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Expression of VHb Improved Lipid Production in *Rhodosporidium toruloides*

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Received: 5 July 2020; Accepted: 24 August 2020; Published: 27 August 2020

Abstract: The oleaginous yeast *Rhodosporidium toruloides* has emerged as a robust host for production of microbial lipids as alternative biofuel feedstocks. Oxygen supply is a limiting factor for microbial lipid production, as lipid biosynthesis is highly oxygen-demanding. *Vitreoscilla* hemoglobin (VHb) is a protein capable of promoting oxygen delivery for anabolism. In this study, we developed *R. toruloides* with VHb expression for improved lipid production. The VHb expression cassette was integrated into the *R. toruloides* chromosome via the *Agrobacterium*-mediated transformation. In shake flask cultures, the engineered strain 4#-13 produced 34% more lipids than the parental strain did. Results obtained under reduced aeration conditions in 3 L bioreactor showed that lipid titer and lipid yield of the engineered strain 4#-13 were 116% and 71%, respectively, higher than those of the parental strain. Under high cell density culture conditions, the engineered strain 4#-13 grew faster and produced 72% more lipids. Our results demonstrated that the VHb gene is functional in *R. toruloides* for promoting lipid production. The strains described here may be further engineered by integrating extra genetic parts to attain robust producers for more valuable products. This should improve the economics of microbial lipids to facilitate a sustainable production of biodiesel and other lipid-based biofuels.

Keywords: microbial lipids; oxygen supply; *Rhodosporidium toruloides*; *Vitreoscilla* hemoglobin

1. Introduction

Microbial lipids are alternative resources for production of foods, chemicals, biofuels, and medicines [1]. Oleaginous microorganisms are those that can produce lipids intracellularly to more than 20% of dry cell weight [2,3]. Strains of the red yeast *Rhodosporidium toruloides* can accumulate lipids up to 70% and use a variety of carbon sources such as glucose, xylose, glycerol, and cellulosic biomass hydrolysates [4–7]. Microbial lipids from oleaginous yeasts are mainly accumulated as triacylglycerides (TAG), of which are composed of C_{16} and C_{18} series of long chain fatty acids. As lipids from diverse sources with a wide range of fatty acid compositional profiles are suitable for biodiesel production [8], microbial lipids from red yeasts have also been demonstrated as feedstocks for biodiesel production [9]. *R. toruloides* also has emblematic characteristics of industrial yeasts, such as being capable of high cell density culture and robust stress resistance [6,10]. High cell density culture of *R. toruloides* has been reported for higher lipid productivity [5,10], however, at the later stage of these cultures, the broth became viscous, which reduced the dissolved oxygen (DO) capacity and inhibited production formation [11]. Conventional methods of

enhancing oxygen transport capacity are to increase aeration rate [12] and use oxygen vector [13] or pure oxygen gas [14], however, those methods are costly.

Vitreoscilla hemoglobin (VHb) is a protein that functions as an oxygen-binder to help bacteria maintain normal growth state even under anoxic conditions [15]. The rate constant of oxygen binding of VHb is 78 μM^{-1} s^{-1}, and its rate constant of oxygen dissociation is 5600 s^{-1}, which is hundreds of times larger than those of other hemoglobins [16,17]. Intracellular oxygen is involved in the oxidative phosphorylation, which participates in adenosine triphosphate (ATP) production, thus, promoting cell growth and product formation [18]. Therefore, VHb is widely used to enhance oxygen transfer efficiency in both prokaryotic and eukaryotic fermentation processes for metabolites accumulation in diminished oxygen environment [19,20]. For example, intracellular expression of VHb improved *Trichoderma reesei* cell growth along with cellulase production during submerged fermentation under hypoxic conditions [21]. Likewise, expression of VHb improved bacterial cellulose production by *Gluconacetobacter xylinus* [19].

The purpose of this study is to integrate the VHb gene into the chromosome of *R. toruloides* and to identify recombinant strains for improved lipid production especially under high cell-density culture conditions. It is assumed that the VHb protein can facilitate oxygen uptake for more ATP production to fuel cell growth and lipid biosynthesis (Figure 1).

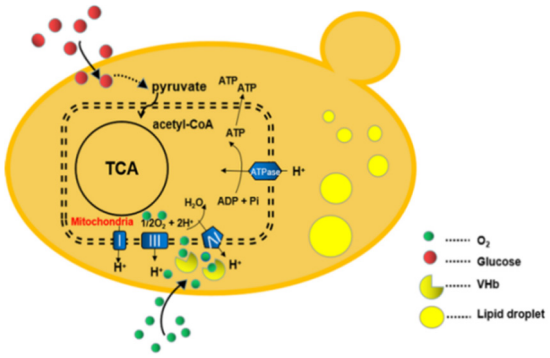

Figure 1. Schematic VHb-expression model regulating lipid production in *R. toruloides*. Oxygen gets into the cell and binds to VHb in the cytoplasm; the oxygen that bound to VHb is released in mitochondria, participates in the electron transport chain and produces more ATP for cell growth and lipid biosynthesis.

