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INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE

ECOLE NATIONALE SUPERIEURE DES INDUSTRIES CHIMIQUES

Ecole Doctorale
Sciences et Ingénierie
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Isolation and structure elucidation of biosurfactant from microorganism and its application model in drug delivery system.

THESE

Présentée pour l'obtention du diplôme de

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CHAPTER I
INTRODUCTION

Most surfactants are synthesized by organic chemical reaction. Their structures comprise hydrophilic and hydrophobic parts which are named as amphiphatic structure. This property reduces the surface tension between 2 different phases and making various advantages in many fields such as agroindustry, cosmetic, waste treatment and pharmaceutics.[1] The disadvantages of chemical surfactants are the toxicity and difficulty in decomposition, so recently biosurfactants are more considered.

Biosurfactants are produced by microorganisms which can be isolated from environment samples, for example *P. aeruginosa* from crude oil-contaminated soil[2] or *B. subtilis* from fermented rice.[3] As biosurfactants are advantageous in structural diversity, biodegradability, less toxicity,[4] low irritancy, and compatibility with human skin,[5] they have also been used in many purposes as food additives (emulsifiers) in food industries, herbicides and pesticides in agriculture industry, including bioremediation, cosmetics and pharmaceutics.[6] For example sophorolipids, a kind of biosurfactant produced by *Torulopsis* sp. was developed for application in cosmetics and health care such as formulation in lipstick and as skin moisturizer and hair product. In pharmaceutical application, there were reports that the biosurfactants have biological activities such as antibiotic, antiviral[7] and antifungal effects.[8] MEL-A and MEL-B, the glycolipid biosurfactants produced by *Candida antarctica* showed high antimicrobial activity particularly against gram-positive bacteria, *Bacillus subtilis*.[8] In addition, the rhamnolipids showed antiphytoviral effect to viral/host combinations of tobacco mosaic virus and potato X virus, and also exhibited zoosporicidal activity of *Phythium aphanidermatum*, *Phytophtora capsici* and *Plasmopara lactucaeradicis*.[9] Moreover, Thanomsub and colleague, 2006 reported anticancer activity of rhamnolipids against human breast cancer cell line.[10]

Cancer is an important disease which encounter with difficulty in treatment due to the problem of drug efficacy and side effect to the normal cell. Therefore, the drug delivery system is the advantage to use for cancer treatment by enhancing the specificity to the cancer cells and prolong drug half life.

The amphipathic structure of biosurfactant renders the self assembly and development of nanoparticles. Therefore nanoparticles can be used in drug delivery system as a vehicle to encapsulate drugs, polypeptides, proteins, vaccines, nucleic acids, genes and others which aim to deliver the substance to the target site.[11] Glycolipid biosurfactant (MEL) was reported to apply in various kinds of drug- and gene- delivery systems by coupling with other carrier materials like phospholipids and polymers.[8] Nowadays the biosurfactant were studied extensively because of the structure diversity and less toxicity, comparing with the chemical surfactants.

So far, liposome is a main encapsulated drug carrier.[12-14] Surfactants or non-ionic surfactants named as niosome have also been used to develop as carriers.[8] Nevertheless, very few of biosurfactant have been used as carrier. A report on mannosylerythritol lipids A (MEL-A), a biosurfactant produced by *Candida antarctica* was used as plasmid DNA carrier, namely MEL-liposome. MEL-liposome (MEL-L) comprised 3β-[N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), dioleoyl phosphatidylethanolamine (DOPE) and mannosylerythritol lipids A (MEL-A). The MEL-L/plasmid DNA complex (MEL-lipoplex) was investigated in the efficiency of transfection in human cervix carcinoma Hela cells. The result showed that MEL-A induced a significantly higher level of gene expression.[15]

In conclusion, not only the property of biosurfactant as amphiphilic structure to encapsulate the drug in drug delivery system application but also their biological activity make them very interesting to be applied in pharmaceutics. The purpose of this study is the isolation of biosurfactant from microorganism, identification of its chemical structure, and the application to use it in drug delivery system.

Aims of thesis

The purposes of this study are as follows.

1. To isolate the microorganisms capable in biosurfactant production from oil contaminated soils collected from Songkhla province, the southern part of Thailand.
2. The biosurfactant producer strain which produced good biosurfactant, i.e. showing high surfactant activity, emulsion activity and emulsion stability was selected and optimized the cultivation condition in order to increase the yield of the production.
3. The biosurfactant producer strain was cultivated in large scale with the optimized condition. The culture broth were extracted and purified according to the surfactant activity.
4. The chemical structure of the pure biosurfactant compound was elucidated.
5. The chemical structure of the biosurfactant isolated was used as a model to synthesize the related structure compound which showed similar physico-chemical property by chemical process.
6. This synthesized product which showed biological activity was used as a drug to be encapsulated in nanoparticle by various processes to determine the best condition for nanoparticle preparing method.

CHAPTER II

REVIEW LITERATURE

1. Surfactants

Surfactants are produced by organic chemical reaction. They consist of hydrophilic and hydrophobic (generally hydrocarbon) moieties which play the role as amphiphatic molecules. They can act at the interface between oil/water or air/water with different degrees of polarity and hydrogen bonding and reduce surface and interfacial tension. Their characteristics of emulsifying, foaming, and dispersing traits make them very useful in many fields.[16-17]

2. Biosurfactants

Biosurfactants are produced by biological processes from microorganisms such as bacteria, fungi and yeast. Their properties are the same as those of surfactants but structure more diverse, biodegradable, less toxicity,[4] low irritancy, and compatibility with human skin.[5]

2.1 Types of biosurfactant

The biosurfactants are classified according to their structures (Table 1) into 5 groups[6] as follows.

2.1.1 Glycolipids

The common structure of this biosurfactant type is a saccharide polar headgroup in combination with hydrocarbon tail (fatty acid). The saccharide can be mono-, di-, tri- or tetrasaccharides of the same microorganism.[18,19]

The best known of glycolipids are rhamnolipids, sophorolipids, trehalolipids and mannosylerythritol lipids.

2.1.1.1 Rhamnolipids

Rhamnolipids consist of rhamnose and 3-hydroxy fatty acids[9,20] (Figure 1). The rhamnolipids were produced by bacteria *Pseudomonas* sp.[21] such as *P. aeruginosa* and *P. putida.*[22] The structure of rhamnolipids produced by *P. fluorescens* is disaccharide of methyl pentose and lipids which are formed by condensing two moles of rhamnose sugar and an acetyl group links to the hydrophobic group. However, the lipid part of the molecule contains ester and carboxyl groups.[6]

2.1.1.2 Sophorolipids

Sophorolipids (SLs) are composed of dimeric sugar (sophorose) and a hydroxyl fatty acid, linked by a β-glycosidic bond,[23] (Figure 1). They are produced by *Torulopsis* sp. or *Candida* sp., such as *T. bombicola*,[24] *T. petrophilum*,[25] *T. apicola*[26] and *Candida bogoriensis*.[27] *Wickerhamiella domercqiae* was also reported to produce sophorolipids.[28]

