as a model for antimicrobial activity testing.

2.2. Raw Materials and Culture Media

GSW was collected from a rice noodle factory in Chiang Mai, Thailand, and it was analyzed for composition, particularly the carbohydrate component, which is an important component for this experiment. CSL was purchased from Friendship Corn Co., Ltd. (Samut Prakarn, Thailand). Other organic nitrogen sources, such as whey, soybean meal, and brewer's yeast were obtained from the local factories in Thailand. Soy milk was purchased from the local market in Chiang Mai, Thailand. All other chemicals used in the investigation were of analytical grade, and were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich (St. Louis, MO, USA), and Ajax FineChem (Seven Hills, Australia). The selective media for microbial cultivation, including MRS agar, SS agar, MacConkey agar, and Eosin methylene blue (EMB) agar were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

2.3. Screening of Nitrogen Sources for Lactic Acid Production

Seed inoculum was prepared by inoculating 5 mL of activated *L. plantarum* S21 culture into 100 mL MRS broth containing GSW as a carbon source, and incubating at $37\text{ }^{\circ}\text{C}$ under static conditions for 18 h. Some selected organic nitrogen sources including whey, soybean meal, soy milk, and brewer's yeast were investigated for their potential to be used as substitutes for high-cost nitrogen sources in the MRS medium (yeast extract, beef extract, and peptone). All nitrogen sources at 10 g/L concentration were used as a sole nitrogen source in the modified MRS formula [25], whereas 10 g/L GSW was used as the sole carbon source. Lactic acid concentration was determined by a high performance liquid chromatography (HPLC) after cultivation at $37\text{ }^{\circ}\text{C}$ for 24 and 48 h. The nitrogen source that produced the highest lactic acid yield was selected for further studies.

2.4. Screening of Factors Influencing Lactic Acid Fermentation, using a Plackett–Burman Design

The important factors in medium composition that affected lactic acid production were screened using the Plackett–Burman design at a 100 mL scale. The main effect of each variable was studied at two levels, low level (-1) and high level ($+1$). The actual lactic acid quantity (g/L) obtained from each designed treatment was determined and analyzed by using the statistical software package Design-Expert 8.0 (Stat-Ease Inc., Minneapolis, MN, USA). The nutritional factors affecting lactic acid production were determined by the significance of the p -value. All experiments were performed in triplicate for the mean calculation.

2.5. Statistical Medium Optimization of Lactic Acid Fermentation by *L. plantarum* S21

A central composite design (CCD) was applied to determine the optimum levels of GSW (X_1) and CSL (X_2), and the effects of their interaction on direct lactic acid production by *L. plantarum* S21. The statistical design matrix was set with five different levels of each variable including -1.682 , -1 , 0 , $+1$, and $+1.682$ (Table 1) and 13 treatments in total were created by the design. The experimental lactic acid data were analyzed by using multiple regression analysis. To evaluate the reliability of the

quadratic model and the best fit, a time course of lactic acid production was performed in 100 mL culture broth containing optimum quantities of the two variables suggested by the quadratic equation. The regression coefficients and model significance were analyzed using Design-Expert 8.0. A second order polynomial model is described as:

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

where Y is the dependent variable, X_i are the independent variables, and β_0 , β_i , and β_{ij} are the model coefficients obtained using the linear least squares method.

Table 1. Central composite design (CCD) experimental ranges and levels of the independent variables.

Variable code	Medium components	Unit	Range and levels				
			−1.682	−1	0	+1	+1.682
X ₁	GSW	g/L	72.50	83.12	108.75	134.38	145.00
X ₂	CSL	g/L	50.00	86.60	175.00	263.39	300.00

2.6. Effect of the Mineral Component Eradication from the Optimized Medium on Lactic Acid Production

In order to reduce the cost of medium, lactic acid production using the optimized medium with and without minerals was compared. The optimized medium (OM) was composed of 134.4 g/L of GSW, 187.7 g/L of CSL, 0.1 g/L of Tween80, 1.0 g/L of K₂HPO₄, 1.0 g/L of CH₃COONa·3H₂O, 0.5 g/L of (NH₄)₂HC₆H₅O₇, 0.35 g/L of MgSO₄·7H₂O, and 0.05 g/L of MnSO₄·7H₂O. The optimized medium without minerals (OM-Mi) was composed of 134.4 g/L of GSW and 187.7 g/L of CSL. Seed inoculums 1% (v/v) was transferred to 800 mL of both OM and OM-Mi broths in a 1 L fermenter (B.E. Marubishi Co. Ltd., Tokyo, Japan) and the temperature was maintained at 37 °C. The pH of fermentation broth was maintained at approximately 6.0 using 10 N NaOH. Lactic acid quantity, total sugar, and amyolytic activities were monitored at 3 h intervals for 72 h.

2.7. Antimicrobial Activity of Crude Lactic Acid Produced by *L. plantarum* S21

Antimicrobial activity was assayed using the filter paper disc diffusion method against some pathogenic bacteria including *S. enterica* serovar Typhimurium TISTR 292, *V. cholerae* TH-001, and *E. coli* ATCC 25922. The culture broth obtained from the cultivation of *L. plantarum* S21 in the OM-Mi at 37 °C for 48 h was harvested and the cell-free culture supernatant (CFCS) was separated by 12,000 × g centrifugation at 4 °C for 10 min. The CFCS was neutralized with 1 N NaOH to a final pH 7.0, and was sterilized by filtration through a 0.2 μm membrane filter. Selective media such as SS agar, MacConkey agar, and EMB agar were prepared as per the standard manual described by the manufacturing company, and freshly prepared cell suspensions of *S. enterica* serovar Typhimurium TISTR 292, *V. cholerae* TH-001, and *E. coli* ATCC 25922 were dispersed on respective selective media, using a sterile swab. A sterile 6 mm paper disc (Schleicher and Schuell, Dassel, Germany) was placed on the solid agar, and then 50 μL of each CFCS prepared previously was gently loaded on the paper disc. A disc loaded with sterile distilled water was included as a control. Inhibition zones were observed after incubation at 37 °C for 18 h.

