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The Production of Lipids Using 5-Hydorxymethy Furfural Tolerant *Rhodotorula graminis* Grown on the Hydrolyzates of Steam Pretreated Softwoods

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Abstract: Acid catalyzed (SO_2) steam pretreated softwoods inevitably contain furans such as 5-hydorxymethy furfural (HMF) and furfural, which are derived from the respective degradation of component hexoses and pentoses. As these materials are known to be inhibitory to fermentation, six oleaginous yeasts were grown on corn steep liquor (CSL) medium containing HMF (0.2%) and furfural (0.1%) to assess their resistance to possible inhibition and its possible influence on lipid production. *R. graminis* showed the highest tolerance to HMF (0.2%) and furfural (0.1%) when they were added individually to the CSL medium. However, when both HMF (0.2%) and furfural (0.1%) were added together, this inhibited the growth of *R. graminis*. Subsequent evaporation of the CSL medium successfully removed furfural from the CSL medium and increased the sugar concentration. However, the residual concentration of HMF (0.4%) still inhibited *R. graminis* growth. To try to improve HMF tolerance, *R. graminis* was slowly acclimatized in medium containing HMF (0.4%) and was eventually able to produce 1.8 g/L of lipids after four days of growth in the HMF containing medium. This was close to the same amount of lipid produced as when *R. graminis* was grown in the CSL medium without HMF and furfural. This indicated that an acclimatization strategy is a promising way to enhance lipids production when *R. graminis* is grown on the hydrolyzates of SO_2-catalyzed steam pretreated lignocellulosic substrates.

Keywords: SO_2-catalyzed steam pretreatment; *Rhodotorula graminis*; HMF tolerant strain

1. Introduction

The production of biodiesel and jet fuel from lignocellulosic materials is one way of reducing the world's dependence on fossil fuels [1,2]. Currently, bio-and-renewable diesels are mainly produced from animal and plant lipids such as tallow, used cooking oil, and soy, rape, and palm oils [3]. Biodiesel is predominantly produced from triacylglycerols (TAGs), and of the various microorganisms that produce TAG, the oleaginous yeasts have been shown to accumulate as much as 50% of the cell dry weight as intracellular lipids [4,5]. Although lignocellulosic materials have the potential to act as the substrates for growing oleaginous yeasts, the recalcitrance of biomass means that typically, some form of pretreatment is required prior to enzymatic hydrolysis of cellulose and hemicellulose [6,7]. SO_2-catalyzed steam pretreatment is a promising pretreatment process due to the limited use of chemicals, relatively low levels of energy, and the recovery of cellulose and hemicellulose in a fermentable form [6]. After the SO_2-catalyzed steam pretreatment, lignocellulosic substrates are recovered as water insoluble fractions, while a part of cellulose and hemicellulose, was depolymerized into oligomeric or monomeric

sugars that dissolved in water soluble fractions [6,8]. In addition to monomeric sugars, the water soluble fractions contain 5-hydorxymethy furfural (HMF) and furfural, which was dehydrated from hexoses and pentoses, respectively [8]. It was reported that furfural and HMF inhibited the growth of microorganisms in a manner of decreasing enzymatic activities such as alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase [9]. In addition, furfural was reported to induce the accumulation of reactive oxygen species in cells which includes damage to mitochondrial and vacuole membranes, the actin cytoskeleton, and nuclear chromatin [10]. Although the production of lipids using oleaginous yeasts from AFEX and SPORL pretreated lignocellulosic biomass has been reported [11–13], the use of the hydrolyzates of SO_2-catalyzed steam pretreated lignocellulosic biomass for the production of lipids by oleaginous yeasts have not been reported. In this study, to produce biodiesel from the hydrolyzates of SO_2-catalyzed steam pretreated lignocellulosic biomass using oleaginous yeast, the effect of HMF and furfural on the growth of six oleaginous yeasts were compared. Of the six strains used in this study, *Rhodotorula graminis*, which had higher tolerance to the inhibitors compared with other strains was selected and the tolerance of *R. graminis* to HMF (0.4%) was attained using acclimation. To evaluate the availability of the HMF (0.4%) tolerant *R. graminis*, the productivity of lipids by the strain in the presence of HMF (0.4%) was examined.

