Utilization of Sugarcane Bagasse as a Substrate for Lipid Production by Aurantiochytrium sp.

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Abstract: Thraustochytrid, Aurantiochytrium sp., produces various lipids such as polyunsaturated and saturated fatty acids, carotenoids, and other hydrocarbons, which are useful in the fields of health foods, cosmetics, fine chemicals, and biofuels. Lignocellulosic biomass, which is abundant and cheap, is a promising feedstock for producing cheaper bulk and high-value-added products using Aurantiochytrium sp. However, the steam explosion of lignocellulosic biomass for efficient enzymatic saccharification generates substances that inhibit the growth of microorganisms. In this study, the inhibitory activities of these by-products on the growth and lipid production of Aurantiochytrium sp. were investigated. Aurantiochytrium sp. was found to be highly sensitive to furfural and vanillin and moderately sensitive to 5-hydroxymethylfurfural and syringaldehyde. Washing steam-exploded bagasse with water, followed by activated charcoal treatment, significantly reduced furfural, which was a major inhibitory component in the saccharified solution.

Key words: Aurantiochytrium sp., biomass conversion, lignocellulosic biomass

1 Introduction
A biorefinery, which converts various biomass to biofuels and fine chemicals using microbial catalysts, is a technology that can be applied to nonuse fossil fuels and recycling society. Among the various microorganisms considered as biocatalysts for biorefineries, thraustochytrids are expected to be key players because they produce various lipids, such as polyunsaturated fatty acids, squalene, and carotenoids by assimilation of organic compounds as well as sugars. Recent research has reported a lipid fermentation system by thraustochytrids using unutilized biomass, including food waste, macroalgae, or waste glycerol. In this study, we attempted to utilize sugarcane bagasse as an abundant terrestrial lignocellulosic biomass as a substrate for lipid production by the thraustochytrid, Aurantiochytrium sp. Sugarcane bagasse is the residue left after squeezing juice from sugarcane and is mainly composed of cellulose, hemicellulose, and lignin. Therefore, pretreatment of bagasse by heating with acids or alkalis is necessary for efficient saccharification using microbial enzymes to convert bagasse to Aurantiochytrium-assimilable sugars such as glucose. However, various by-products such as furans, phenolic compounds, and organic acids generated during the pretreatment process have been reported to inhibit the growth and material production of microorganisms. Therefore, it is necessary to evaluate the sensitivity of biocatalysts to such by-products and establish an efficient procedure for removing harmful compounds. This study investigated the inhibitory effect of substances generated by the steam explosion of sugarcane bagasse on the growth and lipid production of Aurantiochytrium limacinum SR21 and examined pretreatment methods to reduce the inhibitory substances.

2 Materials and Methods
2.1 Microorganisms, culture media, and reagents
A. limacinum SR21 (ATCC MYA-1381) was cultured in GPY medium containing 30 g/L glucose, 6 g/L Hipolypepton (Nihon Pharmaceutical, Tokyo, Japan), 2 g/L yeast extract, and 20 g/L artificial sea salt (Sigma-Aldrich, St. Louis, MO, USA) at pH 6.5, 28°C and 180 rpm for 48 h. Reagents were purchased from Nacalai Tesque (Osaka, Japan), Sigma-Aldrich, and Tokyo Kasei (Tokyo, Japan) unless otherwise indicated.
2.2 Preparation of saccharification liquid from sugarcane bagasse

Sugarcane bagasse containing 50% water was blasted once by the steam explosion at 3 MPa for 5 min. Sugarcane bagasse soaked in 0.5% sulfuric acid was blasted under the same conditions. Steam-exploded bagasse or steam-exploded bagasse washed once with water was treated with 20 mg-protein/g bagasse of the commercial enzyme complex of cellulase and hemicellulase at pH 5.5, 50°C and 120 rpm for 7 days.

2.3 Component analysis of saccharification liquid

The glucose concentration in the saccharification liquid was measured using a glucose (GO) assay kit (Sigma-Aldrich) and a VICTOR NIVO multimode microplate reader (PerkinElmer, Waltham, MA, USA). Furans, phenolic compounds, and organic acids were measured using a high-performance liquid chromatography system (1260 Infinity, Agilent Technology, Santa Clara, CA, USA) equipped with a silica-based reversed-phase column (YMC Carotenoid, 4.6 × 250 mm, YMC, Kyoto, Japan). A mobile phase consisting of 0.05% phosphoric acid/acetonitrile (85:15, v/v) was used at a flow rate of 1 mL/min. All compounds were detected by a diode array detector at 210 nm and identified by comparing their retention times with those of the reference chemicals.

