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A study on the mechanism of subcritical water treatment to maximize extractable cellular lipids

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ABSTRACT

Lipids of microbial origin have gained much attention due to its wide applicability and high productivity. Widely studied microbial lipids are those coming from single cell oils such as microalgae, yeast and other fungi. Many researches have focused on enhancing lipid accumulation as well as biomass productivity with hope to utilize the accumulated lipids as an alternative source for biodiesel production. Unfortunately these biological lipids have often been under utilized due to inefficient extraction technologies. In addition, in order to maximize lipid extraction toxic solvents such as chloroform are often employed. In this study subcritical water (SCW) was employed for the treatment of samples from microbial origin to enhance their extractable intracellular lipids. Optimum temperature and time for SCW treatment of wet microbial cells such as activated sludge and yeast cells was found to be 448 K and 900 s, respectively. After SCW treatment, a 2–4 folds increase in the extractable neutral lipid was observed without the need of using toxic solvent such as chloroform and cyclohexane. An investigation on the possible mechanism on how SCW treatment was able to improve lipid extractability was also carried out in this study.

1. Introduction

Microbial lipids have gained much attention in recent years for its applications in pharmaceutical industries, cosmetics and biodiesel production. In addition to microalgae, two widely studied microbial lipid sources are activated sludge from wastewater treatment systems and oleaginous yeast.

Activated sludge is a by-product of aerobic wastewater treatment, which is often thickened and collected in the secondary settler of a wastewater treatment plant. Activated sludge comprises a community of microbial cells capable of lipid accumulation, which could be tapped as a cheap feedstock for biodiesel production. The continuous industrialization and urbanization worldwide coupled with the growing concerns to environment, wastewater treatment plant has become very common in most production industries, which further makes activated sludge an attractive alternative source of feedstock for biodiesel. In Taiwan it is

Abbreviations: AG, acylglyceride; AOCS, American Oil Chemist Society; FA, fatty acid; FAME, fatty acid methyl ester; FFA, free fatty acid; HTGC, high temperature gas chromatography; PC, phosphatidylcholine; PL, phospholipid; SCW, subcritical water.

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estimated to produce 600,000 Mg of dried sludge with a potential of producing up to 120,000 Mg of biodiesel each year [1].

Yarrowia lipolytica, an oleaginous yeast, is widely studied due to its unique and sophisticated metabolism. It can utilize both hydrophilic and hydrophobic substrates as sole carbon source. Hydrolyzates of agro-industrial lignocellulosic waste such as bagasse and rice bran have been successfully used in inducing lipid accumulation in *Y. lipolytica* up to ~40% by Tsigie et al. [2,3], with comparable or better productivity than microalgae. On the other hand, lipid accumulation up to ~60% was reported [4], utilizing volatile fatty acids, such as acetic, propionic and butyric acid as carbon source. These developments have made *Y. lipolytica* a potential alternative to microalgae for lipid production.

To date the extraction of microbial lipids is mainly conducted using solvents, coupled with mechanical disruption techniques [5]. Depending on the biological features of microorganisms, acid/alkali treatments to soften or weaken the cell walls may be necessary before mechanical treatments. Solvents used include flammable and toxic organic solvents.

Due to difference in polarity of lipids inside the cell, usually a mixture of solvents with different polarity is required to effectively extract the intracellular lipids such as storage lipid that accumulates in lipid bodies in yeast cells. These lipid bodies are made of polar phospholipid (PL) monolayer [6], which is only slightly soluble in solvents such as hexane. The use of polar solvents coupled with non-polar solvents not only aids lipid extraction but also co-extracts other polar substances, which are considered as impurities and could not be utilized in biodiesel production.

Chloroform/methanol extraction protocol is often adopted for cellular lipid extraction but due to its toxicity other binary solvents have been developed. Long and Abdelkader [10] utilized various mixed-polarity solvents for extraction of lipids from microalgae (*Nannochloropsis*) and found azeotropic mixtures of n-hexane and alcohols to be safer and better solvents than conventional extraction solvents. The use of such solvent systems may include the extraction of non-lipid materials as previously stated and would require further purifications.

