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Article

Glucaric Acid Production from *Miscanthus sacchariflorus* via TEMPO-Mediated Oxidation with an Efficient Separation System

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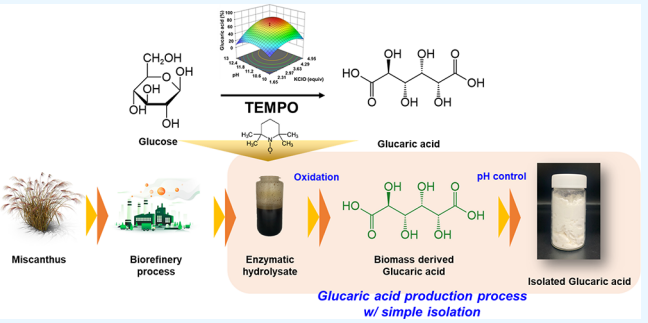
ABSTRACT: In this study, production and isolation of glucaric acid from lignocellulosic biomass were performed via potassium cation-based TEMPO-mediated oxidation for the ease of glucaric acid isolation. To optimize the oxidation conditions, response surface methodology (RSM) was adopted using standard glucose as the raw material. Among the oxidation conditions, the dosage of oxidant and pH of reaction affected the glucaric acid production, and the optimum conditions were suggested by RSM analysis: 5 °C of reaction temperature, 4.23 equiv dosage of KClO per mole of glucose, and pH of 12. Furthermore, glucaric acid was produced from lignocellulosic biomass-derived enzymatic hydrolysate from Miscanthus under optimum conditions. The impurities such as xylose and lignin in enzymatic hydrolysate inhibited the efficiency of glucose oxidation. As a result, more oxidant was required to produce sufficient glucaric acid from the enzymatic hydrolysate compared to standard glucose. The produced glucaric acid was simply isolated by controlling the pH in the form of glucaric acid monopotassium salt, which showed lower solubility in water, and the purity of isolated glucaric acid was over 99%. The overall mass balance of feedstock to glucaric acid was analyzed, suggesting that 86.38% (w/w) glucaric acid could be produced from initial glucan in feedstock.



1. INTRODUCTION

Glucaric acid, which is sugar-based dicarboxylic acid is one of the promising chemicals that could be applied to detergents,[1] chelating agents,[2] and bioplastic monomers.[3] Interestingly, glucaric acid is one of the precursors for producing 2,5-furandicarboxylic acid (FDCA) which is a promising bioplastic monomer that could replace terephthalic acid in polyethylene terephthalate (PET) because glucaric acid is more stable in water or acid than 5-hydroxymethylfurfural (5-HMF) which is a general precursor of FDCA.[4] Glucaric acid can be obtained through the oxidation of glucose, and there are several studies that have reported the oxidation of glucose to glucaric acid. A conventional method for obtaining glucaric acid from glucose is the concentrated nitric acid oxidation method.[5] However, the use of corrosive chemicals and the catalyst recycling requirements of this method have hindered its further application. To overcome these limitations, heterogeneous catalysts have been adopted for the oxidation of glucose to glucaric acid. A previous study reported the oxidation of glucose to glucaric acid over an Au/Al_2O_3 catalyst, in the presence of the oxidant hydrogen peroxide, resulting in a 76% yield of glucaric acid under microwave irradiation at 120 °C for 10 min.[6] However, using noble metal catalysts still causes a problem for economic feasibility.

Meanwhile, (2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (TEMPO) is a stable heterocyclic aminoxyl radical that has been widely employed in organic synthesis for oxidizing primary alcohols to aldehydes.[7] Owing to its chemoselectivity and regioselectivity, TEMPO has also been used in cellulose modification to add carboxyl groups to cellulose.[8] This highly selective oxidation ability could be applied to glucose oxidation, and some studies have been conducted involving glucose oxidation to glucaric acid using TEMPO as a catalyst, sodium hypochlorite (NaClO) as an oxidant, and sodium bromide (NaBr) as a cocatalyst. Through the TEMPO-catalyzed oxidation process at pH 11.5, sodium glucarate was obtained via the selective oxidation of the C1 and C6 positions, and minor amounts of sodium gluconate were produced through the C1 position of glucose, sodium tartarate, and sodium oxalate, which were produced through the further oxidation of sodium glucarate.[9]

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However, sodium glucarate was soluble in water, which required an additional separation step such as evaporation or addition of an organic solvent. Meanwhile, the solubility of glucaric acid was influenced by its countercation. Among them, potassium salts decrease the solubility of glucaric acid in water; specifically, the glucaric acid monopotassium salt showed lower solubility in water.[5] Based on the difference of solubility, glucaric acid could be easily separated when the cation of the reaction was changed from sodium to potassium.

When considering the process commercialization, it is important to select the raw material that is used in the process. Among the various resources, lignocellulosic biomass attracts interest because it is a renewable, abundant, and nonedible carbon neutral resource.[10] Lignocellulosic biomass is composed of polysaccharides including cellulose and hemicellulose and lignin, which is a phenolic macromolecule. For these reasons, different types of value-added products could be produced from each component of lignocellulosic biomass through the fractionation of biomass components and further conversion process, which is called biorefinery.[11] The general biorefinery process consists of pretreatment and enzymatic hydrolysis.[12] Hemicellulose or lignin is separated by various pretreatment processes, and then glucose derived from cellulose was produced by sequential enzymatic hydrolysis. Finally, lignin-rich solid residues are produced from enzymatic hydrolysis.[13]

All biomass components produced from the biorefinery process could be valorized by a further conversion process. For instance, glucose derived from the enzymatic hydrolysis of cellulose could be converted to value-added chemicals such as 5-HMF[14] or levulinic acid.[15] Xylose and xylose oligomer could be valorized by converting xylose to furfural,[16] which is widely applied to resin,[17] solvent,[18] and biofuel[19] by further conversion. Lignin which is a natural aromatic compound could be applied to various industrial fields including biocomposites,[20] bio-oil by pyrolysis,[21] and aromatic compounds such as vanillin.[22]

To improve the efficiency of the biorefinery process, a continuous conversion process from the fractionated biomass component is important. However, the fractionated component contains a minor portion of impurities derived from other biomass-derived components, which could affect the further conversion process.[23] Therefore, it is important to conduct a conversion process using real biomass-derived raw materials and estimate the effect of impurities in raw materials.

