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Statistical Optimization of Alkali Pretreatment to Improve Sugars Recovery from Spent Coffee Grounds and Utilization in Lactic Acid Fermentation

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Abstract: Biorefinery, which utilizes carbon-neutral biomass as a resource, is attracting attention as a significant alternative in a modern society confronted with climate change. In this study, spent coffee grounds (SCGs) were used as the feedstock for lactic acid fermentation. In order to improve sugar conversion, alkali pretreatment was optimized by a statistical method, namely response surface methodology (RSM). The optimum conditions for the alkali pretreatment of SCGs were determined as follows: 75 °C, 3% potassium hydroxide (KOH) and a time of 2.8 h. The optimum conditions for enzymatic hydrolysis of pretreated SCGs were determined as follows: enzyme complex loading of 30-unit cellulase, 15-unit cellobiase and 50-unit mannanase per g biomass and a reaction time of 96 h. SCG hydrolysates were used as the carbon source for *Lactobacillus* cultivation, and the conversions of lactic acid by *L. brevis* ATCC 8287 and *L. parabuchneri* ATCC 49374 were 40.1% and 55.8%, respectively. Finally, the maximum lactic acid production by *L. parabuchneri* ATCC 49374 was estimated to be 101.2 g based on 1000 g of SCGs through the optimization of alkali pretreatment and enzymatic hydrolysis.

Keywords: spent coffee grounds; optimization; alkali pretreatment; enzymatic hydrolysis; fermentation; lactic acid

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1. Introduction

Coffee is one of the most consumed beverages in the world. According to the International Coffee Organization (ICO), global coffee production in 2019 was estimated to be about 170 million bags of 60 kg [1]. Spent coffee grounds (SCGs) are generated from approximately 91 wt% of the whole coffee bean in coffee processing [2]. SCGs are buried in landfills, causing environmental pollution such as soil, water and air pollution and the emission of greenhouse gases [3]. In addition, disposable coffee cups made of plastic have been associated with these forms of pollution [4]. Recently, biorefinery that converts food waste into value-added materials has been attracting attention [5]. SCGs containing carbohydrates, lipids and proteins can be a potential raw material in biorefinery [6]. Various studies have been conducted to produce value-added materials such as biopolymers, biofuels and antioxidants by using the carbohydrates of SCGs as carbon sources for microbial fermentation [7,8].

Lactic acid is a resource for the production of polylactic acid (PLA), a commercial biodegradable plastic [9]. PLA has emerged as a green alternative to petroleum-based plastics because of its properties, such as its biodegradability, biocompatibility, transparency and processability [10]. In addition, 90% of lactic acid utilized in industry is produced

by biological methods through microbial fermentation [11]. Lactic acid synthesized by chemical methods has disadvantages for industrial applications due to its low optical purity, indicating its physical properties and high manufacturing cost [12]. Lactic acid fermented by *Lactobacillus* sp. has a high acid tolerance and selectivity for stereoisomer production [13].

In order to convert biomass to lactic acid, the following three major steps are required: (1) pretreatment of biomass, (2) enzymatic hydrolysis of the pretreated biomass to produce fermentable sugars and (3) fermentation of *Lactobacillus* sp. for lactic acid production [14]. SCGs are composed of 8.6–15.3% cellulose, 31.7–41.7% hemicellulose, 22.2–33.6% lignin and 21.3–40.7% other materials [15]. Carbohydrates (cellulose and hemicellulose) exist in the form of lignin–carbohydrate complexes linked by ether or ester bonds, and lignin inhibits the conversion of carbohydrates to monosaccharides by enzymes [16]. Therefore, the removal of lignin from lignocellulosic biomass during the pretreatment process can improve the enzymatic hydrolysis [17].

Pretreatment of biomass, including chemical (alkali, acid and ionic liquid), physical (pyrolysis and steam explosion) and biological (fungi) pretreatment to remove the lignin, have been carried out [18]. The chemical pretreatment using diluted acid or alkaline is preferred due to its high sugar recovery and low cost [19]. Diluted acid pretreatment leads to fermentation inhibitors, including furfural and 5-hydroxymethylfurfural, which are generated by the decomposition of hemicellulose [20]. Alkaline pretreatment selectively removes lignin, and potassium hydroxide (KOH) is preferred over sodium hydroxide (NaOH) because of its high efficiency in removing recalcitrant components of the plant's cell wall, such as lignin [21]. The pretreatment and enzymatic hydrolysis processes were estimated to be about 35% of the total process cost of biorefinery [22]. For industrial applications, it is required to reduce process costs by establishing optimum reaction conditions for each step. Response surface methodology (RSM) is a mathematical and statistical method to determine the optimum reaction conditions, considering the interaction of parameters [23]. Various studies have been reported to optimize the pretreatment conditions of biomass using RSM to reduce the process cost [14,24,25].

In this study, lactic acid was produced using SCGs as a feedstock for the fermentation of *Lactobacillus* sp. KOH was used to remove the lignin from the SCGs, and various parameters such as the temperature, KOH concentration and time were used in RSM to determine the optimum pretreatment conditions. Pretreated SCGs were enzymatically hydrolyzed with various concentrations of an enzyme cocktail to investigate suitable enzyme loading. SCG hydrolysates were used for the fermentation of *L. brevis* ATCC 8287 and *L. parabuchneri* ATCC 49374 as the carbon sources to produce lactic acid. Finally, the overall process of lactic acid production from biomass was evaluated by the material balance, based on 1000 g of SCGs.

2. Materials and Methods

2.1. Materials

SCGs were obtained from Café Costa (Seoul, Korea). The SCGs were dried at 105 °C for 48 h to remove moisture and stored in plastic bags at room temperature. Potassium hydroxide (KOH), sulfuric acid (H_2SO_4), calcium carbonate ($CaCO_3$), potassium hydrogen phosphate (K_2HPO_4), sodium acetate, ammonium citrate and citric acid monohydrate were purchased from Samchun Chemical (Pyeongtaek, Korea). Peptone, beef extract and yeast extract were purchased from BD Difco (Sparks, MD, USA). Celluclast® 1.5 L (cellulase), Cellic® CTec2 (cellobiase) and Mannaway® 4.0 L (mannanase) were purchased from Novozymes (Krogshoejvej, Bagsværd, Denmark). *Lactobacillus brevis* ATCC 8287, *Lactobacillus parabuchneri* ATCC 49374 and de Man, Rogosa and Sharpe (MRS) medium were purchased from the Korean Collection for Type Cultures (KCTC).

