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Efficient production of sophorolipids from lignocellulosic biomass-based medium

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Abstract

Sophorolipids (SLs) are amphiphilic compounds produced from edible saccharides and vegetable oils by a yeast, *Starmerella bombicola*, and related strains, and are applied as detergents for commercial uses. In present study, SL-production from corn cob hydrolysate (CCH) as non-edible substrates was investigated. According to ultraviolet spectrophotometry and high performance liquid chromatography (HPLC) analyses, frufral (FL) as phenoic compounds were increased along with increase of sulfuric acid concentrations during acid-hydrolysis treatment. In case of use of 3% (w/v) H_2SO_4, 12 mM of FL are detected in the hydrolysate, and over 86% of SL production was inhibited compared to that of 1% (w/v) H_2SO_4. Additional heat treatment led Maillard reaction, colored the media to dark blown, and also stimulated FL generation. Three times of heat treatment (121°C, 20 min) strictly decreased SL production and cell growth, however FL increased to 5 mM. Ammonium nitrate (0.1 g-N/L) recovered the SL production in once heat treated corn cob hydrolysate. The results indicate that the inhibitions of cell growth and SL production were occurred by shortage of nitrogen sources in corn cob hydrolysate. In jarfermentor cultivation, SL production from corn cob hydrolysate and olive oil was reached 49.2 g/L for 4 days. The volumetric productivity was 12.3 g/L/day, which was corresponding to those of previous studies using standard production medium. Waste edible oils did not affect to the SL production and cell growth.

34 Introduction

35 Surfactants and emulsifier are significant materials and widely used in broad ranges of
36 manufactures in pharmaceutical, cosmetic, petroleum and food industries. Chemically synthesized
37 surfactants have been often used in the manufactures. Microbial synthesized surfactants, named
38 biosurfactants (BSs), have been recent used in commercial use due to their excellent surface
39 activities and biocompatibility (1-3). Sophorolipids (SLs) are one of most famous BSs, and are
40 produced from biomass including saccharides and vegetable oils by yeast like fungus, *Starmellela*
41 *bombicola* (4), *Candida batistae* (5), *Candida apicola* (6), *Candida bogoriensis* (7), and
42 *Wickerhamiella domercqiae* (8). *S. bombicola* is one of well-known SL producing yeasts. The SL
43 production yield reaches at the level of 200 g/*l*, and the volumetric productivities are more than 2.4
44 g/L/h (9). The structures have been determined as mixture of lactonic and acidic forms of
45 sophoroside of 17-hydroxyoctadecanoic acid (10) (Fig. 1). The critical micelle concentrations and
46 the surface tension of SL mixtures are 16.6 mg/*l* and 36.4 mN/m (5). SLs shows low-forming
47 surfactants with high detergency, low cytotoxicity, and readily biodegaradable properties (11).
48 Furthermore, SLs show unique pH-dependent supramolecular assemble structure in aqueous (12).
49 SLs and the derivatives show physiological activities including antimicrobial (13), anticancer (8, 14)
50 and anti-virus activities (13).

51 One of significant problems for large-scale production of SLs is cutting the production

cost. The cost of SL production of 10-30 % are occupied by row material account (15). For reducing raw material cost, the compositions of cultivation medium were well studied (16), and the potential of using several alternative raw materials as medium compositions have been described including sweet water (15), deproteinized whey (17), biodiesel co-product stream (18), animal fat (19) and waste frying oil (20). Lignocellulosic materials are one of the most abundant renewable and underutilized resource all over the world. Further developments of the cost-effective utilization of the recourses are desired. Recently, detoxified and delignined corncob residue hydrolysate (DCCR) as low-cost raw material were used for SLs, and contributed to 39.08 g/L of SLs production (21). However, preparation of the detoxified DCCR is necessary to treat alkali treatment and detoxification by activated carbon. These treatments increase the total production costs of SL production process, and induce the amount of waste water. Simplify of the treatment of DCCR were necessary to innovate the efficiency of total cost of SL production.

In order to reduce the raw material cost in SL production cost, methods of corncob hydrolysate (CCH) preparation, and continued effective cultivation are describes in this article. And effects of the phenoic growth inhibitors on SL production are investigated. Furthermore, effects of additional nitrogen source on the SL production are shown.

