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Simultaneous Saccharification and Fermentation for Isobutanol Production from Banana Peel

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Abstract: Each year, near 40 million tons of banana peels are discarded around the world. This plant biomass could potentially be utilized for energy production. Simultaneous saccharification and fermentation (SSF) is an effective method for producing biofuels from plant biomasses. Since SSF with enzymatic hydrolysis and fermentation are performed simultaneously in the same reactor, the production process is simpler than most existing methods. Here, we describe isobutanol production using SSF with hydrothermally treated banana peel samples and an *Escherichia coli* strain able to utilize glucose and xylose to produce isobutanol. To enhance the glucose and xylose concentrations, the reaction conditions for the enzymatic hydrolysis of plant biomass using two kinds of saccharification enzymes were optimized, including the enzyme unit ratio, reaction temperature and sample gram. When the optimized conditions for enzymatic hydrolysis were applied to SSF, the glucose and xylose produced from the hydrothermally treated samples were consumed, producing isobutanol. Moreover, the isobutanol concentration increased with an increasing initial culture pH, reaching 1.27 g/L at pH 6.5, which was consistent with the optimal initial culture pH for isobutanol production by this *E. coli* strain. Taken together, these results indicate that the established method is potentially useful for industrial isobutanol production.

Keywords: banana peel; isobutanol; SSF; *Escherichia coli*; Cellulase ONOZUKA R-10; Macerozyme R-10

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1. Introduction

Fossil fuel is currently the most used energy source in the world, and its consumption is increasing because of economic growth and with increasing population [1]. However, the supply of fossil fuels is finite, and its future depletion is inevitable. Moreover, the increased fossil fuel consumption is causing environmental problems such as global warming and air pollution. Global warming, in particular, has been progressing with the increase in greenhouse gas emissions since the Industrial Revolution, and some geographical areas are already under threat from extreme weather events. Polar ice is melting, caused by rising air and seawater temperatures due to global warming. Three-quarters of the Arctic ice has already melted in the past 40 years, leading to rising sea levels [2]. Moreover, if sea-level rise continues until 2100, it is estimated that more than several thousand locations will be flooded, mainly along the coasts of Asia and Equatorial regions [3]. It is known that a rising temperature due to global warming evaporates soil moisture, causing surface dryness and drought. These dry conditions make wildfires more likely to occur and the damage more serious. For example, wildfires of unprecedented scale have occurred around the world in the past few years. In 2020, wildfires burned 2.7 million hectares on the west coast of the United States, forcing tens of thousands of people from their homes [4]. Also, similar wildfires occurred in British Columbia, Canada, in 2017 and 2018; in the Amazon rainforest of Brazil in 2019 and 2020; and in central Queensland,

Australia, in 2019 and 2020 [4]. Moreover, in Mediterranean Europe, wildfires burn an average of approximately 4500 km^2 each year [5]. In addition to extreme weather events, as mentioned above, tropical storms, floods, extreme heat waves, and cold winter storms are also caused by global warming [6], which leads to ecological imbalance. A report released by the United Nations, in 2020, estimates that more than 1 million species will become endangered in the coming few decades [7]. Therefore, to advance toward a sustainable society, it will be necessary to develop new energy sources that are efficient, renewable and environmentally friendly to replace fossil fuels. One such approach that is being studied around the world is the utilization of plant biomass to produce biofuel. Plants absorb carbon dioxide during photosynthesis for growth, and the amount of carbon dioxide emitted during the combustion of plant biomass is the same as the amount of carbon dioxide previously absorbed for growth. This makes plant biomass a carbon-neutral renewable energy source [1].

Bananas are herbaceous flowering plants widely cultivated in humid tropical and subtropical countries. Since ripe bananas are nutritious and highly digestible, this fruit can be eaten raw, and a variety of snacks, food dyes and insecticides are produced from bananas [8]. On the other hand, unripe bananas are not tasty and are used to produce banana flour. More than 114 million tons of bananas are produced worldwide each year. Notably, banana peels account for about one-third of the fruit's weight, and approximately 39.9 million tons of banana peels are discarded as food waste each year [8]. However, because banana peels contain approximately 50% polysaccharide and 10% protein (Table S1, see Supplementary Data), they can be effectively used as a medium component to cultivate microorganisms [9,10].

