CHAPTER IV
RESULTS AND DISCUSSION

Overexpression and characterization of Candida rugosa lipase isoenzymes in Yarrowia lipolytica

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Abstract

Candida rugosa secretes eight isoenzymes with highly interesting catalytic activities. Nevertheless, due to their high homology and the non-universal codon use of this yeast, the production of pure *C. rugosa* izosymes either by purification from a mixture or by recombinant expression thus represents a challenge. In this study, the synthetic genes encoding Lip1, Lip2, Lip3, Lip4 and Lip5 from *C. rugosa* were designed for optimal expression and secretion in *Yarrowia lipolytica*. Such a system enabled the recovery of highly pure proteins Lip1, Lip3 and Lip4 directly from crude supernatants (67.8 U/mL, 14.9 U/mL, 8.0 U/mL respectively). Lip1 and Lip3 were shown to be specific of medium chain fatty-acids of (C8-C10) whereas Lip4 preferred longer fatty-acids (C16). Moreover, these isoenzymes were found potentially interesting for two pharmaceutical applications. For the first time, the three isoenzymes were shown to enable the enrichment of cis-5, 8, 11, 14, 17-

eicosapentaenoic acid (EPA) and cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA), two health beneficial omega-3 fatty acids, from a mixture of ethyl esters issued from fish oil. The maximal DHA purity ~60 % was obtained with Lip3 and Lip4, with an initial ethyl ester mixture containing 25% DHA.

Introduction

Microbial lipases are an important group of biotechnologically valuable enzymes, mainly for their versatility, diversified specificities and aptitude to catalyse synthesis reactions. For these reasons they are very attractive for a wide range of applications (Jaeger and Eggert 2002; Hasan et al. 2006), and an increasing interest was born in producing these enzymes via recombinant DNA technology. *Candida rugosa* (formerly *Candida cylindracea*) lipases (CRL) have been widely used as biocatalyst for the production of valuable materials in numerous fields such as agro-alimentary, pharmaceutics and white biotechnologies (Jaeger and Eggert 2002; Akoh et al. 2004). CRL are commercially available, however, crude enzyme preparations obtained from various commercial suppliers exhibit notable variation in their catalytic efficiency and specificity. Indeed, *C. rugosa* secrets a mixture of enzymes composed of at least eight isoenzymes with high sequence identity (superior to 70%). Separation of CRL isoenzymes is highly desirable to allow their use under well-defined conditions but their high identity entails technical difficulties in the isolation of individual isoenzymes on a preparative scale for industrial applications (Lee et al. 2002; Chang et al. 2006). The production of highly pure and isolated isoforms of CRL thus represents a challenge. Six of the encoding genes were entirely sequenced (Brocca et al. 1995; Xu et al. 2009) and five CRL were thus intensively studied. Two strategies were then developed. On the one hand, the production of the different isoforms by the wild type strain depending on culture conditions was studied (Rua et al. 1993). On the other hand, the use of recombinant DNA technology has shown to be an alternative way to obtain individual pure CRL isoforms (Akoh et al. 2004).

Unfortunately, *C. rugosa* has an original non-universal codon usage, and the triplet CTG, a universal codon for leucine, is read as a serine. Nearly 20 serines encoding codons on Lip1 to Lip5, including the catalytic Ser, are CTG. Therefore, the heterologous expression of such genes might result in the production of

inactive lipases, and the transposition of most CTG codons into universal serine triplets is required for the expression of a functional lipase protein in a heterologous expression system. This was achieved by the overlap extension PCR-based multiple site-directed mutagenesis, which was used to convert all non-universal serine codons of the CRL genes into universal serine codons and the mutated genes were consequently expressed in *Escherichia coli, Pichia pastoris, Saccharomyces cerevisiae or Candida maltosa*. For instance, Lip1 and Lip3 were expressed either in *P. pastoris* (Chang et al. 2005; Chang et al. 2006) or *S. cerevisiae* (Brocca et al. 1998). In addition, Lip2 was produced by Lee et al., (2002) in *P. pastoris*. Finally, Lip4 could even be produced as an active protein in the prokaryotic host *E. coli* (Tang et al. 2001). Nevertheless, very few studies report an accurate comparison between the different isozymes and the differences both in host for recombinant expression and enzymatic assays makes it difficult to compare CRL isoenzymes.

In the present work, we decided to take advantage of a recently developed expression hostdedicated to high-throughput screening procedure, namely *Y. lipolytica* JMY1212 (Bordes et al. 2007), for the production of CRL. Furthermore, a recent improvement of this system was reported, leading to a highly efficient and reproducible expression system enabling the direct comparison of enzyme variants in the crude supernatant (Piamtongkam *et al.*, submitted). This made it an attractive tool for the direct comparison of CRL isoenzymes. *Y. lipolytica* JMY1212 was successfully used to produce *C. rugosa* lipases Lip1, Lip3 and Lip4. These isoenzymes were then characterised and proved to be highly valuable for industrial applications. Indeed, CRL isoenzymes could be used in the valorisation of fish oils for the purification of cis-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) and cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA), two valuable omega-3 fatty acids.

Results and discussion

Construction of expression plasmids

Genes encoding Lip1, Lip2, Lip3, Lip4 and Lip5 lipases isoenzymes from *C. rugosa* were synthesised by GeneArt for an optimized expression in *Y. lipolytica*. The nucleic sequences encoding mature 534-residue Lip proteins from *C. rugosa* were preceded by the 33-residue prepro peptide from *Y. lipolytica* lipase Lip2 for secretion

of the produced proteins in the culture supernatant. Indeed, this targeting sequence was shown to successfully entail the secretion of recombinant proteins (Nicaud et al. 2002). These genes were subcloned into plasmid JMP62-TEF-*Ura-Ex*, a derivative of JMP62 (Nicaud et al., 2002) where the POX2 promoter, inducible by oleic acid, was replaced by the constitutive TEF promoter (Muller et al. 1998), leading to JMP62-TEF-LipX, where X accounts for 1 to 5. This plasmid contains both a bacterial and a yeast part. The bacterial part enabled the plasmid amplification in *E. coli* and the yeast part, which was further integrated in *Y. lipolytica* genome, enabled the expression of recombinant CRL. The expression cassette is flanked by zeta regions, which permits the integration at the defined locus LEU2 of *Y. lipolytica* strain JMY1212 genome (Bordes et al., 2007). The expression cassette contains the U*ra3d1* marker for selection of *Ura*$^+$ transformants in *Y. lipolytica*. The map of this plasmid is given in figure III-1.

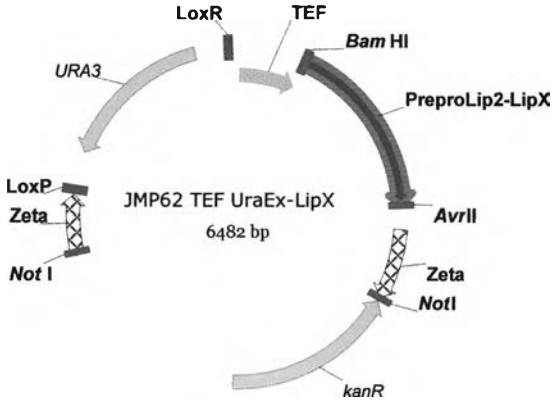

Figure III-1. Schematic map of the expression plasmid. JMP62-TEF-LipX plasmids contain the *ura3d1* marker for selection of Ura$^+$ transformants in *Y. lipolytica,* the kanamycin gene resistance (KanR) for selection in *E. coli*. The gene of interest, LipX encoding for Lip1 to Lip5 of *C. rugosa*, is preceded by the prepro targeting sequence of Lip2 from *Y. lipolytica*, and placed under the control of TEF promoter. The expression cassette in flanked by zeta regions.

Production of recombinant CRL

A medium deprived of uracile was used to select *Ura*$^+$ transformants. As proposed in a recent study from our group (Piamtongkam et al., submitted), three transformants of each strain were cultivated in 10 mL $Y_1T_2O_1$ (10 g/L oleic acid). After complete

consumption of oleic acid, supernatants were recovered and the activity of each transformant was directly evaluated using *p*-NPB hydrolysis reaction.

Table III-1. *P*-nitrophenyl butyrate hydrolysis activity for transformants of JMP62-TEF-LipX in *Y. lipolytica* JMY1212 cultivated in $Y_1T_2O_1$ expression medium. CV, coefficient of variation, is obtained while expressing the standard deviation relative to the mean.

Enzyme	Transformant	*p*-NPB activity (U/mL)	Mean activity (U/mL)	CV (%)
Lip1	1	4.7	5.6	14.3
	2	6.2		
	3	5.8		
Lip2	1	0.1	0.1	/
	2	nd		
	3	nd		
Lip3	1	1.4	1.4	3.5
	2	1.5		
	3	1.4		
Lip4	1	2.3	2.4	6.5
	2	2.5		
	3	2.5		
Lip5	1	nd	nd	/
	2	nd		
	3	nd		

Each experiment was realised in triplicates
nd stands for not detectable

The results are given in Table III-1. This protocol arises from the statistical study of *Y. lipolytica* JMY1212 carried out by our group (Piamtongkam et al., submitted), which demonstrated that the transformation of this strain, thanks to the presence of a zeta docking platform in its genome, enabled the reproducible homologous insertion of an expression cassette flanked by such regions. This insertion reproducibility thus results in an equivalent protein expression level. As shown in table III-1, the coefficients of variation (CV) obtained for three transformants is always inferior to 15%, with a mean CV of 8%, which is in accordance with the 11% reported by Piamtongkam et al..

