Carboxymethyl cellulose as a C-source for lipid accumulation by the oleaginous yeast *Candida orthopsilosis*

Kanti A[1*] and Sudiana IM[1]

[1] Microbiology Division, Research Centre for Biology, Indonesian Institute of Sciences

Abstract

     The objective of this study was to evaluate the possibility of using carboxymethyl cellulose (CMC) as a carbon source for lipid accumulation by oleaginous yeasts: *Candida orthopsilosis* Y09GS34, *Candida oleophila* Y09GS48, and *Lipomyces* sp. 10381. Lipid accumulation in the cells was determined by the Nile Red method and lipid composition was identified by gas chromatography mass spectrometry (GC-MS). During the incubation period, CMC-ase activities were monitored to determine the hydrolytic activities of oleaginous yeasts. The results of the study showed that the cellulase activity of all strains tested (Y09GS34, Y09GS48, and 10381) was higher in N-limited medium with 2% CMC, namely 1.193, 0.633 and 1.233 units per hour, respectively. Y09GS34 showed the highest lipid accumulation (63.75% per cell dry weight). The lipid composition of *Candida orthopsilosis* (Y09GS34) included palmitic acid, stearic acid, linoleic acid, and oleic acid, implying that *Candida orthopsilosis* (Y09GS34) could utilize cellulose as a carbon source for lipid accumulation. Thus, our study demonstrates that cellulose can be used as a carbon source for lipid accumulation by oleaginous yeasts, and Y09GS34 has the potential to be used for bio-fuel research.

Keywords – cellulose – CMC – lipid accumulation

Introduction

    Exploration of renewable energy resources is a common interest of many scientists (Jansson et al.2009, Felice et al. 2012, Sudiana et al. 2014). Exploitation of oleaginous yeast and fungi, which have the ability to accumulate lipids up to > 20% of their dry weight, offer an alternative solution for developing novel renewable energy sources (Liang & Jiang 2013, Kanti et al. 2013). The yeast should be able to use agricultural waste hydrolysate for neutral lipid accumulation to enable their use to the fullest potential (Gao et al. 2014). Oleaginous yeast offer several advantages as lipid sources. These include a rapid cell doubling time, the ability to use various carbon sources for lipid accumulation (Fontanille et al. 2012), and production of large amounts of high biomass in limited space. Unlike plants, their growth is not limited by weather and climate (Cheirsilp et al. 2011, Galafassi et al. 2012) and their intra cellular lipid composition can be manipulated (Sitepu et al. 2013). Agricultural waste is one of the renewable resources that have the potential to be used as a substrate for oleaginous yeasts (Cheirsilp & Louhasakul 2013). Recently, the agricultural sector and business has grown rapidly, which is beneficial to the country. These activities generate large amounts of agricultural wastes, which can be

