Fast Measurement of Lipid Content of Oleaginous Yeast *Trichosporon dermatis* Cultured in Lignocellulosic Hydrolysates Using Fluorescent Method

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(Received on 5th March 2019, accepted in revised form 21st October 2020)

Summary: To avoid complex procedures in measurement of lipid content of oleaginous yeast especially for that can accumulate microbial lipid in lignocellulosic hydrolysates, fluorescent method using Nile Red as fluorescent dye was applied to measure lipid content of oleaginous yeast *Trichosporon dermatis*. The fluorescent method was built by fitting of lipid content identified by both conventional gravimetric method and fluorescence intensity of oleaginous yeast. Within the range of lipid content measured, the fitting curves showed linear relationship with good correlation coefficient (R^2=0.95), showing this method is suitable for measuring lipid content of *T. dermatis* in the simulated medium. To evaluate the applicability of this method for lipid fermentation using lignocellulosic acid hydrolysates as substrate, *T. dermatis* was cultured in corncob acid hydrolysate and rice straw acid hydrolysate and then its lipid content measured by both fluorescent method and gravimetric method were compared. The results showed that the lipid content measured by these two methods were close, therefore, this method was promising for the application in lipid fermentation in lignocellulosic acid hydrolysates.

Keywords: Microbial lipid; Fluorescent method; Lipid content; *Trichosporon dermatis*; Lignocellulosic hydrolysates.

Introduction

Microbial lipid, mainly produced by oleaginous microorganisms, is focused by many researchers because of its important function in both food and energy industry [1, 2]. Generally, oleaginous microalgae and oleaginous yeast are two main microorganisms for microbial lipid production. For microalgae, various microalgae can accumulate microbial lipid with relatively high lipid content and fast rate, and more specially, microalgae can produce microbial lipid by both autotrophic and heterotrophic modes [3]. As for oleaginous yeast, it can accumulate microbial lipid even with a faster rate than that of oleaginous microalgae and more importantly, it can utilize lignocellulosic hydrolysates for lipid accumulation efficiently [4, 5]. Lignocellulosic hydrolysates, the products generated after hydrolysis of lignocellulosic biomass which is one low-cost, renewable, and the most available bio-resource in nature, contain various fermentable sugars and can be one suitable substrate for fermentation. Both acid and enzymatic methods can be utilized for hydrolysis of lignocellulosic biomass to generate lignocellulosic hydrolysates. Enzymatic hydrolysis is one green bioprocess which can be carried out in mild condition, but the cost of cellulase is still too high for large-scale application. Compared with enzymatic hydrolysis, although acid hydrolysis requires high temperature and pressure, it is more efficient and the cost is much lower [6, 7]. Recently, using lignocellulosic hydrolysates to produce microbial oil production has been considered as one attractive method for industrial application because it can reduce the cost of microbial oil production greatly [8].

After lipid fermentation, measurement of lipid content of oleaginous microorganisms is one necessary procedure to determine the lipid yield. For a long time, gravimetric method after extraction by chloroform and methanol is mainly used for determination of lipid concentration [9,10]. Generally,

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this method has high accuracy and wide applicability, but it also has some disadvantages such as requiring long period of measurement, using great amount of organic solvents, and complex operation. Recently, fluorescent method using Nile Red [11, 12] or BODIPY 505/515 [13] as fluorescent dye has attracted much attention for lipid content measurement of oleaginous microorganism due to its advantages of rapid measurement, easy for operation, and avoiding using great amount organic solvents [14,15].

To date, fluorescent method used for lipid content measurement is mainly applied for oleaginous microalgae [16, 17] and merely a few studies used fluorescent method to determine the lipid content of oleaginous yeast [11] especially for that can produce microbial lipid from lignocellulosic hydrolysates. To increase the efficiency of measurement of lipid content for oleaginous yeast, it is necessary and wise to build fluorescent method to measure lipid content of oleaginous yeast especially for that can utilize lignocellulosic hydrolysates for microbial lipid production.