MATERIALS and METHODS

Microorganisms and chemicals

Starrmerella bombicola NBRC 10243 were purchased from NITE Biological Resource Center (NBRC), of National Institute of Technology and Ecaluation (NITE), Japan. For preparing glycerol stocks, the strain cultivated for 48 h at 30°C in YM medium containing 10 g/*l* glucose, 3 g/*l* yeast extract, 3 g/*l* malt extract, and 5 g/*l* peptone. The culture broth were mixed in 20% glycerol. The mixtures were stored at -80°C as glycerol stocks. The glycerol stocks were used for all experiments in this study.

Olive oil was purchased from Wako Pure Chemical Co. (Tokyo, Japan). Waste oils were obtained from a standard home and a food manufacture.

Preparation of CCH

Preparation of CCH was performed by modified previously described method (22) as following. Dried and crushed corncob were purchased from ****, which were harvested at ****, in China. Water contents of the material were from 11.5 to 12.0 %. Corncob and dilute sulfuric acid mixed at 2:10 of solid-liquid ratio, and treated at 121°C for 60 min by using autoclave. The mixture was cooled at room temperature, and neutralizaing by 10 mol/*l* NaOH aqueous to pH 5.0. A commercial cellulose reagent, Meiselase (Meiji Co. Tokyo, Japan), of 5 g par 100 g corncob was

added, and incubated at 40°C for 3 days. The reaction was filtered by Wattman No.1 filter. pH of the filtrate were adjusted to 5.0. And then the sample was filtered again by 0.22 μm of sterile filtration unit (Corning). The filtrate was used as CCH for the following experiments.

Charactarization of CCH

Spectroscopy was carried out by a spectrophotometer (U-1000, Hitachi). The absorbance of CCH was measured during 200 to 600 nm. Phenoic growth inhibitor concentrations were measured by a HPLC system with μBondSphere C18-100Å (3.9 x 150 mm, Waters). Acetonitril and phosphate buffer (pH 7.0) (85 : 15 v/v) was used for mobile phase. The elute was detected by a UV detector at 275 nm. The concentration of flufural (FL), 3-hydroxyflufral (HMF), and vaniline (VA) were calculated from peaks of these standard materials (Wako Pure Chemical Co.). Saccharides concentrations were detected by a HPLC system, equipped TSKgel SCX-H^+ column (7.8 x 300 mm, Tosoh Co., Tokyo Japan). 50 mM perchloric acid was used as mobile phase. Refractive index detector was used for detection saccharides in elute.

Culture conditions

Glycerol stocks (0.5 ml) were inoculated in 20 ml of the YM medium, and cultured at 28°C, 200 rpm for 48 h. In case of flask cultivation, 1 g of olive oil as hydrophobic carbon sources

105 were sterilized in 200 ml baffled Erlenmeyer flasks by autoclave at 121°C for 20 min. the culture
106 broths (1 ml) were inoculated in 20 ml of CCH as production medium, prepared in above 200 ml
107 baffled-Erlenmeyer flasks. Additional medium components were added as 100 fold concentrations
108 stock solutions. Standard SL production medium are composed of 25 g/l glucose, 50 g/l olive oil, 3
109 g/l $NaNO_3$, 0.5 g/l KH_2PO_4, 0.5 g/l $MgSO_4$ $7H_2O$, and 1 g/l yeast extract. In case of jarfermentor
110 cultivation, pre-cultivation was carried out in 20 ml YM medium at 28°C for 2 days. The CCH (500
111 ml) were prepared in 1 L vessels with pH and dissolved oxygen electrodes and autoclaved at 121°C
112 for 20 min. Olive oil (50 g) and additional compositions were separately autoclaved. Separately
113 sterilized components and culture broth (20 ml) were added in the vessels including CCH, when SL
114 productions were started. The cultivating temperature were maintained at 28°C. Agitation speed was
115 controlled between 500 and 1000 rpm to maintain DO above 50% saturation.
116
117 **Quantification of SLs and dry cell weights**
118 SL concentrations and dry cell weight (DCW) were estimated by previously described
119 methods (23), as following. Equal volume of ethyl acetate was added in the culture broth and extract
120 hydrophobic materials from the broth. Ethyl acetate was evaporated by a centrifugation evaporator
121 and the compounds were washed by hexane three times and removed residual olive oil and purified
122 SLs. The residual hexane evaporated again by a centrifugation evaporator. Weights of SLs were

