RESEARCH ARTICLE

Co-utilization of acidified glycerol pretreated-sugarcane bagasse for microbial oil production by a novel *Rhodospiridium* strain

Morteza Hassanpour¹ | Guiqin Cai¹ | Leigh K. Gebbie² | Robert E. Speight² |Valentino S. Junior Te'o³ | Ian M. O'Hara¹ | Zhanying Zhang¹

¹Centre for Tropical Crops and Biocommodities, Queensland University of Technology, Brisbane, QLD, Australia

²School of Chemistry, Physics and Mechanical Engineering, Queensland University of Technology, Brisbane, QLD, Australia

³School of Earth, Environmental and Biological Sciences, Queensland University of Technology, Brisbane, QLD, Australia

Correspondence

Zhanying Zhang GPO Box 2432, 2 George St, Brisbane, QLD 4001, Australia.

Email: jan.zhang@qut.edu.au

Acidified glycerol pretreatment is very effective to deconstruct lignocellulosics for producing glucose. Co-utilization of pretreated biomass and residual glycerol to bio-products could reduce the costs associated with biomass wash and solvent recovery. In this study, a novel strain *Rhodospiridium toruloides* RP 15, isolated from sugarcane bagasse, was selected and tested for coconversion of pretreated biomass and residual glycerol to microbial oils. In the screening trails, *Rh. toruloides* RP 15 demonstrated the highest oil production capacity on glucose, xylose, and glycerol among the 10 strains. At the optimal C:N molar ratio of 140:1, this strain accumulated 56.7, 38.3, and 54.7% microbial oils based on dry cell biomass with 30 g/L glucose, xylose, and glycerol, respectively. Furthermore, sugarcane bagasse medium containing 32.6 g/L glucose from glycerol-pretreated bagasse and 23.4 g/L glycerol from pretreatment hydrolysate were used to produce microbial oils by *Rh. toruloides* RP 15. Under the preliminary conditions without pH control, this strain produced 7.7 g/L oil with an oil content of 59.8%, which was comparable or better than those achieved with a synthetic medium. In addition, this strain also produced 3.5 mg/L carotenoid as a by-product. It is expected that microbial oil production can be significantly improved through process optimization.

KEYWORDS

glycerol, lignocellulose, microbial oils, pretreatment, yeast

1 | INTRODUCTION

Lignocellulosic biomass, such as sugarcane bagasse, mainly composed of cellulose, hemicellulose, and lignin, is the most abundant low-cost renewable bioresource for the production of biofuels and biochemicals. However, due to the recalcitrant nature of lignocellulosic biomass, pretreatment is required to deconstruct biomass structure and to improve the yield of fermentable sugars in a subsequent enzymatic hydrolysis step.

Many biomass pretreatment methods have been reported and well-summarized in some review articles [1,2]. Among these pretreatment methods, the glycerol-based organosolv

pretreatment is very attractive because the pretreatment leads to high cellulose digestibility and glycerol is a high boiling point, nonflammable, nontoxic, and biodegradable solvent [3]. Importantly, glycerol, produced in large quantities from the biodiesel industry (1 mole of glycerol is synthesized for every 3 moles of methyl esters produced), is a surplus solvent/chemical with low commercial value [4]. The annual yield of glycerol was approximately 3.5 billion liters in 2018 based on the projected biodiesel yield of approximately 35 billion liters [4,5]. Application of glycerol for pretreatment will not only improve the utilization efficiency of lignocellulose but also helps to deal with the issues related to overproduction and disposal of glycerol.

Glycerol can be used alone or with acid/alkaline catalysts for pretreatment of lignocellulosic biomass [2].

Abbreviation: HMF, 5-hydroxymethyl furfural

FIGURE 1 Schematic diagram of acidified glycerol pretreatment and downstream processing

Acid-catalyzed glycerol pretreatment requires much lower temperatures and shorter reaction times than standalone alkaline processes [2]. Acid-catalyzed glycerol has been demonstrated at pilot scale with results comparable to those achieved at laboratory scale [3]. Despite the advantages of acidified glycerol pretreatment, a wash step is essential to recover glycerol for recycle in order to reduce the solvent cost (Figure 1). However, complete removal of glycerol is expensive because of the use of large amounts of water while incomplete removal of glycerol may lead to inhibition of enzymatic hydrolysis and fermentation [6]. Fortunately, glycerol is less inhibitory compared to other solvents such as ionic liquids [7,8]. Furthermore, a previous study has shown that the residual glycerol up to 5% did not inhibit cellulosic ethanol production by *Saccharomyces cerevisiae* following pretreatment of sugarcane bagasse [6]. Nevertheless, besides glycerol, biomass degradation and hydrolysis products, such as soluble phenolics from lignin and furans from sugars in the residual pretreatment hydrolysate, may also have inhibitory effects on microorganisms [9–11].

Considering the levels of residual glycerol in the pretreated and washed biomass, it is more industrially attractive to coconvert sugars and residual glycerol to valuable bioproducts such as microbial oils. Indeed, as a biodegradable solvent, glycerol has been used to synthesize a number of bioproducts such as microbial oils (including essential polyunsaturated fatty acids), succinic acid, 1,3-propanediol, and ethanol

PRACTICAL APPLICATIONS

Lignocellulosic biomass is abundant low-cost biomass for the production of renewable biofuels and bioproducts. Pretreatment is required to deconstruct lignocellulosic biomass, subsequently improving the utilization efficiency of biomass. Acidified glycerol pretreatment is a very effective pretreatment technology for enhanced glucose production. However, commercialization of this technology needs cost-effective downstream processing steps, including solvent wash and solvent recovery. Couitilization of pretreated biomass and glycerol-containing residual pretreatment hydrolysate to bioproducts could reduce the costs associated with wash and solvent recovery, improving the economics of glycerol-assisted biorefining of lignocellulosic biomass.

through biological processes [4,12–15]. In terms of microbial oil production, many studies have reported the growth of microorganisms on pure glycerol, crude glycerol, and glycerol–glucose mixture to produce health-beneficial fatty acids, such as arachidonic acid and eicosapentaenoic acid, as well as mixed microbial oils as fuel precursors [14–21].

