Front. Chem. Sci. Eng. 2012, 6(4): 453–460
DOI 10.1007/s11705-012-1210-8

RESEARCH ARTICLE

Repeated batch fermentation with water recycling and cell separation for microbial lipid production

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Abstract Large waste water disposal was the major problem in microbial lipid fermentation because of low yield of lipid. In this study, the repeated batch fermentation was investigated for reducing waste water generated in the lipid fermentation of an oleaginous yeast *Trichosporon cutaneum* CX1 strain. The waste fermentation broth was recycled in the next batch operation after the cells were separated using two different methods, centrifugation and flocculation. Two different sugar substrates, glucose and inulin, were applied to the proposed operation. The result showed that at least 70% of the waste water was reduced, while lipid production maintained satisfactory in the initial four cycles. Furthermore, it is suggested that *T. cutaneum* CX1 cells might produce certain naturally occurring inulin hydrolyzing enzyme(s) for obtaining fructose and glucose from inulin directly. Our method provided a practical option for reducing the waste water generated from microbial lipid fermentation.

Keywords batch fermentation, microbial lipid, *Trichosporon cutaneum* CX1, flocculation, waste water recycle

1 Introduction

Oleaginous microorganisms of bacteria, yeast, fungi, and algae are capable of producing over 20% lipid oils of their weight. These oils are composed of triacylglycerols (TAGs) with long chain fatty acids well-suited for biodiesel production [1]. Various low value added natural resource or waste materials such as Jerusalem artichoke [2], corn stover [3], sorghum bagasse [4], and waste water [5] were used as feedstocks of microbial lipid fermentation. Different fermentation methods were also investigated, including the fed batch fermentation [6], continuous culture [7], and the two stage fermentation of cell propagation and lipid production [8].

Generally, the cell mass of oleaginous yeasts occupies less than 10% of fermentation broth and the rest is disposed as waste water. Therefore, recycling of the fermentation liquid after the harvest of oleaginous yeast cells is important for reducing environmental treatment and fermentation energy input. Another benefit of the spent broth reuse is higher utilization ratio of nutrient substrates. This is of particular interest for fermentations requiring high levels of a particular substrate (either the nitrogen, N, or carbon, C) for product formation [9] as in the amino acid fermentation for high N and in microbial oil fermentation for high C. In microbial lipid fermentation, recycling of the fermentation broth for subsequent fermentation with *Apiotrichurn curvaturn* was tested, 75% of the spent medium was recycled after centrifugation and sterilization [9]. In penicillin amidase production, 40% to 60% of the fermentation broth by recycling continued for three subsequent batches without inhibiting biosynthesis of product [10]. No inhibition was observed for lysine biosynthesis when 10% of the fresh medium was replaced with liquid waste from lysine production after ultrafiltration [11]. The in situ ethanol recovery and the medium recycling in the continuous ethanol fermentation showed that the inhibition of the inhibitor accumulation in the spent on *Saccharomyces cerevisiae* could be overcome [12]. Herein, we report a new fermentation process for microbial lipid production from glucose and inulin by repeated batch fermentation of *Trichosporon cutaneum* (*T. cutaneum*) with water recycling and cell separation. In the repeated batch fermentation, the waste fermentation broth was recycled in the next batch operation after the cells were separated using two different methods. Two different sugar substrates, glucose and inulin, were tested in the proposed operation. The result showed that at least 70% of the waste water was reduced using the proposed operation. The proposed fermentation method provided a practical option for reducing the waste water generation of microbial lipid fermentations.

Received March 12, 2012; accepted July 17, 2012

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2 Material and methods

2.1 Strains and medium

The oleaginous yeast *T. cutaneum* CX1 (China General Microbiological Culture Collection (CGMCC) No. 2527) was cultured in YPD medium containing 20 g/L glucose, 10 g/L peptone, and 10 g/L yeast extract at pH 6.0 for 24 h, and then aliquoted into 1.0 mL vials containing 30% (w/w) glycerol and stored at −80°C freezer. One vial was inoculated into 20 mL medium containing 20 g/L glucose, 0.5 g/L yeast extract, 5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$ with the pH of 5.8–6.0 at each culture. The fermentation medium contained 20–25 g/L glucose or inulin, 0.5 g/L yeast extract, 0.22 g/L $(NH_4)_2SO_4$, 1.0 g/L KH_2PO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$.

