

Bioconversion Studies of Methyl Laurate to Dodecanedioic Acid using a Wild-type of *Candida tropicalis*

Rifkah Akmalina, Ronny Purwadi, and Johnner Sitompul*

Chemical Engineering Department, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung, 40132, Indonesia

Abstract. Production of dodecanedioic acid (DDDA), a platform chemical used as raw material for various commodities and polymers, has been studied through a biological process. This process was conducted by using a wild-type of *Candida tropicalis* which can be obtained easily from natural resources. The aim of this research was to study the characteristics of DDDA production from methyl laurate through batch fermentation process. Growth phase was carried out for 20 h, as the beginning of fermentation, then continued to conversion phase for 5 until 6 days. Utilization of methyl laurate and production of DDDA were analysed using gas chromatography, which proved the ability of *C. tropicalis* in assimilating methyl laurate to convert it become DDDA. The highest value of cells yield ($Y_{x/s}$) and DDDA yield ($Y_{p/s}$) successfully obtained were 0.86 g cells/g methyl laurate and 0.20 g DDDA/g methyl laurate, respectively. This study also showed the possibility of fermentation products accumulation as intermediate, or accumulation of DDDA inside the cells. Thus, this study can be applied as an alternative in addition to the use of mutant microorganism in producing DDDA.

1 Introduction

The long-chain dicarboxylic acids are well known platform chemicals for production of various commodities, such as polymers, perfumes, hot-melting adhesives, and high quality lubricants [1-2]. Dodecanedioic acid (DDDA) is one of dicarboxylic acids produced excessively (>40 million pounds per year) [3]. Currently, crude oil-based DDDAs are still used widely as raw material for polymer production, for example nylon which is produced from crude oil through several steps of chemical reaction [4]. These include limitations in complicated process, generation of unwanted and hazardous byproducts, also dependence on non-renewable petrochemical feedstock [1]. The use of crude oil-based compounds continuously will generate following problem when oil feedstock starts decreasing. A substitution of petrochemical with bio-based synthesis routes is one of alternative [4]. Thus, biotechnology based on renewable resources becomes an interesting process, for example by using fatty acids as raw material.

Several works for dicarboxylic acids production have been studied, with the raw materials or substrates used depend on the long chain of dicarboxylic acid desired. Dodecane [5-6] and lauric acid [7] were widely

used as substrates for DDDA production. A direct comparison between utilization of alkanes and fatty acids as substrates has not been studied in detail. Moreover, the use of methyl ester of lauric acid is still rarely conducted. Hence, this study will compare utilization of alkane and fatty acid methyl ester, in particular dodecane and methyl laurate as substrates in the production of DDDA.

In biological process, the success of dicarboxylic acid production really depends on the microorganisms used. Most of dicarboxylic acids could be produced by utilizing genetically engineered (mutant) microorganisms, for example *Candida tropicalis* 7/34 [6], *C. tropicalis* CGMCC 356 [2], *C. cloacae* MR-12 [8], and *C. cloacae* FERM-P736 [7]. A sophisticated and complicated methods or technologies are needed to obtain mutant microorganisms. In the other hand, there is no research found that elucidate clearly the use of wild-type microorganism for producing dicarboxylic acid, especially DDDA. Whereas, a-wild-type yeast can be easily obtained from natural resources, e.g. palm kernel oil, palm fiber oil, and ambarella fruit. Thus, this work was performed to study the characteristics of wild-type *C. tropicalis* in producing DDDA on dodecane and methyl laurate substrates in general. In this paper, there is also a brief explanation about mechanism of substrate

* Corresponding author: sitompul@che.itb.ac.id

oxidation for producing dicarboxylic acid. Then it can be related to the result of this work for defining more about DDDA production.

2 Experimental

2.1 Materials

Methyl laurate (>60% purity) was obtained from chemical conversion of palm kernel oil. n-Dodecane (99% purity) was a general purpose reagent and obtained from BDH Chemicals Ltd. Poole England. The strain of *C. tropicalis* wild-type was isolated from ambarella fruit without any treatment of mutation.

2.2 Cells cultivation

One loop of *C. tropicalis* grown on potato dextrose agar (PDA) slant was transferred into 50 ml of preculture medium containing 1% yeast extract, 2% peptone, and 2% glucose. Inoculum was incubated for 24 h at 30°C in a shaking incubator at 150 rpm.