The utilization of plant biomass as a source of biofuel entails a multistep process that includes hydrothermal pretreatment, enzymatic hydrolysis and fermentation [11]. In the hydrothermal pretreatment step, powder prepared from plant biomass is hydrothermally treated to break down the tissue into cellulose, hemicellulose and lignin. In the subsequent enzymatic hydrolysis step, the hydrothermally treated samples are treated with several enzymes, including cellulases and hemicellulases, which hydrolyze the cellulose and hemicellulose to produce primarily glucose and xylose. Finally, the hydrolysate is used by microorganisms as a carbon source during fermentation. With this process, biofuels, such as 2,3-butanediol [12], ethanol [13] and isobutanol [14], are being produced by wild-type or genetically engineered microorganisms. However, a number of challenges remain, including the need to reduce production costs, which have made it difficult to put these methods to industrial use. Therefore, we studied simultaneous saccharification and fermentation (SSF) for ethanol production [15,16], which shows the potential to reduce production costs via the simplification of the production process by avoiding enzymatic hydrolysis step.

SSF is one of the fermentation methods that consists in the enzymatic hydrolysis of the hydrothermally treated samples of plant biomass to extract monosaccharides and then the direct utilization of them for biofuel production. This means that SSF enables simultaneous enzymatic hydrolysis and fermentation for biofuel production in a single vessel. Therefore, enzymatic hydrolysis using separate containers is no longer required, and the number of reactors included in the overall process is reduced, which simplifies the production process, reduces the energy input, and contributes to reducing production costs. Moreover, reducing the risk of contamination is another feature. Unlike with the method of performing enzymatic hydrolysis and fermentation for biofuel production separately, the risk of contamination is reduced because the prepared hydrolysate is not added to another reactor for use in fermentation. Because of its superior features, SSF has attracted much interest in the development of industrial bioprocesses [17].

In this study, we demonstrate that a combination of commercial saccharification enzymes such as Cellulase ONOZUKA R-10 [18] and Macerozyme R-10 [19] are effective for the production of glucose and xylose from hydrothermally treated banana peel samples. To enable future scale-up using an isobutanol-producing *Escherichia coli* strain [14], isobutanol was produced using SSF with the hydrothermally treated samples by a method that involves

a magnetic stirrer, which has a similar working configuration to that used in the stirred tank bioreactors [20].

2. Materials and Methods

2.1. Enzymatic Hydrolysis

After drying banana peels (*Musa* sp.) for 3 days at 50 °C in an incubator (SLI-700; EYELA, Tokyo, Japan), the dried samples were milled using a cutter mill (ABSOLUTE3; Osaka Chemical, Osaka, Japan), yielding a powder (particle size: 100–200 μm). On the basis of the findings of our previous study [21], the hydrothermal treatment of the powder was carried out. Briefly, 10 g of the powder was placed in an autoclave with 100 mL water for 30 min at 130 °C, and the resulting hydrothermally treated samples were used as the initial raw material for enzymatic hydrolysis.

The reaction mixture (5 mL) for enzymatic hydrolysis contained 20 mM acetate buffer (pH 5.0), Cellulase ONOZUKA R-10 (10,000 U/g; Yakult, Tokyo, Japan), Macerozyme R-10 (3000 U/g; Yakult), and the hydrothermally treated samples (0.5–4.0 g) in a 10 mL screw-capped vial with a magnetic stirrer. The reaction mixture was incubated for 3 h at 35 °C while stirring at 500 rpm.

Using 0.1 g of the hydrothermally treated banana peel samples, the effects of various enzyme unit ratios were examined. The effects of reaction temperatures were examined ranging from 35 to 50 °C. The effect of the sample gram was examined at ranging from 0.1 to 4.0 g.

2.2. Determination of Isobutanol Productivity

After the isobutanol-producing *E. coli* strain was grown overnight at 37 °C in Luria–Bertani medium, the culture was collected by centrifugation and washed twice with sterilized water. Subsequently, the washed culture was diluted to an OD600 = 0.1 with 15 mL of fresh M9Y medium (pH 6.5) in a 25 mL screw-capped vial with a magnetic stirrer and then cultured under the optimized conditions for isobutanol production [14]. The M9Y medium contained 40 g/L glucose, 8 g/L xylose, 5 g/L yeast extract and M9 minimal salts (17 g/L $Na_2HPO_4 \cdot 12H_2O$, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 0.24 g/L $MgSO_4 \cdot 7H_2O$ and 0.011 g/L $CaCl_2 \cdot 2H_2O$). Isobutanol productivity was examined while varying the stirring speed between 500–1000 rpm. All data shown were obtained from at least from triplicate experiments.

2.3. Simultaneous Saccharification and Fermentation for Isobutanol Production

Using the isobutanol-producing *E. coli* strain, the SSF experiment was also performed in a 25 mL screw-capped vial containing a magnetic stirrer. The washed culture was diluted to an OD600 = 0.1 with 15 mL of the reaction mixture, which contained 9 g of hydrothermally treated banana peel samples, M9 minimal salts, 20 mM acetate (pH 5.0–6.0) or phosphate (pH 6.5) buffers, 15 U Cellulase ONOZUKA R-10 and 15 U Macerozyme R-10. The mixture was stirred at 500 rpm for 120 h at each initial culture pH, which ranged from pH 5.0 to 6.5.