2.2 Reagents and raw materials

The inulin powder extracted from Jerusalem artichoke tuber was purchased from Langrui Fine Chemical Co., Shanghai, China. The composition of inulin was determined according to the method in Gao et al. [13]: the inulin was acidified ($1 < pH < 2$) by 5 mol/L sulphuric acid, and then heated at 100°C for 2 h to hydrolyze the carbohydrates. Then the reducing sugars were determined by HPLC. Glucose (0.23 g) and fructose (0.78 g) were obtained from 1 g inulin.

The chemicals KH_2PO_4, $(NH_4)_2SO_4$, yeast extract, $MgSO_4 \cdot 7H_2O$ were purchased from Lingfeng Chemical Reagent Co., Shanghai, China. Trichloromethane was purchased from Shanghai Chemical Reagent Co. Acetic acid was bought from Shanghai Richjoint Chemical Reagents Co. Methanol and chitosan were from Sinopharm Chemical Reagent Co., Shanghai. Chitosan is biochemical reagent with the deacetylation percentage 80%–95%. All the chemicals and reagents above except chitosan were of analytical grade. Chitosan (10 g/L) dissolved in 1% acetic acid was used as the flocculant reagent.

2.3 Fermentation and cell separation

All lipid fermentations were carried out at 30°C in the fermentor (3 L) with the control of pH and dissolved oxygen (DO) (Baoxing Biotech 4-BG, Shanghai, China). Inoculum culture (100 mL) prepared in a shake flask at 30°C and 180 rpm for 20 h, was inoculated into the bioreactor with 1.0 L of initial culture volume. The pH was maintained at 5.0, and the air flow rate was 0.8 vvm. The DO was maintained at 20% of the air saturation by regulating the agitation rate. Samples were withdrawn at intervals and centrifuged at 10000 $r \cdot min^{-1}$ for 5 min. The supernatant was used for determining the glucose concentration and the precipitant for determining dry cell mass (DCM) and cell lipid content.

In the repeated batch fermentation, 100 mL fermentation broth was drawn out from the fermentor at the end of every run of fermentation as the seeds of the next round fermentation. The fermentation medium composition showed in the part of strains and medium was maintained the same in all rounds of the repeated batch fermentations.

The cell separation was operated by either centrifugation or flocculation. The centrifugation was carried out in the same way as sample taking. The method of flocculation with chitosan was according to those reported by Hughes et al. [14] and Silva et al. [15]. Simply, chitosan flakes were dissolved in 1% (v/v) acetic acid to give the 10 g/L chitosan acetic acid solution, which was used as the flocculant reagent. Then 15 mL flocculant reagent was added to 1 L fermentation broth, the aeration was stopped, and after stirring for 3 min the resulting mixture was sedimented for 30 min to obtain the flocculent cells at the bottom of the bioreactor.

The repeated batch experiments were carried out twice. The control experiment using the synthetic medium was carried out once, because the fermentation of *T. cutaneum* CX1 followed the same procedure as in our previous publication and the same result was obtained as before [3].

2.4 Determination of DCM and lipid concentration

DCM was determined by a combined procedure that culture solution (30 mL) was centrifuged, washed, dried to a constant weight and then measured gravimetrically. The total lipid was extracted using the modified chloroform-methanol method reported by Folch et al. [16]. Briefly, the dry cells were disrupted and homogenized in 6 mL of 4 $mol \cdot L^{-1}$ hydrochloric acid for 30 min, then heated in boiled water for 10 min and quenched in ice water. The cell lysates were stirred with 20 mL methanol-chloroform mixture (1 : 2 by volume ratio) for 30 min, centrifuged at 10000 $r \cdot min^{-1}$ for 5 min to get the lipid-contained solution and then the solvent was removed using rotary evaporation at 80°C, instead of the relatively weak vacuum evaporation suitable for animal fat extraction. Finally, the lipid was measured gravimetrically to obtain lipid concentration.

2.5 Determination of the sugar concentration in the fermentation media

Glucose and fructose were analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-rad Aminex HPX-87H column at the column temperature 65°C [17].

2.6 Inulin hydrolysis with the spent fermentation broth

In 3 L fermentator, 1 L of 5% inulin solution with 0.5 g/L $(NH_4)_2SO_4$ and other nutrition same as the fermentation medium were inoculated with 100 mL *T. cutaneum* seeds

to start the lipid fermentation. After 44 h, the fermentation broth was separated by centrifugation. Then 15 g inulin was added into 45 mL fermentation broth to form 35% (w/v) solid content, and the resulting mixture was hydrolyzed at 60°C and pH 5.0. The citric acid-sodium citrate buffer instead of the fermentation broth was used as the control.