further utilized for many purposes. Many researchers are proposing the utilization of agricultural waste as feed material for renewable energy production (Ahammad et al. 2014, Nallathambi 2009, Ugwuanyi et al. 2004, Bhattarai et al. 2011). However, very little research has been carried out on the bioconversion of agricultural wastes into biodiesel. Biodiesel or microbiodiesel is a diesel fuel processed through transesterification of intracellular lipids produced by oleaginous microbes such as oleaginous yeasts and fungi (Borges & Diaz 2012). Oleaginous microbes are defined as microbes with the ability to accumulate large amounts of lipid in their cells (20-80% of cell dry weight) (Liang & Jiang 2013, Kimura et al. 2004, Beopoulos et al. 2008). Some genera like *Candida, Rhodotorula,* and *Cryptococcus,* are well known oleaginous yeasts (Gong et al. 2012, Amaretti 2010). We intend to utilize oleaginous yeasts with the ability to hydrolyse carboxymethyl cellulose (CMC) to fermentable sugars, which are transformed and accumulated intracellularly in the form of lipids, as stocks for biofuel production. The study was initiated with CMC as a substitute for amorphous cellulose, which is abundant in agricultural wastes. The information obtained from this study is important for studies on the next generation of biofuels. Agricultural wastes such as rice straws, wheat straws, and corncobs have high cellulose content (Cheirsilp & Loahasakul 2013, Virunanon et al. 2013). Plant cell walls are mainly composed of cellulose with a simple structure, which is whitish and insoluble in water but soluble in chlorides, hydroxides, alkalis, and carbon disulfides. The degradation of cellulose is a time consuming process but the cellulase enzymes produced by microorganisms can hydrolyze cellulose. CMC was selected as a model substrate since it has a chemical structure homologous to cellulose (Deswal et al. 2011). CMC can be hydrolyzed by endoglucanase. However, for the complete hydrolysis of cellulose, exoglucanase and β-glucanase is required in addition to endoglucanase (Zhou et al. 2004). Liming & Xueliang (2004) showed that cellulase is a multi-enzyme complex that consists of three major enzymes working synergistically. These include (i) endoglucanase or 1,4 glucan glucanohydrolase (EC 3.2.1.4), which breaks the bonds in amorphous regions, (ii) β-1,4-D-glucan cellobiohydrolase (EC 3.2.1.91) or exoglucanase, which breaks down bonds in crystalline regions, (iii) and β-glucosidase (EC 3.2.1.21) or cellobiase, which hydrolyzes cellobiose to glucose. This hydrolysis end product can be then used as a carbon source for lipid synthesis by oleaginous yeasts. The focus of this study was to assess the possibility of using CMC as a substrate for lipid synthesis by oleaginous yeast. In this study, CMC was used as a carbon source to produce triacylglycerol (TAG) that was converted to fatty acid methyl esters (FAMEs) through a transesterification process, serves as a microbial-based biodiesel. We expect that this work will contribute to sustainable utilization of agricultural wastes for energy generation.

Materials & Methods

Isolates

The yeasts that were used in this experiment are members of the genus *Candida, C. orthopsilosis* and *C. oleophila* (Y09GS34 and Y09GS48) and the genus *Lipomyces* (10381) obtained from Indonesian resources. They were maintained in yeast peptone dextrose (YPD) medium for 24 to 72 hours at 25°C.

Cell growth measurement

The biomass of oleaginous yeasts was determined following a method described previously by Sitepu et al. (2012). The yeasts were grown in 150 ml of medium containing 1% CMC (carboxymethyl cellulose). The medium composition was similar to that described by Kimura et al. (2004), where glucose was substituted with CMC sodium salt (Sigma 9004-32-4). The N-limited medium with 2% CMC contained yeast extract (1.5 g/l), NH_4Cl (0.15 g/l), KH_2PO_4 (7.0 g/l), $Na_2HPO_4.12H_2O$ (5.0 g/l), $MgSO4.7H2O$ (1.5 g/l), $FeCl_3.6H_2O$ (0.08 g/l), $ZnSO_4.7H_2O$ (0.01 g/l), $CaCl_2.2H_2O$ (0.1 g/l), $MnSO_4.5H_2O$ (0.1 mg/l), $CuSO_4.5H_2O$ (0.1 mg/l), and $Co(NO_3)_2.6H_2O$ (0.1 mg/l) with pH 5.5. The yeasts in this medium were then incubated in a Bio Shaker BR-23FP set at 175 rpm for 5 days at 30°C. Cell growth was measured daily by plate count.

CMC-ase analysis

The yeasts were grown according to the method described in the previous section and CMC-ase activity was measured following a protocol described by Ugwuanyi et al. (2004) and Hatano et al. (1991).

Total lipid analysis

The total lipid content of the samples was measured following a protocol described by Sitepu et al. (2013).

Morphological observation of oleaginous yeast

Accumulation of lipids in the yeast cells was observed by fluorescence microscopy (Olympus BX 53) using Nile red stain as described previously by Kimura et al. (2004).