2. Materials and Methods

2.1. Strains, Plasmid, and Medium

Table S1 lists the used strains and plasmids in the current study. The *R. toruloides* strain CGMCC 2.1389 (4#) was availed from China General Microbiological Culture Collection Center. All the used yeast strains were cultured using YPD (yeast extract peptone dextrose) medium that contained (g/L): glucose 20, yeast extract 10 and peptone 20, (pH 6.0) at 30 °C, and the medium (limited nitrogen medium) for lipid production consisted of g/L: glucose 70, ammonium sulfate 0.1, yeast extract 0.75, potassium dihydrogen phosphate 1.0, and magnesium sulfate 1.5, (pH 5.8). *Escherichia coli* DH5α was used for the construction of vectors and was cultivated in Luria-Bertani medium (g/L: tryptone 10, NaCl 10 and yeast extract 5) at 37 °C. *Agrobacterium tumefaciens* (AGL1) was employed for *Agrobacterium*-mediated transformation (AMT) of *R. toruloides*; the strain was cultured at 30 °C in LB medium. The *E. coli* and the electrotransformation transformants of *A. tumefaciens* were selected on kanamycin (50 μg/mL) supplemented LB agar plates. The transformants of yeast were selected on hygromycin B (50 μg/mL) supplemented YPD agar plates.

2.2. Reagents

All DNA polymerases and other restriction enzymes were acquired from TaKaRa (Dalian, China). Kanamycin, ampicillin, hygromycin B, and acetosyringone were acquired from Dingguo Biotech Co. Ltd. (Beijing, China). The peptone, tryptone and yeast extracts were produced by Oxiod (Basingstoke, UK). The primary antibody and goat anti-mouse IgG labelled with HRP were purchased from Beyotime Biotech (Shanghai, China). All of the primers used (Table S2) were manufactured by Synbio Tech (Suzhou, China).

2.3. Plasmid Construction

The *vgb* gene (MT312225) encoding *Vitreoscilla* hemoglobin was optimized based on codon usage in *R. toruloides* [22], and the *vgb* gene was optimized and synthesized by Genewiz Company (Suzhou, China). All the vectors were constructed by using the plasmid pZPK as the framework [23]. To construct the plasmid pZPK-P_{PGK}-*hyg*-T_{NOS}-P_{LDP1}-*vgb*-T_{HSP}, firstly we got the plasmid pZPK-P_{PGK}-*hyg*-T_{NOS}-P_{GPD}-*vgb*-T_{HSP}. Then, we got the target vector by RF cloning method [24], which used Tnos-LDP1-RF1-f and Tnos-LDP1-RF1-r, and the promoter GPD was replaced by promoter LDP1 (Figure S1). All plasmid constructs have been verified by DNA sequencing.

2.4. R. toruloides Transformation and Screening of Clones

Transformation of *R. toruloides* was based on AMT methods [23]. Briefly, *A. tumefaciens* cells were transformed with the correct plasmid by electrotransformation and selected on LB plates supplied with kanamycin (50 μg/mL). Positive *A. tumefaciens* cells were cultivated in LB medium with kanamycin (50 μg/mL) at 30 °C for 24 h. At the same time, the cells *R. toruloides* were also cultivated at 30 °C in YPD medium for 24 h. The bacterial and fungal cells were collected at 13,000 rpm centrifugation for 30 s, washed once and diluted to OD_{600} = 0.6 with sterile water. The cell suspension of 100 μL each was mixed, and co-cultured onto IM agar plates with acetosyringone (200 μM) at 24 °C for 48 h. The cultures were then transferred onto the YPD plate with 50 μg/mL hygromycin B until the clones appeared. The mitotic stability and the genotype of the transformants were confirmed according to the published method [23].

2.5. Western Blot Analysis

Western blot analysis was employed to detect VHb expression in *R. toruloides*. Briefly, protein samples were prepared and separated on 15% SDS-polyacrylamide gel according to our previous work [23]. Then, the samples were shifted to nitrocellulose membrane (Salarbio, Beijing, China). The membrane was blocked at 37 °C in PBS-Tween buffer for 1 h, hatched with the primary anti-his_6 antibody at 37 °C for 1 h, and followed by the secondary antibody HRP Goat Anti-mouse IgG antibody for 1 h. The results were envisioned with Tanon High sig ECL Western Blotting Substrate (Tanon, Shanghai, China).