Trichosporon dermatis CH007 is one oleaginous yeast that can accumulate microbial lipid efficiently from various lignocellulosic hydrolysates [18,19]. To evaluate the possibility of using fluorescent method for measurement of its lipid content, Nile Red was used to stain the cells for *T. dermatis* and also its lipid content was determined by the fluorescence intensity. For building the Nile Red fluorescent method, the linear relationship of Nile Red fluorescence intensity and the lipid content of *T. dermatis* measured by conventional gravimetric method was established. Besides, its applicability and generality for lipid fermentation of *T. dermatis* in lignocellulosic acid hydrolysates was evaluated by measurement of lipid content of its cells obtained from corncob acid hydrolysate and rice straw acid hydrolysate.

Experimental

Oleaginous yeast and cultural conditions

Oleaginous yeast *Trichosporon dermatis* CH007 was obtained from Energy and Chemical Laboratory, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences. Corncob acid hydrolysate and rice straw acid hydrolysates were obtained from by ZHONGKE New Energy Technological Development Co., Ltd (Huai-An, China). The main components and concentration of the lignocellulosic acid hydrolysates are shown in Table-1. Synthetic medium (g/L, glucose 50, peptone 0.25, yeast extract 0.25), corncob acid hydrolysate and rice straw acid hydrolysates were used as lipid fermentation medium. The oleaginous yeast was cultured with 160 rpm and 28ºC for 24 hours firstly in the pre-cultivation medium (pH=6.0) including (g/L) glucose 20, peptone 10, and yeast extract 10. Then 5% (v/v) seed culture solution was inoculated into 50 mL fermentation broth in a 250 mL Erlenmeyer flask with 160 rpm and 28ºC.

Table-1: The main components and concentration of corncob acid hydrolysate and rice straw acid hydrolysate.

	Corncob acid hydrolysate	Rice straw acid hydrolysate
D-glucose (g/L)	2.57	6.10
D-xylose (g/L)	31.95	17.75
L- arabinose (g/L)	3.96	3.25
Formic acid (g/L)	0.81	1.84
Acetic acid (g/L)	3.92	1.38
Levulinic acid (g/L)	0.59	0.47
Furfural (g/L)	0.08	0.14
5-hydroxymethylfurfural (g/L)	0.03	0.02

Observation of oleaginous yeast cell by fluorescent microscope

To prove the possibility of Nile Red fluorescent method for measuring lipid content, the oleaginous yeast cells stained with Nile Red were observed under fluorescent microscope firstly. The fermentation was carried out for 5 days to obtain yeast cells with high lipid content in order to observe the bright fluorescence. After pretreating the fermentation broth by the same way, the cell suspension was stained with Nile Red and incubated in darkness at different periods. Then, the cell suspension was dropped onto the glass slide and observed under the inverted fluorescence microscope (Leica DMI6000B, Germany) in both fluorescent field and light field. The excitation wavelength was set at 488nm. And the 40 objective lens and 10 eye lens were chosen in dry immersion.

Building Nile Red fluorescent method for measurement of lipid content

During fermentation, two samples of fermentation broth were taken out periodically, and the cells in broth were collected by removal of supernatant after centrifugation in 8000 rpm for 10 minutes for twice. Then, the cells were re-suspended with deionized water to adjust the cell density at OD_{600}=1.0 by the ultraviolet and visible spectrophotometer (725, Shanghai Spectral Instrument Co. Ltd, China). Next, the cell suspension solution was stained with Nile Red solution at the same staining condition (showing below) and the fluorescence intensity was measured by fluorescence spectrophotometer (LS-55, Perkin Elmer, US) for three