2.8. Lactic Acid Production in a 10 L Jar Fermenter

Lactic acid production using a low-cost medium or OM-Mi, as achieved in the previous experiment was studied in a 10 L fermenter (B.E. Marubishi Co Ltd., Tokyo, Japan) using a 7 L working volume. Seed inoculums of 70 mL were transferred to 7000 mL of low-cost medium composed of 134.4 g/L of GSW and 187.7 g/L of CSL. The temperature and pH of the culture were controlled at 37 °C and pH 6.0, respectively. An agitation rate of 100 rpm was applied, in order to maintain the homogeneity of

the culture without aeration. The fermentation medium was maintained at pH 6.0 with the addition of 10 N NaOH. Lactic acid, total sugar, and amylolytic activities were monitored at 3 h intervals for 72 h.

2.9. Separation of Maltose-Forming α -Amylase

In total, 7000 mL culture broth was centrifuged at $12,000\times g$ for 10 min at 4 °C to separate the bacterial cells and insoluble particles before being subjected to ultrafiltration. Separation and recovery of the maltose-forming α -amylase enzyme (MW 100 kDa) was carried out at 4 °C under N_2 -pressure filtration in a 400 mL Amicon stirred cell (Millipore, Bedford, MA, USA), using a chemistry regenerated cellulose membrane of 76 mm diameter and 50 kDa MWCO (Millipore, Bedford, MA, USA). The entire 7000 mL was partially dispensed into the chamber until a total of approximately 300 mL of the retained supernatant remained, and it was considered to be the concentrated maltose-forming enzyme fraction. The flow-through supernatant was collected and considered as the lactic acid fraction. Both fractions were examined for lactic acid concentration and amylase activity.

2.10. Lactic Acid Determination and Amylase Activity Assay

Lactic acid content in the culture broth was determined by HPLC using a Rezex ROA-organic acid H^+ column (Phenomenex, Aschaffenburg, Germany). HPLC (Shimadzu Corporation, Kyoto, Japan) was operated using 0.005 N H_2SO_4 , pH 2.2, as the mobile phase with a flow rate of 0.5 mL/min, 55 °C column temperature, and a refractive index detector. Total carbohydrate quantity was determined by using the phenol–sulfuric acid method [27].

Amylase activity was assayed by measuring the amount of reducing sugars released during starch hydrolysis, using the dinitrosalicylic (DNS) acid method [28]. The reaction mixture containing 0.125 mL of appropriately diluted enzyme and 0.125 mL of 10 g/L soluble starch in 0.1 M phosphate buffer (pH 6.5) was incubated at 37 °C for 10 min. The reaction was stopped by adding 0.25 mL of DNS (Sigma-Aldrich, St. Louis, MO, USA), boiled for 10 min, and 2 mL of distilled water was added. Absorbance was measured at 540 nm. Glucose was used as the standard reducing sugar. One unit of amylase activity was defined as the amount of enzyme that released 1 μ mL of reducing sugar per min, under the assay condition.

3. Results

3.1. Screening and Selection of Nitrogen Sources

Organic nitrogen sources, including whey, soybean meal, soy milk, brewer's yeast, and corn steep liquor (CSL) were employed in this study to replace high-cost nitrogen sources in the MRS medium. Figure 1 shows the effects of different nitrogen sources on lactic acid production compared to MRS medium. The results showed that lactic acid production was markedly influenced by the type of nitrogen source. Nitrogen sources in the original MRS (yeast extract, beef extract, and peptone) were the best supplements for efficient lactic acid production. Among the different nitrogen sources tested in this experiment, the highest lactic acid yield was obtained from CSL at 9.3 g/L, which was the closest to the lactic acid yield of the original MRS medium.

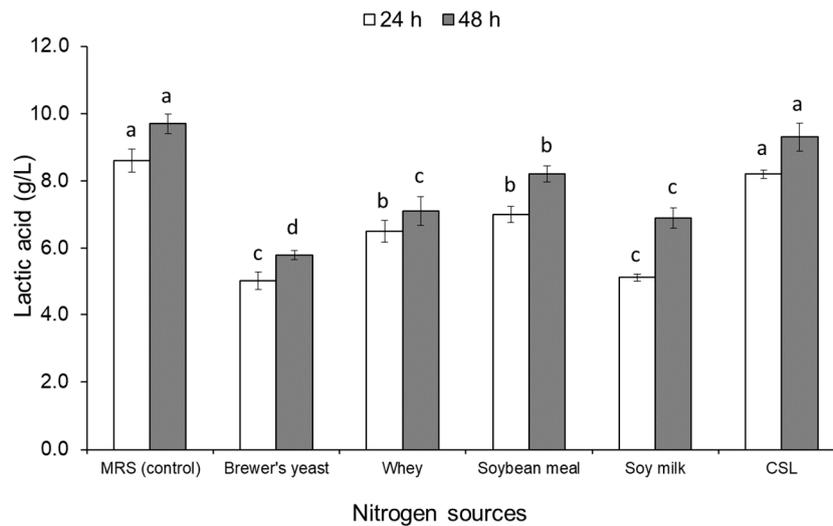


Figure 1. Lactic acid production from various nitrogen sources using *L. plantarum* S21 under static conditions at 37 °C for 24 h (white column) and 48 h (gray column).

