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ORIGINAL PAPER

Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides* Y4

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Abstract Jerusalem artichoke (JA) is a perennial herbaceous plant widely available as non-grain raw material. Microbial lipid has been suggested as a potential feedstock for large scale biodiesel production. This paper describes lipid production using JA tuber processed by oleaginous yeast *Rhodosporidium toruloides* Y4. Batch and fed-batch modes were tested with feeding of concentrated JA extracts or JA hydrolysates. Cultivation of *R. toruloides* Y4 with JA extracts gave a moderate cellular lipid content of 40% (w/w), whereas lipid titer and cellular lipid content reached 39.6 g l^{-1} and 56.5% (w/w), respectively, when JA hydrolysates were fed. Our results suggested that JA tubers may be further explored as raw material for large scale microbial lipid production.

Keywords *Rhodosporidium toruloides* Y4 · Microbial lipid · Jerusalem artichoke · Fed-batch culture · Oleaginous yeast

Introduction

Some microorganisms, including bacteria, yeasts, molds and algae, can accumulate lipids totaling over 20% of their dry biomass [16]. The majority of these microbial lipids are triacylglycerols containing long-chain fatty acids when carbohydrates are applied as feedstocks [17]. As petroleum resources are diminishing, it has been realized that sustainable development requires fuels and chemicals to be produced from renewable resources, such as biomass derived carbohydrates. In this context, microbial lipids have attracted much attention in recent years [23].

A high production cost is one of the major factors limiting a broader use of microbial lipids. Although microbial lipids are currently more costly than vegetable oil, many methods are potentially valuable to improve the techno-economics of lipid production processes. In previous studies, considerable efforts were made to advance the processing strategies using glucose as the feedstock [7, 12]. It is clear that low cost raw materials must be identified to secure a large scale microbial lipid production in the future.

The perennial herbaceous plant *Helianthus tuberosus* L., also known as Jerusalem artichoke (JA), is a widely available non-grain raw material [19]. It grows well on poor land and shows a high resistance to cold climates and various plant diseases. Fresh JA tuber contains nearly 20% (w/w) carbohydrates, of which 70–90% (w/w) is inulin [1]. Inulin is composed of fructose units terminated by a glucose unit, and can be readily hydrolyzed to fructose and glucose. Recently JA has been utilized for the production of ethanol [6, 20], single cell protein [5] and 2,3-butanediol [18]. Production of lipid using JA juice by processing with microalgae *Chlorella protothecodes* was also reported with a final titer of 6.8 g l^{-1} [2].

We previously showed lipid production by oleaginous yeast *Rhodosporidium toruloides* Y4 using JA juice in a shake-flask; cell biomass and lipid were only 25.5 and 10.1 g l^{-1}, respectively [8]. We now report our work on

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microbial lipid production, using JA tuber as the sole feedstock, by *R. toruloides* Y4 with a 15-l bioreactor.

Materials and methods

Microorganism

Yeast *R. toruloides* Y4, a domesticated strain of *R. toruloides* AS 2.1389, was obtained from the China General Microbiological Culture Collection Center [11]. The yeast strain was maintained at 4°C on yeast peptone dextrose (YPD) agar slants (glucose 20 g l^{-1}, peptone 10 g l^{-1}, yeast extract 10 g l^{-1}, agar 20 g l^{-1}, pH 6.0) and sub-cultured twice a month.

Preparation of JA extracts and JA hydrolysates

Fresh JA tubers used were products of year 2008 from Liaoning Province, Northeast of China. The tubers were composed of about 80% moisture, and the remaining 20% was made up of about 85% inulin-type sugars, 4.8% ash, and protein and cellulosic materials. To prepare JA extracts, 1 kg of fresh tubers were washed, peeled and smashed in a mixer in 500 ml of water, followed by heating at 100°C for 1 h. JA extracts were received upon removal of insoluble materials by filtration through eight-layered muslin cloth.

To prepare JA hydrolysates, the JA extracts were adjusted to pH 3.0 with 2 M HCl and incubated at 100°C for 30 min, and then neutralized to pH 6.0 with 10 M NaOH. Precipitates were removed by centrifugation when necessary. The carbon-to-nitrogen (C/N) molar ratios for JA extracts and JA hydrolysates were 42 and 18, respectively. Ion chromatography (IC) analysis indicated that JA extracts comprised glucose, fructose, sucrose and other oligosaccharides with a degree of polymerization (DP) value up to 30, whereas JA hydrolysates included mainly glucose, fructose and a little sucrose (data not shown).

Both JA extracts and hydrolysates were concentrated by evaporation to a final total sugar concentration near 700 g l^{-1}, autoclaved at 121°C for 15 min, and stored at room temperature.