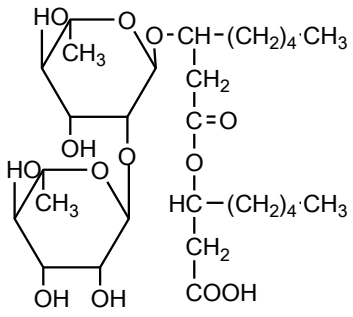
There are two types of SLs namely, the acidic (non-lactonic) SLs and the lactonic SLs. The hydroxyl fatty acid moiety of the acidic SLs has a free carboxylic acid functional group whilst that of the lactonic SLs forms a macrocyclic lactone ring with the 4″ –hydroxyl group of the sophorose by intramolecular esterification.[29]

2.1.1.3 Trehalolipids

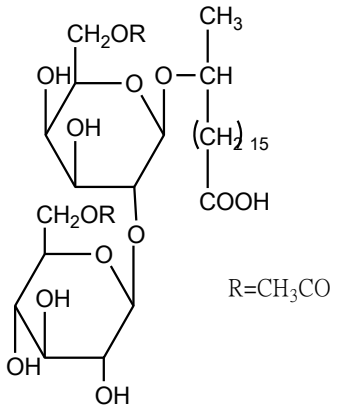
Trehalolipids are composed of disaccharide trehalose linked at C-6 and C6' to mycolic acids which are long-chain, α-branched-β-hydroxy fatty acids (Figure 1). Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms and the degree of unsaturation.[7] The microorganisms which were reported to produce this group of biosurfactants are *Mycobacterium*, *Norcardia* and *Corynebacterium*.[6] Trehalose lipids produced from *Rhodococcus erythropolis* and *Arthrobacter* sp. were also reported.[30]

2.1.1.4 Mannosylerythritol lipids

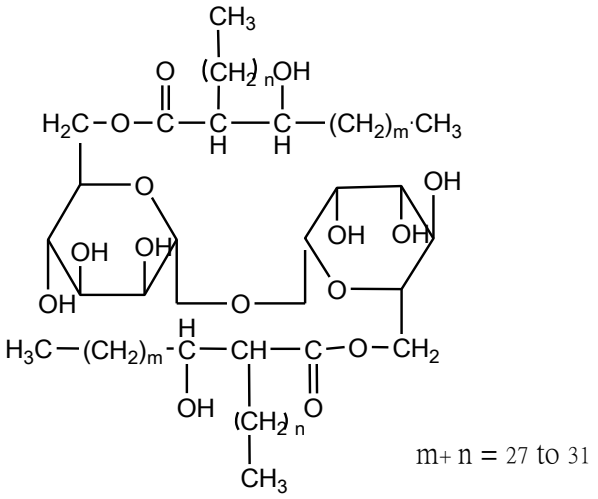
Mannosylerythritol lipids (MELs) consist of 4-O-(mono or di-O-acetyl-di-O-alkanoyl-D-mannopyranosyl)-erythritol. The acyl residues were C8, C10, C12 and C14 fatty acid[31] as the structure shown in Figure 2. They were classified by structural composition into three types; MEL-A MAL-B and MEL-C (Figure 1). MELs were produced by the yeast strains belonging to the genus *Pseudozyma*.[32,8] They showed excellent surface-active.[33] Mannosylerythritol lipids were also reported to be produced by *Pseudozyma siamensis*.[34]



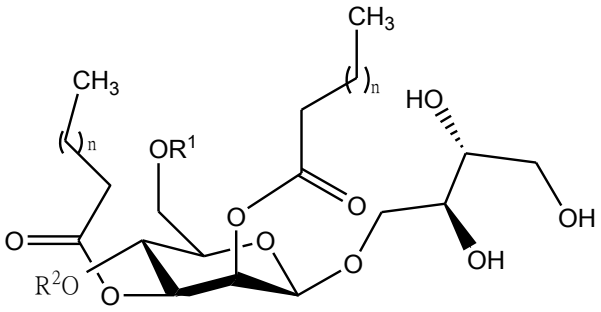
Rhamnolipid



Sophorolipid



Trehalolipid



Mel-A: $R^1 = R^2 = Ac$

Mel-B: $R^1 = Ac$, $R^2 = H$ (n=5-9)

Mel-C: $R^1 = H$, $R^2 = Ac$

Mannosylerythritol lipid

Figure 1 Structure of rhamnolipid, trehalolipid, sophorolipid[7] and mannosylerythritol lipid[32]

2.1.2 Lipopeptides

The common structure of lipopeptides is amino acids linked with long hydrocarbon chain of fatty acid. There are many kinds of lipopeptide produced by microorganisms. Kakinuma and colleague in 1969 isolated surfactin produced by *Bacillus* sp. which contained seven amino acids bonded to a carboxyl and hydroxyl groups of a 14-carbon fatty acid[35] (Figure 2).

Alcaligenes sp. is another microorganism which could produce lipopeptide comprising amino acids liked to aliphatic chain with ester group.[36]

B. subtilis BBK-1 which simultaneously produced three kinds of surface-active compounds: bacillomycin L, plipastatin, and surfactin was isolated and characterized.[37]

In 2008, Lee and colleagues showed that *Klebsiella* sp. produced some kinds of lipopeptide biosurfactant.[38]

Fengycin[39] is a biologically active lipopeptide produced by several *B. subtilis* strains.[40,41] The structure is composed of a β-hydroxy fatty acid linked to peptide part comprising 10 amino acids, where 8 of them are organized in a cyclic structure.

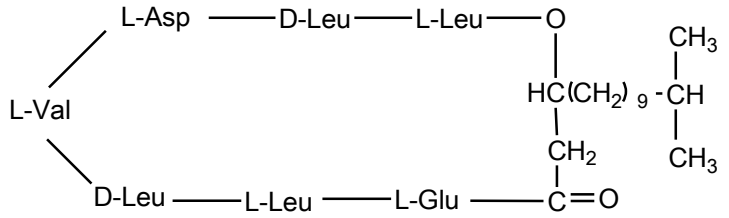


Figure 2 Structure of cyclic lipopeptide surfactin produced by *Bacillus subtilis*[7]

2.1.3 Fatty acids

Their compositions are carboxyl group connecting with long chain hydrocarbon. The examples of this product are corynomycolic acids, spiculisporic acids, etc. The microorganisms which produced this biosurfactant are *Capnocytophaga* sp.*, Corynebacterium lepus, Arthrobacter paraffineus, Talaramyces trachyspermus* and *Nocardia erythropolis*.[42] *Penicillium spiculisporum* was shown by Ban in 1998 on the production of spiculisporic acid aerobically.[43] Rehn and Reiff, 1981 reported that fatty acids produced from alkanes as a result of microbial oxidations have been considered as surfactants by interfacial tension measurement. In addition to the straight-chain acids, micro-organisms produce complex fatty acid containing -OH groups and alkyl branches.[44]

2.1.4 Phospholipids

The quantitive production of phospholipids has also been detected in some *Aspergillus* sp.[45] and *Thiobacillus thiooxidans*[46]. For instance, phospholipids (mainly phosphatidylethanolamine) rich vesicles were produced by *Acinetobacter* sp. grown on hexadecane.[47] Phosphatidylethanolamine also produced by *Rhodococcus erythropolos* grown on n-alkane[6] as the structure shown in Figure 3.