Despite the research progress on microbial oil production using various carbon sources (glucose, xylose, glycerol, and combined carbon sources) for fuels application, the overall economics is still poor due to the high feedstock cost and low fermentation efficiency [22]. In order to improve the process economics, low-cost lignocellulosic biomass has been used to produce microbial oils following pretreatment with various methods including dilute acid, ammonia fiber expansion, and ionic liquid [23–25]. However, the low sugar yield (e.g. dilute acid pretreatment) and/or high pretreatment cost (e.g. ionic liquid pretreatment) may limit the economic production of microbial oils from lignocellulosic biomass. Acid-catalysed glycerol-based pretreatment is more effective than many other pretreatments because of the relatively high glucose yield and relatively low solvent cost [6]. Integration of glycerol-based pretreatment with coconversion of pretreated biomass and residual glycerol to microbial oils may improve the overall process economics further compared to the conversion of pretreated biomass alone. However, such an integrated process has not been proposed and studied. Interestingly, several recent studies reported that addition of crude glycerol into biomass hydrolysates derived from dilute acid or alkaline/oxidative pretreatments could enhance microbial oil production due to the improved C:N ratio [18–21].

Various types of yeasts, including the strains from *Yarrowia*, *Lipomyces*, *Trichosporon Rhodotorula*, and *Rhodospiridium*, have been reported for producing microbial

oils [14,15]. Among these different yeast genera, red yeast strains *Rhodotorula* and *Rhodospiridium* are of particular interest because of their capacity to produce both oils and high-value carotenoids [26–28]. In this study, a total of 10 red yeast strains were first screened for microbial oil production on glucose, xylose, and glycerol, followed by optimization of the C:N molar ratio—the most important factor for microbial oil production with the selected yeast strain. Furthermore, the selected strain was investigated for microbial oil production from glycerol-pretreated sugarcane bagasse and residual glycerol with the aim of demonstration of an integrative pretreatment and fermentation process in order to reduce the overall process cost associated with biomass wash and solvent recovery. In addition, carotenoid produced by the selected yeast was also evaluated during microbial oil production.

2 | MATERIALS AND METHODS

2.1 | Materials

A total of 10 yeast strains were used for the selection experiment, including five *Rhodotorula* (*R.*) strains purchased from FRR Culture Collection (CSIRO, CAN, Australia), one *Rhodospiridium* (*Rh.*) strain from ATCC Culture Collection (Manassas, VA, USA), and four novel *Rhodotorula* and *Rhodospiridium* strains isolated from a local sugarcane bagasse stockpile. All strains were stored in 30% glycerol stock solution at -80°C and streaked onto YPD agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, and 20 g/L agar) for 3 days before selection experiments. All chemicals used in this study were analytical grade or above and purchased from Sigma–Aldrich (St. Louis, MO, USA), except yeast extract (Merck, Darmstadt, Germany) and Accellerase 1500 (Genencor Division, Danisco Inc, USA).

2.2 | Isolation and identification of red yeasts from sugarcane bagasse

Rhodospiridium toruloides RP 2, *Rhodotorula dairenensis* RP 10, and *Rh. toruloides* RP 15 were isolated from a bagasse sample taken from just under the surface of a large bagasse pile at Rocky Point Sugar Mill (QLD, Australia). *Rhodotorula mucilaginosa* MK 6 was isolated from a bagasse sample taken from a storage bin at Racecourse Sugar Mill (QLD, Australia). The yeast strains were isolated as distinct morphotypes from serial dilutions on YPD plates containing chloramphenicol (34 $\mu\text{g/mL}$). They were identified by extracting DNA according to Løoke et al. [29] and sequencing a 550 bp region of the large sub-unit ribosomal DNA. The region was amplified using the primers NL1F 5'-GCATATCAATAAGCGGAGGAAAAG-3' and NL4R 5'-GGTCCGTGTTTCAAGACGG-3' [30] with

Onetaq polymerase (Genesearch, Australia) following the manufacturer's instructions and an annealing temperature of 51°C . Sanger sequencing was carried out by the Central Analytical Research Facility (CARF) at the Queensland University of Technology (QUT).

2.3 | Selection of oleaginous yeast strains

Prior to the selection experiment, the yeast strains were firstly propagated to provide sufficient yeast cell as inoculants using the following procedure. First, a loop of yeast cell was transferred from one agar plate to 10 mL YPD solution in a 50 mL Falcon tube. Preculture was conducted at 28°C for 48 h in a shaking incubator with an agitation speed of 180 rpm. At the end of the cultivation, the tubes were centrifuged, and the supernatants were discarded. The yeast cell masses were washed twice with sterile water (2×10 mL) to remove the residual nutrients from the preculture medium and resuspended in sterile water. The volume of water added was adjusted to give similar yeast cell mass concentrations ($\sim 2\text{--}3$ g/L). These precultures were used as inoculants.

The selection media included a single carbon sources (glucose, xylose, and glycerol, respectively) of 30 g/L, 0.5 g/L yeast extract (8.0% nitrogen and 40.0% carbon), 0.4 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g/L KH_2PO_4 , 0.1 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 8.0 mg/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.1 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 mg/L $\text{CoCl}_2 \cdot \text{H}_2\text{O}$, and 0.1 mg/L $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$. 0.47 g/L $(\text{NH}_4)_2\text{SO}_4$ was added to the media to make a final C:N molar ratio of 100:1 with consideration of the amount of nitrogen in yeast extract. The carbon in the yeast extract, which was only 0.2 g/L compared to the carbon concentrations from sugars (12.0 g/L) or glycerol (11.7 g/L) in the media, was not included for C:N molar ratio calculation. The initial pH of the media was adjusted to pH 5.5 by addition of 2 M HCl or 2 M NaOH. The media was autoclaved at 121°C for 15 min. Selection trials started with the inoculation of 5 mL of each yeast cell mass solution into 250 mL shake flasks containing 45 mL medium. The flasks were incubated at 28°C for 6 days with an agitation speed of 180 rpm in the dark. At the end of cultivation, the yeast cell masses were centrifuged at 4500 rpm for 5 min. A total of 1 mL of the supernatant was filtered for sugar analysis by HPLC analysis. The yeast cell masses were washed a couple of times by distilled water to remove residual medium components, collected, freeze-dried, and stored at 4°C in dark condition for future analyses. The selection trials were conducted in duplicate.

2.4 | Optimization of the C:N molar ratio with the selected yeast strain—*Rh. toruloides* RP 15

Following the selection trials, *Rh. toruloides* RP 15 was identified as the best strain for microbial oil production.

Subsequently, the C:N molar ratio was optimized using single carbon sources (glucose, xylose, and glycerol) for *Rh. toruloides* RP 15 growth. Prior to the optimization trials, 210 mL precultures in 500 mL flasks were grown in YPD at 28°C for 48 h at 180 rpm to prepare sufficient yeast cell masses for inoculations. At the end of the cultivation, yeast cell mass was collected and prepared with the same procedure as that described for the selection experiment.