3.2. Screening of Significant Influential Medium Components, by the Plackett–Burman Design

The Plackett–Burman design was applied to screen significant medium components that affect lactic acid production by *L. plantarum* S21. The limits of the variables, and a summary of the analysis of variance (ANOVA) are shown in Table 2. According to the Plackett–Burman design, GSW, CSL, and MgSO₄·7H₂O showed positive effects, and thus, their +1 levels would be expected to improve the production of lactic acid. K₂HPO₄, CH₃COONa·3H₂O, (NH₄)₂HC₆H₅O₇, and MnSO₄·7H₂O showed negative effects, and their –1 levels would be helpful for the high production of lactic acid. A low *p*-value indicates a significant effect. From our results, GSW and CSL were the most significant nutritional factors (*p*-value < 0.05) that affected lactic acid production. In the results listed in Table 2, an R² of 0.9969 means that model could explain 99.69% of the total variations in the system. Therefore, GSW and CSL were selected for further optimization by CCD.

Table 2. The Plackett–Burman design for screening significant variables in lactic acid production at 37 °C under static conditions for 18 h.

Variables	Code	Low level (–1)	High level (+1)	Coefficient	<i>p</i> -value
Model	-	-	-	0.0897	0.0011 ^a
GSW	X ₁	1	15	0.7344	<0.0001 ^a
CSL	X ₂	1	10	0.1875	0.0153 ^a
K ₂ HPO ₄	X ₃	1	5	–0.2079	0.0901
CH ₃ COONa·3H ₂ O	X ₄	1	10	–0.0627	0.1919
(NH ₄) ₂ HC ₆ H ₅ O ₇	X ₅	0.5	3.5	–0.1705	0.2259
MgSO ₄ ·7H ₂ O	X ₆	0.05	0.35	2.3500	0.1273
MnSO ₄ ·7H ₂ O	X ₇	0.05	0.35	–2.1277	0.1543
Tween80	X ₈	0.1	2	–0.0271	0.8878
R-square (R ²)		0.9969			
Adjust R-square		0.9888			

^a significance level at *p*-value < 0.05.

3.3. Quantitative Optimization of Significant Components by CCD

GSW (X₁) and CSL (X₂) screened by the Plackett–Burman design were further studied by CCD to establish their optimum levels. To examine the combinatorial effect of these components on lactic acid production, 13 experiments were conducted, including the CCD of a 2² full factorial design, 2 × 2 axial star points, and five center points. The design matrix of CCD, the variables, and the corresponding

results, are presented in Table 3. Data were analyzed by linear multiple regression the Design-Expert 8.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA), and the following equation was obtained.

$$Y = -86.178 + 1.476X_1 + 0.740X_2 + 1.049 \times 10^{-3}X_1X_2 - 4.947 \times 10^{-3}X_1^2 - 2.348 \times 10^{-3}X_2^2$$

where Y is the predicted lactic acid concentration, and X₁ and X₂ are the concentrations of GSW and CSL, respectively.

Table 3. The CCD experimental design matrix with experimental and predicted values of lactic acid production at 37 °C under static conditions for 18 h.

Run	Coded values and actual values		Lactic acid (g/L)	
	GSW (g/L)	CSL (g/L)	Experimental	Predicted
1	−1 (83.12)	−1 (86.61)	57.15	56.44
2	1 (134.38)	−1 (86.61)	75.41	81.62
3	−1 (83.12)	+1 (263.39)	59.03	57.44
4	1 (134.38)	+1 (263.39)	86.80	92.14
5	−1.682 (72.50)	0 (175.00)	63.23	75.28
6	+1.682 (145.00)	0 (175.00)	115.38	105.22
7	0 (108.75)	−1.682 (50.00)	55.67	72.28
8	0 (108.75)	+1.682 (300.00)	62.56	78.04
9	0 (108.75)	0 (175.00)	90.33	93.50
10	0 (108.75)	0 (175.00)	91.42	93.50
11	0 (108.75)	0 (175.00)	95.26	93.50
12	0 (108.75)	0 (175.00)	98.00	93.50
13	0 (108.75)	0 (175.00)	92.49	93.50

Table 4 presents an ANOVA for the quadratic response surface model. According to the regression analysis of the experimental design, the interactive model term X₁X₂, with a *p*-value of more than 0.05, was insignificant, whereas all the other model terms (*p* < 0.05) were significant. The fitness of the polynomial model equation was judged by R². The R² was 0.9594, indicating the model could explain 95.94% of the variability in lactic acid production.

Table 4. Analysis of variance for the quadratic response surface model.

Source	Coefficient estimate	SS	df	MS	F-value	<i>p</i> -value (Prob > F)
Intercept/Model	93.50	4229.12	5	845.82	33.09	<0.0001
X ₁ : GSW	14.97	1793.98	1	1793.98	70.91	<0.0001
X ₂ : CSL	2.88	66.24	1	66.24	2.59	0.1515
X ₁ X ₂	2.38	22.63	1	22.63	0.89	0.3781
X ₁ ²	−3.25	73.52	1	73.52	2.88	0.1337
X ₂ ²	−18.34	2341.07	1	2341.07	91.59	<0.0001
Residual		178.92	7	25.56		
Lack of fit		140.16	3	46.72	4.82	0.0814
Pure error		38.76	4	9.69		

R² = 0.9594; adjusted R² = 0.9304; CV = 6.30%

In order to display the results visually, a three-dimensional surface response plot was generated, to indicate the interactions of GSW and CSL (Figure 2). A predicted highest lactic acid production of 105.6 g/L could be obtained at a concentration 134.4 g/L GSW and 187.7 g/L CSL. To verify the predicted results, an experiment was performed under optimized nutrient levels, and the experimental lactic acid production was found to be 102 g/L, suggesting that the experimental and predicted values were in good agreement.