123 measured by a electric balance. Three different experiments were carried out and calculated the
124 means of SL concentration. DCWs were determined from weight of cells washed by ethyl acetate,
125 methanol, and deionized water.
126
127 **Isolation of major SL component**
128 To purify major component, crude SLs were obtained by ethyl acetate extraction. The
129 organic layer was evaporated and dissolved in small amount of ethyl acetate again. The major
130 component of SLs were purified by open-column chromatography with silica-gel (Wako gel C-200)
131 using a gradient elution of chloroform-acetone (10:0 to 7:3, v/v). The purity of SLs were checked by
132 TLC on silica gel plate (silicagel 60, Merck) with solvent system consisting of chloroform /
133 methanol (8:2). The compounds on the plates were located by heating at 110°C for 5 min, after
134 spraying the anthrone/ sulfuric acid reagent.
135
136 **Structural determination**
137 Structure of purified major component of SLs were determined by nuclear magnetic
138 resonance (NMR) analyses (^{1}H-NMR, and ^{13}C-NMR) with ECA-600 spectrometer (600 MHz, Nihon
139 denshi) using chloroform-*d*.. The molecular weight of the compounds was measured by
140 matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS)

141 (Ultraflex III, Bruker) with a α-cyano-4-hydroxycinnamic acid matrix.

142

143

144 **RESULTS**

145 **Effects of sulfuric acid concetntration in CCH preparation**

146 Saccharification of lignocellulosic biomass is often carried out combination of chemical
147 and biological reaction including acid and heat treatments and cellulase digestion (24). In
148 preliminary experiments, a combination of acid treatment using sulfuric acids and cellulose
149 saccharification procedure were suitable for microbial fermentations (25). Therefore, effects of the
150 concentrations of sulfuric acid during acid treatments on characters of CCH and the following SL
151 production were investigated. Fig. 2A indicates spectrographs of CCH prepared using from 1 to 5%
152 (w/v) H_2SO_4. In case of 1 and 2% (w/v) H_2SO_4, broad peak was observed between 260 to 350 nm of
153 wave length. In case of 3 to 5%(w/v) H_2SO_4, single peak was observed around 280 nm. Increase of
154 H_2SO_4 concentration caused peak shift the broad band to single peak at 280 nm, which indicated
155 benzene ring including various phenoic compounds. Therefore, FL, HMF, and VA as representatives
156 of phenoic compounds, which are often reported as growth inhibitors, were measured by a HPLC
157 method. Fig. 2B indicates the results of HPLC analysis. Concentrations of HMF and VA were
158 maintained under 2 mM in all experimental conditions. Using 1 and 2%(w/v) of H_2SO_4, FL

159 increased to approximately 3 mM. The concentration were increased to 12 mM when using 3% (x/v)
160 H_2SO_4.This results indicated more than 3%(w/v) of H_2SO_4 introduces the degradation of lignin as
161 phenoic polymer in lingocellulose. Glucose concentrations of CCH after 1, 2, 3, 4, and 5% H2SO4
162 treatments were 45.0, 34.9, 29.1, 29.9, and 27.1 g/l, respectively. Fig. 2C indicates effect of H_2SO_4
163 concentrations on SL production and cell growth. In case of using more than 3% (w/v) of H_2SO_4, SL
164 production and cell growth were strictly inhibited by toxicity compounds caused by acid
165 saccarification reaction. Although 2% H_2SO_4 treatment gave the no longer inhibition to cell growth,
166 the treatment slightly affect on the SL production, and reduced SL production to 18.6 ± 0.9 g/L. In
167 case of 1% H2SO4 the SL production and cell growth were reached to 33.7±1.0 g/L, and 8.60 ± 0.24
168 g/L, respectively. The SL production and cell growth were corresponding to those in standard SL
169 production medium (pH 5.0), which were 33.9 ± 3.8 g/L and 3.6 ± 0.3 g/L, respectively.
170
171 **Effect of additional heat treatment**
172 For SL production using jar-fermentor, the prepared SSH with vessel is necessary to be
173 sterilized at 121°C for 20 min by autoclave. Effects of the additional heat treatment were examined
174 on UV absorbance of CCH, phenoic compounds concentrations and SL production. Fig. 3A indicates
175 results of spectrometric analysis of CCH after different times of heat treatment. Increase of times of
176 heat treatments induced the absorbance at wave length lower than 300 nm. Fig. 3B indicates the

