

Synthesis of Monoacylglycerol from Glycerolysis of Crude Glycerol with Coconut Oil Catalyzed by *Carica Papaya* Lipase

P. Pinyaphong, P. Sriburi, and S. Phutrakul

Abstract—This paper studied the synthesis of monoacylglycerol (monolaurin) by glycerolysis of coconut oil and crude glycerol, catalyzed by *Carica papaya* lipase. Coconut oil obtained from cold pressed extraction method and crude glycerol obtained from the biodiesel plant in Department of Chemistry, Uttaradit Rajabhat University, Thailand which used oils were used as raw materials for biodiesel production through transesterification process catalyzed by sodium hydroxide. The influences of the following variables were studied: (i) type of organic solvent, (ii) molar ratio of substrate, (iii) reaction temperature, (iv) reaction time, (v) lipase dosage, and (vi) initial water activity of enzyme. High yields in monoacylglycerol (58.35%) were obtained with molar ratio of glycerol to oil at 8:1 in ethanol, temperature was controlled at 45°C for 36 hours, the amount of enzyme used was 20 wt% of oil and initial water activity of enzyme at 0.53.

Keywords—Monoacylglycerol, crude glycerol, coconut oil, *Carica papaya* lipase.

I. INTRODUCTION

MONOACYLGLYCEROL (MAG) constitutes the major type of food emulsifiers used in many food systems and are also important as a basic starting material to prepare several other derivatives of modified functional properties [1]. In particular, MAGs have been used as surface-active agents in many consumer and industrial cleaning products such as detergents, shampoos, lotions, and toothpastes or as raw materials for the synthesis of chemical compounds such as alkyd resins [2]. MAG can be prepared by two processes; direct esterification of glycerol with fatty acids or glycerolysis of glycerol with oils or fats (indirect esterification). Current MAG manufacture involves continuous glycerolysis of fats and oils using inorganic alkaline catalysts at high temperature (220-250°C) under nitrogen atmosphere [3]. However, this process only yields 30-40% MAG [4] and has several drawbacks such as low yield, dark color and burnt taste [5]. Lipases can be used as biocatalysts for the glycerolysis process and they have many advantages over the chemical process such as mild reaction condition, high catalytic efficiency and stereo- and positional specificities [5]-[6]. Different lipase-catalyzed reaction systems have been

reported, using organic solvents in monophasic, biphasic [7]-[9] or solvent-free systems [10]-[12]. Unfortunately, cost of bacterial lipase is relatively high for industrial scale. In this paper, some efforts have been made to explore the possibility to use a relatively cheap lipase from *Carica papaya* latex. It has been reported that the particulate part of *C. papaya* latex possesses lipolytic activity with 1(3)-regiospecificity [13]. *C. papaya* lipase has potential as a biocatalyst in lipid transformation [14].

Glycerol is an alcohol with three hydroxyl groups, which can be produced as a by-product of biodiesel production through transesterification of oil with alcohol [15]. According to the crisis of diesel fuel price, biodiesel is expected to be an alternative fuel. In the production of biodiesel, large amounts of crude glycerol are generated. With the demand for biodiesel predicted to increase greatly, the amount of crude glycerol generated will also rise. In this work, crude glycerol was utilized for production of more valuable products, such as MAG, instead of dumping in the landfills.

Monolaurin is monoacylglycerol that also presents biological activity. It is effective against several lipid-coated viruses, a class which includes the AIDS virus, and against certain bacteria [16]. Therefore, glycerolysis of crude glycerol derived from biodiesel production with coconut oil for monolaurin synthesis by *C. papaya* lipase was investigated.

II. MATERIALS AND METHODS

A. Preparation of Purified Glycerol from Crude Glycerol

Crude glycerol was obtained from the biodiesel plant in Department of Chemistry; Uttaradit Rajabhat University, Thailand which used oil was used as raw material for biodiesel production through transesterification process catalyzed by sodium hydroxide. Crude glycerol (1 kg) was acidified by the addition of 1.19 M H₂SO₄ to pH 2, left until phase separated into three distinct layers, which is a top layer of free fatty acids, the middle glycerol-rich layer and the bottom inorganic salt rich layer. The top layer was removed by slow decantation and the middle layer then harvested and filtered. After that, the middle layer was neutralized with 12.5 M NaOH and evaporated at 105°C for 2 hours and followed by filtration. The enriched glycerol layer was then extracted with excess ethanol for 10 min. The glycerol-ethanol solution was harvested and then filtered. The obtained purified crude glycerol was evaporated to eliminate ethanol at 80°C for 20 min [17] and then was used in MAG synthesis.