3 Results and discussion

3.1 Repeated batch fermentation using glucose as feedstock with fermentation liquid recycling

The batch fermentation using glucose as feedstock with fermentation liquid recycling was repeated for 6 successive rounds. The *T. cutaneum* cells were removed from the fermentation broth by centrifugation, and the supernatant was used in the next batch operation. The batch fermentation using the fresh medium without fermentation liquid recycling was performed as a control.

Figure 1 shows that the lipid productions in the repeated batch fermentation with and without fermentation liquid recycling had essentially no difference at least in the first 5 rounds of the batch operations. Figure 1(a) shows that the lipid production with the fermentation liquid recycling was approximately constant at 1.6 – 1.9 g/L in the initial 5 rounds, but decreased in the 6th round (below 1.5 g/L). Figure 1(b) shows that the lipid production without fermentation liquid recycling was almost constant in all the 6 rounds of the batch fermentation tested (1.4 – 1.5 g/L), although the first round batch was a little bit higher than other ones. The glucose consumption rate in the repeated batch fermentations with and without fermentation liquid recycling decreased with increasing

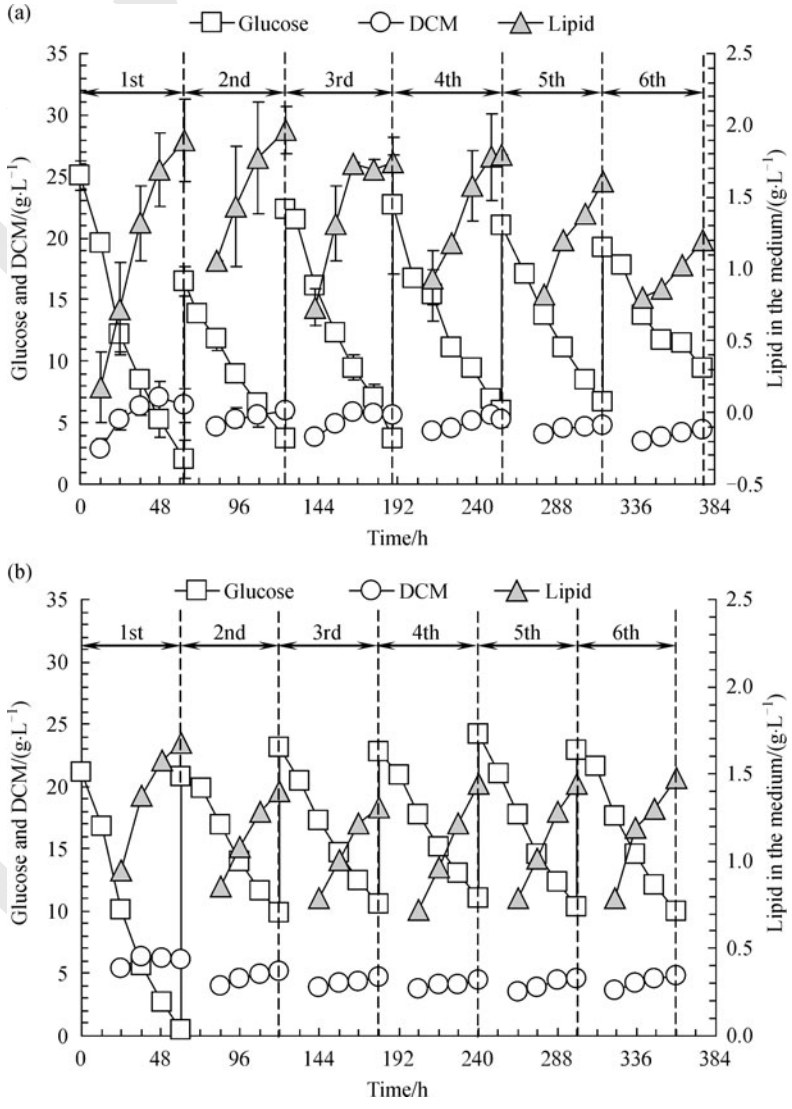

Fig. 1 Repeated batch fermentation of glucose for lipid production with water recycling and cell separation. (a) With cell separation using centrifugation and water recycling. Cells were separated and the fermentation liquid was recycled. The same fermentation nutrition was added to the recycled water. (b) Without cell separation and water recycling