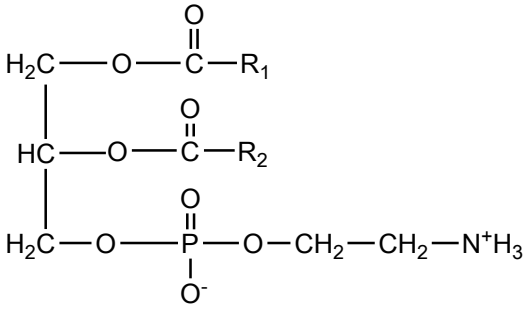


Figure 3 Structure of phosphatidylethanolamine, a potent biosurfactant produced by *Acinetobacter* sp. R1 and R2 are hydrocarbon chains of fatty acids[7]

2.1.5 Polymeric biosurfactants

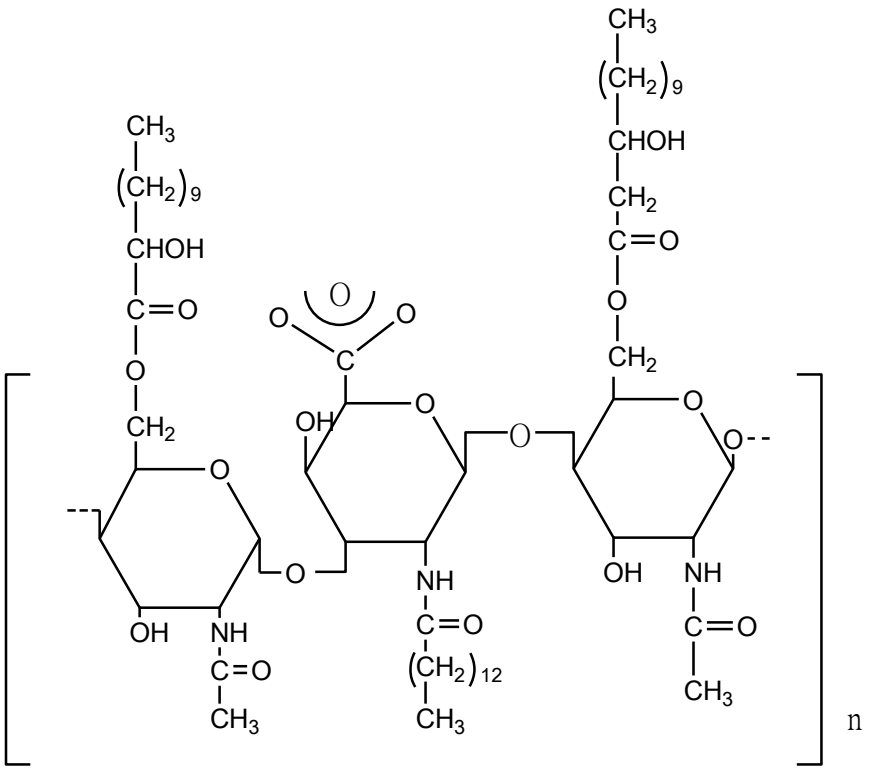
This biosurfactant can be carbohydrate and/or protein based linked to lipids. These polymers have high molecular weight, ranging from 50,000 to greater than 1,000,000. Emulsan, liposan, mannoprotein, polysaccharide-protein complexes, biodispersan, alasan, food emulsifiers, protein complexes and insectides emulsifiers belong to the polymeric biosurfactant group. Rosenberg and colleagues isolated a potent polyanionic amphiphatic heteropolysaccharide bioemulsifier called emulsan from *Acinetobacter calcoaceticus* RAG-1.[48] The heteropolysaccharide backbone contains a repeating trisaccharide of *N*-acetyl-D-galactosamine, *N*-acetylgalactosamine uronic acid, and an unidentified *N*-acetyl amino sugar. Fatty acids are covalently linked to the polysaccharide through *o*-ester and amide linkages[7] as the structure shown in Figure 4.

Acinetobacter calcoaceticus A2 could also produce biodispersan extracellulary. It is a repeating anionic heteropolysaccharide containing four reducing sugars, namely 6-methylaminohexose, glucosamine, galactosamine uronic acid, and an unidentified amino sugar.[7,6]

Navonvenezia et al., 1995 described the isolation of alasan, an anionic alanine-containing heteropolysaccharide-protein biosurfactant from *Acinetobacter radioresistens* KA-53.[49,7,6]

Candida lipolytica produced liposan; an extracellular water-soluble emulsifier. It is composed of 83% carbohydrate consisting of glucose, galactose, galactosamine, and galacturonic acid and 17% protein.[7,6]

Saccharomyces cerevisiae was reported in producing large amounts of mannoprotein and showed excellent emulsifier activity toward several oils, alkanes, and organic solvents.[50]



Emulsan

Figure 4 Structure of emulsan[7]

The compositions of various biosurfactants are summarized in table1.

Table 1 The chemical structures of 5 biosurfactants

Biosurfactant	Hydophilic part	Hydrophobic part
1.Glycolipids	Saccharide	long hydrocarbon chain of fatty acid
Rhamnolipids	Rhamnose	long hydrocarbon chain of 3-hydroxy fatty acid
Sophorolipids	Sophorose	long hydrocarbon chain of hydroxyl fatty acid
Trehalolipid	Trehalose	long hydrocarbon chain of mycolic acid
Mannosylerythritol lipid (MELs)	4-O-(mono or di-O-acetyl-diO-alkanoyl-D-mannopyranosyl)-erythritol	long hydrocarbon chain of fatty acid
2.Lipopeptide	Amino acids	long hydrocarbon chain of fatty acid
3.Fatty acid	carboxyl group	long hydrocarbon chain
4.Phospholipids	Phosphate group	long hydrocarbon chain of fatty acid
5.Polymeric biosurfactant	repeating saccharide and/or repeating protein	long hydrocarbon chain of fatty acid

In another aspect, biosurfactants are also classified according to their molecular masses into 2 groups[38]; low molecular weight and high molecular weight as summarized in table 2.

Table 2 Biosurfactants produced by the microorganisms (Table is adapted from Jonathan et al, 2006) [51]

Biosurfactant	Microorganisms
Low molecular weight	
-Rhamnolipids	*Pseudomonas aeruginosa, Serratia rubidea, Pseudomonas putida Pserdomonas fluorescens*
	Pseudomonas aeruginosa
-(Hydroxyalkanoyloxy)alkanoic	
acids (HAAs) Rhamnolipid precursor	*Arthrobacter paraffineus, Rhodococcus erythropolis,*
-Trehalose lipids	*Mycobacterium, Norcardia, Corynebacterium*
	Candida lipolytica, Torulopsis bombicola
-Sophorose lipids	*Ustilago maydis*
-Cellobiose lipids	*Pseudomonas fluorescens*
-Viscosin	*Bacillus subtilis, Bacillus pumilus*

