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Heterotrophic growth and lipid accumulation of *Chlorella protothecoides* in whey permeate, a dairy by-product stream, for biofuel production

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HIGHLIGHTS

- Whey permeate, a waste stream, was used to produce microalgae for biofuels.
- High lipid-algal biomass was obtained with whey permeate and yeast extract at low C/N.
- Batch, fed-batch and SSF bioprocesses modes were investigated for algal culture.
- Algal fatty acid profile when grown in whey permeate was similar to that of glucose.

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ABSTRACT

This study proposes a novel alternative for the utilization of whey permeate, a by-product stream from the dairy industry, as the feedstock for the biomass and lipid production of the microalgae *Chlorella protothecoides*. Glucose and galactose from the pre-hydrolyzed whey permeate were used as main carbon sources in a base mineral media for establishing batch and fed batch cultures. Batch cultures reached a biomass production of 9.1 ± 0.2 g/L with a total lipid accumulation of 42.0 ± 6.6% (dry weight basis), while in the fed batch cultures 17.2 ± 1.3 g/L of biomass with 20.5 ± 0.3% lipid accumulation (dry weight basis) were obtained. A third strategy for the direct utilization of whey permeate was investigated by simultaneous saccharification and fermentation (SSF), wherein, 7.3 ± 1.3 g/L of biomass with 49.9 ± 3.3% lipid accumulation (dry weight basis) was obtained in batch mode using immobilized enzyme.

1. Introduction

The use of microalgae for producing biofuels has been highlighted for its potential to be easily integrated into a biorefinery for obtaining diverse value added products with priority towards lipids. Broadly, the advantages that some microalgae offer include high lipid productivity, negligible competition for arable land, and adaptability to grow in inexpensive media including waste water streams (Chisti, 2007; Rodolfi et al., 2009; Stephens et al., 2010; Xavier Malcata, 2011; Menetrez, 2012). Heterotrophic systems offer technological advantages for the cultivation of microalgae including homogeneous, consistent and reproducible processes with overall higher rates of biomass and lipid production (Bumbak et al., 2011; Perez-Garcia et al., 2011). One of the best lipid producing microalgae to date is *Chlorella protothecoides* (Bumbak et al., 2011), which is able to use several carbon sources with preference towards glucose since it promotes the highest respiration and growth rates (Perez-Garcia et al., 2011). However, the use of pure glucose as carbon source can represent around 60–75% of the total cost of the culture media (Li et al., 2007; Menetrez, 2012). An effective strategy to lower the cost of culturing *C. protothecoides* is the utilization of inexpensive glucose-containing substrates (Cheng et al., 2009; Gao et al., 2010; Lu et al., 2010, 2011; Yan et al., 2011; Sun et al., 2013). An ideal scenario for the cost-effective heterotrophic cultivation of microalgae would be the utilization of abundant industrial by-product streams that have limited competing applications.

One such by-product stream is the whey permeate from the dairy industry. Whey permeate is obtained by ultrafiltration and removal of protein from whey that is generated during cheese manufacturing and represents about 85% of the total milk used in the process (Panesar and Kennedy, 2012). Whey permeate displays an overall composition of mostly lactose along with salts and non-protein nitrogen (Jelen, 2009). Considering the large amounts of whey permeate generated, its utilization and disposal becomes crucial. The use of whey permeate as a direct lactose

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source has been neglected due to the extensive processing required for its recovery such as demineralization and dewatering (Jelen, 2009).

Whey derivatives have been used to grow some microalgae for evaluating their potential to decrease organic matter and deplete nutrients from dairy and other manufacturing industrial effluents (Durmus et al., 1999; Woertz et al., 2009). More recently, a dual approach for treating a dairy waste stream from a specific medium-scale industry and producing algal biomass for biofuel was proposed (Kothari et al., 2012). However, the direct use of whey permeate, a consistent and readily available feedstock from the dairy industry, for the controlled heterotrophic microalgal cultivation has not been reported.

The aim of this study was to evaluate the use of whey permeate as the main carbon source for the heterotrophic growth and lipid accumulation of the microalgae *C. protothecoides*. The potential application of pre-hydrolyzed whey permeate as feedstock for batch and high cell density fed-batch microalgal cultures was investigated along with the direct use of non-hydrolyzed whey permeate by simultaneous saccharification and fermentation with immobilized hydrolysing enzyme.

2. Methods

2.1. Materials

C. protothecoides (UTEX 256) was directly obtained from The Culture Collection of Algae (UTEX; University of Texas, Austin, TX). Axenic stocks were stored at room temperature (23 ± 2 °C) with 25 μmol/m^{2}s at 12/12 h light/dark cycles as the long-term storage conditions (4 weeks; new agar slants were made every 4 weeks). All chemicals used for media preparation were purchased from Sigma–Aldrich (St. Louis, MO). Sulphuric acid and hexane (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ). Whey permeate was procured from a large dairy producer (liquid form; pH 5.5) with lactose concentration of 182 g/L and nutrient composition: phosphorous (0.8% dry weight basis), magnesium (0.2% dry weight basis) and calcium (0.8% dry weight basis). Whey permeate was used 'as is' or hydrolyzed as described below.