2.4. Quantification of Sugar and Isobutanol

After each sample was clarified by centrifugation and filtration, the resultant supernatant was subjected to HPLC using an Aminex HPX-87H cationic exchange column (Bio-Rad Labs, Richmond, CA, USA). The concentrations of glucose, xylose and isobutanol were determined using a refractive index indicator. The chromatographic conditions were as follows: mobile phase, 1.5 mM H_2SO_4; flow rate, 0.6 mL/min; column oven temperature, 60 °C.

3. Results and Discussion

3.1. Use of Cellulase ONOZUKA R-10 and Macerozyme R-10 for Enzymatic Hydrolysis

To enable the industrial production of inexpensive chemicals such as biofuels, low production costs are essential. For example, acetone–butanol–ethanol fermentation has already been industrialized to use edible biomass, such as sugarcane as the raw material, and the production cost of biofuels is significantly affected by the raw material's price, which accounts for 60–65% of the total production cost [22]. When edible biomass is used as raw material for biofuel production, a large amount of monosaccharides available to biofuel-producing microorganisms can be easily extracted from the raw material, as well as hydrothermal pretreatment and enzymatic hydrolysis are not necessary. Thus, the production cost is reduced by simplifying the total production process. On the other hand, when plant biomass is used as raw material, the cost of the raw material is lower than that of edible biomass, but the production process increases because monosaccharides cannot be easily extracted from plant biomass. In the production of biofuels using plant biomass as the raw material, the extraction of monosaccharides such as glucose and xylose that can be used by genetically engineered microorganisms is essential. Glucose and xylose can be extracted from the breakdown of cellulose and hemicellulose in plant biomass tissue. To extract the monosaccharides, several kinds of commercial saccharification enzymes are available on the market (Table 1). In particular, Acremonium cellulase, Optimash BG and Novozyme188 have been used extensively, because these enzymes exhibit several high-level activities that degrade hydrothermally treated materials prepared from various biomass [23–26]. On the other hand, Cellulase ONOZUKA R-10, which is prepared from *Trichoderma viride*, shows high cellulase, α-amylase, hemicellulase and pectinase activities [18]. Macerozyme R-10, which is prepared from *Rhizopus* sp., shows high pectinase and hemicellulase activities [19]. In other words, Cellulase ONOZUKA R-10 and Macerozyme R-10 are among the commercially available enzymes with hemicellulase activity, and the combination of these enzymes is useful for the degradation of banana peel tissue, which contains cellulose and hemicellulose as its main components.

Table 1. List of commercial saccharification enzymes.

Saccharification Enzyme	Activity	Source	Supplier
Accellerase 1500	Cellulase, xylanase	*Trichoderma reesei*	Genencor
Acremonium cellulase	β-Glucosidase, cellulase, xylanase	*Acremonium cellulolyticus*	Meiji Seika
Biocellulase A	β-Glucosidase	*Aspergillus niger*	Quest Intl
Bio-feed Beta L	β-glucosidase, cellulase, xylanase	*Trichoderma longibrachiatum*, *T. reesei*	Novozymes
Cellubrix	β-Glucosidase, cellulase, xylanase	*T. longibrachiatum*, *A. niger*	Novozymes
Cellulase 2000 L	Cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	Rhodia-Danisco
Cellulase AP	β-Glucosidase, cellulase	*A. niger*	Amano Enzyme
Cellulase ONOZUKA R-10	α-Amylase, cellulase, hemicellulase, pectinase	*Trichoderma virid*	Yakult
Cellulase TRL	β-Glucosidase	*T. longibrachiatum*, *T. reesei*	Solvay Enzymes
Cellulyve 50 L	Cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	Lyven
Energex L	β-Glucosidase, cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	Novozymes
GC220	β-Glucosidase, cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	Genencor
GC440	β-Glucosidase, cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	Genencor
GC880	β-Glucosidase, cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	Genencor

Table 1. *Cont.*

Saccharification Enzyme	Activity	Source	Supplier
Macerozyme R-10	Hemicellulase, pectinase	*Rhizopus* sp.	Yakult
Novozyme 188	α-Amylase, β-glucosidase, cellulase, glucoamylase, xylanase	*A. niger*	Novozymes
Optimash BG	β-Glucosidase, cellulase, xylanase	Not defined	Genencor International
Rohament CL	β-Glucosidase, cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	AB Enzymes
Spezyme CP	Cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	Genencor
Ultraflo L	β-Glucosidase, cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	Novozymes
Ultraflo XL	α-Amylase	*Bacillus amyloliquefaciens*, *Humicola insolens*	Novozymes
Viscoferm	β-Glucosidase, cellulase, xylanase	Not defined	Novozymes
Viscostar 150 L	β-Glucosidase, cellulase, xylanase	*T. longibrachiatum*, *T. reesei*	Dyadic
Viscozyme L	β-Glucosidase, cellulase, xylanase	*Aspergillus* sp.	Novozymes