2.2. Alkali Pretreatment of SCGs

Alkali pretreatment of the SCGs was performed to remove lignin from the SCGs with a 250 mL Erlenmeyer flask in a water bath, and 5 g of dried SCGs were reacted with 50 mL KOH of various concentrations (0, 1, 2, 3 and 4% w/w) at different temperatures (0, 25, 50, 75 and 100 °C) for various times (0, 1, 2, 3 and 4 h). The pretreated SCGs were neutralized using deionized water (DW) until the pH reached 7.0 and were dried at 105 °C for 48 h. All samples were quantified on a dry weight basis.

2.3. Optimization of Alkali Pretreatment of SCGs Using Response Surface Methodology

In order to optimize the alkali pretreatment of SCGs, a central composite design (CCD) of the response surface methodology (RSM) was performed using Design-Expert 7 software (Stat-Ease Inc., USA). The three factors affecting the alkali pretreatment of SCGs were divided into five levels (−2, −1, 0, 1 and 2; see Table 1). The factors and their ranges were as follows: temperature (X_1; 0–100 °C), KOH concentration (X_2; 0–4%) and time (X_3; 0–4 h). The experimental results were analyzed by analysis of variance (ANOVA). The effects of these factors and their interactions were explained by applying the following quadradic equation:

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

where Y is the predicted response (Y_{GC} = glucan content, Y_{MC} = mannan content and Y_{ED} = enzymatic digestibility), X_i and X_j are input factors affecting the response Y, β_0 is the offset term, β_i is the first-order model coefficient, β_{ii} is the quadratic coefficient for factor i and β_{ij} is the linear model coefficient for the interaction between factors i and j [26,27]. The glucan content (GC), mannan content (MC) and enzymatic digestibility (ED) were taken as the response and calculated according to the following equations:

$$\text{Glucan content, GC (\%)} = \text{weight of glucan in dried SCG/weight of dried SCG} \times 100 \quad (2)$$

$$\text{Mannan content, MC (\%)} = \text{weight of mannan in dried SCG/weight of dried SCG} \times 100 \quad (3)$$

$$\text{Enzymatic digestibility, ED (\%)} = \text{weight of glucose and mannose released/(weight of glucan and mannan} \times 1.1) \times 100 \quad (4)$$

where 1.1 is the conversion factor of glucan to glucose and mannan to mannose.

Table 1. Factors and their levels in the response surface methodology (RSM).

Factors	Unit	Symbol	Coded Factor Levels				
			−2	**−1**	**0**	**1**	**2**
Temperature	°C	X_1	0	25	50	75	100
KOH concentration	%	X_2	0	1	2	3	4
Time	h	X_3	0	1	2	3	4

2.4. Enzymatic Hydrolysis of SCGs

Enzymatic hydrolysis was performed according to the National Renewable Energy Laboratory's (NREL) analytical procedure (TP-510-42629) [28]. One filter paper unit (FPU) and cellobiase unit (CBU) were determined as the amount of enzyme that released 1 μmol of glucose per min under a standard assay. One mannanase unit (MNU) was determined as the amount of enzyme that released 1 μmol of mannose per min under a standard assay. Enzymatic hydrolysis was carried out by soaking 0.3 g of pretreated SCGs in a 10 mL 50 mM sodium citrate buffer (pH 4.8) and hydrolyzed with various loadings (biomass weight (g) per 7.5–60 FPU cellulase, 3.75–30 CBU cellobiase and 12.5–100 MNU mannanase) of an enzyme cocktail at 50 °C for 144 h in a shaking incubator at 180 rpm (SI-100R, Hanyang Science Lab, Seoul, Korea).

2.5. Lactic Acid Production Using SCG Hydrolysates

Lactic acid was produced by fermentation of *L. brevis* ATCC 8287 and *L. parabuchneri* ATCC 49374, using SCG hydrolysates as carbon sources. Seed cultures (preculture) of *L. brevis* ATCC 8287 and *L. parabuchneri* ATCC 49374 were carried out in an MRS broth (KCTC media No. 178) at 30 °C for 24 h in a shaking incubator at 150 rpm. In the main culture to produce lactic acid, 10% (v/v) of the seed cultural broth was inoculated on the main medium (production medium). The fermentation was performed with 50 mL of the main medium at 30 °C for 24 h in a shaking incubator at 150 rpm. The main medium was composed as follows: 20 g/L carbon source (glucose and mannose mixtures for control and SCG hydrolysates for the experimental group), 10 g/L peptone, 10 g/L beef extract, 5 g/L yeast extract, 1 g/L tween 80, 2 g/L K_2HPO_4, 5 g/L sodium acetate, 2 g/L ammonium citrate, 0.1 g/L $MgSO_4$, 0.05 g/L $MnSO_4$ and pH = 6.5. Cell growth was measured by optical density (OD) at 600 nm using a UV spectrophotometer (DU® 730, Beckman Coulter, Brea, CA, USA), and the pH was measured using a pH meter (EcoMet P25, Istek, Seoul, Korea). Lactic acid conversion was determined by following equation:

$$\text{Lactic acid conversion (\%)} = (\text{weight of lactic acid released/weight of monosaccharide consumed}) \times 100 \quad (5)$$

2.6. Analytical Methods

The chemical composition of the SCGs was determined by the NREL laboratory analytical procedure (TP-510-42618) [29], in which 0.3 g of SCGs were soaked in 30 mL of 72% (*w*/*w*) H_2SO_4 at 30 °C for 2 h. The mixture was diluted with 84 mL DW to a concentration of 4% and reacted at 121 °C for 1 h. After the reaction, the mixture was neutralized using $CaCO_3$ powder until reaching a pH of 7, and the supernatant was filtered with a 0.22 μm syringe filter for high-performance liquid chromatography (HPLC) analysis.

