Lignocellulose-derived inhibitors improve lipid extraction from wet *Rhodococcus opacus* cells

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ABSTRACT

Extracting lipids from oleaginous microbial cells in a cost effective and environmentally compatible manner remains a critical challenge in developing manufacturing paradigms for advanced liquid biofuels. In this study, a new approach using microbial growth inhibitors from lignocellulose-derived feedstocks was used to extract lipids efficiently from wet **cell mass** of the oleaginous bacterium *Rhodococcus opacus* MITXM-61. Nine common lignocellulose-derived inhibitors for **treatment of cells** prior to solvent extraction were used and evaluated for their efficiency of lipid extraction from the cells. When the inhibitors were individually examined, formic acid and furfural showed the highest extraction efficiency of lipids from wet **cell mass**. Multiple extractions of lipids with methanol from wet **cell mass** pretreated with combined common inhibitors or hardwood hydrolysate comprising lignocellulose-derived inhibitors resulted in lipid recovery of greater than 85% of total lipids, a 1.7-fold increase of lipid extraction as compared to those in the absence of the inhibitors.

Keywords:

Rhodococcus opacus

Triacylglycerol

Lipid extraction

Wet cell extraction

Lignocellulose-derived inhibitor

1. Introduction

Triacylglycerols (TAGs) are neutral lipids, and are used as precursors for producing lipid-based renewable biofuels, such as diesel, gasoline and jet fuel (Furimsky, 2013; Lestari et al., 2009; Yen et al., 2008). TAGs are found extensively as major carbon storage molecules in animals, plants, algae and microorganisms (Hu et al., 2008; Murphy, 2001). Oleaginous microorganisms that utilize miscellaneous substrates provide advantages for TAG production from biomass residues such as agricultural and industrial operation wastes (Kosa and Ragauskas, 2011; Rittmann, 2008). Nonetheless, microbial technologies for cost-effective conversion of available resources to renewable fuels have not been fully developed to large-scale worldwide operations (Peralta-Yahya et al., 2012).

Renewable fuel production process from microorganisms is composed of many steps including microbial cultivation, harvest of microbial cells from fermentation broth and lipid extraction from the cells as well as synthesis of biodiesel or hydrocarbon biofuels. Downstream processes for product recovery have also been recognized as being an important issue for developing efficient manufacturing paradigms (Zhang et al., 2014). Studies in recent decades

have shown the use of organic solvents and the use of supercritical carbon dioxide to extract lipids from microbial cells, but **the technologies have been identified as a major bottleneck for large-scale operations (Jin et al., 2012)**. Since microbial lipids are accumulated intracellularly, they must be extracted from the cells, which include barriers to permeation by extraction solvents. The chloroform/methanol mixture is the most commonly used solvent system for lipid extraction from microorganisms and is known to be effective (Halim et al., 2012). Chloroform is highly toxic, which is disadvantageous owing to the safety concerns accompanied with its usage, and thus, alternative solvent systems with low-toxicity and environmental friendliness have been explored (Axelsson and Gentili, 2014; Cheng et al., 2014). In addition, the presence of moisture in microbial biomass poses a significant and costly challenge as it prevents organic solvents from reaching the intracellular lipids. To accomplish optimal lipid extraction, microbial biomass is usually dried to a powder and disrupted to disengage the lipids in order to have solvents to come into contact with the stored lipids. An extensive range of approaches, such as microwave irradiation, sonication, hot acid hydrolysis, milling, high-pressure homogenization and enzyme treatment, have been used for cell disruption to improve lipid recovery (Jin et al., 2012). **However, such approaches are not efficient and cost-effective enough for industrial-scale activities since they require high energy inputs and use of expensive infrastructure facilities, chemicals and enzymes (Wang et al., 2012)**. To reduce cost and simplify downstream processes, there is a need for novel extraction techniques that are effective with wet microbial biomass containing lipids (Willis et al., 2014).

