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SELECTIVITY OF CANDIDA RUGOSA LIPASE IMMOBILIZED ONTO LAYERED DOUBLE HYDROXIDES AS CATALYST IN SYNTHESIS OF FATTY ACID ESTERS

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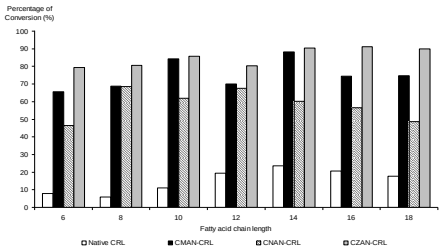
Graphical abstract



Abstract

The enzymatic selectivity of Lipase from *Candida rugosa* immobilized onto a calcined layered double hydroxide (CLDHs-CRL) towards the chain-length of fatty acids and alcohols in the synthesis of fatty acid esters was investigated. The results showed that CMAN-CRL catalyzed the esterification process with fatty acids of medium chain lengths (C10-C14) effectively while, CNAN-CRL and CZAN-CRL exhibited high percentage conversion in fatty acids with carbon chain lengths of C8-C12 and C10-C18, respectively. In the alcohol selectivity study, CMAN-CRL showed high selectivity toward alcohols with carbon chain lengths of C4, C6 and C10. On the other hand, both CNAN-CRL and CZAN-CRL exhibited rather low selectivity towards longer carbon chain length of alcohols.

Keywords: Immobilized lipases, esterification, layered double hydroxides

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1.0 INTRODUCTION

Fatty acid esters are important ingredients in the production of consumer products. They are utilized extensively as additives and flavoring agents in food, as fragrances, surfactants and specialty emollients in cosmetics and as plasticizers, lubricants and solvents in pharmaceutical products. Conventionally, fatty acid esters are manufactured either by extraction from natural sources or by chemical synthesis. However, nature's derived fatty acid esters are limited and costly [1-4].

On the other hand, the chemical synthesis of fatty acid esters by using corrosive acid catalysts at high temperature are hazardous and often leads to high overall production cost due to the higher energy cost, additional repair work on the corroded equipment as well as the multiple steps including removal of by-products [5-7].

Recently, the demand in the use of natural products and the move towards safer and 'environmentally benign manufacturing' processes has made the use of enzymes as biocatalysts in the fatty acid esters production more preferable [8]. Enzymes are naturally produced by living cells to catalyze hundreds of biochemical reactions which are important to the physiological functions of all organisms [9]. Thus, by using the isolated enzymes in the synthesis, these nature's catalysts may give 'natural' label to the products. Moreover, since most enzymes are of plant or animal origin, they are safe and can be transported and disposed in the soil with very little safety precautions [10-11].

Enzymatic synthesis offers various advantages over chemical synthesis for industrial processing. Enzymes are biological catalysts that able to operate under mild reaction conditions, hence reduces the cost of energy and capital-equipment as well as reducing the requirements of nonrenewable energy. In addition, enzymes are highly specific compared to inorganic catalysts and it may be possible that by choosing the right enzyme, the variety of products generated are controlled and the unwanted side reactions are minimized, thus simply the downstream process. As a result, the plant using enzymatic reactions can be operated at a lower by-products removal and effluent treatment costs [12-14].

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are the most important biocatalysts in enzymatic organic synthesis because of their catalytic ability, satisfactory availability, broad substrate specificities and good stability in aqueous to non-aqueous organic solvents, wide range of pH and temperatures [15-16]. In spite of the many advantages, the application of lipases in industrial processes is still lacking due to the high cost of the enzymes and the lack of an effective scheme for their multiple uses [17-18]. However, these problems can be tackled via immobilization of the enzymes. Immobilization allows reutilization of the enzymes and makes it possible to operate enzymatic processes continuously [19].

Lipases have been immobilized onto LDHs and it is noteworthy that in the synthesis of fatty acid esters such as butyl oleate, the adsorbed CRL onto LDHs showed higher yield and stability compared to its native CRL [20-21]. LDHs are low-cost minerals and relatively environment friendly synthetic materials consisting structurally of positively charged brucite-type layers of mixed metals hydroxides with charge-compensating anions together with water molecules in interlayer spaces. There are generally represented by the formula: $[M^{2+}(1-x)\ M^{3+}x\ (OH)_2]^{x+}(A^{n-}\ x/n).mH_2O$ where, M^{2+} is a bivalent cation, M3+ is a trivalent cation and A^{n-} is an interlayer anion [22-23].