2. Materials and Methods

2.1. Microorganisims

Six oleaginous yeasts that have been studied for the production of lipids from lignocellulosic biomass were used. *Trichosporon oleaginosus* ATCC 20509, *Rhodotorula graminis* DBVPG 4620, *Lipomyces starkeyi* NRRL Y-1389, *L. starkeyi* NBRC 10381, *L. spencer-martinsiae* NRRL Y-7042, and *Yarrowia lipolytica* CBS 7504 were maintained at 4 °C on YM agar (3 g/L yeast extract; 5 g/L hipolypepton; 3 g/L malt extract; 10 g/L glucose; 15 g/L agar) plates.

2.2. Yeast Screening

For preculture, a loopful of cells from an isolated colony on YM agar plate was aseptically transferred to 50 mL of YM medium (3 g/L yeast extract; 5 g/L hipolypepton; 3 g/L malt extract; 10 g/L glycerol) in a 300-mL Erlenmeyer flask. The culture was incubated at 26 °C with shaking at 100 rpm until OD_{600} was reached to 10. For oleaginous yeasts screening, 50 mL of corn steep liquor (CSL) medium (35 g/L glucose; 15 g/L xylose; CSL 8 g/L) with or without 5-hydroxymetyl furfural (HMF, 0.2%) and furfural (0.1%) were prepared in a 100-mL Erlenmeyer flask. The aliquots of preculture was inoculated in the CSL medium and incubated at 26 °C with shaking at 100 rpm. Initial OD_{600} of 0.1 was used for all fermentation experiments. All flask culture experiments were performed in triplicate.

2.3. Removal of Furfural and Concentration of Monomeric Sugars by Rotary-Evaporation

CSL medium (35 g/L glucose; 15 g/L xylose; CSL 8 g/L) with HMF (0.2%) and furfural (0.1%) was rotary evaporated (40 °C, 50 torr) to remove furfural and concentrate monomeric sugars in the medium.

2.4. Analytical Methods

Biomass was measured as the optical density at 600 nm (OD_{600}) using a UV mini-1240 spectrophotometer (Shimadzu, Japan). HMF and furfural contents in the medium was measured by HPLC (JASCO LC-2000 plus series equipped with UV-2075 UV-vis detector) using an inert ODS-3 reverse phase column (4.6 mm × 150 mm L, GL sciences Inc. Japan). The chromatographic zones were visualized under UV light (254 nm). The mobile phase was methanol/water (2/1, v/v) with a flow rate of 1.0 mL/min. Cell dry weight and total lipids in the cell were measured according to the reported methods [14].

2.5. Acclimation of R. Graminis to HMF(0.4%)

Preculture of *R. graminis* incubated in the YM medium was inoculated into 50 mL of the CSL medium with HMF (0.4%) in a 300-mL Erlenmeyer flask. The culture was incubated at 26 °C with shaking at 100 rpm and the OD_{600} of the medium was measured.