-Surfactin	*Bacillus polymyxa*
-Polymixins	*Bacillus brevis*
-Gramicidin S	*Acinetobacter, Thiobacillus thiooxidans, Rhodococcus erythropolos*
-Phospholipids	*Flavobacterium sp.*
-Flavolipids	*Bacillus subtilis (Iturin A), Bacillis pumilus, Bacillus licheniformis, Pseudomonas syringae,*
-Lipopeptides	*Pseudomonas fluorescens*
	Gluconobacter cerinus, Thiobacillus thiooxidans, Streptomyces tendae
-Ornithin, lysine peptides	*Rhodotorula glutinis, Rhodotorula graminis*
-Polyol lipids	*Serretia marcescens*
-Serrawettin	*Nocardia erythropolis, Arthrobacter parafineus, Corynebacterium lepus, Penicillium*
-Fatty acids	*spiculisporum, Talaromyces trachyspermus*
	Capnocytophaga, Corynebacterium
-Sulfonolipids	*Rhizobium trifolii*
-Diglycosyl diglycerides	
High molecular weight	*Acinetobacter radioresistens*
-Alasan	*Acinetobacter calcoaceticus*
-Emulsan	*Acinetobacter calcoaceticus*
-Biodispersan	*Candida lipolytica*
-Liposan	*Candida utilis*
-Food emulsifier (bioemulsifiers utilisan)	*Pseudomonas tralucida*
-Insecticide emulsifier	
(production by alpha	
Hexachlorocyclohexane (HCH) utilizing	
P. tralucida)	*Halomonas eurihalina*
-Sulfated polysaccharide	*Sphingomonas paucimobilis*
-Acetyl heteropolysaccharide	
N-acetyl and O-pyruvil	*Pseudomonas fluorescens*
-Heteropolysaccharide	

3. Monoacylglycerol

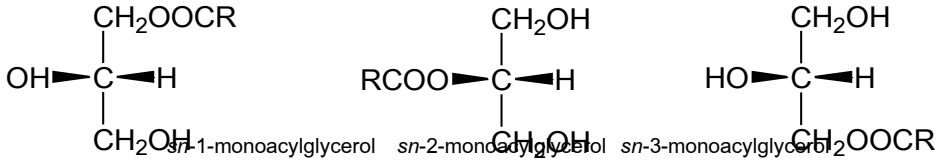
Monoacylglycerols (MAGs) (or monoglyceride) are mono- fatty acid esterified with only one of the hydroxyl group glycerol, and they can exist in three stereochemical forms (Figure 5). The terms of 1- and 3-isomers are alike, as α-monoacylglycerols, because they are not distinguished from each other, while the 2-isomers are β-monoacylglycerols.

1- and 3-Monoacylglycerols tend to be minor components only of most plant and animal tissues. They show strong surfactant properties as detergent which would not be expected to accumulate and would have a disruptive effect on membranes. In commercial, this monoacylglycerol are synthesized and used as constituents of detergent which are recently important..[52]

2-Monoacylglycerols are usually as a major end product of the intestinal digestion of dietary fats in animals by pancreatic lipase enzyme. Before being transported in lymph to the liver, they can be taken up directly by the intestinal cells and converted to triacylglycerols by monoacylglycerol pathway.[52]

Monoacylglycerols (MAGs) are amphiphilic molecules which exhibit the surfactant activity and belong to glycolipid biosurfactants. In 2004, Thanomsub and colleagues isolated *Candida ishiwadae*, a thermotolerant yeast from plant material in Thailand. This microorganism produced biosurfactants which exhibited surfactant activity higher than chemical surfactants such as SDS and Tween 80. Their

structures were elucidated as olelylglycerol and linoleylglycerol and classified as monoacylglycerols.[53] As a biosurfactant, MAGs is potentially applicable in drug delivery system by its low toxicity and biodegradability properties.



sn-1-monoacylglycerol *sn*-2-monoacylglycerol *sn*-3-monoacylglycerol

Figure 5 Structure of monoacylglycerol[52] (R = hydrocarbon chains of fatty acids)

3.1 Biological activity of monoacylglycerol

Monoacylglycerols (MAGs) are used in food and pharmaceutical industries. In addition to emulsifying properties, they exibit inhibitory effects against some types of microorganisms. This property makes them able to be used in various fields of food industry.[54]

Kabara and colleague, 1977 reported that the number of carbon atom and double bond in their hydrocarbon chains presenting on MAGs, are somewhat potent to the antimicrobial activity of them.[55]

In many research works, it was proved that monoacylglycerols have a broad spectrum of microbicidal activity. They inhibited various bacteria such as group A *Streptococci* (GAS), group B *Streptococci* (GBS),[56] *Staphylococcus aureus*,[57] *Neisseria gonorrhoeae*, *Clamydia trachomatis* and *Helicobacter pylori*.[58] In addition, monoacylglycerols were reported to inhibit growth of yeast, *Candida albicans*,[59] and herpes simplex virus (HSV).[60,61] The antimicrobial effects of these monoacylglycerols are summarized in table 3.

Table 3 The antimicrobial effects of monoacylglycerols

Monoacylglycerol	Structure	Inhibited microbes
Monocaprin (C10:0)	Capric acid esterified with glycerol	- group A *Streptococci* (GAS), group B *Streptococci* (GBS), *Candida albicans*[56,59] -*Neisseria gonorrhoeae*[62], *Clamydia trachomatis*[63] -*Helicobacter pylori*[64] -Herpes simplex virus (HSV), vesicular stomatitis virus[60], human immunodeficiency virus (HIV)[61]
Monolaurin (C12:0)	Monolauric acid esterified with glycerol	-*Staphylococcus aureus*[57] - *Neisseria gonorrhoeae*[62] -*Clamydia trachomatis*[63] -*Helicobacter pylori*[64] -*Candida albicans*[59]
Monomyristin	Myristic acid	-*Helicobacter pylori*[58,63]

(C14:0)	esterified with glycerol	

In addition to antimicrobial activity, monoacylglycerols have been reported on antileukemic property by showing ability to induce apoptosis (programmed cell death) of murine leukemic cell.[65] The unsaturated MAGs, 1-C16:1, 1-C18:1, and 1-C18:2 MAG were the best inducers of murine leukemic cell death. However, some saturated MAGs such as 1-C14:0 MAG and 1-C16:0 MAG were also able to induce cell death which the others (1-C10:0 MAG, 1-C12:0 MAG, 1-C18:0, and 1-C8:0 MAG) were inactive.[65]

In 2003, Philippoussis and colleagues found that monoacylglycerols had cytotoxicity effect against malignant but not normal human cells. 1-monooleoylglycerol (1-C18:1), 1-monopalmitoleyl glycerol (1-C16:1) and 3-monopalmitoyl glycerol (3-C16:0) were able to induce cell death in human leukemic cell lines by which 1-monooleoylglycerol is the most effective.[66]

In 2008, Barta et al. studied the effect of monoacylglycerol on activity and expression of P-glycoprotein (P-gp) in Caco-2 cells, a human colon adenocarcinoma cell line. The results from this study suggested that non-cytotoxic concentrations of 1-monoolein and 1-monostearin are inhibitors of P-gp in Caco-2 cells, a human colon adenocarcinoma cell line.[67]

