Bioresource Technology 157 (2014) 214–222

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of feeding strategies on lipid production by *Lipomyces starkeyi*

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HIGHLIGHTS

- *Lipomyces starkeyi* exhibited the highest productivities using undetoxified H-H.
- The simultaneous utilization of glucose and xylose for lipid production was observed.
- High cell mass and lipid concentrations were obtained at repeated fed-batch strategy.
- Highest cell mass and lipid yields were obtained at a low dilution rate of 0.03 h^{-1}.
- The fatty acid composition profile was notably similar to that of palm oil.

ARTICLE INFO

Article history:
Received 29 November 2013
Received in revised form 19 January 2014
Accepted 24 January 2014
Available online 4 February 2014

Keywords:
Biodiesel
Oleaginous yeast
Sugarcane bagasse hydrolysate
Fed-batch
Continuous culture

ABSTRACT

The aim of this study was to produce microbial oil from *Lipomyces starkeyi* DSM 70296 grown in hemicellulose hydrolysate (H-H). Glucose and xylose were used for batch, fed-batch, repeated fed-batch, and continuous cultures, and H-H was tested at continuous culture. The highest cell and lipid concentrations of 85.4 and 41.8 g/L, respectively, were obtained using repeated fed-batch strategy. Continuous culture with dilution rate of 0.03 h^{-1} presented the highest overall cell (0.443 g/g) and lipid yields (0.236 g/g). At 0.06 h^{-1} were obtained the highest cell and lipid productivities. Continuous cultivation using H-H at 0.03 h^{-1} resulted in higher cell productivity than that obtained using glucose:xylose. Gas chromatography analysis of the esterified lipids indicated that the major constituents of this complex are palmitic acid, stearic acid, oleic acid, and linoleic acid with an estimated cetane number (approximately 61) similar to that of palm biodiesel, which is important for biofuel production.

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1. Introduction

The oleaginous yeast *Lipomyces starkeyi* is a species belonging to the Saccharomycetales order that was originally isolated from soil. The available information regarding how nutrient-derived signals regulate cell growth and how these nutritional signals are integrated with growth factor pathways in eukaryotes is limited. Nutrient limitation evokes a complex change in the physiology of yeast cells, which allows them either to re-program their metabolism such that they can cope with the change in nutrient supply or to activate a survival program to outlive sustained periods of starvation (Gray et al., 2004).

In general, lipid production by oleaginous microorganisms is classified into two steps: the growth phase and the lipid production phase (Ratledge and Cohen, 2008). The fed-batch process is an important strategy because it makes it possible to control the growth and lipid accumulation phases by modifying the feed throughout the fermentation process. In theory, during the late stage of a fed-batch process, nitrogen sources are exhausted, which leads to a reduction in the cell mass production rate and the channeling of the flux of carbon toward lipid biosynthesis. However, in the repeated fed-batch culture process, fresh medium containing nitrogen is fed to restart exponential cell propagation (Zhao et al., 2011).

In addition to glucose, pentoses (mainly xylose) are the main carbon components of hemicellulose hydrolysate (H-H) derived from agricultural wastes (lignocellulose-rich material), and microbial lipid production from xylose has recently been explored (He et al., 2010). Because it can be used directly for hydrolysis following fermentation in sugar plants, sugar cane bagasse is inexpensive and thus appears to be more competitive for lipid production than other lignocellulosic biomasses (Huang et al., 2012).

Because the lipid contents of oleaginous yeast are generally influenced by the media components, particularly the carbon

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http://dx.doi.org/10.1016/j.biortech.2014.01.104
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source, nitrogen source, and minerals, it is logical to identify good conditions for lipid production from glucose and xylose. Some oleaginous yeasts can assimilate glucose and xylose simultaneously to accumulate intracellularly a considerable amount of lipid with a good lipid coefficient, in both artificial and real hydrolysates (Hu et al., 2011). The fermentation of mixed sugars is not only significant for the improvement of the overall economics associated with microbial lipid production but also holds an interesting route for effective cell mass conversion (Zhao et al., 2008). However, few studies have investigated process analysis with the aim of evaluating different feeding strategies. Therefore, the present study focused on evaluating the effect of alternative feeding strategies using a mixture of glucose and xylose, as well as H-H, as carbon sources on cell mass and lipid production.

2. Methods

2.1. Strain and media

The oleaginous yeast *L. starkeyi* DSM 70296 was used throughout this study. Agar slants (YPD medium) were stored in a refrigerator and propagated monthly or stored through cryopreservation at −80 °C with 10% (v/v) glycerol. The composition of the inoculum and fermentation medium was (per liter): 1.92 g of yeast extract (0.192 g of nitrogen), 1.36 g of $(NH_4)_2SO_4$ (0.288 g of nitrogen), 1 g of Na_2HPO_4, 1 g of KH_2PO_4, 0.4 g of $Mg_2SO_4{\cdot}7H_2O$, 0.04 g of $CaCl_2{\cdot}2H_2O$, 0.08 g of $ZnSO_4{\cdot}7H_2O$, 0.001 g of $CuSO_4{\cdot}5H_2O$, 0.001 g of $CoCL_2{\cdot}6H_2O$, and 0.005 g of $(NH_4)_2Mo_2O_7$ (pH 5.5).