Table 1 Effect of the culture time of seeds on lipid accumulation

Seeds culture time	After 20 h		After 54 h	
Inoculation ratio /(v·v^{-1})	10%	5%	10%	20%
DCM /(g·L^{-1})	8.75	6.36	6.33	6.89
Lipid content /(g per 100 g)	19.2	30.7	30.1	29.1

batches of the fermentation. The DCM in the repeated batch fermentation decreased with the batch round increasing, while the decrease in the control was negligible. The declined lipid and DCM in the later batch rounds of the repeated batch fermentation with fermentation liquid recycling may be due to the decreased viability of the cells with the recycled successive batch operations. When the fermentation broth at the end of the first run was used as the seeds of the successive batches, equivalently the seeds cultured for 54 h in fermentation broth in Table 1, the lipid content of *T. cutaneum* CX1 was 30.1%, which was 10.9% higher than that using seeds newly cultivated in the fresh seed medium for 20 h (Table 1). The seeds precultured for 54 h gave higher lipid content at different inoculation ratios than that using the newly cultured seeds. The cell viability was reduced after 5 successive batches with the lipid and DCM decreasing, as observed in the Fig. 1. In the batch fermentation with fermentation liquid recycling, the metabolites from the early batch fermentation may accumulate in the repeated batch operations and inevitably inhibited the cell growth and the metabolism rate of substrate such as glucose.

3.2 Repeated batch fermentation using inulin as feedstock with fermentation liquid recycling

Inulin is a linear β-2,1-linked *D*-fructofuranose and used as the feedstock for microbial lipid production [18]. The repeated batch fermentation of inulin with fermentation liquid recycling was tested. Figure 2 shows that both the lipid production and DCM in the batch operations with fermentation liquid recycling were lower than that without liquid recycling for the recycling rounds, but the differences were not significant (lipid was all around 1.5 g/L and the DCM was around 5 g/L, respectively, for both the fermentation modes). The same reason of the metabolites accumulation in the repeated batch fermentation as using glucose may be responsible for the differences in the two repeated batch fermentations.

An interesting phenomenon was that inulin was directly used as feedstock without addition of inulinase enzyme for hydrolysis of inulin to get the fermentable fructose and glucose. Figure 3 shows that fructose and glucose were produced steadily and quickly, indicating the existence of the certain inulin hydrolyzing enzyme(s) in the fermentation system. It is highly possible that *T. cutaneum* CX1 produced certain natural occurring inulin hydrolyzing enzyme(s) and catalyzed the degradation of inulin to fructose and glucose, and then these sugars were fermented into microbial lipid simultaneously. Figure 3 also shows that the fructose concentration at the late rounds of the repeated batch with fermentation liquid recycling was somewhat greater than that without the fermentation liquid recycling. The reason might be that the inulin hydrolyzing enzyme(s) accumulated in the fermentation broth were recycled, and thus resulted in the greater inulinase activity than that without the water recycling. When the inulin was added to the supernatant of the fermentation broth after the cells were removed, the total sugar concentration was 58.5 g/L; after the inulin solution was incubated at 60°C and pH 5.0 for 74 h, the total sugar increased to 164.3 g/L, 2.8-fold of its original sugars. On the other hand, when same amount of inulin was added to the citrate buffer at the same incubation conditions (60°C and pH 5.0) for 74 h, the sugar concentration only increased 11% to its original concentration. The result indicates that there existed certain types of inulin hydrolyzing enzyme(s) in the fermentation broth for inulin hydrolysis. However, the inulin hydrolyzing enzyme(s) in the broth were not characterized and quantitatively assayed. The further study on the inulin hydrolyzing enzyme(s) is on the way.

When inulin was used as the feedstock of microbial lipid fermentation, the inulinase hydrolyzed it into fermentable monosaccharide sugars, which were then used for cell growth and accumulation of lipid oil such as the microbial lipid fermentation of inulin hydrolysate using a natural *R. mucilaginosa* TJY15a [18], and an engineered *Yarrowia lipolytica* expressing inulinase gene [19]. Zhao et al. reported that *Rhodosporidium toruloides* Y4 accumulated the lipid directly from Jerusalem artichoke without inulinase addition [20]. To our best knowledge, there was no report on microbial lipid production by *T. cutaneum* using inulin as the substrate. Our study showed that *T. cutaneum* can use inulin directly to produce single cell oil, and the lipid content was up to 32 g lipid/100 g DCM."