3. Results and Discussion

3.1. Screening of Oleaginous Yeasts for the Production of Lipids in the Presence of HMF and Furfural

The growth rates of six oleaginous yeasts, *T. oleaginosus, R. graminis*, two *L. starkeyi*, *L. spencer-martinsiae*, and *Y. lipolytica*, which were reported as the promising strains for producing lipids from the pretreated lignocellulosic biomass [5], were compared to determine the strain that is adequate for the production of lipids from the hydrolyzates of SO_2-catalyzed steam pretreated lignocellulosic biomass. It was known that the presence of inhibitors (HMF, furfural), monomeric sugar concentrations, and sugar compositions affected the production of lipids from the pretreated lignocellulosic biomass [5]. For the inhibitors, when lignocellulosic biomass is pretreated under acidic condition, cellulose and hemicelluloses are hydrolyzed into monomeric sugars such as glucose and xylose and further dehydrates into HMF and furfural, respectively [15]. The produced HMF and furfural decrease the growth of microorganisms in a manner of enzyme activity inhibition [9] and the generation of active oxygen [10]. When beetle-killed lodgepole pine was pretreated by SO_2-catalyzed steam pretreatment at medium severity, the hydryzates contained 0.2% of HMF and 0.1% of furfural, respectively [8]. The HMF concentration was increased to 0.3% at high severity that decreased the fermentability of *Saccharomyces cerevisiae*, while furfural concentration was almost 0.1% even the severities of SO_2-catalyzed steam pretreatment were increased [8]. Other factors affecting the growth of oleaginous yeasts were the concentration and compositions of sugars [5,16]. It was reported that when Douglas-fir was SO_2-catalyzed steam pretreated, the water soluble fraction contained about 3.5% of glucose and 1.5% of xylose, respectively [17]. In this study, to observe the applicability of the oleaginous yeasts for the production of lipids from the hydrolyzates of SO_2-catalyzed steam pretreated lignocellulosic biomass at medium severity, the effects of HMF (0.2%) and furfural (0.1%) on the growth of six oleaginous yeasts were compared in the corn steep liquor (CSL) medium supplemented with glucose (3.5%) and xylose (1.5%). CSL, a by-product of the corn wet milling industry, was used for the nitrogen source in the medium due to its containing a rich complement of important nutrient to support robust microorganism growth, and it is thought to be inexpensive [18,19]. The effects of HMF and furfural on the growth of the oleaginous yeasts were different. The OD_{600} of *T. oleaginosus* with HMF (0.2%) was decreased by 23% compared with that without inhibitors after seven days. In addition, the presence of furfural (0.1%) completely inhibited the growth of *T. oleaginosus* (Figure 1). Of the six oleaginous yeasts used in this study, it seemed that *R. graminis* was the most promising strain for the production of lipids from the hydrolyzates of the SO_2-catalyzed steam pretreated lignocelluloses, because the growth of *R. graminis* was not affected by HMF (0.2%). The OD_{600} of *R. graminis* in the presence of HMF (0.2%) reached to 11.7 after seven days, which was the same OD_{600} as without the inhibitors (Figure 2). The growth of *R. graminis* was reported to be decreased by 35% when *R. graminis* was inoculated in the minimal medium in the presence of HMF (0.15%) [20], while the growth of *R. graminis* with HMF (0.2%) was not decreased in the CSL medium (Figure 2). This suggests that the CSL medium was preferable for *R. graminis* growth even in the presence of HMF. Another reason that *R. graminis* was thought to be preferable for producing lipids from the hydrolyzates of SO_2-catalyzed steam pretreated lignocellulosic biomass is that although induction time was observed, the OD_{600} of *R. graminis* in the presence of furfural (0.1%) was almost same as the OD_{600} without inhibitors (Figure 2). The OD_{600} of *R. graminis* in the presence of either HMF (0.2%) or furfural (0.1%) was not decreased after seven-days incubation, but *R. graminis* did not grow when both furfural (0.1%) and HMF (0.2%) were added in the CSL medium (Figure 2). Two *L. starkeyi* and *L. spencer-martinsiae* species showed similar growth patterns (Figure 3). The presence of HMF (0.2%) or furfural (0.1%) in the

CSL medium increased the induction period and the presence of both HMF and furfural in the CSL medium inhibited the growth of the strains. It was reported that *L. starkeyi* could grow in up to 0.2 g/L furfural and 0.63 g/L HMF, respectively, in chemically defined medium [21]. As three *Lipomyces* species grew even in the presence of 0.1% furfural and 0.2% HMF, respectively (Figure 3), it is likely that CSL medium is preferable for the growth of *Lipomyces* species in the presence of furfural and HMF. Of the six oleaginous yeasts, only *Y. lypolytica* grew in the presence of both HMF (0.2%) and furfural (0.1%) after induction period (Figure 4). However, the OD_{600} of *Y. lypolytica* even without the inhibitors was lower than those of other five strains. Thus, *R. graminis* was thought to be the promising strain for the production of lipids using the hydryzates from SO_2-catalyzed steam pretreated lignocellulosic biomass, because the growth of the strain in the presence of either HMF (0.2%) or furfural (0.1%) was almost same as the OD_{600} without the inhibitors after seven-days incubation (Figure 2). However, *R. graminis* could not grow when both HMF (0.2%) and furfural (0.1%) were presented in the medium. The removal of both HMF and furfural or the removal of furfural from the medium was required to produce lipids from the hydryzates of SO_2-catalyzed steam pretreated lignocellulosic biomass.