Culture conditions

The inoculum of yeast *R. toruloides* Y4 was prepared using JA extracts with a total sugar concentration at 40 g l^{-1}. Cells were cultured at 30°C for 36 h on a shaking incubator at 200 rpm. The inoculum was diluted tenfold in the bioreactor to initiate the lipid production culture.

Batch and fed-batch cultures were performed in a 15-l stirred-tank bioreactor (FUS-15L (A), Shanghai Guoqiang Bioengineering Equipment Co. Ltd., Shanghai, China) with an initial volume of 9 l at 30°C. The dissolved oxygen was maintained at 40–50% of air saturation by automatically varying with the agitation speed. The minimum and maximal agitation speeds were set at 200 and 600 rpm, respectively. The pH value was maintained at 6.0 by automatic addition of 10 M NaOH or 2 M HCl. The gas flow rate was set at 0.8 vessel volume per minute. Both JA extracts and JA hydrolysates with total sugar concentration at 700 g l^{-1} were employed as the feeding substrates, and about 400–500 ml of the feeding substrates were introduced into the bioreactor upon the dissolved oxygen burst to above 70% of air saturation when the agitation speed decreased to the minimum.

Analytical methods

Samples were withdrawn at different time intervals and divided into small portions. The following experiments were done in triplicate. The samples were centrifuged at $7{,}000 \times g$ for 5 min. The supernatant was saved for total sugar analysis. The pellets were washed twice with distilled water and dried in an oven at 105°C to a constant weight to determine cell biomass, or the dry cell weight (DCW). Lipid was extracted using a mixture of chloroform and methanol [13], and estimated gravimetrically. Cellular lipid content was expressed as grams lipid per grams DCW. The total lipid was expressed as grams lipid per liter.

Total sugar was determined by the anthrone-sulfuric acid method [9] as total fructose equivalents using standard fructose solutions. To determine the sugar patterns in the fermentation broth, the supernatant was also analyzed by an ion chromatography system (Dionex, Sunnyvale, CA, USA) using a CarbonPac PA100 analytical column (4 × 250 mm) and a CarbonPac PA100 guard column (4 × 50 mm). The column temperature was kept at 30°C. The broth was diluted with Milli-Q water and filtered through a 0.45 μm Satorius cellulose acetate membrane before injection. The gradient was established by mixing eluant A (Milli-Q water) and eluant B (200 mM sodium hydroxide) with eluant C (1 M sodium acetate) using a flow rate of 1.0 ml min^{-1}.

Results and discussion

Batch fermentation in bioreactor

One of the most important tasks for large scale microbial lipid production is to identify reliable yet cheap feedstock. While waste materials, including glycerol and whey, have been applied [14, 22], it remains in high demand to explore raw materials based on agricultural practice with no arable land requirements. JA has a potential to support industrial

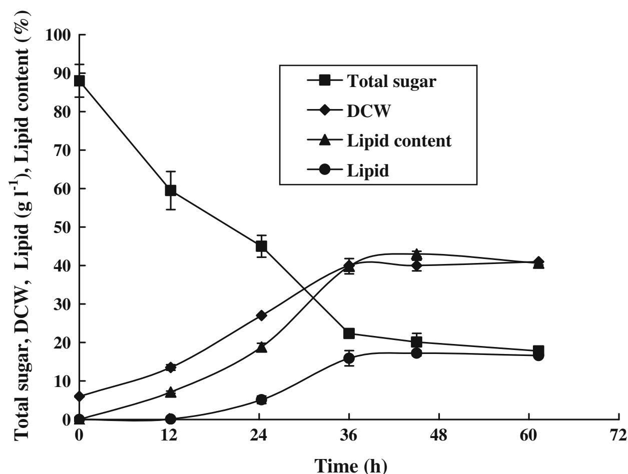

Fig. 1 Time courses of lipid production by *R. toruloides* Y4 on JA extracts in a 15-l bioreactor using a batch mode

biotechnology, because it can grow on poor land and has high biomass productivity [19]. One of the most attractive features is that fresh JA tuber contains about 17% (w/w) inulin, oligosaccharides composed of fructose and glucose with a relative lower DP values [1]. Therefore, JA can be readily hydrolyzed to release monosaccharides for microbial cultivation. In addition, JA tuber also contains minerals and other integrants that are potential nutrients for cell growth.