4. Isolation and screening of the biosurfactant producing microorganisms

Microorganisms capable in producing biosurfactants were isolated from various types of samples such as marine sediments with a history of hydrocarbon exposure, sludge oil and petroleum-contaminated soil.[68-69] Most of them were isolated from oil contaminated soil.[70]

To isolate microorganisms producing biosurfactants, various kinds of media and cultivation methods were used. The isolated microorganisms were then screened for surfactant activity of by various methods. In 2009, Nayak and colleague reported the isolation of *Pseudoxanthomonas* by using PMS medium (EDTA, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, NH_4Cl, K_2HPO4, KH_2PO_4, sodium pyruvate, trace element solution, biotin, vitamin B_{12}) supplemented with 50mg of sodium salicylate cultivated at 37°C for 5 days on rotary shaker at 180 rpm.[71]

To produce lipopeptide biosurfactant from *Alcaligenes* sp., Huang and colleagues used MMSW medium (NH_4NO_3, K_2HPO4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and trace mineral solution: $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$ and EDTA) supplemented with 4% (v/v) liquid paraffin. The strain was cultivated in shaker incubator at 35°C and 140 rpm for seven days, then the culture was transferred to fresh screening medium for seven days, repeated for cultivation three times.[39] The culture at the end of the third cultivation was spread on agar plate medium containing 5.0 g/L beef extract, 10.0 g/L peptone, 5.0 g/L NaCl and 15g/L agar powder (pH 7.0) and incubated at 35°C. The individual colony was picked up from the plate after cultivation and streaked on a fresh agar plate and incubated. This process was repeated to obtain the pure biosurfactant producing strain.

4.1 Screening of biosurfactant producing microorganisms

The microbial surfactant research is a rapidly developing field. The method for screening biosurfactant producing microorganisms is also increased in attention. In general, the screening methods for biosurfactant producer depend on the purpose in using of the biosurfactant. The methods used to screen the biosurfactant producers are as follows.

4.1.1 Axisymmetric drop shape analysis (ADSA)

Axisymmetric drop shape analysis (ADSA) is the method to asses the potential biosurfactant-producing bacteria. Drops of culture broth are placed on a fluoroethylene-propylene surface and the profile of the droplet is evaluated by a contour monitor. The biosurfactant-producing bacteria suspensions display reduction in surface tension which is calculated from the droplet profiles by ADSA.[72]

4.1.2 Colorimetric test

Colorimetric test is the estimation of biosurfactant production described by Shulga and colleague, 1993.[73] This technique is specific for anionic surfactant and based on the ability of the anionic surfactants to react with the cationic indicator to form a chloroform-extractable colored ion-association complex. The intensity of the extracted color complex is measured spectrophotometrically. This method was used for rapid screening of rhamnolipid-producing microorganisms[74] and also hydrocarbon-degrading microorganisms.[75]

4.1.3 Drop-collapsing test

Drop-collapsing test was described by Jain and colleague, 1991.[76] A microbial cell suspension is dropped on an oil-coated surface. The drop of suspension which contains biosurfactant will collapse but the drop containing no or less surfactant activity remains stable.

4.1.4 Estimation of emulsification index value (*E-24*)

Estimation of emulsification index value (*E-24*) is the method used for detecting emulsifying property of biosurfactant described by Cooper and Goldenberg, 1987.[25] This method is suitable for the research purposed to use the emulsion properties of biosurfactant for application. The technique is done by mixing the culture broth sample with an equal volume of kerosene and measuring the percent emulsification after 24 h.

4.2 Surfactant activity determination

The methods used to determine the surfactant activity of the biosurfactant are surface and interfacial tensions tests, measurement of stabilization and destabilization of emulsions, including evaluation of hydrophobic-lipophilic balance (HLB).

4.2.1 Surface and interfacial tensions

Surface and interfacial tensions is used for measuring the property of the amphiphilic molecules, like surfactants. The amphiphillic molecules associate readily to form the structure as micelles, bilayers, and vesicles. The tensiometer is the instrument used to measure the surface tension at the air/water and oil/water interfaces. The surface tension of distilled water value is 72 mN/m at

room temperature. With surfactant, this value is lowered down to 30 mN/m. In addition, a reduction of surface tension is observed up to a critical level when a surfactant is added to air/water or oil/water systems at increasing concentrations. At low concentrations surfactants will favor arrangement on the surface and at some concentrations the surface become completely loaded with surfactant and any further additions must arrange as micelles. This concentration is known as the critical micelle concentration (CMC). The biosurfactant will be diluted and measured their surface tension and the CMC is calculated by plotting a graph of surface tension versus logarithm of concentration of surfactant. The CMC is found as the point at which two lines intersect; the baseline of minimal surface tension and the slope where surface tension shows linear decline (in a semi-logarithmic plot).

4.2.2 Stabilization of emulsions

An emulsion is the formation of the liquid phase which is dispersed as microscopic droplets in another liquid continuous phase. The emulsification activity is defined by ability of biosurfactant to generate turbidity due to suspended hydrocarbons such as kerosene[25] and a hexadecane-2-methylnaphthalene mixture.[77,48] In 1997 Patel and Desai described this method by adding 3 ml of hydrocarbon or oil to 2 ml of testing sample and vortexing at high speed for 2 min.[78] After 24 h, emulsification activity was calculated as the (height of emulsion layer/total height) x 100. The stability of the emulsion was evaluated after 48 h.

4.2.3 Hydrophobic-lipophilic balance (HLB)

This is the value indicating that a biosurfactant promotes water-in-oil or oil-in-water emulsion.[7] In 1957, Davies suggested a method calculating a HLB value based on the chemical groups composition of the molecule, strongly and less strongly hydrophilic groups.[79] The calculation of HLB value uses the formula:

$$HLB = 7 + m * Hh - n*Hl$$

m - Number of hydrophilic groups in the molecule
Hh - Value of the hydrophilic groups
n - Number of lipophilic groups in the molecule
Hl - Value of the lipophilic groups

The HLB value between:
0-3 indicates an antifoaming agent
3-6 indicates a W/O emulsifier
7-9 indicates a wetting agent
8-28 indicates an O/W emulsifier
12-15 is a typical detergent

11-18 indicates a solubiliser or hydrotope

5. Production and purification of biosurfactant

In the past several years, the study on the biosurfactant production by various microorganisms has been extensively reported. The disadvantages of the microbial surfactant or biosurfactant, comparing with synthetic surfactant are low yield and high production cost.[80] So, the method trying to improve the biosurfactant production in large scale with low cost is of interest.

The main parameters in biosurfactant production with low cost are the carbon source or nitrogen source substrates, so the amount and type of them are extensively considered. A variety of cheap raw materials, such as plant-derived oils, oil wastes, starchy substances, lactic whey and distillery wastes have been used as substrates for cultivation of microorganisms. The list of microorganisms and substrates used for the production of various kinds of biosurfactant is shown in table 4.

