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Omega-3 fatty acid production from enzyme saccharified hemp hydrolysate using a novel marine thraustochytrid strain

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HIGHLIGHTS

- Sugar hydrolysate obtained from pretreated hemp biomass was used for PUFA production.
- Novel thraustochytrid was used for omega-3 fatty acid production by growing on lignocellulose biomass.
- Lowest sugar hydrolysate led to increased PUFAs accumulation.
- DHA as % TFA was determined by FAMEs to be 38% in 2% SH.

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ABSTRACT

In this work, a newly isolated marine thraustochytrid strain, *Schizochytrium* sp. DT3, was used for omega-3 fatty acid production by growing on lignocellulose biomass obtained from local hemp hurd (*Cannabis sativa*) biomass. Prior to enzymatic hydrolysis, hemp was pretreated with sodium hydroxide to open the biomass structure for the production of sugar hydrolysate. The thraustochytrid strain was able to grow on the sugar hydrolysate and accumulated polyunsaturated fatty acids (PUFAs). At the lowest carbon concentration of 2%, the PUFAs productivity was 71% in glucose and 59% in the sugars hydrolysate, as a percentage of total fatty acids. Saturated fatty acids (SFAs) levels were highest at about 49% of TFA using 6% glucose as the carbon source. SFAs of 41% were produced using 2% of SH. This study demonstrates that SH produced from lignocellulose biomass is a potentially useful carbon source for the production of omega-3 fatty acids in thraustochytrids, as demonstrated using the new strain, *Schizochytrium* sp. DT3.

1. Introduction

The microbial biosynthesis of sustainable biochemicals or biofuels from lignocellulose biomass obtained from agricultural feedstock, industrial and urban bio-residue or woody biomass, has attracted significant attention (Wu et al., 2014). Lignocellulose biomass (LCB) can serve as an inexpensive carbon source for growing microbes including oleaginous microorganisms thus reducing the total production cost of biofuels, value added metabolites and co-products. LCB consists mainly of lignin, cellulose and hemicellulose that requires pretreatment for the opening of the compact structure by breaking cross-linked bonds (Puri et al., 2012; Cao et al., 2013). Pretreatment increases the hydrolysability of complex materials and maximizes enzyme penetration by increasing the cellulose surface area, thus producing sugar hydrolysate (SH) that can be used for producing ethanol and lipids (Pakarinen et al., 2012; Abraham et al., 2014). Reducing sugars such as glucose, xylose, mannose, arabinose, glucan, rhamnose, and cellobiose are the main constituents in the sugar hydrolysate and their concentration vary based on the biomass composition (Toquero and Bolado, 2014).

In addition to biofuel production, LCB can be utilized for the production of nutraceuticals or other chemicals of industrial value. A recent report has described the potential of LCB for producing fatty acids and their derivatives (Liu et al., 2014). Utilization of inexpensive and abundant lignocellulose feedstocks such as rice straw, sugarcane bagasse, corn stover, and corncob for the production of microbial lipids using oleaginous yeast has been investigated in the previous studies (Gao et al., 2014). Metabolic engineering has also been applied to produce engineered microbes capable of metabolising multiple sugars from LCB to produce biofuels and other important metabolites (Yao and Shimizu, 2013).

Thraustochytrids are oleaginous marine protists found in mangrove and estuarine environments with the ability to accumulate higher amounts of polyunsaturated fatty acids (PUFAs) (Gupta

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et al., 2012). These microbes originating from marine environments are promising sources of industrially important long chain fatty acids. The utilization of various carbon sources by thraustochytrids for fatty acid production has been reported previously, including the use of conventional hexose sugars and complex cellulosic matter (Hong et al., 2012).

In the present study, a novel isolate named *Schizochytrium* sp. DT3 was employed to utilize the SH obtained after the enzymatic saccharification of pretreated hemp biomass (*Cannabis sativa*), for the production of omega-3 fatty acids. Hemp is an annual dicotyledonous angiosperm plant under Cannabaceae family and includes bast, xylem and marrow. It is mainly grown for the outer covering or bast fiber and the applications extend across many industries. Hemp stalks are used for industrial purpose due to the higher volume of stalk (two times higher) than bast fiber. Interest in the use of hemp biomass is due to its high cellulose content (cellulose ~55%, hemi-cellulose ~16%, pectin ~18% and lignin ~4%) (Jaldon et al., 1998), easier availability, high biomass yield, low need of fertilization and insensitivity to frost (Pakarinen et al., 2012; Abraham et al., 2013). Hemp has been used as a potential biomass for the production of bioenergy such as ethanol, biogas, electricity and methane (Barta et al., 2013). Thus production of PUFAs was investigated using SH from hemp biomass as the sole carbon source, in comparison to glucose.