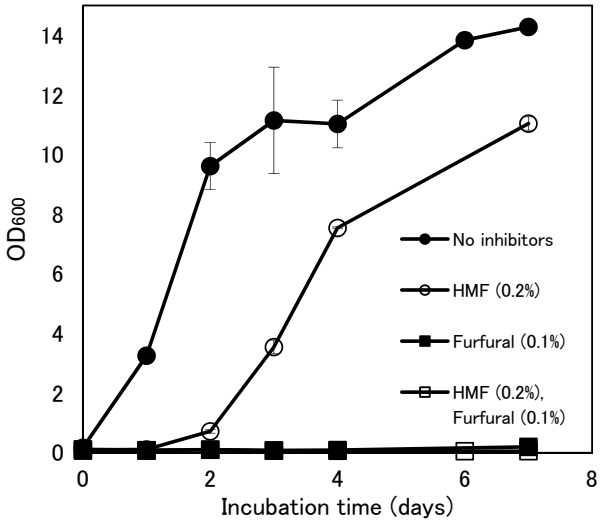

Figure 1. Effect of HMF (0.2%) and furfural (0.1%) on the growth of *T. oleaginosus.* Error bars shows the standard deviation of triplicate experiments.

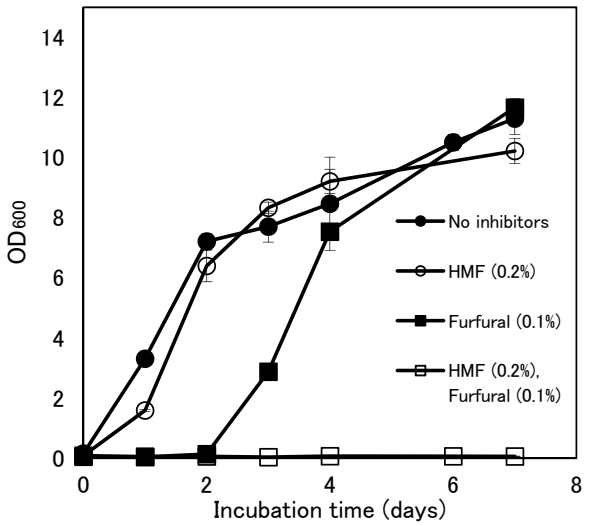

Figure 2. Effect of HMF (0.2%) and furfural (0.1%) on the growth of *R. graminis.* Error bars shows the standard deviation of triplicate experiments.