The use of SCW to enhance and maximize extractable lipids in activated sludge and *Y. lipolytica* grown in bagasse hydrolyzate has been reported by Huynh et al. [1] and Tsigie et al. [3], respectively. The utilization of this technology may possibly avoid the use of toxic and hazardous solvents in maximizing lipid extraction.

Activated sludge comprises mix microbial communities and other organic materials. Compositions of sludge may be widely different due to different sludge sources and microbial communities present. To identify and isolate the most commonly found oleaginous microorganism in activated sludge and cultivate them in an environment to promote lipid accumulation would be very difficult. *Y. lipolytica* is a well studied and known oleaginous microorganism, thus in this study, it was chosen as the model microorganism to understand the possible mechanism on why SCW treatment can increase its extractable lipids.

2. Materials and methods

2.1. Materials

Dewatered activated sludge were acquired from wastewater treatment facilities of 4 different industrial plants in Taiwan, sludge A (Chung-Hua Picture Tubes LTD Taoyuan Factory, Taoyuan, Taiwan; +24° 58′ 12.27″, +121° 19′ 32.37″); sludge B (Jhunan Brewery, Jhunan, Taiwan; +24° 42′ 40.19″, +120° 52′ 41.17″); sludge C (Hsin-Tung-Yang Taoyuan Factory, Taoyuan, Taiwan), sludge D (Uni-President Enterprises Corp. Ltd., Chung Li Bakery Factory, Taoyuan, Taiwan; +24° 59′ 36.14″, +121° 14′ 42.55″). PL, 98% phosphatidylcholine (PC) and soybean lecithin (40% PC, 30% inositol choline, 20% acylglycerides (AGs) and 10% free fatty acids (FFAs)) were obtained from Nacali Tesque Inc. (Kyoto, Japan). Standards of fatty acid (FA) and fatty acid methyl esters (FAMEs) were obtained from Supelco (Bellfonte, PA). All solvents and reagents used were either high performance liquid chromatography (HPLC) or analytical reagent grade, obtained from commercial sources.

2.2. Characterization of sludge samples

The dewatered sludge samples were collected from various plants and kept at 200 K prior to use. Water content of the sludge was measured by putting sludge sample (10 g) in a predried glass tube. The sludge loaded tube was put into a freeze drier (Labconco FreeZone 2.5 dm^3 Model: 7670520, Kansas City, MO) operated at 229 K and 11.0 Pa for 48 h. Water content of the sludge was calculated based on difference in weight of sludge sample before and after freeze-drying.

Crude lipids from the various dried activated sludge were extracted with n-hexane, unless other wise specified. Extraction was carried out for 24 h using a Soxhlet extractor.

FFA content in the crude lipid was determined by the titrimetric method following AOCS official methods (Method Ca 5a-40) and by high temperature gas chromatography (HTGC). Analyzing known samples of FA with varied concentrations showed that the results of two methods differ from each other by less than 0.5%. Amount of unsaponifiable matter in the lipid sample was analyzed using AOCS official methods (Method 6b-53).

Saponified lipids obtained after determination of unsaponifiable matter were collected and acidified to pH 2 with concentrated sulfuric acid and continually stirred at 333 K until the solution was clear. The solution was then allowed to settle until 2 phases were formed. The upper layer consisting of FAs was extracted with hexane and further reacted with BF_3-methanol for later analysis of FA profile using HTGC.

Dewaxing and deguming of the extracted lipids was first carried out before its AG composition was analyzed using HTGC. In brief, 1 g crude lipid was dissolved in 0.05 dm^3 acetone and heated in a 333 K water bath until the solution was clear. The solution was allowed to cool to room temperature and then stored at 277 K for 3 h. The solution was then filtered (0.22 μm pore size) to remove the precipitated wax and gum. The procedure was repeated twice to ensure total removal of wax and gum.