Nevertheless, no activity was found in the supernatants from *Y. lipolytica* JMY1212 transformed with JMP62-TEF-Lip2 and JMP62-TEF-Lip5.

The low CV found for each set of three transformants was interpreted as an identical lipase expression level, and very probably as genetic identity between these transformants. Therefore, one transformant was selected for each strain and cultivated with a higher amount of carbon source (either 30 g/L oleic acid or 50 g/mL glucose) in baffled flasks. Results of *p*-NPB activity for Lip1 to Lip5 are shown in Table III-2.

Table III-2. *P*-nitrophenyl butyrate hydrolysis activity of recombinant CRL produced under optimal carbon source and aeration conditions. *Y. lipolytica* strains were grown at 28 °C in baffled 500 mL erlenmeyer flasks containing 50 mL $Y_1T_2O_3$ or $Y_1T_2D_5$ medium until complete substrate consumption.

	Carbon source	**Enzyme**				
		Lip1	**Lip2**	**Lip3**	**Lip4**	**Lip5**
p-NPB activity (U/mL)	Oleic acid	26.2	0.1	3.6	4.2	nd
	Glucose	67.8	0.1	14.9	8.0	nd

each experiment was realised in triplicates
nd stands for not detectable

As shown in Table III-3, the production of isoenzymes with optimised conditions led to an important increase in protein production both with oleic acid and glucose as carbon source. Indeed, a 1.8/3-fold, 2.6/10.6-fold and 4.6/12.2-fold increase was found for specific activities of Lip4, Lip3 and Lip1, respectively, produced with oleic acid/glucose. Due to differences either in lipase activity assays, production conditions and so on, the production of Lip1, Lip3 or Lip4 in *Y. lipolytica* is hardly comparable to other production obtained in different hosts. The recombinant production of Lip3 in *P. pastoris* led to 1.2 units (calculated with *p*-NPB activity) per mL of culture supernatant before any purification step (Chang et al., 2006), which could be enhanced by one order of magnitude with production in *Y. lipolytica*. The activity found for Lip1 expressed by our *Y. lipolytica* strain is highly higher than the one reported for production in *S. cerevisiae* (5 U/mL, Brocca et al. 1998) and inferior to the 150-250 U/mL reported previously for recombinant Lip1 produced in *P.*

pastoris (Brocca et al., 1998; Chang et al. 2005, 2006), thus showing the importance of the expression host. Nevertheless, these former activities were reached after more than ten days fermentation of a multicopy variant (150 U/mL, Brocca et al., 1998) or when using a medium optimised by statistical experimental design methods (250 U/mL, Chang et al., 2005, 2006). Moreover, Chang et al. (2005, 2006) assayed their recombinant lipase on triglycerides, while it was shown that Lip1 is nearly 30-fold more active on natural triglycerides than on generic *p*-NP esters (Lopez et al. 2004).
The SDS-PAGE analysis of Lip1, Lip3 and Lip4 revealed a major proteic band for each lipase (Figure III-2A). Their electrophoretic mobilities were in accordance with calculated molecular masses of 57 kDa incremented by post-translational glycosylations for Lip1 and Lip3. Indeed, Lip1 and Lip3 carry three consensus sequences for N-glycosylation at N291, N314 and N351, whereas Lip4 only contains the N351 site for potential N-glycosylation. Moreover, detailed analysis of Lip1 glycosylation profile showed that only N314 and N351 were glycosylated and that the carbohydrate modification consisted of two *N*-acetyl-glucosamines units followed by a variable number of mannose residues (Brocca et al. 2000; Natalello et al. 2005).

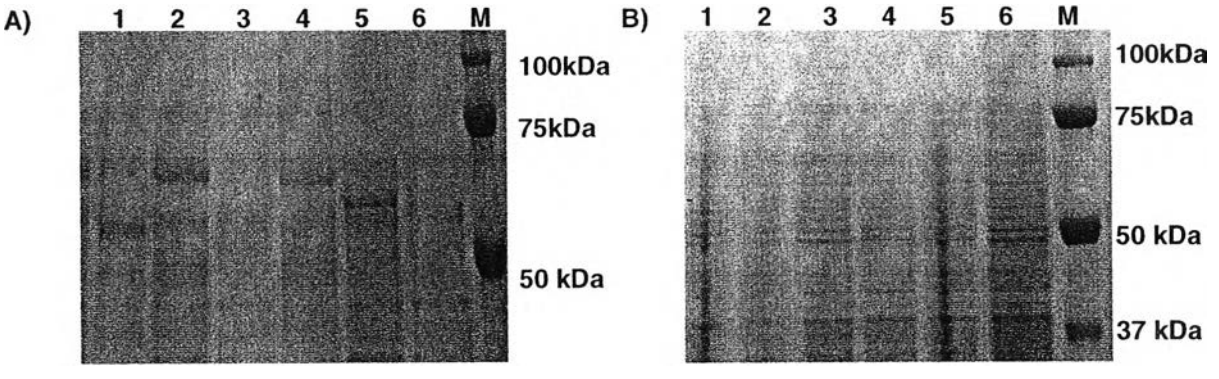

Figure III-2. SDS-PAGE analysis of supernatants (**A**) and cells (**B**) of *Y. lipolytica* JMY1212 transformed with JMP62-Tef- LipX. Lane 1: JMY1212, lanes 2-6: JMY1212 transformed with JMP62-Tef-Lip1, -Lip2, -Lip3, -Lip4, and -Lip5; M, precision plus protein standards (Bio-Rad).

Few studies report the recombinant production of Lip2, and Lip5 has never been produced as recombinant. Here, we could not detect any lipasic activity in the

supernatant of *Y. lipolytica* JMY1212 transformed with JMP62-Tef- Lip2 and -Lip5. This was correlated with the absence of band visualisation by SDS-PAGE analysis (figure III-2A). As it had been shown that both the leader peptide and the nature of the gene could interfere with the production of extracellular CRL isoenzymes (Fusetti et al. 1996; Brocca et al. 1998), we searched for intracellular lipase accumulation. The SDS-PAGE analysis shown in figure III-2B could not highlight such accumulation neither for Lip2 nor for Lip5. Further analysis of mRNA by RT-PCR should determine if this problem comes from transcription level, the translatability of specific mRNA or the stability of the recombinant protein. An alternative could be the production of a fusion product as was done for Lip1 (Passolunghi *et al.*, 2003).

Enzyme characterization

Hydrolysis of methyl esters

Lip1, Lip3 and Lip4 were further analysed for their chain length specificity. As quantifying the hydrolytic activity of lipases against single esters would only have provided an initial view of substrate specificity, we decided to assay our lipases directly by competitive hydrolysis assays utilizing a mixture of methyl esters of various chains lengths (C6-C18).

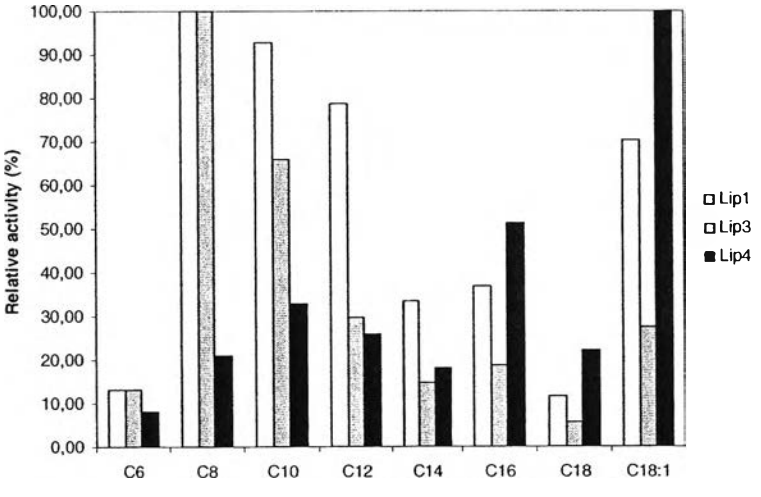

Figure III-3. Substrate specificity of recombinant Lip1, Lip3 and Lip4 from *C. rugosa*. Relative activities towards methyl esters of various chain lengths (C6-C18) were determined at pH 7.2 and ambient temperature after 4 h hydrolysis.

As shown in Figure III-3, Lip1, Lip3, and Lip4 demonstrated different preferences for the acyl part of the ester substrates. Lip1 and Lip3 are specific of medium chain fatty acid esters with a maximum activity towards methyl octanoate (C8). Indeed, both Lip1 and Lip3 hydrolyse C8 to C12 methyl esters preferentially, although the difference in relative activity is highly noticeable with Lip3 compared to Lip1 for these three esters. The results resembled those previously reported on *p*-nitrophenyl esters of various chain lengths for native or recombinant lipases (Brocca et al. 1998; Pernas et al. 2000; Lopez et al. 2004). Conversely to the former isoenzymes, Lip4 showed the highest conversion rate for methyl palmitate (C16) and methyl oleate (C18:1). As highlighted by previous study by Lee and collaborators (Lee et al. 2007), Lip4 also showed a broadest specificity compared to Lip1 and Lip3. Indeed, all saturated methyl esters ranging from C8 to C18 were hydrolysed to a comparable extend (24%) except C16. Finally all three isoenzymes displayed a lipolytic activity inferior to 15% towards methyl hexanoate (C6). This is difficult to explain although similar results for *C. rugosa* isoenzymes were also achieved in previous studies.

To take advantage of the difference between the substrate specificities of the produced recombinant isozymes, we investigated their potential in the purification of highly valuable pharmaceutical compounds.