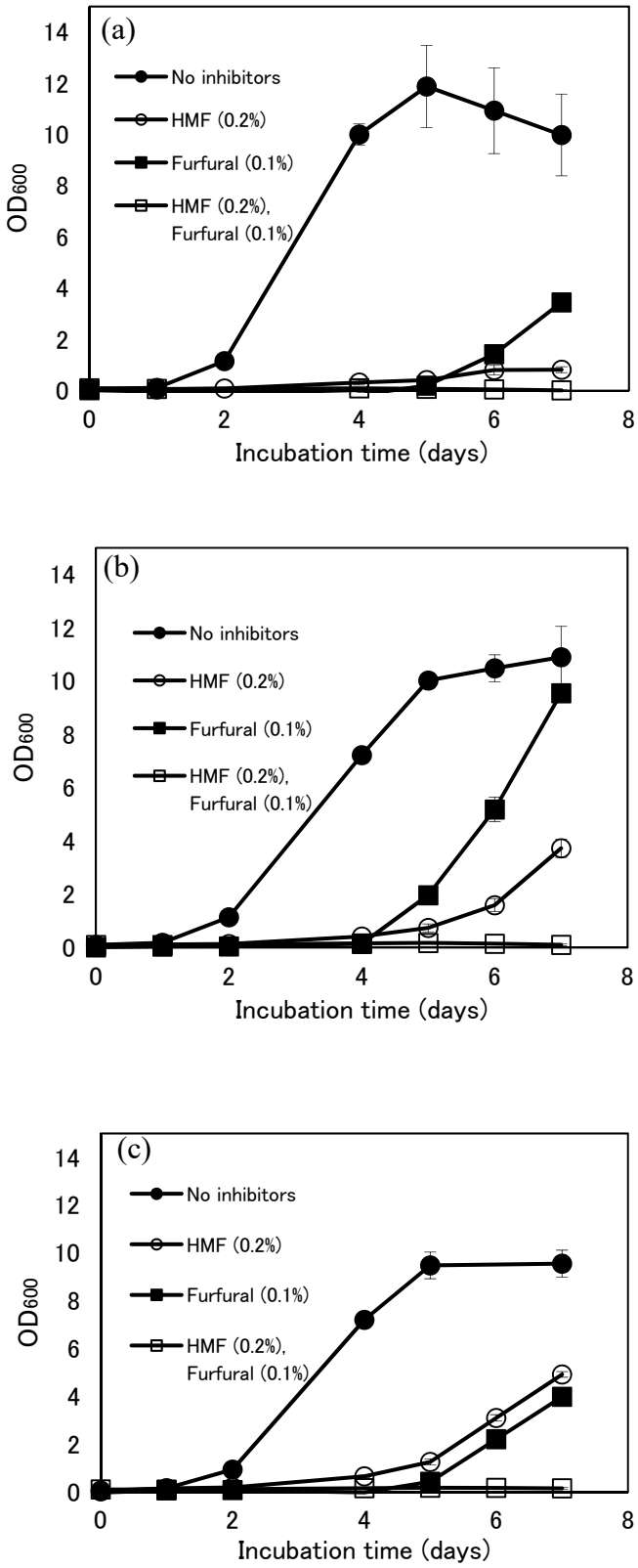

Figure 3. Effects of HMF (0.2%) and furfural (0.1%) on the growth of *Lipomyces* species. **(a)** *L. starkeyi* NRRL Y-1389, **(b)** *L. starkeyi* NBRC 10381, **(c)** *L. spencer-martinsiae*. Error bars shows the standard deviation of triplicate experiments.

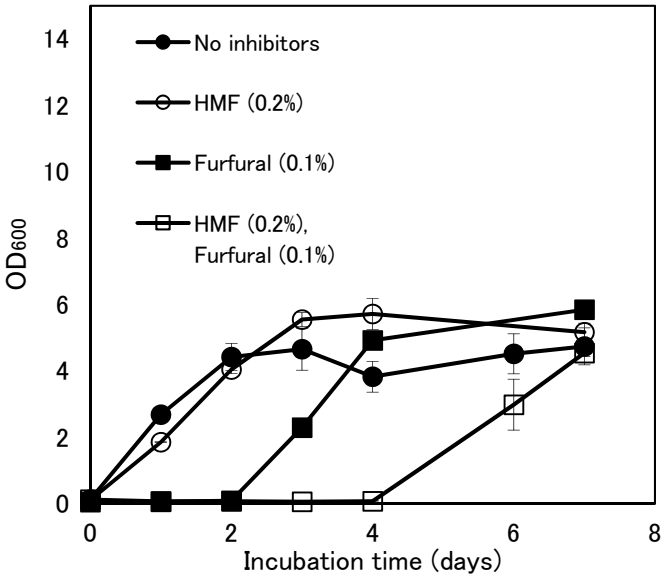

Figure 4. Effect of HMF (0.2%) and furfural (0.1%) on the growth of *Y. lipolytica.* Error bars shows the standard deviation of triplicate experiments.