The concentrations of glucose, mannose and lactic acid were determined by HPLC equipped a refractive index detector (RID-10A, Shimadzu, Japan) and a Shodex SUGAR SH1011 H^+ ion exclusion column (300 mm × 8 mm, Shodex, Japan). The HPLC analysis conditions were as follows: mobile phase of 0.005 N H_2SO_4, flow rate of 0.6 mL/min, temperature of column at 50 °C and an injection volume of 20 μL.

3. Results and Discussion

3.1. Optimization of KOH Pretreatment Conditions for SCGs Using RSM

In order to optimize the reaction conditions for SCG pretreatment, a CCD of the RSM was performed. RSM is a statistical and mathematical tool to reduce the number of experiments and obtain reliable data [30]. Table 2 shows the 20 experiments and their responses designed for the KOH pretreatment of SCGs. The selected three factors (X_1 = temperature, X_2 = KOH concentration and X_3 = time) were divided into five levels (temperature = 0, 25, 50, 75 and 100 °C; KOH concentration = 0, 1, 2, 3 and 4%; and time = 0, 1, 2, 3 and 4 h). A KOH concentration of 0% ($X_2 = -2$) meant that the SCGs were pretreated with DW, and a time of 0 h ($X_3 = -2$) meant that the SCGs were not pretreated. The range of each response was a GC of 9.3–19.0%, MC of 23.3–41.6% and ED of 26.7–48.1%. The GC, MC and ED of the untreated SCGs (Std no. 13) were found to be 9.3%, 23.3% and 35.2%, respectively.

The CCD results were estimated using the following second-order polynomial equation by analyzing a quadratic regression ANOVA for the experimental response:

$$Y_{GC} = 14.53 + 0.76\,X_1 + 1.46\,X_2 + 0.82\,X_3 + 0.17\,X_1X_2 + 0.25\,X_1X_3 + 0.20\,X_2X_3 + 0.20\,{X_1}^2 - 0.30\,{X_2}^2 - 0.42\,{X_3}^2 \quad (6)$$

$$Y_{MC} = 36.51 + 1.62\,X_1 + 2.27\,X_2 + 1.81\,X_3 + 0.55\,X_1X_2 + 0.14\,X_1X_3 + 0.36\,X_2X_3 + 0.67\,{X_1}^2 - 1.36\,{X_2}^2 - 1.49\,{X_3}^2 \quad (7)$$

$$Y_{ED} = 40.42 + 2.68\,X_1 + 4.56\,X_2 + 1.55\,X_3 - 0.34\,X_1X_2 - 0.59\,X_1X_3 - 1.36\,X_2X_3 + 0.68\,{X_1}^2 - 0.49\,{X_2}^2 - 0.53\,{X_3}^2 \quad (8)$$

where Y_{GC} is the glucan content, Y_{MC} is the mannan content and Y_{ED} is the enzymatic digestibility, respectively. X_1, X_2 and X_3 are the independent factors, and they are the mean

temperature, KOH concentration and time, respectively. Tables 3–5 show the ANOVA results for the response surface quadratic model.

Table 2. The central composite design and their responses for five-level, three-factor response surface analysis.

Std	Coded Factor Levels			Response		
	X_1	X_2	X_3	GC [1] (%)	MC [2] (%)	ED [3] (%)
1	−1	−1	−1	13.5	32.6	26.7
2	1	−1	−1	14.0	35.0	37.3
3	−1	1	−1	16.4	35.2	41.1
4	1	1	−1	16.2	37.5	47.3
5	−1	−1	1	13.7	34.3	34.3
6	1	−1	1	13.8	34.9	39.4
7	−1	1	1	16.1	36.0	40.2
8	1	1	1	18.2	41.1	47.1
9	−2	0	0	14.2	33.8	43.5
10	2	0	0	19.0	41.6	47.6
11	0	−2	0	9.8	23.8	32.6
12	0	2	0	15.5	35.4	48.1
13	0	0	−2	9.3	23.3	35.2
14	0	0	2	14.9	34.8	46.3
15	0	0	0	16.2	38.9	41.1
16	0	0	0	14.4	33.8	45.2
17	0	0	0	14.6	36.1	43.3
18	0	0	0	14.3	36.4	35.5
19	0	0	0	14.2	36.0	38.4
20	0	0	0	14.1	35.9	40.8

[1] GC = glucan content. [2] MC = mannan content. [3] ED = enzymatic digestibility.

Table 3. Analysis of variance (ANOVA) for the response surface quadratic model of the glucan content.

Source	Sum of Square	Degree of Freedom	Mean Square	F-Value	*p*-Value	Remarks
Model	78.83	9	8.76	5.21	0.0083	significant
X_1	9.15	1	9.15	5.44	0.0419	significant
X_2	34.05	1	34.05	20.25	0.0011	significant
X_3	10.63	1	10.63	6.32	0.0307	significant
X_1X_2	0.24	1	0.24	0.14	0.7118	
X_1X_3	0.49	1	0.49	0.29	0.6021	
X_2X_3	0.33	1	0.33	0.20	0.6675	
X_1^2	12.13	1	12.13	7.22	0.0228	significant
X_2^2	2.19	1	2.19	1.30	0.2800	
X_3^2	4.49	1	4.49	2.67	0.1335	

Coefficient of determination (R^2) = 0.8242. Adjusted R^2 = 0.6660. Coefficient of variation (CV) = 8.93. Adequate precision = 9.151.

A high F-value and low *p*-value ($p < 0.05$) indicate that the model and the model terms are significant [31]. The F-values of each model (GC, MC and ED) were found to be 5.21, 5.45 and 16.87, respectively. This means that each model had only a 0.83%, 0.70% and 0.01% chance to obtain a large F-value due to noise, respectively. Each model was significant, showing *p*-values of 0.0083, 0.0070 and <0.0001, respectively. X_1, X_2 and X_3 were significant model terms ($p < 0.05$) for the GC, MC and ED. X_1^2 was a significant model term ($p < 0.05$) for the GC, and X_2^2 and X_3^2 were significant model terms ($p < 0.05$) for the MC. The coefficient of determination (R^2) indicates the reliability of the model and is recommended to be higher than 0.9 for high model quality [32]. The adjusted R^2 is a value adjusted to the number of factors of the model relative to the number of points in the design, and the difference with R^2 should be less than 0.2 [33]. The difference with R^2 and adjusted R^2 in each model were less than 0.2. The coefficient of variation (CV) refers

to the variance of the data, and a CV of less than 10% means that the results are accurate and reliable [34]. The CV of each model was found to be 8.93, 7.15 and 4.68, representing that the results of each model had accuracy and reliability. The adequate precision of each model was found to be 9.151, 9.049 and 14.673, respectively. The adequate precision determines the signal-to-noise ratio, and a value higher than 4 means that the predicted model is useful to investigate the designed space [35].