The optimization of enzymatic hydrolysis is essential to achieve maximum isobutanol production from hydrothermally treated banana peel samples at a minimum cost. Thus, to optimize the conditions for enzymatic hydrolysis, the effects of the enzyme unit ratio, reaction temperature and sample gram were evaluated. The reaction pH was set at 5.0, which is optimal pH for both enzymes. Examination of the enzyme unit ratio showed that glucose and xylose concentrations were highest when the enzyme unit ratio was 5:5 (Figure 1A). In particular, the glucose concentration achieved using the two enzymes together was about 1.1 times higher than that achieved using Cellulase ONOZUKA R-10 or Macerozyme R-10 alone. When the enzyme unit ratio was kept at 5:5, the highest glucose and xylose concentrations were obtained at 40 °C (Figure 1B). The glucose and xylose concentrations decreased at temperatures above 45 °C (Figure 1B), likely due to the thermal denaturation of the enzymes. Consistent with this idea, both enzymes are mesophilic and are reportedly denatured and inactivated above 45 °C [18,19]. On the other hand, the optimal temperature for isobutanol production using the isobutanol-producing *E. coli* strain is 32 °C [14]. Therefore, to avoid denaturation and inactivation of both enzymes, as well as to activate the *E. coli* strain during SSF, the reactions were run at 35 °C. When the effect of the sample gram was assessed, the glucose and xylose concentrations increased with an increasing sample gram up to 3.0 but were markedly reduced at 4.0 g (Figure 1C). The enzyme reaction is more favorable in the presence of higher substrate concentrations because of the increased frequency of enzyme–substrate contact. However, for highly viscous substrates such as the hydrothermally treated banana peel samples, the reaction is suppressed by the viscosity of the reaction solution at substrate concentrations above the optimal. In the present study, a sample weight of 3.0 g was found to be optimal. In sum, the optimized reaction conditions for SSF for isobutanol production were as follows: enzyme unit ratio, 5:5; reaction temperature, 35 °C; sample gram, 3.0 g.