3.2. Removal of Furfural from CSL Medium

Furfural (0.1%) was more toxic for *R. graminis* compared with HMF (0.2%), because furfural (0.1%) increased induction time (Figure 2), while the growth rate of *R. graminis* with HMF (0.2%) was the same as that without inhibitors. Thus, it was thought that removing furfural was more efficient compared with removing HMF (0.2%) from the medium. To remove furfural from the CSL medium, rotary-evaporator was used, as furfural is known to form azeotrope with water. It was reported that rotary-evaporation could remove 96% of furfural dissolved in water in the presence of other inhibitors (HMF, acetic acid, formic acid, and levulinic acid) when the initial concentration of each inhibitor was 0.1 g/L, while HMF was concentrated almost proportionately with concentration factor [22]. In this study, although the concentration and the kinds of inhibitors were different from the previous study [22], furfural was successfully removed by two-times concentration using rotary-evaporation. In addition, increasing of the monomeric sugars concentrations in the medium was possible by rotary-evaporation. Thus, it was shown that the evaporation of the hydryzates from the SO_2-catalyzed steam pretreatment would be a beneficial way to remove furfural and increase sugar concentrations.

3.3. Acclimating of R. graminis for HMF(0.4%)

The removal of furfural and increasing of monomeric sugar concentration were attained by the concentration of the CSL medium by rotary-evaporation. However, HMF concentration was simultaneously increased from 0.2% to 0.4%, as HMF is not volatile compound. Although *R. graminis* had a higher HMF tolerance compared with the other strains (Figures 1–4), *R. graminis* did not grow in the medium in the presence of HMF (0.4%). This result was the same as the previous research in which wild type of *R. graminis* could grow in up to 0.35% of HMF [20]. It was assumed that acclimation was one of the best ways to produce lipids from the medium containing 0.4% of HMF, because the acclimation was effectively used for other oleaginous yeasts, *L. tetrasporoous* and *Y. lipolytica*, which increased the productivity of lipids due to the decreasing of induction time from SPORL enzymatic hydrolyzates containing HMF (0.06%) [12]. In addition, when *S. cerevisiae* was inoculated in a medium containing HMF and furfural, it started to grow when these inhibitors were reduced to their less toxic alcohol derivatives during the growth lag time [10]. Thus, it was considered that obtaining HMF (0.4%) tolerant *R. graminis* could be possible with longer incubation time in the CSL medium with HMF (0.4%) due to acclimation. As the result, after 140 h incubation of *R. graminis* with HMF (0.4%), increasing

of OD_{600} was observed. The aliquot of the medium was inoculated on the YMA with HMF (0.4%) and then the strain was incubated in the CSL medium with HMF (0.4%). Although the OD_{600} of wild type *R. graminis* was 1.3 times higher than that of HMF (0.4%) tolerant *R. graminis* after seven-days incubation, the OD_{600} of the two strains were almost same until four-days incubation (Figure 5). The previous study showed that the growth rate of *R. graminis* was decreased by about 70% in the presence of HMF (0.35%) [20]. In this study, the growth rate of the HMF (0.4%) tolerant *R. graminis* was similar to that of wild type (Figure 5), showing that acclimation was a useful way for obtaining oleaginous yeast even at higher HMF concentration (0.4%). Further study is required to reveal the mechanisms for the acclimation of *R. graminis* to HMF (0.4%), but one possible mechanism would be the increasing of alcohol dehydrogenase activity that reduces HMF to 5-hydroxymethylfurfuryl alcohol [23].

