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Sequential recycling of enzymatic lipid-extracted hydrolysate in fermentations with a thraustochytrid

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HIGHLIGHTS

- Nutrient recycling was conducted with *Thraustochytrium* sp. (T18) in fermentors.
- Recycling enzymatic hydrolysate with T18 is possible without drop in production.
- Lipid production is slightly reduced due to excessive nitrogen available.
- Sequential recycling showed equivalent biomass and improved lipid content.
- Fatty acid profiles in twice-recycled hydrolysate had improved DHA content.

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ABSTRACT

This study extends the findings of prior studies proposing and validating nutrient recycling for the heterotrophic microalgae, *Thraustochytrium* sp. (T18), grown in optimized fed-batch conditions. Sequential nutrient recycling of enzymatically-derived hydrolysate in fermentors succeeded at growing the tested thraustochytrid strain, with little evidence of inhibition or detrimental effects upon culture health. The average maximum biomass obtained in the recycled hydrolysate was 63.68 ± 1.46 g L^{-1} in 90 h the first recycle followed by 65.27 ± 1.15 g L^{-1} in 90 h in the subsequent recycle of the same material. These compared to 58.59 g L^{-1} and 64.92 g L^{-1} observed in fresh media in the same time. Lipid production was slightly impaired, however, with a maximum total fatty acid content of $62.2 \pm 0.30\%$ in the recycled hydrolysate compared to 69.4% in fresh control media.

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1. Introduction

Nutrient recycling – the strategy of recirculating microalgae production wastewaters into successive cultures – is an increasingly important topic as research and development advances toward commercialization of microalgae technologies. Regardless of the specific metabolic state (i.e. photoautotrophic, heterotrophic, or mixotrophic), species selection, culture system or feedstock selection, all microalgae production systems require enormous quantities of nutrients and water (Brennan and Owende, 2010; Chisti, 2013; Pate et al., 2011). The magnitude of that resource consumption at a commercially relevant scale demands a significant allocation of natural resources to the production process (Wigmosta et al., 2011). Additionally, any unused media elements and potentially toxic waste material (e.g. organic solvents) represent a serious environmental hazard if not treated properly prior to discharge.

The overarching conclusion from prior research is that nutrient recycling does offer potential to increase utilization efficiency of nutrients and water; however, mitigating the likely negative effect of inhibitory conditions in the secondary cultures is the most serious challenge (Biller et al., 2012; Discart et al., 2014; Hadj-Romdhane et al., 2012; Lowrey et al., 2015). To avoid inhibition during nutrient recycling, a critical aspect to address is the potential for accumulation of micronutrients, salts or inhibitory compounds in the substrate after repeated recycles of media. As repeated nutrient additions are made into the same recycled substrate, residual concentrations of unused media elements can accumulate to levels that are hazardous to the microalgae or the stainless steel production equipment (González-López et al.,

Abbreviations: T18, *Thraustochytrium* sp.; AOM, algogenic organic matter; FAA, free amino acids; DHA, docosahexaenoic acid; FAME, fatty acid methyl esters; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid.

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2013; Lowrey et al., 2015). Specific compounds of concern include salts, chloride ions and Maillard intermediaries such as hydroxymethylfurfural (HMF) that are formed during exposure to high temperatures (Nursten, 2005; Patil and Gogate, 2015).

Our study explores sequential nutrient recycling with T18 using enzymatic lipid-extracted hydrolysate that was supplemented with media elements. Building upon successful results from single recycle in batch-scale flasks (Lowrey et al., 2016a,b), this investigation specifically addresses the prospect and challenge of scaling up to highly controlled fermentors. Parallel fermentations using fresh media and identical growth conditions provided a baseline for assessing the recycled hydrolysate as a secondary fermentation substrate. To assess practical viability as well as the potential accumulation of inhibitors, it was also necessary to conduct at least two sequential secondary fermentations using the same recycled material with all media ingredients added. Fermentors were operated in fed-batch conditions to provide commercially-applicable data for sequential nutrient recycling using heterotrophic microalgae. Differences observed in biomass yields and substrate usage provided some insight into the viability of a more extensive application of nutrient recycling for thraustochytrids in enzymatically hydrolyzed waste.

2. Methods

2.1. Primary fermentation and initial hydrolysate production

To meet the volume requirements for hydrolysate in 2-liter secondary fermentations it was necessary to conduct the primary fermentation with T18 in a 500-liter glucose fed-batch fermentation. At the end of the 187-h fermentation, enzymatic lipid extraction was conducted as described by Dennis and Armenta (2015). Specifically, the extraction method used 0.1% (v/v) of the enzyme Alcalase (Novozymes, Bagsvaerd, Demark). Hydrolysis conditions were 55 °C and pH 8.0 with 100 rpm mixing for 12 h resulting in an 87.7% extraction efficiency (Dennis and Armenta, 2015). The resultant emulsion was then separated using a Westfalia ASD-2 continuous disc centrifuge (Rheda-Wiedenbrück, Germany) operating at 10,000 rpm. The heavy-phase waste product, henceforth designated as the hydrolysate, was then collected and frozen for subsequent recycling experiments.