Our previously study found that *R. toruloides* Y4 can accumulate substantial amounts of intracellular lipid when JA extracts are used as the sole carbon and nitrogen sources [8]. In a typical shaking flask culture experiment, biomass and lipid content were 25.5 g l^{-1} and 39.5% (w/w), respectively, after 136 h. To further understand the lipid production profile on JA tubers by this strain, we decided to scale up the process using a 15-l stirred tank bioreactor. We first conducted batch culture of *R. toruloides* Y4 on JA extracts. The time courses of total sugar, DCW and lipid are shown in Fig. 1. The maximal DCW of 40 g l^{-1} and lipid of 17.2 g l^{-1} were obtained after 45 h. This batch mode gave 57 and 70% increments in DCW and lipids, respectively, compared to those of the shaking flask culture. This was likely due to an improved control over culture conditions, such as pH and dissolved oxygen, by the bioreactor unit. However, DCW remained almost constant at 40 g l^{-1} till the end of the culture, and the residual total sugar decreased very slowly after 36 h. The residual total sugar was about 20 g l^{-1} in the end of fermentation. Furthermore, dissolved O_2 increased during the culture process after 36 h, indicating that cell growth ceased, likely due to substrate limitations [14]. Thus, it seemed that there were sugar components in JA extracts that could not be assimilated under such conditions.

Fed-batch fermentation in bioreactor

As our early results demonstrated that a fed-batch culture mode could significantly improve lipid production [12], we next performed fed-batch cultivation of *R. toruloides* Y4. Concentrated JA extracts with a total reducing sugar concentration of 700 g l^{-1} were used as the feeding material. In this experiment, cell cultures were started with an initial total sugar concentration of 120 g l^{-1}. After 35.8 h, total sugar concentration dropped to 19.5 g l^{-1}, while dissolved O_2 increased to above 70%. At that moment, 500 ml of concentrated JA extracts were fed. The feeding was done for 6 times during the cultivation process within 119.8 h. The DCW, lipid and lipid content reached 113, 45.4 g l^{-1} and 40% (w/w), respectively (Fig. 2a). The fed-batch mode gave 182 and 164% increments in DCW and lipid, respectively, compared to those of the batch mode. However, cellular lipid content of 40% (w/w) was even lower than that of batch mode. Because concentrated JA extracts had a C/N ratio of 42, the feeding should actually supply excess nitrogen sources that had detrimental effects on lipid accumulation, as *R. toruloides* prefers a nitrogen-deficient environment for lipid production [3, 4]. It should be noted that the residual total sugar was almost equal to the batch mode, indicating that some robust components escaped from microbial assimilation. These components may be oligosaccharides with higher DP values. Therefore, we decided to prepare JA hydrolysates for the lipid production.

We next performed fed-batch cultivation of *R. toruloides* Y4 on concentrated JA hydrolysates. The culture was started with an initial total sugar concentration of 95 g l^{-1}. It was found that dissolved O_2 burst to above 70% after 45.2 h, indicating that carbon sources were diminishing. Thus, 500 ml of concentrated JA hydrolysates were fed. The feeding was done 6 times within 118.4 h. Figure 2b shows the time course of this lipid production experiment. The DCW, lipid and lipid content reached 70, 39.6 g l^{-1} and 56.5% (w/w), which represent increases of 75, 130 and 31%, respectively, compared to those of the batch mode.

It was interesting to note that the residual total sugar was reduced to 0.7 g l^{-1} at 45.2 h when JA hydrolysates were employed. This phenomenon suggests that carbohydrates of the JA tubers were made available to the hydrolysis process, i.e., treatment at 100°C, pH 3.0 for 30 min. However, the anthrone-sulfuric acid method indicated that the residual total sugar increased progressively during the fed-batch process (Fig. 2b). To solve this apparent discrepancy, we analyzed the fermentation broth using IC. No detectable monosaccharides or oligosaccharides with DP values within 30 were found in the broth samples collected at 45.2 h and 118.4 h (data not shown). Thus, we believe that the anthrone-sulfuric acid method did not reveal real total sugars in these cases. One possible reason may be that the