times. Before measurement, the sample was pre-scan and the emission wavelengths (570/10 nm) and excitation wavelengths (488/10 nm) were chosen, respectively. For the measurement, the detail procedure is shown as follows: Firstly, 2.4 mL cell suspension solution was added, then 300 μL dimethyl sulfoxide (DMSO) was mixed with the cell suspension and standing for 3 minutes to improve cell permeability. After that, 300 μL Nile Red stock solution (dissolved in the acetone to a concentration of 0.05 mg/mL) was put into cell suspension solution after shaking about 10 seconds with the final concentration of Nile Red at 5 μg/mL. After incubating five minutes in darkness, the fluorescence intensity of cell suspension was determined by fluorescence spectrophotometer immediately for three times. Besides, the fluorescence intensity of cell suspension and auto-fluorescence of Nile Red were also detected by the same way. The value of fluorescence intensity for intracellular lipid was calculated by the total fluorescence intensity subtracting the fluorescence intensity of cell suspension and Nile Red auto-fluorescence intensity. The cuvette was washed twice with distill water before measuring each time. The values were the averages of three measurements and expressed as a mean ± standard deviation.

To build Nile Red fluorescent method for lipid content measurement, the lipid content of oleaginous yeast was measured using the conventional gravimetric method. In detail, fermentation broth was withdrawn and the cell biomass of *T. dermatis* was collected after centrifugation. Then the cell biomass was dried to constant weight and the intracellular lipid was extracted from dry cell mass using the modified procedures according to previous studies [10,20] with a mixture of chloroform/methanol (v/v, 2:1). The intracellular lipid was extracted and purified after removing the solvent by vacuum rotatory evaporator (RE52CS-1, Shanghai YaRong Biochemical Instrument Factory). Lipid content was defined as the percentage of lipid to cell dry mass (%, w/w). The values were the averages of two measurement.

Finally, the linear relationship between lipid content measured by traditional gravimetric method and fluorescence intensity was built, and this was used to determine the lipid content by evaluating the fluorescence intensity of cellular lipid.

Measurement of lipid content of T. dermatis by fluorescent method in lignocellulosic hydrolysates

To evaluate the Nile Red fluorescent method built above was suitable for the actual fermentation system or not, the oleaginous yeast *T. dermatis* was cultured in different lignocellulosic hydrolysates (corncob acid hydrolysate and rice straw acid hydrolysate). The lipid content of *T. dermatis* was measured by both fluorescent and gravimetric methods described above. If both lipid content were close, it could be considered that the Nile Red fluorescent method was suitable to be applied for lipid fermentation in lignocellulosic hydrolysates.

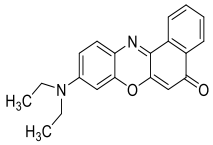
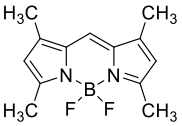
Results and discussion

Observation of oleaginous yeast cell staining with Nile Red in fluorescence microscope

Fluorescence probes such as Nile Red or BODIPY 505/515 which structure are shown as Table-2 have a good hydrophobic property and the capacity of lipid binding [21,22]. As shown in Table-2, the structural formula of BODIPY 505/515 was symmetrical distributing with the four methyl resulting in its good hydrophobicity and stability. As for optical properties, both of two fluorescence probes could combine with lipid especially intracellular lipid and generate certain fluorescence when it was exposed to different lights [23]. Therefore, they could be applied for measuring lipid content according to the fluorescence intensity. However, compared with Nile Red, BODIPY 505/515 is usually applied for the visual observation of intracellular lipids because its fluorescence intensity is more stable in water solution leading the value of background fluorescence intensity to be a little higher with the application of measuring lipid content. Hence, in generally, Nile Red is always applied in the quantitative determination of intracellular lipids and BODIPY 505/515 is better for visual observation of cellular lipids [24]. Also, besides the determination of lipid content, Nile Red could be applied for staining certain protein [25, 26].

In this study, Nile Red was used for staining oleaginous yeast *T. dermatis* in order to measure its lipid content because Nile Red can combine with cellular lipid and generate certain fluorescent light [16]. The process of the combination of Nile Red and cellular lipid has been elucidated in some researches [27, 28]: in generally, the penetration process of Nile Red had two different steps including the fast insertion/dissociation into plasma membrane (1s and 2s) and slowly transferred between the plasma membrane and the lipid droplets (30s to 2 mins). The possible detail process was as followed: some fluorescence probe molecule firstly adhered to the cell surface by the molecular diffusion because of the dissolution principle of similar phase when it was added into cell suspension. Then, some molecule of fluorescence probe without quenching permeated across cell membranes into plasma membrane based on the fluidity of cell membrane. Last, the fluorescence molecule was slowly diffused from the plasma membrane to the lipid droplets.