177 concentrations of phenoic compounds. FL concentrations were gradually increased along with
178 increase of times of treatments, and reached to 6 mM in case of forth times of the treatments. The
179 concentrations are corresponding to the half amount of that of 5% H_2SO_4 treated CCH (Fig. 2B).
180 Glucose concentrations without additional heating and after once, twice, three and four times heating
181 were26.2, 28.3, 31.6, 30.1 and 32.0 g/l, respectively. The absorbance at 400 nm of samples without
182 additional heating and after once, twice, three and four times heating were 3.16, 3.33, 4.54, 5.35, and
183 6.28, respectively. This result implied that the heat treatment stimulated non-enzymatic browning
184 reaction including Mailard reaction. Fig. 3C shows the SL productions and cell growths in cases of
185 different times of heat treatments. Once heat-treatment reduced 36% SL production and 40 % cell
186 growth compared to no-treatment, respectively. Twice heat-treatment further decreased the SL
187 production to 8.3 ± 0.3 g/L. In cases of three and four times, SL productions and cell growths were
188 strictly inhibited and the concentrations were below 5 g/l.
189
190 **Effects of additional nitrogen**
191 In order to examine the shortage of nitrogen sources in the CCH, the effects of additional
192 nitrogen source on the SL productions in CCH with and without additional heat treatment. Figure 4A
193 indicate the effects of additional nitrogen concentrations on the SL production and cell growth in
194 CCH media without additional heat treatment. Ammonium nitrate was used as additional nitrogen

source. Although additional 0.05 g-N/l of nitrogen show no significant effects on the SL production and cell growth, additional 0.1 g-N/l of nitrogen decreased the SL production from approximately 30 g/l to 12 g/l, and seemed to be no significant effect on the cell growth. Further amounts of nitrogen caused inhibition of SL production and cell growth. Figure 4B indicates the effects of additional nitrogen on SL production and cell growth in CCH media with once heat treatment of 121°C for 20 min. Interestingly, increasing of additional nitrogen gradually increased the amount of produced SL, and additional 0.1 g-N of nitrogen increase the SL to 30 g/l, which are corresponding to the amount of SL production in CCH without heat treatment. The recoveries of cell growth were not observed in the experimental conditions. The dry cell weights of the culture broths were approximately 5 g/l, which were slightly lower than those with CCH without heat treatment.

Production of SL from CCH in jar-fermentor

To examine the efficiencies of SL production in jar fermentor, SL production were performed in different three experimental conditions. Figure 5A indicates time courses of produced SL, DCW, residual glucose, and residual hydrophobic substrates during cultivation using CCH. Here, hydrophobic substrates indicates materials extracted by hexane including tri-, di-, and tri-glyceride, free fatty acids, and the other hexane-extracted minor compounds. The CCH was treated once additional heat treatment with a jarfermentor vessel. Decreases of the residual glucose and

213 hydrophobic substrates were not observed during early two days. The residual substrates gradually
214 decreased after third day in cultivation period. Glucose was completely consumed for 5 days and
215 hydrophobic substrates remained 26.2 g/l at seventh day. SL gradually increased to 43.8 ± 0.6 g/l for
216 7 days. DCW was reached to approximately 5.0 g/l and maintained the level during the cultivation.
217 The volumetric productivity of SL was calculated to 6.25 g/l/day. Figure 5B indicates time courses
218 of produced SL, DCW, residual glucose, and residual hydrophobic substrates during cultivation
219 using CCH with 5.0 g-N/l $NH_4(NO_3)_2$. Residual glucose and hydrophobic substrates decreased from
220 second day after inoculation, and were completely consumed for 2 and 5 days, respectively. DCW
221 increased to approximately 11 g/l for 3 days and gradually increased to 11.9 g/l for 7 days, which
222 were slightly larger than those without additional nitrogen. SL induced to 50.5 ± 2.3 g/l for 5 days.
223 The volumetric productivity reached to 10.1 g/l/day. Figure 5C indicates time courses of produced
224 SL, DCW, residual glucose, and residual hydrophobic substrates during cultivation using CCH with
225 5.0 g-N/l $NH_4(NO_3)_2$ and using pH control at 5.0. The amount of initial glucose was unfortunately
226 smaller than those of the other experiment, since the efficiency of enzyme saccharification would be
227 slight worse. Residual glucose and hydrophobic substrates were immediately consumed after 1st day.
228 In this case, almost of hydrophobic substrates was consumed during 4 days cultivation. SL and DCW
229 reached to 49.2 ± 3.3 g/l and 10.3 ± 0.1 g/l, respectively. The volumetric productivity of SL was
230 reached to 12.3 ± 0.8 g/l/day. The volumetric productivity marked the best in the experiments,

231 although the initial glucose concentration was the lowest.