Growth phase was started by transferring 15 ml of inoculum to a 500-ml conical flask containing 150-ml medium which had the following composition per litre: 5 g glucose, 2 g (NH₄)₂SO₄, 2 g NH₄NO₃, 1 g K₂HPO₄, 1.5 g KH₂PO₄, 1.5 g Na₂HPO₄·2H₂O, 0.2 g MgSO₄·7H₂O, 0.2 g KCl, 4 mg ZnSO₄·7H₂O, 4 mg FeSO₄·7H₂O, and 10 g yeast extract. The culture was aerobically grown at 30°C, 150 rpm in a shaking incubator. Cultivation was carried out until the beginning of stationary phase.

2.3 Dodecanedioic acid production

Methyl laurate was added into the culture at the beginning of conversion phase, followed by the addition of tween 80 (0.01% v/v). Medium pH was raised to 8.0 by adding 1 N NaOH and maintained using phosphate buffer. Fermentation process was continued for 5 until 6 days at 30°C in a shaking incubator at 150 rpm.

Samples (6 ml) were taken manually at regular intervals. Analysis of cells concentration, methyl laurate consumption, and DDDA production was carried out for each sample by these following methods.

2.4 Analysis

Cell growth was determined by measuring of the turbidity. Samples (0.5 ml) were 10x diluted and measured using UV-vis spectrophotometer at 600 nm. The absorbance data obtained were calibrated with cell dry weight data. Fermentation samples (5 ml) were centrifuged at 5000 rpm, 5°C for 10 min. The precipitated cells were dried in oven at 100°C, and weighted.

Glucose concentration in growth phase was measured by phenol-sulphuric method. To 1 ml of diluted free-cell samples, 1 ml of 5% phenol solution and 5 ml of 95-97% H₂SO₄ were added respectively. The absorbance of the solution was measured using UV-vis

spectrophotometer after 10 min at 488 nm, and the data were compared with calibration curve of glucose standard solution.

Methyl laurate consumed was determined by gas chromatography (GC). A half ml of free-cell samples was extracted using 1 ml of hexane. The mixture was centrifuged at 5000 rpm, 25°C for 5 min. Aliquot taken from organic phase was injected into a Shimadzu gas chromatograph. The operating conditions were as follow: injection temperature of 280°C, FID-detector temperature of 300°C, and quartz capillary column coated with RTX-5 (crossbond® 5% diphenyl/95% dimethyl polysiloxane). Initial column temperature was set at 70°C for 3 min, followed by 35°C/min temperature increase to 210°C, followed by 3°C/min temperature increase to 240°C with helium carrier gas and column flow of 1 ml/min.

DDDA produced from fermentation process was also determined by GC. To 0.25 ml of free-cell samples added 0.25 ml of 6 N H₂SO₄. DDDA then was extracted into 1 ml of diethyl ether. The mixture was centrifuged at 5000 rpm, 25°C for 5 min. An aliquot of 200 µl from organic phase was transferred to eppendorf tube, followed by the addition of BSTFA (100 µl) for silylation process. Aliquot from the mixture was injected into a Shimadzu gas chromatograph with these following operating conditions: injection temperature of 300°C, column coated with RTX-5 (crossbond® 5% diphenyl/95% dimethyl polysiloxane), temperature of 190°C (hold for 30 min) with helium carrier gas and column flow of 1 ml/min, and FID-detector temperature of 250°C.

3 Results and discussion

3.1 Growth phase of fermentation

When *C. tropicalis* is grown in a medium containing glucose as a carbon source, there was a significant increase in cell concentration. The highest concentration was reached at 20-h-cultivation which produced about 2.8 g dry biomass/l. Further cultivation until 24 h resulted in an equal value, indicating a stationary phase of cell growth (Fig. 1).

