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Nitrogen limitation, oxygen limitation, and lipid accumulation in *Lipomyces starkeyi*

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HIGHLIGHTS

- Measurements of nitrogen concentration enabled monitoring of C:N ratios over time.
- Lipid production was optimal in flasks with an initial C:N ratio of 72:1.
- Alcohols including ethanol, mannitol, arabitol, and 2,3-butanediol were produced.
- Increased agitation rates significantly reduced alcohol and lipid accumulation.
- Corn stover hydrolysate was converted to lipids similar in composition to palm oil.

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ABSTRACT

Lipid production by oleaginous yeasts is optimal at high carbon-to-nitrogen ratios. In the current study, nitrogen and carbon consumption by *Lipomyces starkeyi* were directly measured in defined minimal media with nitrogen content and agitation rates as variables. Shake flask cultures with an initial C:N ratio of 72:1 cultivated at 200 rpm resulted in a lipid output of 10 g/L, content of 55%, yield of 0.170 g/g, and productivity of 0.06 g/L/h. All of these values decreased by ≈50–60% when the agitation rate was raised to 300 rpm or when the C:N ratio was lowered to 24:1, demonstrating the importance of these parameters. Under all conditions, *L. starkeyi* cultures tolerated acidified media (pH ≈ 2.6) without difficulty, and produced considerable amounts of alcohols; including ethanol, mannitol, arabitol, and 2,3-butanediol. *L. starkeyi* also produced lipids from a corn stover hydrolysate, showing its potential to produce biofuels from renewable agricultural feedstocks.

1. Introduction

Replacing non-renewable fossil fuels with environmentally sustainable biofuels remains a major challenge for our civilization. Oleaginous yeasts, defined by their ability to accumulate from 20% to 70% of their total dry biomass as lipids, are being investigated for their potential to produce a variety of valuable chemicals (Sitepu et al., 2014b). Metabolic engineering and bioprocess optimization efforts may lead to economically competitive manufacturing of biodiesel fuel using these organisms. *Lipomyces starkeyi* is a particularly well suited host given its impressive native abilities, including the capability to utilize a wide variety of carbon sources and to grow in media without vitamin supplementation (Sitepu et al., 2014a). *L. starkeyi* is capable of simultaneously utilizing mixed sugars, including glucose and xylose (Anschau et al., 2014), glucose and mannose (Yang et al., 2014), and cellobiose and xylose (Gong et al., 2012). This species is also known for its considerable ability to degrade extracellular polysaccharides by secreting glycosidases (Kang et al., 2004; Angerbauer et al., 2008; Wild et al., 2010), a trait that could be useful for generating valuable oils from low-cost food waste substrates (Ratledge and Cohen, 2008). Finally, *L. starkeyi* has a sequenced genome (Grigoriev et al., 2011) and a recently developed transformation system (Calvey et al., 2014), providing tools for genetic manipulation which are lacking in most oleaginous yeast species.

The future of biofuels depends upon developing strains that are capable of growth on affordable and environmentally sustainable feedstocks. Lignocellulosic hydrolysates primarily contain glucose and xylose, so microbial conversion of pentose sugars is essential

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(Jin et al., 2015). Previous studies have demonstrated that *L. starkeyi* can produce lipids from hydrolysates derived from spent yeast cells (Yang et al., 2014), sewage sludge (Angerbauer et al., 2008), wheat straw (Yu et al., 2011), sugarcane bagasse (Anschau et al., 2014), corncobs (Huang et al., 2014), and corn stover (Sitepu et al., 2014c). Lipid accumulation proceeded despite the presence of moderate concentrations of inhibitors released during the hydrolysis of hemicellulosic biomass; including acetic acid up to 4 g/L (Yu et al., 2011), hydroxymethylfurfural up to 2 g/L (Sitepu et al., 2014a), and up to 0.5 g/L furfural (Chen et al., 2009). The ability of *L. starkeyi* to produce lipids despite the presence of these inhibitors fulfills an essential prerequisite for "second generation" biofuel production.

Oleaginous yeasts are known to accumulate lipids when grown in a nitrogen-deficient or other nutrient-limited medium (Ratledge and Wynn, 2002). Nitrogen limitation can be induced by growing cultures in media with a high molar ratio of carbon to nitrogen; typical C:N ratios reported in the literature are in the range of 50:1–150:1 (Subramaniam et al., 2010). In experiments that investigate multiple C:N ratios, lipid accumulation commonly increases as the C:N ratio increases (Angerbauer et al., 2008; Wild et al., 2010). However, nitrogen concentrations are rarely measured directly in these studies. Here, several *L. starkeyi* batch shake flasks with defined minimal media were studied, in which the nitrogen content was altered while keeping all other variables constant. Additionally, a persulfate digestion method for measuring media nitrogen levels was employed, to directly investigate the relationship between nitrogen limitation and lipid production over time.