B. Preparation of Cold Pressed Coconut Oil

Grated coconut meat was put into a clean white nylon mesh bag. The coconut meat in the nylon bag was pressed with hand

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to extract coconut milk and pressed it again to extract as much milk as possible. The coconut milk was poured in the plastic bag and soaked for 24 hours in a refrigerator. After that, two layers in the bag were obtained, top cream layer and bottom sour water layer. The top cream layer was carefully collected and soaked for 36 hours in freezing compartment. The hard cream was defrosted at room temperature. The coconut milk was allowed to settle in the separating funnel. After that, three layers were separated; top cream layer, middle oil layer and bottom sour water. The middle coconut oil layer was collected and filtered. The oil is colorless and will then be used in the next steps.

C. Preparation of *C. Papaya* Lipase

Papaya latex was obtained by making a longitudinal incision on the unripe fruit (70-100 days) of Thai papaya tree. The latex was scraped and collected in plastic bottle and then was stored at -20°C until be usable. Defrosted hard latex at room temperature and then centrifuged at 9,500×g for 15 min. The insoluble particulate was washed with distillate water and lyophilized for using as *C. papaya* lipase. The water activity of *C. papaya* lipase was determined by a Thermoconstanter TH200 Novasina (Novasina, Switzerland), respectively. The hydrolysis activity of *C. papaya* lipase on coconut oil was investigated by colorimetric method [18].

D. Determination of Cold Pressed Coconut Oil Composition

The 40 mg of coconut oil (from B) was weighted into a small Erlenmeyer flask and then 3 ml of 0.5 M methanolic sodium hydroxide was added. The mixture was heated over a steam bath in hood until a homogeneous solution was obtained. For saponification reaction, BF₃-methanol (5 ml) was added to the reaction mixture and then boiled for 2 to 3 min. The solution was cooled and transferred into a separator funnel containing 25 ml of hexane and 20 ml of saturated NaCl solution. The solution was gently shake well and allowed the layers to separate. The hexane layer, containing the fatty acid methyl esters, was dried with about 1 g of anhydrous MgSO₄ and filtered into a small vial. The solution was concentrated on the steam bath until the volume was reduced to 0.5 ml. This solution of fatty acid methyl esters was analyzed by gas chromatography-mass spectrometry as described in F.

E. Glycerolysis Reaction

A reaction mixture consisted of 20 g coconut oil; 15 g purified crude glycerol and 3 g of *C. papaya* lipase. The reaction carried out at 40°C in shaking incubator with 250 rpm for 24 hours. Sample (2 ml) of the reaction mixture was withdrawn at 6, 12, 18 and 24 hours for further analysis.

The factors that effect on lipase-catalyzed glycerolysis to be investigated were type of organic solvent, molar ratio of glycerol to coconut oil, temperature, time, amount of lipase, and water activity of enzyme. Firstly, an effect of organic solvents type were investigated, glycerol was mixed with coconut oil in molar ratio of 6:1 and various organic solvent (25 wt% of original oil) such as *tert*-butanol, *n*-hexane, 2-