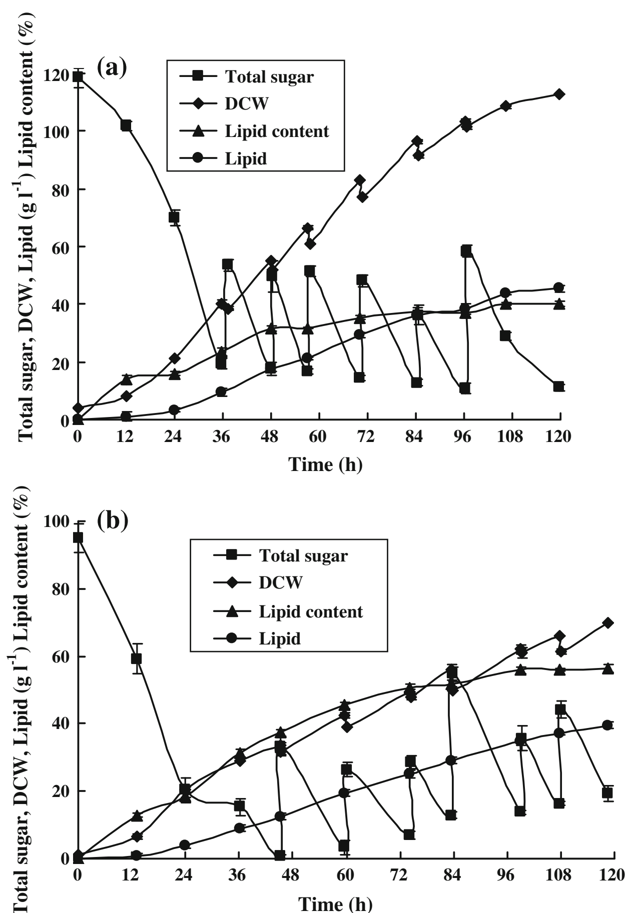

Fig. 2 Time courses of lipid production by *R. toruloides* Y4 on JA extracts (**a**) and JA hydrolysates (**b**) in a 15-l bioreactor using a fed-batch mode

hydrolysis process produced compounds such as 2-hydroxymethylfurfural (HMF) or difructose anhydride [21], which are known to interfere with the anthrone-sulfuric acid method [9]. Although the DCW was lower, cellular lipid content was improved substantially when JA hydrolysates were utilized. There was another phenomenon worthwhile mentioning. JA hydrolysates had a total C/N molar ratio of 18, which was pretty low compared to known conditions for lipid production. Yet, *R. toruloides* Y4 accumulated substantial amounts of lipid on JA hydrolysates. These results imply that a portion of the nitrogen sources seemed inert, i.e., unavailable as nutrients for cell growth. Indeed, we noted that total nitrogen was consumed very slowly during the culture and accumulation of nitrogen sources occurring after repeated feeding of JA hydrolysates (data not shown).

Table 1 shows a comparison of lipid production by *R. toruloides* Y4 using JA tubers with different cultivation modes. It was clear that cultures using a bioreactor gave much better results, especially for lipid productivity, than those using a shaking flask. Specifically, lipid productivity was improved over fivefold when JA extracts were used. Also, higher DCW and lipid titers were obtained in the fed-batch mode. The highest DCW of 113 g l^{-1} and the highest lipid titer of 45.4 g l^{-1} were obtained in fed-batch mode with concentrated JA extracts. However, the highest lipid content of 56.5% (w/w) was observed in fed-batch mode with concentrated JA hydrolysates. When concentrated JA extracts were applied, lipid productivity was 0.38 g l^{-1} h^{-1} for cultures using either batch mode or fed-batch mode, whereas the fed-batch mode with concentrated JA hydrolysates gave a slightly lower lipid productivity of 0.33 g l^{-1} h^{-1}. The concentrated JA hydrolysates were prepared by heating at 100°C, pH 3.0 for 30 min. Such harsh conditions led to nearly complete hydrolysis of inulin to monosaccharides; yet, fructose may be dehydrated to HMF, a compound known to have inhibitory effects on cell-growth and lipid production [10].

An early study proposed that a lipid productivity of 1.0 g l^{-1} h^{-1} should be reached to make microbial lipid technology costs competitive for commercialization [15]. Our earlier study reached a lipid productivity of 0.54 g l^{-1} h^{-1} using glucose and yeast extracts as the carbon source and nitrogen sources, respectively [12]. In this work, lipid productivity was 0.33–0.38 g l^{-1} h^{-1}. However, commercialization of microbial lipids should be highly dependent on the costs of the feedstock and the quality of the lipid per se. When JA extracts were used as feedstocks, almost no auxiliary ingredients were required during the cultivation process. This should reduce the production costs significantly. On the other hand, strategies should be developed to make the pretreatment processes more efficient,

Table 1 Comparison of lipid production by *R. toruloides* Y4 using JA tubers with different cultivation modes

Culture mode	DCW (g l^{-1})	Lipid content (%)	Lipid (g l^{-1})	Lipid productivity (g l^{-1} h^{-1})	References
Shake-flask	25.5	39.5	10.1	0.07	[8]
Batch	40	43.3	17.2	0.38	This study
Fed-batch[a]	113	40.1	45.4	0.38	This study
Fed-batch[b]	70	56.5	39.6	0.33	This study

[a] Concentrated JA extracts feeding

[b] Concentrated JA hydrolysates feeding

preferrably without generation of inhibitory compounds. Moreover, studies are surely required for optimization of the cultivation process to further improve lipid productivity.

In conclusion, we demonstrated that microbial lipids can be effectively produced by culturing oleaginous yeast *R. toruloides* Y4 using extracts or hydrolysates of JA tubers as the feedstocks. In the future, it should be more viable to incorporate biorefinery concepts in exploitation of JA tubers as feedstock for biofuels and bio-based chemicals.

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