The cultivation media for optimization of the C:N ratio contained 30 g/L of single carbon sources (glucose, xylose, and glycerol), and 0.5 g/L yeast extract and other nutrients at the same levels as the media for selection trials. The C:N molar ratios were adjusted to 60:1, 80:1, 100:1, 120:1, 140:1, 160:1, 180:1, and 200:1 through addition of 0.91, 0.64, 0.47, 0.36, 0.28, 0.22, 0.18, 0.14 g/L (NH₄)₂SO₄, respectively into the cultivation medium with the consideration of the amount of nitrogen from yeast extract. The pH of the cultivation media was adjusted to 5.5 by 2 M HCl or 2 M NaOH prior to autoclave at 121°C for 15 min. Optimization trials started with the inoculation of 5 mL of each yeast cell mass solution into 250 mL shake flasks containing 45 mL working volume. At the end of the cultivation, the yeast cell mass and fermentation broth were collected and processed using the same procedure as that described for the selection experiment.

2.5 | Microbial oil production by *Rh. toruloides* RP 15 using acidified glycerol pretreated bagasse

2.5.1 | Preparation of production media from pretreated sugarcane bagasse

Sugarcane bagasse was pretreated in a horizontal reactor (Andritz, USA) at the Mackay Renewable Biocommodities Pilot Plant, a Queensland University of Technology facility located on the site of the Racecourse Sugar Mill (Mackay, QLD, Australia). The pretreatment was conducted at 150°C for 15 min using a procedure similar to that described previously [6]. The initial liquid/solid mass ratio was 5:1 (50 kg:10 kg) and the liquid included approximately 42 kg glycerol and 2.4 wt.% H₂SO₄ on bagasse. Following the pretreatment, the liquid and solid were separated by the press filtration and collected for later use and analysis.

A total of 2 kg-pretreated bagasse was thoroughly washed with plenty of water to remove all the residual pretreatment hydrolysate as the presence of glycerol would affect biomass composition analysis. Based on the biomass composition data, glycerol-containing residual pretreatment hydrolysate was mixed back with the completely washed biomass to simulate the glucan:glycerol ratio of 30:20 for an incomplete wash operation according to a previous publication [6]. Enzymatic hydrolysis was conducted in 500 mL shake flasks containing 200 g reaction mixture (pH 4.8) consisting of 3 wt.% glu-

can, 2 wt.% glycerol, and 0.5 mL Accellerase 1500/g glucan. Enzymatic hydrolysis was conducted at 50°C for 72 h in a shaking incubator with an agitation speed of 140 rpm. After hydrolysis, the reaction mixture was filtered to collect the hydrolysate.

Nutrients were added to the enzymatic filtrates to prepare cultivation media. Except for nitrogen, the nutrient composition and concentrations were the same as those used for the selection and C:N optimization media. The C:N molar ratio was controlled at 140:1 (based on the C:N optimization results) with the addition of (NH₄)₂SO₄. Synthetic medium containing similar amounts of pure glucose, xylose, and glycerol, same C:N molar ratio, as well as the same amounts of nutrients was used as the control. Unlike the previous trials, yeast extract was not used in any of the production media. The pH of the media was adjusted to 5.5 prior to autoclaving at 121°C for 15 min.

2.5.2 | Microbial oil production

Prior to microbial oil production by the *Rh. toruloides* RP 15 strain using the bagasse-derived media, yeast cell mass was prepared using the same preculture described for the C:N ratio optimization trials. Microbial oil production was conducted in 250 mL flasks containing 45 mL bagasse and synthetic media, respectively, and 5 mL yeast cell mass solution. Cultivation was conducted at 28°C for 14 days with an agitation speed of 180 rpm. Two flasks from each medium were withdrawn every 2 days to collect cell mass and fermentation broth. The collection procedure was similar to that described for the selection and C:N ratio optimization trials. Microbial oil production was conducted in duplicate.

2.6 | Determination of microbial oil content

Microbial oil content was determined using a modified Bligh and Dyer method [31]. Briefly, 40 mg freeze-dried yeast cell mass was lysed in a 2 mL capped tube containing stainless steel beads using a bead mill (Tissue Lyser II, Qiagen, Germany) at 30 Hz for 4 min. Afterwards, 0.24 mL water, 0.3 mL chloroform, and 0.6 mL methanol were added sequentially to the same tube. The cell mass was lysed for 2 min at 30 Hz. After centrifugation, 0.3 mL chloroform was added to the tube and mixed for 0.5 min at 30 Hz, followed by centrifugation, addition of 0.3 mL water, and mixing for 0.5 min at 30 Hz. The tube was then centrifuged and the organic phase was collected in a preweighed glass vial using a syringe and needle. The cell mass was re-extracted three times more. The pooled extracts in the preweighed vial were dried in a 45°C oven under a vacuum and the amount of microbial oils was determined gravimetrically and was used to calculate the total content of oils. Microbial oil extraction was conducted in duplicate.

2.7 | Determination of carotenoids in *Rh. toruloides* RP 15

Carotenoids are soluble in microbial oils and were simultaneously extracted from yeast cell mass during the microbial oil extraction process. The total content of carotenoids was estimated using a modified spectrophotometric method described previously [32]. Following determination of the amount of oils using the gravity method, carotenoids in the extraction vial were dissolved and extracted in pure ethanol. The absorbance of carotenoids-containing ethanol solution was measured at 450 nm using a spectrophotometer and calculated using a previously used method [32]. Since the content of carotenoids was very low (≤ 0.5 mg/g), their effect on total oil content was negligible.

2.8 | Determination of total nitrogen

A TOC-V_{CSH} (Shimadzu, Japan) coupled with TN detector was used to measure the total nitrogen in the enzymatic hydrolysates. The sample was transferred to a combustion chamber using air and all nitrogen atoms were converted to NO at 720°C through catalytic oxidation. Then, the gas containing NO was transferred to the chemiluminescence detector to measure the NO concentration.