butanol, toluene, *n*-heptane, water, acetone and ethanol were added in the substrate mixtures and followed by adding *C. papaya* lipase (5 wt% of original oil) into the reaction mixtures. All of mixtures were incubated in orbital shaker fitted with temperature of 40°C for 24 hours. A reaction medium (0.3 ml) was taken and separated composition by TLC. Secondly, glycerolysis of the coconut oil was investigated using the glycerol: oil molar ratio at 2:1, 4:1, 6:1, 8:1, 10:1 and 12:1 with adding 95% ethanol at 40°C for 24 h using 5% lipase. Thirdly, the effect of temperatures on enzymatic glycerolysis were investigated, the reactions consisted of the glycerol and coconut oil in molar ratio of 8:1 adding 95% ethanol and were conducted at 40, 45, 50, 55 and 60°C for 24 hours using 5% lipase. For investigation of the reaction times, the reaction consisted of the glycerol and coconut oil in molar ratio of 8:1 with 95% ethanol and were conducted at 45°C for various time intervals such as 6, 12, 18, 24, 30, 36, 42 and 48 hours. To study the effect of enzyme amount, the *C. papaya* lipase (5-25 wt% of oil used) was incorporated into the reaction mixture that consisted of glycerol and coconut oil in molar ratio of 8:1 and 95% ethanol. All of reactions were carried out at 45°C for 36 hours. Finally, the effect of initial water activity of enzyme was examined, before starting of the reaction; *C. papaya* lipase was pre-equilibrated with the water vapor of saturated salt solution. Pre-equilibration was done at 25°C for 5 days. The saturated salt solution used were prepared with LiCl (water activity, $a_w = 0.11$), CH₃COOK ($a_w = 0.23$), MgCl₂ ($a_w = 0.33$) and Mg(NO₃)₂ ($a_w = 0.53$) [19]. A water activity of the bio-catalyst was determined using a Thermoconstanter TH 200 Novasina. The enzyme 20 wt% of original oil with various a_w was added to the reaction mixture consisted of glycerol and coconut oil in molar ratio of 8:1 adding 95% ethanol. The reaction was carried out at 45°C for 36 hours.

F. Analysis of Reaction Product

The reaction product (MAG, DAG, TAG and fatty acid) was identified by Thin Layer Chromatography (TLC). Aliquots of the reaction mixtures were applied on a Silica gel 60 plate (Merck) and developed with a solvent mixture of chloroform, acetone and methanol (95:4.5:0.5, by vol). Pure 1(3)-monolaurin, 1,3-dilaurin, lauric acid and trilaurin (Sigma) were used as reference glycerides. Spots of each lipid were visualized by spraying the plate with iodine vapor [20]. Fractions corresponding to each lipid type were scraped from the plates and then saponification with BF₃-methanol as described in D. After that, methyl ester of each glyceride was analysis by GC-MS.

The reaction products were analyzed by a gas chromatograph (GC 6850, Agilent Technologies) fitted with a capillary column (HP-1MS, 30 m×0.25 mm, 0.25 μm thickness) and equipped with mass spectrometer (MSD 5973 (EI), Agilent Technologies). The chromatographic conditions were as follow: on temperature of MS Quadrupole and MS Source were 150°C and 230°C, respectively, helium as a carrier gas at flow rate 1.0 ml/min, and an injector temperature of 250°C. Separations were made using the following oven temperature profile: initial temperature 140°C, programmed to 240°C at 10°C/min, and final temperature held for 15 min.

III. RESULTS AND DISCUSSION

A. Characteristics of the Purified Crude Glycerol

The original crude glycerol from biodiesel plant was a black liquid with a high pH (11.85 ± 0.59) and low density and viscosity compared with commercial glycerol. Moreover, it was found to only contain 38 ± 1.90 % (w/w) glycerol with a high content of ash and water, as summarized in Table I. The ash content was largely composed of inorganic matter, such as sodium salt, that originated from the utilized catalyst (NaOH) in the transesterification process, while the water content might be attributed to the absorption of moisture from its surrounding during the production process [21].

After the addition of H_2SO_4 to the original crude glycerol to pH 2, it automatically phase separated into three distinct layers, the inorganic salt layer on the bottom, a glycerol-rich layer in the middle and the free fatty acid layer on the top. This might be explained that the strong acid condition could neutralize the alkaline catalyst, which precipitated out as the bottom layer, and also achieve the hydrolysis to insoluble free fatty acids as the top layers [21]. After the glycerol-rich layer was neutralized with NaOH and extracted with ethanol, it obtained high purity with neutral pH (7.11 ± 0.35), increase in glycerol content (80.04 ± 4 % w/w) and density, content of ash and water decreased and the color of purified crude glycerol was light brown, as shown in Table I.