Table-2: Characteristics and application of fluorescence probe Nile Red and BODIPY 505/515.

	Nile Red	BODIPY 505/515
Chemical formula	$C_{13}H_{15}BF_2N_2$	$C_{20}H_{18}N_2O_2$
Molecular weight	248.08	318.37
Structural formula		
Hydrophobic/hydrophilic property	Hydrophobic	Hydrophobic
Structural symmetry	Dissymmetric	Bilateral symmetry
λex/λem[a]	488 ~ 530/575 ~ 580 nm	488/510 nm
Solution	Acetone/ Glycerinum	DMSO[b]
Fluorescence	Orange yellow ~ Red	Green
Application	Better for quantitative analysis	Better for qualitative observation

Notes: [a] the values of excitation and emission wavelength are from reference [16]
[b] dimethyl sulfoxide

In order to make clear the mechanism of dyeing and build the fluorescent method, the Nile Red staining in the cell of oleaginous yeast *T. dermatis* was observed using fluorescence microscope firstly. As shown in Fig. 1, Nile Red had been entered into the cell of oleaginous yeast *T. dermatis* and combined with the cellular lipid where the golden yellow fluorescence was generated clearly at the same place under both fluorescent field and bright field. To evaluate the stability of Nile Red staining, the images of fluorescence microscope for staining time ranged from 5 to 30 mins were compared (Fig. 1 A-D). Interestingly, there was no obvious difference for these images in the Fig. 1, indicating that the staining of Nile Red for oleaginous yeast was stable during the period of 5 to 30 mins. Overall, the fluorescence image of Nile Red partly proved that Nile Red could penetrate into oleaginous yeast.

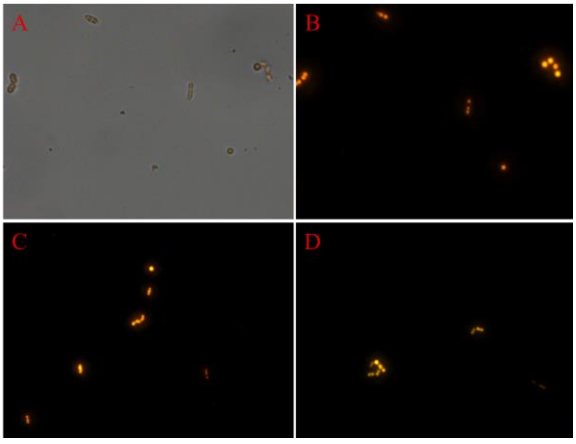


Fig. 1: The images of fluorescence microscope for *T. dermatis* stained with Nile Red at different staining time both in bright field and fluorescent field (A stained for 5minutes in bright field; B stained for 5 minutes in fluorescent field; C stained for 10 minutes in fluorescent field; D stained for 30 minutes in fluorescent field).

Evolution of fluorescence intensity and lipid content during lipid fermentation of T. dermatis

In many previous studies, the Nile Red fluorescence intensity and the intracellular lipid content of oleaginous microorganisms (mainly for microalgae) had certain linear correlation and therefore the lipid content of oleaginous microorganisms can be calculated using the fluorescent method by measuring the value of Nile Red fluorescence intensity [12, 15]. It is meaningful because the fluorescent method can overcome the disadvantages of the conventional gravimetric methods such as complicated operating steps, more toxicant, time-consuming, etc. For lipid fermentation of *T. dermatis* in the synthetic medium, both the Nile Red fluorescence intensity and the lipid content of *T. dermatis* (measured by gravimetric method) were evaluated during the fermentation to initially show the relationship of the Nile Red fluorescence intensity and intracellular lipid content. As depicted in Fig. 2, oleaginous yeast *T. dermatis* with various lipid content could be obtained at different fermentation time. In addition, a clear positive correlation between fluorescence intensity of Nile Red and intracellular lipid content means that higher lipid content results in higher fluorescence intensity of Nile Red. Therefore, it is possible to measure the Nile Red fluorescence intensity to calculate the intracellular lipid content of *T. dermatis*.