In this concept, the TEMPO-mediated oxidation of glucose to glucaric acid was conducted in a potassium-cation-salt system. The influence of the reaction factor was analyzed, and the optimum condition for performing the TEMPO-mediated glucose oxidation was proposed. Furthermore, TEMPO oxidation was conducted using lignocellulosic biomass-derived glucose as a raw material. Then, the effect of impurities in the lignocellulosic biomass-derived glucose to glucose conversion was estimated.

2. RESULTS AND DISCUSSION

2.1. Glucaric Acid and Gluconic Acid (Intermediate) Production from TEMPO-Mediated Oxidation of Glucose. During the TEMPO-mediated oxidation of glucose, the aldehyde and primary alcohol groups of glucose are oxidized to convert glucose to glucaric acid. Table 1 shows the results of the analysis of variance (ANOVA) for glucaric acid production from glucose. The *p*-value of the model is 0.0193, which is lower than 0.05, implying that the model is significant.[24] Among the independent variables considered, the oxidant dosage (X2) shows the lowest *p*-value (0.0093), meaning that the dosage of KClO strongly influences the glucose conversion to glucaric acid. In the KClO-KBr-TEMPO oxidation, KClO initiates and controls the redox chemistry of TEMPO and KBr, resulting in glucose oxidation.[1] One molecule of KClO oxidizes the aldehyde to a carboxyl group or the primary alcohol to an aldehyde group.[25] This means that at least 3 equiv moles of KClO per glucose is required to produce glucaric acid from glucose. Therefore, as the oxidant dosage increases, oxidation of the aldehyde and primary alcohol groups of glucose continues to occur to yield increasing amounts of glucaric acid. However, as shown in Figure 1a, when the oxidant dosage exceeds 4.3 equiv, the glucaric acid yield decreases because of the degradation of glucaric acid by the oxidant.[9] The pH of the reaction (X3) also influences glucaric acid production, with the pH between 10 and 13 causing a decrease in the glucaric acid yield. It was assumed that oxidation of the primary hydroxyl group and aldehyde group in glucose required a specific pH condition to suppress side reactions including oxidative cleavage.[26] Therefore, controlling the pH in the TEMPO-mediated oxidation of glucose is crucial for suppressing the degradation of glucaric acid, which is the target product.

Table 1. Results of ANOVA for Glucaric Acid Production from Glucose via TEMPO-Mediated Oxidation

source	sum of squares	DF	mean square	*F*-value	*p*-value
model	13425.24	9	1491.69	5.31	0.0193
X1[a]	161.26	1	161.26	0.57	0.4732
X2[b]	3536.99	1	3536.99	12.60	0.0093
X3[c]	879.86	1	879.86	3.13	0.1199
X1 × 2	12.01	1	12.01	0.04	0.8420
X1 × 3	3.65	1	3.65	0.01	0.9124
X2 × 3	831.51	1	831.51	2.96	0.1289
$X1^2$	158.86	1	158.86	0.57	0.4764
$X2^2$	3410.01	1	3410.01	12.15	0.0102
$X3^2$	4403.41	1	4403.41	15.69	0.0055
residual	1964.74	7	280.68		
lack of fit	1903.94	5	380.79	12.53	0.0756
pure error	60.80	2	30.40		
total	15389.97	16			

[a]X1: Reaction temperature (°C). [b]X2: Dosage of oxidant (KClO) (equiv per mole of glucose). [c]X3: pH of the reaction.

Meanwhile, gluconic acid is produced during the oxidation of the aldehyde group of glucose to the corresponding carboxyl group. Typically, glucose is first oxidized to gluconic acid; then, gluconic acid is oxidized to glucaric acid in the TEMPO-mediated oxidation.[27] This means that gluconic acid is an intermediate of the glucaric acid production process from glucose. Therefore, it is important to investigate the effect of different reaction conditions on gluconic acid production and conversion during the TEMPO-mediated oxidation of glucose.

Table S1 shows the results of ANOVA for gluconic acid production from glucose. The *p*-value of the model is 0.0142, which is lower than 0.05, suggesting that the model is significant. Similar to glucaric acid production, the oxidant dosage (X2) is the factor that results in the lowest *p*-value (0.0003). This phenomenon is shown in Figure 1b, where the gluconic acid yield decreases significantly when the KClO dosage increases. Specifically, when low amounts of the oxidant (KClO) are used, only the aldehyde group of glucose is

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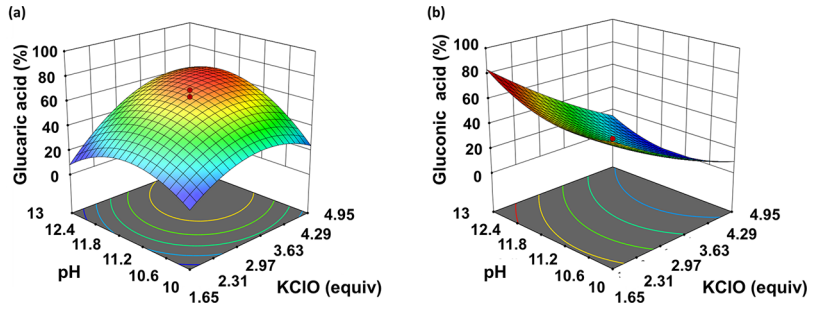

Figure 1. Three-dimensional plots of the RSM analyses of glucaric acid and gluconic acid production during the TEMPO-mediated oxidation of glucose: (a) glucaric acid and (b) gluconic acid production as a function of the KClO dosage and pH at 5 °C.