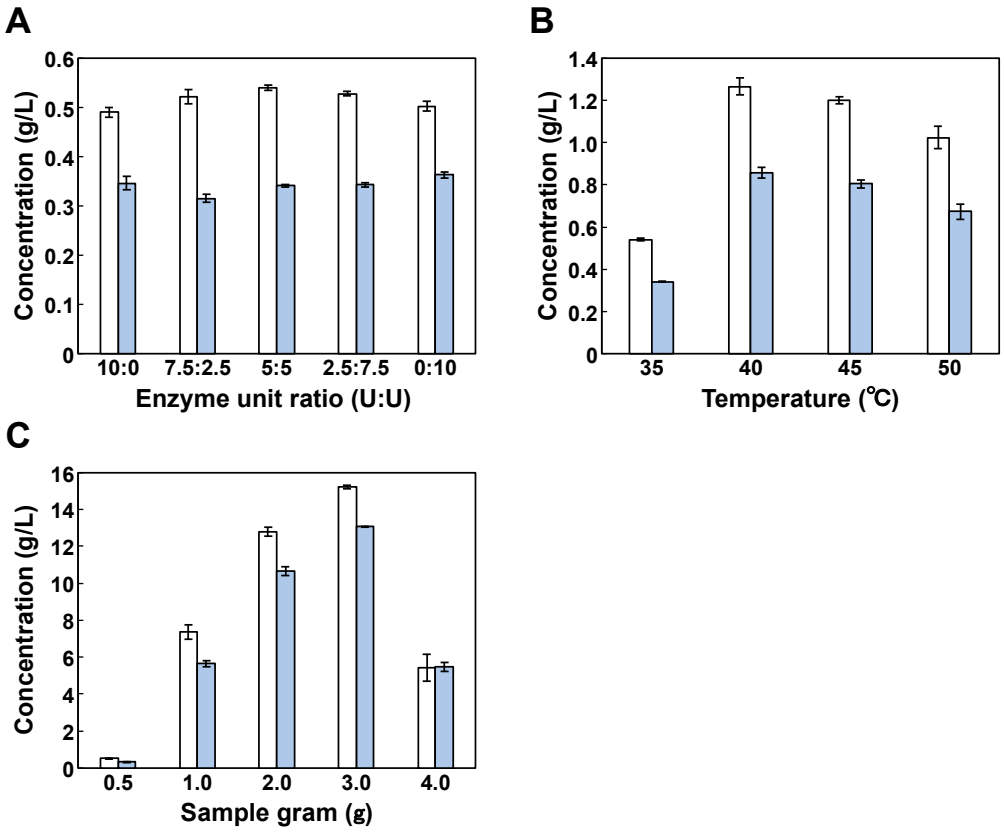

Figure 1. Effects of reaction conditions on enzymatic hydrolysis yielding glucose and xylose. The concentrations of glucose and xylose are shown as white and blue bars, respectively: (**A**) effect of the enzyme unit ratio (Cellulase ONOZUKA R-10: Macerozyme R-10); (**B**) effect of reaction temperature; (**C**) effect of sample gram. Error bars indicate SE (n = 3).

3.2. Effect of Stirring Speed on Isobutanol Production

Akita et al. previously developed an isobutanol-producing *E. coli* strain [14]. To enable isobutanol production, five heterologous genes, including *alsS*, *ilvCD*, *kivd* and *adhA*, were introduced into the genome of the *E. coli* MG1655 strain which are expressed by xylose induction [14]. In this strain, the five enzymes promote sequential reactions to produce isobutanol from pyruvate (Figure 2). Firstly, acetolactate synthase (encoded by *alsS*) converts two molecules of pyruvate to 2-acetolactate, and then 2-acetolactate is converted to 2,3-dihydroxyvalerate by ketol-acid reductoisomerase (encoded by *ilvC*). Subsequently, 2,3-dihydroxyvalerate is converted to 2-ketoisovalerate by dihydroxy-acid dehydratase (encoded by *ilvD*), and then 2-ketoisovalerate is further converted to isobutyraldehyde by 2-keto acid decarboxylase (encoded by *kivd*), yielding isobutyraldehyde. Finally, isobutanol is produced from isobutyraldehyde by alcohol dehydrogenase (encoded by *adhA*). Although *E. coli* has a native alcohol dehydrogenase (encoded by *yqhD*), alcohol dehydrogenase from *Lactococcus lactis* is used for isobutanol production, scince this enzyme is advantageous because of the utilization of NADH rather than NADPH as a cofactor. In the artificial pathway of the isobutanol-producing *E. coli* strain, the five enzymes are derived from mesophiles, so the reaction can proceed at room temperature.

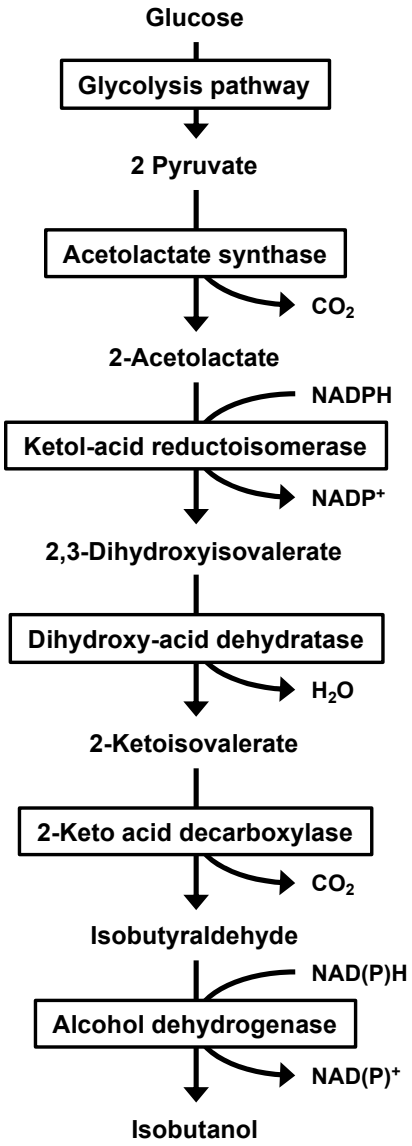

Figure 2. Artificial pathway for isobutanol production. Acetolactate synthase (EC 2.2.1.6) is from *Bacillus subtilis*. Ketol-acid reductoisomerase (EC 1.1.1.86) and dihydroxy-acid dehydratase (EC 4.2.1.9) are native enzymes of *E. coli*. 2-Keto acid decarboxylase (EC 4.1.1.72) and alcohol dehydrogenase (EC 1.1.1.1) are from *L. lactis*.