***Rhodococcus opacus* MITXM-61 is an oleaginous bacterium capable of producing large quantities of lipids on high concentrations of glucose and xylose present in lignocellulosic hydrolysates (Fei et al., 2015; Kurosawa et al., 2014). The lipids are stored intracellularly as lipid**

bodies and are mainly composed of TAGs, consisting primarily of C16 and C18 series of long chain fatty acids. Meanwhile, previous studies have shown that *R. opacus* is highly sensitive to certain materials present in lignocellulosic hydrolysates that interfere with the growth and metabolic activity of microbial cells (Brigham et al., 2011; Kurosawa et al., 2015).

In this study, an approach using lignocellulose-derived fermentation inhibitors for pretreatment of cells with the objective to more efficiently extract lipids from *R. opacus* was explored. The recovery of lipids from wet cell mass after pretreatment with lignocellulose-derived inhibitors with solvents as environmentally safer than chloroform was evaluated. The effects of the lignocellulose-derived inhibitor pretreatment, including effects of individual and combined inhibitors, and treatment conditions in terms of incubation temperature and time, on lipid extraction with methanol from the wet cell mass were investigated.

2. Materials and methods

2.1. Materials

A hydrolysate from hardwood using dilute-acid pretreatment was kindly provided by Sweetwater Energy Inc. (Rochester, NY). LB broth and agar were purchased from BD Diagnostic Systems (Sparks, MD). Acetic acid, methanol and isopropanol were purchased from EMD Millipore (Billerica, MA). Lignin (soluble, 471003 Aldrich), furfural, vanillin, 4-hydroxybenzaldehyde (4-HB), syringaldehyde and 5-hydroxymethyl-2-furaldehyde (HMF), levulinic acid, formic acid and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

2.2. Strain and media

R. opacus MITXM-61 was constructed in our previous study, and deposited with the ATCC patent depository (PTA-12196) (Kurosawa et al., 2014). The strain was preserved in 20% (w/v) glycerol at -80°C . The culture media used were LB broth, a hardwood hydrolysate, and a phosphate-buffered defined medium. The defined medium contained per liter: 16 g glucose, 1.0 g $(\text{NH}_4)_2\text{SO}_4$ and mineral components consisted of 1.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.015 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 35.2 ml 1.0 M phosphate buffer (pH 7.0), 1.0 ml trace element solution and 1.0 ml stock A solution. The trace element solution and stock A solution were the same as those described by (Kurosawa et al., 2010). Modifications of the defined medium are described in the text. Solid media were supplemented with 2% (w/v) agar.

2.3. Culture conditions

R. opacus was grown at 30°C . Cell growth was determined by the optical density (OD) at 660 nm (Thermo Scientific GENESYS 20, Waltham, MA). Submerged cultivations were carried out using baffled flasks on a rotary shaker at 200 rpm (Multitron, Infors, Bottmingen, Switzerland). Cultures were inoculated with a seed culture to an initial OD of 1.0 (2.5×10^8 cfu/mL). To prepare the seed culture, cells from colonies grown on a LB agar plate for 3 days were inoculated into a flask of defined medium and cultivated for 2 days, until late exponential growth phase.

2.3.1. Growth experiments

Shake flask fermentations were carried out in 250-mL flasks containing 50 mL of various media on a shaker. The half maximal inhibitory concentrations (IC_{50}) of lignocellulose-derived compounds that cause 50% inhibition of the growth of *R. opacus* MITXM-61 were estimated from the growth profiles through a series of experiments as described (Franden et al., 2013).

2.3.2. Preparation of bacterial cell mass

For cell mass production, strain MITXM-61 was cultivated in 500-mL flasks containing 100 mL of modified defined medium containing a mixture of 8 g/L glucose and 8 g/L xylose on a shaker for 4 days. The final OD and cellular lipid content were 18.6 (± 1.7) and 41.6 (± 3.2) % cell dry weight, respectively. The cells were harvested by centrifugation at 8,000 g for 15 min, washed twice with an equal volume of deionized water, resuspended in deionized water to obtain an OD of 40.0 (11.1 ± 0.4 g/L of cell dry weight), and adjusted to pH = 4.0 with HCl to suppress the microbial metabolism. Aliquots of 2 mL the cell suspension were poured into 16-mL tubes for further study.