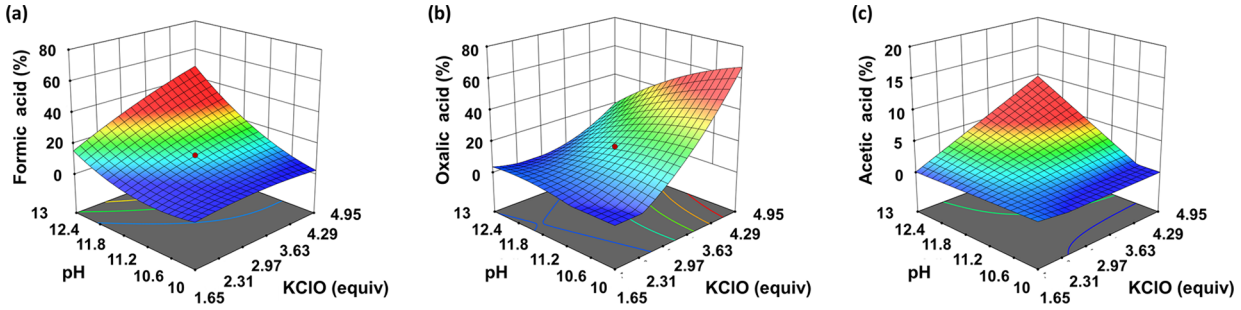

Figure 2. Three-dimensional plots of the RSM analyses of byproducts produced during the TEMPO-mediated oxidation of glucose: (a) formic acid, (b) oxalic acid, and (c) acetic acid production as a function of the KClO dosage and pH at 5 °C.

oxidized, and not the hydroxyl group at the end of the glucose molecule, leading to significant amounts of gluconic acid being produced.[28] However, when the dosage of KClO is increased, the primary hydroxyl group of gluconic acid is oxidized to the corresponding carboxyl group to form glucaric acid. Therefore, gluconic acid production is promoted at low KClO dosages regardless of the pH of the reaction. However, further oxidation to glucaric acid or byproducts is highly dependent on pH.

2.2. Byproduct Formation from TEMPO-Mediated Oxidation of Glucose. In the TEMPO-mediated oxidation of glucose to glucaric acid, glucose was first oxidized to gluconic acid favorably at pH between 10 and 13. However, further oxidation of gluconic acid to glucaric acid was achieved in certain pH conditions (Figure 1a), meaning that undesirable side reactions occurred depending on the pH of the reaction. Therefore, exploring the tendency of byproducts (organic acid) which were produced from the oxidative degradation of gluconic acid was required. Formic acid can be produced via the oxidative C−C cleavage of glucose or glucaric acid,[29] making it one of the byproducts of the glucose-oxidation-to-glucaric acid process. Therefore, it is important to investigate the influence of different reaction conditions on formic acid production in TEMPO-mediated glucose oxidation.

Tables S2−S4 show the results of the AVOVA of formic acid, oxalic acid, and acetic acid production from glucose in the TEMPO-mediated oxidation of glucose. Among the independent variables considered, the pH of the reaction (X3) is the factor that influences byproduct formation the most. This is supported by the three-dimensional (3D) plots of the RSM analyses of byproduct formation as a function of pH (Figure 2). In Figure 2a, formic acid production sharply increases as the pH increases over 12, with a yield of over 50% being reached at pH 13. In the oxidative C−C cleavage of glucose and glucose-derived sugar acids, different products can be obtained, depending on the position of the cleavage. For example, after the oxidation of the aldehyde group (−CHO) of glucose to a carboxyl group to form gluconic acid, the C1−C2 cleavage (α-scission) can occur, which would lead to the formation of formic acid.[30] On the other hand, the C2−C3 cleavage (β-scission) can occur after the oxidation of glucose to gluconic acid. As a result of the C2−C3 cleavage, oxalic acid (C1−C2) and tartaric acid (C3−C6) can be formed. Previous studies have been conducted on the oxidation of glucose to different organic acids using hydrogen peroxide as an oxidant in alkaline media.[31] In this study, the formic acid yield increases as the sodium hydroxide (NaOH) dosage increases, which provides an alkaline reaction medium. This means that at a high reaction pH (high NaOH dosage), the C1−C2 cleavage is favored more than the C2−C3 cleavage. Additionally, the stability of oxalic acid in alkaline oxidative media has also been investigated in previous studies, and it has been revealed that the oxalic acid produced via the C2−C3 cleavage has very good stability in alkaline oxidative media. However, in this study, the C1−C2 cleavage of gluconic acid dominates at a high pH, with formic acid being produced as the main product. At pH below 11, the production of glucaric acid through the oxidation of the primary hydroxyl group of gluconic acid or the production of oxalic and tartaric acids via the C2−C3 cleavage of gluconic acid can occur (Figure 2b). Therefore, the organic acids produced through the oxidative cleavage of gluconic acid can vary, depending on the pH of the oxidation reaction medium.

The KClO dosage is also a significant factor for formic and oxalic acid production. The influence of KClO is shown in Figure 2a,b. When the KClO dosage is low, the formic acid yield is much lower than that under the high KClO dosage condition. This is because glucose molecules are oxidized by reacting with KClO, implying that a lack of KClO limits the yields of gluconic acid or formic acid.[32] On the other hand,

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when the KClO dosage is sufficient, glucose is oxidized to glucaric, formic, and oxalic acids. However, when KClO is in excess, glucaric acid is oxidized and converted to formic or oxalic acid, depending on the reaction pH.

Meanwhile, acetic acid is produced in a pathway different from that of formic, tartaric, and oxalic acids. In the glucose-to-acetic-acid conversion, glucose first isomerizes to fructose, and then fructose is split into two trioses via the retro-aldol reaction. The two trioses, namely, dihydroxyacetone and glyceraldehyde, are subsequently converted to lactic acid through dehydration and hydration.[33] Thereafter, the produced lactic acid is converted to acetic acid via oxidation.[34] As shown in Figure 2c, acetic acid production is promoted at a pH greater than 11.5, which is similar to formic acid production. Previous studies have shown that the lactic acid yield increases as the NaOH dosage increases.[35] The increase in the NaOH dosage results in an increase in pH; therefore, acetic acid production is promoted at a high pH because lactic acid production is favored, which is the precursor of acetic acid. However, in this study, the acetic acid yield is lower than those of formic acid and oxalic acid. It is worth mentioning that before lactic acid production, the isomerization of glucose to fructose is required, and the presence of transition-metal ions, such as Ni^{2+}, Zn^{2+}, or Co^{2+}, accelerate glucose isomerization via the 1,2-hydride shift.[36] In the TEMPO-mediated oxidation of glucose, the transition metals that can catalyze the 1,2-hydride shift of glucose are absent. Instead, the amine group of 4-acetamido-TEMPO could catalyze the isomerization of glucose to fructose and produce lactic acid at a high pH even though formic acid production via the C1−C2 cleavage of glucose is dominant.[37] Moreover, formic acid can also be produced after the oxidation of lactic acid, resulting in one acetic acid molecule for every formic acid molecule,[38] still leading to a larger production of formic acid than that of acetic acid.