2.6. VHb Activity Detection

The transformants grown at 30 °C for 24 h in YPD medium were collected at 8000 rpm centrifugation for 5 min, and washed twice with ultrapure water and one time with 100 mM potassium phosphate buffer (pH 7.4). Before putting on the pre-cooling super high pressure equipment, all the cells were resuspended in the buffer. Then, the cells suspension was broken three times at 27 kpsi pressure (1800 bar), then collected, centrifuged at low temperature, and the supernatant was assayed using carbon monoxide difference spectra to detect the VHb as previously described [25].

2.7. Shaking Flask Cultures

The seed cultures of *R. toruloides* were prepared by inoculation of 50 mL YPD medium in a 250 mL Erlenmeyer flask with a loopful of fresh cells and holding at 30 °C and 200 rpm shaking for 24 h. For lipid production, 5% (v/v) seed culture was transferred into the limited nitrogen medium, and the culture was held at 200 rpm and 30 °C. To compare the effects of oxygen availability on cell growth,

cultures were performed within 100 mL YPD medium in 250 mL Erlenmeyer flasks with *R. toruloides* 4#, *R. toruloides* 4#-13 and their mixtures at an identical initial cell density. The cultures were sampled every 24 h for analysis of cell density, lipids and residual glucose. An optimal strain was selected for further lab scale lipid production in a 3 L bioreactor.

2.8. Two-Stage Fed-Batch Flask Cultures

Two-stage culture strategy was employed with a high initial cell density [26]. Shortly, cells of both *R. toruloides* 4# and *R. toruloides* 4#-13 were prepared in a media that contained 20 g/L glucose, 10 g/L peptone and 10 g/L yeast extract, at 30 °C, 200 rpm for 36 h, collected by centrifugation at 5000 rpm for 5 min, and washed twice with deionized water. The cells, corresponding to an initial concentration of 27.0 g/L, were transferred into 250 mL Erlenmeyer flasks and resuspended in 50 mL of 70 g/L glucose solution without any other auxiliary nutrients containing. The cultures were held at 30 °C and 200 rpm, sampled every 24 h for analysis of cell density and residual glucose. When residual glucose dropped below 10 g/L, the cultures were fed with glucose powder to a final concentration of 70 g/L. The lipid production was terminated after 216 h.

2.9. Fed-Batch Fermenter Culture

To compare lipid production profile under oxygen-limited conditions, both *R. toruloides* 4# and *R. toruloides* 4#-13 were grown in a 3 L fermenter at the same DO 30% of air saturation. Firstly, 20 mL of 24-h-old precultures were shifted into 180 mL of YPD media in 1000 mL shaking flasks and held at 30 °C, 200 rpm for 24 h. Then, all of the seed culture was introduced into a 3 L fermenter for lipid production at 30 °C, pH 5.5 maintained via 1.0 M HCl or 1.0 M NaOH as per demand [26]. The lipid production medium contained; glucose (70 g/L), yeast extract (0.75 g/L), $(NH_4)_2SO_4$ (0.1 g/L), KH_2PO_4 (1.0 g/L) and $MgSO_4$ (1.5 g/L).

2.10. Lipid Extraction and Quantification

At the end of fermentation, the yeast cells were collected at 8000 rpm centrifugation for 5 min, washed twice with de-ionised water and dried at 105 °C to constant weight. The cell mass was measured gravimetrically and mentioned in g/L (Total dry cells produced per litre). To extract the lipid, first the digestion of the dried cells was carried with 4 M HCl at 78 °C for 1 h with 200 rpm shaking, and then extracted with 1:2, *v/v* (methanol/chloroform). The extracts were then washed with 0.1% NaCl and passed via a pad of anhydrous Na_2SO_4; the solvents were evaporated under reduced pressure. The concentrated residues were dried at a constant weight at 105 °C; lipids were measured gravimetrically. The total lipid produced was indicated as lipid titer and expressed in g/L, whereas the cellular lipid content was expressed in percentage as gram lipid produced per gram cell mass. The lipid yield was calculated as gram lipid produced per gram substrate consumed.

2.11. Microscopy

We also checked the cellular morphology of the engineering strain 4#-13 and the wild strain 4#, both grown in a 3 L fermenter with the same DO level of 30%. Cellular morphological changes were detected by the EVOS microscope (Thermo Fisher, Waltham, MA, USA).