2.4. Lignocellulose-derived inhibitor treatment of bacterial cell mass

Aliquots of 2 mL the cell suspension with an OD of 40.0 were collected by centrifugation at 8,000 g for 15 min. The cell pellets were resuspended with 2 mL of deionized water containing the inhibitor at appropriate concentrations, and adjusted to pH = 4.0 with NaOH or HCl to eliminate artifacts caused by pH change. The suspensions were incubated on a rotary shaker at 150 rpm (Multitron, Infors, Bottmingen, Switzerland) at various temperature-time combinations.

2.5. Extraction of lipids

Aliquots (2 mL) of cell suspension, treated with or without microbial growth inhibitors from lignocellulosic feedstocks, were centrifuged at 8,000 g for 15 min, and the supernatants were removed completely. In the case of lipid extraction from dried cell mass, the pellets were lyophilized. The moisture content of the wet cell mass pellets was 67.0 (± 3.3) % as determined by measuring the mass differences before and after lyophilization. Each extraction was carried out in 15-ml glass tubes with screw caps on a rotary shaker at 150 rpm (Multitron, Infors,

Bottmingen, Switzerland). The dried **cell mass** (approximately 22 mg) or the wet **cell mass** (approximately 67.3 mg, equivalent to 22 mg of dried **cell mass**) was mixed with 2 mL of the desired organic solvent at appropriate temperatures. After incubation for designated times, the mixture obtained was centrifuged at 4000 g for 15 min, and the organic phase was transferred into a glass tube. The organic phase was then evaporated using a heat block, and the lipids were recovered. **The lipid extraction efficiency of each treatment was quantified as by the lipid recovery.** All experiments were performed in triplicate.

2.6. Analytical methods

The cell dry weight (CDW) was determined by lyophilizing cell pellet after centrifuging 10 ml of culture broth at 8,000 g for 15 min and washing the cell pellet twice in deionized water. **To determine the lipid content of the cells, the composition of lipids and the lipid recovery, the whole cells or the extracted lipids were subjected to acid-catalyzed transesterification and the resulting fatty acid methyl esters (FAMES) were analyzed by gas chromatography (GC-FID) as described in detail (Kurosawa et al., 2013).** GC analysis of FAMES was carried out using an Agilent 6850 series II GC system equipped with an Agilent DB-Wax column (30 m by 0.32 mm, 0.5 μ m film) and a flame ionization detector (Agilent Technologies, Santa Clara, CA) with hydrogen as the carrier gas. The fatty acids were identified and quantified by comparison to standard FAMES: methyl myristate (C14:0), methyl pentadecanoate (C15:0), methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl heptadecanoate (C17:0), methyl *cis*-10-heptadecenoate (C17:1), methyl stearate (C18:0), methyl oleate (C18:1) and methyl linoleate (C18:2). For the identification of intact lipids, thin-layer chromatography (TLC) experiments were performed using a two-step resolution method as previously described (Kurosawa et al., 2015). The extracted lipids were mixed with 1.85 mL of 20:17 chloroform/methanol, and 2 μ L of