Table 4 Use of inexpensive raw materials for the production of biosurfactants by various microbial strains (Table is adapted from Mukherjee et al., 2006).[80]

Low cost or waste raw material	Biosurfactant type	Microbial strain producer
Rapeseed oil	Rhamnolipids	*Pseudomonas species* DSM 2874
Babassu oil	Sophorolipids	*Candida lipolytica* IA 1055
Turkish corn oil	Sophorolipids	*Candida bombicola* ATCC 22214
Sunflower and soybean oil	Rhamnolipids	*Pseudomonas aeruginosa* DS10-129
	Lipopeptide	*Serratia marcescens*
Sunflower oil	Mannosylerthritol	*Candida* sp. SY16
Soybean oil	lipid	
	Rhamnolipids	*Pseudomonas aeruginosa 47T2 NCIB 40044*
Waste frying oils		
(sunflower and olive oil)	Rhamnolipids	*Pseudomonas aeruginosa LBI*
Soybean soapstock waste	Rhamnolipids	*Pseudomonas aeruginosa LBI*
Sunflower oil soapstock	Glycolipids	*Candida antarctica and/or Candida*
Oil refinery wastes		*apicola*
	Rhamnolipids	*Pseudomonas aeruginosa* AT10
Soybean oil refinery wastes	Rhamnolipids	*Pseudomonas aeruginosa strain* BS2
Curd whey and distillery	Lipopeptide	*Bacillus subtilis*
wastes	Lipopeptide	*Bacillus subtilis* ATCC 21332 *and*

Potato process effluents Cassava flour wastewater		*Bacillus subtilis* LB5a

Approaches used to reduce the cost of production are exploitation of cheaper or waste materials as substrate of cultivation. Moreover, development of efficient bioprocesses, such as optimization of the culture conditions, i.e. the carbon source, nitrogen source and the environment of cultivation were examined.

5.1 Factor affecting biosurfactant production

5.1.1 Carbon source

The nature of carbon source substrate influences the type, quality and quantity of biosurfactant.[81-82] Glycerol, glucose, mannitol and ethanol are the examples of the water-soluble carbon sources for *Pseudomonas* sp.. Moreover, the water-immiscible substrates such as n-alkanes and olive oil were also reported as the carbon sources of producing biosurfactant.[7] The composition of biosurfactant production in *Pseudomonas* sp. was affected by different carbon sources, but in glycolipids, there is no effect on the chain lengths of fatty acid moieties when using different chain lengths of substrates.[83-84] Duvnjak and colleagues used D-glucose, supplemented with hexadecane in the medium during the stationary growth phase of growing *Arthrobacter paraffineus* ATCC 19558 and showed a significant increase in biosurfactant yield.[85]

5.1.2 Nitrogen source

Nitrogen source is also important factor in biosurfactant production. *Arthrobacter paraffineus* preferred to use ammonium salts and urea as the nitrogen source for producing biosurfactant[86] The maximum surfactant production of *P. aeruginosa*[87-89] and *Rhodococcus* sp.[90] were supported by nitrate. The addition of L-amino acids such as asparagine, aspartic acid, glycine, and glutamic acid to the medium increased the biosurfactant production by *A. paraffineus.*[86] To produce either Val-7 or Leu-7 surfactin, this structure was influenced by the L-amino acid concentration in medium.[91] *B. licheniformis* BAS50 produced lichenysin-A production which was enhanced two- and four- fold by addition of L-glutamic acid and L-asparagine, respectively, in the medium.[92]

5.1.3 Environmental factors

The factors which are reported to affect biosurfactant production were pH, salinity, temperature, agitation and oxygen availability.[7,6] The sophorolipid which was produced by *T. bombicola* depended on the pH of the medium culture.[93] *Pseudomonas* sp. could highly produce rhamnolipids at pH range from 6 to 6.5 and decreasing sharply above pH7.[87] The composition of biosurfactant was altered when changing the temperature of cultivation of *A. paraffineus*[94] and

Pseudomonas sp. strain DSM-2874. [83] When the agitation and aeration rates of *Pseudomonas aeruginosa* and the yeast cultivation were increased, the increasing biosurfactant production was observed.[95] The surfactin produced by *B. subtilis* involved with oxygen transfer which is one of the key parameters for the process optimization and scale-up of this product.[96] Another effect of biosurfactant production is salt concentration which depends on its effect on cellular activity.[90]

5.2 Purification of biosurfactant

The most widely used technique on the first step of biosurfactant purification is the extraction the culture medium of biosurfactant producing microorganism with organic solvent. The solvents generally used are chloroform-methanol, dichloromethane-methanol, ethyl acetate, hexane, hexane-methanol, butanol, pentane, acetic acid or ether, etc.[7] In addition, acid precipitation and ultrafiltration were also used in isolation of some biosurfactant.[7] After the extraction, the column chromatography is the prefered method, such as silica-gel column chromatography, sephadex gel filtration, ODS column chromatography and HPLC for purification, which depend on the type, properties and the purpose of the biosurfactant application. Thin layer chromatography with the various specific sprays was used for detection of the purity of the product.

The example of purification of lipopeptide from *Alcaligenes sp.* was reported by using 150 ml chloroform-ethanol (2:1, by volume) for extraction of biosurfactant.[36] The crude product was diluted to 20 g/l with chloroform followed by silica gel column chromatography with the gradient eluent system. The fractions were collected and evaporated with rotary evaporator and then the product was dissolved in chloroform before oil-spreading test and TLC analysis.

6. Application of biosurfactant

6.1 Biodegradation and bioremediation

Biosurfactant has received considerable interest in the field of environmental remediation process because of their efficacy as remediation agents and dispersion and their environment-friendly properties such as low toxicity and high biodegradability.[42] Moreover, it has been reported to enhance the dispersion and biodegradation of hydrocarbons.[97-99]

Oil dispersants is one of the applications of biosurfactant in oil removal or oil spill remediation. The dispersants are usually composed of the mixtures of surfactant, solvent and additives. The dispersants enhance the rate of natural dispersion of oil and its removal from the contaminated surface.[100] In addition, the dispersants increase surface area of oil as a result of its dispersion into small droplets that is expected to stimulate its biodegradation via the activity of naturally occurring microorganisms.[101]

Pseudomonas strains produced rhamnolipids have received much attention due to their remarkable tensioactive and emulsifying properties.[102,42] Rhamnolipids are proper to use in bioremediation of oil pollutants since they have high emulsification activity. Moreover, comparing with

non-ionic or cationic chemical surfactants, they are less strongly retained on negatively charged soil particles.[42]

Emulsan is the emulsifying agent for hydrocarbons in water which is very effective even at a concentration as low as 0.001-0.01%. It is a powerful emulsion stabilizer because of the ability to resist inversion even at a water-to-oil ratio of 1:4.[103]

6.2 Food

Biosurfactant was usually applied in food industry as food additive in emulsifying oil and water ingredients of food. In bakery products, they can improve loaf volume and create a smooth and soft crumb. Fatty acid containing glycerol, sorbitan or ethylene glycol, lectin and its derivatives, ethoxylated derivatives of monoacylglycerols including recently synthesized oligopeptide were used for instance.[104] *Candida utilis* produced a novel bioemulsifier which has shown potential use in salad dressing.[105]

6.3 Cosmetics

The acetylated lactonic sophorolipids have been applied in cosmetics as antidandruff bacteriostatic agent and deodorants.[106]