2.9 | Analysis of sugars and metabolites

Sugars in the enzymatic hydrolysis solutions, as well as the sugars and potential sugar alcohols, such as glycerol, mannitol, xylitol, and sorbitol, in the fermentation broths were measured using a Waters HPLC system equipped with a refractive index detector (Waters 410, VA, USA) and a SP0810 carbohydrate column (300 mm $\times$ 8.0 mm, Shodex, Japan). The column temperature was set at 85°C and water was used as a mobile phase at a flow rate of 0.5 mL/min. Furans (furfural, 5-hydroxymethyl furfural [HMF]) in the pretreatment/enzymatic hydrolysates as well as citric acid in the fermentation broths were analysed by the other Waters HPLC system equipped with a refractive index detector (Waters 410) and Aminex HPX-87H column (300 mm $\times$ 8.0 mm, Bio-Rad, CA, USA). The column temperature was set at 60°C and 5 mM H₂SO₄ was used as a mobile phase at a flow rate of 0.5 mL/min.

3 | RESULTS AND DISCUSSION

3.1 | Selection of oleaginous yeasts

Table 1 shows microbial oil production with 10 yeast strains on 30 g/L of glucose, xylose, and glycerol. The highest oil

content and cell mass concentration were achieved with *Rh. toruloides* RP 15 (a novel strain from bagasse stockpile) on all the three carbon sources. Consequently, the highest oil concentrations were also achieved with this strain. The oil concentrations of *Rh. toruloides* RP 15 reached 3.1 g/L with glucose, 2.0 g/L with xylose, and 3.6 g/L with glycerol, respectively. The corresponding oil contents were 43.4, 36.0, and 44.6%, respectively. The second-best strain, however, varied depending on the carbon source. For example, on glucose and glycerol, *Rh. toruloides* ATCC 204091 appeared to be the second-best strain though the difference was not obvious compared to *R. mucilaginosa* FRR 1295 on glucose. However, on xylose, the best producer was *R. mucilaginosa* FRR 4309.

Under the tested condition in this study, *Rh. toruloides* RP 15 had much higher oil production capacity on all the carbon sources than the two previously reported strains—*Rh. toruloides* (also reported as *Rhodotorula glutinis* ATCC 204091) ATCC 204091 and *R. mucilaginosa* FRR 2406 [33,34]. With *Rh. toruloides* ATCC 204091, a previous study reported a high microbial oil concentration of 43.2 g/L with a cell mass concentration of 60 g/L and oil content of 77% in a two-step cultivation process with nitrogen limitation [34]. The higher oil production capacity of *Rh. toruloides* RP 15 over *Rh. toruloides* ATCC 204091 as observed in this study suggests that there is the potential for even higher oil concentrations to be achieved with *Rh. toruloides* RP 15 through process optimization.

Table 1 also shows the residual carbon sources concentrations with different strains. The concentrations of residual glucose, xylose, and glycerol after 6 days' cultivation were the lowest for *Rh. toruloides* RP 15, at only 5.1, 7.1, and 5.0 g/L, respectively. The second lowest glucose concentrations were approximately 9.9 g/L, observed with *Rh. toruloides* ATCC 204091 and *R. dairenensis* RP 10. The second lowest glycerol concentration was 8.0 g/L, again observed with *Rh. toruloides* ATCC 204091. The second lowest xylose concentrations were 10.9 g/L with *R. dairenensis* RP 10. Considering the error, there was no obvious difference from *R. glutinis* FRR 4522 (10.7 g/L). The strains *R. mucilaginosa* FRR 1295, *Rhodotorula minuta* FRR 5053, and *R. mucilaginosa* MK 6 consumed the least amounts of glucose, xylose, and glycerol, respectively.

In addition, the yields of cell mass and oils on consumed carbon sources with different strains were calculated and shown in Table 1. The yields on consumed glucose and glycerol varied more significantly than those on consumed xylose. *R. mucilaginosa* FRR 1295 and *R. glutinis* FRR 4522 had the highest cell mass yields and relatively high oil yields on consumed glucose. However, because of their low oil contents and low glucose consumption rates, the total oil concentrations were 1.9 g/L and 1.7 g/L, respectively. There were no obvious differences in the cell mass yields and oil yields on xylose and the oil concentration mainly depended on

TABLE 1 Microbial oil production on glucose (A), xylose (B), and glycerol (C) with different strains. Initial substrate concentration of 30 g/L, 6 days of cultivation in shake flasks; $Y_{\text{Cell/S}}$, cell mass yield based on consumed substrate; $Y_{\text{Oil/S}}$, oil yield based on consumed substrate