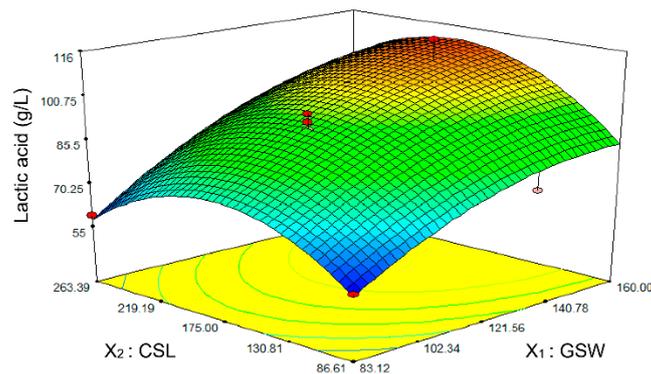


Figure 2. The three-dimensional response surface plot demonstrates the effects of CSL and GSW on lactic acid production by *L. plantarum* S21 at 37 °C after 24 h fermentation.

3.4. Comparison of Lactic Acid Fermentation on OM and OM-Mi in a 1 L Fermenter

Time course of lactic acid fermentation in an optimized medium (OM) in a 1-L fermenter was presented in comparison to that of the optimized medium without additional minerals (OM-Mi) (Figure 3). The total carbohydrate contents of both fermentation broths were decreased rapidly from 130 g/L to 20 g/L during the initial 30 h, and remained stable until the end of fermentation (Figure 3a), and the lactic acid produced from both media reached a maximum of 102 g/L at 30 h (Figure 3b) whereas the cells in both media grew quickly and reached a maximum of around 5.6 g/L dry cell weight at 18 h, and decreased after they had reached the highest values in both fermentations (Figure 3c). Amylase activity from both media was increased to a maximum of around 8 U/mL at 15 h; however, the enzyme activity of the OM increased much faster than that in OM-Mi, as shown in Figure 3d.

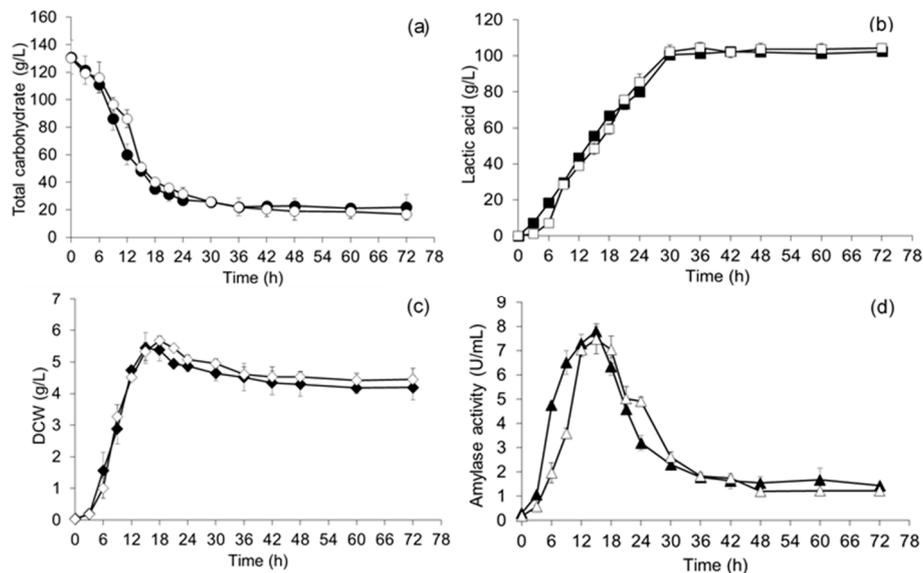


Figure 3. Comparison of lactic acid fermentation by *L. plantarum* S21 using the optimized medium with minerals (OM) (closed symbol) and without minerals (OM-Mi) (open symbol) in a 1 L fermenter at 37 °C. Time course of total carbohydrate concentration (a), lactic acid concentration (b), dry cell weight (DCW) (c), and amylase activity (d).

3.5. Antimicrobial Activity of Crude Lactic Acid Produced by *L. plantarum* S21

The cell free culture supernatant (CFCS) obtained from this experiment was tested for antimicrobial activity and the result is presented in Figure 4. The CFCS containing approximately 102 g/L lactic acid with different final pH values clearly showed antimicrobial activity against all

of the pathogenic bacterial strains tested in different capacities. Regarding the size of the clear zone formed surrounding the paper disc, non-neutralized CFCS (pH 6.0) showed the highest antimicrobial activity against *E. coli* 25922 and *S. enterica* serovar Typhimurium TISTR 292, whereas a smaller clear zone was found with neutralized CFCS (pH 7.0). The widest clear zone against *V. cholerae* TH-001 was observed clearly, and intriguingly, both neutralized CFCS and non-neutralized CFCS showed almost the same levels of antimicrobial activity.