LDHs have also been reported as human health friendly material as the possibility of using the LDHs as an injectable drug delivery vehicle has also been suggested [24-25]. Calcination of LDHs at higher temperatures (around 450°C and above) affords layered double oxides (LDO), which have been paid more attention as support for enzyme immobilization owing to their larger surface area, less diffusion resistance, porous structure and abundance basic sites to bind with an enzyme than those LDHs [26-28].

Meanwhile, the use of LDHs calcined at a lower temperature (150°C), as support for lipase immobilization has also shown to exhibit higher lifetime and thermal stability of the biocatalyst. Plus, the high catalytic activities of these immobilized lipases in esterification reactions and their capability to retain high activities after repeated use will positively improve the cost-effectiveness of the enzyme catalyzed process [29-30].

Native CRL is reported to display a preference for fatty acids of chain lengths (C4, C16, and C18) and alcohols of medium chain lengths (C6, C8 and C12) in esterification reaction [31]. However, it has been demonstrated that the substrate selectivity can also be altered by immobilization [32-33]. Hence, it is important to characterize the immobilized lipases concerning their substrate selectivity in view of the fact that the substrate selectivity of lipases is one of the crucial properties which determine their ultimate usage [34].

2.0 EXPERIMENTAL

2.1 Immobilization of CRL onto CLDHs

Mg/Al-NO_3 LDH (CMAN) were prepared by co-precipitation method similarly as described by Hussein *et al*. (2004). $Mg(NO3)_2.H_2O$ and $Al(NO_3)_3.9H_2O$ were dissolved in distilled water to make an aquoeus solution with molar ratio of Mg^{2+}:Al^{3+} = 4:1. NaOH (2.0 M) was added dropwise into continuously stirred Mg^{2+}:Al^{3+} solution. The addition was monitored by benchtop pH meter and ended at the pH of 10.00. Then, the resulted slurry was aged in the horizontal water bath shaker at temperature of 70oC and 110rpm agitation speed for 18hr. The precipitates were filtered, washed, dried overnight in oven at 80°C and grinded into powder form. Same method was applied to prepare Ni/Al-NO_3 LDH (CNAN) and Zn/Al-NO_3 LDH (CZAN). All LDHs were calcined (150°C, 5hrs) to increase the surface area and the porosity of the LDH and also to eliminate the CO_3^{2-} anion which may have trapped inside the LDH's interlamella during the precipitation

Crude CRL (2.0g) was mixed with distilled water (20mL), stirred for 1hr and centrifuged at 10,000rpm for 15min. The suspended solid was discarded. The supernatant was used directly or stored at -4°C. Immobilization was carried out by continuous shaking at 110rpm of CMAN (1.0g) with lipase supernatant (7.5mL) for 1hr at room temperature in horizontal shaker bath. The immobilized lipase (CMAN-CRL) was filtered and washed with distilled water (10mL) and saline solution (10mL, 1M) to remove the unadsorbed lipase. Washings were carried out several times (3 to 5 times) till no soluble protein was detectable in the washing solutions as determined by Bradford's method [35-36]. Immobilizations of CRL onto CNAN and CZAN were also carried out in a similar way.

2.2 CLDHs-CRL Activity in Esterification

The enzymatic reaction consisted of fatty acid (2mmole), alcohol (4mmole), biocatalyst (contained 1mg protein) and hexane (2mL). The reaction mixture was incubated at 50°C for 6hr, with continuous shaking at 150rpm in a horizontal water bath shaker. The reaction was terminated by the addition of 10mL of acetone: ethanol (50: 50, v/v). The esterification activities were expressed as percentage of conversion by determining the remaining free fatty acid in the reaction mixture by titration with 0.1M NaOH using an autotitrator to an end point at pH10. All experiments

were tested in triplicates and control experiments were carried out without biocatalyst.

2.2.1 CLDHs-CRL Fatty Acid Carbon Chain-Length Selectivity in Esterification

The selectivity of the CLDHs-CRL prepared towards fatty acid in esterification reactions was tested using similar method as described in section 2.2. Adipic acid (C6), caprylic (C8), lauric acid (C12), myristic acid (C14), palmitic acid (C16) and stearic acid (C18) were reacted separately with methanol. The enzymatic activities were expressed as percentage of conversion of fatty acid to fatty acid ester.

2.2.2 CLDHs-CRL Alcohol Carbon Chain Length Selectivity in Esterification

The selectivity of the CLDHs-CRL prepared towards alcohol in esterification reactions was tested using similar method as described in section 2.2. Methanol (C1), butanol (C4), hexanol (C6), octanol (C8) and decanol (C10) were reacted separately with caprylic acid. The enzymatic activities were expressed as percentage of conversion of fatty acid to fatty acid ester.