Table 4. ANOVA for the response surface quadratic model of the mannan content.

Source	Sum of Square	Degree of Freedom	Mean Square	F-Value	*p*-Value	Remarks
Model	302.26	9	33.58	5.45	0.0070	significant
X_1	41.92	1	41.92	6.80	0.0262	significant
X_2	82.26	1	82.26	13.34	0.0044	significant
X_3	52.61	1	52.61	8.53	0.0153	significant
X_1X_2	2.45	1	2.45	0.40	0.5426	
X_1X_3	0.15	1	0.15	0.024	0.8801	
X_2X_3	1.05	1	1.05	0.17	0.6891	
X_1^2	11.19	1	11.19	1.81	0.2078	
X_2^2	46.70	1	46.70	7.57	0.0204	significant
X_3^2	55.87	1	55.87	9.06	0.0131	significant

Coefficient of determination (R^2) = 0.8305. Adjusted R^2 = 0.6780. Coefficient of variation (CV) = 7.15. Adequate precision = 9.049.

Table 5. ANOVA for the response surface quadratic model of the enzymatic digestibility.

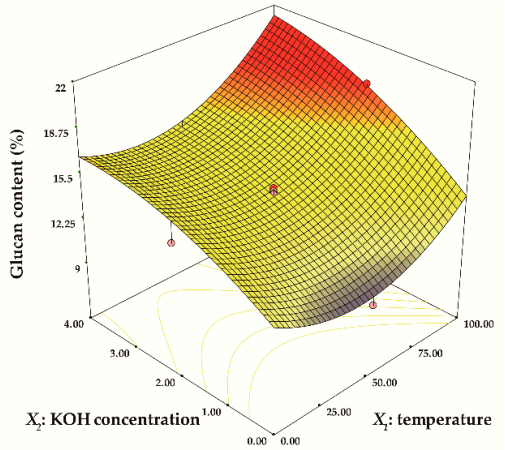
Source	Sum of Square	Degree of Freedom	Mean Square	F-Value	*p*-Value	Remarks
Model	535.21	9	59.47	16.87	<0.0001	significant
X_1	114.68	1	114.68	32.53	0.0002	significant
X_2	332.85	1	332.85	94.42	<0.0001	significant
X_3	38.48	1	38.48	10.92	0.0080	significant
X_1X_2	0.90	1	0.90	0.26	0.6244	
X_1X_3	2.79	1	2.79	0.79	0.3944	
X_2X_3	14.72	1	14.72	4.18	0.0682	
X_1^2	11.59	1	11.59	3.29	0.0999	
X_2^2	5.98	1	5.98	1.70	0.2220	
X_3^2	7.05	1	7.05	2.00	0.1877	

Coefficient of determination (R^2) = 0.9382. Adjusted R^2 = 0.8826. Coefficient of variation (CV) = 4.68. Adequate precision = 14.673.

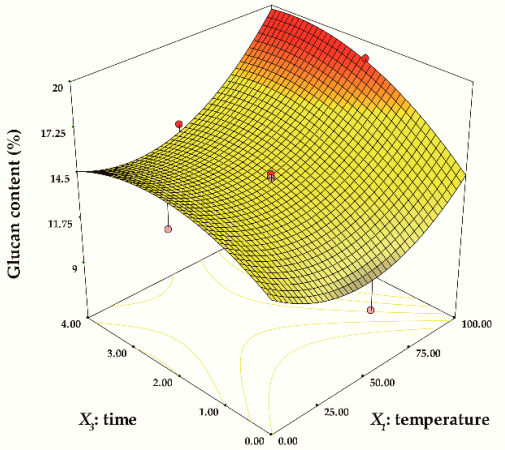
Figures 1–3 show a three-dimensional response surface plot, made to investigate the effect of the interactions between the experimental factors on each response based on the predicted regression model. The three-dimensional response surface plot was suitable to represent the possible independence of factors in relation to the response [36]. The effect of the factors on the GC is represented in Figure 1. In Figure 1a, the GC reached the maximum point (21.3%) at 100 °C and 4% KOH, and it decreased as the temperature and KOH concentration decreased. Regardless of the time level, the GC was low at 50 °C and increased as the temperature and time increased (Figure 1b). The GC was at its minimum point (7.9%) at 0% KOH for 0 h and enhanced drastically with increasing KOH concentrations and times (Figure 1c). Figure 2 shows the effect of the factors on the MC. The maximum MC was determined to be 44.5% at 100 °C and 4% KOH, and the MC decreased as the temperature approached 50 °C and the KOH concentration decreased (Figure 2a). Figure 2b shows that the MC was high at 100 °C and 2.5 h and decreased with the change of temperature and time. In Figure 2c, the minimum MC was obtained with 18.4% at 0% KOH for 0 h, and the MC increased significantly as the KOH concentration and time reached 3% and 3 h, respectively. The effect of the factors on the ED is shown in Figure 3. The highest ED was determined to be 54.3% at 100 °C and 4% KOH, and the ED decreased drastically

as the temperature and KOH concentration decreased (Figure 3a). In Figure 3b, the ED was high at 100 °C for 3 h and decreased as the temperature and time approached 0 °C and 0 h, respectively. The lowest ED was found to be 18.7% at 4% KOH for 0 h. and the ED increased sharply with the increasing KOH concentration and time (Figure 3c).