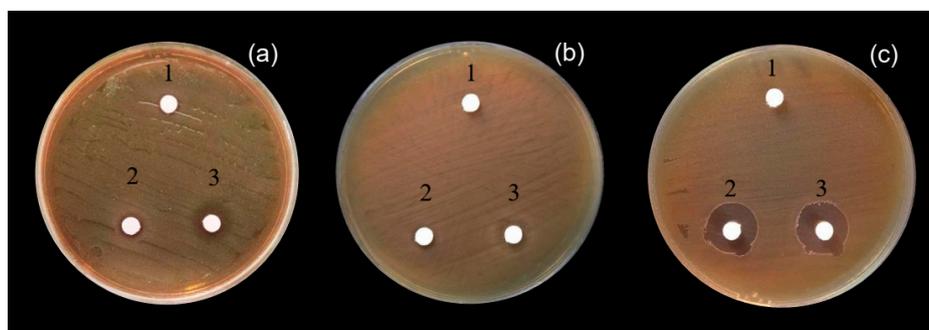


Figure 4. Antimicrobial activity of cell-free culture supernatant (CFCS) obtained from *L. plantarum* S21 cultivation in the optimized medium at 37 °C for 48 h against *E. coli* ATCC 25922 (a), *S. enterica* serovar Typhimurium TISTR 292 (b), *V. cholerae* TH-001 (c); deionized water (1), neutralized CFCS (2), and non-neutralized CFCS (3).

3.6. Trial Experiments for the Separation of Maltose-Forming α -Amylase from CFCS

The separation of maltose-forming α -amylase from CFCS was performed by using an ultrafiltration unit with a cellulose membrane of 50 kDa MWCO and the resulting recovery efficiency is demonstrated in Table 5. From the initial activity of the enzyme found in the CFCS of 49,140 U, the enzyme activity was retained in the unfiltered CFCS was recovered up to 47,343 U, which is calculated to be 96.3%, whereas the lactic acid yield in the filtrated supernatant was also recovered to a high level of up to 92.4%.

Table 5. Amylase and lactic acid recovery in an ultrafiltration system with 50 kDa MWCO.

	Volume (mL)	Amylase (U/mL)	Total amylase (U)	Recovery of amylase (%)	Lactic acid (g/L)	Total lactic acid (g)	Recovery of lactic acid (%)
Initial CFCS	7000	7.02	49,140	100.0	84.9	594,300	100.0
Retained CFCS	300	157.81	47,343	96.3	83.1	24,930	4.2
Filtrated supernatant	6600	0	0	0	83.2	549,120	92.4

4. Discussion

The result of the nitrogen source screening clearly confirmed the importance of the nutritional requirements for the growth of LAB [14,29], as the highest yield of lactic acid was achieved from the original MRS medium tailored for LAB development, containing peptone, beef extract, and yeast extract as the main organic nitrogen source. Among the nitrogen sources tested in this experiment, CSL is considered to be a feasible and inexpensive alternative nitrogen source, regarding the high yields of lactic acid that were close to the value obtained from the original MRS. This corresponds to the previous reports in the cultivation of *Streptomyces pilosus* [30] and *Lactobacillus* spp. [9,18,31]. Wee, Kim and Ryu [24] reported that 85% of CSL comprises of a nitrogen source such as crude proteins and amino acids. In addition, CSL is also a rich source of natural vitamins, organic acids, minerals, and other elemental nutrients [9,30]. Therefore, we concluded that CSL is possibly able to be used efficiently as an inexpensive nitrogen source for *L. plantarum* S21 cultivation, instead of the high-cost components such as yeast extract, beef extract, and peptone in MRS medium. However, the CSL components possibly vary depending on the manufacturer, which may introduce undesirable components for

microbial fermentation [32,33]. In addition, a high content protein of CSL may lead to the formation of Maillard reaction products (MRPs) during the media heat sterilization process, which has been reported to inhibit the growth of some lactobacilli, whereas two strains of *L. plantarum* (*L. plantarum* La-1203 and *L. plantarum* La-1210) are found to be non-sensitive strains [33]. *L. plantarum* S21 may also have attributes of this characteristic. Based on our results, CSL obtained from a domestic company in Thailand (Friendship Corn Starch Company) was a good alternative nitrogen source for the growth and metabolism of *L. plantarum* S21, and it did not show any negative effects on the formation of the targeted fermentative product.

Regarding the maximum lactic acid yield with a high efficiency of bioconversion as our target, statistical optimization methodologies, including the Plackett–Burman design, the central composite design, and the response surface plot were applied, and these statistic experimental designs were very helpful for obtaining an optimized medium that was mainly comprised of GSW and CSL with other six mineral salts, which was able to stimulate an increased conversion of GSW to lactic acid (approximately 102–106 g from 134 g GSW). The validation by our experiment, performed under optimized nutrient levels, and experimental lactic acid production, was found to be 102 g/L (%), suggesting that the experimental and predicted values were in good agreement. The statistical experimental design has been successfully utilized in medium optimization for the microbial fermentation process in recent decades [34–36]. Even the optimization was successfully achieved by helpful experimental designs, but the production medium and the expensive organic nitrogen sources, such as beef extract, peptone, and yeast extract, were replaced by CSL, but the optimized production medium is based on the MRS medium, and is still composed of other mineral salts such as K_2HPO_4 , CH_3COONa , and $MnSO_4$, which might be cost-effective substrates if fermentation is performed on a larger scale. The almost identical capabilities for *L. plantarum* S21 growth and lactic acid production in an optimized medium (OM), and the optimized medium without additional minerals (OM-Mi) (Figure 3) indicated that CSL not only serves as an excellent alternative nitrogen source, but it also be able to compensate for other mineral salts in MRS-based medium, and it can also be omitted from the production medium. This finding leads to an achievement for low-cost media for lactic acid production, which is composed of only two agricultural wastes, GSW and CSL.