232

233 **Use of waste oils**

234 To examine the possibility of usage of waste oil for SL production in lignocellulitic
235 material based media, SL were produced from two different waste oils in shaking flasks. Home-used
236 waste oil and manufacture-used waste oil were not significantly effect on yield of SL for four days
237 cultivation. The SL yields from home and manufacture waste oils were 33.8 ± 1.39 and 30.3 ± 1.06
238 g/l, respectively. The yields were corresponding to that from olive oil (32.0 ± 0.9 g/l). No significant
239 differences in cell growth were observed (data not shown).

240

241 **Structure determination of major component**

242 To check the quality of produced SL, molecular structure of major component of SLs
243 produce from olive oil and CCH in jar fermentor were examined by MALDI-TOF/MS and NMR
244 analyses. Figure 6A indicates results of TLC analysis of crude and purified SL. Several spots were
245 observed in crude SL sample, and single spot were detected in purified sample. The purified sample
246 was analyzed by MALDI-TOF/MS and NMR analyses. Table 2 indicates the summarized results of
247 purified SL by ^{1}H- and ^{13}C-NMR analyses. The spectrograms were well corresponded to those of
248 previous report (5, 26). Figure 6B demonstrated the spectrogram of MALDI TOF-MS analysis of

249 purified SL. Major $[M+Na]^+$ ion was observed at *m/z* 712. These results indicated that the major
250 product was assigned as lactone-form of di-*O*-acetyl SL, that is 6',
251 6"-di-*O*-acetyl-β-D-glucopyranosyl-2-*O*-β-D-glucopyranosiloxy-octadecenoic acid.
252
253 **DISCUSSION**
254 In this study, the possibility of efficient production of SL from lignocellulosic materials
255 based simple media and additional nutrients were demonstrated. One of serious problems for
256 microbial production from lignocellulosic materials are unknown growth and production inhibitors
257 generated during chemical and enzymatic saccharification of biomass. In many cases, the inhibitors
258 often removed by activated charcoals and the treatment increase the total production cost.
259 Optimization of sulfuric acid concentration during chemical saccharification (Fig. 2) indicated
260 1 %(w/v) of H_2SO_4 would not inhibit SL production. Increase of H_2SO_4 concentrations increased
261 phenoic compounds and decreased glucose yield during the following enzymatic saccharification.
262 High H_2SO_4 concentration would cause excess decomposition of saccharides and generate phenoic
263 compounds including frufral and their derivatives. Therefore, acid saccharification by 1% (w/v)
264 H_2SO_4 is suitable for the following SL production. Although further optimization of H_2SO_4
265 concentration can be reduce the amount of H_2SO_4 use in the process, the optimization will not be
266 expected to increase of SL production, because SL production using CCH treated by 1% (w/v)

267 H_2SO_4 were corresponding to that of general SL production medium (data not shown). Additional
268 heat treatments (Fig. 3) increased glucose concentration, FL, and blowing compounds in CCH, and
269 reduced SL production in the following fermentation. Heating would cause Maillard reaction
270 between reducing end of saccharides and amino groups of nitrogen sources including inorganic and
271 organic compounds. An evidence of the Maillard reaction during heat treatments was demonstrated
272 to recover the SL production by SL producing experiments with additional nitrogen (Fig. 4). This
273 point is significant for not only handling for CCH but also design procedures for manufacture scale
274 production. SL productions by jar fermentor demonstrated the efficiency of additional nitrogen and
275 advantage of pH control. The best yield and volumetric productivity of SL were 49.2 ± 3.3 g/l and
276 12.3 ± 0.8 g/l/day from simple lignocellulosic based medium composed of only CCH, oil, and small
277 amount of nitrogen (Fig. 5C). The productivity was higher than that from corncob residue by
278 *Wicherhamiella domrercquiae* var *sophorolipids* CGMCC 1576 and *Cryptococcus curvatus* ATCC
279 96219 (21). Moreover, waste oils can be used for SL production along with the lignocellulosic based
280 medium. *Starrmerella bombicola* NBRC 10243can utilize various hydrophobic various vegetable
281 oils (27), animal fats (19), substrates alkanes (28), industrial fatty acid residues (29) and biodiesel
282 co-product stream (18). Supply of the fatty acid brocks would be important for efficient production
283 of SL. There are possibilities that the various hydrophobic substrates can be use for efficient SL
284 production along with CCH-based media. These results indicate that SL is possible to being

produced from non-edible biomass. The quality of SL produced from CCH and olive oil was maintained according to structural determination of major product (Fig. 6). Therefore, CCH seems not to be affected to the quality of SLs.