(**a**)



(**b**)



(**c**)

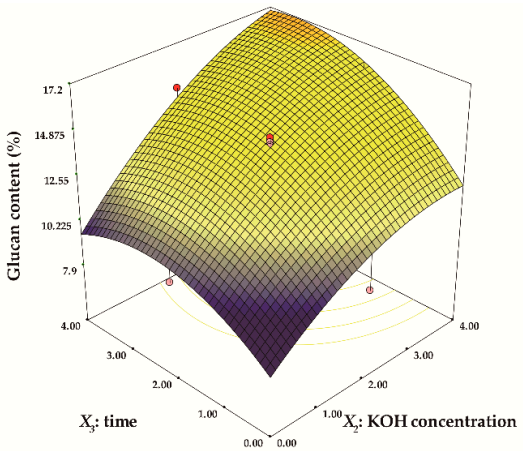
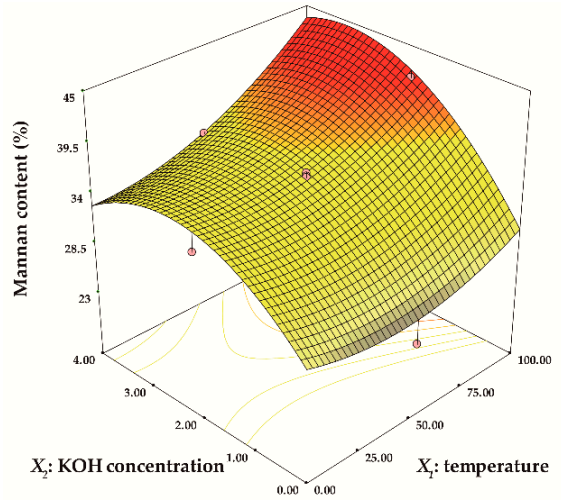


Figure 1. The three-dimensional response surface plot for the effect of the experimental factors on glucan content. Effects of temperature and KOH concentration (**a**), temperature and time (**b**) and KOH concentration and time (**c**).

(**a**)



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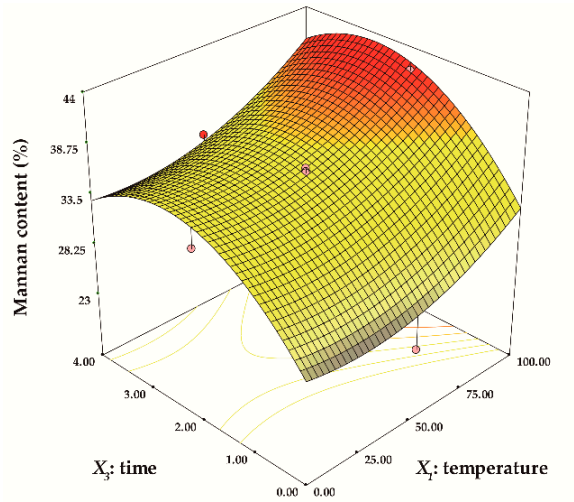


(**c**)

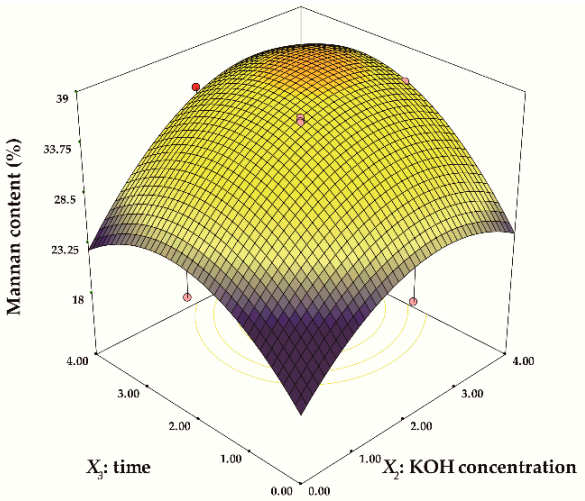
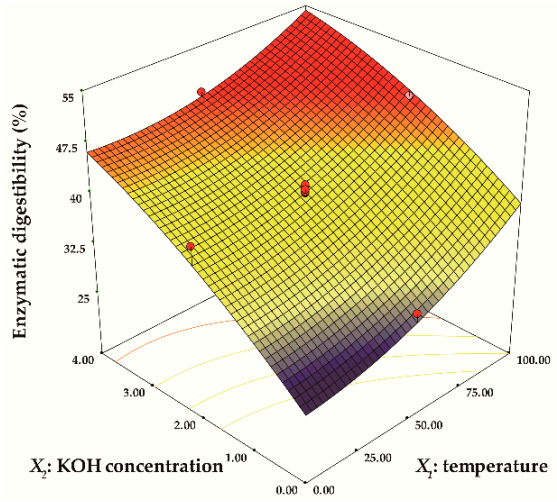


Figure 2. The three-dimensional response surface plot for the effect of the experimental factors on mannan content. Effects of temperature and KOH concentration (**a**), temperature and time (**b**) and KOH concentration and time (**c**).

(**a**)



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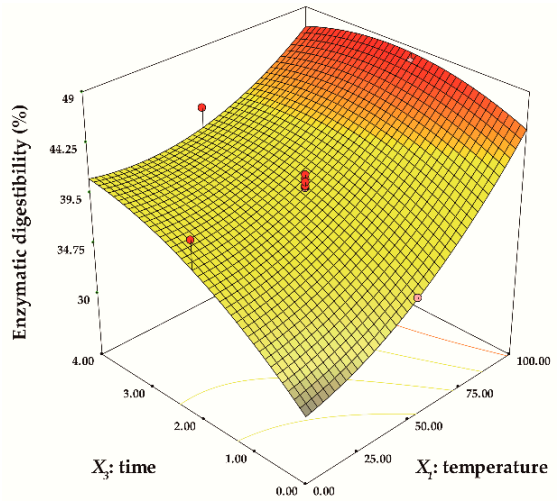


(**c**)

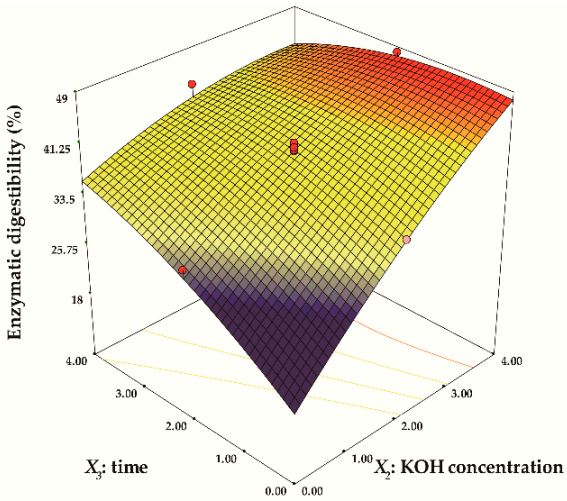


Figure 3. The three-dimensional response surface plot for the effect of the experimental factors on enzymatic digestibility. Effects of temperature and KOH concentration (**a**), temperature and time (**b**) and KOH concentration and time (**c**).