Purification of DHA from Fish oil

Yet in the fifties, Sinclair and collaborators (1953) explained the rarity of coronary heart disease in Greenland Eskimos by a diet high in whale, seal, and fish. This report was then supported by various studies signalling the benefit effects of polyunsaturated fatty acids (PUFAs) in prevention and treatment of cardiovascular diseases (von Schacky 2009). Amongst long chain polyunsaturated fatty acids (PUFAs), cis-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) and *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) are of interest due to the wide range of health benefits that they present (Okada et al., 2007).

Given their difference in specificities, the aptitude of Lip1, Lip3 and Lip4 to purify DHA and EPA from esterified fish oil was assessed in 1.5 mL tubes. The hydrolysis reactions were carried out with Ethyl Ester of Fish Oil (EEFO), containing 25% DHA and 5% EPA ethyl esters, as starting material.

Table III-3. Percentage of long chain fatty acid ethyl esters after 24h hydrolysis with lipases from *C. rugosa*. 16:00, 18:01, 18:02, 20:05, 22:06 stand for palmitate, oleate, linoleate, EPA and DHA ethyl esters, respectively. The percentage recovery of EPA and DHA is indicated in parenthesis.

Enzyme	Fatty acid ethyl esters composition (%)				
	16:00	**18:01**	**18:02**	**20:05**	**22:06**
EEFO	20.2	13.9	1.6	5.7	26.2
Lip 1	16.42	6.5	0.9	7.0 (85.9)	37.8 (100)
Lip 3	8.5	5.5	1.1	9.0 (93.1)	44.7 (99.9)
Lip 4	17.2	9.2	1.5	5.7 (77.4)	35.6 (100)

As shown in Table III-3, the three lipases were able to concentrate DHA ethyl ester. This is in accordance with previous studies that showed the ability of CRL to increase the DHA content in the non-hydrolysed fraction of marine oils (Wanasundara and Shahidi 1998b; Okada and Morrissey 2007). The best results concerning DHA were obtained with Lip3, which enable the almost entire recovery of DHA ethyl ester (99.9%) with a 1.7 concentration factor. Lip 1 and Lip 3 could also concentrate EPA while the percentage of EPA remained constant during the hydrolysis of EEFO by Lip4. In order to increase the final concentration of DHA and EPA in EEFO, the reaction was scaled up and two supplementary hydrolysis were performed, as described previously (Shimada et al. 1994). The reaction was carried out at 30°C, in 10 mL tubes and a reaction time of 24 h for each hydrolysis. After each hydrolysis EEFO was recovered, free fatty acids were discarded by saponification and fresh enzyme was added. The percentage of DHA and EPA ethyl esters after three rounds of hydrolysis are shown in Figure III-4.

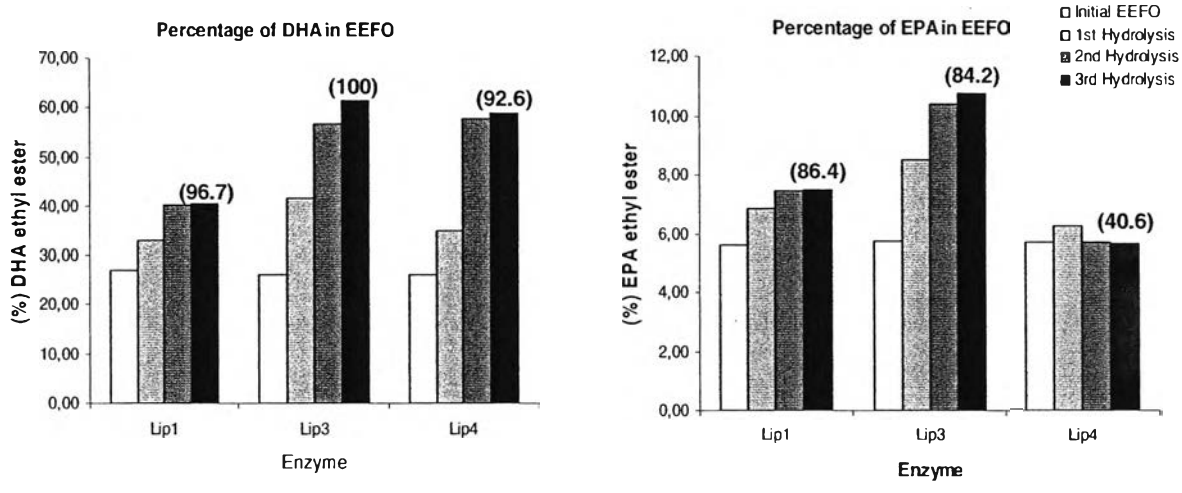

Figure III-4. Percentage of DHA (left) and EPA (right) after 3 rounds of EEFO hydrolysis with *C. rugosa* Lip1, Lip3 and Lip4. The percentages recovery of EPA and DHA after three hydrolysis are indicated in parenthesis.

The second and third hydrolysis permitted to further increase the percentage of DHA, Lip3 being the most efficient enzyme. Indeed, the final EEFO after hydrolysis with Lip3 contained 61.5% of DHA. Final concentrations of DHA in EEFO were reached after the second hydrolysis by Lip1 and Lip4, and were of 40.5 and 59.1%, respectively. The third hydrolysis with Lip1 and Lip4 did not considerably increase the amount of DHA.

A considerable difference between these isoenzymes is their relative hydrolytic performance towards EPA compared to DHA. As observed in 1.5 mL tubes, Lip3 was able to concentrate EPA ethyl ester, reaching 10.75% final concentration after three rounds of hydrolysis. This is in accordance with the discrepancy observed concerning the hydrolysis of EPA by Lip1, Lip3 and Lip4. Indeed, the hydrolysis of EEFO by Lip4 is far more important (59.4%) than by Lip1 and Lip3 (13.6% and 15.8%). Although the first round of hydrolysis with Lip4 leaded to EPA concentration (5.7% to 6.3%), an extended hydrolysis with Lip4 decreased the concentration of EPA ethyl ester. This situation was formerly observed during the hydrolysis of fish oils with commercial preparations (Hoshino and Yamane 1990; Shahidi and Wanasundara 1998). The DHA percentage of recovery with all enzymes

was over 90% while the recovery of EPA was approximatively 85% for Lip1 and Lip3 lipases and only 40% with Lip4.

These promising results open the door to attractive enzyme evolution perspectives. *Geotrichum candidum* lipases (GCL) consist of two sub-domains whose association creates an acyl binding tunnel (Cygler et al. 1993). Similarly to GCL, X-ray data showed that CRL, which display 40% identity with CRL, also possess an acyl binding tunnel which starts at the catalytic serine and protrudes through the entire lipase, enabling the entrapping of the ester acyl moiety (Grochulski et al. 1994). Holmquist et al. (Holmquist 1998) showed that mutations in GCL of residues located at the mouth of the acyl binding tunnel could modify the chain length specificity. Indeed, mutations of C379 and S380 in GCL izoenzyme 1 by bulky amino acids (F and Y, respectively) entailed a 4-fold decrease of the hydrolysis ratio of trioleoin to trioctanoin. The corresponding amino acids in CRL (F367-V/I368), and to a larger extend several amino acids of the acyl binding tunnel (7 positions differing in Lip1, Lip3 and Lip4 on segment D336-T393 containing the fatty acid specificity determinants in GCL) could be engineered to customize the acid chain length specificity of CRL. Such a study was initiated by Schmitt et al. who reported the alteration of chain length specificity of Lip1 variants mutated in the acyl binding tunnel (Schmitt et al. 2002).

Conclusion

For many years, the use of *C. rugosa* lipases for industrial purposes was impeded by the difficulties in obtaining pure isolated forms of the different isoenzymes secreted by the wild type strain. The purification by conventional chromatographic techniques was indeed rendered extremely difficult by the high identity between these isoenzymes. An alternative appeared with recombinant DNA technology. Nevertheless, the non-conventional use of the genetic code by *C. rugosa* makes the production of recombinant CRL in heterologous host tricky. In the present study, we describe the successful cloning of three isoenymes from *C. rugosa* and their production in *Y. lipolytica*. Lip1, Lip3 and Lip4 were obtained with yields ranging from 8 to 68 U/mL. Their substrate typospecificity was compared. Lip1 and Lip3

were shown to be specific of medium chain fatty acid esters whereas Lip4 showed a marked preference towards long chain fatty acid esters. This difference in typoselectivity was exploited for the purification of DHA and EPA, two omega-3 fatty acids with undeniable pharmaceutical properties, from pre-treated fish oil. The maximal DHA purity (~60 %) was obtained with Lip3 and Lip4, starting from an initial ethyl ester mixture containing 25% DHA, with a final percentage recovery superior to 90 %.

A single amino acid located in the substrate binding site of Candida rugosa Lipases Lip1, Lip3 and Lip4, direct their enantioselectivity towards 2-bromo phenyl acetic acid octyl esters

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Abstract

Enzyme discrimination is a very accurate mechanism where in general few amino acids, even sometimes a single amino acid, are implicated in the selectivity. The family of *Candida rugosa* Lipases are a very good tool to study this phenomenon because they present high homology (>80%) and their enantioselectivity was often demonstrate. We demonstrated that 3 Lipases from C. rugosa (Lip1, Lip3 and Lip4) present high enantioselectivity during resolution of 2-bromo-arylacetic acid esters, an important class of chemical intermediates in the pharmaceutical industry. Lip1 and Lip3 present enantioselectivity > 200 and the most interesting result is that Lip4, a very homologue Lipase, prefers the *R*-enantiomer (E-value=15). The analysis of the alignment of the 3 primary structures of the 3 Lipases suggests that positions 296 could be crucial for the discrimination. Different variants of the three enzymes at these positions were built by site-directed mutagenesis. The steric hindrance of the amino acid at position 296 appeared to be crucial. In Lip1, change of phenylalanine in a small amino acid, glycine or alanine, leads to selectivity inversion. On the contrary, in Lip4 change at this position of alanine in valine or phenylalanine, leads to reversed selectivity, whereas a glycine increases the R preference. A preliminary modelling study of the tetrahedral intermediates formed by Lip1 and Lip4 with the (*R*, *S*)-2-

bromo-phenylacetic acid octyl ester enantiomers enabled discrimination phenomenon to be better understood.