Establishment of the fluorescent method for lipid content measurement

As mentioned above, Nile Red can enter into the cell of oleaginous yeast *T. dermatis* and generate fluorescence light after stable combination with its cellular lipid. Also, the lipid content had obviously positive correlation with the Nile Red fluorescence intensity. Therefore, the fluorescent method for lipid content measurement of *T. dermatis* can be built. In this part, various points of different lipid content of *T. dermatis* and its corresponding fluorescence intensity of Nile Red were linear fitted. As shown in Fig. 3, a linear

equation (Y=36.84X-337.14) was built and the data points of fluorescence intensity distributed closely to this equation. Overall, the correlation coefficient (R^2) of this equation was high and therefore it is suitable for measurement of lipid content of *T. dermatis*.

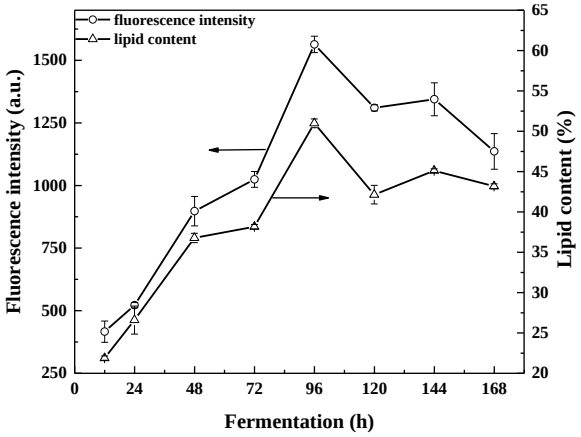


Fig. 2: The relationship of lipid content (*white triangle*) and fluorescence intensity (*white circle*) for *T. dermatis* cultured in the synthetic medium at different fermentation time.

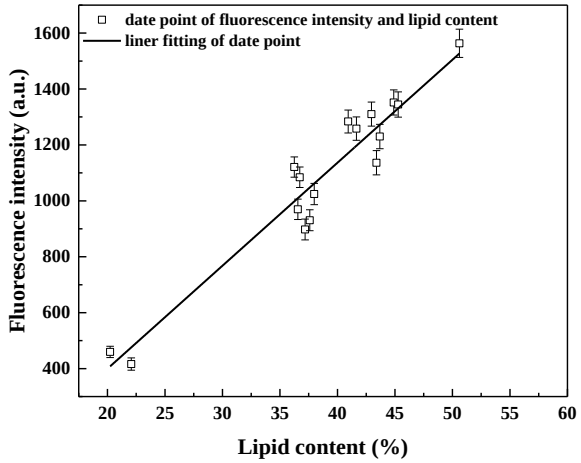


Fig. 3: The fitting curve of lipid content and fluorescence intensity for *T. dermatis* cultured in the synthetic medium. (R^2=0.95).

To further show the potential of applying this equation for lipid content measurement of *T. dermatis*, the R^2 of this equation was further compared with those of other equations in previous studies which also used Nile Red fluorescent method to evaluate the lipid content of other oleaginous microorganisms (Table-3). As shown in Table-3, the Nile Red fluorescent method could be applied for both microalgae and yeast lipid content measurement. Compared with oleaginous yeast, the Nile Red fluorescent method was applied more for oleaginous microalgae. Also, the R^2 of equation for lipid content measurement for microalgae was a little higher than that of yeast. Therefore, it is necessary to obtain some equations with high R^2 for lipid content measurement of yeast. To date, Nile Red fluorescent method was merely applied for a few oleaginous yeasts such as *Rhodosporidium diobovatum* UCD-FST, *Saccharomyces cerevisiae* UCD-FST [11], *Lipomyces starkeyi* IFO and *Rhodosporidium toruloides* [29]. In this study, Nile Red fluorescent method was used for measuring the lipid content of *T. dermatis*, one oleaginous yeast that could accumulate microbial lipid efficiently from several of lignocellulosic hydrolysates [18,19]. In Table-3, the lipid content of oleaginous microorganisms, such as *Scenedesmus obliquus*, *Neochloris oleoabundans* [30] and *Rhodosporidium toruloides* IFO-0559 [29] *Rhodotorula glutinis* CCMI 145 [31], was different from that of oleaginous yeast *T. dermatis*. Overall, the R^2 of equation for oleaginous yeast *T. dermatis* was higher than other kinds of oleaginous yeast. Obviously, Nile Red was shown to be suitable for lipid content measurement of *T. dermatis* that the R^2 of equation was comparable and even higher than most R^2 shown in Table-3, thus it was possible to use fluorescent method for lipid content measurement of this oleaginous yeast and overcome the disadvantages of traditional gravimetric method.