2.2. Methods

2.2.1. Whey permeate hydrolysis

Whey permeate was neutralized using 2 M KOH and hydrolyzed at 30 °C for 24 h in shake flask at 200 rpm using 65 U of Lactozyme 3000 (Sigma–Aldrich, St. Louis, MO) per g lactose quantified in whey permeate. Hydrolyzed whey permeate (HWP) with a final composition of 95 g/L glucose and 85 g/L galactose was filtered (0.22 μm) and stored at 2 °C.

2.2.2. Analytical methods

Lactose, glucose and galactose content was determined using an Agilent 1200 series High Performance Liquid Chromatography (HPLC) instrument (Agilent, Santa Clara, CA), with a refractive index detector, Bio-Rad HP87H column (Bio-Rad Laboratories, Hercules, CA) at 60 °C (300 × 7.8 mm), using 0.005 M sulfuric acid as mobile phase with a flow rate of 0.5 mL/min. Algal growth was monitored by measuring $OD_{600\ nm}$ with a standard curve for dry biomass, in which $y = 2.2899\ x + 0.0523$ ($R^2 = 0.9944$); where $y = OD_{600\ nm}$ and x = dry weight (g/L). Lipid accumulation kinetics was determined by measuring fluorescence using Nile Red with a reference standard curve for lipid content hexane extraction; lipid content is reported as percent dry weight basis (DelaHoz Siegler et al., 2012). Briefly, freeze dried algal biomass was crushed with mortar and pestle followed by hexane washes of the resulting paste. Lipid percentage was determined gravimetrically from hexane supernatants. Fluorescence with Nile red was measured in reference algal biomasses of known lipid content (6.3–48.9% dry weight basis) at specific concentration, which were used to build up a standard curve with fluorescence signal vs. lipid percentage for each experiment. Total fatty acid composition in freeze-dried algal biomass was determined by esterification using methanolic hydrochloric acid 3 N (Sigma–Aldrich, St. Louis, MO) according to manufacturer instructions, with nonadecanoic acid methyl ester as internal standard. GC–FID for fatty acid quantification analysis was done in a 30 m × 0.25 mm × 0.25 μm, a column from SGE Analytical Science (Melbourne, Australia) was used. The initial injection temperature was 50 °C held for 0.2 min before ramping to 230 °C in the following program: 50–170 °C/20 min; 170 °C/5 min; 170–230 °C at rate 10 °C/min; 230 °C for 13 min. The injection volume was 1 μL in splitless mode. Total nitrogen and total carbon were measured by the Dumas Combustion Method at the Natural Resources Analytical Laboratory, University of Alberta.

2.2.3. Batch fermentations

C. protothecoides was cultured in 250 mL Erlenmeyer flasks with 100 mL base mineral media (Siegler et al., 2011). The amount of glucose, galactose, lactose, whey permeate or HWP used as main carbon sources was standardized based on total C mole amount of sugar from 0.33 C mol/L to 1 C mol/L which corresponds to 10 g/L and 30 g/L monomeric sugar, respectively. To trigger lipid accumulation, low nitrogen concentration was used in the growth media: $NaNO_3$ (2.5 g/L) for preliminary experiments and yeast extract for all other experiments (Xiong et al., 2008). Yeast extract amounts were varied from 1 to 1.2 g/L ($7.8E^{-3}$ mol/L–$1E^{-2}$ mol/L; based on results from preliminary experiments) for establishing a carbon to nitrogen ratio from 50/1 to 100/1 (DelaHoz, 2012). Flasks were inoculated with 5% (v/v) starter inoculum obtained from a shake flask culture at the late exponential phase (DelaHoz, 2012). Cultures were grown in the dark at 25 °C, 150 rpm with 24 h sampling intervals for a total of 168 or 216 h. pH of the cultures was monitored and adjusted to 6.4 with 2 M KOH. Each experimental unit was monitored for contamination by microscopic examination and streaking on Luria Bertani (LB) agar plates with subsequent incubation. All experiments were done in triplicate.

2.2.4. Fed batch fermentations

2.2.4.1. Small scale. The carbon sources used for fed batch fermentations, done in 250 mL Erlenmeyer flasks, were either HWP or a control mixture of glucose and galactose prepared at the same concentration as HWP. Initial C mole from sugar was standardized to 0.33 C mol/L (10 g/L of each monomeric sugar) and N was supplied at 2 g/L yeast extract added at the beginning. Inoculation, incubation, pH control and contamination checks were done as described for batch cultures. Feeding with HWP or control mixture along with yeast extract (0.5 g/L) was done as required. Triplicate cultures were monitored for a total of 240 h.

2.2.4.2. Scale up. 5 L bioreactors (Infors, Einsbach, Germany) were used for the scale-up of fed batch fermentations done with either HWP or glucose control, which was prepared at the same concentration as HWP. Initial fermentation volume was 3 L with a C mole amount from sugar doubled from the small scale experiments (HWP: 10 g/L glucose, 10 g/L galactose; glucose control: 20 g/L glucose) and initial yeast extract at 4 g/L. Inoculation, pH control and contamination checks were done as indicted for batch cultures. HWP or glucose along with yeast extract (2 g/L) was fed as required. During fermentation, aeration rate and stirrer speed were varied between 1–2 vvm and 100–200 rpm, respectively, to keep