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Synthesis of Sugar Ester by Local Yeast Lipase in Solvent Free System

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ABSTRACT

Lipase was produced from yeast isolation located in petroleum contaminated soil. This lipase has good stability at pH between5.0-7.0 and at temperature up to 70 ^{o} C for 30 minutes. It could tolerate in many organic solvents as acetonitrile or hexane. The highest activity throughput the hydrolysis, as well as, the esterification reaction was expressed at pH 6.0 and temperature at 37 ^{o} C. For instant, the application to synthesis of sugar ester from sugars and fatty acids were carried out in various solvent by incubation at 37 ^{o} C for 24 hours with agitation at 600 rpm. The effect of types of substrates as sugar (glucose, fructose, sucrose, xylose and sorbitol), and fatty acid (oleic acid, palmitic acid, myristic acid, lauric acid, and palm fatty acid distillate) were conducted. The molar ratio of substrates, as well as the percentage of enzyme involved in the reaction, disclosed to the percentage of yield of sugar ester was recorded. After incubation, the product was separated and analysis with HPLC. The results revealed the successfully catalysis the reaction to sugar ester was achieved in aqueous solution. The suitable initial substrates were glucose and oleic acid. The optimum molar ratio of sugar to fatty acid was at 3 to 1 with the maximum percentage of yield at 94.31 % within 24 hours.

Keywords: *Lipase, Yeast, Sugar ester, Solvent free system*

1. INTRODUCTION

Sugar esters are bio-surfactant that can be easily degraded [1]. Since the good stabilizing and conditioning properties, they are widely used in many proposes. One is used as emulsifying agent in food processing, as a conditioning in cosmetic products, as a stabilizing in detergents industries, for example [2]. Sugar esters are normally synthesized from esterification reaction of sugar or sugar alcohol with fatty acids under chemically or biologically catalysis [3].

Biological catalysis plays advantages over chemical reaction by mean of it performs under mild condition that consequently save energy. As well as, a selectivity of enzymatic reaction avoid its side reaction and by products [4, 5]. The enzymatic synthesis of sugar ester is based on esterification reaction. Water is one of products of esterification which plays an important role to reverse the reaction back resulting to lower the ester yield [6].

Some methods to maximize the ester yield were reported, for example, water was removed out of the system using molecular sieve [6], or by reduction the pressure to splash out of water [7]. Pervaporation dehydration technique was also applied as well [8].

Nonetheless, some difficulty of the reaction of sugar and fatty acid to become sugar ester is met due to the different solubility of two initial substrates, sugar and fatty acid [9].

One substrate is high soluble in aqueous solution, in opposite of one has high solubility in organic solvents. To overcome this problem, the solvent which induce the partial solubility of two substrates are preferred. Some synthesis media was changed from conventional system to ionic liquids system [10]. As well as, the application of supercritical fluid to enhance the solubility of the substrates was also reported [11]. However, the challenge for seeking the solvent of non toxic are needed due to the carefulness to the environment; solvent free system has become preferable process to carry out.

In this study, the synthesis of sugar ester by local yeast lipase in solvent free system was carried on. The effect of types of substrates (sugar and fatty acid) was performed. The molar ratio of two substrates, including of the percentage of enzyme involved in the reaction were also conducted. The percentage of yield of sugar ester was analyzed. As well as, the initial characterization and the activity of local yeast lipase were investigated before applied as catalyst.

2. MATERIALS AND METHOD

2.1 Characterization of Local Yeast Lipase

Lipase from selected yeast isolation was separated from the supernatant of induced culture media. The enzymatic assay for lipase activity was analyzed with a spectrophotometric method using p-nitrophenyl palmitate (*p*NPP) as an initial substrate followed Maria et al. method. The absorbance measured optically at 410 nm was followed. One unit of lipase activity was defined as the amount of enzyme that liberated 1/µmol of *p*-nitrophenol per min under the assay conditions described [12].

The suitable pH for lipase stability from pH 3- 10 for remaining enzymatic activities within 24 hours was tested.