Table 2 shows the reduction of the water when the fermentation broth was recycled after the cell separation. From the second cycle, 90% of the fermentation liquid in the flocculation separation and 95% in the centrifugation separation mode were recycled. The lipid production remained satisfactory without significant changes on fermentation performance. However, the repeated batch fermentation was carried out under relatively low initial sugar concentration (20 – 25 g/L). The higher initial sugar concentration (higher than 100 g/L), which was preferred for initiating the lipid accumulation in the cells, was not

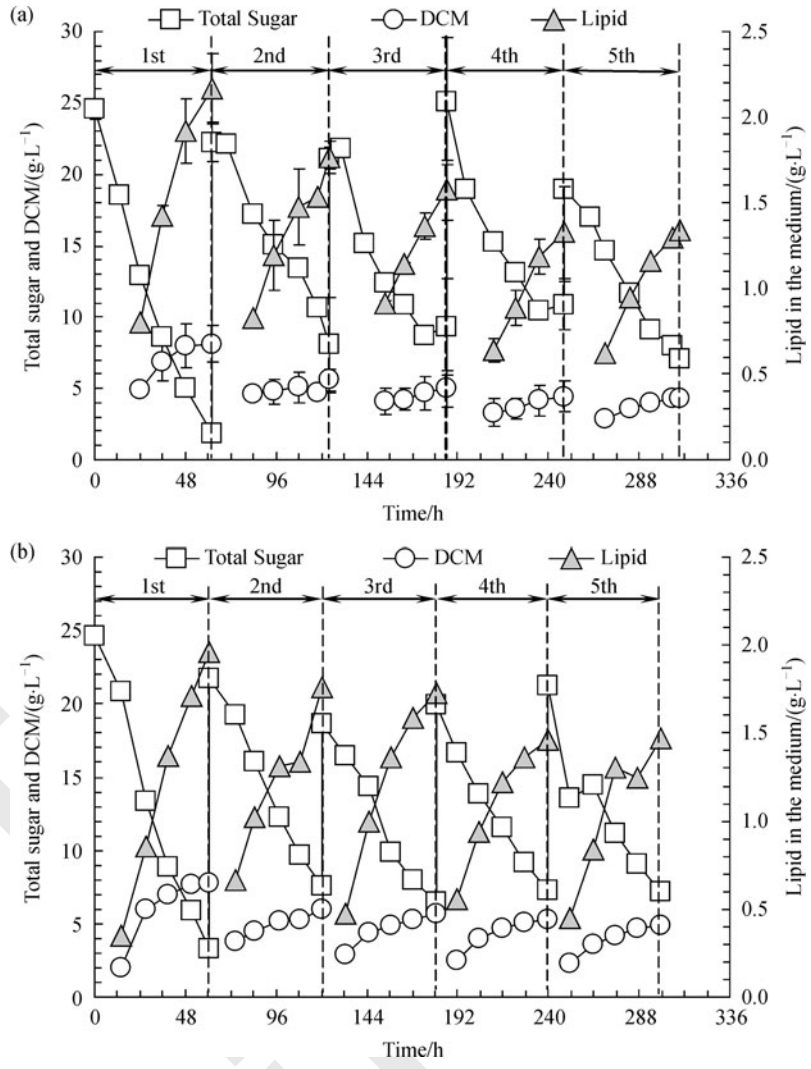

Fig. 2 Repeated batch fermentation of inulin for lipid production with cell separation by centrifugation and water recycling. (a) With cell separation by centrifugation and water recycling. (b) Without cell separation and water recycling

tested [21]. The similar or even better results might be obtained under high initial sugar concentration and the study will be carried out in our next research.

3.3 Repeated batch fermentation with the cell separation by flocculation

Cell separation by centrifugation is efficient but energy intensive, and suffers from bacterial contamination if the fermentation liquid is recycled. To assist the proposed repeated batch fermentation, the flocculation method was used for cell separation by adding chitosan as flocculant reagent. The flocculation rate increased to 99% when the flocculant was increased to 15 mL/L broth. The continuous lipid fermentation process was generated combined the repeated batch fermentation and the flocculation method with the flocculant at 15 mL/L broth.

Figure 4(a) shows the fermentation of glucose for lipid production using flocculation method for cell separation. By using flocculation method, the total lipid production was 2.0 g/L in the first batch operation, approximately 1.3 g/L in the second to the fourth batch sessions, and less than 0.5 g/L in the fifth session. Compared with the centrifugation method used in the fermentation of glucose, the lipid production was lower than the constant lipid concentration of 1.7 g/L in the five batches. Figure 4(b) shows that when inulin was used with flocculation, a high lipid concentration of 2.35 g/L was achieved in the first round, followed by sharply decreasing to 0.81 g/L in the fourth round, and even 0.28 g/L in the fifth cycle. Figure 4(c) shows that a certain amount of fructose was not applied since the second batch operation. These results indicated the declined lipid production may be due to the effect of flocculant reagent (chitosan) on the cell growth and lipid production indicated by the extended lag phase. Besides, the hard agglomeration of cells was observed in