2.4 Evaluation of cell growth

The growth of A. limacinum SR21 in the culture fluid was determined by measuring the optical density at 590 nm (OD_{590}). The half-maximal inhibitory concentration (IC_{50}) of each chemical was defined as the concentration at which OD_{590} was half that of the control.

2.5 Lipid extraction and fatty acid analysis

The cells of the strain SR21 from 1 mL of culture were washed with distilled water and vigorously vortexed with 1 mL of tert-butylmethyl ether/methanol (2:1, v/v) and glass beads (0.5 mm) using a bead crusher µT-12 (Taitech, Aichi, Japan). Five hundred microliters of distilled water and 50 μg of arachidic acid as an internal standard were added, and the mixed solutions were vortexed for a short time. Organic layer was collected by centrifugation at 12,000 × g for 10 min and transferred into a screw cap test tube. Methanolysis of fatty acids was performed by adding 10% methanolic hydrogen and heating at 60°C for 90 min. Fatty acid methyl esters (FAMEs) were extracted with n-hexane. A gas chromatography system (GC2025, Shimadzu, Kyoto, Japan) equipped with a capillary column (TC-70, GL Science, Tokyo, Japan) was used to analyze the FAMEs. FAMEs were identified by comparing their retention times with those of 37-compounds (Spelco, Bellefonte, PA, USA) and quantified by comparing the peak area with that of arachidic acid.

2.6 Statistical analysis

Student's t-test was used to determine the differences in experimental values between the control medium and the medium containing inhibitors. p < 0.05 was considered significant.

3 Results

3.1 Growth and fatty acid production of A. limacinum SR21 cultured in the medium containing saccharified solutions

The saccharified solutions, Sol-exp, Sol-exp-W, and Sol-exp-S, were prepared by enzymatic saccharification of bagasse blasted by steam explosion, bagasse blasted and washed with water, and bagasse pretreated with 0.5% sulfuric acid followed by steam explosion (Fig. 1A), respectively. Sol-exp, Sol-exp-W, and Sol-exp-S contained 98.8 g/L, 110.5 g/L, and 80.5 g/L of glucose, respectively. A. limacinum SR21 was cultured in the GPY medium containing each saccharified solution (Fig. 1B). The cell growth in the Sol-exp medium containing glucose at 50 and 80 g/L were OD_{590} values of 22.9 ± 4.6 and 22.7 ± 1.6, which were 24.6% and 24.8% lower than those in the control GPY medium, respectively. The total fatty acid production also decreased by 3.0 ± 1.9 and 2.6 ± 0.8 g/L, which were 28.6 and 41.1% lower than control, respectively. In addition, the cell growth and total fatty acid production in the Sol-exp-S medium containing glucose at 50 g/L was OD_{590} of 0.6 ± 0.01 and 0.03 ± 0.02 g/L, which were 97.9% and 99.2% lower than those of control medium, respectively. These results indicate the presence of some substances inhibiting the growth and fatty acid production of strain SR21 in Sol-exp and Sol-exp-S. The particularly strong inhibitory activity of Sol-exp-S suggests that the formation of these inhibitors may be enhanced in the presence of sulfuric acid. In contrast, strain SR21 cultured in a medium containing Sol-exp-W did not significantly reduce cell growth and fatty acid production. Hence, the growth inhibitors contained in the steam-exploded bagasse were presumed to be watersoluble.

3.2 The sensitivity of A. limacinum SR21 against substances generated by the heat treatment of lignocellulosic biomass

The inhibitory substances generated by the pretreatment of lignocellulosic biomass were examined to determine the sensitivity of strain SR21. As shown in Fig. 2A, the presence of 60 mM 5-hydroxymethylfurfural completely inhibited growth and fatty acid production. A significant inhibitory effect was also observed with the addition of furfural at concentrations higher than 15 mM (Fig. 2A). In phenol derivatives from lignin (Fig. 2B), syringaldehyde at 30 mM showed significant inhibition, and vanillin at con-
Fig. 1 Preparation of hydrolysate from sugarcane bagasse and cultivation of *A. limacinum* SR21 in the media containing sugarcane bagasse hydrolysate. Sol-exp, Sol-exp-W, and Sol-exp-S were prepared by enzymatic saccharification of sugarcane bagasse pretreated with the respective methods (A). *A. limacinum* SR21 was cultured in the medium containing each sugarcane hydrolysate for 48 h, and OD$_{590}$ (open circle) and the fatty acid yield (cumulated bar graphs) were measured (B).