In conclusion, we developed an eco-friendly SL production combined optimization both saccharification of corncob and fermentation conditions, without a costly activated charcoal treatment. Furthermore, we first described that H_2SO_4 concentration during acid saccharification and heating during CCH preparation are significant factors for the following SL production efficiency.

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373 Figure Legends

374 Fig. 1. Molecular structures of SLs. A: lacton-form SL, B: acidic-form SL

375 Fig. 2. The effects of sulfuric acid concentration for CCH preparation. A: spectrophotometric
376 profiles of CCH perpetrated by using different concentrations of H2SO4, B:phenoic
377 compounds concentrations. Open, solid, and gray bars indicate HMF, FL, and VA,
378 respectively. C: SL production and cell growth. Shaded and open indicate SL concentration
379 and DCW after 4 days cultivation. Bars indicate standard deviation.

380 Fig. 3. The effects of additional autoclave treatment for CCH preparation. A: spectrophotometric
381 profiles of CCH perpetrated by using different concentrations of H2SO4, B:phenoic
382 compounds concentrations. Open, solid, and gray bars indicate HMF, FL, and VA,
383 respectively. C: SL production and cell growth. Shaded and open indicate SL concentration
384 and DCW after 4 days cultivation. Bars indicate standard deviation.

385 Fig. 4. Effects of supplemental nitrogen source concentration on the SL production in CCH media.
386 A: CCH prepared using 1%, w/v H_2SO_4 without additional autoclave treatment. B: CCH
387 prepared using 1%, w/v H_2SO_4 and once additional autoclave treatment.

388 Fig. 5. Jarfermentor cultivations for SL production in CCH prepared using 1% w/v H_2SO_4. Time
389 cources of SL, DCW, residual glucose, and residual hydrophobic substrates, in A: CCH
390 without additional nitrogen source and pH control, in B: CCH with 5.0 g-N/L of $NH_4(NO_3)_2$

391 without pH control, and C: CCH with CCH with 5.0 g-N/L of $NH_4(NO_3)_2$ with pH control

392 (pH 5.0). Symbols: closed circles, SL; open circles, DCW; closed triangles, hydrophobic

393 substrates; open triangles, glucose.

394 Fig. 6. Structure analyses of major SL component. A: TLC analysis of crude SLs and purified major

395 component. Lane C and P indicates crude SL and purified major component, respectively. B:

396 Spectrogram of MALDI-TOF/MS analysis of SL major component.

397

Table 1 Summary of NMR data for major products

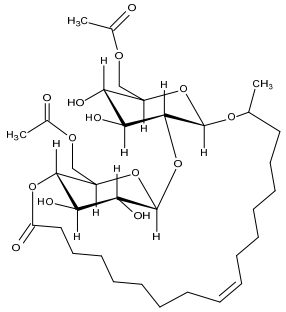
	^{13}C-NMR	^{1}H-NMR
	δ (ppm)	δ (ppm)
Saccharides		
C-1’	102.5	4.45 *d*
C-2’	82.3	3.52 *m*
C-3’	75.8	3.54 *m*
C-4’	69.7	3.39 *t*
C-5’	72.6	3.66 *m*
C-6’	62.1	4.12 *dd*
		4.17 *d*
C-1”	104.1	4.57*d*
C-2”	75.4	3.45 *m*
C-3”	74.0	3.65 *m*
C-4”	70.5	4.98 *t*
C-5”	73.2	3.68 *t*
C-6”	63.7	4.31 *b*

(Table 1 continued)

		4.33 *b*
Acetyl groups		
-C=O (C-6', 6")	171.8, 170.6	
-CH_3 (C-6', 6")	20.9, 20.1	2.08 *s*
Acyl groups		
-C=O (C-1)	173.5	
-CO-$\underline{C}H_2$- (C-2)	34.4	2.34 *m*
-CO-CH_2-$\underline{C}H_2$- (C-3)	24.5	1.58 *m*
-$(CH_2)_n$- (C-4-7, C-12-4)	28.2-30.2	1.27 *b*
-CH=CH-$\underline{C}H_2$- (C-8, 11)	26.9, 27.2	2.03 *m*
-CH=CH- (C-9, 10)	129.8, 130.1	5.34 *m*
-O-$CH_2CH_2\underline{C}H_2$(C-15)	25.4	1.49 *b*
-O-$CH_2\underline{C}H_2$(C-16)	37.6	1.58 *m*
-O-$\underline{C}H_2$-(C-17)	79.4	3.76 *m*
-$OCH_2\underline{C}H_3$- (C-18)	21.1	1.23 *d*