3. Results

3.1. Construction and Confirmation of VHb Expression in R. toruloides

VHb gene was optimized, synthesized, and used to construct the vector pZPK-P_{PGK}-hyg-T_{NOS} -P_{LDP1}-vgb-T_{HSP} through RF cloning [24]. Then the vector was transformed into the *R. toruloides* 4# genome by AMT. The transformants were screened on the hygromycin B plates and verified by PCR and sequenced. The genomic DNA samples of different transformants were used as templates for PCR reaction. Results showed a band at 0.7 kb for the recombinant strain, indicating successful integration

of the VHb gene into the chromosome (Figure 2a). The expression of VHb protein was verified by SDS-PAGE and Western blot method. The results showed that a 15.7 kDa band was observed in each transformant sample, which was indiscernible in the control sample (Figure 2b). Many studies showed CO binding to VHb, which is expressed in prokaryotes and eukaryotes, could form an absorption peak at 419 nm [25]. CO-difference spectra analysis was carried to verify the function of VHb. The expressed VHb was shown to be biochemically active, as higher absorption at 419 nm was observed in the CO-difference spectra of the cell extracts of the recombinant strain than those of the wild type strain 4# (Figure S2).

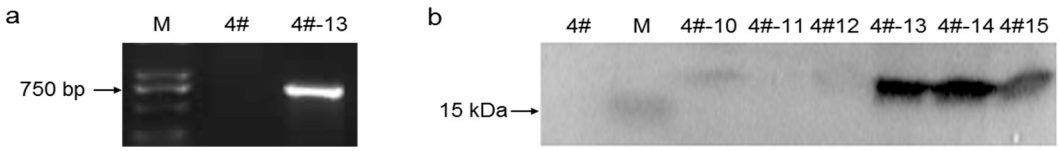

Figure 2. Results of expression of VHb in *R. toruloides* strains. (**a**) PCR amplification of VHb gene. M, DNA maker. (**b**) Western blot analysis of VHb expression. M. Protein molecular weight marker.

3.2. Lipid Production with Shaking Flask Cultures

It has been demonstrated that the expression of VHb could improve the production arachidonic acid by *Mortierella alpina* [27]. In this study, we intended to test whether the expression of VHb had any effects on lipid production by *R. toruloides*. The recombinant *R. toruloides* strains 4#-13, 4#-14, 4#-15 and the wild-type strain were cultured at 200 rpm in 50 mL culture in 250 mL shaking flasks for 96 h. It was found that all strains had similar profiles in terms of glucose consumption, cell growth and lipid accumulation (Figure 3a,b). It should be pointed out that the culture conditions were considered without oxygen limitation, under which neither advantages nor disadvantages were high upon the expression of VHb in *R. toruloides*.

As oxygen availability changes during the culture by which cell growth and lipid production may be affected differently, we hypothesized that effects of VHb might be more apparent at different culture stages. To verify this, we compared the culture processes of *R. toruloides* stains 4# and 4#-13 by analyzing residual glucose, cell mass and lipids at different time points. It was clear that *R. toruloides* 4#-13 consumed glucose more rapidly at the late stage of the culture (Figure 3c). Also, *R. toruloides* 4#-13 produced more cell mass (20.6 g/L) and lipids (12.7 g/L) than the wild type strain did (Figure 3d,e). The lipid content of the strain 4#-13 was 61%, roughly 22% higher than that of the strain 4# (Figure 3f).

To determine whether VHb expressed strains gain benefits due to improved oxygen utilization, we performed cultures in 250 mL shaking flasks at a volume of 100 mL, in which DO should be limiting. Results showed that *R. toruloides* 4#-13 consumed glucose much faster than *R. toruloides* 4# did (Figure 4). Interestingly, the mixed culture of 4# and 4#-13 also outperformed the pure culture of 4#. After 204 h, *R. toruloides* 4#-13 and 4# used glucose of 69 g/L and 58 g/L and produced cell mass of 25.6 g/L and 19.6 g/L, respectively. These data further confirmed that VHb expression in *R. toruloides* led to improved substrate consumption and cell mass formation, which might be attributed to the fact that VHb protein can facilitate oxygen assimilation for cell growth [18–20].