2. Methods

2.1. Chemicals and culture maintenance

All chemicals used in this study were of analytical grade obtained from Sigma–Aldrich, (St. Louis, MO, USA) and Merck Chemicals (Frankfurter Strabe, Darmstadt, Germany). Instant ocean sea salts, was obtained from Aquarium Systems Inc. (Blacksburg, VA, USA). The biomass for this study was obtained from Commins stainless manufacturing, Whitton, NSW (Australia). A new thraustochytrid strain, *Schizochytrium* sp. DT3 isolated in our lab was used for this study (Genbank accession number KF682125). It was maintained on sterile (autoclaved) GYP agar medium containing glucose 5 g/l, yeast extract 2 g/l, peptone 2 g/l, agar 12 g/l in 50% artificial seawater (ASW) and incubated at 25 °C for further use. Subculturing was performed every 3 weeks.

2.2. Biomass growth at different glucose concentrations

The seed medium consisted of glucose 5 g/l, yeast extract 2 g/l, peptone 2 g/l in 50% artificial seawater (ASW) and was incubated at 25 °C and150 rpm for 48 h. Various glucose concentrations (2%, 4% and 6%) were used in the fermentation medium (200 ml) to evaluate their effect on biomass and lipid production, including impact on the fatty acid profile. The medium contained: yeast extract 0.1%, mycological peptone 0.1%, artificial seawater 50% and initial pH was maintained at 7. The shake flask fermentation was performed at 150 rpm and 25 °C for 5 days. For growth monitoring, 20 ml culture was harvested at the interval of 24 h up to 120 h. $OD_{600\ nm}$ and cell dry weight was measured at each 24 h. Increase in OD value as a function of incubation correlates to increased biomass growth.

In addition, xylose and cellobiose medium was prepared along with other components as described above.

2.3. Preparation of sugar hydrolysate (SH)

The cellulosic biomass used in this study was Ukrainian variety hemp (*C. sativa*) and the inner core of the hemp (hemp hurds) biomass was procured as an industrial waste. The hemp hurd biomass (HHB) was dried at 70 °C to obtain a constant weight and milled to reduce the size. Milled HHB was sieved to obtain a size of about 300 μm and then pretreated at 120 °C using 10 g of HHB in 1 l of aqueous sodium hydroxide (0.5%, w/v) (Abraham et al., 2013). The enzyme hydrolysis of alkaline pretreated HHB was performed at 50 °C with shaking at 100 rpm for 72 h using cellulase from *Trichoderma reesei* (EC 3.2.1.4; 700 units (Sigma–Aldrich, St. Louis, MO, USA). The sugar analysis was performed using reverse phase-high performance liquid chromatography (RP-HPLC).

Different concentrations of SH were prepared (2%, 4% and 6%) and used in the fermentation medium along with other medium components as described in Section 2.2. The flasks were incubated at 150 rpm, 25 °C for 5 days. The biomass growth was monitored after every 24 h by measuring the optical density (OD) at 600 nm.

2.4. Cell dry weight (CDW) determination, lipid extraction and fatty acid methyl esters (FAMEs) production

The isolate was grown in media with different glucose and SH concentration as described above (50 ml fermentation medium) and the biomass was harvested at the end of five days and centrifuged at 10,000*g* for 10 min. The resultant cell pellet was washed three times with distilled water before freeze drying. The thraustochytrids cell pellet was weighed after freeze drying and the dry cell weight of the pellet was recorded. The freeze dried cells were stored at −20 °C before proceeding with lipid extraction. Results are presented as mean ± SD of samples prepared in duplicates.

Lipid was extracted and quantified from freeze dried biomass following a previously described protocol (Gupta et al., 2013a) with some modifications. 10 mg freeze dried biomass was added in 600 μl solvent (chloroform and methanol in 2:1 ratio) and vortexed for 2 min followed by centrifugation at 13,000*g* for 15 min. This extraction process was repeated three times. The three supernatants were collected and dried in oven at 50 °C. Lipid percentage was measured gravimetrically. Fatty acids were converted to methyl esters by acid-catalyzed trans-esterification as follows. 500 μl toluene was added into glass vials followed by the addition of 10 μl internal standard (methyl nonadecanoate, C19:0) and

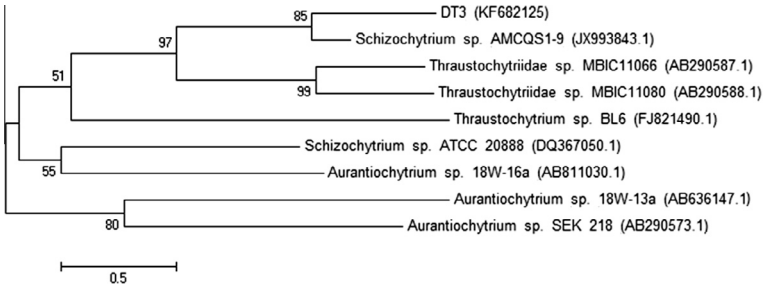

Fig. 1. Phylogenetic tree showing the close relationship of the isolate DT3 to related thraustochytrid sequences.