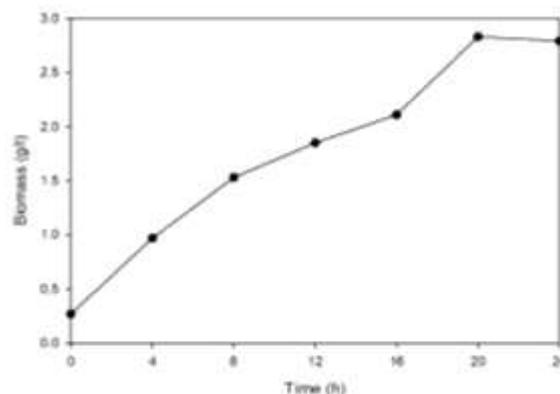


Fig. 1. Cell growth of *C. tropicalis* in medium containing glucose as a carbon source

Table 1. Growth phase in several studies using different medium and carbon source

Yeast	Medium composition	Carbon source	Growth phase	References
<i>C. tropicalis</i> (wild-type)	(NH ₄) ₂ SO ₄ , NH ₄ NO ₃ , K ₂ HPO ₄ , KH ₂ PO ₄ , Na ₂ HPO ₄ ·2H ₂ O, MgSO ₄ ·7H ₂ O, KCl, ZnSO ₄ ·7H ₂ O, FeSO ₄ ·7H ₂ O, yeast extract	Glucose	20 h	This work
<i>C. tropicalis</i> ATCC 20987	(NH ₄) ₂ SO ₄ , K ₂ HPO ₄ , KH ₂ PO ₄ , corn steep liquor, MgSO ₄ , NaCl, CaCl ₂	Glucose	18 h	[3]
<i>C. tropicalis</i> 7/34	(NH ₄) ₂ SO ₄ , NH ₄ NO ₃ , K ₂ HPO ₄ , KH ₂ PO ₄ , Na ₂ HPO ₄ ·H ₂ O, MgSO ₄ ·7H ₂ O, KCl, MnCl ₂ , ZnSO ₄ ·7H ₂ O, FeSO ₄ ·7H ₂ O, corn steep liquor, polypropylene glycol	Glycerol	24 h	[6]
<i>C. tropicalis</i> CGMCC 356	Yeast extract, corn steep liquor, urea, KH ₂ PO ₄ , CH ₃ COONa, NaCl, MgSO ₄ ·7H ₂ O, polypropylene glycol	Sucrose	18 h	[2]

Growth phase in fermentation process aims to increase cells amount, so that a carbon source which can be easily utilized by microorganism is needed. Glucose is one of carbon source that frequently used for yeast growth [9]. In line with the result given by this study, it showed that glucose was an appropriate carbon source used for increasing cell concentration.

Growth phase can be carried out in slightly different times. It depends on medium composition, type and amount of carbon source, also type of yeast used in fermentation, as shown in Table 1. In general, a mutant and wild-type of *C. tropicalis* have the same capability in assimilating sugar.

3.2 Growth on dodecane and methyl laurate

Candida tropicalis grown in dodecane and methyl laurate resulted in a different tendency. Those substrates could be assimilated and converted into cells (biomass). At the beginning of substrates addition, there was a decrease in cell concentration, indicating an adaptation phase of cell growth (Fig. 2). Microorganisms need to rearrange their enzyme to assimilate dodecane or methyl laurate after assimilating glucose as carbon source. A significant growth was shown after 24-h-fermentation in both substrates, but further fermentation until 72 h showed that *C. tropicalis* could assimilate methyl laurate better than dodecane.

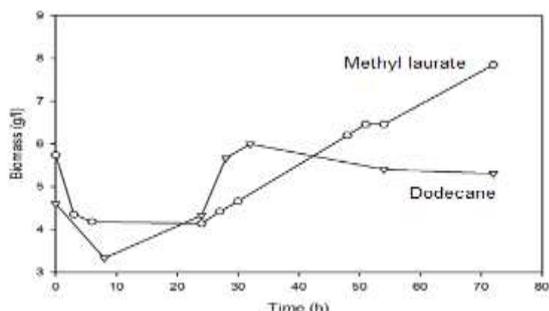


Fig. 2. Growth of *C. tropicalis* in dodecane and methyl laurate during 72-h-fermentation with substrates concentration of 5% v/v respectively

Dodecane is a long-chain alkane that supplies carbon source alternative for growth, while short-chain alkanes can be toxic that inhibit the yeast growth [7]. In addition, short-chain fatty acids can also inhibit the growth of microorganisms by disturbing basal cell functions [10]. The results shown in Fig. 2 are in an agreement with the theories above, in which dodecane and methyl laurate were able to be used as substrates for growth.