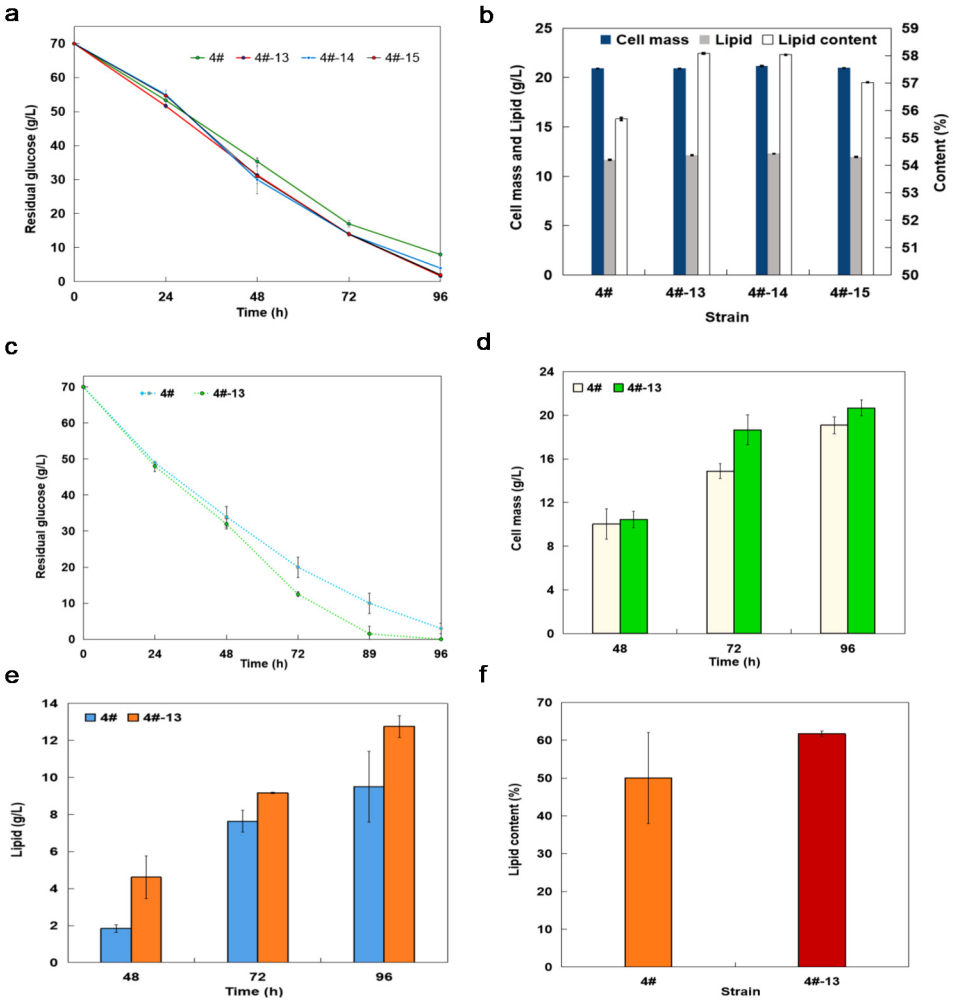

Figure 3. Lipid production with 50 mL cell culture in 250 mL shaking flasks by *R. toruloides* strains. Results of (**a**) residual glucose and (**b**) cell mass, lipids and lipid contents of initial screening cultures. Experiments were done in three technical replicates for 4# and with three colonies in three technical replicates for each recombinant strain. Data are means ±SD of three replicates. Results of (**c**) residual glucose, (**d**) cell mass, (**e**) lipids, and (**f**) lipid contents of lipid production cultures are done in two technical replicates. Data are means ±SD of two replicates.

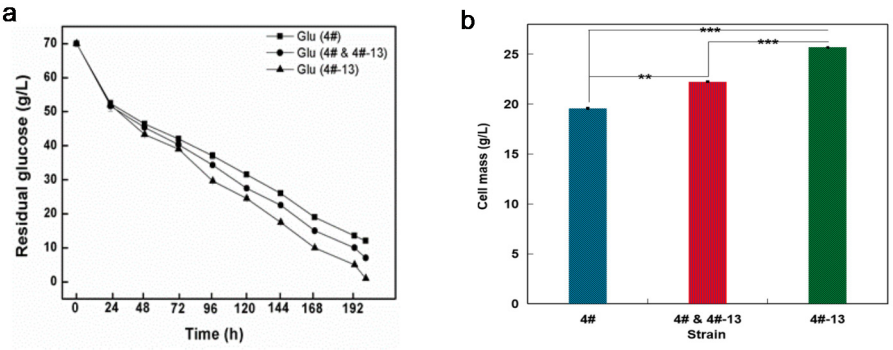

Figure 4. Lipid production with 100 mL cell culture in 250 mL shaking flasks by *R. toruloides* strains. (**a**) Evolution of residual glucose. (**b**) Cell mass of cultures with different strains. The experiments were done in three technical replicates. Statistical significance (** $p < 0.01$; ***, $p < 0.001$) was determined by one-tailed Students, *t*-test. Data are means ±SD of three replicates.