Lipid accumulation is the dominant metabolic activity of oleaginous yeasts during nitrogen limitation (Ratledge and Wynn, 2002). However, few studies have examined the influence of aeration on lipid productivity in these species (Sitepu et al., 2014b). Further, the effects of oxygen limitation appear to differ widely among lipogenic yeasts. For example, in *Apiotrichum curvatum* (*Cryptococcus curvatus*) grown on casein whey, lipid yields decreased with lower oxygenation rates (Davies et al., 1990). In one *L. starkeyi* study, dissolved oxygen concentrations had no significant effect on lipid yields (Naganuma et al., 1985). Conversely, in *Yarrowia lipolytica* cultivated with stearin, lipid accumulation occurred at low oxygen saturation, while cell mass was produced predominantly in highly aerated cultures (Papanikolaou et al., 2002). High dissolved oxygen concentrations also consistently reduced lipid accumulation in *Rhodotorula glutinis* (Yen and Zhang, 2011). Compared to cells grown in airlift bioreactors, which typically exhibit low aeration rates, *R. glutinis* cells cultivated in highly aerobic agitated fermenters had a faster growth rate and higher cell mass, yet a nearly 50% reduction in average lipid content (Yen and Liu, 2014). In the following studies, evidence indicates that oxygen limitation plays an important role in promoting lipid accumulation in *L. starkeyi*.

2. Methods

2.1. Strains and media

L. starkeyi NRRL Y-11557 (ATCC 58680, CBS 1807) was used throughout these experiments. Unless otherwise noted, reagents were purchased from Sigma–Aldrich (St. Louis, MO). *L. starkeyi* cultures were maintained on YPD plates (m/v: 1% yeast extract, 2% peptone, 2% glucose, 2% agar) with subculturing once per month.

All starter cultures were grown in 50 mL of defined minimal media (DMM) with 20 g/L of glucose, as described by (Long et al., 2012). Lipid accumulation experiments were carried out in modified DMM, containing 60 g/L of glucose and nitrogen provided as a mixture of urea and ammonium chloride with a 1:1 ratio of atomic nitrogen (Section 3.4). Carbon-to-nitrogen (C:N) ratios were varied as indicated: media with an initial C:N ratio of 24:1 contained 1.07 g/L of urea and 1.91 g/L of NH_4Cl, corresponding to a total atomic nitrogen content of 1.0 g/L. Media with an initial C:N ratio of 48:1 contained 0.54 g/L of urea and 0.96 g/L of NH_4Cl, corresponding to a total atomic nitrogen content of 0.5 g/L. Media with an initial C:N ratio of 72:1 contained 0.36 g/L of urea and 0.64 g/L of NH_4Cl, corresponding to a total atomic nitrogen content of 0.33 g/L. All media were sterilized by passage through a 0.2 μm filter.

Corn stover hydrolysate treated using the ammonia fiber expansion (AFEX™) process was obtained from Michigan State University (Balan et al., 2009). The AFEX-treated corn stover hydrolysate (referred to herein as "AFEX") contained approximately 49 g/L glucose and 20.5 g/L xylose. Nitrogen content of the hydrolysate was not determined, but the C:N ratio was estimated to be approximately 20:1 based on the composition of synthetic AFEX as described by Sitepu et al. (2014c).

2.2. Culturing conditions

All shake flask experiments were conducted in 500 mL Erlenmeyer flasks containing 200 mL of media, incubated at 30 °C and shaken at 200 rpm (rpm), unless otherwise noted. For experiments in defined media, starter cultures were grown in DMM for 48–72 h, and cells were inoculated at 1–2% by volume in order to yield initial OD_{600} readings targeted at 0.75. AFEX flasks were also inoculated with DMM cultures, to a starting OD_{600} of 5. No increase in OD_{600} was observed after 4 days, and the flasks were reinoculated with a second starter culture to a higher density (OD_{600} of 8) and a final inoculation volume of ≈13% (v/v).

2.3. Analytical methods

For all experiments, analytical methods described below were performed once per day. Cell concentration was determined with an Agilent 8453 spectrophotometer (Santa Clara, CA) by taking optical density readings at 600 nm (OD_{600}). Dry cell weight (DCW) was determined by filtering 5–7 mL of culture through a pre-weighed PALL Corporation 0.2 μm pore size polyethersulfone membrane (Port Washington, NY), drying the cells by microwaving at low power for 10–15 min, and weighing. All sugar (glucose, xylose) and alcohol (ethanol, mannitol, arabitol, 2,3-butanediol) concentrations were determined by high performance liquid chromatography (HPLC) as described by Su et al. (2014). Media pH was measured using a Fisher Scientific Accumet AB15 Basic meter (Hampton, NH).

To determine lipid content, 2 mL of cells were washed, centrifuged, and frozen at −20 °C prior to extraction. Thawed cell pellets were resuspended in 2 mL of water, and lipids were liberated by addition of 200 μL of concentrated HCl, with heating at 95 °C for 1 h. Lipids were collected using a Bligh & Dyer type extraction with 7.5 mL of 2:1 (v:v) methanol:chloroform and three extractions in 2 mL of chloroform (Bligh and Dyer, 1959). Pooled chloroform layers were evaporated to dryness in a 40 °C heating block under a constant stream of N_2 prior to transesterification. To convert lipids to fatty acid methyl esters (FAMEs), 1.0 mL of anhydrous HCl in methanol was added to the dried extract, and the mixture was heated at 50 °C overnight. Then, 1.0 mL n-Hexane and 10 mL of 50 mg/mL $NaHCO_3$ were added to the samples, vortexed thoroughly, and centrifuged to separate the layers. The hexane layer was collected and the extracted FAMEs were analyzed immediately using a LECO Corp. Inc., (Saint Joseph, MI) Pegasus 4D GCxGC TOFMS with an Agilent Technologies (Santa Clara, CA) 7890A gas chromatograph and a Stabilwax-DA 30 m × .25 mm I. D., × 0.25 μm primary column. Helium was used as the carrier gas with a flow rate of 1.0 mL/min. Inlet temperature was 225 °C. The primary GC oven temperature program began at 100 °C held