6.4 Pharmaceutics and Medicine

Pseudozyma sp. produced mannosylerythritol lipids (MELs) from vegetable oils substrate.[32,8] They showed not only excellent surface-active,[33] but also versatile biochemical actions, such as antitumor against rat pheochromocytoma[107] and mouse melanoma cells,[108-109] including activation of cell differentiation activity of human leukemia cell line HL60.[110]

Streptococcus thermophilus A could produce glycolipids rich fraction which was found to be a potent antimicrobial agent and anti-adhesive against several microbial strains isolated from explanted voice prostheses.[109]

Lactonic sophorolipids have measurable biocide activity.[111]

The mixture of seven homologues of newly identified rhamnolipids, AT10 showed excellent growth inhibition activities on gram positive and gram-negative bacteria and filamentous fungi.[112]

Trehalose lipids inhibited growth of the conidia germination of the fungus *Glomerella cingulata,* herpes simplex virus and influenza virus.[7,113]

Sophorolipids showed growth inhibition on *Bacillus subtilis*, *Staphylococcus epidermidis* and *Streptococcus faecium* and inhibited the germination of conidia of fungus *Glomerella cingulata*.[113] Moreover, Chen et al., 2006 reported that sophorolipids produced by *Wickerhamiella domercqiae* could induce apoptosis of liver cancer cell line, H7402.[114]

Surfactin was also inhiibited the proliferation of human colon carcinoma cell line.[115]

Rhamnolipids biosurfacant produced by *Pseudomonas aeruginosa* B189 showed significant antiproliferative activity against human breast cancer cell line (MCF-7).[10]

7. Drug delivery systems

Drug delivery system means the process bringing drug to the target site by a vehicle, including the method of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals. The goal of drug delivery system is to carry the medication intact to the specific target in the body to avoid toxicity to normal cells. To achieve this goal, researchers are turning to advance in micro- and nanotechnology,[116] especially, the nanotechnology which is trying to adapt the system of drug vehicle (drug delivery system) in the form of particle in nanometer size which is called as nanoparticle. The drug encapsulated nanoparticle is advantage for drug targeting because of the alteration in the drug's biodistribution favors drug accumulation at the desired site. The improvement to pass of the drug across various barriers may allow alternative administration routes.[117]

For the drug release, some kinds of nanoparticle are gastric-resistant dosage forms which drug release occurred mainly in the colon. They cannot release in the gastric part.[118]

The development of drug vehicles had begun many years ago. The vehicles were classified into three generations.[119] The first generation vectors are liposomes and nanoparticles (nanospheres and nanocapsules).

The liposome was the first drug vehicle which has been developed forty years ago.[120] It is small spherical vesicle constituted by phospholipids bilayers and aqueous phase in central cavity. They can carry the active compound by dissolving the hydrophilic compound in the aqueous phase and the hydrophobic in the bilayer. The liposomes have a good biocompatibility because of their raw materials composition such as natural phospholipids, sterols, glycerolipid. However, these vesicular structures are unstable in chemical (oxidation and hydrolysis of phospholipids) and physical (aggregation, fusion and loss of contents) environments.[120]

The nanospheres are spherical polymeric matrix which were first developed in 1976 by Birrenbach and Speiser[121] using the cross-linked polyacrylamide, a non-biodegradable polymer. Thereafter, the system (bio) degradable based PACA, were developed by Couvreur and colleague, 1979.[122] The active ingredient is either incorporated within the polymer network but not chemically bonded to the matrix during the formation of nanospheres, or adsorbed on their surface through hydrophobic-type bonds, electrostatic or covalent. It is released by simple diffusion or as a result of the (bio) degradation of the polymer in the body.

The nanocapsules are spherical structures generally consisting of an oil core surrounded by a thin wall polymer. The active ingredient is usually dissolved in the oil core, but can also be adsorbed on the surface of the nanocapsules.[123]

Hydrophobic polymer such as aliphatic polyesters, are generally hydrophobic and a negative surface charge. After intravenous administration, the opsonins, cationic plasma proteins, adsorb rapidly to the surface of these particles which facilitates their capture by the organs of mononuclear phagocyte system (MPS) such as the liver, spleen, kidney and bone marrow.[124-125] The particles are then

internalized by endocytosis to reach in lysosomes where they can potentially be degraded by the lysosomal enzyme. The drug is released primarily in the cells of the liver, spleen and kidney. This accumulation, mainly liver, can be an advantage to improve the treatment of certain infections of MPS.[126-127] The degree of hydrophobicity of the particles influence the quantity and composition of plasma proteins adsorbed on the surface of particles.[128-129] So, the modification of the hydrophobic surface of the particles for reducing their accumulation in the liver and extend their time in the bloodstream. Surface hydrophilisation is one effective way to minimize both the hydrophobic and electrostatic interactions between proteins and particle. The hydrophilic layer can act as a steric barrier that hides the hydrophobicity and surface charge,[130] and renders the particles hydrophobic "invisible" MPS ("stealth" effect).[131] This development of particles hydrophobic "invisible" MPS is the second generation which can extend the time of drugs movement in blood, and control their release. However, specific targeting of drugs remains elusive; so, the third generation vectors are occurred. This is an advantage especially for cancer treatment which is necessary to use the relatively toxic drugs and to monitor the quantity administered. The supramolecular structure-ligands (antibodies, peptides, sugars, folic acid) are attached to the surface of vectors[132] which bind selectively the specific markers (antigens or receptors) that are hyperexpressed on the surface of target cells (cancer cells, infected cells, etc.).

Recently, Suzawa et al., 2002 reported of using an adriamycin-conjugated poly(ethylene glycol) linked with enzymatically cleavable peptide sequences (alanyl-valine, alanyl-proline, and glycyl-proline). The result showed a greater selectivity cleavage at tumor cells.[133]

7.1 Type of drug delivery vehicle[134]

7.1.1 The liposome group

7.1.1.1 Liposome

The structure of liposome is a phospholipids bilayer and cholesterol. The cholesterol is used for increasing stability because of raising bilayer rigidity. They sizes are about 50 nm to several micrometers.

7.1.1.2 Virosomes

Virosomes consist viral phospholipids and viral membrane proteins (Hemagglutinin, HA). HA acts as a targeting device.

7.1.1.3 Transfersomes

The components of this vehicle are phospholipids (phosphatidylcholine, PC) and cholate (9:2 molar ratios). This type of vehicle performs better than the classical liposome after epicutaneous administration.

7.1.1.4 Archaeosomes

The compositions of this vehicle type are the lipid parts in the archaeobacteria membrane. The archaeosomes show the physical and chemical stability properties.

7.1.1.5 Niosomes

They are non-phospholipids liposomes which are the combination of non-ionic surfactants and cholesterol forming as small unilamellar vesicle. The chemical stability of this vehicle is the same as archaeosomes but is higher than phospholipids liposome.

7.1.1.6 Cochleate

The structure is phospholipids (phosphatidylethanolamine), negatively charged phosphatidylserine and cholesterol, intercalating with calcium ions between bilayers. Its appearance is non-vesicular bilayer sheets.

7.1.1.7 Proteosomes

The main structures of proteosomes consist of protein from outer membrane of bacteria. The average size of the vesicles is about 100 nm.