The overall TEMPO-mediated oxidation of glucose is suggested in Figure 3. The selectivity of TEMPO oxidation products was strongly influenced by the KClO dosage and pH of the reaction. Glucose was preferentially oxidized to gluconic acid, which is the intermediate of glucaric acid, and other oxidation products when the dosage of KClO was low, at relatively all pH ranges in this study. On the other hand, glucaric acid and organic acids including formic acid and oxalic acid were produced, preferably when the dosage of KClO was high, meaning that the additional oxidant oxidized gluconic acid to further oxidation products. However, the selectivity of glucaric acid, formic acid, and oxalic acid was strongly influenced by the pH of the reaction. First, glucaric acid was favored when the pH range was between 11 and 12. At this pH, oxidation of the primary hydroxyl group of gluconic acid was preferred, and conversion of gluconic acid to glucaric acid occurred. On the other hand, when the pH was higher than 12, oxidative C1−C2 cleavage of gluconic acid was preferred, leading to formic acid production mainly. The oxalic acid production was favored when pH was lower than 11, the tendency of which was opposite to formic acid, because oxidative C2−C3 cleavage occurred at a relatively lower pH than C1−C2 cleavage. Meanwhile, acetic acid which was a minor byproduct was produced in a different pathway compared to formic and oxalic acids. Acetic acid was produced in a sequential retro-aldol reaction and oxidation of fructose, which was the isomerization product of glucose.

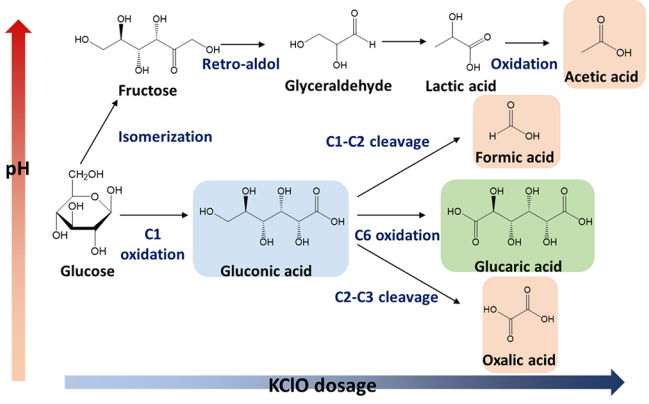

Figure 3. Overall scheme of TEMPO-mediated oxidation of glucose when KClO was the oxidant.

2.3. Optimization of Reaction Conditions. To optimize the reaction conditions for glucaric acid production, a regression analysis was performed by using a central composite design matrix with the corresponding glucaric acid yield. A quadratic equation (eq 1) was generated based on the outcomes of the regression analysis:

$$\begin{aligned}\text{Glucaric acid yield}(\%) &= -423.84025 + 4.08067X1 + 40.959X2 + 14.4117X3 \\ &\quad - 0.112999X1X2 - 0.010062X1X3 + 0.75624X2X3 \\ &\quad - 0.009982X1^2 - 6.84226X2^2 - 0.929452X3^2 \end{aligned} \quad (1)$$

Based on eq 1, the optimum conditions of maximizing glucaric acid production were as follows: reaction temperature of 5 °C, 4.23 equiv dosage of KClO per mole of glucose, and pH of 12. The predicted and actual product yields are shown in Table 2. The actual glucaric acid yield at optimum conditions was 69.22 ± 2.78%, which was similar to the predicted yield (69.49%). The main byproducts were formic and oxalic acids, which were produced by the C−C cleavage of gluconic acid. Interestingly, gluconic acid was not detected after the TEMPO-mediated oxidation of glucose in optimum conditions, meaning that gluconic acid was fully converted to glucaric acid or other byproducts. Meanwhile, acetic acid was also not detected under optimum conditions because acetic acid was a minor byproduct produced from the isomerization and retro-aldol reaction of glucose. In the suggested optimum conditions, byproducts were mainly produced from the C−C cleavage of gluconic acid, resulting in formic or oxalic acid formation.

Table 2. Predicted and Actual Yields of Products at Optimum Conditions after the TEMPO-Mediated Oxidation of Glucose to Glucaric Acid

yield (%)	glucaric acid	gluconic acid	formic acid	oxalic acid	acetic acid
predicted	69.49	13	23.33	25.28	4.66
actual	69.22 ± 2.78	0	25.46 ± 3.45	14.13 ± 0.16	0

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2.4. Glucaric Acid Production from the Enzymatic Hydrolysate of Miscanthus. As a result of RSM analysis, the optimum condition was suggested to produce glucaric acid from standard glucose in a TEMPO-mediated oxidation system. However, it was crucial to use biomass-derived sugars such as enzymatic hydrolysate as a raw material to produce glucaric acid for further commercialization or establish a biomass-derived biorefinery process. The main difference between the standard sugar and enzymatic hydrolysate was purity. The enzymatic hydrolysate contained various by-products which could cause the inhibition of oxidation reaction derived from the biomass component including hemicellulose or lignin.[39] Therefore, it is important to explore the influence of biomass-derived inhibitors on the oxidation of glucose in the TEMPO system. To screen the inhibitor in enzymatic hydrolysate, the chemical composition of enzymatic hydrolysate was analyzed (Table 3). The main component is glucose, which is approximately 38% (w/v (g/mL)). Xylose, which is derived from the enzymatic hydrolysis of hemicellulose, remains as a solid residue after the autohydrolysis pretreatment and is the second dominant component of enzymatic hydrolysate.[40] Additionally, TPC also shows a high concentration (10.6 g/L) when compared with those of other impurities except xylose. TPC is derived from the degradation of lignin to water-soluble phenolic compounds during autohydrolysis.[41] Acetic acid is also present in enzymatic hydrolysate and is derived from the dissociation of the *O*-acetyl group in hemicellulose.[42]

Table 3. Chemical Composition of Enzymatic Hydrolysate

	glucose	xylose	acetic acid	5-HMF	furfural	TPC[a]
concentration (g/L)	382.35	51.75	2.36	0.26	0.32	10.6

[a]TPC: total phenolic compounds (analyzed according to the Folin−Denis method[43]).