3.3. Lipid Production in 3 L Fermenter under a Reduced DO Level

We further hypothesized that the engineered strain *R. toruloides* 4#-13 might attain advantages when cultured under well-controlled yet limited oxygen supply. To testify this, we compared the cultures in a 3 L fermenter with DO at 30% of air saturation. It should be noted that lipid production in fermenters was normally run with DO at above 40% of air saturation [6,10,28]. The results are shown in Figure 5. It was found that *R. toruloides* 4#-13 consumed glucose much faster than the wild type strain, and after 72 h, the residual glucose was 7.0 g/L for the former (4#-13) and 21.5 g/L for the later (4#). In addition, the cell mass of the recombinant and the wild type strain reached 24.2 g/L and 14.6 g/L, respectively (Figure 5b). Lipid titer of 15.1 g/L of the recombinant strain was 116% higher than that (7.0 g/L) of the wild-type strain (Figure 5c). Thus, lipid productivity of the wild type and recombinant strain was 0.10 g/L/h and 0.21 g/L/h, respectively, indicating 110% improvement for the recombinant strain. Lipid yields were estimated as 0.24 g/g and 0.14 g/g, respectively, by the recombinant and wild type strain, indicating a 71% improvement for the recombinant strain. These results confirmed that, with DO at 30% saturation, the recombinant strain 4#-13 outperformed the wild type strain 4# in terms of substrate consumption, cell growth and lipid production, suggesting that VHb expression in *R. toruloides* improved oxygen utilization for more robust lipid production under oxygen insufficient conditions. Moreover, it was noted that lipid droplets of the recombinant strain were larger than those of the wild type strain (Figure S3, Table S3). Similarly, it has been known that expression of VHb can enhance cell size [29].

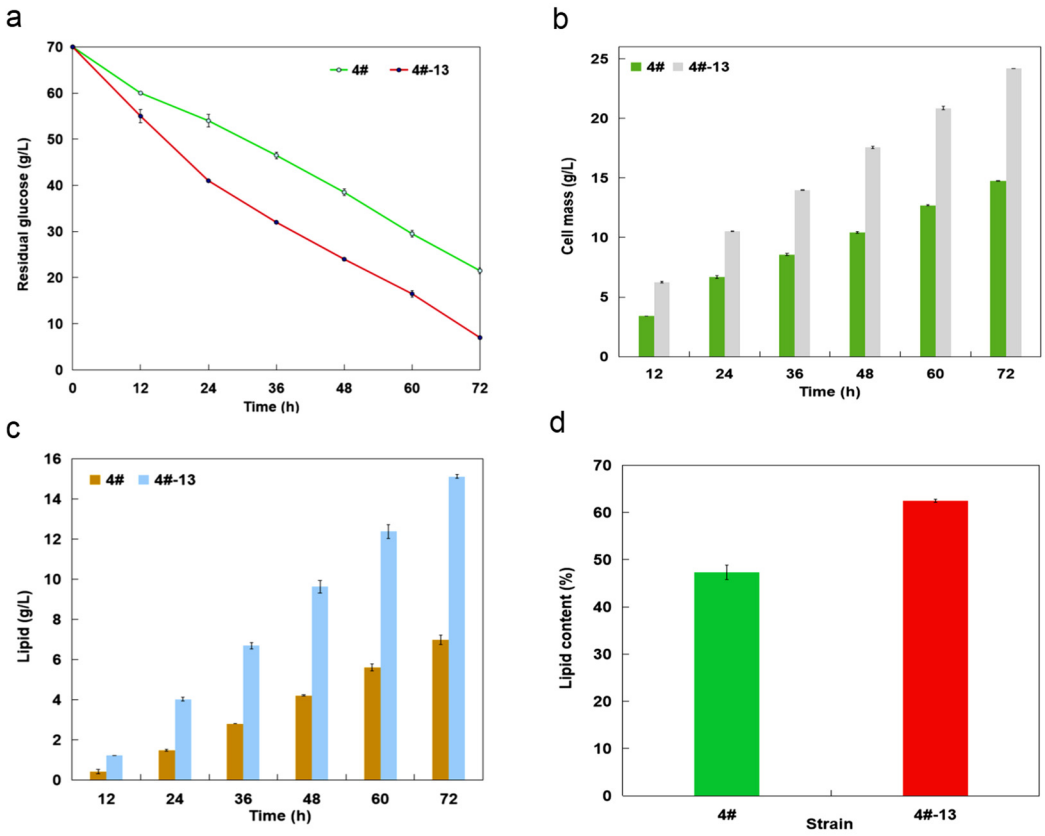

Figure 5. Lipid production in 3 L fermenter at a DO level of 30% by *R. toruloides* strains. Evolution of (**a**) residual glucose, (**b**) cell mass, and (**c**) lipids. (**d**). Cellular lipid contents. The experiments were done in two technical replicates. Data are means ±SD of two replicates.