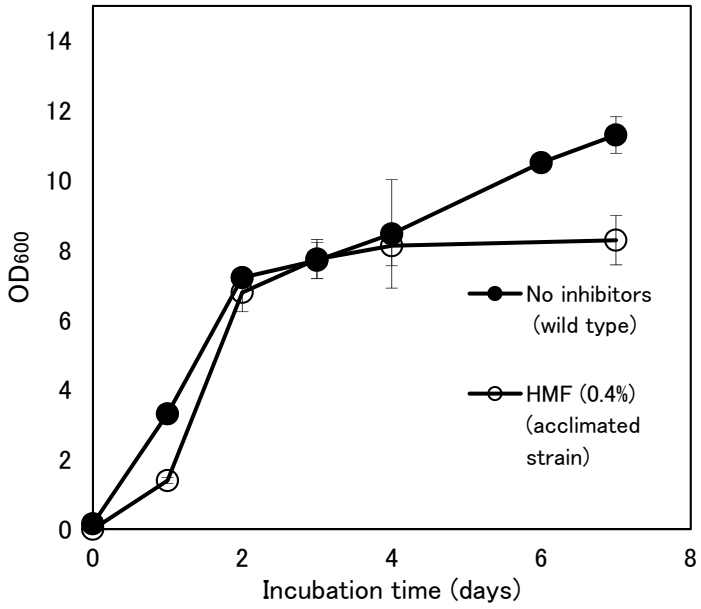

Figure 5. Comparison of the growth of acclimated *R. graminis* in the two-times concentrated CSL medium containing HMF (0.4%) and that of wild type in the CSL medium without inhibitors. Error bars shows the standard deviation of triplicate experiments.

3.4. Production of Lipids Using 2 Times Concentrated CSL Medium

The total lipids of acclimated *R. graminis* and wild type *R. graminis* were compared after four-days incubation in the two-times concentrated CSL medium with HMF (0.4%) and CSL medium without HMF, respectively (Figure 5). The acclimated *R. graminis* produced 1.8 g/L of lipids after four days when the strain was incubated in two-times concentrated the CSL medium by rotary-evaporation which contained 0.4% of HMF, while wild type *R. graminis* produced 2.0 g/L of lipids after four days in the CSL medium without HMF and furfural. Previous study showed that the lipids concentration produced by *R. graminis* was increased by 1.4 times when 0.15% of HMF was added into the medium [20]. In this study, the increasing of lipids concentrations was not observed when 0.4% of HMF was added in the CSL medium. One of the possible reasons could be that the higher HMF concentration used in this study negatively affected the biosynthetic pathway of lipids. However, considering the result that wild type *R. graminis* did not grow in the CSL medium containing HMF (0.4%) after four-days incubation (data not shown), the use of HMF (0.4%) tolerant *R. graminis* was shown to be effective for the production of lipids from the hydrolyzates of the SO_2-catalyzed steam pretreatment of lignocellulosic biomass.

4. Conclusions

Six oleaginous yeasts were compared to produce lipids from the hydrolyzates of SO_2-catalyzed steam pretreated lignocellulosic biomass using the CSL medium with HMF (0.2%) and furfural

(0.1%). The presence of both HMF (0.2%) and furfural (0.1%), which was inevitably contained in the hydrolyzates of SO_2-catalyzed steam pretreated lignocellulosic biomass, decreased the growth of the oleaginous yeasts showing that the removal of the inhibitors from the medium was required. Of the six oleaginous strains used in this study, *R. graminis* was the most promising strain for higher tolerant to the inhibitors. The removal of furfural from the CSL medium was conducted by rotary-evaporation because furfural was more toxic for *R. graminis*.

However, HMF concentration was increased from 0.2% to 0.4% by rotary-evaporation that resulted in the growth inhibition of *R. graminis*. As the result of acclimation, HMF (0.4%) tolerance *R. graminis* was obtained, and this strain produced lipids as much as that produced by wild type of *R. graminis* without inhibitors. This result suggests that the acclimation is an effective way to obtain HMF tolerant *R. graminis* and this strain is applicable for the production of lipids from the hydrolyzates of the SO_2-catalyzed steam pretreated lignocellulosic biomass. Further study is required to observe the productivity of lipids by the HMF (0.4%) tolerance *R. graminis* from the concentrated hydrolyzates of SO_2-catalyzed steam pretreated lignocellullosic biomass such as lodgepole pine and Douglas-fir.

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