The TEMPO-mediated oxidation was conducted using enzymatic hydrolysate as the glucose source, with the glucose concentration of 10% (w/v, g/mL), which is the same condition used for the TEMPO-mediated oxidation of a standard glucose solution. The results of the TEMPO-mediated oxidation of enzymatic hydrolysate are shown in Figure 4a. The glucaric acid yield from enzymatic hydrolysate was 60.76%, which is lower than that achieved in the oxidation of the standard glucose solution conducted under the optimum condition suggested by RSM analysis (KClO dosage: 4.23 equiv per mole glucose; red box in Figure 4a,b). However, when the KClO dosage increased from 4.23 to 5.1, the glucaric acid yield increased to 71.92%, which is similar to the result of standard glucose. It was assumed that impurities contained in the enzymatic hydrolysate inhibited the oxidation of glucose by the competitive consumption of the oxidant. In the presence of impurities, the glucose oxidation rate slackened relatively compared to standard glucose. It was clearly shown when comparing the result of standard glucose that the glucaric acid yield sharply dropped as the dosage of KClO increased to 5.1. It was because glucose oxidation to glucaric acid was completed at the optimum condition; then, the excess oxidant oxidized glucaric acid to further degradation products including organic acids. Meanwhile, a significant amount of gluconic acid remained when the dosage of KClO was 3.3 after the TEMPO-mediated oxidation of enzymatic hydrolysate compared to standard glucose. Then, gluconic acid was further oxidized to glucaric acid as the dosage of KClO increased. It could be explained by the presence of impurities, which reduced the oxidation of glucose, showing a similar trend to the production of glucaric acid in the enzymatic hydrolysate.

Meanwhile, formic acid production was similar to the results of standard glucose solution even though gluconic acid remained in enzymatic hydrolysate at the optimum condition (red box in Figure 4b). Considering that formic acid was mainly derived from the C−C cleavage of gluconic acid, formic acid production was expected to be lower than standard glucose because the rate of oxidation in enzymatic hydrolysate was slower than that of standard glucose. Therefore, the similar formic acid production might imply that formic acid was produced from not only glucose-derived gluconic acid but also impurities in the enzymatic hydrolysate. In oxidation condition, xylose in enzymatic hydrolysate also underwent C3−C4 cleavage to produce glyceraldehyde and glycoxal.[44] Glycoxal and glyceraldehyde are further oxidized to formic or oxalic acid. As a result, a similar formic acid production tendency was shown due to the oxidative degradation of xylose to formic acid.

On the other hand, oxalic acid production was different when comparing enzymatic hydrolysate and standard glucose. Oxalic acid production sharply increased to 88.47% at the dosage of KClO increasing to 5.1 when glucose was used as the raw material. Considering the significant decrease of glucaric

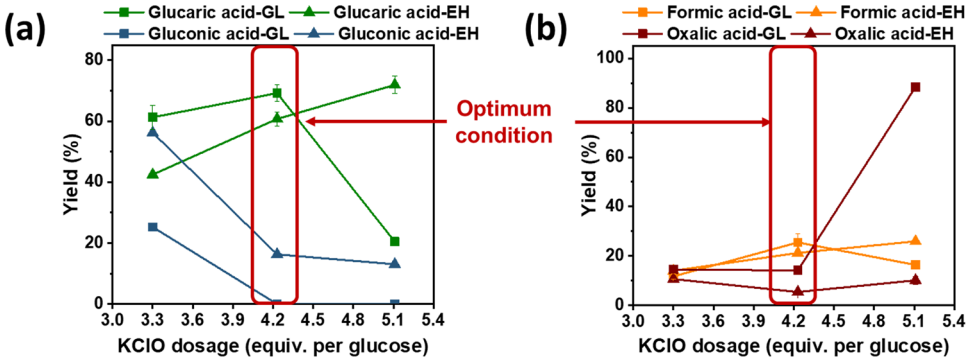

Figure 4. Results of TEMPO-mediated oxidation of standard glucose (−GL, -■-) and enzymatic hydrolysate (−EH, -▲-) depending on the dosage of KClO: (a) glucaric and gluconic acids, (b) formic and oxalic acids (reaction temperature: 5 °C, pH: 12, red box indicates the optimum condition suggested by RSM analysis).

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Table 4. TEMPO-Mediated Oxidation of Model Compounds (Glucose + Xylose and Glucose + Coniferyl Alcohol) in Optimum Conditions Suggested by RSM Analysis

yield (%)	glucaric acid	gluconic acid	formic acid	oxalic acid
glucose + xylose	64.16 ± 1.95	6.59 ± 0.63	22.86 ± 1.46	7.44 ± 0.52
glucose + coniferyl alcohol	63.53 ± 0.88	2.79 ± 0.88	26.15 ± 1.97	11.71 ± 0.36

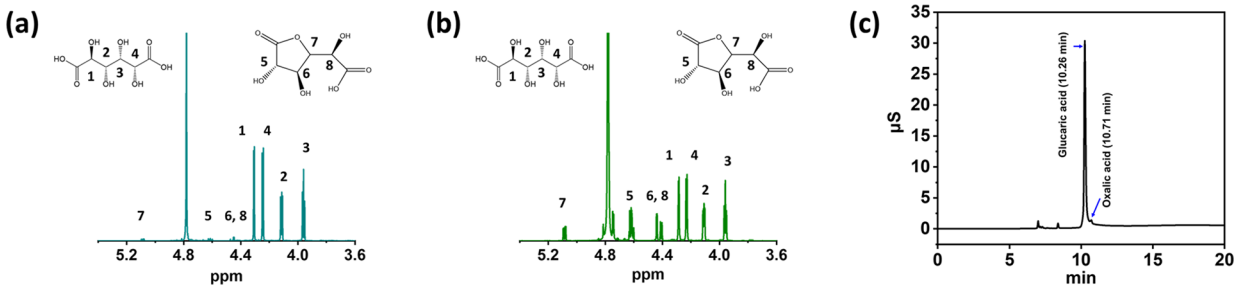

Figure 5. ^{1}H NMR spectrum of (a) isolated glucaric acid from enzymatic hydrolysate and (b) standard glucaric acid monopotassium salt (b). (c) Chromatogram of isolated glucaric acid from enzymatic hydrolysate.

acid at excess oxidant (Figure 4a), oxalic acid was mainly produced from the oxidative degradation of glucaric acid. Therefore, it is important to set an appropriate dosage of oxidant to minimize the degradation of glucaric acid in TEMPO-mediated oxidation. On the other hand, 25.92% and 10.11% of formic and oxalic acid were produced from enzymatic hydrolysate when the KClO dosage was 5.1, the result of which was similar to that of formic and oxalic acid production from glucose at optimum conditions (Table 2), meaning that the impurities in enzymatic hydrolysate prevented the further degradation of glucaric acid via competitive consumption of the oxidant. To sum up, impurities in the enzymatic hydrolysate decreased the glucose oxidation rate by consuming the oxidant instead of glucose, resulting in the requirement of more oxidant than the TEMPO-mediated oxidation of standard glucose to achieve the maximum glucaric acid production.