the sample was spotted onto silica gel 60 plates (EMD Chemicals Inc., Gibbstown, NJ). Samples were resolved using an initial polar solvent system consisting of 60:35:5 chloroform/methanol/water (v/v/v), followed by a second solvent system containing 70:30:1 hexane/diethyl ether/acetic acid (v/v/v). Resolved lipids were visualized by charring. Plates were sprayed with an 8% (v/v) aqueous phosphoric acid solution containing 3% (v/w) cupric acetate followed by baking in a 200°C oven for 10 min, and imaged under visible light in an EpiChem3 Darkroom (UVP Bioimaging System, Upland, CA). Cellular lipid content was defined as the percentage of the ratio of fatty acids to cell dry weight (% CDW). The lipid recovery was calculated as the percentage lipids (% FAMES) obtained with respect to total lipids present in *R. opacus* cells (weight of lipids extracted/average total weight of lipids in the lyophilized cell pellet x 100). Glucose, xylose, arabinose, acetic acid, formic acid and levulinic acid concentrations were determined by high-performance liquid chromatography (HPLC, Agilent 1100 system) fitted with an Aminex HPX-87H column (300 x 7.8 mm, BIO-RAD) coupled to a refractive index (RI) detector as previously described (Kurosawa et al., 2013). HMF, furfural, 4-HB, vanillin and syringaldehyde were analyzed using HPLC (Agilent 1260 infinity analytical-scale purification system) fitted with a XTerra MS C18 column (150 x 2.1 mm, Waters) (Kurosawa et al., 2015). The column was eluted with a gradient of water and acetonitrile, both of which contained 3 mM formic acid, at 25°C and a flow rate of 0.4 ml/min. The gradient design consisted of three steps: (a) 2% (v/v) acetonitrile was applied for 7 min, (b) the concentration of acetonitrile was increased linearly to 20% during 28 min, and (c) 100% acetonitrile was applied for 5 min. Compounds were detected by UV at a wavelength of 284 nm. Soluble lignin content was calculated by using UV absorbance of the filtrate at 205 nm (Agilent 8453 spectrophotometer).

3. Results and discussion

3.1. Lipid extraction from wet cell mass treated with individual lignocellulose-derived microbial growth inhibitors

In order to efficiently extract lipids from wet cell mass, an approach using lignocellulose-derived inhibitors for treatment of cells prior to solvent extraction was sought to apply. The inhibitors, as by-products formed during the hydrolysis of lignocellulosic biomass with dilute acid, have significantly negative effects on microbial cell growth and metabolism (including lipid production), likely via cell membrane damage, nucleic acid damage and enzyme inhibition (Mills et al., 2009). The major inhibitors include furans (e.g., 5-hydroxymethyl-2-furaldehyde (HMF) and furfural), phenols (e.g., 4-hydroxybenzaldehyde (4-HB), soluble lignin (lignosulfonates), syringaldehyde and vanillin), and organic acids (e.g., acetic, formic and levulinic acids) (Palmqvist and Hahn-Hagerdal, 2000). The effects of the inhibitor treatment prior to solvent extraction, using nine representative inhibitors of lignin, furfural, HMF, vanillin, 4-HB, syringaldehyde, levulinic acid, formic acid and acetic acid detected in lignocellulosic hydrolysates, on lipid extraction from wet cell mass of *R. opacus* MITXM-61 were investigated. The cell mass was pretreated with individual inhibitors at 18-fold of its IC₅₀ value (Supplementary Table S1) following 3-day exposure in the growth, and the treated cell mass was extracted once with methanol. As shown in Fig. 1, all the inhibitors tested exhibited effective lipid extraction, but the efficacy depended on the inhibitor used. Pretreatment with formic acid, furfural, acetic acid, 4-HB and levulinic acid to the cell mass significantly improved the extraction and resulted in increases of 71%, 65%, 59%, 54% and 53% in lipid recovery, respectively, compared to the untreated group. Furthermore, when the nine inhibitors were mixed into the suspension at a 2-fold concentration of their respective IC₅₀ values, the lipid recovery

was increased by 78%, as compared with that of the untreated group. In view of the data obtained in experiments using individual inhibitors, the combination of the representative inhibitors resulted in greater lipid recovery and synergistic positive effects on lipid extraction.