However, consumption rate of methyl laurate by *C. tropicalis* was faster than dodecane. It can be seen from Fig. 2 that until 72 h fermentation was carried out, cell concentration kept increasing significantly with methyl laurate as substrate. This study also proved that the use of fatty acid methyl ester (FAME) did not inhibit cell growth. It is presumably because the concentration of methanol contained in FAME is relatively low, so that it supplies more carbon source for yeast growth.

By using dodecane as substrate, the highest cell concentration attained was 6.0 g dry biomass/l at 32-h-fermentation. Further fermentation showed a gradual decrease in cell amount because there was insufficient carbon source to be consumed afterwards.

Theoretically, dicarboxylic acids can be produced from alkanes and fatty acids. Alkanes were oxidized into fatty alcohols, fatty aldehydes, and fatty acids. Further oxidation of fatty acids will produce dicarboxylic acids [11]. This study showed that biooxidation of alkanes was slower than biooxidation of fatty acids, indicating that oxidation step of alkanes into fatty acids was a rate limiting step. Using methyl laurate as substrate can accelerate oxidation process for producing DDDA, which is more advantageous than using dodecane as substrate.

3.3 Production of dodecanedioic acid

Growth of *C. tropicalis* was initially analysed in methyl laurate with concentration of 5 g/l, 10 g/l, and 15 g/l. In general, *C. tropicalis* was able to assimilate methyl laurate in various concentrations and convert it into biomass. At the end of fermentation process, there was a little amount of methyl laurate remained in medium: 0.5, 1.6, and 2.1 g methyl laurate/l from the initial concentration, respectively (Fig. 3). Theoretically, the

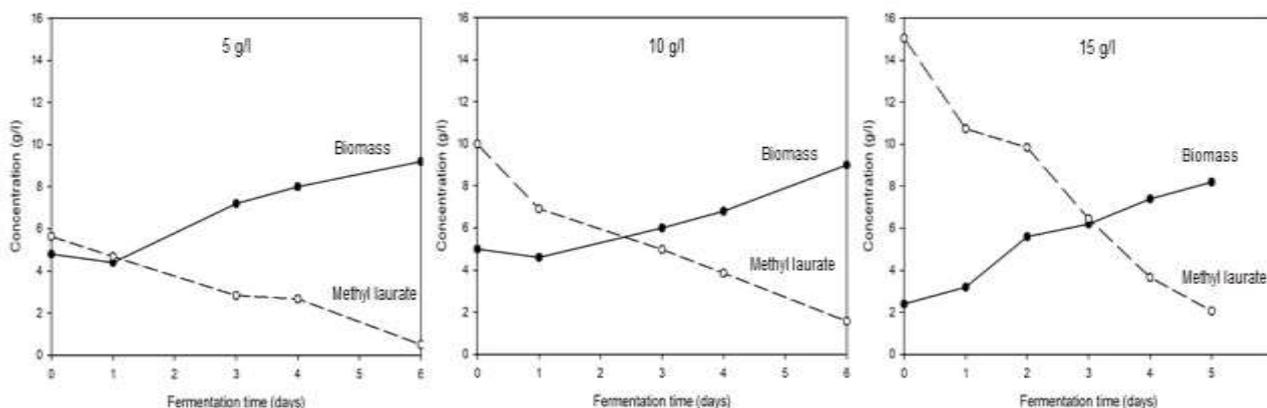


Fig. 3. Assimilation of methyl laurate in various concentrations with the production of biomass

Table 2. Biomass and product yield from various concentrations of methyl laurate

Parameters	Initial concentration of methyl laurate		
	5 g/l	10 g/l	15 g/l
Y _{x/s} (g cells/g methyl laurate)	0.86	0.48	0.45
Y _{p/s} (g DDDA/g methyl laurate)	0.20	0.11	0.08

more methyl laurate consumed, the more biomass produced. In actual, the biomass produced was relatively equal among three variations of concentration (Table 2).

The equal value of biomass yield indicates other products that might also be produced. There was a probability for DDDA produced from fermentation process. Further analysis was conducted to prove the ability of wild-type *C. tropicalis* in producing DDDA. Gas chromatography analysis showed the existence of DDDA in fermentation broth as shown in Fig. 4.