Strain	Cell mass (g/L) _S	Oil content (%)	Oil (g/L)	Residual glucose (g/L)	$Y_{\text{Cell/S}}$ (g/g)	$Y_{\text{Oil/S}}$ (g/g)
<i>R. minuta</i> FRR 5053	3.7 ± 0.0	22.5 ± 2.5	0.8 ± 0.1	15.3 ± 0.2	0.23 ± 0.00	0.05 ± 0.01
<i>R. glutinis</i> FRR 4522	6.8 ± 0.0	25.0 ± 0.8	1.7 ± 0.1	16.6 ± 0.0	0.46 ± 0.00	0.11 ± 0.00
<i>R. mucilaginosa</i> FRR 2406	5.9 ± 0.2	23.2 ± 1.5	1.4 ± 0.0	12.9 ± 1.1	0.31 ± 0.01	0.07 ± 0.01
<i>R. mucilaginosa</i> FRR 1295	6.3 ± 0.1	30.0 ± 5.0	1.9 ± 0.3	17.8 ± 0.1	0.46 ± 0.01	0.14 ± 0.02
<i>R. mucilaginosa</i> FRR 4309	6.2 ± 0.2	28.3 ± 0.1	1.8 ± 0.0	11.4 ± 0.3	0.31 ± 0.01	0.09 ± 0.00
<i>R. mucilaginosa</i> MK 6	6.2 ± 0.1	19.7 ± 2.8	1.2 ± 0.1	13.6 ± 1.4	0.35 ± 0.03	0.07 ± 0.00
<i>R. dairenensis</i> RP 10	5.7 ± 0.1	24.8 ± 1.4	1.4 ± 0.2	9.9 ± 0.5	0.27 ± 0.00	0.07 ± 0.01
<i>Rh. toruloides</i> RP 2	4.0 ± 0.2	27.1 ± 2.1	1.1 ± 0.0	15.1 ± 0.1	0.24 ± 0.01	0.07 ± 0.00
<i>Rh. toruloides</i> RP 15	7.2 ± 0.1	43.4 ± 3.2	3.1 ± 0.1	5.1 ± 0.2	0.27 ± 0.00	0.12 ± 0.01
<i>Rh. toruloides</i> ATCC 204091	5.5 ± 0.0	36.4 ± 2.3	2.0 ± 0.1	9.9 ± 0.6	0.26 ± 0.01	0.09 ± 0.00
<i>R. minuta</i> FRR 5053	3.7 ± 0.0	30.4 ± 2.9	1.1 ± 0.1	14.5 ± 0.9	0.22 ± 0.01	0.07 ± 0.00
<i>R. glutinis</i> FRR 4522	4.8 ± 0.0	27.0 ± 0.8	1.3 ± 0.0	10.7 ± 0.4	0.23 ± 0.00	0.06 ± 0.00
<i>R. mucilaginosa</i> FRR 2406	5.0 ± 0.0	26.3 ± 0.5	1.3 ± 0.0	11.3 ± 0.1	0.25 ± 0.00	0.07 ± 0.00
<i>R. mucilaginosa</i> FRR 1295	4.8 ± 0.3	25.6 ± 1.9	1.2 ± 0.0	11.4 ± 0.2	0.24 ± 0.01	0.06 ± 0.01
<i>R. mucilaginosa</i> FRR 4309	5.0 ± 0.0	30.5 ± 4.2	1.5 ± 0.2	11.6 ± 0.0	0.25 ± 0.00	0.08 ± 0.00
<i>R. mucilaginosa</i> MK 6	4.6 ± 0.1	21.1 ± 0.4	1.0 ± 0.0	11.7 ± 0.3	0.23 ± 0.01	0.05 ± 0.00
<i>R. dairenensis</i> RP 10	5.0 ± 0.0	25.6 ± 0.1	1.3 ± 0.0	10.9 ± 0.1	0.24 ± 0.00	0.06 ± 0.00
<i>Rh. toruloides</i> RP 2	4.1 ± 0.1	23.8 ± 0.4	1.0 ± 0.0	12.3 ± 1.0	0.21 ± 0.00	0.05 ± 0.00
<i>Rh. toruloides</i> RP 15	5.5 ± 0.2	36.0 ± 2.9	2.0 ± 0.1	7.1 ± 0.4	0.22 ± 0.01	0.08 ± 0.00
<i>Rh. toruloides</i> ATCC 204091	4.1 ± 0.1	26.8 ± 0.4	1.1 ± 0.0	12.7 ± 0.1	0.22 ± 0.01	0.06 ± 0.00
<i>R. minuta</i> FRR 5053	2.8 ± 0.4	30.4 ± 0.6	0.9 ± 0.1	12.8 ± 0.3	0.15 ± 0.02	0.05 ± 0.00
<i>R. glutinis</i> FRR 4522	5.4 ± 0.0	25.3 ± 0.1	1.4 ± 0.0	12.2 ± 0.0	0.28 ± 0.00	0.07 ± 0.00
<i>R. mucilaginosa</i> FRR 2406	3.9 ± 0.2	21.2 ± 0.7	0.8 ± 0.0	19.8 ± 1.0	0.34 ± 0.04	0.07 ± 0.01
<i>R. mucilaginosa</i> FRR 1295	3.6 ± 0.0	19.3 ± 0.3	0.7 ± 0.0	21.7 ± 0.3	0.37 ± 0.02	0.07 ± 0.00
<i>R. mucilaginosa</i> FRR 4309	3.6 ± 0.0	17.7 ± 3.0	0.6 ± 0.1	23.1 ± 0.3	0.42 ± 0.02	0.07 ± 0.01
<i>R. mucilaginosa</i> MK 6	2.6 ± 0.0	16.7 ± 1.2	0.4 ± 0.0	23.8 ± 0.5	0.33 ± 0.03	0.06 ± 0.01
<i>R. dairenensis</i> RP 10	3.7 ± 0.1	21.9 ± 0.9	0.8 ± 0.1	20.6 ± 0.1	0.34 ± 0.01	0.07 ± 0.00
<i>Rh. toruloides</i> RP 2	6.4 ± 0.1	34.3 ± 0.7	2.2 ± 0.1	10.1 ± 0.2	0.30 ± 0.01	0.10 ± 0.00
<i>Rh. toruloides</i> RP 15	8.2 ± 0.1	44.6 ± 0.1	3.6 ± 0.0	5.0 ± 0.3	0.31 ± 0.00	0.14 ± 0.00
<i>Rh. toruloides</i> ATCC 204091	6.6 ± 0.0	39.3 ± 0.1	2.6 ± 0.0	8.0 ± 0.1	0.28 ± 0.00	0.11 ± 0.00

the xylose consumption rate. Although *R. mucilaginosa* FRR 4309 had the highest cell mass yield (0.42 g/g) on glycerol, the oil concentration was only 0.6 g/L because of its low oil content and relatively slow glycerol consumption rate. Although the cell mass yields of *Rh. toruloides* RP 15 were not the highest on all carbon sources, its oil concentrations were the highest because of the rapid growth and the highest oil contents. The theoretical microbial oil yields on glucose, xylose, and glycerol were estimated to be 0.30–0.32 g/g, 0.32–0.34 g/g, and 0.29–0.30 g/g, respectively [35,36] and the real oil yield on glycerol is generally higher than that on glucose [36]. However, several strains tested in this study did not show higher oil yields on glycerol, possibly due to relatively poor glycerol

assimilation and oil producing capacity (e.g. FRR 1295 and FRR 4309; Table 1).

Analysis of cultivation media for sugar alcohol production at the end of cultivation revealed the production of xylitol and mannitol by some strains as shown in Supporting Information Table S1. It is known that yeast and bacteria can produce sugar alcohols in response to increased external osmotic pressure and the sugar alcohol production capacity varies among the strains [37,38]. Among these strains, *R. minuta* FRR 5053 produced the highest concentrations of mannitol on glucose and glycerol, as well as the highest concentration of xylitol on xylose. This strain was also the only one to produce mannitol on glycerol, up to 6.8 g/L, indicating it is a potential

TABLE 2 Microbial oil production on glucose (A), xylose (B), and glycerol (C) with different C:N molar ratios by *Rh. toruloides* RP 15. Initial substrate concentration of 30 g/L, 6 days of cultivation in shake flasks; $Y_{\text{Cell/S}}$, cell mass yield based on consumed substrate; $Y_{\text{Oil/S}}$, oil yield based on consumed substrate