Abbreviation, s*:* singlet, *d*: doublet, *dd*: double doublet, *m*: multilet, *b*: broad

A



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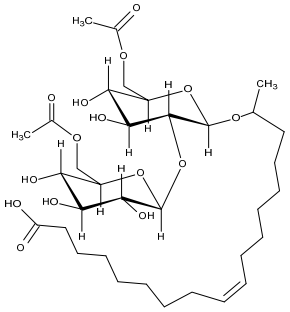
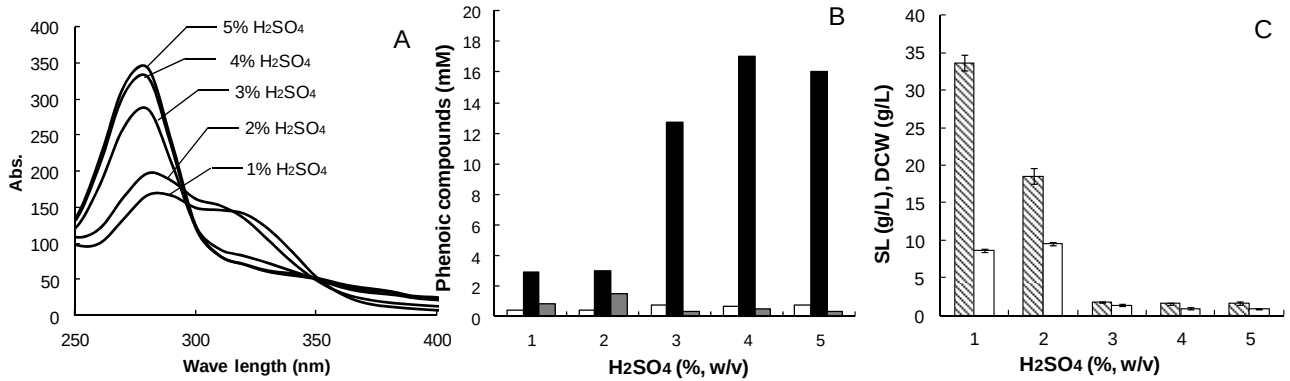


Konishi et al. Fig. 1



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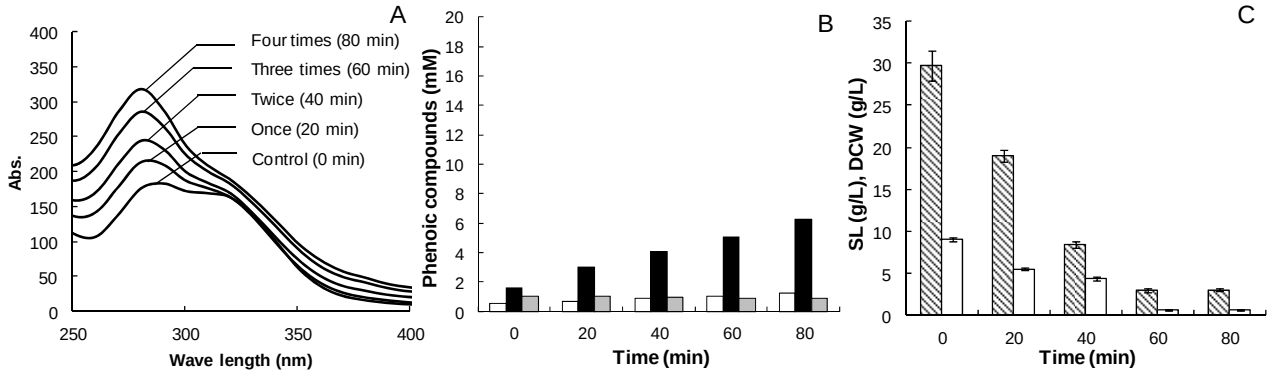