3.4. Lipid Production on Glucose under a Two-Staged Process

Our previous study established a two-staged process where cultures were set at high initial cell densities for lipid production on glucose solution without auxiliary nutrients [26]. However, oxygen deficiency

might occur and lipid production was limited when cells were cultivated at a high cell density. To test our hypothesis that the expression of VHb facilitates lipid production on glucose under a two-staged process, we conducted trials in shaking flasks with initial cell density of 27.0 g/L (OD_{600} 78.6). Indeed, *R. toruloides* 4#-13 gave much better results than the wild type strain 4# (Figure 6). Specifically, the culture of *R. toruloides* 4#-13 reached an OD_{600} of 127.6 and used 68.6 g/L glucose within 96 h, while 4# afforded an OD_{600} of 105.3 and used only 56.3 g/L glucose. After 96 h, each culture was fed with appropriate amounts of glucose powder to a final glucose concentration of 70.0 g/L glucose concentration, and the experiments continued (Figure 6a). At the time point 156 h, the OD_{600} of 139.8 and glucose consumption of 59.0 g/L were achieved with the recombinant strain, however, the wild type strain showed an OD_{600} of 116.1 and glucose consumption of 43.0 g/L. Again, glucose powder was fed to each culture to a final glucose concentration of 70.0 g/L, and the experiment was stopped after 216 h. The final OD_{600} reached 144.8 for the recombinant strain while it was 119.7 for the wild type strain. Likewise, *R. toruloides* 4#-13 consumed more glucose (62.0 g/L) after 216 h, while the wild type strain 4# consumed less glucose (47.0 g/L). In total, *R. toruloides* 4#-13 consumed 189.0 g/L glucose whereas the wild type strain 4# only consumed 147.0 g/L glucose. Final cell mass, lipid titer and lipid content of the culture with *R. toruloides* 4#-13 were all considerably higher than those with the wild type strain 4# (Figure 6b). Based on the lipid titer of 49.0 g/L, lipid yield reached 0.25 g/g consumed glucose by *R. toruloides* 4#-13, whereas a lower lipid titer of 28.4 g/L and a lower lipid yield of 0.19 g/g were observed for the wild type strain 4#. Thus, the culture with *R. toruloides* 4#-13 gave improved lipid titer and lipid yield by 72% and 31%, respectively. Together, these results clearly indicated that VHb expression in *R. toruloides* offered advantages for high cell density cultures.

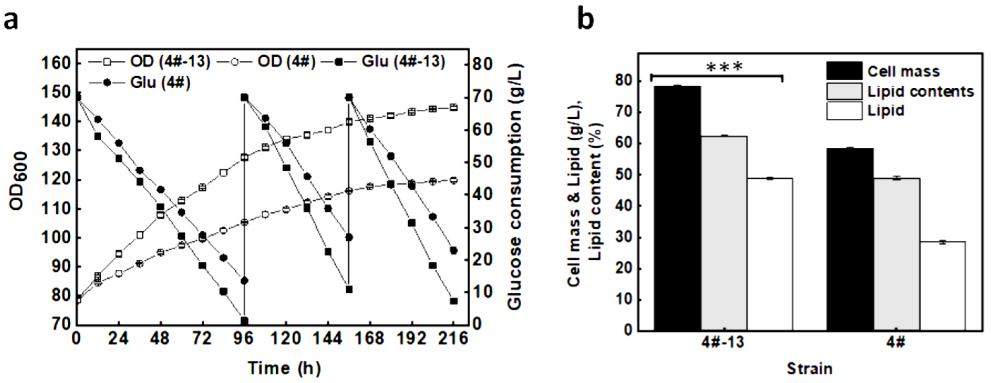

Figure 6. Lipid production on glucose solution at high cell density in 250 mL shaking flasks by *R. toruloides* strains. (**a**) Evolution of cell growth and residual glucose. (**b**) Results of final cell mass, lipids and lipid contents. The experiments were done with three technical replicates. Statistical significance (***, $p < 0.001$) was determined by one-tailed Students *t*-test. Data are means ±SD of three replicates.

4. Discussion

In this study, the VHb expression vector was constructed and transformed into *A. tumefaciens*. Transformation through the AMT method was done to integrate the VHb expression cassette into the genome of *R. toruloides* AS 2.1389 (4#). Positive recombinant strains were confirmed by PCR analysis and sequencing. Initial lipid production cultures of recombinant strains in shaking flasks revealed that the strain *R. toruloides* 4#-13 produced cell mass with much higher lipid content. Further comparison experiments showed that *R. toruloides* 4#-13 consumed glucose faster and produced more lipids than the wild type strain did. When cultivated in 3 L fermenter under reduced aeration conditions, the recombinant strain 4#-13 produced 116% higher lipid titer with 71% higher lipid yield than the wild type strain did. These results suggested that the expression of VHb in *R. toruloides* was beneficial to cell growth and lipid production, likely due to improved oxygen utilization capacity. Moreover, when an initial cell mass of 27.0 g/L was employed for two-staged cultures, cell mass and lipid titer of the recombinant strain 4#-13 were improved by 34% and 72%, respectively, compared to those of the

wild type strain. Previous studies showed that cell mass could reach over 100 g/L when *R. toruloides* 4# was cultivated in advanced 15 L stirred-tank bioreactor with enhanced aeration [6,7]. We expected that *R. toruloides* 4#-13 should give even better results because it outperformed *R. toruloides* 4# in different culture conditions described here.