Identification of lipid with *Gas Chromatography-Mass Spectrometry* (GC-MS)

Total lipid composition was determined following the method of Guan et al. 2010. GCMS QP 2010 (Shimadzu, Japan) was used for the analysis raw lipids and biodiesel. The GC was equipped with a DB-1ht capillary column (30 m × 0.25 mm; J&W Scientific, USA) and a flame ionizing detector (FID). The temperatures of the injector and detector were set at 350°C and 360°C, respectively. The profile of the column temperature was raised from 100°C to 180°C at 15°C/min, then raised to 230°C at 10°C/min, and finally raised to 330°C at 20°C/min and was maintained for 5 min. Helium was used as the carrier gas and heptadecanoic acid methyl ester purchased from Sigma was used as the internal standard.

Results and Discussion

Oleaginous Yeast Growth

The first indicator of substrate utilization is the assimilation of the C-source reflected as biomass growth. We found that the yeasts were able to grow in medium containing CMC and that the growth of the isolate Y09GS34 in 1% CMC was the highest among other isolates (Fig.1). The growth of isolate Y09GS34 was lower in N-limited medium with 2% CMC compared to 1% CMC containing medium, because excessive amounts of the C-source trigger lipid synthesis while small amounts of N (nitrogen) limit cell growth (Liang & Jiang 2013). Fig.1 indicates that the isolates hydrolyzed the CMC and absorbed it as a carbon source for cell growth and energy. Cellulose being the dominant component of agricultural wastes, can be used by cellulolytic microorganisms (Zhu et al. 2010), and the assimilation of CMC by yeast may indicate their ability to use agricultural waste hydrolysates as substrates.

Correlation between cellulase activities and lipid accumulation

Fig. 2 shows that the highest CMC-ase activity was achieved by isolate 10381, when grown in N-limited medium with 2% CMC, which implies that enzyme activity was stimulated by excessive CMC. However, all isolates grown in 1% CMC showed lower CMC-ase activity than in N-limited medium with 2% CMC, implying that N-limited conditions may also stimulate CMC-ase activity. Cellulase is an induced enzyme (Ahamed & Vermette 2008), and high amounts of reduced sugars in bulk solutions may inhibit cellulase production, whereas cellulose induces cellulase activity (Ugwuanyi et al. 2004).