In addition, the cell-free culture supernatant (CFCS), containing approximately 102 g/L lactic acid with different final pH values, clearly showed antimicrobial activity against the critical restricted pathogenic bacteria in food and feed industries as *Vibrio* spp., *Salmonella*, and *E. coli*. [37]. Our results suggested that the antimicrobial activity of CFCS obtained from the cultivation of *L. plantarum* S21 in OM-Mi is mainly attributed to the acidic condition of the CFCS. However, a clear zone was still found in a sample with the pH adjusted to 7.0, particularly in the case of *V. cholerae* TH-001. The result indicated that besides lactic acid, which is the main product of starch fermentation by *L. plantarum* S21, the CFCS possibly contains other antimicrobial agents, such as a bacteriocin called plantaricin [38,39]. Since the prohibition of antibiotics used in animal nutrition has been announced in 2006 by the EU, the use of organic acids as non-antibiotic alternatives in animal nutrition has increased in the feed industry, due to safety concerns for animals and humans [1,4]. Lactic acid is one of the organic acids that are approved and accepted for use as a functional feed ingredient [3,40], and our results confirm the beneficial effects of CFCS from *L. plantarum* S21 against pathogenic bacteria from the genus *Salmonella* and *Vibrio*, which often cause contamination problems, particularly in feed for broiler chickens and swine [40]. Therefore, the CFCS containing lactic acid as the main fermentative product obtained from this experiment is feasible for various applications, especially in food and feed products. The high recovery of maltose-forming α -amylase might arise from the suitable pore size of the ultrafiltration membrane (50 kDa MWCO) used in this experiment and the molecular weight of maltose-forming α -amylase from *L. plantarum* S21, which is reported to be 100 kDa [26]. However, the process and the apparatus used in this experiment may not be the most suitable for practical application, as the capacity of the chamber is too small (400 mL), which caused difficulties in dealing with a large sample of 7000 mL. Furthermore, the filtration system was operated by a vertical

flow system, which can easily encounter membrane fouling caused by the deposition of biological suspensions or macromolecules onto the membrane surface [41]. Fortunately, a top-drive magnetic stirrer equipped with the ultrafiltration chamber was helpful for overcoming a serious problem such as the membrane fouling problem, until the total sample volume had been filtrated. However, the application of this bioseparation model on a large scale requires modifications for a simpler and highly efficient performance. However, in large-scale production, this limitation may rise from some natural properties of the substrate. The gelatinized starchy waste is the cooked starch remaining in the noodle processing. The viscosity of this substrate can cause a problem of mixing, but it will be better after the rapid growth of *L. plantarum* S21, which is originally isolated from fermented starch. In addition to the problems caused from the substrate viscosity, the properties of CSL available from factories might also be a problem, due to the differences in composition, particularly protein and metal ion contents. Lower protein contents may effect either the lactic acid yield or its conversion efficiency. Some undesirable metal ions may inhibit the growth of microbes. Therefore, the standardization of CSL before its usage is a necessary requirement.

The overall results from this research confirm the high feasibility of using the amylolytic lactic acid bacterium *L. plantarum* S21 in the large-scale production of lactic acid for food and feed industries, using a low-cost medium, and the food-applicable enzyme is also able to produce as a by-product. Moreover, this lactic acid bacterium originates from Thai fermented rice noodles [25], and due to its high capability for growth on variety starchy materials, this bacterial strain is also attractive for applications in the processing of various food, such as pasta [23] and bread production [20].

5. Conclusions

The main purpose of this research is to find the proper processes for the treatment and utilization of starchy wastes from the noodle manufacturing process, and the direct bioconversion of GSW to lactic acid was first targeted. CSL was selected as the most suitable low-cost nitrogen source. The statistical optimization methodology, including the Plackett–Burman design, central composite design, and response surface plot, were helpfully applied, to obtain an optimized medium that mainly comprised GSW and CSL with six other mineral salts that were able to produce lactic acid, (approximately 102–106 g from 134 g GSW). A low-cost medium containing only two agricultural wastes, GSW and CSL was also discovered, and no effect on lactic acid yield was observed when omitting all of the other six components from the optimized medium. The bioconversion of GSW to lactic acid and its by-product was successfully produced in 1 L and 10 L fermenters. Crude lactic acid obtained from this process demonstrated the additional benefits of antimicrobial activity against some important pathogenic bacteria in food and feed industries, whereas a maltose-forming α -amylase was also successfully separated, with a high recovery yield of up to 96%. This study clearly demonstrates a promising bioprocess for the simultaneous production of lactic acid, and a value-added maltose-forming α -amylase from *L. plantarum* S21, using only two industrial wastes, GSW and CSL, as the medium components.