Recent studies have reported the isolation and identification of the cellulolytic strain *Bacillus coagulans* MA-13, suggesting that it is a useful strain to produce biofuels by SSF [27]. However, the cellulolytic activity of *B. coagulans* MA-13 is relatively low, the addition of saccharification enzymes is required at the start of SSF. Thus, in addition to growing well under the optimal conditions for the activity of saccharification enzymes, the host must be able to metabolize the wide range of sugars produced by plant biomass. On the other hand, the isobutanol-producing *E. coli* strain is able to metabolize mixed sugars by eliminating carbon catabolite repression, which is a global regulatory mechanism that depresses the expression of genes required for the utilization of other sugars when glucose is present. Consequently, this strain can produce isobutanol through effective utilization of both glucose and xylose, which are the main components of plant biomass. When isobutanol was produced using the isobutanol-producing *E. coli* strain in a previous study, the shaking culture method was used with a conical flask with a screw cap [14]. Although this method is effective for lab-scale production, when considering a scale-up to industrial production, a bioreactor is more effective. In the present study, therefore, isobutanol production was

performed in a screw-capped vial with a magnetic stirrer, which is the basic working configuration of a bioreactor.

An earlier investigation in our study found that when the isobutanol-producing *E. coli* strain is cultured in M9Y medium, isobutanol productivity reaches its maximum within 24 h [14]. For that reason, the effects of different stirring speeds on isobutanol concentration and productivity were investigated after incubating cultures for 24 h in the present study. The highest level of production was observed with a stirring speed of 500 rpm (Table 2). This result confirms that the used method is effective for isobutanol production by the strain, which is fundamental for a potential scale-up. These increases in concentration and productivity were likely caused by an increase in the NADH production under micro-anaerobic conditions.

Table 2. Comparison of isobutanol concentration and productivity after 24 h of incubation using M9Y medium.

Stirring Speed (rpm)	Concentration (g/L)	Productivity [(g/(L·h)]
500	4.62 ± 0.122	0.193 ± 0.00512
750	4.26 ± 0.184	0.178 ± 0.00768
1000	3.79 ± 0.369	0.158 ± 0.0154

During isobutanol production, acetolactate synthase, ketol-acid reductoisomerase, dihydroxy-acid dehydratase, 2-ketoacid decarboxylase and alcohol dehydrogenase are overexpressed in the strain cells [14]. Ketol-acid reductoisomerase and alcohol dehydrogenase require NADH as a cofactor for the catalysis, and the levels of NADH are higher under anaerobic conditions than aerobic conditions. Moreover, under aerobic conditions, NADH is used to generate ATP for cell synthesis and maintenance rather than to generate secondary metabolites such as isobutanol [28]. We speculate that when isobutanol was produced at a stirring speed of 500 rpm, the productivity was enhanced because the culture was kept micro-anaerobic conditions.

3.3. SSF for Isobutanol Production

We next applied Cellulase ONOZUKA R-10, Macerozyme R-10 and the *E. coli* strain for SSF to produce isobutanol from hydrothermally treated banana peel samples. When the strain was incubated with the hydrothermally treated banana peel samples and the saccharification enzymes under the optimized reaction conditions, isobutanol production was observed at pH 5.0–6.5 (Figure 3A). Moreover, the isobutanol concentration increased with an increasing initial culture pH, and the highest concentration (1.27 g/L; Figure 3A), which is consistent with the optimal initial culture pH for isobutanol production by this *E. coli* strain [14]. Correspondingly, the concentrations of both glucose and xylose decreased with isobutanol production, and the lowest concentrations were observed at pH 6.5 (Figure 3B,C). These results indicate that the saccharification enzymes have sufficient hydrolytic activity at pH 6.5 to catalyze the production of glucose and xylose. It thus appears that it will be more important to increase the isobutanol productivity of the *E. coli* strain than to increase the activity of the saccharification enzymes.

Although the highest concentration of isobutanol was observed after incubation for 72 h at pH 6.5, the isobutanol concentration after 48 h when incubated at pH 6.0 (0.361 ± 0.000906 g/L) was 7.4-fold higher than that when incubated at pH 6.5 (0.0486 ± 0.0134 g/L; Figure 3A). The details of this result are unknown, but it may be because protein folding was slowed by incubation at pH 6.5, which may reduce the isobutanol productivity of the initial culture. According to Coutard et al., the folding of protein derived from foreign organisms is promoted at around pH 5.0–6.0 when the protein is expressed in *E. coli* cells [29]. On the other hand, the five enzymes necessary for isobutanol production show the maximum activity at pH 6.0–8.0 [30–34]. So, when cultured at pH 6.5, the isobutanol concentration was initially low due to the slow folding of the enzymes, but the amounts of the enzymes in the *E. coli* strain cells increased with the

passage of incubation time, and the concentration increased as those enzymes reacted near their optimal pHs.