The optimum temperature for its activity was investigated at 25, 37, 40, 50 and 60 ^{o}C. As well as, the enzymatic stability for 30 min at different temperatures (50, 60, 70, 80 and 90 ^{o}C) was detected. Besides, the toleration of enzyme in different solvents was also processed, under the method described by Schmid et al., 1992 [13]. The remaining activities of lipase were determined. This crude enzyme was desired as a catalyst in following experiments.

2.2 Synthesis of Sugar Ester

Five representatives of sugars (glucose, fructose, sucrose, xylose and sorbitol) were applied to synthesize of sugar ester. All chemicals in this work were analytical grade and purchased from Fluka, Switzerland.

Five representatives of fatty acids studied in this research were oleic acid, palmitic acid, myristic acid, lauric acid, and palm fatty acid distillate (PFAD). All fatty acids were purchased from Fluka, Switzerland, except PFAD was supported by Pure Energy Company limited, Thailand.

A typical experiment was composed of reaction mixture of 1:1 molar ratio of desired fatty acid and sugar adding of 100 mg of crude lipase. The mixture was well shaking and incubated at 40°C for 24 hours. The supernatant product was withdrawn for HPLC analysis. In further experiments, the changing parameters of molar ratio of substrates and amount of enzyme were performed due to the determination of the effect of molar ratio, effect of the amount of enzyme, as well as, the effect of synthesis time.

2.3 Analytical Methods

Esterification product was separated and purified by chromatography on silica gel (grade 60, 230-400 mesh). Chloroform/ methanol/ water (64/ 10/ 1) mixture was used as eluent followed Ducret's methods [7].The percentage of conversion was compared to the corresponding fatty acid. The ester in the upper phase was further analyzed by HPLC Shimazu, Japan equipped with a column of Apollo Silica 5µm (250 mm × 4.6 mm). The evaporative light scattering detector (ELSD) was applied with the drift tube temperature of ELSD at 40 °C. Mobile phase was run at 1.5 mL/ min of flow rate. An eicosane was used as an internal standard.

3. RESULTS AND DISCUSSION

3.1 Characterization of Yeast Lipase

The lipase activity was noticeable by the releasing of *p*-nitrophenol from *p*-nitrophenyl palmitate (*p*NPP) substrate. The highest activity of this lipase was exhibited of 2.30±0.104 Unit/ ml.

The maximum lipase activity was observed at pH 6.0, as well as the stability of this lipase was in the range of pH 3-10. The stability temperature for this lipase was from 30 ^{o}C to 70 ^{o}C. Nevertheless, the suitable temperature was at 40 ^{o}C as the result shown in Table 1. The stability of yeast lipase have been mentioned as well to have the optimum pH from 4 to 8 [14] and also found to exhibit the highest activity at 40 ^{o} C with a residual lipase activity at 87.08% [15].

Table 1: The Characteristics of Local Yeast Lipase

Stability pH	Suitable pH	Stability Temperature (^{o} C)	Suitable temperature (^{o} C)
3.0 -10.0	6.0	30-70	40

The toleration of enzyme in many organic solvents plays an important role in enzymatic syntheses regarding of many initial organic substrates have low dissolubility in water. This lipase played an advantage of having a good stability in water and water -immiscible organic solvents (high log P values) as acetonitrile, t-butanol. In the other hand, it was discovered to have a poor stable in less polar organic solvent as hexane or acetone, and ethyl acetate as shown in Figure1. Lipase from *C. rugosa* was also known to well catalysis in polar solvent which suggested keeping enzyme to have an open conformation and the active site was flexible to catalyst [16].