To ensure the effect of impurities clearly, model compounds were prepared which contained glucose and xylose and glucose and coniferyl alcohol, which represented the phenolic compounds derived from lignin (Table 4).

As shown in Table 4, glucaric acid yield decreased in both model compounds compared to the result of standard glucose (69.22%), indicating that xylose- or lignin-derived phenolic compounds in enzymatic hydrolysate inhibited the efficiency of glucose oxidation to glucaric acid. The composition of gluconic acid, which was fully converted to glucaric acid or organic acids when glucose was oxidized by TEMPO without adding impurities, in the model compounds indicated that xylose or lignin inhibited the conversion of gluconic acid to glucaric acid, resulting in a decrease in glucaric acid production. Based on the result of full conversion of xylose, xylose in the model compound participated in TEMPO-mediated oxidation instead of glucose by consuming the oxidant, leading to a decrease in glucose conversion to glucaric acid.[45] Lignin-derived phenolic compounds also could undergo depolymerization by TEMPO oxidation via the cleavage of ether and C−C bond.[46] Previous research conducted TEMPO oxidation of thermomechanical pulp which contained a high lignin content.[47] The results revealed that the NaClO-NaBr-TEMPO oxidation system in pH 10.5 not only oxidized the primary alcohol of cellulose but also degraded lignin by cleaving the β−O−4 bond in lignin. Based on these previous results, lignin-derived phenolic compounds were one of the oxidant consumers and decreased glucaric acid yield. Meanwhile, formic and oxalic acid contents in model compounds showed results similar to those in the absence of impurities. It was assumed that xylose- and lignin-derived phenolic compounds could be converted to formic acid or oxalic acid via the oxidative C−C cleavage of xylose[29] and oxidative ring-opening of lignin.[48] Therefore, formic acid and oxalic acid were produced sufficiently due to the oxidation of xylose and lignin, even though glucose was not oxidized sufficiently.

2.5. Isolation of Glucaric Acid. After the TEMPO-mediated oxidation of enzymatic hydrolysate (reaction temperature: 5 °C, dosage of KClO per mole of glucose: 5.11, and pH: 12), glucaric acid could be isolated by a simple pH control because all cations in the oxidation system was potassium. In this system, the glucaric acid potassium salt was converted to the glucaric acid monopotassium salt, which showed low solubility in water. After the pH was decreased to 3.8, 1.82 g of precipitation products including glucaric acid monopotassium salt was precipitated from 2 g of glucose. The isolated products were characterized by using a 600 MHz NMR spectrometer. Figure 5a shows the proton (^{1}H) NMR spectrum of the glucaric acid monopotassium salt. The hydrogen signals derived from the C2−C5 atoms of glucaric acid are shown at 3.8−4.2 ppm. Meanwhile, small peaks are observed at 4.3, 4.5, and 5 ppm. These small peaks can be assigned to D-glucaro-1,4-lactone, which is produced via the intramolecular lactonization of the C1 carboxyl group and C4 hydroxyl group. The lactonization of glucaric acid occurs in an acidic medium or during heating.[49] Glucaric acid lactone is easily formed during heating, even though the heating was mild in this study.[50] Therefore, it was assumed that D-glucaro-1,4-lactone is formed during the NMR sample preparation process. Because the solubility of the glucaric acid monopotassium salt in water is low, the dissolution of glucaric acid in D_2O was conducted at 65 °C. This phenomenon is also reflected by the small peaks observed at 4.3, 4.5, and 5 ppm in the ^{1}H NMR spectrum of the standard glucaric acid monopotassium salt (Figure 5b), which are derived from D-glucaro-1,4-lactone. Therefore, the ^{1}H NMR spectra of the isolated glucaric acid and standard glucaric acid are identical, implying that glucaric acid, which is produced during the TEMPO-mediated

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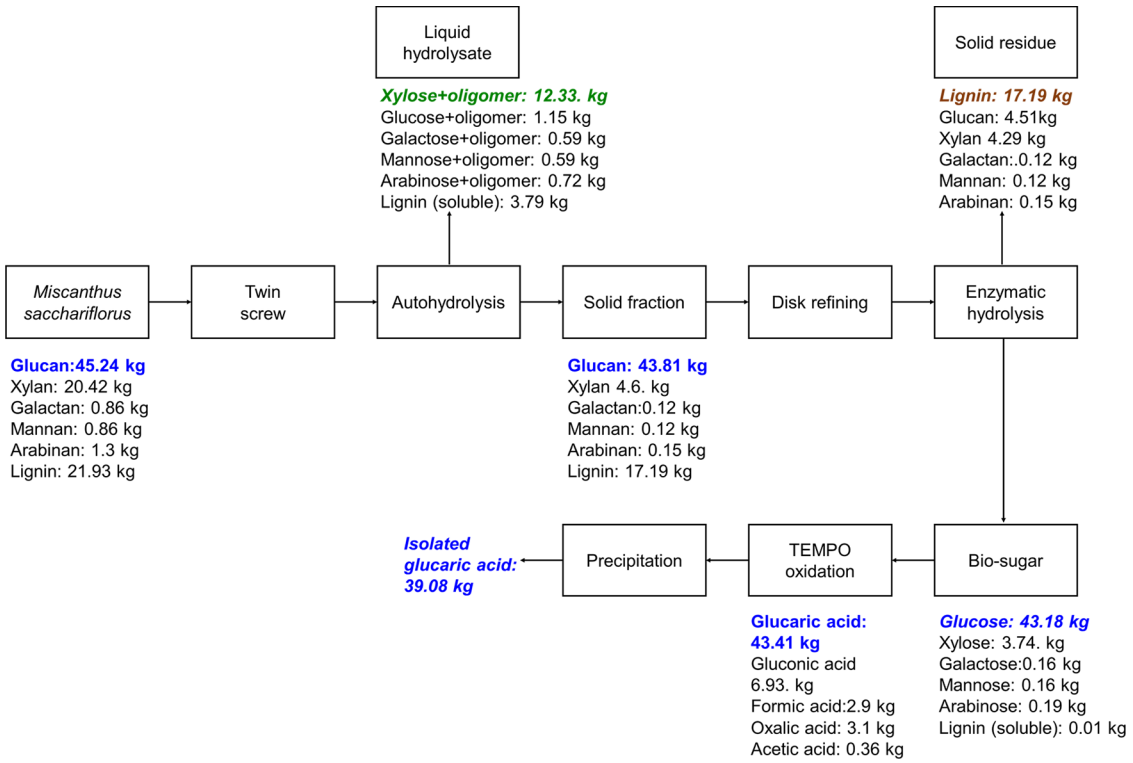

Figure 6. Mass balance of feedstock to glucaric acid production.

oxidation of glucose, was successfully isolated from the reaction medium by decreasing the pH of the solution.