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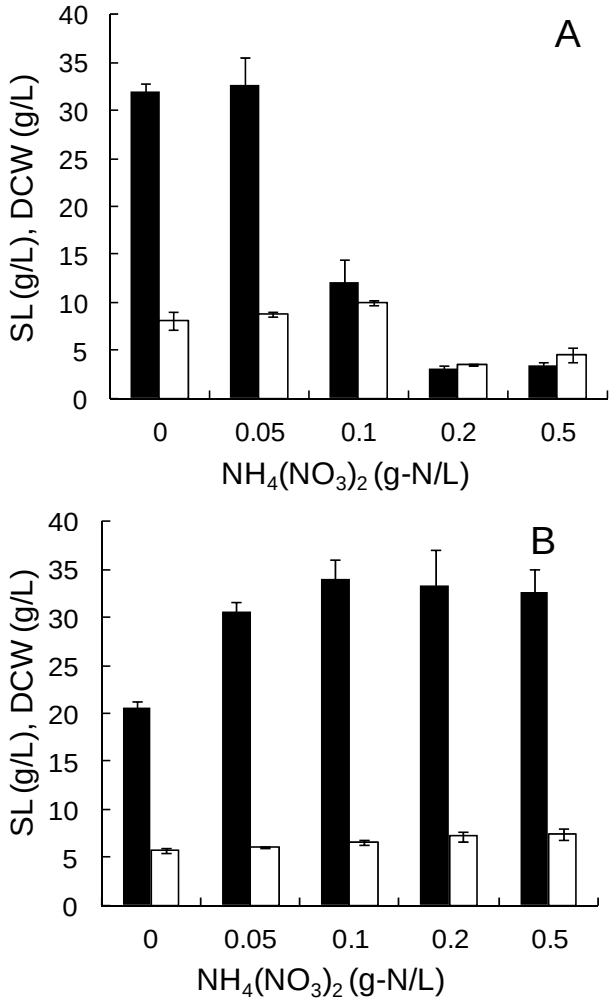

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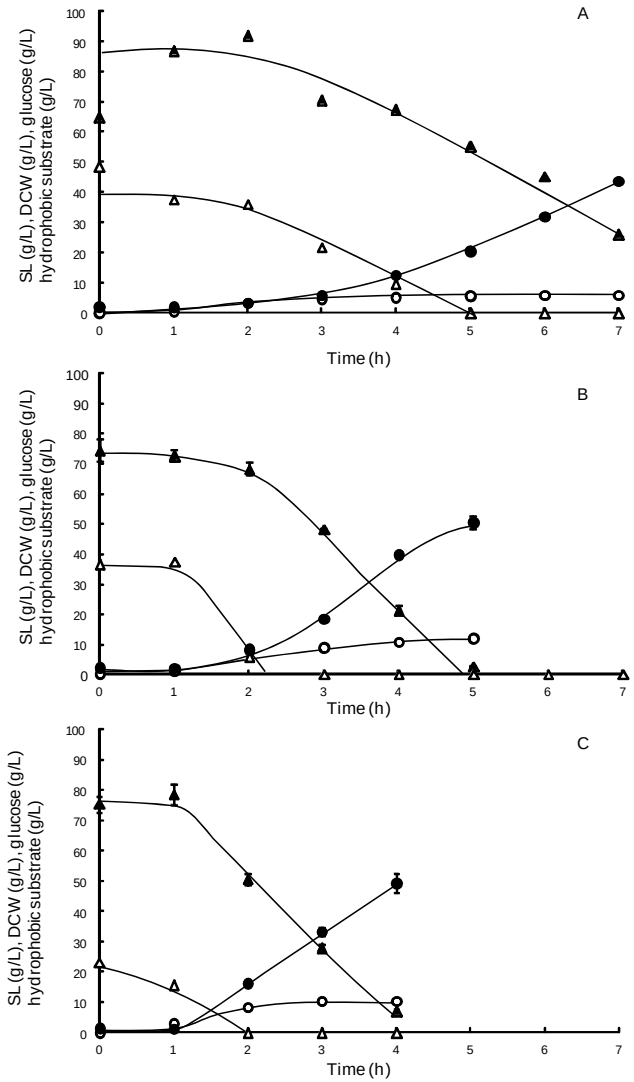

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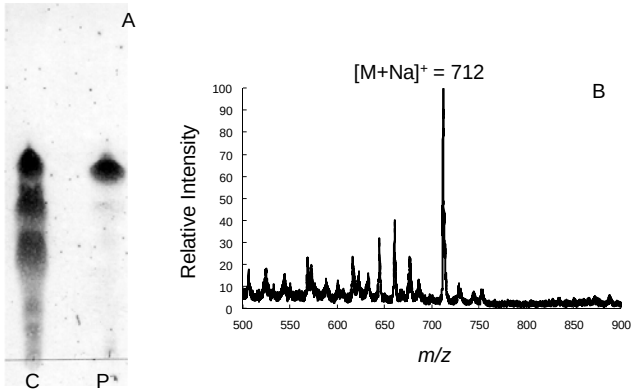

Konishi et al. Fig. 6