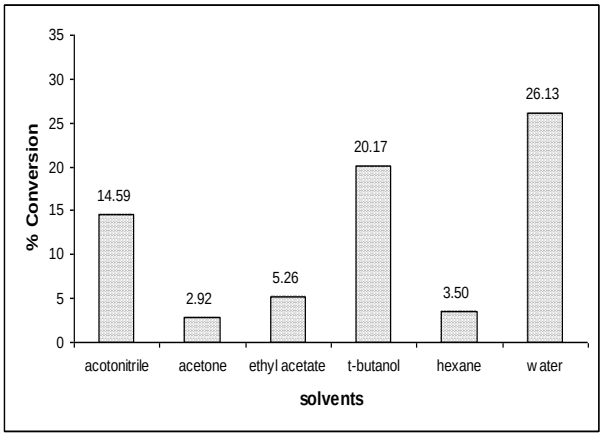

Fig. 1: The stability of lipase in various solvents showed to give the percentage of conversion from the reaction of oleic acid and glucose

3.2 Effect of Types of Sugar and Fatty Acid

Resulting from this lipase was preferred and was stable in polar solvents as water. In consequently, the synthesized sugar ester in water was obtained the highest yield. This ester synthesis was addressed more advantage than the other previous works as it was environmental friendly and low cost process.

In this study, the enzymatic synthesis of sugar ester was performed to preliminary selection of sugar molecule for further study. The reaction was conducted with a typical condition for 24 hours. The various types of sugars reacted with oleic acid was conducted. Five representative types of sugars were provided. (xylose, an adolpentose group;

fructose, a furanose; glucose, an aldohexose; sucrose, a disaccharide including of sorbitol, a sugar alcohol). The results showed in Figure 2 that the highest percentage of conversion was achieved when applied with glucose. Xylose was nearly un-reacted as the lowest yield of product was achieved.

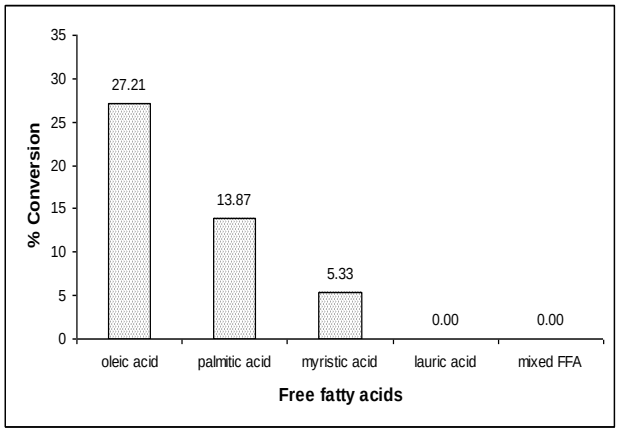

Fig 3: The percentage of conversion of sugar ester from different fatty acids react with glucose.

Further, sucrose could give a little bit higher conversion than fructose, another monosaccharide. This was an attractive result achievement because sucrose is a cheaper substrate than fructose and is available in many countries. Moreover, sucrose is easily soluble in water, a preferable solvent for this enzyme. Relatively, the possibility to get a reasonable price of product is not so far.

However, in this study, the percentage of conversion to be a product when using sorbitol as substrate was higher than when using sucrose as substrate. Sorbitol is a sugar alcohol which could dissolve well in an aqueous solution and the acyl group was donate easily and directly to the molecule of fatty acid became an ester.

Since oleic acid reacted with the other carbohydrates based as fructose, sorbitol and sucrose were also expressed. The high performance of the reaction between oleic acid and sorbitol was also reported to be better than reacted with sucrose [6]. In contrast, this result was disagreed to the study of Seino and Uchibori that the reaction between oleic acid and glucose to become ester was less efficiency than oleic acid reacted with sucrose representing the lower yield achievement [17].

In further study, the selection of suitable fatty acids was further processed with different type of fatty acids reacted to glucose. The result was expressed in Figure 3 that the esterification of glucose with oleic acid (C18:1) was achieved relatively highest yield of product. Palmitic acid (C16:0) and myristic acid (C14:0) reacted with glucose in less amount resulting to obtain of much lower yield of product. Nevertheless, the obvious studies of synthesis of glucose and palmitic acid were successful [18] and presented the opposite result to this work.

Otherwise, neither lauric acid (C12:0), the other shorter chain fatty acid, nor PFAD, a mixed FFA, was observe any product yield from the unsuccessful synthesized. This could be noticed that this lipase preferred a longer chain fatty acid than a shorter chain one.