Introduction

The use of racemic mixtures in pharmaceutical drugs is strongly regulated because one enantiomer can be the active molecule whereas the other enantiomer can be toxic. Thorough investigations are consequently requested on the toxicological effects of each enantiomer before they are accepted in drug composition. The commercialisation of a sole pure active enantiomer is thus often privileged. Classical methods used to separate enantiomers, such as chemical asymmetric synthesis, stereoselective crystallization or chiral chromatography, are usually expensive. The use of enantioselective enzymes appeared as an appealing alternative to separate enantiomers. However, to obtain purities compatible with pharmaceutical legislation, biocatalysts with high enantioselectivity are required (*E*-value $\geq$200).

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) have attracted great interest in the pharmaceutical applications because of their wide range of substrate specificities, especially their ability to discriminate between enantiomers (Kim 2000). Lipases have been successfully used for the resolution of various racemic alcohols via hydrolysis, esterification or transesterification but only a limited amount of information exists on the chiral resolution of 2-halogeno-substituted acids. Most of the studies concern the resolution of 2-arylpropionic acids, an important group of non-steroidal anti-inflammatory drugs, or 2-hydroxy carboxylic acids that are also important intermediates for synthetic pathways of a number of drugs, such as prostagrandin, prostacyclin, semi-synthetic penicillin and thaiazolium salts (Guieysse *et al.*, 2001). The Lipase of *Candida rugosa* have been successfully employed for the aminolysis of esters using (*R, S*) l-phenylethylamine (Soledad de Castro and Sinisterra Gago 1998). The results showed an important interaction between aromatic ring of Phe-296 and the substrate to explain the preference of *S*-enantiomer. Manetti *et al.*, reported a tailor-made enzyme to modify the molecular recognition of 2-arylpropionic esters by recombinant Lip1 using site-derected mutagenesis of Phe 344 and Phe 345. Their results suggested that Phe345 plays an important role in the S-enantiomer

preference of ketoprofen and naproxen, which are non-steroidal anti-inflammatory drugs (Manetti et al. 2000).

In this study, the objective is to test, for resolution of a racemic mixture of 2-bromo -phenyl acetic acid octyl esters, three of the *Candida rugosa* Lipases, Lip1, Lip3 and Lip4, recently cloned in the oleaginous yeast, *Yarrowia Lipolytica*. More precisely, they were cloned in the strain JMY1212 described elsewhere (Bordes et al. 2007, Piamtongkam, 2010). This strain enables single integration of the expression cassette into the genome at a defined locus: the "zeta docking platform". It is the first expression system which enables activities of enzymes to be directly compared from the supernatant.

Results and discussion

Production of recombinant *Candida rugosa* in *Yarrowia Lipolytica*

In a previous work, the main Lipases produced by *Candida rugosa*, Lip1, Lip3 and Lip4 were cloned in the yeast *Yarrowia Lipolytica*. For this purpose, synthetic genes were used to circumvent the non-canonical codon–usage of *C. rugosa* in which the triplet CUG, coding normally for leucine, is read as serine and also to adapt triplets to *Y. Lipolytica* codon-usage. To allow a direct comparison of the supernatant activity, the *Y. Lipolytica* strain JMY1212 was used (Bordes et al. 2007, Cambon et al. 2010) It favours the integration of the expression cassette into the genome at a defined locus: the "zeta docking platform", avoiding multi-insertion or insertion at locus poorly or highly expressed. High reproducibility between expression levels of different transformants is by this way obtained and activities of different variants can be directly compared from their supernatants without purification steps. Activities follow a normal distribution with a 9.1 % coefficient of variation for the whole process (transformation, growth, protein expression and activity measurement) (Cambon et al. 2010). The strategy proposed and used in this work was to choose randomly 3 transformants, to express the proteins and to compare their activities. If activities present a coefficient of variation lower than 15%, the 3 transformants can be considered as identical and one is chosen to continue experiments. If not, 3 other transformants are tested.

Table III-4 presents *p*NPB hydrolytic activities of Lip1, 3 and 4. On this *p*NPB substrate, Lip1 is the most active enzyme, whereas Lip3 and 4 present only approximately 15% of Lip1 activity.

Table III-4. *P*-nitrophenyl butyrate hydrolysis activity of wild-type Lip1, Lip3 and Lip4 from *Y.Lipolytica*.

Enzyme	Lip1	Lip3	Lip4
Initial rate*	26.2	3.6	4.2
Coefficient of variation (%)	4.7	6.8	5.4

*µmol of *p*-nitrophenol liberated per min and mL of enzyme.
each experiment was realised in triplicates.

Racemic resolution of 2-bromo-phenylacetic acid octyl ester with wild-type Lipases

The three Lipases, Lip1, Lip2 and Lip3, were tested for their hydrolytic activity on a racemic mixture of 2-bromo-phenylacetic acid octyl ester in a biphasic medium (water/decane v/v) at 25°C (Table III-5). Even if these three Lipases are very similar presenting high homology among then (>70%) (figure III-5), surprisingly, they present difference in their enantiomer preference. Lip1 and Lip3 are totally selective versus the *S*-enantiomer (E-value>200), almost no conversion of the *R*-enantiomer being observed after 60h of reaction. On the contrary, Lip4 better recognizes the *R*-enantiomer (E-value of 15). From the point of view of activity, Lip1 is the most active enzyme. Lip3 and Lip4 present 46 and 64 % respectively of Lip1 if the most active enantiomer is considered.

The chain length of the ester function, from C2 to C8, has no influence on both enantioselectivity and activity.

Table III-5. 2-bromo-phenylacetic acid octyl ester hydrolysis activity of wild-type Lip1, Lip3 and Lip4 *Candida rugosa* Lipases.

Enzyme	Lip1	Lip3	Lip4
Preferred enantiomer	S	S	R
Enantioselectivity [a]	370	300	15
Initial rate [b] ($\mu mole.h^{-1}.L^{-1}$)	518	236	331

[a] E-value = ViS/ViR or ViR/ViS according to enantiomer preference; viR, viS: initial rates.

[b] µmol of the preferred enantiomer of 2-bromo-phenylacetic acid liberated per hour and L of enzyme.

In order to understand this difference in enantio-discrimination by the three enzymes, a special attention was turned to the amino acids which composed their active sites. The multiple sequence alignment of the 3 proteins is shown in Figure III-5. The binding pockets of *CR* Lipases are exceptional with a L shape long tunnel of nearly 25 Å and the catalytic triad at the mouth of the tunnel. The substrate binding site is formed by amino acids G124 (oxyanion hole), F125, the catalytic S209, A210 (oxyanion hole), M213, V245, P246, F or A296, S301, L302, R303, L304, L307, F345, Y361, F362, S365, F366, V409, L410, L413, G or A414, F415, F532 and V534 (Pleiss *et al.*, 1998; Akoh *et al.*, 2004). Amino acid 414 is a glycine for Lip1 and an alanine for Lip3 and Lip4 and consequently is not a good candidate to explain the difference of discrimination observed. On the contrary, amino acid at position 296 is a phenylalanine in Lip1 and Lip3, but is an alanine in Lip4.

To test the hypothesis that amino acid present at position 296 is crucial for enantiomer discrimination, the phenylalanine residues in Lip1 were changed into glycine, alanine and leucine residues, whereas alanine in Lip4 was changed into glycine, valine and phenylalanine. Lip3, which presents the same behavior than Lip1, was not retained for further experiments because it present a lower activity.

Figure III-5. Multiple sequence alignment of Lip1, Lip3 and Lip4 Lipases from *Candida rugosa* (*CRL1, CRL3* and *CRL4* respectively).

After transformation of JMY1212 *Y. Lipolytica* strain with the expression cassette, three transformants were randomly chosen. All the variants satisfied the criterion of coefficient of variation inferior to 15 %, (data not shown) and one clone was chosen and enzyme production was realized in 500 mL Erlenmeyer flasks containing 50 mL medium $Y_1T_2O_3$ was realized (Table III-6)

Table III-6. *p*Nitrophenol butyrate hydrolysis activity of wild-type *C. rugosa* Lip1 and Lip4 and their variants.

Enzyme	Lip1	Lip1-296G	Lip1-296A	Lip1-296L	Lip4	Lip4-296G	Lip4-296V	Lip4-296F
Initial rate [a]	26.2	0.8	0.4	29.2	4.2	2.1	4.4	4.9

[a] µmol of pNP liberated per minute and mL of enzyme. Each experiment was realised in triplicates.

Lip4 activity is not largely influenced by the chosen amino acid present at position 296. Only the presence of a glycine leads to a half decrease in activity. Lee et al., 2007 obtained by saturation mutagenesis targeted at position 296 of Lip4 a variant A296I presenting an activity enhancement by one order of magnitude. On the contrary, the presence at position 296 of both a glycine and a alanine in Lip1 is very detrimental to the activity (less than 5% of the corresponding wild-type activity). On the contrary, variants Lip1-F296L presents approximately the same activity than the wild type enzyme.