C:N ratio	Cell mass (g/L)	Oil content (%)	Oil (g/L)	Residual glucose (g/L)	$Y_{\text{Cell/S}}$ (g/g)	$Y_{\text{Oil/S}}$ (g/g)
60:1	7.9 ± 0.0	34.2 ± 2.7	2.7 ± 0.2	0.0 ± 0.0	0.26 ± 0.00	0.09 ± 0.01
80:1	8.0 ± 0.2	36.0 ± 0.9	2.9 ± 0.1	1.7 ± 0.1	0.28 ± 0.01	0.10 ± 0.01
100:1	7.7 ± 0.0	41.0 ± 0.5	3.2 ± 0.0	3.4 ± 0.1	0.29 ± 0.00	0.12 ± 0.00
120:1	7.6 ± 0.2	49.7 ± 0.2	3.8 ± 0.1	3.4 ± 0.2	0.29 ± 0.01	0.14 ± 0.01
140:1	7.1 ± 0.2	56.7 ± 1.6	4.0 ± 0.2	4.7 ± 0.4	0.28 ± 0.01	0.16 ± 0.01
160:1	6.7 ± 0.1	58.0 ± 0.5	3.9 ± 0.0	5.6 ± 0.5	0.28 ± 0.01	0.16 ± 0.00
180:1	6.3 ± 0.1	61.3 ± 3.0	3.9 ± 0.2	6.0 ± 0.2	0.27 ± 0.00	0.16 ± 0.01
200:1	5.9 ± 0.1	59.4 ± 1.6	3.5 ± 0.2	7.4 ± 0.0	0.26 ± 0.01	0.16 ± 0.01
60:1	6.5 ± 0.0	26.0 ± 0.5	1.7 ± 0.0	4.1 ± 0.1	0.25 ± 0.00	0.07 ± 0.00
80:1	5.8 ± 0.1	29.7 ± 1.2	1.7 ± 0.1	6.5 ± 0.1	0.25 ± 0.00	0.07 ± 0.00
100:1	5.5 ± 0.1	34.3 ± 0.2	1.9 ± 0.0	6.6 ± 0.2	0.24 ± 0.00	0.08 ± 0.00
120:1	5.4 ± 0.4	37.9 ± 2.0	2.0 ± 0.2	7.7 ± 0.3	0.24 ± 0.01	0.09 ± 0.01
140:1	5.1 ± 0.2	38.3 ± 1.9	2.0 ± 0.0	8.9 ± 0.2	0.24 ± 0.01	0.09 ± 0.00
160:1	4.8 ± 0.2	41.7 ± 1.9	2.0 ± 0.2	10.0 ± 0.3	0.24 ± 0.00	0.10 ± 0.01
180:1	4.2 ± 0.1	43.8 ± 0.4	1.8 ± 0.1	12.5 ± 0.4	0.24 ± 0.00	0.10 ± 0.00
200:1	3.8 ± 0.1	47.9 ± 1.6	1.8 ± 0.1	12.6 ± 0.1	0.22 ± 0.00	0.11 ± 0.01
60:1	8.5 ± 0.4	39.3 ± 0.5	3.4 ± 0.1	0.0 ± 0.0	0.29 ± 0.00	0.11 ± 0.00
80:1	8.3 ± 0.1	38.1 ± 1.9	3.2 ± 0.2	2.2 ± 0.1	0.30 ± 0.01	0.11 ± 0.01
100:1	8.2 ± 0.1	45.5 ± 1.8	3.7 ± 0.2	3.3 ± 0.1	0.31 ± 0.00	0.14 ± 0.01
120:1	8.0 ± 0.1	47.0 ± 0.4	3.8 ± 0.0	5.7 ± 0.0	0.33 ± 0.00	0.16 ± 0.00
140:1	7.6 ± 0.2	54.7 ± 1.1	4.1 ± 0.2	6.1 ± 0.6	0.32 ± 0.00	0.17 ± 0.00
160:1	7.1 ± 0.2	57.4 ± 0.8	4.1 ± 0.0	6.9 ± 0.4	0.32 ± 0.00	0.18 ± 0.00
180:1	6.3 ± 0.4	56.2 ± 0.7	3.6 ± 0.1	8.7 ± 0.6	0.30 ± 0.01	0.17 ± 0.01
200:1	5.9 ± 0.2	56.5 ± 0.5	3.3 ± 0.1	8.7 ± 0.6	0.28 ± 0.00	0.16 ± 0.00

mannitol producer on glycerol. With glucose, *R. dairenensis* RP 10 also produced low levels of mannitol. With xylose, all strains except for *Rh. toruloides* RP 2 and *Rh. toruloides* ATCC 204091 produced low levels of mannitol and xylitol. It was not clear how mannitol was produced from xylose may be through the conversion of xylose to glycerol, followed by conversion of glycerol to mannitol. Xylitol and mannitol are important building block chemicals [39,40]. It is worth investigating the production capacity of these chemicals by the strains, such as *R. minuta* FRR 5053, in future.

In summary, all the test strains could accumulate significant amounts of oils in their cell biomass on glucose, xylose, and glycerol with oil contents ranged from 16.7 to 44.6%. *Rh. toruloides* RP 15 had the highest oil production capacity with the highest cell mass, oil content, oil concentration, as well as high consumption rates on all the three carbon sources. Therefore, *Rh. toruloides* RP 15 was selected for further investigation.

3.2 | Optimization of the C:N molar ratio by *Rh. toruloides* RP 15

It is well-known that nitrogen limitation leads to the accumulation of microbial oils and increasing the C:N ratio can increase the content of microbial oils. However, the optimum C:N ratios vary, depending on strains and substrates. Table 2 shows the effect of C:N ratios on RP 15 cell mass concentration, oil content, and oil concentration on glucose, xylose, and glycerol. As expected, increasing the C:N ratio not only led to a decrease in the cell mass concentration but also an increase in the oil content. With glucose and glycerol, the increase in oil content was more significant when increasing the C:N ratio from 60:1 to 140:1 compared to from 140:1 to 200:1. The maximal oil concentrations were achieved around C:N ratio of 140:1 as further increases in C:N ratio not only led to a marginal increase in the oil content (on glucose and glycerol) but also a significant reduction in cell mass and oil

concentrations (on all carbon sources). At the C:N ratio of 140:1, the cell mass concentration with glycerol was the highest at 7.6 g/L, followed by 7.1 g/L with glucose, and 5.1 g/L with xylose while the highest oil content was observed with glucose (56.7%), followed by glycerol (54.7%) and xylose (38.3%). As a result, the oil concentrations with glucose and glycerol reached 4.0 g/L and 4.2 g/L, respectively, almost double of that with xylose.