TABLE I
CHARACTERISTICS OF CRUDE GLYCEROL AND PURIFIED CRUDE GLYCEROL

Parameters	Crude glycerol	Purified crude glycerol
pH	11.85 ± 0.59	7.11 ± 0.35
Glycerol content (%)	38 ± 1.90	80.04 ± 4
Ash content (%)	3.20 ± 0.16	1.90 ± 0.09
Water content (%)	6.30 ± 0.31	2.80 ± 0.14
Density at $20^\circ C$ (g/cm^3)	1.105 ± 0.05	1.205 ± 0.006
Viscosity at $40^\circ C$ (cSt)	49.50 ± 2.47	108.40 ± 5.42
Color	Black	Light brown

B. Fatty Acid Composition in Coconut Oil

The fatty acid contents of the coconut oil were 0.5% C6:0, 7.3% C8:0, 6.5% C10:0, 49.2% C12:0, 17.4% C14:0, 7.8% C16:0, 3.0% C18:0, 6.5% C18:1 and 1.8% C18:2, as shown in Table II. The composition of extracted coconut oil was similar to Codex standard [22]. When coconut oil was verified by thin-layer chromatography (TLC), it showed that neither of them contained partial acylglycerols. The saponification value and the iodine value of the extracted coconut oil were determined to be 256 ± 12 mg KOH/g oil and 8.4 ± 0.42 , respectively.

TABLE II
CHARACTERISTICS OF COCONUT OIL

% Fatty acid composition	Extracted oil	Codex Alimentarius [22]
C6:0	0.5	ND-0.7
C8:0	7.3	4.6-10.0
C10:0	6.5	5.0-8.0
C12:0	49.2	45.1-53.2
C14:0	17.4	16.8-21.0
C16:0	7.8	7.5-10.2
C18:0	3.0	2.0-4.0
C18:1	6.5	5.0-10.0
C18:2	1.8	1.0-2.5
C18:3	0	ND-0.2

C. Lipase Activity and Physical Properties of C. Papaya Lipase

Fresh papaya latex contained 80 ± 35 u of lipase/g of latex. High speed centrifugation was required to separate the particulate part from the latex. This part of latex possessed all lipase activities which were found to be 200 ± 55 u of lipase/g of particulate fraction, whereas no activities were found in a clear solution. The lipase activity of dried lyophilized *C. papaya* lipase on the coconut oil hydrolysis was 925 ± 23 u of lipase/g of lyophilized particulate. The water activity and water content of crude *C. papaya* lipase was 0.395 ± 0.02 and 3.62 ± 0.17 , respectively. The crude *C. papaya* lipase was used as biocatalyst in further glycerolysis of the coconut oil without purification fraction.

D. Glycerolysis of Coconut Oil and Purified Crude Glycerol Catalyzed by C. Papaya Lipase

The results of glycerolysis between coconut oil and purified crude glycerol (molar ratio at 1:6) in presence of lipase from *C. papaya* latex as catalyst at $40^\circ C$ were initially investigated. The compositions of reaction products were 28.69% monoacylglycerol, 20.53% diacylglycerol, 9.96% fatty acid and 40.82% residual triacylglycerol. In order to select the more suitable reaction system, various factors that affect on this reaction were investigated. The use of organic solvents can improve the poor solubility in glycerol of substrate. To select the most suitable solvent for glycerolysis reaction system, the effect of organic solvents on the catalytic activity of lipase was examined. The results were shown in Fig 1. It was found that ethanol gave the highest yield of MAG (35.71%) at 24 hours. When the ratio 2:1 which is the stoichiometric molar ratio of glycerol to triacylglycerol required for the formation of MAG was employed, the MAG content was found to be very low (18.96%). The MAG yield increased with increasing the molar of glycerol as shown in Fig. 2. At the molar ratio 8:1 of glycerol to coconut oil gave the maximum yield of MAG (40%). When the molar ratio of glycerol to coconut oil was more than 8:1, the yield of MAG was slightly decreased. It may be explained that increasing of glycerol will decrease the amounts of available substrate at the interface between oil and glycerol and hence decreased the MAG yield [23].

For the effect of temperature on MAG production, the temperature was controlled at $40-60^\circ C$. When the temperature was controlled in the range $40-45^\circ C$ the MAG production

increased with increasing temperature (Fig. 3). It may be due to the rate of reaction increased as the temperature increased. In contrast, when increasing the temperature from 45 to 60°C the yield of MAG was decreased. It can be explained that *C. papaya* lipase can be deactivated by higher temperature. The effects of reaction time on glycerolysis were determined and the results were shown in Fig. 4. When increasing the reaction time the MAG production was also increased. However, no benefit came from increasing reaction time above 36 hours. Therefore, the reaction time at 36 hours was used in further experimentations.