Accumulation of lipids in isolate 10381 was less than that observed in other strains (Fig. 3). Therefore, 10381 is preferred as a cellulolytic yeast. On the other hand, Y09GS34 was able to hydrolyze CMC and accumulate more lipid than the others, establishing itself as a cellulolytic yeast with lipid accumulating capabilities. Cellulose hydrolysis produces monosaccharides, and an excess of glucose and limited nitrogen affect cell division, finally leading to accumulation of lipids in the form of triacylglycerol (TAG). *C. orthopsilosis* (Y09GS34), grown in N-limited medium with 2% CMC was able to accumulate lipids up to 63.75% of cell dry weight, which was higher than the other isolates. In
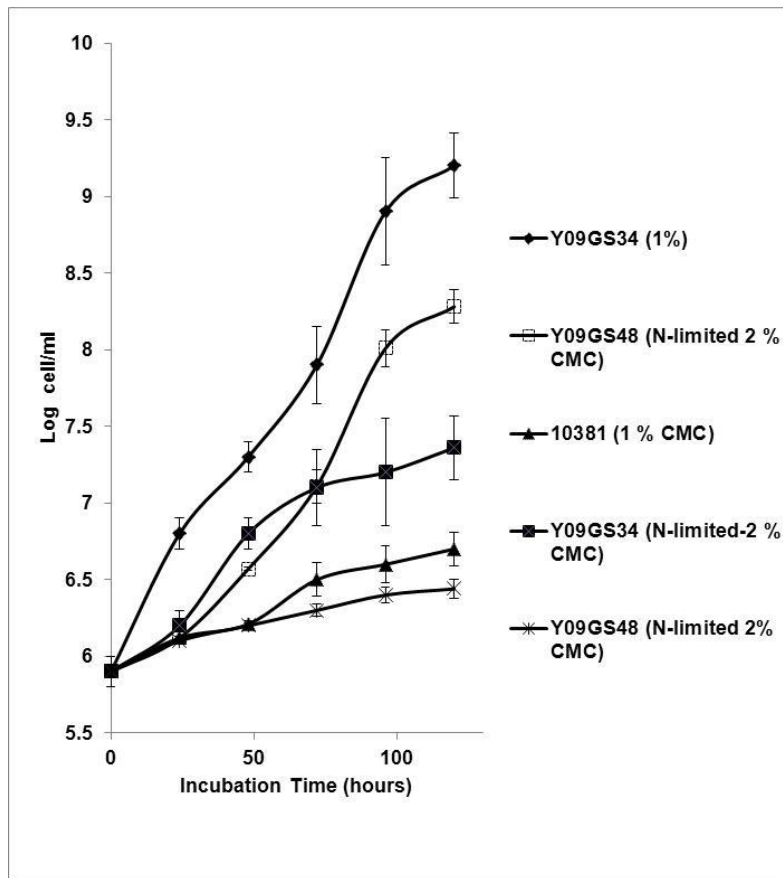
Fig. 1 – Biomass growth of yeasts, Y09GS34, Y09GS48 and 10381 in 1% CMC or N-limited medium with 2% CMC for 120 hours
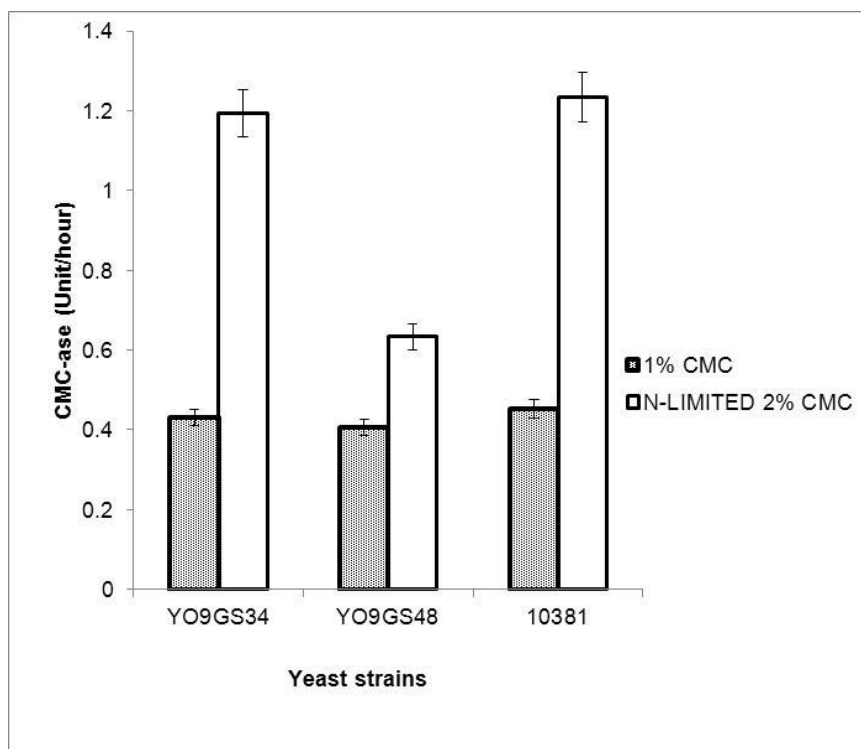