The numerical optimization for KOH pretreatment of SCGs was performed using Design-Expert 7 software based on multiple regression model analysis (Table 6). All factors

(X_1 = temperature, X_2 = KOH concentration and X_3 = time) were used for the numerical optimization because all factors significantly affected all responses (GC, MC and ED). The GC, MC and ED were selected as responses because the carbohydrates in SCGs mainly consist of glucan and mannan, and the purpose of alkali pretreatment is to improve ED. The optimum conditions for KOH pretreatment of SCGs are as follows: a temperature of 75.0 °C, a KOH concentration of 3.0% and a time of 2.8 h. Under the optimum conditions, the GC, MC and ED were predicted to be 18.1%, 41.1% and 46.8%, respectively. In order to validate the reliability of the predicted model, the pretreatment of SCGs was performed under the optimum conditions. The GC, MC and ED were determined to be 18.9%, 47.5% and 42.0%, respectively. These results demonstrate that our predicted model was suitable for optimizing the pretreatment conditions of SCGs.

Table 6. Numerical optimization for KOH pretreatment of spent coffee grounds (SCGs) based on multiple regression model analysis.

Factors	Coded Levels	Actual Levels
Temperature	1.0	75.0 °C
KOH concentration	1.0	3.0%
Time	0.8	2.8 h
Response	**Predicted**	**Experimental**
GC (%)	18.1	18.9
MC (%)	41.1	47.5
ED (%)	46.8	42.0

3.2. Profiling for Enzymatic Hydrolysis of SCGs

Profiling of enzymatic hydrolysis was performed to investigate the appropriate enzyme loading and time for the conversion of pretreated SCGs into monosaccharides. The enzyme loading was 7.5–60 FPU/g biomass of Celluclast® 1.5 L, 3.75–30 CBU/g biomass of Cellic® CTec2 and 12.5–100 MNU of Mannaway® 4.0 L, and each sample was hydrolyzed for 144 h. Untreated SCG were used as the control group and SCG pretreated under the optimum conditions were used as the experimental group.

The enzymatic hydrolysis profiling of SCG by various enzyme loadings was shown in Figure 4. The maximum ED of the experimental group (SCGs pretreated under the optimum conditions) was found to be 68.4% at 144 h, with enzyme loading of 60 FPU/g biomass, 30 CBU/g biomass and 100 MNU/g biomass. It was estimated that the complex structure of microcrystalline cellulose and hemicellulose, which inhibit enzyme activity, influenced the incomplete enzymatic hydrolysis of the experimental group [37]. Compared with various biomass pretreated with alkali and hydrolyzed using enzymes, the maximum ED of the experimental group was higher than that of poplar (41.5%), sugarcane bagasse (55.1%) and oil palm mesocarp fiber (60.0%) and similar to that of switchgrass (69.3%), *Imperata cylindrica* (70.0%) and rice straw (71.1%) [24,37]. These results mean that SCGs have potential to be used as the feedstock for biorefinery. Under the same conditions, the ED of the control group (untreated SCGs) was found to be 43.9%, showing that the ED was improved by 1.6-fold with KOH pretreatment. The economic efficiency of the saccharification process is significantly affected by the amount of enzyme and time required for enzymatic hydrolysis [38]. After 96 h of enzymatic hydrolysis, the ED was not significantly affected by time and increased slightly. At 144 h, the ED of the experimental groups, except for enzyme loading of 7.5 FPU/g biomass cellulase, 3.75 CBU/g biomass cellobiase and 12.5 MNU/g biomass mannanase, was not significantly improved by the enzyme loading. These results indicate that enzyme loading of at least 30 FPU/g biomass cellulase, 15 CBU/g biomass cellobiase and 50 MNU/g biomass mannanase and a time of 96 h are recommended for efficient enzymatic hydrolysis of the experimental group. Therefore, the optimum conditions for enzymatic hydrolysis that we suggest for an economic saccharification process are as follows: enzyme loading of 30 FPU/g biomass

cellulase, 15 CBU/g biomass cellobiase and 50 MNU/g biomass mannanase and a time of 96 h at 50 °C and 180 rpm (ED of the control group = 31.6%, and ED of the experimental group = 59.4%).