3.0 RESULTS AND DISCUSSION

3.1 CLDHs-CRL Fatty Acid Carbon Chain-Length Selectivity in Esterification

Figure 1 shows the effect of immobilization of CRL onto CLDHs to the selectivity of the enzyme towards carbon chain-length of the fatty acid in the esterification reaction. Results showed that native CRL favored esterification of fatty acids with medium chain-length (C12-C14) compared to shorter chain fatty acids but the activity decreased with longer chain fatty acids (C16-C18). It has been explained before that that the decrease of activity may be due to the bulky chain moiety which may restrain the molecule from free rotation in the acyl binding site cavity of the enzyme active site or impose hindrance to attack by the nucleophile (alcohol) [37]. In addition, it was also reported that CRL has low activity towards long, polyunsaturated fatty acid [38].

In contrast to those previously reported, CRL immobilized onto CLDHs exhibited rather high activity in all cases of fatty acids with CMAN-CRL catalyzed effectively the esterification process with fatty acids of C10 and C14. While CNAN-CRL and CZAN-CRL exhibit higher percentage conversion in fatty acids of chain lengths from 8 to 12 and 10 to 18, respectively. These results showed that CLDHs-CRL may be used in the synthesis of fatty acid ester with long chain fatty acids.

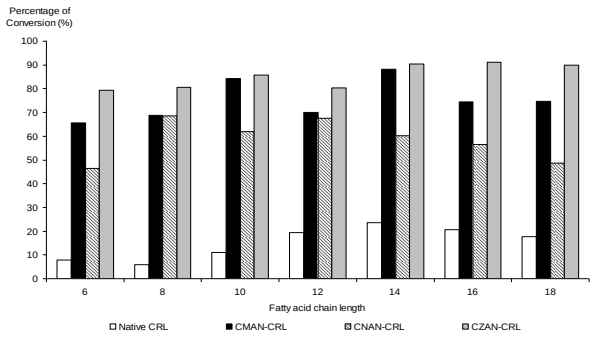

Figure 1 Effect of fatty acid carbon chain-length (C6-C18) on the synthesis of fatty acid esters by native CRL and CLDHs-CRL

3.2 CLDHs-CRL Alcohol Carbon Chain Length Selectivity in Esterification

Figure 2 shows effect of immobilization of CRL onto CLDHs to the selectivity of the enzyme towards alcohol carbon chain-length in the esterification reaction. Generally, immobilized lipases exhibited high activities with all alcohols compared to native CRL. Results also showed that native CRL showed higher activities towards alcohol with longer chain length. This may be due to the different affinity in terms of the binding energy that was released when a substrate binds at the active site of lipases. Only a few of the many substrates that bind at the active site can release a sufficient amount of binding energy required for effecting a change in conformation of lipase to a form that is a more efficient catalyst [39].

It is also reported that, substrates such as methyl and ethyl alcohols, which are too small, are not able to release enough energy, so that the change in conformation of the native lipase to the desired catalytically active form does not occur [40]. Results showed that CRL immobilized onto CMAN was more accessible to a longer alcohol chain length; C4, C6 and C10. On the other hand, both CNAN-CRL and CZAN-CRL exhibited rather lower activities towards longer chain alcohols.

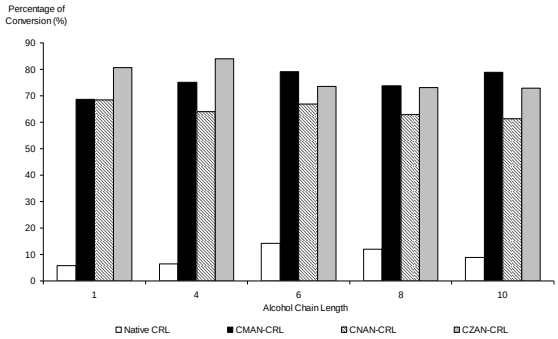

Figure 2 Effect of alcohol carbon chain-length (C1-C10) on the enzymatic synthesis of caprylic esters by native CRL and CLDHs-CRL

4.0 CONCLUSION

In conclusion, the immobilization of CRL onto CLDHs has increased the biocatalytic activities of CRL in the synthesis of fatty acid esters. The CLDHs-CRL offers relatively cheaper price compared to the commercial available immobilized enzyme due to the low cost of CLDHs preparation. These carriers are preferred due to their broad substrate selectivity in the synthesis of various fatty acid esters. Immobilized lipase onto CLDHs also showed better stability when used in the synthesis with polar short chain alcohol. The environmentally benign process using CLDHs-CRL has very good prospect due to its biodegradability and the safety of CLDHs towards human and environment.

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