Racemic resolution of 2-bromo-phenylacetic acid octyl ester with variant Lipases

Our hypothesis was verified: position 296 appears to be crucial for enantiomer recognition (Table III-6). Whatever the enzyme, there is a perfect correlation between the size of the amino acid present at position 296 and enantioselectivity. A phenylalanine at position 296 leads to the higher preference for the *S* enantiomer. However, Evalue is much lower with variant Lip4296F than the one for WT Lip1 (E-value 7 and 350 respectively). The presence of a bulky atom, such as a valine or a leucine leads to the same preference, but the E-values are then lower (E-value 5 and 42 respectively).. On the contrary, a smaller amino acid, glycine or alanine leads to discrimination inversion and smaller the amino acid, higher the enantioselectivity. In Lip4, changing the alanine in the WT enzyme by a glycine leads to an increase in enantioselectivity from 15 to 40, without detrimental effect on activity.

Table III-7. 2-bromo-phenylacetic acid octyl ester hydrolysis activity of wild-type *C. rugosa* Lip1, Lip3 and Lip4 and their variants.

Enzyme	Lip1 position 296				Lip4 position 296			
	Gly	Ala	Leu	Phe	Gly	Ala	Val	Phe
Preferred enantiomer	R	R	S	S	R	R	S	S
E-value [a]	45	3	42	350	40	15	5	7
Initial rate [b] ($\mu mole.h^{-1}.L^{-1}$)	266	37	154	518	249	331	75	291

[a] E-value = ViS/ViR or ViR/ViS according to enantiomer preference; viR, viS: initial rates.

[b] µmol of the preferred enantiomer of 2-bromo-phenylacetic acid liberated per hour and L of enzyme.

In order to get some structural insight about the effect of the mutations on the enantio-discrimination by the enzyme, we analyzed models of the tetrahedral intermediates formed by Lip1 and Lip4 with the (R, S)-2-bromo-phenylacetic acid octyl ester enantiomers.

The three-dimensional model of the Lip4 Lipase was built by homology modelling techniques using as template the structure of the open Lipase from Lip1 (pdb code 1LPN, identity x%, homology Y%). The lowest energy model of Lip4 built using MODELLER was further refined using the CFF91 force field implemented within the DISCOVER module of InsightII suite of programs (Accelrys, San Diego, CA, USA).

The two enantiomers were covalently docked in the two wild-type enzymes (Figure III-6). In Lip1, the phenylalanine residue at position 296 provided a good favourable stacking interaction onto the phenyl group of both the *S* and *R*-enantiomers. This Stacking is not the explanation of the difference in discrimination of this enzyme. The Bromine atom of both enantiomers points towards the interior of the active site. The explanation of the discrimination of Lip1 lies probably in the stabilisation of the tetrahedral intermediate by the oxyanion hole. Distance between the N of glycine 124 and the O^- of the carbonyl is 2.89 A for the S enantiomer, whereas it is 3.5 A for the R enantiomer. For the second residue of the oxyanion hole, Ala210, it is 3.94 and 2.95 A respectively.

In Lip4, the positioning of the 2 enantiomers is different, the phenyl group points towards the tunnel, whereas the bromine atom is turned toward amino acid 296, and without significant difference between the 2 enantiomers. In this case, oxyanion hole residues stabilize more efficiently the *R*-enantiomer. These modelling studies are always carried on to better understand enzyme discrimination.

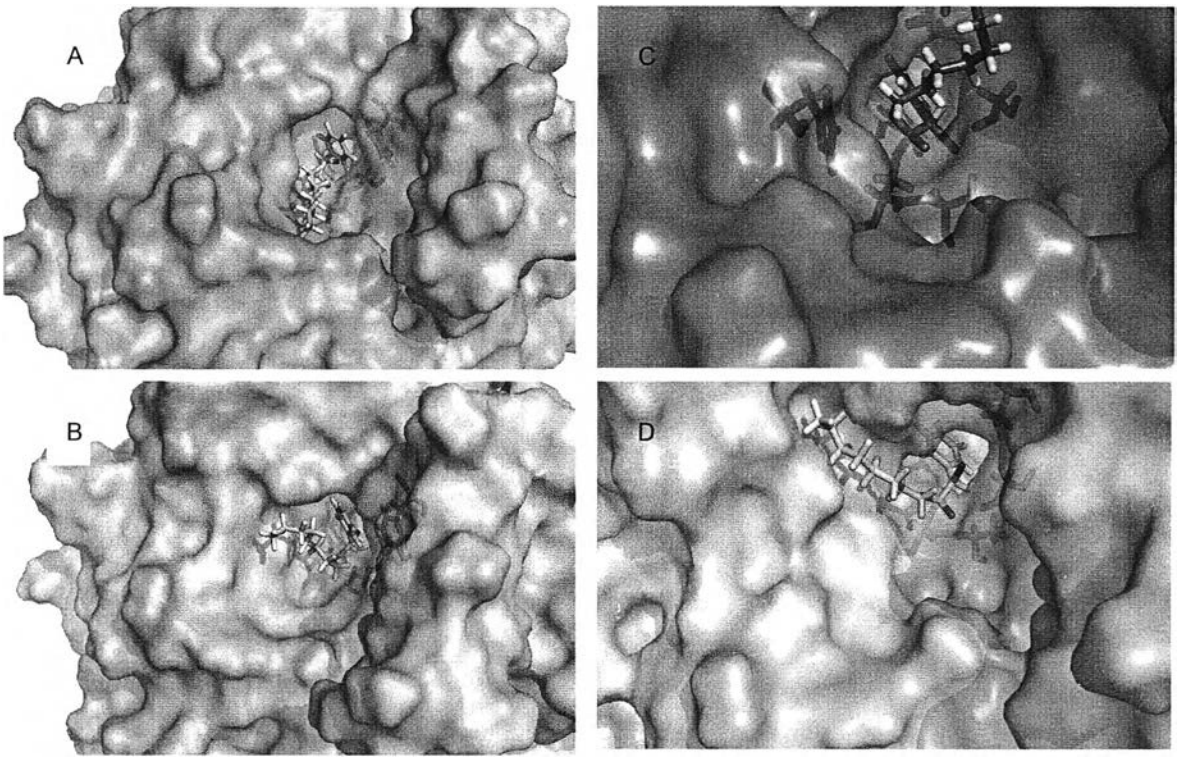

Figure III-6. Representation of the highest-scored docking modes of (R, S)-2-bromo-phenylacetic acid octyl ester enantiomers covalently bound to catalytic Ser 209 of A and B- CRL1 with *S* and *R* enantiomers respectively; C and D- CRL4 with *S* and *R* enantiomers respectively.

Biodiesel production catalysed by immobilized *Candida rugosa* lipase

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Keywords: *Candida rugosa* lipase, immobilization, hydrolysis, transesterification, biodiesel production

Abstract

Lipase is the enzyme which catalyzes the hydrolysis of oil to free fatty acids. In this research, lipase isolated from yeast, *Candida rugosa* was studied for hydrolytic and synthetic activities. The optimal conditions for the hydrolysis of palm oil catalyzed by the obtained crude lipase were determined as followings: 0.17%(w/w of oil) protein loading with 3 grams of palm oil, 100% (v/v of oil) reaction water content at 40°C for 48 hours. The maximal yield of free fatty acids was approximately 97%. When crude lipase were immobilized by adsorption on 5 types of macroporous resins namely, AB-8, H103, NKA-9, NKA and D4020 in the presence of heptane for comparison, the immobilized lipase adsorbed on nonpolar resin, H103, revealed the highest activity and the conversion to free fatty acids was 92.35%. In addition, the obtained immobilized *Candida rugosa* lipase could be reused twice and the conversion to free fatty acid were 44.57 and 2.68% respectively. For transesterification of palm oil and methanol, the results showed that immobilized lipase on NKA-9 could catalyze transesterification better than the others. The optimum conditions were as follows: weight ratio of lipase powder to resins equals to 1:1, 1 to 4 molar ratio of palm oil to methanol, 40% water content (volume/oil volume) at 30 °C for 24 hours yielding the obtained conversion to methyl ester 75.11%. Finally, immobilized lipase washed by heptane could be reused twice in

transesterification and one time in hydrolysis. The methyl esters retained were 31.88 and 9.42 percent and the free fatty acid conversion was 28.37 percent, respectively.

Introduction

Nowadays, lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) stand out as a versatile group of biocatalysts among a number of enzymes commercially available (Hasan et al. 2006). The reactions catalyzed by lipases are very important in many situations, such as in food, pharmaceutical, and chemical applications. Lipases can be used in hydrolysis of oils and fats to produce important fatty acids (FAs) as polyunsaturated FAs of omega-3 and omega-6 series. In organic media, esterification reactions are important to produce flavor esters, monoglycerides, or other compounds of clinical interest. Moreover, lipases can catalyze interesterification reactions, which can be used as a method to modify the physical properties of chemical compound. Hydrolysis of oil and fat is an important industrial operation. The product, fatty acids and glycerol are basic raw materials for a wide range of applications. Fatty acids are used as feedstock for the production of oleochemicals such as fatty alcohols, fatty amines and fatty esters. These oleochemicals are used as lubricant greases, anti-block agents, plasticizers, and emulsifiers and as ingredients in the manufacture of soaps, detergents, and animal feed. (Sulaiman et al., 2003). At present, Industrial hydrolysis of triglyceride is usually carried out with a continuous high pressure uncatalyzed counter-current splitting method. But this is an energy-consuming type process in chemical industry. The use of lipase for production of fatty acid from triacylglyceride has been attempted as one of the energy-saving methods (Szczesna Antczak et al., 2008). For example, biodiesel production by enzymatically.