To evaluate the purity of the isolated glucaric acid, ion chromatography (ICS-3000) was conducted. The isolated glucaric acid monopotassium salt showed high purity, which consisted of 99.67% glucaric acid. The only byproduct is oxalic acid, which made up 0.33% of the isolated glucaric acid (Figure 5c). Oxalic acid was formed as potassium hydrogen oxalate at pH 3.8 which showed a relatively low solubility in water at a lower temperature. as similar as glucaric acid, and potassium hydrogen oxalate was coprecipitated with glucaric acid as impurities. Considering the purity of glucaric acid, 95.48% of glucaric acid was isolated from the produced glucaric acid by the pH control.

2.6. Mass Balance. The mass balance of feedstock to glucaric acid production was calculated as shown in Figure 6. The enzymatic hydrolysate was produced by sequential autohydrolysis, refining, and enzymatic hydrolysis, which was 95.45% of glucose yield based on initial glucan in the feedstock. A minor portion of glucose remained in the hemicellulose-rich liquid hydrolysate due to the hydrolysis of cellulose during autohydrolysis and a form which remained in the lignin-rich solid residue by incomplete enzymatic hydrolysis. Then, glucaric acid was produced by TEMPO-mediated oxidation of enzymatic hydrolysate, resulting in 39.08 kg of glucaric acid that could be produced from 100 kg of initial biomass. These results indicated that 86.38% (w/w) of glucose in the initial feedstock could be converted to glucaric acid, which is a value-added chemical that could be applied as a chelating agent for organic contaminants,[51] a biodegradable polymer including hydroxylated nylon,[52] and an antiplasticizer.[53]

3. CONCLUSIONS

In this study, glucaric acid was produced from enzymatic hydrolysate via TEMPO-mediated oxidation as a concept of lignocellulosic biomass-based biorefinery. The statistical analysis was performed to optimize the oxidation condition using standard glucose as a raw material. Based on statistical analysis, the relationship between the oxidation condition and glucose oxidation products was suggested. When the dosage of the oxidant was low, gluconic acid which is aldonic acid of glucose was mainly produced. Gluconic acid was further converted as the dosage of the oxidant increased, but the final oxidation product was dependent on the pH of the reaction. The selectivity of glucaric acid was high in the pH between 11 and 12. On the other hand, when the pH was higher than 12, formic acid was mainly produced due to the C1−C2 cleavage of gluconic acid at a higher pH. Unlike formic acid, the selectivity of C2−C3 cleavage of gluconic acid increased when the pH was lower than 11, resulting in the production of oxalic acid.

The TEMPO-mediated oxidation was finally adopted by the glucose-rich enzymatic hydrolysate of Miscanthus to valorize the biorefinery concept, revealing that more oxidant was required to produce glucaric acid compared to standard glucose. This difference was derived from the competitive consumption of oxidant caused by the impurities in enzymatic hydrolysate including xylose- and lignin-derived phenolic compounds, which consumed the oxidant and then oxidized to further oxidation products.

Finally, glucaric acid was simply isolated as a form of glucaric acid monopotassium salt by pH control without the addition of organic solvent or ion-exchange resin because of the potassium cation-based TEMPO oxidation system. The purity of isolated glucaric acid was over 99%; only a small amount of oxalic acid remained as an impurity. Furthermore, the mass balance of

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initial feedstock to glucaric acid was proposed to suggest the potential of the lignocellulosic biomass-based biorefinery process. If the valorization of another biomass component including hemicellulose and lignin was proposed, a more detailed and highly efficient biorefinery process could be suggested.

4. MATERIALS AND METHODS

4.1. Materials. Standard glucose (>99.5%) and (4-acetamido-2,2,6,6-tetramethylpiperidin-1-yl)oxyl (4-acetamido-TEMPO, 97%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Calcium hypochlorite ($Ca(ClO)_2$, 70%), potassium bromide (KBr, 99%), potassium hydroxide (KOH, 95%), and potassium carbonate (K_2CO_3, 99.5%) were purchased from Samchun Pure Chemicals Co., Ltd. (Pyeongtaek, Republic of Korea).

4.2. TEMPO-Mediated Glucose Oxidation. *4.2.1. Synthesis of Potassium Hypochlorite (KClO).* To create a potassium-rich condition, NaBr was replaced with KBr, NaOH, which was used for pH regulation, was replaced with KOH, and NaClO was replaced with potassium chlorite (KClO). KClO was synthesized using the following procedure:[25] First, 200 g of 70% $Ca(ClO)_2$ was dissolved in 600 mL of deionized (DI) water in a 1000 mL beaker. Thereafter, 40 g of KOH and 140 g of K_2CO_3 were dissolved in 250 mL of DI water in another 500 mL beaker. The KOH-K_2CO_3-containing solution was poured into the $Ca(ClO)_2$ solution, followed by vigorous stirring with a mechanical stirrer. After stirring for 30 min, a semifluid gel was formed, which was filtered using a filter paper under reduced pressure. The filtered liquid was KClO solution, and the concentration of KClO was measured via titration to be 1.8−2.2 M.