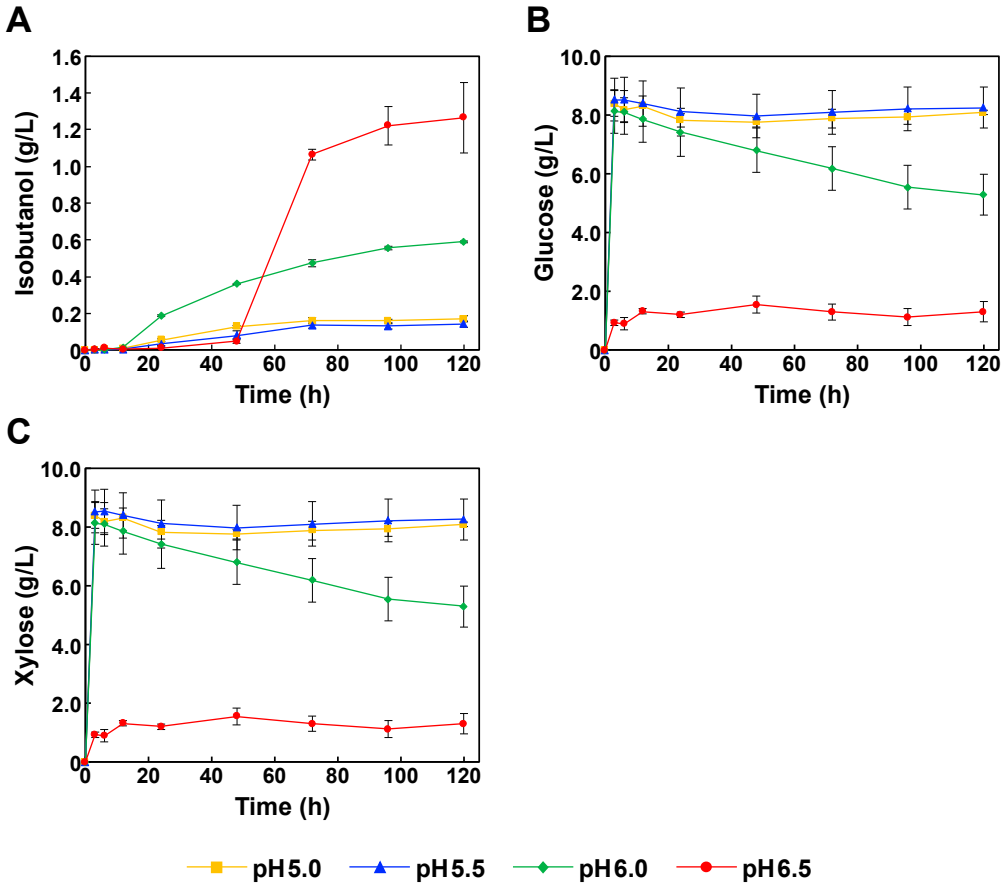

Figure 3. Comparison of the time courses of isobutanol production by SSF with hydrothermally treated banana peel samples: (**A**) isobutanol concentration; (**B**) glucose concentration; (**C**) xylose concentration. Error bars indicate SE (n = 3).

SSF using Cellulase ONOZUKA R-10, Macerozyme R-10 and an isobutanol-producing *E. coli* strain has several noteworthy features, which may be a potentially economical method for isobutanol production from plant biomass. First, the total production process of this method is simple. To date, several effective methods for isobutanol production have been developed [35], but they require expensive reagents to maintain the expression plasmids and induce gene expression for providing the isobutanol production capacity to the host. Moreover, plasmid-based expression requires genetic transformation to incorporate plasmids into the host cells, which takes approximately two days. By contrast, the *E. coli* strain does not require genetic transformation with expensive reagents for isobutanol production, because the five heterologous genes necessary for isobutanol production are all knocked into the genome, and the expressions of those genes are induced by xylose. Second, the implementation of this method is easy. In this method, isobutanol can be produced by reacting the isobutanol-producing *E. coli* strain, Cellulase ONOZUKA R-10 and Macerozyme R-10 in a synthetic medium containing hydrothermally treated banana peel samples as the main component. Moreover, since the basic working configuration of this method is similar to that of bioreactors, it can be easily applied to bioreactors, which can be expected to increase the production scale. However, to discuss production costs in more detail, demonstration tests based on plant-scale production are essential [36]. Moreover, to apply this method to industrial production, it is necessary to follow the regulatory guidelines for recombinant DNA experiments, such as the Cartagena Protocol. Thus, bioreactors

must be prepared to prevent the leakage of genetically modified bacterium such as this *E. coli* strain.