Fig. 2 – Cellulase activities of Y09GS34, Y09GS48 and 10381 in 1% CMC, and in N-limited medium with 2% CMC at 5 days incubation, error bar indicates SD, n = 3
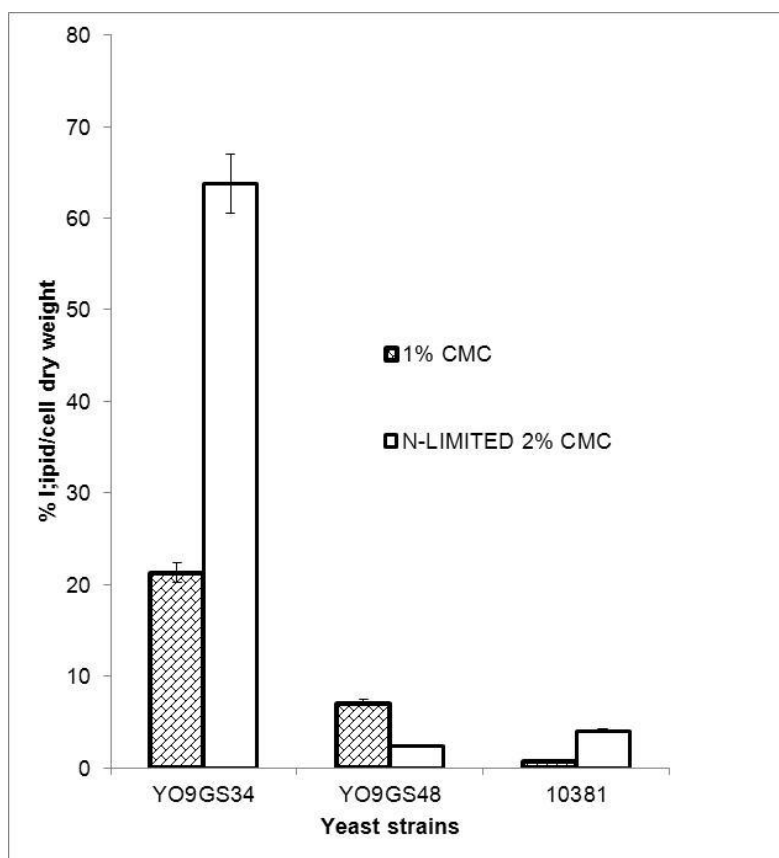
Fig. 3 – Lipid accumulation by Y09GS34, Y09GS48 and 10381, error bar indicates SD, n=3

comparison, *Trichosporon fermentans* grown in agricultural waste only produced 40.1% lipids of cell dry weight (Huang et al. 2011). This would indicate that *C. orthopsilosis* Y09GS34 is a potential oleaginous yeast for efficient use of agricultural waste hydrolysates.

Intracellular lipid accumulation was also observed with a fluorescence microscope using Nile red staining (Fig. 4). Nile red has been used for detection and quantification of intracellular lipid droplets in various biological systems. It is highly soluble and fluoresces strongly in a wide range of organic solvents, but its solubility and fluorescence are negligible in water. While Nile red can stain most lipids, its fluorescence characteristics vary depending on the class of lipids. Its emission is at a shorter wavelength when bound to neutral lipids as compared to polar compounds. Unsaturated fatty acids exhibit stronger fluorescence intensity than saturated fatty acids.
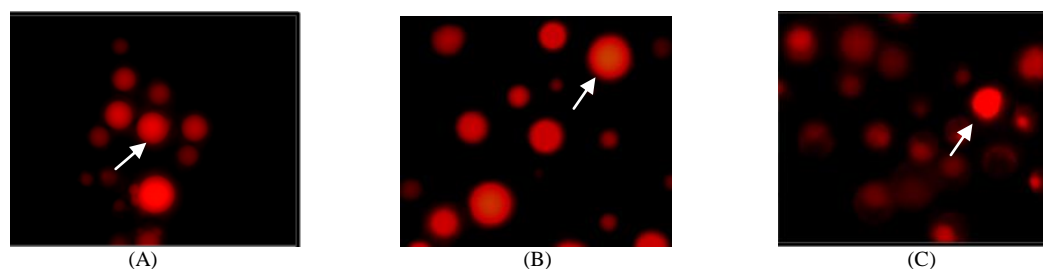

Fig. 4 – Intracellular lipid accumulation observed by Nile Red staining under a fluorescence microscope at magnification (10 x 100), (A) yeast strain 10381(B) yeast strain Y09GS34, and (C) yeast strain Y09GS48.

The lipid bodies were stained orange to red. Oleaginous yeasts store lipids as lipid bodies accounting for >20% of cell dry weight. Oleaginous yeasts are more suitable for biofuel production than microalgae since they are easy to harvest and show faster cell division, which is advantageous for biodiesel production.