The result obtained in this work was presented the same achievement of Soultani et al., that the esterification reaction of fructose with longer chain length of fatty acid was easier taken place than with shorter chain of fatty acid [19]. However, the disagreement result was appeared from Pedersen et al., that lipase from *C. antartica* had high catalytic activity for short and medium chains fatty acids (C4- C10) and low active for long chain fatty acids regarding to the shape and the dimension of the binding site of enzyme was specific for each structure of substrates [20].

3.3 Effect of Molar Ratio of Sugar to Fatty Acid

Many molar ratios of glucose and oleic acids were studied in further. The conversion of product was found to be higher when the higher molar ratio of glucose to oleic acid was applied (Fig 4). The excess amount of sugar enhanced the productivity of ester while the amount of fatty acid reduced the production. Particularly, the excess of acyl accepter (sugar) was relatively more effective to the reaction than with the large amount of acyl donor (fatty acid). This was described the higher molar ratio of sugar to fatty acid involved; the higher conversion of product was achieved. The same result was obtained [6].

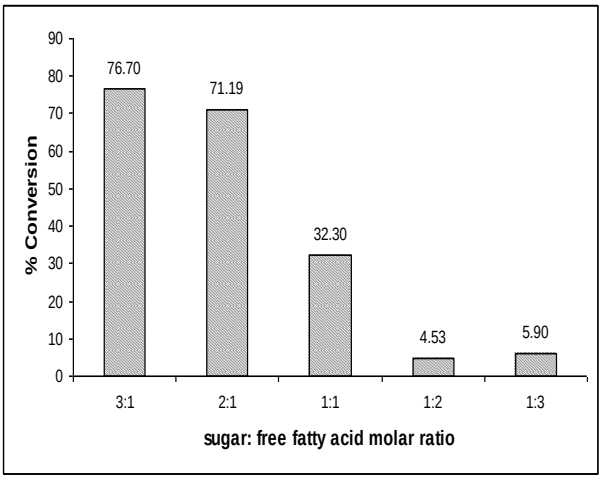

Fig 4: Effect of molar ratio of glucose to oleic acid on the percentage of conversion in water

Nevertheless, the molar ratio of glucose to oleic acid at 3:1 and 2:1 revealed an insignificantly in different percentage of yields of product. However, the desirable molar ratio was depended on the cost base and the details of separation, especially in industrial production.

The difference in the suitable molar ratio of sugar to fatty acid referred to the obviously result of Seino and Uchibori that the optimum molar ratio of glucose to oleic acid was 1:4

[17]. Contrarily, this was said to have the opposite result to this study due to a large amount of fatty acid was required.
In the other hand, the higher molar ratio of sugar to fatty acid was successfully synthesized to obtain the higher yield of product as well as in this result. The conversion of 90% up was achieved from molar ratio of sorbitol to oleic acid of 3:1, as the conversion of 62.7% was obtained when applied with molar ratio of sorbitol to oleic acid of 1:2 [21].

3.4 Effect of the Amount of Enzyme

The amount of enzyme is one of the affecting parameters of the synthesis of sugar ester. The different amount of enzyme induced the percentage of conversion of ester shown in Figure 5.

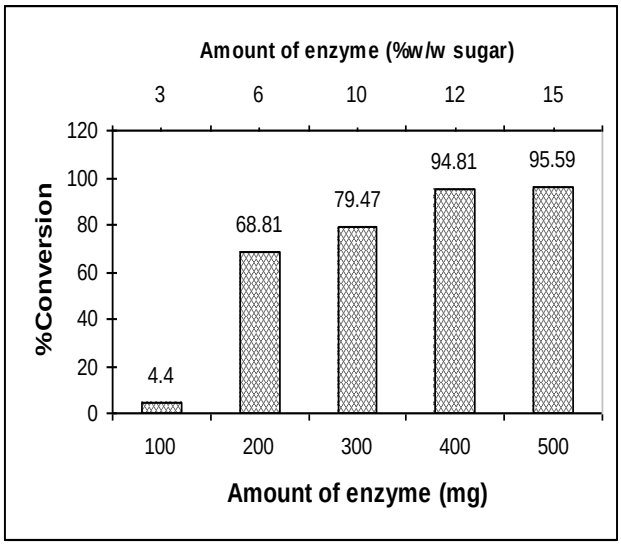

Fig 5: The effect of the amount of enzyme to the percentage of product (gluco- oleate)