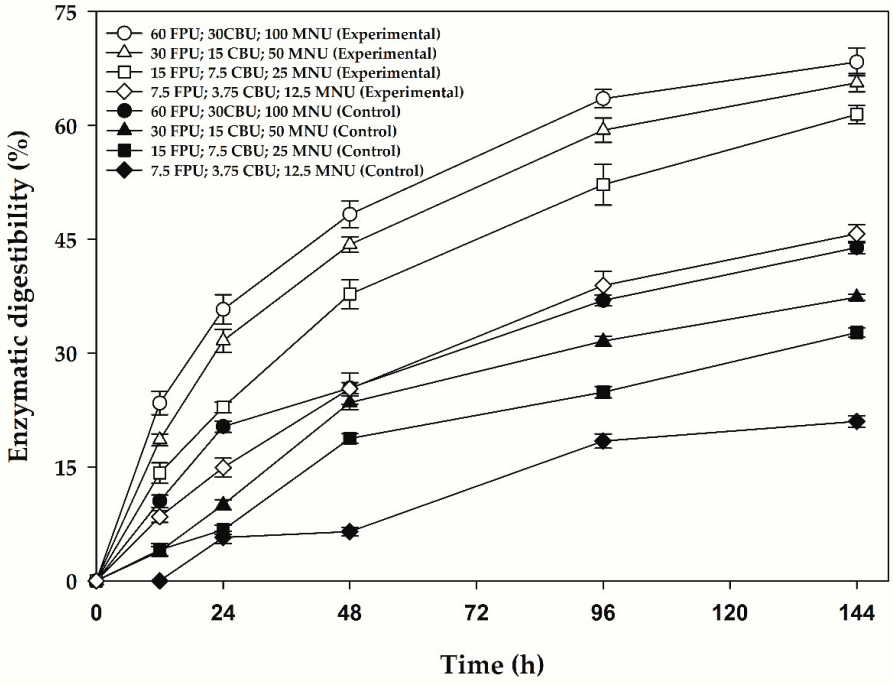


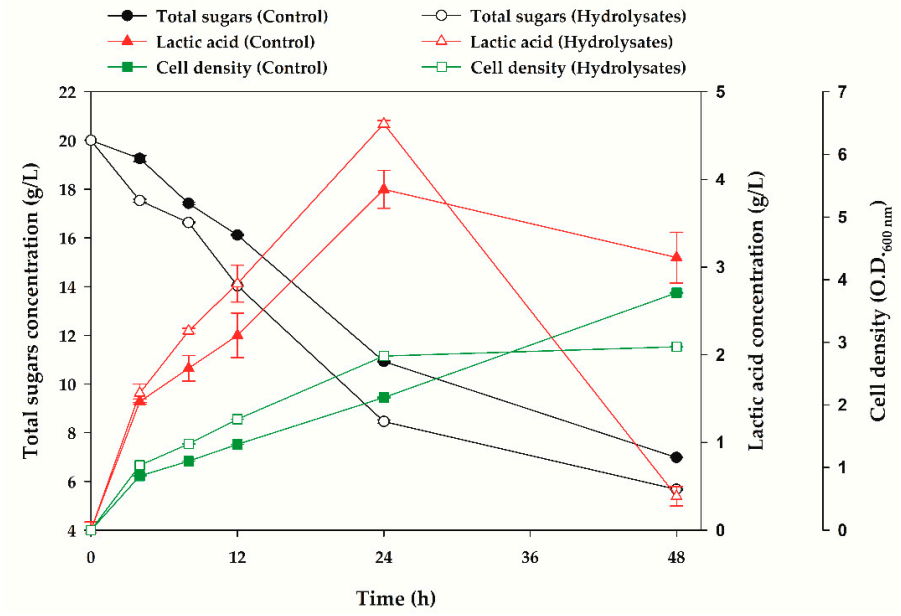
Figure 4. Enzymatic hydrolysis profiling of SCGs by various enzyme loadings (control group = filled symbol, and experimental group = hollow symbol).

3.3. Lactic Acid Production Using SCG Hydrolysates

Lactic acid was produced by the fermentation of *L. brevis* ATCC 8287 and *L. parabuchneri* ATCC 49374, using SCG hydrolysates as the carbon source. The carbon source of the control medium was mixtures of glucose and mannose prepared in the same composition as the SCG hydrolysates.

Figure 5a shows the lactic acid production by *L. brevis* ATCC 8287. *L. brevis* ATCC 8287, rapidly converting sugar to lactic acid until 24 h, and the sugar consumption rate significantly decreased after 24 h. The concentration of lactic acid decreased drastically after 24 h, especially in the SCG hydrolysates medium. It was estimated that *L. brevis* ATCC 8287 used the produced lactic acid instead of the remaining sugar for cell growth because the cell density steadily increased. *L. brevis* ATCC 8287 produced 3.9 g/L of lactic acid in the control medium at 24 h, and the lactic acid conversion was determined to be 41.3%. In the SCG hydrolysates medium at 24 h, the maximum lactic acid production was found to be 4.6 g/L, which was 1.2-fold higher than that of the control group, and the lactic acid conversion rate was determined to be 40.1%.

(**a**)



(**b**)

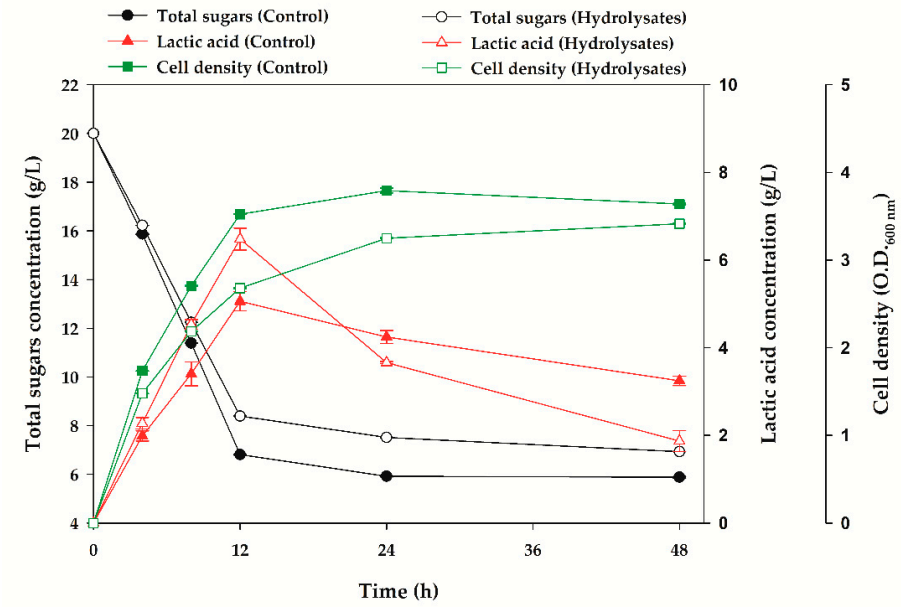


Figure 5. Lactic acid production by fermentation of *L. brevis* ATCC 8287 (**a**) and *L. parabuchneri* ATCC 49374 (**b**) using SCG hydrolysates (control medium: filled symbol and SCG hydrolysate medium: hollow symbol).