DDDA produced from various concentrations of methyl laurate was relatively equal as fermentation time prolonged (Fig. 4). The highest DDDA concentration that successfully attained was only 1 g/l in approximate. Relating this result with assimilation data shown in Fig. 3 have not yet answered the question related to products obtained beside of biomass. More DDDA should be produced as more methyl laurate was consumed.

When n-dodecane used as a substrate for producing DDDA, 12-hydroxydodecanoic acid, n-dodecanol, and n-dodecanoic acid were excreted and detected in the medium [5] as intermediate products. In this study, fatty acid was used as a substrate. There is a possibility of fermentation products accumulated in the form of hydroxy-fatty acid and fatty acid aldehyde as intermediate products of fatty acid oxidation. Hence, there was insufficient DDDA accumulated in fermentation broth.

Another possibility related to the process occurred in utilization of a wild-type of *C. tropicalis* is the accumulation of DDDA inside the cells (DDDA as intracellular product). This phenomenon might cause in low DDDA concentration in fermentation broth, as shown in Fig. 4. The use of mutant strain successfully

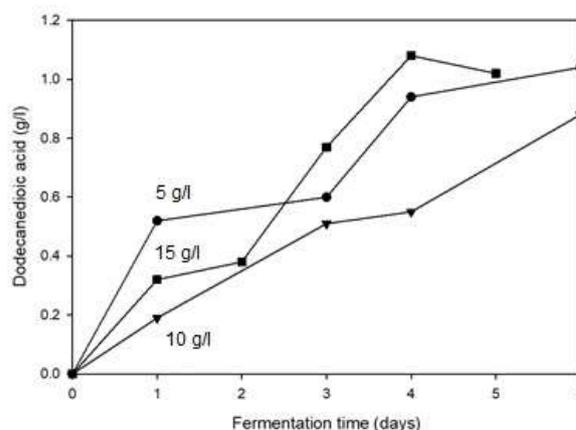


Fig. 4. Dodecanedioic acid produced from several concentrations of methyl laurate

improved the ability to produce and secrete dicarboxylic acid through cell membrane [12]. Extracellular pH is a factor influencing acid secretion. In a higher pH, cells had to secrete more acid to neutralize OH⁻ in order to maintain the micro-environment balance inside cells [12]. The appropriate pH adjustment is important to make secretion process becomes effective while using a wild-type microorganism.

Comparing biomass and DDDA yield (Table 2), the amount of biomass was about four times higher than DDDA. In other word, methyl laurate was converted to biomass more than DDDA.

Several studies had successfully proved that mutant microorganisms were able to produce dodecanedioic acid (Table 3). Compared with this study, the use of mutant microorganism resulted in higher yield of DDDA in shorter fermentation time. DDDA yield of 0.20 g/g methyl laurate was obtained in 144-h-fermentation from this study. It means that the use of mutant yeast was more advantageous because the fermentation process was more effective than the use of wild-type yeast.

Table 3. Comparison of DDDA yield from several researches using mutant yeasts

Substrate	Microorganism	Fermentation time	Yield (g DDDA/g substrate)	References
Dodecane	<i>C. tropicalis</i> 7/34	48 h	0.91	[6]
Dodecane	<i>C. tropicalis</i> CGMCC 356	120 h	0.73	[2]
Dodecane	<i>C. cloacae</i> MR-12	48 h	0.26	[8]
		72 h	0.41	
		48 h	0.20	
		72 h	0.29	
Dodecane	<i>C. cloacae</i> FERM-P736	72 h	0.20	[7]
Lauric acid			0.28	
Dodecane	<i>C. tropicalis</i> S-76	36 h	0.23	[5]
		48 h	0.28	

3.4 Mechanism of Fatty Acid Oxidation

The alkane or fatty acid metabolism of *C. tropicalis* is divided into three steps [12]: (1) substrate uptake, (2) metabolism of substrate in *C. tropicalis* cells, and (3) secretion of dicarboxylic acid. Substrate uptake into the cell is the first important step before starting oxidation process. In this study, tween 80 was used as surfactant to emulsify methyl laurate into fermentation medium, thus reducing mass transfer limitation between methyl laurate and aqueous phase of medium.