A recent review article summarizes microbial contents of a number of red yeast *R. glutinis* strains including *R. glutinis* ATCC 204091 (also reported as *Rh. toruloides* ATCC 204091) used in this study [[28] and references therein]. The highest oil contents of more than 60–66% on glucose and glycerol were achieved with three *R. glutinis* strains (T216, TISRT 5159, and IIP 30) under optimized conditions [[28] and references therein.]. Another recent review article summarizes oil contents with various yeast and yeast-like fungal strains from *Aureobasidium*, *Cryptococcus*, *Lipomyces*, *Rhodospiridiobolus*, *Rhodospiridium*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces*, *Trichosporon*, and *Yarrowia* [[41] and references therein]. It was reported that two *Rh. toruloides* strains and one *Rhodospiridium sphaerocarpum* accumulated 70–76% in cell mass on glucose and inulin [[41] and references therein]. Another commonly reported yeast genera, *Yarrowia* strains accumulated 40–50% oils in cell mass while most of the other yeast strains had the oil contents of 40–66% on various carbon sources [[41] and references therein]. In contrast, *Rh. toruloides* RP 15 in this study accumulated approximately 55–57% on glucose and glycerol under semioptimized conditions. It is worth noting that the strain *R. glutinis* ATCC 204091 (also reported as *Rh. toruloides* ATCC 204091 and used in this study) only accumulated 25–42% oils in the cell mass from different studies [[28] and references therein]. However, a recent study reported a 77% oil content with *R. glutinis* ATCC 204091 through a two-step cultivation strategy [34]. As shown previously, in this study under the same conditions, *R. glutinis* ATCC 204091 accumulated less oils than *Rh. toruloides* RP 15. These results indicate *Rh. toruloides* RP 15 selected from this study is one of the top oil-producing yeast strain; and oil production by this strain can be further improved through the process optimization.

Table 2 also shows the residual carbon source concentrations. In general, the consumption of glucose was the fastest, followed by glycerol. The consumption of xylose was the slowest and significant amounts of xylose were observed at all C:N ratios. Even at the lowest C:N ratio of 60:1, there was still 4.1 g/L xylose left after 6 days of cultivation. At a C:N ratio of 140:1, the residual xylose was 8.9 g/L, almost 30% of the initial concentration. In contrast, glucose and glycerol were consumed completely at low C:N ratios and the residual carbon sources increased at higher C:N ratios. At the C:N ratio of 140:1, the residual glucose and glycerol concentrations were 4.7 g/L and 6.1 g/L, respectively.

TABLE 3 Composition of medium derived from pretreated bagasse and synthetic medium

Component	Medium (g/L)	
	Pretreated bagasse ^a	Synthetic
Glucose	32.5	30.2
Glycerol	23.4	21.7
Xylose	1.1	1.0
N-hydrolysate ^b	0.10	–
(NH ₄) ₂ SO ₄	0.44	0.82
C:N ratio	140	140

^aBagasse medium also contained 0.5 g/L acetic acid, 0.1 g/L furfural, and 0.01 g/L 5-hydroxymethyl furfural; soluble phenolics were not determined.

^bTotal nitrogen in hydrolysate prior to (NH₄)₂SO₄ addition.

Because of the production of different amounts of cell mass, oils, and residual sugar concentrations, the yields of cell mass and microbial oils on consumed carbon sources vary as shown in Table 2. Cell mass yields were quite stable in the studied range of C:N ratios and higher cell mass and oil yields on consumed carbon sources were achieved on glycerol, followed by glucose and xylose. This observation was in line with previous reports because of the higher theoretical oil yield on glycerol than those on glucose and xylose [35,36]. As expected, oil yields on glucose and xylose increased significantly with increasing C:N ratios from 60:1 to 200:1 though a slight decrease in oil yields was observed on glycerol at the highest C:N ratios. The highest oil yields on consumed glucose, xylose, and glycerol at the optimal C:N ratio of 140:1 were 0.16 g/g, 0.09 g/g, and 0.17 g/g, respectively, leading to the highest oil concentrations.

3.3 | Microbial oil production by *Rh. toruloides* RP 15 using glycerol-pretreated sugarcane bagasse

Microbial oil production using glycerol pretreated bagasse was assessed with the selected strain *Rh. toruloides* RP 15. The bagasse medium contained glucose from pretreated bagasse and glycerol from residual pretreatment hydrolysate. A glucose:glycerol mass concentration ratio of approximately 30:20 was utilized to simulate the composition from incomplete wash operation, as reported previously [6]. The residual pretreatment hydrolysate in enzymatic hydrolysate accounted for approximately 4.5% of the total pretreatment hydrolysate.

Table 3 shows the composition of bagasse and synthetic media. The concentration of xylose was low (~1.0 g/L). This was because the majority of hemicellulose sugars remained in the pretreatment hydrolysate and the incompletely washed biomass only contained 4.5% of the total pretreatment hydrolysate. Because of this, the levels of sugar degradation products, such as furfural and HMF were low, 0.1 g/L and 0.01 g/L, respectively.

FIGURE 2 Comparison of microbial oil production by *Rh. toruloides* RP 15 using glycerol-pretreated bagasse medium and synthetic medium. (A) Consumption of carbon sources and production of cell mass. (B) Contents of microbial oils and carotenoids. (C) Concentration of microbial oils and carotenoids

Figure 2 compared carbon source consumption and microbial oil production with bagasse and synthetic media. It is worth noting that the pH of the media was not controlled during the cultivation and the cultivation time was long. In addition, since the xylose concentrations were low, they were not shown in Figure 2. Interestingly, glucose and glycerol were consumed simultaneously but glucose was consumed much faster than glycerol (Figure 2). Simultaneous consumption of glucose and glycerol for microbial oil production by *Yarrowia lipolytica* IBT 446 was also observed previously but glycerol was consumed more rapidly than glucose [42]. In general, diauxic growth on glucose and other carbon sources, such as xylose and glycerol, is common among microorganisms. For example, *S. cerevisiae* shows

diauxic growth on glycerol and glucose due to the complete repression of transcription of the genes encoding glycerol kinase (*GUT1*) and the FAD-dependant glycerol-3-phosphate dehydrogenase (*GUT2*) in the presence of glucose [43]. In other studies, *Rh. toruloides* DSMZ 4444 exhibited diauxic growth on glycerol and glucose [36,37]. However, glycerol kinase and FAD-dependant glycerol-3-phosphate dehydrogenase were not down-regulated in the presence of glucose [37]. These different observations on consumption of glycerol and glucose with different strains may indicate different inherent metabolic mechanisms. Process and metabolic engineering approaches may help to improve glycerol consumption rate of *Rh. toruloides* RP 15. For example, a two-step cultivation process consisting of a nitrogen-rich step for the growth of cell