3.4. Comparison of Isobutanol Productivity

Using plant biomass as the only carbon source, several methods for the microbial production of isobutanol have been developed on a laboratory scale (Table 3). When the hydrolysate prepared from Japanese cedar without supplementation with glucose and xylose was used as the medium, the isobutanol-producing *E. coli* strain produced 3.70 g/L isobutanol [14]. The concentration and productivity in the present study were about half those seen in the previous study. However, the method used in the present study reduces the number of steps required for isobutanol production, which would be expected to reduce production costs, making it a potentially effective approach to producing industrial isobutanol from plant biomass.

Table 3. Comparison of isobutanol concentration and productivity when using plant biomass as the only carbon source.

Strain	Use of Expression Plasmid	Source Material	Production Process	Concentration (g/L)	Productivity [(g/(L·h)]	Reference
Corynebacterium crenatium MA11C	Yes	Duckweed	2 steps	5.61	0.0688	[37]
E. coli mlcXT7-LAFC-AAKCD	No	Banana peel	2 steps	1.27 ± 0.191	0.0148 ± 0.00200 *	This study
E. coli mlcXT7-LAFC-AAKCD	No	Japanese cedar	3 steps	3.70 ± 0.0356	0.0386 ± 0.000376	[14]
Pichia pastoris PPY0311	Yes	Sugarcane trash	3 steps	0.0482 ± 0.00170	0.000669 ± 0.0000236	[38]

* The value was calculated from the concentration after 72 h of incubation.

To enhance the efficiency of isobutanol production, the application of batch fermentation is considered an effective method. Since isobutanol is toxic to *E. coli*, the procedure for removing products from the hydrolysate is important. In fact, it has been reported that more than 50 g/L isobutanol can be produced from a synthetic medium using batch fermentation [39]. On the other hand, further improvement in the isobutanol-producing *E. coli* strain is also required. This may be achieved through the elimination of competing pathways of byproducts. According to Jung et al., the accumulation of acetate is an influential factor that decreases isobutanol productivity [40]. In *E. coli* cells, the starting material for isobutanol production is pyruvate, which is converted to acetate by pyruvate oxidase (encoded by *poxB*). This suggests that *poxB* knockout may improve isobutanol productivity by preventing pyruvate conversion to acetate. Another effective method is the utilization of an engineered enzyme. The activity of acetolactate synthase from *B. subtilis* when used in isobutanol production is improved by Gln487Ser mutation [41]. The mutant enzyme can catalyze both the condensation of two molecules of pyruvate to form 2-acetolactate and the decarboxylation of 2-ketoisovalerate to form isobutyraldehyde [42]. Consequently, the utilization of the mutant enzyme shortens the artificial isobutanol pathway by eliminating the need for 2-ketoacid decarboxylase, which would contribute to improving isobutanol productivity.

Changing the saccharification enzymes may also be effective in enhancing the efficiency of isobutanol production. Cellulase ONOZUKA R-10 and Macerozyme R-10 show the maximum activity at pH 5.0, but the optimal pH of the *E. coli* strain for isobutanol production is pH 6.5 [14]. Moreover, when isobutanol was produced at pH 5.0–6.5, the maximum concentration was found at pH 6.5 (Figure 3A). This result means that enhancing the activity of the *E. coli* strain rather than the activity of the saccharification enzymes leads to higher isobutanol concentrations in SSF with Cellulase ONOZUKA R-10, Macerozyme R-10 and the isobutanol-producing *E. coli* strain. Thus, isobutanol concentration can be enhanced if saccharification enzymes with a maximum activity at around pH 6.5 can be applied to SSF. Cellulase from *Acetivibrio thermocellus* [43], hemicellulase from *Aspergillus*

ochraceus [44] and xylanase from *B. subtilis* [45] are known to show maximum activities at pH 6.5. To enhance isobutanol productivity, studies are planned on the production of isobutanol using batch fermentation, the aforementioned mutation to our isobutanol-producing *E. coli* strain and the use of the saccharification enzymes with maximum activity at around pH 6.5 in future studies.

4. Conclusions

In the present study, we used hydrothermally treated banana peel samples to determine the optimal conditions for the enzymatic hydrolysis of plant biomass. Subsequently, we demonstrated the efficacy of SSF for isobutanol production using an isobutanol-producing *E. coli* strain, Cellulase ONOZUKA R-10 and Macerozyme R-10 in a screw-capped vial with a magnetic stirrer. Under the optimized conditions, the highest isobutanol concentration (1.27 g/L) and productivity [0.0165 g/(L·h)] were obtained at pH 6.5, which indicates that glucose and xylose were produced by the enzymatic hydrolysis of the hydrothermally treated samples, and those sugars were consumed by the *E. coli* strain for isobutanol production. Although the highest concentrations and productivities achieved in this study were about half those seen in earlier studies, our method may be much more cost-effective and potentially advantageous for industrial use, as it does not require an enzymatic hydrolysis step or special expertise.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10030161/s1. Tables S1: Component of banana peel.

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