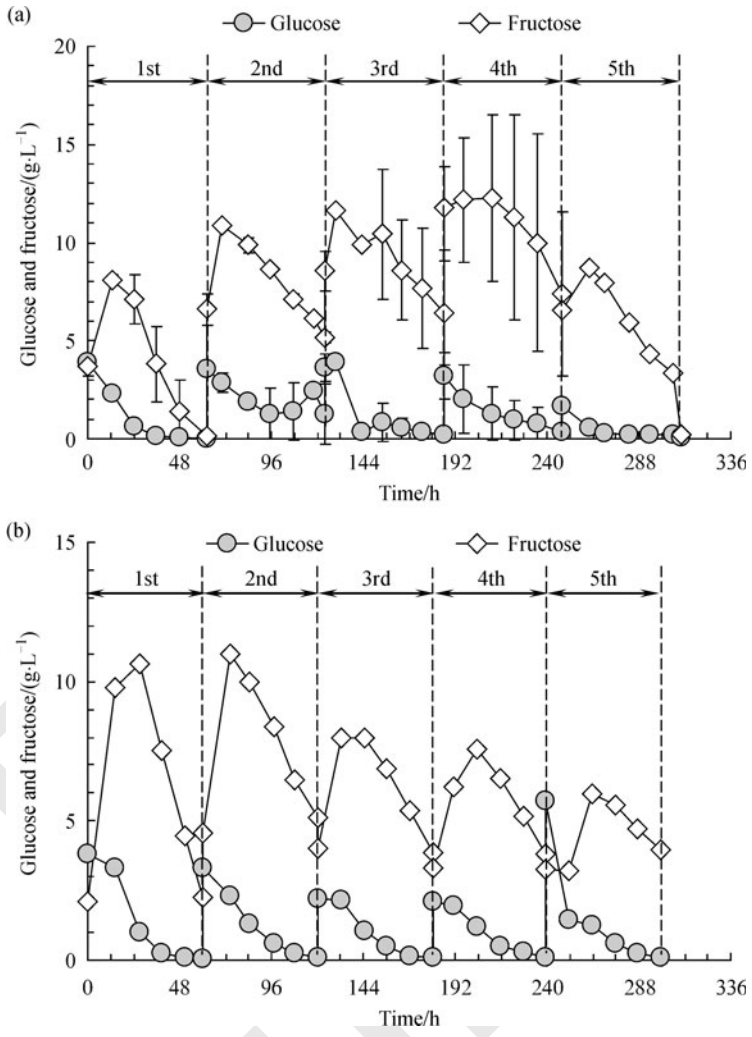

Fig. 3 Time course of glucose and fructose using inulin for lipid fermentation. (a) With cell separation by centrifugation and water recycling. (b) Without cell separation and water recycling

Table 2 Fermentation parameters under different modes*

Parameters	Cell separation method	Glucose	Inulin
Lipid production /(g·L^{-1})	Control	1.46	1.68
	Centrifugation	1.70	1.53
	Flocculation	1.30	1.34
Lipid content /(g·g^{-1})	Control	0.30	0.28
	Centrifugation	0.32	0.32
	Flocculation	0.28	0.28
Lipid yield ($Y_{L/S}$) /(g·g^{-1})	Control	0.10	0.11
	Centrifugation	0.11	0.11
	Flocculation	0.10	0.07
Water consumed /L	Control	5.00	5.00
	Centrifugation	1.20	1.20
	Flocculation	1.36	1.36

* Control, repeated batch without cell separation and recycling liquid; Centrifugation, repeated batch fermentation with cell separation by centrifugation; Flocculation, repeated batch fermentation with cell separation by flocculation. Lipid production, lipid content, lipid yield, water consumed were calculated on the basis of the total five operation cycles. For water consumed, each batch consumed 1 L water in control and the first batch in centrifugation mode and flocculation mode; every batch after only added the volume of water with 50 mL and 90 mL, lost during the process of centrifugation and flocculation respectively