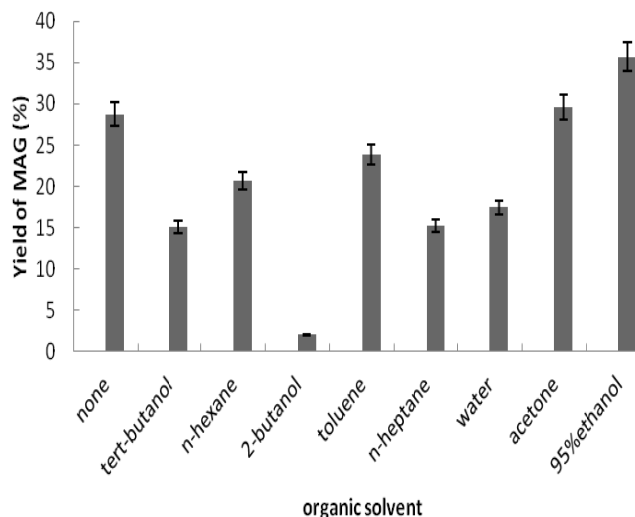


Fig. 1 Effect of organic solvents on MAG production by *C. papaya* lipase. The reaction mixture contained glycerol and coconut oil at molar ratio of 6:1 in organic solvent. The amount of *C. papaya* lipase used was 5% of original oil. The reaction was carried out at 40°C for 24 hours

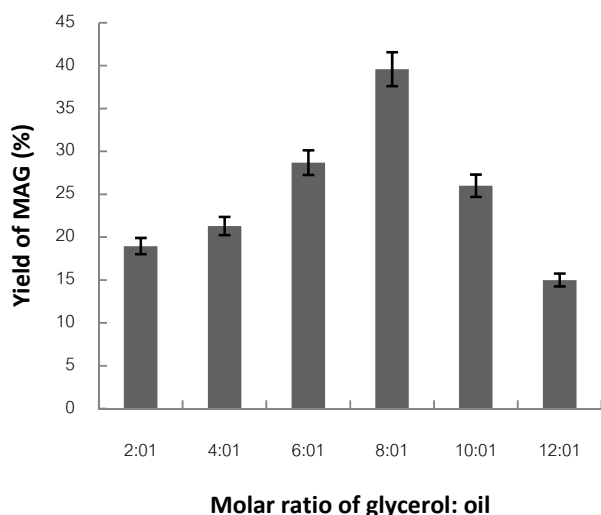


Fig. 2 Effect of molar ratio of substrate on MAG production by *C. papaya* lipase. The reaction mixture contained glycerol and coconut oil at various ratios in ethanol (25 wt% of oil). The amount of *C. papaya* lipase used was 5% of original oil. The reaction was carried out at 40°C for 24 hours

The effect of *C. papaya* lipase loading on MAG production was studied. The results were demonstrated in Fig 5. When increasing the amount of enzyme in the reaction mixture the production of MAG was also increased. The amount of enzyme at 20% by weight of oil gave the maximum yield of MAG (58.35%). Therefore, the amount of enzyme 20% of coconut oil was used in the next experiment. For the effect of initial water activity of enzyme, the results were shown in Fig. 6. When increasing the initial water activity of enzyme the MAG production was also increased. Since glycerol phase can dissolve some water from enzyme, hydrolysis reaction cannot be occurred when too much water of enzyme. The other authors found that 10-12% water in glycerol was suitable for glycerolysis of butter oil and beef tallow [24]-[26].