3.2. Lipid extraction from wet cell mass pretreated with combined lignocellulose-derived inhibitors

3.2.1. Effects of organic solvents on lipid extraction

In order to further assess the efficacy of the inhibitor pretreatment on lipid extraction from the oleaginous microbial biomass, lipids from wet and dried cells of *R. opacus* MITXM-61 treated with or without the combined nine representative inhibitors (at a 2-fold concentration of their respective IC₅₀ values) were extracted **once** using nine different solvent systems. As shown in Fig. 2, the chloroform-based solvent system provided reasonably good lipid-extraction efficiency, and the solvent type used exhibited a large impact on the effectiveness of lipid extraction from *R. opacus* cells. **The extraction efficacy with methanol, isopropanol, ethanol, methyl ethyl ketone (MEK), methanol/MEK, chloroform, acetone, methanol/chloroform, *n*-hexane to the wet inhibitor-treated cell mass resulted in 85%, 60%, 50%, 48%, 40%, 32%, 23%, 22% and 18% increases, respectively, of the lipid recovery, compared to those to the wet untreated cell mass (Fig. 2a). In contrast, there were no significant differences in lipid extraction efficacy using *n*-hexane, chloroform-based, and MEK-based solvent systems between the untreated and inhibitor-treated cell mass, although the extractions with methanol, isopropanol, acetone and ethanol to the treated dry cell mass lead to 57%, 54%, 51% and 32% increases of lipid recovery, compared to those to the untreated dry cell mass (Fig. 2b). These results suggested that polar solvents allowed for better inhibitor-assisted lipid extraction from *R. opacus***

cells in comparison with non-polar solvents. In order to determine whether differences in the conditions had effects on the profiles of lipids extracted from the treated wet cell mass, analyses of thin-layer chromatography and gas chromatography were carried out (Supplementary Fig. S1 and Supplementary Table S2). The identities and the fatty acid compositions of lipids obtained via different extraction systems from the treated wet cell mass were similar to one another. The major component of the intact lipids was TAGs, and the FAME profiles consisted primarily of palmitic acid, oleic acid and *cis*-10-heptadecenoic acid, although methanol extraction led to a slightly higher percentage of linoleic acid, comparing with other solvents used.

3.2.2. Effects of multiple extractions on lipid recovery

As described above, although methanol exhibited excellent potential for inhibitor-assisted lipid extraction from the wet cell mass, the chloroform-based solvent system recovered the highest levels of lipids on the extraction (Fig. 2). The effects of multiple extractions on the amount of lipids extracted from *R. opacus* were examined by applying two solvents of methanol and chloroform/methanol on the wet cell mass treated with or without the mixed nine inhibitors (at a 2-fold concentration of their respective IC₅₀ values). As shown in Fig. 3, the multiple extractions were more efficient than a single extraction in both solvent systems. The total recovery of five extractions from the wet cell mass with chloroform/methanol and methanol was 92.8 (±2.9) % and 87.0 (±4.2) % of total lipid with the inhibitor pre-treatment (Fig. 3a), and 83.5 (±3.5) % and 48.1 (±4.5) % without the inhibitor pre-treatment (Fig. 3b). The lipid recovery of multiple extractions with methanol on the treated cell mass was significantly higher than that on the untreated cell mass, and was almost the same as those with methanol/chloroform. These results indicated that multiple methanol extractions of the inhibitor pretreated cell mass which is

less toxic and more environment-friendly than chloroform, is a promising alternative of conventional chloroform-based extractions.

3.2.3. Effects of incubation temperature and time on lipid extraction

The use of methanol for the extraction of lipids from **cell mass** has advantages over the traditional chloroform/methanol method (Paudel et al., 2015). In order to improve performance of the lignocellulosic inhibitor-assisted methanol extraction of wet **cell mass**, the effects of temperature and time in the pretreatment and extraction processes on lipid extraction were examined. Fig. 4 shows the effects of pretreatment conditions on lipid recovery. As shown in Fig.4a, the lipid recovery increased from 17.3 (± 2.1) % to 32.5 (± 4.3) % when the pretreatment temperature was increased from 20°C to 30°C, but raising the temperature to 40°C or 50°C did not increase the recovery. The results of lipid recoveries from the **cell mass** pretreated with different incubation times are shown in Fig. 4b. Although only marginal increase in the recovery was observed in the first 3 h, the recovery increased from 18.8 (± 2.2) % to 34.3 (± 2.0) % when the treatment time was extended from 3 h to 12 h. Accordingly, the optimum conditions of temperature and duration for the pretreatment to achieve maximum lipid extraction efficiency was determined to be in the range of 30-40°C and 12-16 h. **The effects of extraction conditions on lipid recovery are shown in Supplementary Fig. S2.** The results showed that it depended on incubation temperature and time and required moderate temperatures of 50-60°C and adequate times of 12-24 h to generate the optimal lipid extraction efficiency with methanol. These were in reasonable agreement with the findings on lipid extraction with methanol from other oleaginous microorganisms (Zhang et al., 2014).