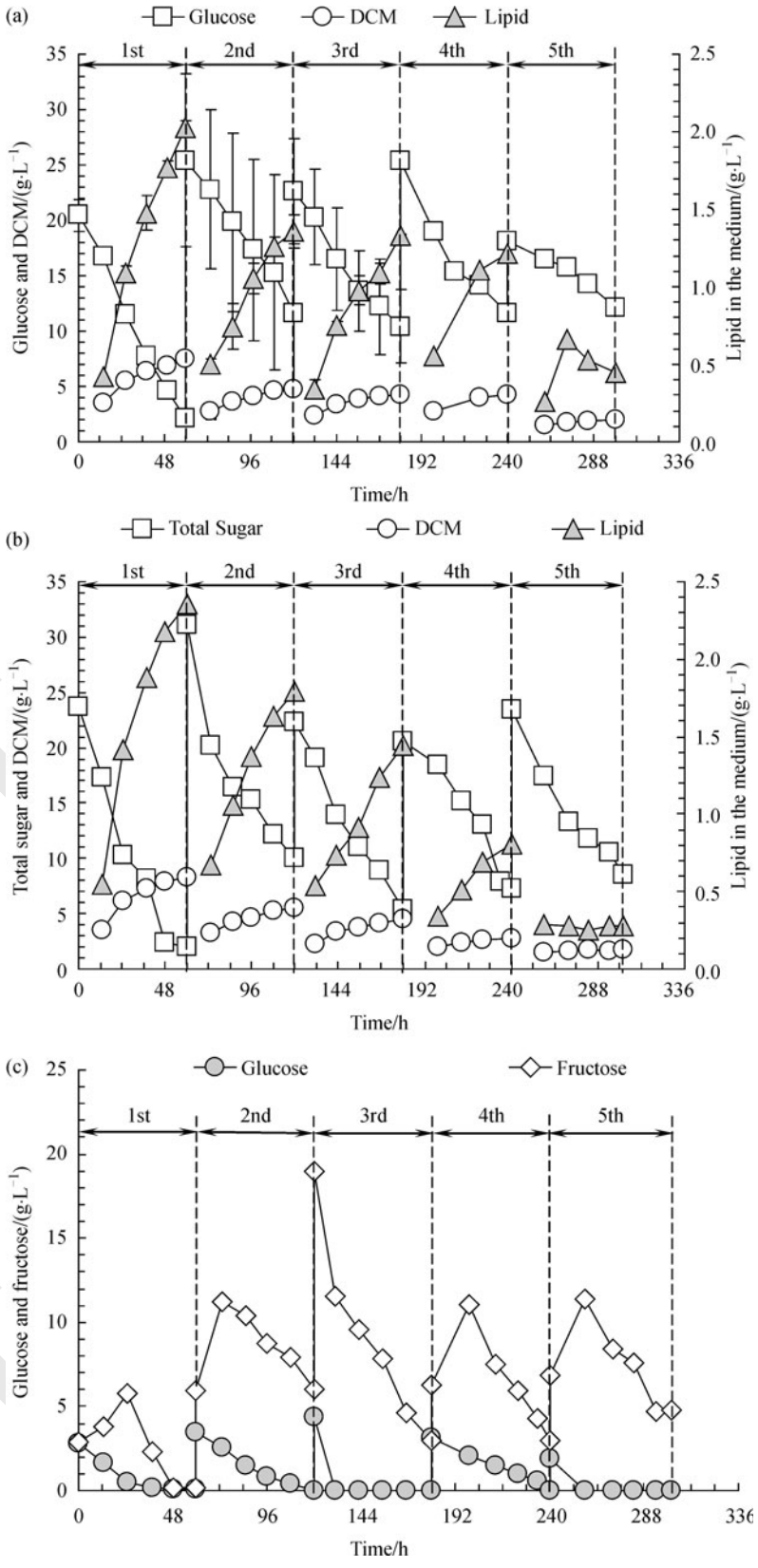

Fig. 4 Time course of lipid fermentation with cell separation by flocculation. (a) With cell separation by flocculation and water recycling when glucose was used. (b) With cell separation by flocculation and water recycling when inulin was used. (c) The time course of glucose and fructose concentrations in the fermentation process of the substrate of inulin

the fifth circle, which may be responsible for the almost ceased fermentation. As for inulin, the stress of producing inulinase and lipid fermentation simultaneously except for the flocculant damage should be the main reason for the dramatic decrease of the lipid production. The high concentration of residue fructose and glucose in the later rounds in Fig. 4(c) illustrates that the inulinase activity accumulation while the sugar utilization rate is not lower than that shown in Fig. 4(a).

4 Conclusions

The repeated batch fermentation with cell separation mode was suitable both in glucose and inulin substrates. Lipid production did not decrease at the initial five circles with recycling the fermentation liquid under this fermentation mode, and the amount of waste water disposal greatly decreased. The flocculation method was practical for cell harvest in the lipid production and greatly decreased the water usage in the process.

Acknowledgements This research was supported by the National Basic Research Program of China (No. 2011CB707406), the National Natural Science Foundation of China (Grant No. 20976051), the Fundamental Research Funds for the Central Universities of China (WF0913005/1114054), and the Shanghai Leading Academic Discipline Project (B505).

