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Lipid production by eukaryotic microorganisms isolated from palm oil mill effluent

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ABSTRACT

Microbial oil production combined with wastewater management is one option for a more sustainable future. Micrographs of microbial cultures enriched from palm oil mill effluent (POME) showed lipid inclusion in the eukaryotic cells, indicating the cells can accumulate lipids. However, enriching the culture did not increase the total lipids. Therefore, eukaryotic microorganisms were isolated from POME to investigate whether these microorganisms are potential lipid producers. Four strains were isolated, and their lipid synthesis capabilities were compared with known oleaginous yeasts in a synthetic oil-free medium. Two strains (identified as *Galactomyces geotrichum* and *Graphium penicillioides*) had the potential to accumulate lipid accumulation based on the increase in triacylglycerol content. *G. penicillioides* was the most promising strain for lipid production as this strain accumulated more lipids than the well-known oleaginous yeast *Cryptococcus curvatus* (29.1 ± 3.0 wt% vs. 20.2 ± 2.9 wt%). To our knowledge, oil synthesis and accumulation by *G. penicillioides* have not previously been reported.

1. Introduction

Due to the increasing global demand for energy, fossil fuels must be substituted, or their use must be combined with alternative energy sources [1]. In the future, the ideal wastewater treatment plant would conserve the energy content of wastewater into a useful form instead of consuming energy. For example, wastewater sludge is used as a source of biogas, electricity, or liquid biofuels [2]. One possibility is to use wastewater to produce oleaginous biomass, from which lipids can then be extracted and used to produce biodiesel or renewable diesel [3].

Several oleaginous microorganisms have been studied for lipid accumulation. Known oleaginous yeasts include genera such as *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Trichosporon*, and *Lipomyces* [4]. Oleaginous microorganisms typically accumulate more than 20% of their dry weight as lipids [5]. The occurrence and activity of oleaginous yeasts in wastewater environments have not been systematically studied.

The palm oil industry produces a large volume of effluents with high organic carbon content [6]. Palm oil mill effluent (POME) is a colloidal suspension containing water (95–96%), oil (0.6–0.7%), and solids (4–5%) [7]. The estimated palm oil production of the two largest palm oil producers, Indonesia and Malaysia, was 33.5 and 21.25 million tons in 2014, respectively [8]. Each ton of crude palm oil produces at least 2.5 t of POME [6]. Thus, the estimated POME production in Indonesia and Malaysia was at least 80 and 50 million tons, respectively. POME is biodegradable. Gobi and Vaidivelu [9] reported that POME is a potential substrate for microbial biomass production.

The objective of this study was to produce lipids to generate biofuel by utilizing the carbon and nutrients of POME. Our hypothesis was that the microorganisms that most efficiently use POME are found in it, due to the long-term selective enrichment in this environment. Lipid production was studied with a mixed culture of indigenous microorganisms enriched from POME and pure cultures of microorganisms isolated from this mixed culture. The biomass production and lipid accumulation ability of isolated strains were compared with known oleaginous yeasts

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(*Yarrowia lipolytica* DSMZ8212, *Cryptococcus curvatus* DSMZ70022, and *Cryptococcus albidus* DSMZ70197) in a synthetic medium. Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) was used to characterize the microbial community composition of the POME enrichment culture and to identify the isolated strains.

2. Materials and methods

2.1. Palm oil mill effluent (POME) and culture enrichment

The POME used in this study originated in a Malaysian palm oil mill, and then was transported and stored frozen. The POME had a high organic matter load (38–39 g BOD/L, 43–50 g COD_{tot}/L) and contained a significant amount of lipids (19–20 wt% of total dry weight, 7.2–9.6 g/L) [10]. The soluble COD:N:P mass ratio of POME was 200:4:1 [10].

To increase the number of POME-degrading microorganisms, the microorganisms present in the POME were enriched with a series of shake flask incubations. The enrichment was conducted by incubating 100 mL of non-sterilized POME (which acted as the source of the microorganisms and the substrate) in 250 mL Erlenmeyer flasks on an orbital shaker at 250 rpm and 27 °C. Thereafter, 10 mL of enrichment culture was transferred once a week into 90 mL of fresh non-sterilized POME for several months. The microbial community composition of the culture enrichment was characterized with PCR-DGGE to detect possible oleaginous microorganisms.

2.2. Growth kinetics of indigenous organisms on POME

A batch experiment studying the ability of indigenous organisms to use POME and accumulate lipids was conducted in 250 mL Erlenmeyer flasks with 150 mL initial culture volume. The POME enrichment culture (10% v/v) was used as inoculum to grow a microbial biomass on the POME (90% v/v) at 27 °C and with 250 rpm mixing. The culture pH was not adjusted.