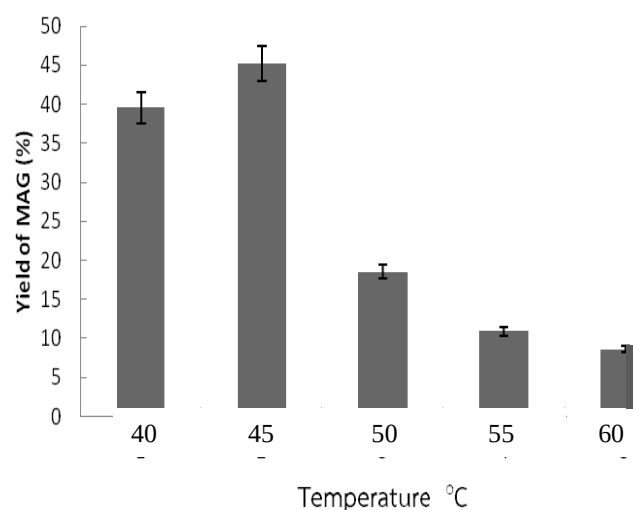


Fig. 3 Effect of temperature on MAG production by *C. papaya* lipase. The reaction mixture contained glycerol and coconut oil at molar ratio of 8:1 in ethanol (25 wt% of oil). The amount of *C. papaya* lipase used was 5% of original oil. The reaction was carried out at various temperatures for 24 hours

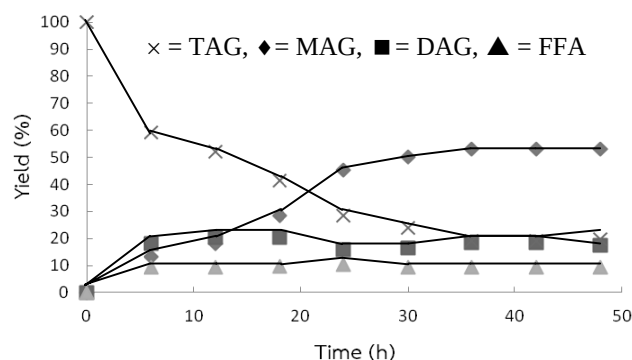


Fig. 4 Effect of reaction time on MAG production by *C. papaya* lipase. The reaction mixture contained glycerol and coconut oil at molar ratio of 8:1 in ethanol (25 wt% of oil). The amount of *C. papaya* lipase used was 5% of original oil. The reaction was carried out at 45°C for 24 hours

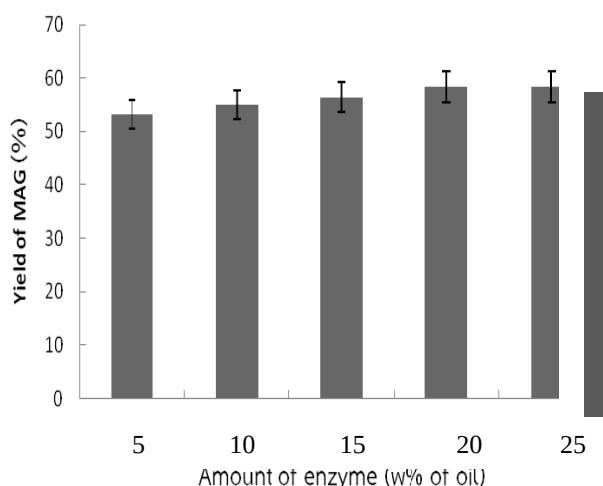


Fig. 5 Effect of enzyme loading on MAG production. The reaction mixture contained glycerol and coconut oil at molar ratio of 8:1 in ethanol (25 wt% of oil). The amount of *C. papaya* lipase used was 5-25 wt% of original oil. The reaction was carried out at 45°C for 36 hours

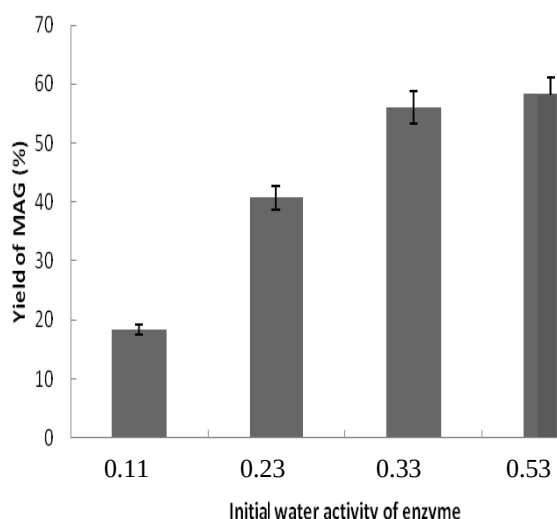


Fig. 6 Effect of initial water activity of enzyme on MAG production. The reaction mixture contained glycerol and coconut oil at molar ratio of 8:1 in ethanol (25 wt% of oil). The amount of *C. papaya* lipase used was 20 wt% of original oil. The reaction was carried out at 45°C for 36 hours

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