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References

1. Adil, S.; Banday, T.; Bhat, G.A.; Mir, M.S.; Rehman, M. Effect of dietary supplementation of organic acids on performance, intestinal histomorphology, and serum biochemistry of broiler chicken. *Vet. Med. Int.* **2010**, *2010*, 479485. [[CrossRef](#)] [[PubMed](#)]
2. Özogul, F.; Hamed, I. The importance of lactic acid bacteria for the prevention of bacterial growth and their biogenic amines formation: A review. *Crit. Rev. Food Sci. Nutr.* **2018**, *58*, 1660–1670. [[CrossRef](#)] [[PubMed](#)]
3. Roth, F.; Kirchgessner, M. Organic acids as feed additives for young pigs: Nutritional and gastrointestinal effects. *J. Anim. Feed Sci.* **1998**, *7*, 25–33. [[CrossRef](#)]
4. Rychen, G.; Aquilina, G.; Azimonti, G.; Bampidis, V.; Bastos, M.d.L.; Bories, G.; Chesson, A.; Cocconcelli, P.S.; Flachowsky, G.; Kolar, B.; et al. Safety of lactic acid and calcium lactate when used as technological additives for all animal species. *EFSA J.* **2017**, *15*, 4938. [[CrossRef](#)]
5. Wang, Y.; Tashiro, Y.; Sonomoto, K. Fermentative production of lactic acid from renewable materials: Recent achievements, prospects, and limits. *J. Biosci. Bioeng.* **2015**, *119*, 10–18. [[CrossRef](#)]
6. Ghaffar, T.; Irshad, M.; Anwar, Z.; Aqil, T.; Zulifqar, Z.; Tariq, A.; Kamran, M.; Ehsan, N.; Mehmood, S. Recent trends in lactic acid biotechnology: A brief review on production to purification. *J. Radiat. Res. Appl. Sci.* **2014**, *7*, 222–229. [[CrossRef](#)]
7. Abdel-Rahman, M.A.; Tashiro, Y.; Sonomoto, K. Recent advances in lactic acid production by microbial fermentation processes. *Biotechnol. Adv.* **2013**, *31*, 877–902. [[CrossRef](#)] [[PubMed](#)]
8. Bernardo, M.P.; Coelho, L.F.; Sass, D.C.; Contiero, J. L-(+)-lactic acid production by *Lactobacillus rhamnosus* B103 from dairy industry waste. *Braz. J. Microbiol.* **2016**, *47*, 640–646. [[CrossRef](#)]
9. Thakur, A.; Panesar, P.S.; Saini, M.S. L (+)-lactic acid production by immobilized *Lactobacillus casei* using low cost agro-industrial waste as carbon and nitrogen sources. *Waste Biomass Valor.* **2017**, 1–11. [[CrossRef](#)]
10. Brock, S.; Kuenz, A.; Prüße, U. Impact of hydrolysis methods on the utilization of agricultural residues as nutrient source for D-lactic acid production by *Sporolactobacillus inulinus*. *Fermentation* **2019**, *5*, 12. [[CrossRef](#)]
11. Palmerín-Carreño, D.M.; Hernández-Orihuela, A.L.; Martínez-Antonio, A. Production of D-lactate from avocado seed hydrolysates by metabolically engineered *Escherichia coli* JU15. *Fermentation* **2019**, *5*, 26. [[CrossRef](#)]
12. Komesu, A.; de Oliveira, J.A.R.; da Silva Martins, L.H.; Maciel, M.R.W.; Maciel Filho, R. Lactic acid production to purification: A review. *Bioresources* **2017**, *12*, 4364–4383. [[CrossRef](#)]
13. Kanpiengjai, A.; Lumyong, S.; Pathom-aree, W.; Khanongnuch, C. Starchy effluent from rice noodle manufacturing process as feasible substrate for direct lactic acid production by *Lactobacillus plantarum* S21. *J. Korean Soc. Appl. Biol. Chem.* **2014**, *57*, 217–220. [[CrossRef](#)]
14. Altaf, M.; Naveena, B.J.; Reddy, G. Use of inexpensive nitrogen sources and starch for L(+) lactic acid production in anaerobic submerged fermentation. *Bioresour. Technol.* **2007**, *98*, 498–503. [[CrossRef](#)]
15. Yousuf, A.; Bastidas-Oyanedel, J.R.; Schmidt, J.E. Effect of total solid content and pretreatment on the production of lactic acid from mixed culture dark fermentation of food waste. *Waste Manag.* **2018**, *77*, 516–521. [[CrossRef](#)]
16. Tang, J.; Wang, X.; Hu, Y.; Zhang, Y.; Li, Y. Lactic acid fermentation from food waste with indigenous microbiota: Effects of pH, temperature and high OLR. *Waste Manag.* **2016**, *52*, 278–285. [[CrossRef](#)]
17. Kona, R.; Qureshi, N.; Pai, J. Production of glucose oxidase using *Aspergillus niger* and corn steep liquor. *Bioresour. Technol.* **2001**, *78*, 123–126. [[CrossRef](#)]
18. De la Torre, I.; Ladero, M.; Santos, V. Production of D-lactic acid by *Lactobacillus delbrueckii* ssp. *delbrueckii* from orange peel waste: Techno-economical assessment of nitrogen sources. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 10511–10521. [[CrossRef](#)]
19. Panesar, P.S.; Kaur, S. Bioutilisation of agro-industrial waste for lactic acid production. *Int. J. Food Sci. Tech.* **2015**, *50*, 2143–2151. [[CrossRef](#)]
20. Russo, P.; Fares, C.; Longo, A.; Spano, G.; Capozzi, V. *Lactobacillus plantarum* with broad antifungal activity as a protective starter culture for bread production. *Foods* **2017**, *6*, 110. [[CrossRef](#)]
21. Huang, W.-C.; Wei, C.-C.; Huang, C.-C.; Chen, W.-L.; Huang, H.-Y. The beneficial effects of *Lactobacillus plantarum* PS128 on high-intensity, exercise-induced oxidative stress, inflammation, and performance in triathletes. *Nutrients* **2019**, *11*, 353. [[CrossRef](#)]