3.3. Lipid extraction from wet **cell mass** using lignocellulosic hydrolysate

It was found from Figs. 1-3 that an approach using lignocellulose-derived fermentation inhibitors for treatment of cells prior to solvent extraction was a promising technique for lipid extraction from the wet cell mass. The use of these inhibitors at gram-scale per liter to more efficiently recover lipids produced by *R. opacus* can be costly because of additional chemicals cost. The impact of hardwood hydrolysate comprising lignocellulosic inhibitors on lipid extraction with methanol from wet cell mass of *R. opacus* MITXM-61 was explored.

The hydrolysate was composed of 213 g/L glucose, 124 g/L xylose and 6 g/L arabinose with 38 g/L lignin, 0.032 g/L furfural, 0.94 g/L HMF, 0.085 g/L vanillin, 0.007 g/L 4-HB, 0.21 g/L syringaldehyde, 1.6 g/L levulinic acid, 2.2 g/L formic acid and 30 g/L acetic acid (Supplementary Fig. S3). As shown in Supplementary Fig. S4, using even of a 20% (v/v) solution of the hydrolysate (1:4 hydrolysate/water, 68.6 g/L of lignocellulosic sugars) resulted in complete growth inhibition on *R. opacus* MITXM-61, while the strain expeditiously reached the stationary state within 3 days post-inoculation when grown in modified defined liquid medium containing refined sugars of equivalent doses of the sugars found in the 20% hydrolysate, but in the absence of any inhibitory compounds.

The efficacy of the pretreatment on lipid extraction from the wet cell mass using the undiluted hardwood hydrolysate was evaluated by multiple extractions with methanol. As shown in Fig. 5, the total recovery of the multiple extractions was 85.1 (± 2.7) % by the pretreatment with the hydrolysate, 72.9 (± 4.1) % by the pretreatment with equivalent amounts of nine common inhibitors found in the hydrolysate, and 48.9 (± 3.8) % by the untreated group. The application of the lignocellulosic hydrolysate in pretreatment process provided a 1.7-fold increase in the lipid recovery, as compared to control. Of note, the lipid extraction efficacy on the cell mass treated with the hydrolysate was significantly higher than that on the cell mass

treated with the combined inhibitors. This indicated that the hardwood hydrolysate apparently contained certain cytotoxic compounds, other than the nine common inhibitors focused on in this study, which contributed to the pretreatment process. Very little is known about the cytotoxic effects on *R. opacus* of specific lignocellulose-derived compounds (Kurosawa et al., 2015; Wang et al., 2014). To investigate whether some of the other compounds act synergistically or additively to damage the cells would be likely to contribute to further improvement in the lipid extraction efficiency from the wet cell mass.

Lignocellulosic biomass has been expected to play an important role as a renewable non-food source for production of biofuels (Fairley, 2011). The hydrolysis of lignocellulose into fermentable sugars is essential for assimilation of the carbon feedstock by microbes (Weber et al., 2010). The feedstock used should consist of highly concentrated sugars to minimize the cost of fermentations, but there will be concomitantly increased the total amount of lignocellulose-derived inhibitors with increasing the sugar content in the hydrolysates (Hawkins and Doran-Peterson, 2011). Toxicity of the inhibitors is a serious obstacle in the feasibility of biological conversion of lignocellulosic biomass to biofuels, and various detoxification strategies, including physical, chemical and biological methods prior to fermentations, have been investigated to address negative effects of the inhibitors (Parawira and Tekere, 2011). Results of Fig. 5 showed that the application of lignocellulosic hydrolysates on lipid extraction from wet cell mass could be a useful approach, while the inhibitors at concentrations found in the hydrolysate exhibited potent cytotoxic effects on *R. opacus* (Supplementary Fig. S4). In addition, the wet cell mass was effective for the adsorptive removal of certain inhibitors from the hydrolysate with minimal loss of the sugars (data not shown). These findings suggest that the strategy using lignocellulosic hydrolysates comprising fermentation inhibitors is a valuable means for lipid extraction from wet