2.3. Isolation of eukaryotic microorganisms from POME

Eukaryotic microorganisms were isolated from the POME using four types of agar plates (potato dextrose [PD: 4 g/L potato extract = 200 g/L potato infusion, 2 g/L dextrose, 15 g/L agar], universal medium for yeast [YM: 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone from soybeans, 10 g/L glucose, 15 g/L agar], YM with chloramphenicol [50 mg/L] and modified YM [with xylan used as carbon source instead of glucose]) at 27 °C. An array of different agar plates was used to isolate as many different eukaryotes as possible. The agar plates were inoculated by taking 10 μL POME enrichment culture after 7 days of incubation with a sterile disposable plastic cultivation loop and spreading the culture on one quarter of the agar plate. To obtain single colonies, the sample was diluted with the streak plate method using a flame-sterilized cultivation loop on the three other quarters of the plate. Three replicate plates for each plate type were inoculated. The colonies on the plates were checked daily for 3 weeks. All dissimilar colonies were isolated and transferred to new similar agar plates (two separate colonies of each type on two separate agar plates). To confirm whether the colonies were bacteria or eukaryotes, the colonies were monitored with phase contrast microscopy. Eukaryotic colonies were transferred to new corresponding agar plates at least four consecutive times to ensure the purity of the strain.

2.4. Known lipid accumulating microorganisms used as reference

The following yeast strains were obtained from the culture collection of Deutsche Sammlung von Mikroorganismen und Zellkulturen GMBH (DSMZ): *Y. lipolytica* DSMZ8212, *C. curvatus* DSMZ70022, and *C. albidus* DSMZ70197. These strains, which accumulate high concentrations of lipids [4], were used as reference organisms for the isolated strains to study lipid production capability.

2.5. Biomass production and lipid composition of isolated strains

Four strains isolated from POME (Strains 1–4) were cultivated in batch mode to delineate their biomass and lipid production abilities. The strains were pre-incubated in YM at 27 °C on an orbital shaker at 250 rpm for 3 days. These cultures were used as inoculum for cultivation in GA medium (40 g/L glucose; 2.5 g/L yeast extract; 1 g/L $(NH_4)SO_4$; 0.83 g/L $MgCl_2{\cdot}6H_2O$; 1 g/L K_2HPO_4; 0.5 g/L KH_2PO_4; 0.2 g/L $CaCl_2{\cdot}2H_2O$). In each flask (250 mL), the initial culture volume was 100 mL with 98% (v/v) GA medium and 2% (v/v) inoculum. The cultivations were conducted at 27 °C with 250 rpm mixing. Duplicate flasks with each strain were incubated for 7 days.

2.6. Lipid production and fatty acid profiles of isolated strains and oleaginous strains

The biomass production and lipid accumulation capability of the two strains isolated from POME (Strains 1 and 4) were compared with the three strains ordered from the culture collection (*Y. lipolytica* DSMZ8212, *C. curvatus* DSMZ70022, and *C. albidus* DSMZ70197). All strains were pre-incubated in YM at 27 °C on an orbital shaker at 250 rpm for 3 days. Using these cultures as inoculum, each strain was incubated separately in 250 mL of GA medium at 27 °C with 250 rpm mixing. In each flask, the initial culture volume was 100 mL with 98% (v/v) GA medium and 2% (v/v) inoculum. Duplicate flasks of each strain were incubated for 15 days.

2.7. Analyses

Culture pH was measured with a WTW pH330i meter and a WTW SenTix 41 electrode. The dissolved oxygen (DO) concentration was measured with the WTW Oxi 330i meter and the CellOX 325 electrode.

The biological oxygen demand ($BOD_{7,ATU}$) with 7 days of incubation was determined according to Finnish standard SFS 3019 [11]. Allyl-thiourea (ATU) was used in the BOD determination to inhibit nitrification. The total chemical oxygen demand (COD_{tot}) and the COD of soluble compounds (COD_s) were determined with the closed tube dichromate method according to Finnish standard SFS 5504 [12]. The COD_s samples were filtrated through an Acrodisc PSF syringe filter with a 0.45 μm Supor (PES) membrane before the analysis.

Soluble nitrogen (N_s) and phosphorus (P_s) were determined with commercial Hach Lange LCK 238 and LCK 349 kits (Dusseldorf, Germany), respectively. Before the N_s and P_s analyses, the samples were filtrated with the 0.45 μm Acrodisc PSF syringe filter. The Hach Lange DR 200 Dry Thermostat Reactor (Dusseldorf, Germany) was used to heat the samples, and the results were analyzed with the Hach Lange DR 2800 Portable Spectrophotometer.

Samples for lipid extraction (5–10 mL) were centrifuged (Sigma 4K15, Osterode, Germany) at 5000 × *g* for 10 min. For gravimetrical lipid analysis, lipids were extracted with the modified Bligh and Dyer method using chloroform, methanol, and phosphate buffered saline (PBS, pH 7.4) as described by Santala et al. [13]. Extracted lipids were analyzed with thin-layer chromatography (TLC) using silica gel glass plates described by Efimova et al. [10].