Biodiesel is an alternative fuel for diesel engine, where it is produced by chemically reacting a vegetable oil or animal fat with an alcohol such as methanol and ethanol. These mixtures of fatty acids (FFA) and triglycerides (TAG) need to be chemically altered to fatty acid alkyl esters (FAAE) to be useful as biodiesel fuel for currently used diesel engines (Fjerbaek et al., 2009). However, the chemical esterification of free fatty acids requires application of the acid catalyst. Other drawbacks are the necessity of application of large amounts of alcohols, complete

removal of water and separation of glycerol by product are needed (Szczesna Antczak et al., 2008).

Enzymatic synthesis of biodiesel has been usually conducted at temperature between 20 and 60 °C. On completion of transesterification process, the glycerol phase is simply separated from the biofuel phase and neither deodorization nor neutralization of the product is necessary. This in turn decreases duration of the batch. A small excess of alcohol provides high yield of biodiesel synthesis and the biocatalyst can be used several times, particularly the immobilized lipase. Lipases have a great potential for commercial applications, especially microbial, due to their stability, selectivity and broad substrate specificity. There are a certain number of lipases produced by yeasts, most of them belonging to the *Candida* genus, that have been used for biotechnological proposes. (Cardenas et al., 2001) However, high cost of lipase makes enzymatically driven processes economically unattractive. The use of immobilized lipase is a possible solution to this problem because the enzyme can be recovered from the product and reused. The reuse of lipase provides cost advantages that are often an essential prerequisite for establishing a lipase-catalyzed process. Furthermore, easy separation of lipase from the product simplifies lipase applications and provides the basis for a reliable and efficient technology (Lee et al., 2006).

This study was to investigate optimization condition for produce free fatty acid from hydrolysis reaction of palm oil by free lipase from yeast *Candida rugosa* and immobilized lipase by physical adsorption in aqueous and non-aqueous media (heptane) onto macroporous resins for use in hydrolysis reaction.

Results and discussion

Protein determination and activity test of *Candida rugosa* lipase

The *Candida rugosa* were culture in production medium (Kamini et al., 2000) using palm oil as an inducer for lipase production. After 5 days supernatant was collected and lyophilized. Lipase activity was determined by monitoring the *p*-nitrophenol released from *p*-nitrophenyl palmitate (*p*NPP) as a substrate according to a method described by Maia et al. (2001) with slightly modifications for assay in microplate reader. The protein concentration was determined by micro lowry method

using BSA as a protein standard. The lipase specific activity and lipase production were calculated and showed in table III-8.

Table III-8. Protein content, lipase activity and specific activity of crude and lyophilized lipase from *C. rugosa.*

Lipase	**Vol. (ml)**	**Protein (mg/ml)**	**Lipase activity (U/ml)**	**Specific activity (U/mg protein)**
Crude supernatant	2500	0.36±0.069	1.43±0.161	3.97
Lyophilized lipase	250	3.68±0.185	10.12±1.33	2.83

Hydrolysis ability of *C. rugosa* lipase

The ability for produced free fatty acid from hydrolysis reaction of lipase from *C. rugosa* was tested by hydrolysis reaction in vial under magnetic stirring for 48 h, reaction was composted of 3 g of palm oil, 50 % distilled water (v/oil w) and 10.5 mg protein of free lipase. Sample were taken at time interval and analyzed by TLC (Figure III-7) and HPLC, the results show that the highest free fatty acid yield was 97.92 % after 48 h (Figure III-8.).

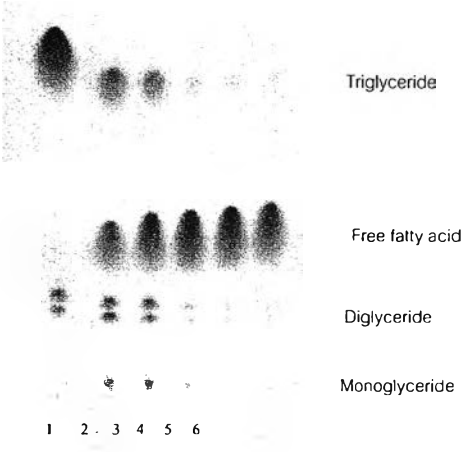

Figure III-7. Thin-layer chromatography analysis of the hydrolysis reaction mixture catalyzed by free lipase from ***C. rugosa*** lipase at various reaction time, Lane 1-6; 0, 6,12, 18, 24, 48 h, respectively.

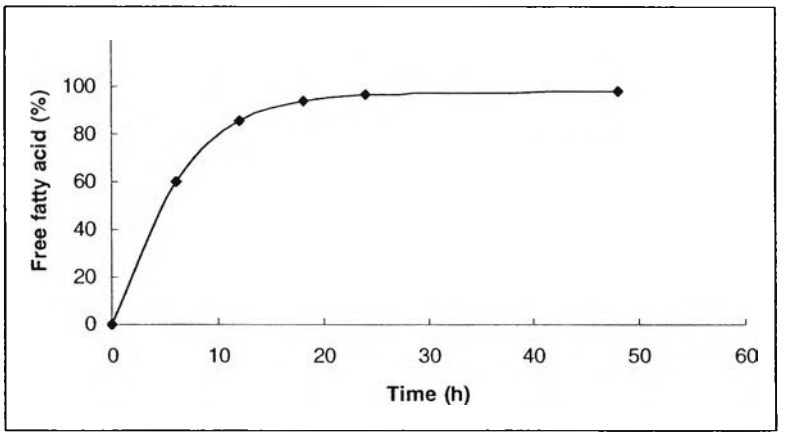

Figure III-8. Free fatty acid conversion hydrolyzed by free lipase from *C. rugosa.*

Transesterification reaction catalyzed by *C. rugosa* lipase

Transesterification reaction carried out in vial with lipase 10.5 mg. proteins, 3 g. of palm oil and methanol, 1:3 mole ratio of oil and methanol. Methanol was added 3 steps every 8 h of reaction (Shimada et al., 2002), with 600 rpm stirring at 40 °C. After 24 and 48 h, samples were taken and centrifuged, upper phase were analyzed by HPLC, the results show that the highest fatty acid methyl ester synthesis was 35.22 % after 48 h (figure III-9.).

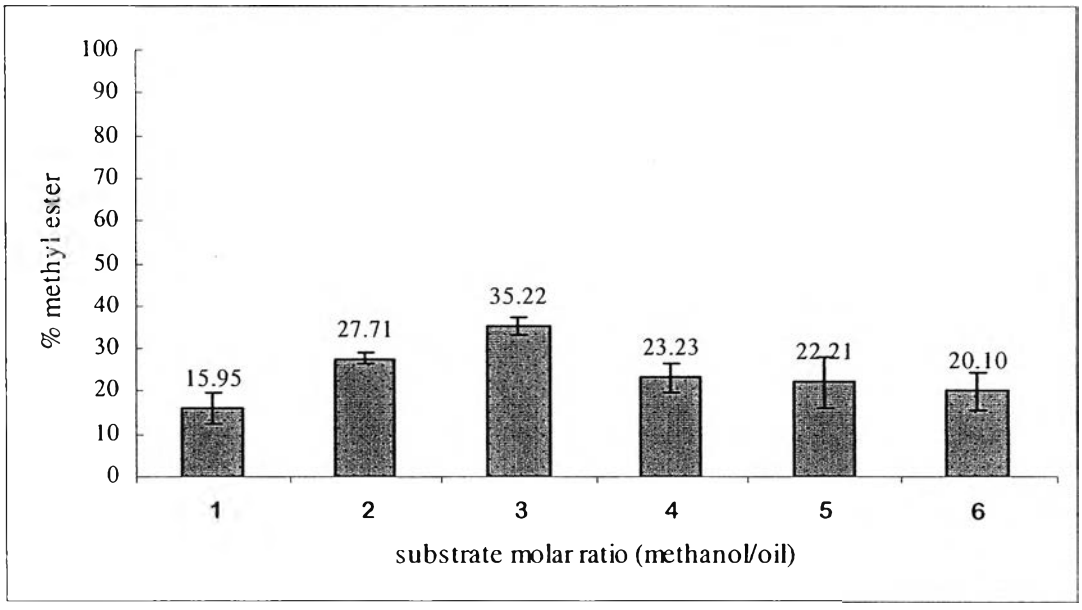

Figure III-9. Effect of ratio of methanol to palm oil in transesterification reaction catalyezed by free lipase of ***C. rugosa***, after 48 h of reaction at 40 °C.

Optimization condition for hydrolysis and transesterification reaction catalyzed by free and immobilized *C. rugosa* lipase.

Effect of amount of enzyme

Lipase was used as catalyst in hydrolysis and synthesis reaction. Thus, the reaction mixture was consisted of oil, lipase and water. Generally, lipase activity depends on the available surface area at the oil-water interface. Thus, free fatty acid production depended on the amount of enzyme. From the results at reaction time 48 h, free fatty acid production slightly increase when amount of lipase increase from 5-20 mg protein (Figure III-10) and subsequent were carried out with 5 mg protein. Results obtained from transesterification were increased and the highest conversion yield showed in figure III-11 with 50.65 % conversion to fatty acid methyl ester.