4.2.2. TEMPO-Mediated Oxidation of Glucose. TEMPO-mediated oxidation was performed based on previous research[28] but with some modifications. The reaction was performed in a 100 mL beaker. First, 2 g of glucose was dissolved in 20 mL of DI water. Thereafter, 0.3 g of KBr and 0.04 g of 4-acetamido-TEMPO were added to the glucose solution. The pH of TEMPO added to the glucose solution was adjusted using a 45% KOH solution prior to the reaction. To investigate the effects of the reaction temperature, amount of oxidant, and pH, a response surface methodology (RSM) was adopted. The analysis was performed based on a central composite design (CCD) using the Design Expert 11.1.0.1 software (Stat-Ease, Inc., Minneapolism MN, USA). The reaction temperature (X1, °C), oxidant (KClO) dosage (X2, equiv per mole glucose), and pH (X3) were regarded as independent variables. The glucaric acid, gluconic acid, formic acid, and acetic acid yields (%, mol/mol per glucose) were regarded as dependent variables. The coded level of the CCD from each run was applied to real independent variables as follows:

> Variables: value of the central point/variation of the coded level per one point.
>
> Reaction temperature (°C): 5/5, oxidant dosage (equiv per mole glucose): 3.3/1.65, and pH: 11.5/1.5.

The reaction conditions suggested by the RSM analysis are listed in Table 5. The temperature of TEMPO added to the glucose solution and amount of the KClO solution were set to the reaction temperature before use. The temperature was kept at reaction temperature using an ice bath (if the reaction temperature was below 0 °C, sodium chloride was added to decrease the temperature below 0 °C), and the KClO solution was slowly added using a syringe pump (NE-300, New Era Pump Systems, Inc., Farmingdale, NY, USA) at a rate of 0.2 mL/min. During the reaction, the pH was maintained by adding a particular amount of the 45% KOH solution. After adding the required amount of KClO solution, the glucose solution was allowed to stand for 1 h until a pH change was no longer observed. The solution was then transferred into a 70 mL screw-capped glass vial and kept in a refrigerator for future analysis and glucaric acid isolation.

Table 5. Central Composite Design (CCD) for Varying Independent Variables

	independent variables		
no	reaction temperature (°C)	oxidant dosage (equiv per mole glucose)	pH
1	0	1.65	10
2	10	1.65	10
3	0	4.95	10
4	10	4.95	10
5	0	1.65	13
6	10	1.65	13
7	0	4.95	13
8	10	4.95	13
9	−3.41	3.3	11.5
10	13.41	3.3	11.5
11	5	0.53	11.5
12	5	6.07	11.5
13	5	3.3	8.98
14	5	3.3	14.02
15	5	3.3	11.5
16	5	3.3	11.5
17	5	3.3	11.5

4.3. TEMPO-Mediated Oxidation of Lignocellulosic Biomass-Derived Glucose. In this study, glucose produced from enzymatic hydrolysis Miscanthus (*Miscanthus sacchariflorus*) was used as the raw material for TEMPO-mediated oxidation. The enzymatic hydrolysate containing glucose was provided by the Center for Biobased Chemistry of the Korea Research Institute of Chemical Technology (Ulsan, Republic of Korea). The glucose production process was accomplished via extrusion, autohydrolysis pretreatment, refining, and enzymatic hydrolysis.[54]

The TEMPO-mediated oxidation of enzymatic hydrolysate was conducted by a similar method with standard glucose. Briefly, enzymatic hydrolysate was diluted to 10% (w/v), and the same amount of KBr and 4-acetaamido-TEMPO were added to 20 mL of diluted enzymatic hydrolysate. Reaction temperature, dosage of the oxidant, and pH were set as the same as the optimum conditions suggested by the statistical analysis.

4.4. Glucaric Acid Isolation from the TEMPO-Mediated Oxidation Reaction. Glucaric acid can be produced and dissolved in solution as a glucaric acid dipotassium salt during the TEMPO-mediated oxidation of glucose. To isolate glucaric acid from solution, the solution was acidified using concentrated hydrochloric acid until the pH was 3.8 while in an ice bath. At this pH, the glucaric acid dipotassium salt was converted to a glucaric acid monopotassium salt, which is less soluble in water. Thereafter, the crystalline glucaric acid monopotassium salt precipitated to the bottom of the solution, and the solution was kept at 4 °C

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overnight. The precipitated glucaric acid monopotassium salt was then collected via filtration (ADVANTEC Micro No. 5C 110 mm, ADVANTEC, Tokyo, Japan) and washed with cold DI water. The filtered precipitates were dried overnight at 65 °C in an oven.

4.5. Analysis of the TEMPO-Mediated Oxidation Products. The concentrations of monomeric sugars (glucose and xylose) and organic acids (formic, acetic, oxalic, and tartaric acids) produced during the TEMPO-mediated oxidation of glucose were determined using a high-performance liquid chromatography instrument (HPLC, Ultimate-3000, Thermo Dionex, CA, USA) equipped with an Aminex 87H column (eluent: 0.01 N sulfuric acid, oven temp: 40 °C, flow rate: 0.5 mL/min, and injection volume: 10 μL).

The concentrations of sugar acids, including aldonic, uronic, and aldaric acids, formed during the TEMPO-mediated glucose oxidation were determined using an ion chromatography instrument (ICS-3000, Thermo Dionex, CA, USA) equipped with a Dionex IonPac AS20 column (4 mm × 250 mm) and a column guard (Dionex IonPac AG20, 4 mm × 50 mm). The flow rate was set to 1 mL/min, and the column temperature was maintained at 30 °C. The mobile phase was sodium hydroxide. A conductivity detector was used to analyze sugar acids at a detector temperature of 35 °C.

Peaks were identified based on retention times, and the quantification of each compound was achieved by comparing the areas of the identified peaks with those of standard peaks. The yields of each product were calculated according to eq 2:

$$\text{product yield (\%)} = \frac{\text{produced product (mole)}}{\text{initial glucose (mole)}} \times 100 \quad (2)$$

The total phenolic compound (TPC) content in enzymatic hydrolysate was analyzed by the Folin−Denis method.[43] The TPC content was observed by changing the absorbance which was detected by a UV−vis-spectrophotometer, and gallic acid was used as a standard for the calibration curve.

The identification of glucaric acid was made using a 600 MHz nuclear magnetic resonance (NMR) spectrometer (AVANCE 600, Bruker, Germany) equipped with a 14.095 T superconducting 51 mm bore magnet and 5 mm BBO BB-H&F-D CryoProbe prodigy. The purity of glucaric acid was determined via ion chromatography (ICS-3000).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08924.

Results of analysis of variance (ANOVA) for gluconic acid, formic acid, oxalic acid, and acetic acid from glucose via TEMPO-mediated oxidation (PDF)

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Notes

The authors declare no competing financial interest.

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