The lactic acid production by *L. parabuchneri* ATCC 49374 iss represented in Figure 5b. *L. parabuchneri* ATCC 49374 converted sugar to lactic acid with a higher efficiency than *L. brevis* ATCC 8287. In the control medium, the maximum lactic acid production by *L. parabuchneri* ATCC 49374 was found to be 5.1 g/L at 12 h, showing a lactic acid conversion rate of 38.4%. In the SCG hydrolysates medium, *L. parabuchneri* ATCC 49374 produced 6.5 g/L of lactic acid with a lactic acid conversion rate of 55.8%, which was a 1.3-fold enhancement compared with the control medium. After 12 h, *L. parabuchneri* ATCC 49374 showed a similar tendency to *L. brevis* ATCC 8287, in which lactic acid was consumed even in the presence of sugar in the SCG hydrolysates medium. These results indicated that *L. parabuchneri* ATCC 49374 was more suitable for lactic acid production using SCG hydrolysates than *L. brevis* ATCC 8287.

However, both strains had low lactic acid production and did not completely utilize sugar in the SCG hydrolysates medium. In addition, the lactic acid conversion rate was lower compared with other biomass, such as corn stover (73.0%) and orange peel (83.6%), which are used for lactic acid production [39,40]. This was estimated to be the effect of polyphenols contained in the SCGs. Hudeckova et al. reported that removing the polyphenols from SCGs could improve the yields of the biotechnological process [41]. Therefore, further studies are required to investigate how to improve low lactic acid conversion rates and utilize completely all the sugars in the SCG hydrolysates medium.

The material balance was established based on 1000 g of SCGs to evaluate the overall process of biomass conversion to lactic acid (Figure 6). A total of 380 g of solid was recovered after pretreatment of 1000 g of SCGs under the optimum conditions and consisted of 79 g glucan and 198.6 g mannan. The pretreated SCGs were enzymatically hydrolyzed to 51.6 g glucose and 129.8 g mannose at 50 °C and 180 rpm for 96 h, with an enzyme loading of 30 FPU/g biomass cellulase, 15 CBU/g biomass cellobiase and 50 MNU/g biomass mannanase. The fermentable sugar from the SCGs was used as the carbon source to produce lactic acid. The hydrolysates of pretreated SCGs were converted to 101.2 g lactic acid by *L. parabuchneri* ATCC 49374 fermentation, showing a 1.6-fold improvement compared with the control (untreated SCG hydrolysates).

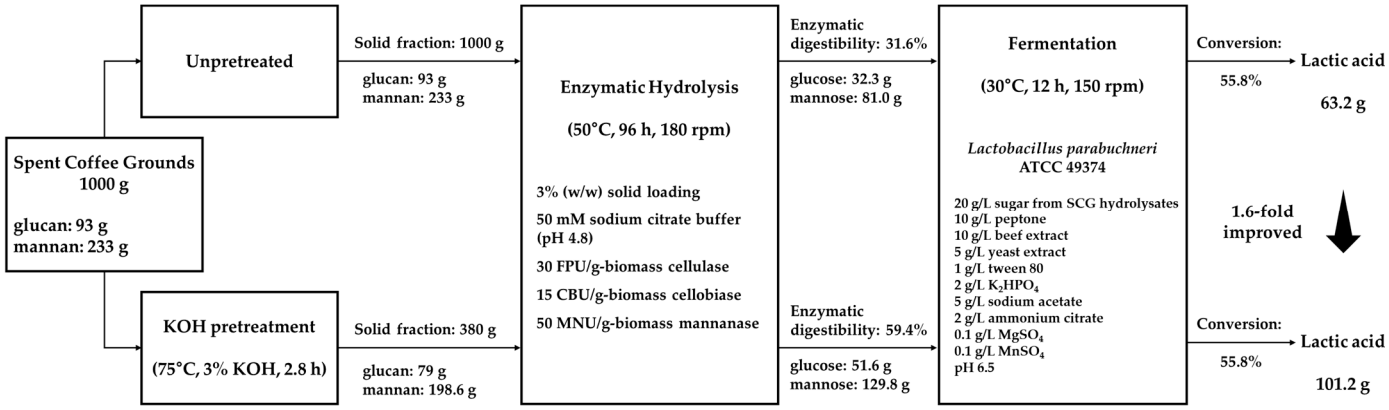


Figure 6. Material balance of lactic acid production based on 1000 g of SCGs.

4. Conclusions

In this study, we suggested the optimum conditions for alkaline pretreatment and enzymatic hydrolysis to convert SCGs to lactic acid. KOH pretreatment was performed to improve the enzymatic hydrolysis efficiency of SCGs, and the derived optimum conditions by statistical methods were as follows: 75 °C, 3% (w/w) KOH and 2.8 h. Under the optimum conditions, the predicted GC, MC and ED were 18.1%, 41.1% and 46.8%, respectively, and the experimental GC, MC and ED were determined to be 18.9%, 47.5% and 42.0%, respectively. The appropriate enzyme loading and time for the saccharification process to convert the pretreated SCGs into fermentable sugar were as follows: an enzyme loading of 30 FPU/g-biomass cellulase, 15 CBU/g-biomass cellobiase and 50 MNU/g-biomass mannanase and 96 h. Through the optimization of alkali pretreatment and enzymatic hydrolysis, 1.6-fold enhanced fermentable sugar was recovered compared with the untreated SCGs. The converted sugar was used as the carbon source for fermentation of *L. brevis* ATCC 8287 and *L. parabuchneri* ATCC 49374. The maximum lactic acid productions fermented by *L. brevis* ATCC 8287 and *L. parabuchneri* ATCC 49374 were 4.6 g/L and 6.5 g/L, respectively, and the lactic acid conversion rates at this time were 40.1% and 55.8%, respectively. Finally, through our study, 101.2 g lactic acid was produced based on 1000 g of SCGs. Our study proposed a biorefinery concept using SCGs as the feedstock for lactic acid production. The recovery of fermentable sugar was improved through the optimization of the pretreatment and saccharification process, and it was confirmed that there were no fermentation inhibitory compounds in the SCG hydrolysates. The aim of our further study

is to investigate how to completely utilize the fermentable sugars from SCG hydrolysates and improve the efficiency of lactic acid production.

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