cell mass, from the viewpoint of cost-effective use of the resource as a by-product, and provides an important clue to improve the robustness of lignocellulose-to-TAG bioprocess. The present inhibitor-assisted extraction methodology requires the long process time to extract lipids efficiently from wet cell mass, but it may have some advantages in terms of low-cost scalability, low energy inputs and environment-friendliness in the downstream processes, compared to conventional physical, mechanical and biological approaches, such as microwave irradiation, sonication, hot acid hydrolysis, milling, high-pressure homogenization and enzyme treatment, used for cell damage prior to solvent extraction.

3. Conclusions

A potentially cost-effective and green technological process for lipid extraction from the oleaginous bacteria *R. opacus* was investigated. This study demonstrated that lignocellulose-derived inhibitor pretreatment can be successfully employed to increase the effectiveness of the organic extraction of lipids from wet *R. opacus* cells. The lipid recovery of multiple extractions with methanol from inhibitor-pretreated wet cell mass was of the same level as that of conventional chloroform/methanol extraction. The lipid extraction methodology using lignocellulose-derived inhibitors based on the pretreatment applicable to wet cell mass will be valuable for developing a cost-effective downstream process to generate advanced renewable fuels.

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Figure captions

Fig. 1. Effects of individual lignocellulose-derived inhibitors on lipid recovery, in the extraction of lipids with methanol at 50°C for 12 h from wet **cell mass** of *R. opacus* MITXM-61 pretreated with or without individual inhibitors (64.8 g/L lignin, 39.6 g/L furfural, 41.4 g/L HMF, 23.4 g/L vanillin, 6.3 g/L 4-HB, 6.12 g/L syringaldehyde, 165.6 g/L levulinic acid, 59.4 g/L formic acid or 133.2 g/L acetic acid) at 30°C for 12 h. Combined inhibitors: 7.2 g/L lignin, 4.4 g/L furfural, 4.6 g/L HMF, 2.6 g/L vanillin, 0.7 g/L 4-HB, 0.68 g/L syringaldehyde, 18.4 g/L levulinic acid, 6.6 g/L formic acid and 14.8 g/L acetic acid were mixed. Data represent the mean and s.d. of triplicate experiments.

Fig. 2. Lipid extraction from wet (a) and dried (b) **cell mass** of *R. opacus* MITXM-61 treated with or without mixed nine lignocellulose-derived inhibitors (2-fold of their respective IC₅₀) at 30°C for 12 h using different solvent systems at 50°C for 12 h. Data represent the mean and s.d. of triplicate experiments.

Fig. 3. Effects of multiple extractions on lipid recovery, in the extraction of lipids with methanol-chloroform (1:1, v/v) and methanol from wet **cell mass** of *R. opacus* MITXM-61 pretreated (a) with or (b) without inhibitor cocktail (2-fold of their respective IC₅₀) at 30°C for 12 h. **The treated cell mass was subjected to five extractions with a further equal volume of the solvent each time (3, 6, 12, 24 and 36 h) during 36 h of incubation at 50°C.** Data represent the mean and s.d. of triplicate experiments.

Fig. 4. Effects of pretreatment conditions of (a) incubation temperature for 12 h and (b) exposure duration at 30°C on lipid recovery, in the extraction of lipids from wet **cell mass** of *R. opacus* MITXM-61 pretreated with inhibitor cocktail (2-fold of their respective IC₅₀). **The treated cell mass was extracted once with methanol at 50°C for 12 h.** Data represent the mean and s.d. of triplicate experiments.

Fig. 5. Effects of multiple extractions on lipid recovery, in the extraction of lipids with methanol from wet cell mass of *R. opacus* MITXM-61 pretreated with hardwood hydrolysate, and with or without common lignocellulose-derived inhibitors found in the hydrolysate at 30°C for 16 h. The methanol extraction was repeated five times with a further equal volume of the solvent each time (3, 6, 12, 24 and 36 h) during 36 h of incubation at 50°C. Data represent the mean and s.d. of triplicate experiments.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5