Fig. 2 The effect of typical by-products generated by the heat, acid, and alkali treatment of lignocellulosic biomass on the growth and the fatty acid production of *A. limacinum* SR21. *A. limacinum* SR21 was cultured in the GPY media containing furans (A), phenols (B), or organic acids (C) for 48 h, and OD$_{590}$ (open circle) and the fatty acid yield (cumulated bar graphs) were measured.
centrations higher than 15 mM completely inhibited growth. Vanillic acid and coniferyl alcohol had moderate effects. As for organic acids (Fig. 2C), acetic acid and formic acid are also major by-products generated by the heat treatment of lignocellulosic biomass and have been reported to reduce the growth of *Saccharomyces cerevisiae*\(^\text{20}\) and *Trichoderma reesei*\(^\text{21}\). However, these organic acids did not show any inhibitory activity against SR21. The addition of acetic acid tended to increase fatty acid production, which suggested to be assimilated, at least in part, as a nutrient.

### 3.3 Identification of the toxic substances in Sol-exp and Sol-exp-S

HPLC analysis of Sol-exp and Sol-exp-S revealed three major peaks with retention times consistent with those of furfural, 5-hydroxymethylfurfural, and acetic acid (Figs. 3B, 3D). Their concentrations were 4.8, 0.7, and 13.7 mM in Sol-exp and 11.2, 2.9, and 51.8 mM in Sol-exp-S, respectively, whereas these substances were not detected in Sol-exp-W (data not shown). Among these substances, furfural was expected to be the main growth inhibitor according to the sensitivity test of *A. limacinum* SR21 (Fig. 2A). To remove inhibitory substances, activated charcoal was added to Sol-exp and Sol-exp-S at 5% (w/v) and agitated for 30 min. This resulted in a significant reduction in furfural, 5-hydroxymethylfurfural, and acetic acid (Figs. 3C, 3E), whereas 92-94% of glucose remained (data not shown). The strain SR21 was also sensitive to furfural (\(\text{IC}_{50} = 7.5\) mM) and 5-hydroxymethylfurfural.

### 4 Discussion

Fermentative production of lipids from unutilized biomass is important to ensure the sustainability and economic viability of the production process, and some efforts are underway to utilize biomass, such as non-edible plants\(^\text{9}\), food waste\(^\text{7, 22-24}\), and industrial waste\(^\text{25-27}\) for lipid production by *Aurantiochytrium* sp. The utilization of lignocellulosic biomass for lipid production by *Aurantiochytrium* sp. is meaningful in expanding the field of lipid product application. In this study, the inhibitory effects of typical by-products generated by the heat, acid, and alkali treatment of lignocellulosic biomass\(^\text{13, 15}\) on the growth and fatty acid production of *A. limacinum* SR21 were investigated. In addition, a procedure to remove substances from the saccharified solution prepared from sugarcane bagasse was developed.

The dehydration of hexose and pentose in hemicellulose generates furan derivatives such as furfural and 5-hydroxymethylfurfural\(^\text{28, 29}\). Furan derivatives are major microbial growth inhibitors in lignocellulosic hydrolysates and have been reported to inhibit glycolysis, induce oxidative stress, damage cell membrane homeostasis, and repress protein translation\(^\text{30-32}\). The strain SR21 was also sensitive to furfural (\(\text{IC}_{50} = 7.5\) mM) and 5-hydroxymethylfurfural (\(\text{IC}_{50}\)
Lipid Production from Lignocellulosic Biomass by Aurantiochytrium sp.

Fig. 4  Cultivation of A. limacinum SR21 in the media containing the activated-charcoal treated sugarcane bagasse hydrolysate. A. limacinum SR21 was cultured in the media, containing untreated Sol-exp and Sol-exp-S (A) or the activated charcoal-treated hydrolysates (B), with 80 g/L-glucose for 48 h, and OD590 (open circle) and the fatty acid yield (cumulated bar graphs) were measured.