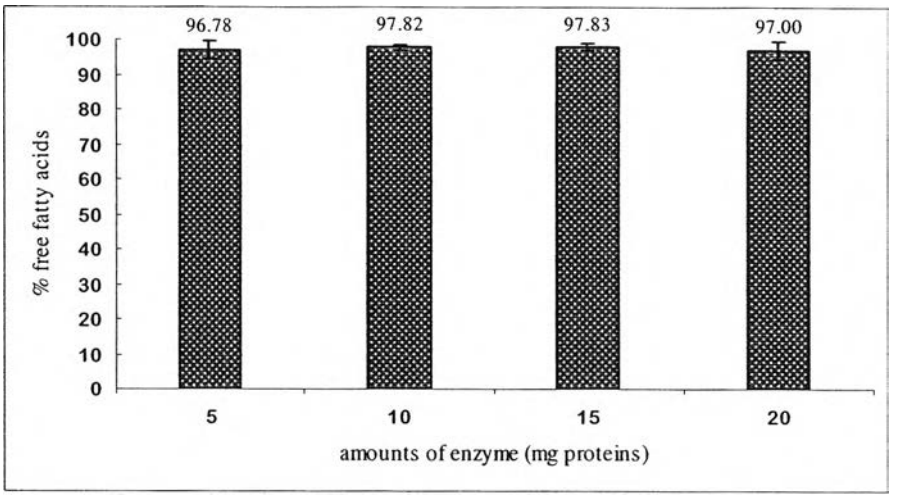

Figure III-10. Effect of amount of enzyme on hydrolysis reaction catalyzed by free lipase of ***C. rugosa*** at 40 oC and after 48 h of reaction.

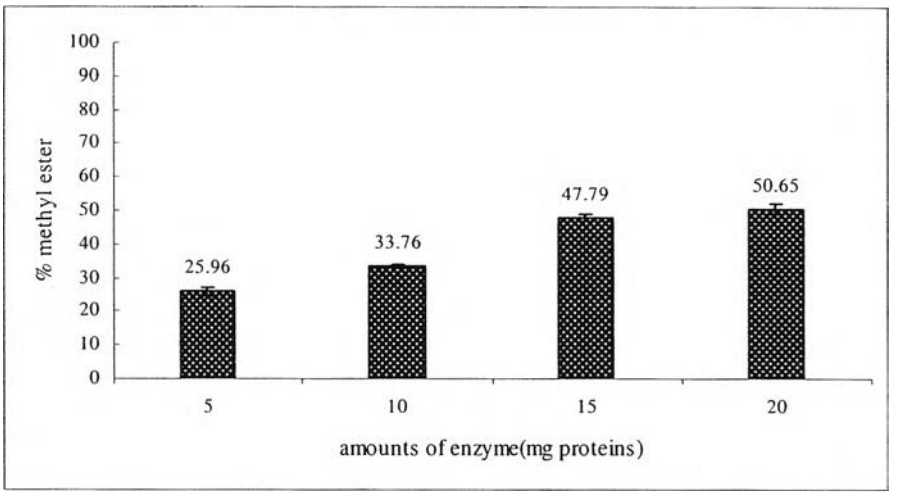

Figure III-11. Effect of amount of enzyme on transesterification reaction catalyzed by free lipase of ***C. rugosa*** at 30 oC and after 48 h of reaction.

Immobilization lipase from *Candida rugosa*

Immobilization of lipase by adsorption method in heptane (non-aqeous phase) was carried out with five support materials: AB-8, H103, NKA-9, NKA, D4020. After used in hydrolysis reaction of palm oil for produced free fatty acids, The results showed that the non-polar resin H103 was the best carrier (Figure III-12 and III-14). The highest percent free fatty acid content could be achieved in the first used was 92.6% at 48 hr. (Table III-8) This may be explained by H103 resin has high specific

area for adsorption and has suitable pore diameter, helping to interact between enzyme and substrate (Table III-9). The stability of immobilized enzyme is extremely important for any subsequent industrial applications. Immobilized lipase on H103 resin was reused 2 times, %free fatty acid from hydrolysis reaction when reused first time was 44.57% and second reused was 2.68%. Simple adsorption as a simple method and use mild condition that not make enzyme damage, then still attracts attention (Minovska et al., 2005) and adsorption in a medium of low polarity (heptane, log P = 40) lipophilic domains of lipase may interact with heptane. The effect seems to help induce conformational changes of lipase resulting in the active form. Therefore, this would allow free access of the substrate to active site of the immobilized lipase and increase activity of the lipase via 'interphase activation mechanism'. (Gao et al, 2006). On the other hand for transesterification, NKA-9 showed the best support for immobilization of *C. rugosa* lipase for biodiesel production (Figure III-13).

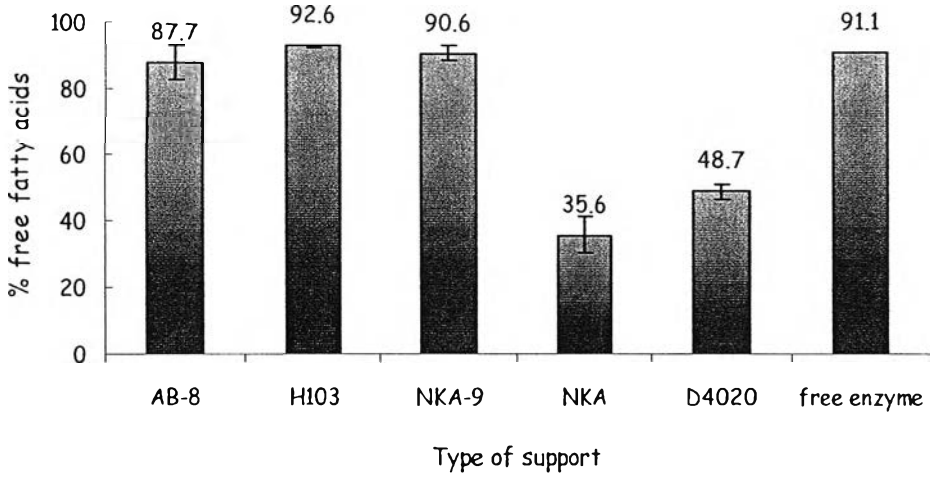

Figure III-12. Comparision of support for immobilization of lipase for hydrolysis of palm oil.

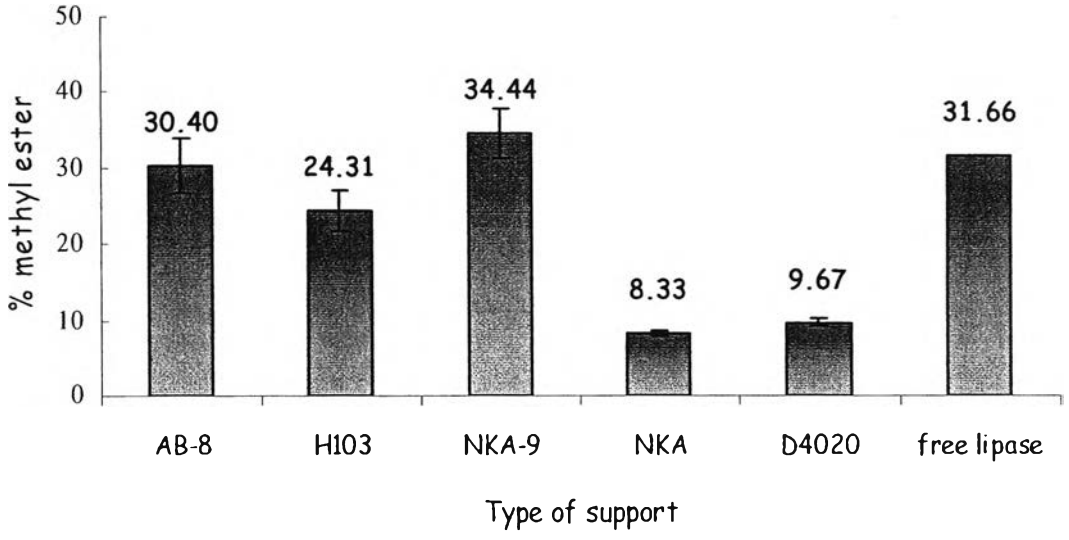

Figure III-13. Comparision of support for immobilization of lipase for transesterification of palm oil and methanol.

Table III-9. Effect of resin on immobilization of lipase.

Resin	**Polarity**	**Specific area (m^2/g)**	**Pore diameter (nm)**	**Lipase activity of immobilized lipase(U/g)**
AB-8	weakly polar	480-520	13-14	0.191
H103	nonpolar	1000-1100	8.5-9.5	0.199
NKA-9	polar	250-290	15.5-16.5	0.104
NKA	nonpolar	570-590	20-22	0.09
D4020	nonolar	540-580	10-10.5	0.145

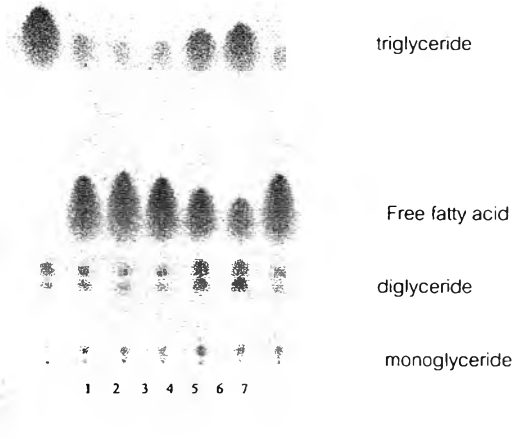

Figure III-14. Thin-layer chromatography analysis of the hydrolysis reaction mixture catalyzed by immobilized lipase from ***C. rugosa*** on different resins at reaction time 48 h; Lane 1: standard triglyceride, Lane 2-6: lipase immobilized on AB-8, H103, NKA-9, D4020 and NKA, respectively, Lane 7: free lipase.