References

1. Kosa M, Ragauskas A J. Lipids from heterotrophic microbes: advances in metabolism research. Trends in Biotechnology, 2011, 29(2): 53–61
2. Zhao X, Wu S G, Hu C M, Wang Q, Hua Y Y, Zhao Z K. Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides* Y4. Journal of Industrial Microbiology & Biotechnology, 2010, 37 (6): 581–585
3. Huang X, Wang Y M, Liu W, Bao J. Biological removal of inhibitors leads to the improved lipid production in the lipid fermentation of corn stover hydrolysate by *Trichosporon cutaneum*. Bioresource Technology, 2011, 102(20): 9705–9709
4. Liang Y N, Tang T Y, Umagiliyage A L, Siddaramu T, McCarroll M, Choudhary R. Utilization of sorghum bagasse hydrolysates for producing microbial lipids. Applied Energy, 2012, 91(1): 451–458
5. Xue F Y, Miao J X, Zhang X, Luo H, Tan T W. Studies on lipid production by *Rhodotorula glutinis* fermentation using monosodium glutamate wastewater as culture medium. Bioresource Technology, 2008, 99(13): 5923–5927
6. Zhao X, Hu C M, Wu S G, Shen H W, Zhao Z K. Lipid production by *Rhodosporidium toruloides* Y4 using different substrate feeding strategies. Journal of Industrial Microbiology & Biotechnology, 2011, 38(5): 627–632
7. Evans C T, Ratledge C. A comparison of the oleaginous yeast, *Candida curvata*, grown on different carbon sources in continuous and batch culture. Lipids, 1983, 18(9): 623–629
8. Lin J T, Shen H W, Tan H D, Zhao X, Wu S G, Hu C M, Zhao Z K. Lipid production by *Lipomyces starkeyi* cells in glucose solution without auxiliary nutrients. Journal of Biotechnology, 2011, 152(4): 184–188
9. Hsiao T Y, Glatz C E, Glatz B A. Broth recycle in a yeast fermentation. Biotechnology and Bioengineering, 1994, 44(10): 1228–1234
10. Babu P, Panda T. Effect of recycling of fermentation broth for the production of penicillin amidase. Process Biochemistry (Barking, London, England), 1991, 26(1): 7–14
11. Hsiao T Y, Glatz C E. Water reuse in the *L*-lysine fermentation process. Biotechnology and Bioengineering, 1996, 49(3): 341–347
12. Converti A, Perego P, Lodi A, Fiorito G, Borghi M, Ferraiolo G. In-situ ethanol recovery and substrate recycling during continuous alcohol fermentation. Bioprocess and Biosystems Engineering, 1991, 7(1): 3–10
13. Gao J, Xu H, Li Q J, Feng X H, Li S. Optimization of medium for one-step fermentation of inulin extract from Jerusalem artichoke tubers using *Paenibacillus polymyxa* ZJ-9 to produce R,R-2,3-butanediol. Bioresource Technology, 2010, 101(18): 7076–7082
14. Hughes J, Ramsden D, Symes K. The flocculation of bacteria using cationic synthetic flocculants and chitosan. Biotechnology Techniques, 1990, 4(1): 55–60
15. Silva A C, Guimarães P M R, Teixeira J A, Domingues L. Fermentation of deproteinized cheese whey powder solutions to ethanol by engineered *Saccharomyces cerevisiae*: effect of supplementation with corn steep liquor and repeated-batch operation with biomass recycling by flocculation. Journal of Industrial Microbiology & Biotechnology, 2010, 37(9): 973–982
16. Folch J, Lees M, Sloane-Stanley G. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 1957, 226(1): 497–509
17. Chen X, Li Z H, Zhang X X, Hu F X, Ryu D D Y, Bao J. Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. Applied Biochemistry and Biotechnology, 2009, 159 (3): 591–604
18. Zhao C H, Zhang T, Li M, Chi Z M. Single cell oil production from hydrolysates of inulin and extract of tubers of Jerusalem artichoke by *Rhodotorula mucilaginosa* TJY15a. Process Biochemistry (Barking, London, England), 2010, 45(7): 1121–1126
19. Zhao C H, Cui W, Liu X Y, Chi Z M, Madzak C. Expression of inulinase gene in the oleaginous yeast *Yarrowia lipolytica* and single cell oil production from inulin-containing materials. Metabolic Engineering, 2010, 12(6): 510–517
20. Zhao X, Wu S G, Hu C M, Wang Q, Hua Y Y, Zhao Z B K. Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides* Y4. Journal of Industrial Microbiology & Biotechnology, 2010, 37 (6): 581–585
21. Li Y H, Zhao Z B, Bai F W. High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. Enzyme and Microbial Technology, 2007, 41(3): 312–317