TABLE 4 Comparison of microbial oil production using sugars–glycerol mixtures by oleaginous yeasts

Lignocellulosic biomass	Pretreatment	Strain	Carbon source	Substrate (g/L)	Oil (g/L)	Oil content (%)	Oil yield (g/g)	References
-	-	<i>Rh. toruloides</i>	Glucose+glycerol	~50	~8-9	~50%	0.17–0.20	[37]
Rice straw	Dilute acid	<i>R. glutinis</i>	EH ^a +PH ^b +crude glycerol (air-lift reactor)	60	10.8	58.5	0.3 ^c	[18]
Sugarcane top	Alkali and oxidation	<i>Rh. fluvialis</i>	EH+crude glycerol (shake flaks)	~92	13.9	66.6	0.19	[19]
			EH+crude glycerol (stirred tank reactor)		18.2	75	0.25	
Corn stover	Dilute acid	<i>C. curvatus</i>	EH+PH+crude glycerol	~63	10.8	49.7	0.18	[20]
Sugarcane bagasse	Dilute acid	<i>Rh. toruloides</i>	PH+crude glycerol (shake flask)	~62	3.3	41.7	–	[21]
			PH+crude glycerol (stirred tank reactor)	~78	3.9	55.5	–	
Sugarcane bagasse	Acidified glycerol	<i>Rh. toruloides</i>	EH+ residual PH (containing glycerol) (shake flaks)	57.1	7.7	59.8	0.15	This study

^aEH, enzymatic hydrolysate.^bPH, pretreatment hydrolysate.^cLikely overestimated.

mass and a subsequent nitrogen-limiting step for the accumulation of microbial oils may improve the oil concentration and productivity [34]. Metabolic network analysis has revealed the role of oxygen in the improvement of microbial oil production with the various and mixed carbon sources including glycerol [36,44], which can be used to guide process optimization (e.g. well-controlled oxygen supply and pH) in bioreactors instead of shake flasks.

Figure 2 also shows that pretreated bagasse medium did not negatively affect carbon source consumption and cell mass production compared to synthetic medium because of the low levels of biomass hydrolysis and degradation products (e.g. 0.1 g/L furfural and 0.01 g/L HMF). The consumptions of glucose and glycerol with bagasse medium were even slightly faster than that with synthetic medium, possibly due to the presence of an organic nitrogen source and more micronutrients in bagasse media. As shown in Table 3 prior to addition of nitrogen to bagasse medium, it already contained 0.10 g/L nitrogen, respectively. These low levels of nitrogen in the bagasse medium were probably protein-derived nitrogen due to the use of saccharification enzymes to hydrolyse pretreated bagasse and the presence of trace amount of protein in pretreated sugarcane bagasse. The cell mass concentrations reached 12.9 g/L with the two media at the end of cultivation.

Figure 2 shows that the oil contents with bagasse medium were higher than those with synthetic medium after 4 days of cultivation. The oil content was 59.8% with bagasse medium at the end of the cultivation, 7% higher than that with synthetic medium. The higher microbial oil contents could possibly be attributed to the presence of organic nitrogen, which was observed in previous studies to lead to increased

oil accumulation in *Rh. toruloides* [45]. In addition, trace amounts of citric acid (less than 1.0 g/L, data not shown) were detected during cultivation with bagasse media while citric acid was not detected in synthetic medium. The production of citric acid was possibly associated with the presence of organic nitrogen as well [45]. This red yeast strain also accumulated carotenoids during the cultivation and the contents of carotenoids with the two media were at similar levels. At the end of cultivation, carotenoids content of 274 µg/g dry cell mass was observed with bagasse medium. Figure 2 shows the kinetics of production of microbial oils and carotenoids. A maximal oil concentration of 7.7 g/L was achieved with bagasse medium, higher than that of 6.8 g/L with synthetic medium at the end of cultivation. The maximum carotenoid concentrations of 3.2–3.5 mg/L were achieved at the end of cultivation.

Table 4 summarizes and compares microbial oil production using sugars–glycerol mixtures as carbon sources. It is worth noting that the results in this study were obtained in shake flasks, and pH and oxygen supply were not well-controlled. Although the oil yield achieved in this study appeared to be the lowest (possibly due to unoptimized conditions), the oil content from this study was one of the top ones. Among these previous studies, one study used glucose-rich enzymatic hydrolysate with the addition of crude glycerol [23], one study used xylose-rich pretreatment hydrolysate with glycerol [25], and the other two studies used both hydrolysates with glycerol [22,24]. The oil concentration varied significantly because of the variations in initial carbon source concentrations [22–24] (Table 4). The low oil concentration with the pretreatment hydrolysate (xylose-rich)-glycerol mixture

was because of the slow consumption of both xylose and glycerol [25].

In these previous studies with the use of sugars-glycerol mixtures, glycerol was mainly used as a low-cost carbon source to increase the C:N ratios in order to improve the microbial oil production [22–25]. In this study, glycerol was an additional carbon source left from glycerol-based pretreatment process. The use of glycerol for biomass pretreatment could lead to higher glucose yields from lignocellulosic biomass. Moreover, the integrative use of glycerol could improve overall economics of biomass-based biorefinery process because of the reduced cost associated with biomass wash and solvent recovery. However, the glycerol-based pretreatment process may generate some compounds with types and concentrations being different from those generated from conventional pretreatment processes (e.g. dilute acid and alkali pretreatments). Therefore, demonstration of the feasibility for co-conversion of biomass pretreated by glycerol and residual pretreatment hydrolysate (containing glycerol) is very important prior to further process development and optimization.

4 | CONCLUDING REMARKS

A novel red yeast strain, *Rh. toruloides* RP 15 was selected and optimized for microbial oil production. Cultivation of this strain with medium containing glucose from pretreated bagasse and glycerol from residual pretreatment hydrolysate led to the production of both microbial oils and carotenoids at higher concentrations and higher contents than that with synthetic medium. Demonstration of the feasibility of this integrative process for glycerol-based biomass pretreatment and coutilization of pretreated biomass and residual glycerol will lead to further investigations on the development of process and metabolic engineering approaches to improve both microbial oil and carotenoid production.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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