At the end of reaction (24 hours), the conversion of higher yield was obtained at the higher added amount of enzyme. In this present work, the suitable dosage of enzyme was at 12 % of sugar based weight. The lower dosage applied, the less conversion was achieved. A large amount of enzyme could induce to reach more rapidly in an equilibrium state resulting to the higher conversion of product was achieved. Nevertheless, the more amount of enzyme added, the higher cost to be concerned.

Finally, the reaction time was determined to establish for the maximum conversion. In Figure 6, the maximum conversion was expressed nearly completed of 100% of conversion within 24 hours. After this time up, the percentage of conversion was not showed any significantly different. The equilibrium time was reached within 24 hours.

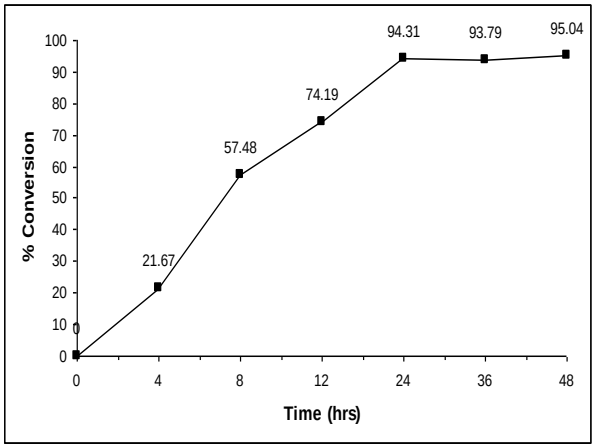

Fig 6 The reaction time for enzymatic synthesis of glucose/ oleic acid ester in batch process in solvent free system

Regarding to types of substrates, enzyme activity, including of the solvent system influence the reaction of saccharide esters, the equilibrium times and percentage of conversion were mentioned in difference. This research was showed the better efficiency than the obviously works of Seino and Uchibori which only 28% of conversion of glucose and oleic acid was found within 72 hours [17].

In the other side, the synthesis of other sugars and fatty acids, e.g. the reaction of glucose with stearic acid in this work was found to be less efficiency when compared to other research [22]. Cao et al. revealed the esterification of D (+) glucose with palmitic acid was given up to 86% conversion after 48 hours of reaction [23]. Whereby, Tarahomjoo found the conversion of glucose with palmitic acid was 41.18% - 54.27% at 111 hours [18]. Including of the reaction between sorbitol and oleic acid was presented the equilibrium time within 3 hours and obtained of more than 90% of conversion [6].

In the conclusion, this research was provided the green synthesis of sugar ester catalyzed by local yeast lipase. This lipase was investigated to have an excellent characteristic as its stability was reached 70 ^{o}C and pH between 3- 10. The highest activity was at pH 6.0 and at temperature 40 ^{o}C and tolerated in many high log P organic solvents. This crude enzyme was successfully catalyzed esterification reactions of sugar and fatty acid in aqueous solution. The suitable sugar was glucose and suitable fatty acid was oleic acid. The efficiency of this lipase to catalyze the esterification with the longer chain fatty acid was higher than with the shorter chain one. The optimum molar ratio of glucose to oleic acid was 3: 1 catalyzed with 12% of enzyme based sugar weight and achieved the maximum percentage of yield of 94.31 % within 24 hours.

For the environmental concern, the sugar ester catalysis with this lipase have a much advantage regard to the non toxic solvent was applied. The further studies were needed to identify the species of this local yeast by 16s RNA, as well as the possibility to synthesis in an upscale reactor.

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