The different result obtained from using mutant and wild-type yeast in producing DDDA was related to the oxidation mechanism of substrate used. A reaction of β -oxidation might cause in low yield of DDDA when using wild-type *C. tropicalis* in fermentation process.

Fatty acids can be oxidized through reactions of β -oxidation, α -oxidation, and ω -oxidation. Beta-oxidation, which produces acetyl-CoA in peroxisome is the main reaction of fatty acid oxidation. Further oxidation of acetyl-CoA through citric acid cycle generates CO₂, and energy in the form of ATP is produced through oxidative phosphorylation (Fig. 5) [13].

In biological process of dicarboxylic acid production, ω -oxidation is more desired, but it occurs in small amount. Methyl group of fatty acid is hydroxylated into ω -hydroxy-fatty acid. Further oxidation of that compound leads to the production of fatty acid aldehyde and dicarboxylic acid (Fig. 5) [11,13]. Dicarboxylic acids produced from this reaction are only intermediate compounds, and will further be degraded by β -oxidation resulting in shorter-chain dicarboxylic acids.

Naturally, a wild-type yeast will oxidize fatty acids through β -oxidation reaction. This reaction is avoided in dicarboxylic acid production due to CO₂ and energy generation as the main product, so that insufficient amount of dicarboxylic acids is accumulated in fermentation broth.

A genetic engineering through deletion of genes involved in β -oxidation and overexpression of ω -oxidation enzyme can be applied to block β -oxidation and make ω -oxidation as the main pathway for fatty acid oxidation, resulting high yield of dicarboxylic acids [11,14].

Genetically engineered microorganisms might also have improved ability to secrete dicarboxylic acids produced from oxidation process [12], so

that dicarboxylic acids could be obtained easily from fermentation broth in sufficient amount. In contrast, a wild-type microorganism needs distinctive condition to make an effective secretion process, for instance by raising extracellular pH [12]. As a result, there are differences in DDDA yield by using mutant and wild-type microorganisms as shown in Table 3.

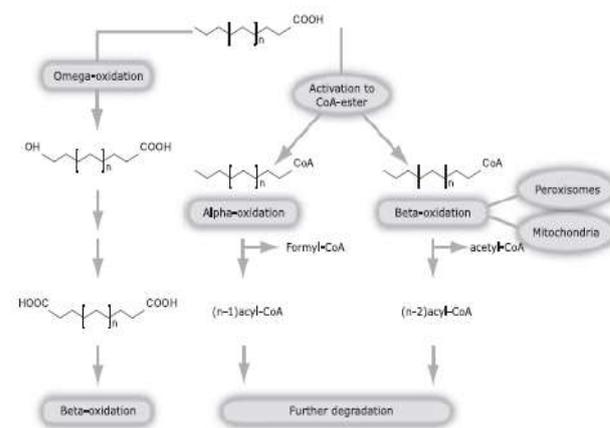


Fig. 5. Mechanism of fatty acid oxidation [15]

4 Conclusions

This study has shown that a wild-type of *Candida tropicalis* is able to produce dodecanedioic acid from methyl laurate. However, the amount of DDDA produced is less than the use of mutant yeast. In addition, dodecane can also be used as a substrate for DDDA production. However, cell growth analysis indirectly showed that oxidation of dodecane was slower than oxidation of methyl laurate.

Various concentrations of methyl laurate used in fermentation process do not affect the biomass and DDDA yield. The possibility of fermentation products accumulation in the form of hydroxy-fatty acid and fatty acid aldehyde may cause the low yield of DDDA. Hence, it is important to do further analysis of compounds contained in fermentation broth using gas chromatography-mass spectrometry (GC-MS). In the other hand, DDDA may also be accumulated inside the cells. Using wild-type microorganisms did bring the disadvantage of poor DDDA secretion from the cells, so that pH adjustment should be performed well to improve the ability of secretion process. Moreover, an experiment

for disrupting cells can also be carried out to obtain more DDDA from the cells.

In general, using mutant microorganisms gives more advantages than using a wild-type one. It is related to β -oxidation reaction that may occur, which also produce CO₂ and energy as main metabolism products of wild-type microorganisms. However, this preliminary study could be useful for the following research and might be applied as an alternative in addition to the use of mutant microorganism in producing DDDA.

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