Phenolic compounds generated by degrading lignin in plants under acidic conditions \(^{36}\) have been reported to inhibit the growth and substance production of fungi and microalgae by harming enzyme activities and homeostasis of the cell membrane \(^{37}\). Among the 4-hydroxybenzoic acid and its derivatives tested in this study, chemicals other than 4-hydroxybenzoic acid inhibited growth and lipid production of SR21. Since vanillin showed significant inhibitory activity \(^{38}\), it was necessary to reduce its concentration in the hydrolysate. In addition, the result that the inhibitory activity of vanillin (IC\(_{50}\) = 8.3 mM) is higher than that of vanillic acid (IC\(_{50}\) = 27 mM), an oxide of vanillin, is consistent with a report that phenols with aldehyde groups show a higher inhibitory effect on the growth of S. cerevisiae than phenols with carboxyl groups \(^{39}\).

Organic acids such as acetic acid, formic acid, and levuric acid have been reported to inhibit yeast cell growth by lowering the intracellular pH or inducing the accumulation of reactive oxygen species \(^{39, 40}\). In contrast, the inhibitory effects of these organic acids were marginal or negligible under the conditions tested in this study \(^{41}\). Moreover, with acetic acid at concentrations higher than 50 mM, an increased fatty acid content was observed. Strain SR21 has been reported to grow and produce lipids using up to 40 g/L acetic acid as the sole carbon source \(^{39, 41}\). Therefore, acetic acid in the saccharified solution is presumed to positively affect growth and the fatty acid production.

As a result of culturing the strain SR21 in the medium containing the hydrolysates prepared from hydrothermally treated sugarcane bagasse, remarkable growth inhibition was observed in the medium containing Sol-exp and Sol-exp-S compared with the GPY medium containing glucose at the same concentration, whereas Sol-exp-W showed no growth inhibitory effect \(^{42}\). Furfural, 5-hydroxymethylfurfural, and acetic acid were identified as major by-products in Sol-exp and Sol-exp-S \(^{36, 43}\). Considering the sensitivity of SR21 to each compound \(^{44}\), furfural was expected to be the primary inhibitor of cell growth and fatty acid production. Sol-exp-W, which contained only trace amounts of furfural and 5-hydroxymethylfurfural, showed no inhibitory effect. Therefore, washing steam-exploded bagasse with water effectively removes growth-inhibiting substances. In addition, enriching the recovery of inhibitory components in the saccharified solution should also be considered because washing treatment requires wastewater disposal. The by-products in Sol-exp and Sol-exp-S, including furfural, were effectively removed by the activated charcoal treatment \(^{45}\). Activated charcoal treatment has advantages over other procedures, such as distillation and ion exchange \(^{46}\) in terms of removal efficiency and energy cost. Furfural absorbed on activated charcoal can be collected using organic solvent and used as a material for lubricating oil and solvent in the chemical industry. When Sol-exp and Sol-exp-S were treated with activated charcoal, cell growth and lipid productivity were equal to or higher than those in the control (Fig. 4). In particular, charcoal-treated Sol-exp induced significantly higher fatty acid productivity, suggesting sugarcane bagasse hydrolysate contains substances that promote not only cell growth \(^{44}\) but also lipid production,
such as plant hormones\textsuperscript{45, 46}.

5 Conclusion

The inhibitory effect of substances generated by heat treatment of lignocellulosic biomass on the growth and lipid production of \textit{Aurantiochytrium} sp. was elucidated for the first time. Furfural was speculated as the primary harmful substance in the saccharified solution of sugarcane bagasse, and efficient lipid production was possible by removing it by water-washing and activated charcoal treatment. These results provide an efficient strategy for preparing a saccharified solution from lignocellulosic biomass suitable for lipid fermentation by \textit{Aurantiochytrium} sp. and contribute to the development of bioprocesses that convert lignocellulosic biomass to valuable lipids.

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Author Contributions

K.W. designed this research, analyzed the data, and drafted the original manuscript. M.N. performed the experiments and analyzed the data. S.M. designed this research, performed the experiments, and analyzed the data. T.A. designed this research and revised the draft critically for important intellectual content. All authors reviewed and revised the manuscript draft and approved the final version for submission.

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