22. Berbegal, C.; Garofalo, C.; Russo, P.; Pati, S.; Capozzi, V.; Spano, G. Use of autochthonous yeasts and bacteria in order to control *Brettanomyces bruxellensis* in wine. *Fermentation* **2017**, *3*, 65. [[CrossRef](#)]
23. Capozzi, V.; Russo, P.; Fragasso, M.; De Vita, P.; Fiocco, D.; Spano, G. Biotechnology and pasta-making: Lactic acid bacteria as a new driver of innovation. *Front. Microbiol.* **2012**, *3*, 94. [[CrossRef](#)]
24. Wee, Y.J.; Kim, J.N.; Ryu, H.W. Biotechnological production of lactic acid and its recent applications. *Food Technol. Biotechnol.* **2006**, *44*, 163–172.
25. Kanpiengjai, A.; Rieantrakoonchai, W.; Pratanaphon, R.; Pathom-aree, W.; Lumyong, S.; Khanongnuch, C. High efficacy bioconversion of starch to lactic acid using an amyolytic lactic acid bacterium isolated from Thai indigenous fermented rice noodles. *Food Sci. Biotechnol.* **2014**, *23*, 1541–1550. [[CrossRef](#)]
26. Kanpiengjai, A.; Lumyong, S.; Nguyen, T.H.; Haltrich, D.; Khanongnuch, C. Characterization of a maltose-forming α -amylase from an amyolytic lactic acid bacterium *Lactobacillus plantarum* S21. *J. Mol. Catal. B Enzym.* **2015**, *120*, 1–8. [[CrossRef](#)]
27. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [[CrossRef](#)]
28. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [[CrossRef](#)]
29. Altaf, M.; Naveena, B.J.; Venkateshwar, M.; Kumar, E.V.; Reddy, G. Single step fermentation of starch to L(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using inexpensive nitrogen sources to replace peptone and yeast extract—Optimization by RSM. *Process Biochem.* **2006**, *41*, 465–472. [[CrossRef](#)]
30. Chiani, M.; Akbarzadeh, A.; Farhangi, A.; Mehrabi, M. Production of desferrioxamine B (Desferal) using corn steep liquor in *Streptomyces pilosus*. *Pak. J. Biol. Sci.* **2010**, *13*, 1151–1155. [[CrossRef](#)]
31. Yu, L.; Lei, T.; Ren, X.; Pei, X.; Feng, Y. Response surface optimization of L-(+)-lactic acid production using corn steep liquor as an alternative nitrogen source by *Lactobacillus rhamnosus* CGMCC 1466. *Biochem. Eng. J.* **2008**, *39*, 496–502. [[CrossRef](#)]
32. Hull, S.R.; Yang, B.Y.; Venzke, D.; Kulhavy, K.; Montgomery, R. Composition of corn steep water during steeping. *J. Agric. Food Chem.* **1996**, *44*, 1857–1863. [[CrossRef](#)]
33. Senz, M.; Keil, C.; Schmach, M.; Palinski, S.; Cämmerer, B.; Hageböck, M. Influence of media heat sterilization process on growth performance of representative strains of the genus *Lactobacillus*. *Fermentation* **2019**, *5*, 20. [[CrossRef](#)]
34. Avci, A.; Saha, B.C.; Dien, B.S.; Kennedy, G.J.; Cotta, M.A. Response surface optimization of corn stover pretreatment using dilute phosphoric acid for enzymatic hydrolysis and ethanol production. *Bioresour. Technol.* **2013**, *130*, 603–612. [[CrossRef](#)]
35. Cingadi, S.; Srikanth, K.; Arun, E.; Sivaprakasam, S. Statistical optimization of cassava fibrous waste hydrolysis by response surface methodology and use of hydrolysate based media for the production of optically pure d-lactic acid. *Biochem. Eng. J.* **2015**, *102*, 82–90. [[CrossRef](#)]
36. Zhang, J.; Dong, Y.C.; Fan, L.L.; Jiao, Z.H.; Chen, Q.H. Optimization of culture medium compositions for gellan gum production by a halobacterium *Sphingomonas paucimobilis*. *Carbohydr. Polym.* **2015**, *115*, 694–700. [[CrossRef](#)]
37. Mor-Mur, M.; Yuste, J. Emerging bacterial pathogens in meat and poultry: An overview. *Food Bioprocess Technol.* **2010**, *3*, 24. [[CrossRef](#)]
38. Gupta, A.; Tiwari, S.K. Plantaricin LD1: A bacteriocin produced by food isolate of *Lactobacillus plantarum* LD1. *Appl. Biochem. Biotechnol.* **2014**, *172*, 3354–3362. [[CrossRef](#)]
39. Holo, H.; Jeknic, Z.; Daeschel, M.; Stevanovic, S.; Nes, I.F. Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. *Microbiology* **2001**, *147*, 643–651. [[CrossRef](#)]
40. Khan, S.H.; Iqbal, J. Recent advances in the role of organic acids in poultry nutrition. *J. Appl. Anim. Res.* **2016**, *44*, 359–369. [[CrossRef](#)]
41. Mohammad, A.W.; Ng, C.Y.; Lim, Y.P.; Ng, G.H. Ultrafiltration in food processing industry: Review on application, membrane fouling, and fouling control. *Food Bioprocess Technol.* **2012**, *5*, 1143–1156. [[CrossRef](#)]