2.2. Seed preparation

For each sequential secondary fermentation in the three 2-liter fermentors, a total seed volume of 300 mL was required to inoculate each of them using 100 mL (6.25% v/v). This required preparing a 2-liter seed flask with a fermentation medium volume of 500 mL. Inoculum was obtained from refrigerated cultures of T18 grown on standard fermentation media agar on petri dishes for 4 weeks. The cells were used to inoculate the 2-liter Erlenmeyer flasks with 500 mL of the following medium (per liter): 60 g glucose, 4 g soy peptone, 1 g yeast extract, 4 g $MgSO_4{\cdot}7H_2O$, 2 g NaCl, 6.8 g $(NH_4)_2SO_4$, 1.6 g KH_2PO_4, 1.75 g K_2HPO_4, 0.5 mL $FeCl_3{\cdot}6H_2O$, 1.5 mL trace elements, 0.5 mL $CaCl_2{\cdot}2H_2O$, and 1 mL vitamin solution. The trace elements and vitamin solutions were prepared to the following recipe (per mL): trace elements were 2 mg $CuSO_4{\cdot}5H_2O$, 1 mg Na_2MoO_4, 2 mg $ZnSO_4$, 1 mg $CoCl_2$, 1 mg $MnCl_2$ and 1 mg $NiSO_4(H_2O)_6$; vitamins were 0.01 mg vitamin B_{12}, 0.01 mg biotin and 2 mg thiamin hydrochloride. All medium components were sourced from Sigma Aldrich (St. Louis, USA) and Fisher Scientific (Waltham, USA) and the soy peptone was obtained from Tekniscience Inc. (Terrebonne, Canada).

Once the seed medium was prepared and autoclaved, the seed flask was aseptically inoculated with one full loop of plated T18 biomass and placed on the New Brunswick Scientific Excella E25 orbital shaker (Edison, USA) at 200 rpm and 25 °C. The seed was allowed to grow for three days prior to evaluation for further use. After the incubation period the seed was evaluated prior to use to ensure that acceptable growth occurred and no morphological issues or contamination existed.

2.3. Secondary fermentations and recycle

2.3.1. 2-Liter fermentors

Three Sartorius Biostat B-DCU 2-liter fermentors (Göttingen, Germany) were used for the experiments, operated in fed-batch mode. Sartorius MFCS/DA 3.0 software continuously measured the pH, temperature, dissolved oxygen (DO), agitation rate and the volume of acid and base additions. Temperature was controlled by pumping heated or chilled water from the VWR International 1197P Programmable Temperature Controller (Radnor, USA) through the double-walled vessel. Aeration was provided through a flow meter into two 0.20 μm disc filters and through the stainless steel air sparger at the bottom of the fermentor vessel.

2.3.2. Experimental design

Using three vessels, one was designated as a control and the other two were duplicates of the supplemented hydrolysate (AH-1 and AH-2). All vessels were setup identically and conditions across all three were set to the same values. To match the conditions in the previous flask fermentations, the hydrolysate was diluted by adding 800 mL (50% v/v) into 600 mL of fermentation media (prepared to 1.6 L recipe amounts as shown in Table 1) (Lowrey et al., 2016b) In the fermentor 100 mL of buffer and 100 mL of seed were introduced, bringing the total secondary fermentation starting volume to 1.6 L.

2.3.3. General fermentation conditions

The secondary fermentations were designed to promote high productivity and to reveal differences between the recycled hydrolysate and a standard fermentation media that may not have been evident at a smaller scale (Lowrey et al., 2016a,b). Accordingly, to maximize T18 growth in the 2 L fermentations, operating fermentor conditions included a maintained vessel temperature of 25 °C with 600 rpm of mixing, 0.6 slpm of aeration and a pH of 4.50. During fed-batch operation, a calculated volume of 70% glucose solution was fed throughout the fermentation to restore the concentration of glucose to approximately 60 g L^{-1}. The secondary fermentations were conducted with the goal of feeding the culture with glucose at least twice, while reaching carbon and nitrogen depletion prior to termination. For equivalence, both the control

Table 1
Fermentation media recipe for 2-liter fermentations using recycled hydrolysate prepared to a total volume of 1.6 L. Acid and base additions are based upon the pH control and added as needed throughout the fermentation.

Ingredient	Amount (L^{-1})	Total
Glucose	60 g	96 g
Soy peptone	2 g	3.2 g
$MgSO_4{\cdot}7H_2O$	4 g	6.4 g
NaCl	9 g	14.4 g
$FeCl_3{\cdot}6H_2O$ (0.01 g mL^{-1} stock)	0.5 mL	0.8 mL
Trace solution (stock)	1.5 mL	2.4 mL
Antifoam	0.1 mL	0.16 mL
KH_2PO_4	2.2 g	3.52 g
K_2HPO_4	2.4 g	3.84 g
$(NH_4)_2SO_4$	10 g	16 g
Vitamin solution (stock)	3 mL	4.8 mL
$CaCl_2{\cdot}2H_2O$ (0.2 g mL^{-1} stock)	0.5 mL	0.8 mL
200 g L^{-1} NaOH solution	80 mL	128 mL
250 g L^{-1} H_3PO_4 solution	30 mL	48 mL

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