Table-3: The correlation coefficient (R^2) of the fitting curves of Nile red fluorescent method for lipid content measurement of different microalgae and oleaginous yeast.

Stain	Slope	Total lipid	R^2	Reference
Saccharomyces cerevisiae	null	10~15%	0.56	[11]
Rhodosporidium toruloides	null	0~4 mg/ml	0.86	[29]
Mortierella nana	null	0~4 mg/ml	0.87	[29]
Scenedesmus obliquus	0.79	10~15%	0.80	[30]
Necochloris oleoabundans	0.40	10~16%	0.84	[30]
Rhodotorula glutinis	0.014	5~15%	0.83	[31]
Trichosporon dermatis	36.83	10~50%	0.95	This study

Using Nile Red fluorescent lipid content measurement method for lipid fermentation in lignocellulosic hydrolysates

As mentioned above, oleaginous yeast *T. dermatis* can accumulate microbial lipid efficiently in lignocellulosic hydrolysates [4,5]. Namely, lignocellulosic biomass, the most available and renewable bio-resources in nature, can be utilized to produce microbial lipid. In this study, the Nile Red fluorescent method for lipid content measurement of *T. dermatis* was built by fitting the intracellular lipid content determined by both traditional gravimetric method and Nile Red fluorescent method in the synthetic medium. To verify the Nile Red fluorescent method built in this study is suitable for measuring the lipid content of *T. dermatis* in lignocellulosic hydrolysates or not, two typical lignocellulosic hydrolysates including the

corncob acid hydrolysate and the rice straw acid hydrolysate were used to cultivate *T. dermatis* and its lipid content was measured by both fluorescent method built in this study and traditional gravimetric method during different periods in fermentation.

During lipid fermentation, the intracellular lipid content of oleaginous yeast *T. dermatis* was higher in the corncob acid hydrolysate than that in the rice straw acid hydrolysate, possibly due to the existence of more inhibitors in the rice straw acid hydrolysate [32]. In this study, both high and low lipid content was measured by the fluorescent and gravimetric analysis methods in these two lignocellulosic hydrolysates (Fig. 4). As it depicted, no matter the lipid content was high or low, the lipid content of *T. dermatis* in both the corncob acid hydrolysate and the rice straw acid hydrolysate could be measured by fluorescent method with high accuracy that the results are close to the value measured by traditional gravimetric method. As shown in Fig. 5, the data points of fluorescence intensity and lipid content for the oleaginous yeast cultured in corncob acid hydrolysate and rice straw acid hydrolysate was well distributed on the standard curve established in the simulated medium. Not only the low lipid content in the rice straw acid hydrolysate but also the high lipid content in the corncob acid hydrolysate could be measured by the Nile Red fluorescent method built in this study. Therefore, Nile Red fluorescent method is also an effective and fast method for measuring the lipid content of oleaginous yeast *T. dermatis* in lignocellulosic hydrolysates. More importantly, it also provides a good example for measuring lipid content of other oleaginous yeasts which can accumulate microbial lipid in lignocellulosic hydrolysates.