Effect of water content

The effect of water content added on hydrolysis of palm oil is shown in Figure III-15. The percent conversion to free fatty acid was increased from 91 to 97% upon increasing the water content from 40 to 160 % (v/v), after 48 h, and there was no significant difference in free fatty acid content when water content in reaction increase from 80 to 120 % (v/v). Then in subsequent were carried out with 120 % (v/v). For transesterification of palm oil and methanol, water content added driven to production of fatty acid methyl ester from 23% to 58 % by added 40 to 160 % v/v of water. In figure III-16, result showed that added of 40 % water (v/ oil v) to transesterification of palm oil and methanol gave highest conversion to methyl ester after 48h.

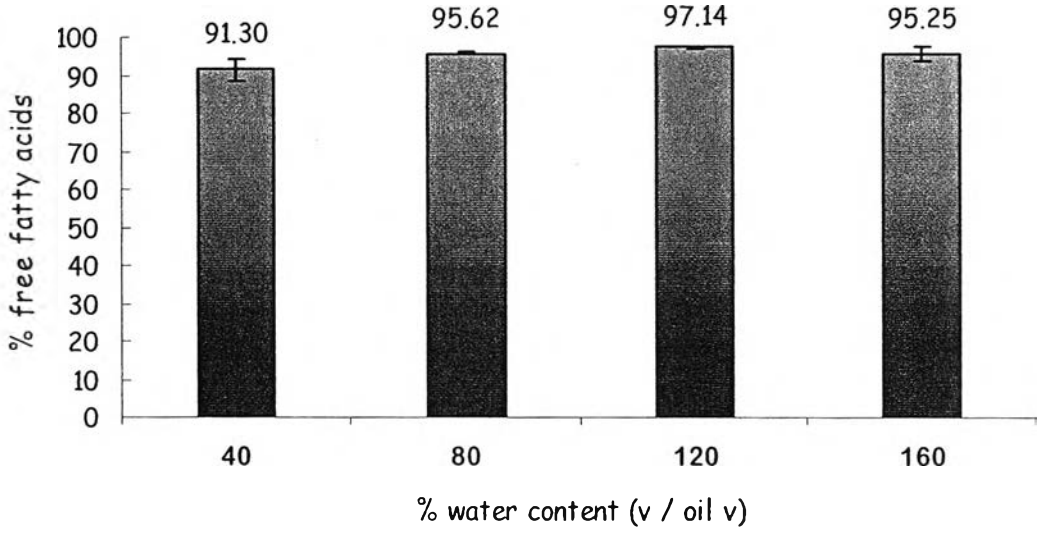

Figure III-15. Effect of water content on hydrolysis reaction catalyzed by free lipase from ***C. rugosa*** after 48 h of reaction.

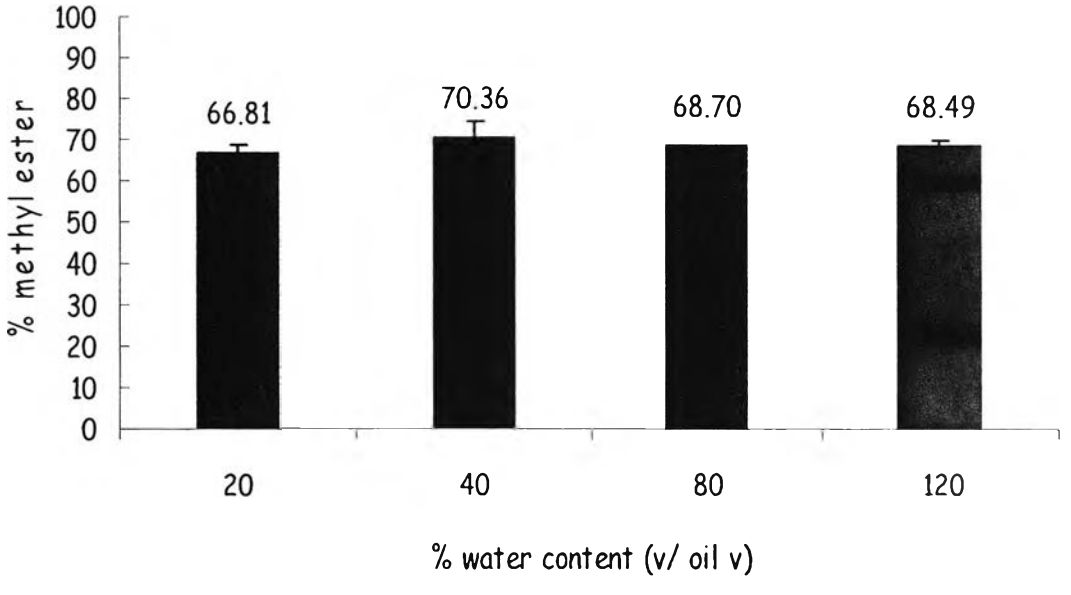

Figure III-16. Effect of water content on transesterification reaction catalyzed by free lipase from C. rugosa after 48 h of reaction.

Effect of temperature

To investigate the effect of temperature on hydrolysis and transesterification of palm oil, the reaction was conducted over the temperature range of 30-60°C. The free fatty acid content at 48 hr decreased with increasing temperature, with the optimal reaction temperature from 40-60°C.(Figure III-17) The percent free fatty acid content was highest at 40°C and 30 °C for transesterification reaction (Figure III-17).

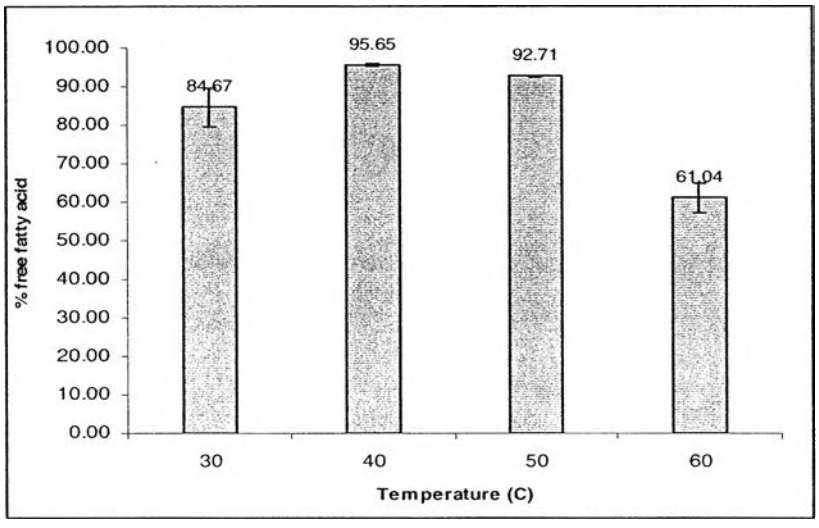

Figure III-17. Effect of temperature on hydrolysis reaction at reaction time 48 h by free lipase of *Candida rugosa*.

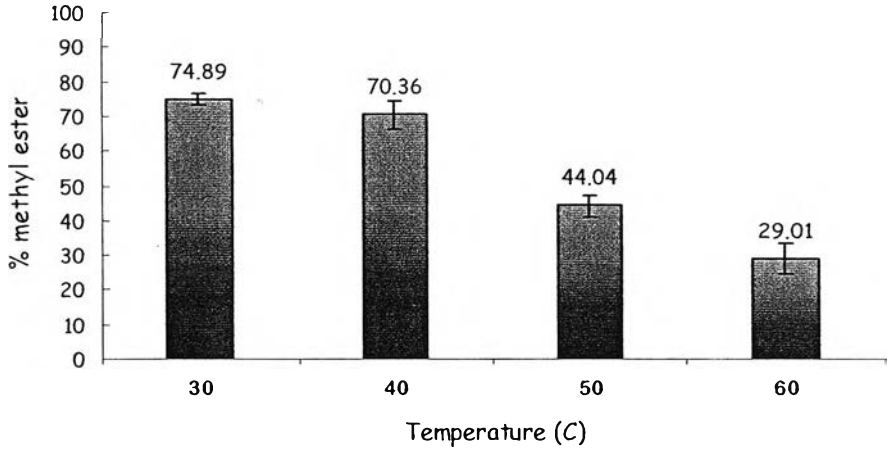

Figure III-18. Effect of temperature on transesterification reaction at reaction time 48 h by free lipase of *Candida rugosa*.

Table III-10. High performance liquid chromatography analysis of hydrolysis reaction mixture catalyzed by Immobilized lipase from C. rugosa on different resins after 48 h.

Immobilized resin	**% Free fatty acid at reaction time 48 hr**		
	1st used	**2nd used**	**3rd used**
AB-8	81.89	3.38	0
H103	92.34	44.57	2.68
NKA-9	87.75	28.65	1.46
NKA	22.1	0	0
D4020	46.09	0	0
Free enzyme	91.07	-	-

Conclusion

The present work suggests that *Candida rugosa* can produce lipase for catalyze hydrolysis reaction of palm oil to be free fatty acid and optimization condition for hydrolysis reaction by free lipase was amount of enzyme 5 mg protein, water content 150%(v/woil) and temperature 40°C at the reaction time 48 hr. The

highest percent free fatty acid is 95.65%. The macroporous adsorption resins are well desirable to immobilize lipase from *Candida rugosa*. The remarkable immobilization effect was found when the coupling procedure was performed in the presence of heptane. Additionally, lipase adsorbed onto non-polar resin H103 could effectively catalyze the free fatty acid production via hydrolysis of palm oil. The percent free fatty acid was 92.6 % at first used and can be reused 2 time, percent free fatty acid was 44.57 and 2.68%, respectively, at reaction time 48 h. In parallel, transesterification reaction for biodiesel production from immobilized *C. rugosa* lipase obtained was 75 % of methyl ester conversion of palm oil to methanol as 1 to 3 molar ratio with supplemented 40 % of water in the reaction at 30 ^{o}C after 48h.

We hope that the application of the novel biocatalyst for producing free fatty acid will expand in the future.