It should be noted that recombinant *Aureobasidium melanogenum* strain with VHb expression was found to grow faster and produce more pullulan in shaking flask cultures than the wild type strain [30]. Similarly, a *Yarrowia lipolytica* strain with VHb expression produced 24% more cell mass when cultivated in 3 L fermenter under oxygen limited condition (DO 30% of saturation) [29], while in this study, *R. toruloides* 4#-13 produced 66% more cell mass than that of the wild type strain. More interestingly, we found that cellular lipid droplets of *R. toruloides* 4#-13 were larger than those of the wild type strain, suggesting that the expression of VHb improved oxygen utilization, which further coordinated many other biological processes such as lipid droplet homeostasis. To gain more detailed insights into the molecular basis of VHb function in *R. toruloides*, a systems approach may be employed as has been done for the analysis of the mechanism of lipid production under phosphate limitation [31].

We and others have demonstrated that lipids produced from sugars by wild-type and engineered *R. toruloides* species are isolated mainly as neutral lipids and that the fatty acid compositions of these products are relatively conserved and mainly C_{16} and C_{18} long chain fatty acids [8,31–33]. It should be noted that biodiesel could be prepared from lipids of diverse origins with major fatty acid compositional variations [8]. Recently, *R. toruloides* has emerged as an excellent host for the production of functional lipids, carotenoids and related metabolites [34,35]. For example, engineered *R. toruloides* strains have been documented for the production of nutraceuticals such as linoleic acid-rich lipids [36], and terpenoids such as bisabolene [37] and *ent*-kaurene [38]. Notably, to scale up the production of those intracellular products, it is essential to operate with high cell density cultures, which means that the processes are subjected to restriction due to inefficient oxygen supply. Also, lipid production has been well-known as an oxygen-demanding process [39], and oxygen availability has been identified as a major limiting factor for lipid production from lignocellulosic biomass [40]. Therefore, co-expression of the VHb gene with those functional genes may provide advanced strains.

In summary, here we developed *R. toruloides* strains with heterogonous expression of VHb and achieved better lipid production profiles. Further researches may focus on lipid production on other substrates at a larger scale with these strains. In addition, these strains may be further engineered by integrating additional genetic elements to attain robust producers for more valuable products. This should improve the economics of microbial lipids to facilitate a sustainable production of biodiesel and other lipid-based biofuels.

Supplementary Materials: The following are available online at http://www.mdpi.com/1996-1073/13/17/4446/s1, Figure S1: Vector construction, Figure S2: CO differential chromatograms, Figure S3: Cell morphological differences, Table S1: Strains and plasmids used in this study, Table S2: Primer used in this study, Table S3: Quantifications of lipid droplets.

Author Contributions: S.W. and R.K. conducted the experiments and wrote the manuscript. Y.Z. assisted the designing part of experiments. Y.Z., R.Z., L.L. and Q.H. revised the manuscript. S.Z., S.Q. and Z.K.Z. conceived the study, designed the experiments and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by National Natural Science Foundation of China (31870042 and 51761145014), and Dalian Institute of Chemical Physics, CAS (DICP I201947 and DMTO 201701).

Acknowledgments: The authors thank Wenliang Zhu from Dalian Institute of Chemical Physics, CAS, China for providing CO gas.

Conflicts of Interest: The authors declare no conflict of interest in this paper.

Abbreviations

AMT: *Agrobacterium* mediated transformation; ATP: Adenosine triphosphate; CGMCC: China General Microbiological Culture Collection Center; CO: Carbon monoxide; DNA: Deoxyribonucleic acid; DO: Dissolved oxygen; GPD: Glyceraldehyde-3-phosphate dehydrogenase; LB: Luria-Bertani medium; LDP1: Lipid droplet protein 1; OD: Optical

density; PBS: Phosphate buffered solution; PCR: Polymerase chain reaction; SDS: Sodium lauryl sulfate; VHb: *Vitreoscilla* hemoglobin; YPD: Yeast extract peptone dextrose medium; TAG: triacylglycerides.

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