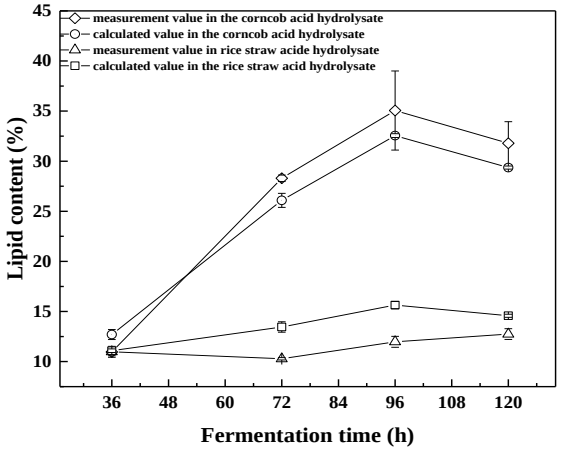


Fig. 4: Measurement of lipid content of *T. dermatis* cultured in corncob acid hydrolysate and rice straw acid hydrolysate by conventional gravimetric method and Nile Red fluorescent method.

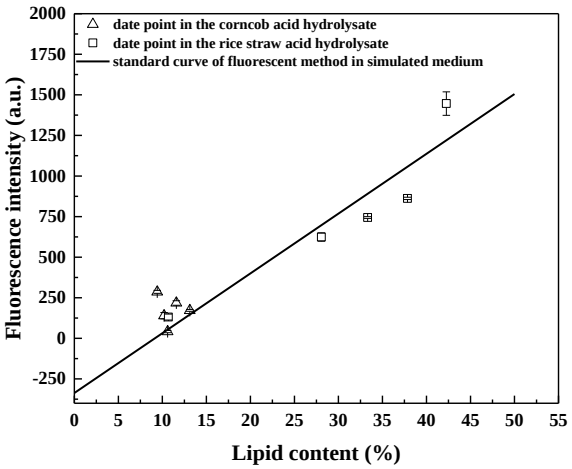


Fig. 5:Distribution of data point of fluorescence intensity and lipid content for *T. dermatis* cultured in corncob acid hydrolysate (*white circle*) and rice straw acid hydrolysate (*white triangle*) around the fitting curve established in the synthetic medium.

The issues and possible improvement of the Nile Red fluorescent method

In this study, the Nile Red fluorescent method for lipid content measurement of *T. dermatis* has been initially built. Specially, this method is suitable for the lipid fermentation in lignocellulosic hydrolysates. Although this method has a good correlation coefficient, some issues are still existed. Firstly, as for current Nile Red fluorescent method of lipid content measurement, the fluorescence probe is usually dissolved in hydrophobic organic solvents such as acetone, DMSO and they are not so environmentally friendly and also bring some environmental problems. Moreover, the combination mechanism of hydrophobic fluorescent probe (Nile Red) and hydrophilic environment (cell suspension) should be further explored. To explore above, finding some hydrophilic systems such as modified surfactant which can dissolve Nile Red is one promising direction to solve this issue [33]. Besides this, the effect of different factors related to fluorescent analysis on the Nile Red fluorescence intensity and lipid content measurement should be learned to build some methods suitable for lipid content measurement of different oleaginous yeasts [34].

Conclusion

In this study, the Nile Red fluorescence intensity has an obvious linear relationship with the

lipid content of oleaginous yeast *T. dermatis* and a fast and effective Nile Red fluorescent method for determining the lipid content of *T. dermatis* was successfully established. Specially, this method was suitable for determining lipid content of *T. dermatis* cultured in different lignocellulosic hydrolysates. By this method, the disadvantages of traditional gravimetric method can be overcome and thus the efficiency for the study of lipid fermentation of *T. dermatis* can be improved. Further study should focus on building more efficient fluorescent system and well controlling the related factors affecting the fluorescent measurement.

Acknowledgments

The authors acknowledge the financial support of National Natural Science Foundation of China (21606229), project of Pearl River S&T Nova Program of Guangzhou (201610010014), the Science and Technology Project of Guangdong Province (2016A010105016), the Foundation Project of Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development (y809jj1001), and the Foundation of Director of Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences (y407r41001).

Conflict of interest

No conflict of interest declared.

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