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FAME chemical composition

Feed substrate for biodiesel production is commonly comprised of C-16 (palmitic acid) and C-18 (stearate acid) (Vasudevan & Briggs 2008). FAMEs produced by Y09GS34 grown in N-limited medium with 2% CMC medium are shown in Fig. 5. The major lipid components identified were methyl caprylate, methyl butanoate, and methyl oleate: 22.2%, 32.71%, and 28.26% respectively. Thus, the use of Y09GS34 can be further explored as biodiesel feedstock. This study shows that oleaginous yeasts can use the cellulose from agricultural waste hydrolysates as a carbon source for lipid accumulation, and can then serve as potential feedstock for biodiesel as an alternative renewable energy source.
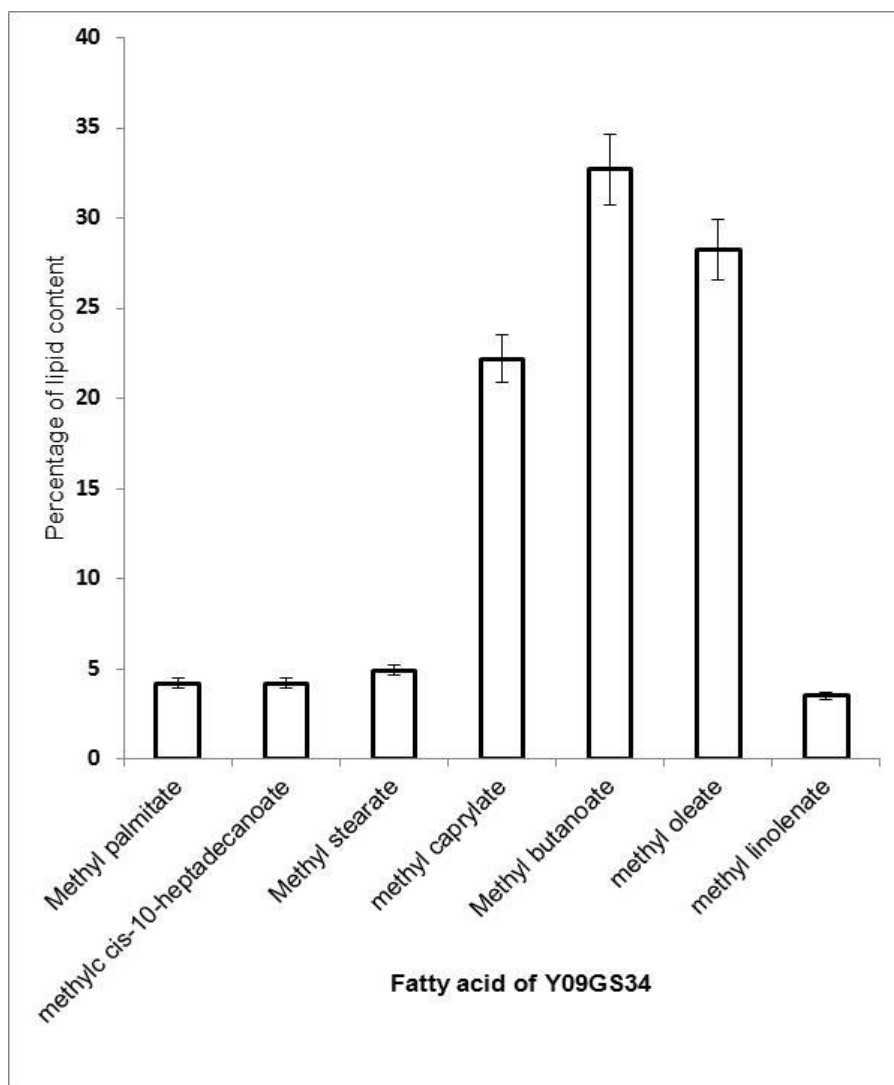
Fig. 5 – Fatty acid methyl ester composition of Y09GS34 in N-limited medium with 2% CMC

Conclusions

Three yeast isolates were able to hydrolyze cellulose and accumulate lipids in N-limited medium with 2% CMC. Thus, cellulose can be used as a carbon source for lipid accumulation by oleaginous yeasts. We found that *C. orthopsilosis* Y09GS34 is potential oleaginous yeast for efficient use of agricultural waste hydrolysates.

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