For the carbon and energy sources, sugars (glucose:xylose) at 60 g/L (24 g of carbon) were mixed at different proportions to produce an artificial lignocellulosic (70:30) or hemicellulose hydrolysate (30:70). The initial carbon-to-nitrogen (C/N) ratio was fixed at 50 for all operation modes. To avoid medium darkening due to the reaction of the sugars (caramelization and Maillard reactions), the sugar solutions were sterilized in separate flasks. All of the chemicals and reagents used were of analytical grade.

2.2. Raw material

Sugarcane bagasse was kindly provided by CTBE (National Laboratory of Bioethanol Science and Technology, Campinas-SP, Brazil), and its chemical composition on a dry basis 49.5% cellulose, 24.3% hemicellulose, 22.7% lignin, and 0.3% ash. The sugarcane bagasse was hydrolyzed with 1.5% sulfuric acid (w/v) at a solid-to-liquid ratio of 1:10 in an autoclave at 120 °C for 20 min. After hydrolysis, the mixture was separated by centrifugation at 2980*g* and vacuum filtered, and the liquid fraction (H-H) was stored at −18 °C until its use in the continuous cultivation experiments. The composition of H-H obtained was 13.1 g/L xylose, 2.9 g/L glucose, 2.6 g/L acetic acid, 2.1 g/L arabinose, 0.04 g/L furfural, and 0.02 g/L hydroxymethylfurfural (HMF).

2.3. Culture conditions

The inoculum was prepared through two successive cell propagations in liquid media at 28 °C and 150 rpm in an orbital shaker. The first was incubated for 48 h, and the second was incubated for 30 h until a cell mass of 10 g/L (equivalent to 1×10^8 cells/mL) was obtained. The experiments using glucose:xylose were conducted in a 3.0-L bioreactor (New Brunswick, USA) with an initial working volume of 1.0 L, and the experiments using H-H medium were conducted in a 1.3-L bioreactor (New Brunswick, USA) with working volume of 0.5 L. For all cultivations, the aeration rate, agitation, and temperature were set to 1 vvm, 400 rpm, and 28 °C, respectively. The pH was maintained at 5.5 through the automatic addition of 2 M NaOH. Aliquots were collected at various intervals and stored at −20 °C until their analysis for substrate concentrations, cell dry weight, and lipid content. Concentrated sugar solutions (600 g/L) were used for the fed-batch modes, and the concentration of the nutrient solution (with nitrogen sources and salts) was tenfold higher than that of the culture medium. The fed-batch cultures were started with an initial sugar concentration of 60 g/L in the bioreactor, which was fed using a peristaltic pump. To determine an appropriate sugar and nutrient feeding strategy, different modes were studied, as described in Table 1.

In Study 1, concentrated solutions of glucose:xylose and nutrients were added into the bioreactor three times to increase the sugar concentration to 60 g/L immediately after the residual sugar concentration decreased to 0–5 g/L. The main goal of Study 2 was to evaluate the relationship between the cell growth and the lipid content at a constant C/N ratio throughout the cultivation. For this reason, different volumes of both feeding solutions (sugar and nutrients) were added based on the analysis of the sugar and nitrogen contents in the broth during the cultivation. After the analysis of each sample, the sugar concentration was adjusted to 60 g/L, and nitrogen and salts were also added to reach a C/N of 50 or 900 to induce lipid accumulation, as suggested by Sattur and Karanth (Sattur and Karanth, 1989). We previously found that this C/N value allows cell growth and high lipid storage. The C/N ratio was maintained constant (at 50) during the first 48 h of cultivation and was then increased to 900. For Study 3, the first three feeding pulses contained sugar and nutrient solutions, and the last three pulses only included sugar. The single-batch stage of this study represents Study 4. A batch experiment (Study 5) was also conducted with an inoculum concentration of 3 g/L to reduce the lag phase of growth in the bioreactor. This inoculum concentration was used for the next cultivations. Another fed-batch mode (Study 6), in which the first three feeding pulses contained sugar and nutrient solutions and the last pulse involved only sugars, was also investigated.

A repeated fed-batch culture (Study 7) with two cycles was performed after Study 6. At the end of the first fed-batch cycle, half of the fermentation broth was removed such that only 500 mL remained in the fermenter. The fermenter was then filled with 500 mL of fresh medium to accelerate the cell growth phase of the second cycle. In the first cycle, the feeding pulses were the same as those used in Study 6, but the feeding pulses used in the second cycle contained only sugar solutions.

For continuous culture with glucose:xylose, a sugar solution containing 60 g/L (30:70, w/w) was used, and the C/N ratio was adjusted to 50. For continuous culture with H-H medium, a C/N of 50 was used, and yeast extract (10% of N) and $(NH_4)_2SO_4$ (21% of N) were added into the medium according to the concentration of the carbon sources (xylose, glucose, arabinose, and acetic acid) present in the H-H. The dilution rate (D, volume of incoming medium per unit time/volume of medium in the culture vessel) was attained by varying the medium flow. According to Gill et al. (1977), maximum lipid accumulation requires the yeast to be grown at a dilution rate equal to one-third the value of the maximum growth rate. In this order, dilution rates of 0.03 h^{-1} (Study 8) and 0.06 h^{-1} (Study 9) were studied with glucose:xylose, and the continuous culture was started after 24 h of batch cultivation. Study 10 was performed at a D value of 0.03 h^{-1}, and the continuous culture was started after 15 h of batch cultivation. Steady-state conditions were obtained after a continuous flow of at least four working volumes of the culture medium.

2.4. Analytical methods

The cell optical density was measured at 600 nm with a nanophotometer (Implen GmbH, Germany). The cell dry weight (CDW)

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