7.1.2 ISCOMs (Immunostimulating complex)

The components of this vehicle are saponin (Quil A), cholesterol and phospholipids (PE) by ratio 4:1:1 (Quil A:cholesterol: phospholipids(PE)) . Its shape is spherical, micellar assemblies of about 40 nm.

7.1.3 Emulsions

The examples of this vehicle are MF59 (o/w), montanide (w/o), Freund's adjuvant (w/o) and emulsomes.

- MF59 emulsion consists of Tween 80, SPAN 85 and squalene.

- Montanide emulsion is a mixture of oil and water which combined with a specific antigen to boost the immune response to that antigen.[135]

- Freund's adjuvant is a water-in-oil emulsion consisting of a mineral oil, an antibody stimulator such as *tubercle bacilli*, and an emulsifying agent such as lanolin or Arlacel A.[135]

- Emulsome's structure is a solid fat core surrounded by phospholipids bilayers. They are nanoemulsions and physically considered as between oil in water emulsion and liposome.[135]

7.1.4 Microparticle and Nanoparticle

The examples of this carrier are poly(lactic-*co*-glycolic acid)(PLGA), chitosan, alginate, etc..[135]

Due to the small size, nanoparticles are suitable for intravenous administration. On the contrary, the microparticles which are larger size are used via alternative routes.[117]

7.1.5 Inorganic insoluble salt

Aluminium phosphate, Aluminium hydroxide and calcium phosphate are classified in this group.[135]

7.2 Advantages of using nanoparticle in drug delivery system

7.2.1 To prolong the action of drugs or biomolecules: The particle size is ultra fine, so they can pass through the smallest capillary vessels and these particles promote avoidance the rapid clearance by phagocytes, therefore their duration in blood stream is greatly prolonged.

7.2.2 To arrive at the specific target: They can penetrate the gap of the cells and tissue to arrive target organs such as liver, spleen, lung, spinal cord and lymph.

7.2.3 To be able of controlled–release properties: The biodegradability, pH, ion and/or temperature sensitivity of materials are properties of these particles which are the advantages to control the release of the inner molecule.

7.2.4 To reduce drug toxicity: They can affect cell and organ specifically, so the utility of the drugs can be improved and reduce toxic side effects.[11]

As drug delivery system, the drugs or biomolecules can be entrapped into their interior structures and/or absorbs drugs or biomolecules onto their exterior surfaces of the nanoparticle. Recently, nanoparticles have been widely used to deliver drugs, genes, vaccines, polypeptides, proteins, nucleic acids, etc..[136]

7.3 Nanoparticle delivery system in pharmaceutics

Over the years, nanoparticle drug delivery systems are very potential in biological, medical and pharmaceutical applications.[135] Currently, the direction to study nanoparticle drug delivery is

- To find and combine of carrier which produce the best drug release rate profile.
- To modify the surface of nanoparticle in order to improve their targeting ability
- To optimize the preparation of nanoparticle to receive the better drug delivery capacity
- To develop the nanoparticle for application in clinics and the possibility of industrial production
- Investigate *in vivo* dynamic process to reveal the nanoparticle interaction with blood and targeting tissues and organs, etc..[136]

In 1998, a nanoparticle formulation of ciprofloxacin-loaded polyethylbutylcyanoacrylate (PEBCA) was investigated and optimized. Ciprofloxacin with nanoparticles was performed by emulsion polymerization. The ciprofloxacin nanoparticle suspension was stable when preparing only with acetone in the polymerization medium.[137]

Chan and colleague in 2009 prepared core-shell nanoparticle which consists of a poly (D,L-lactide-co-glycolide) hydrophobic core, a soybean lectin monolayer and a poly(ethylene glycol) shell. They tried to use this vehicle to carry a model chemotherapy drug, docetaxel. The process to prepare nanoparticle is a modified nanoprecipitation method and self-assembly.[138]

Tacrine was the first acetylcholinestearse inhibitor licensed for treatment of Alzheimer's disease. It was encapsulated by using magnetic chitosan microparticles which were prepared by emulsion cross-linking. *In vivo* study on evaluating the concentration of the tacrine at the target and non-target organ was investigated. The result is the tacrine concentration was significantly increased in brain with magnetic chitosan microparticle comparing with free drug.[139]

The delivery of insulin was investigated by Singh and Chauhan in 2009 by preparing psyllium polysaccharide and methacrylamide based hydrogels with *N*, *N'*-methylenebisacrylamide as crosslinker and evaluated the mechanism and drug release.[140]

For antibacterial therapy, drug carrier was used to control antibiotic drugs efficacy by hiding and protecting the molecule from degradation and delivering it to accessible target cells. Liposomes and nanoparticles are the main carriers developed for these logistic targeting strategies.[12] So, there are many researchers who studied the potential of liposomes and nanoparticles in treatment of bacterial infections. Vladimirsky and coworkers studied on streptomycin liposomes treated mice infected with *Mycobacterium tuberculosis*. The result showed a significant inhibition of bacterial growth in the liver and spleen and prolongation the survival of mice.[140]

For vaccine delivery, combination of heat-inactivated influenza virus with LTR192G adjuvant (a whole virus influenza vaccine) was incorporated in Amioca® starch / poly (acrylic acid) powder and nasally administered to rabbits. Different ratio of powder formulations based on spray-dried mixtures of Amioca® / poly(acrylic acid) (Carbopol®974P) was investigated and tested *in vivo* with a rabbit model. Individual rabbit sera were tested against hemaglutinin (HA), the major surface antigen of influenza. From this study, the carriers facilitated the induction of systemic anti-HA antibody response after intranasal vaccination with a whole virus influenza vaccine.[141]

In gene delivery, chitosan is used but the chitosan-based complexes may lead to difficulties in DNA release when they arrive at the target site. Therefore in 2009, Peng and colleague, reported to use a negatively charged poly(γ-glutamic acid) incorporated in chitosan/DNA complex nanoparticles. They found a significant increase in its tranfection efficiency and enhanced the cellular uptake. The nanoparticles containing chitosan, DNA and poly(γ-glutamic acid) were examined the characteristics of the compound by dynamic light scattering (DLS), transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS). Fluorescence and luminance spectrometry and flow cytometry were used to evaluate the potential of gene expression and transfection efficiency of tested nanoparticles. The internalization efficiency was examined using a confocal laser scanning microscope (CLSM) and a flow cytometer.[142]

For the pharmaceutics, glycolipid biosurfactant, mannosylerythritol lipids-A (MELs-A) produced by yeast, *Candida antarctica* was demonstrated to dramatically increase the efficiency of gene transfection which mediated by liposomes with a cationic cholesterol derivative.[143] Recently, the cationic liposome bearing MEL has been shown to increase the efficiency of gene transfection into mammalian cells dramatically.[8]

8. Nanoparticle preparation

8.1 Methods of nanoparticle preparation

The techniques used to prepare nanoparticles are numerous. They are generally classified into two groups. In the first group, the nanoparticles are formed by polymerization of monomers in emulsion. The drug loaded nanoparticles can be obtained by incorporation of active ingredients during or after the preparation of the polymer dispersion. In the other group, nanoparticles are prepared from preformed polymers. These polymers can be soluble or insoluble in water, and they may be synthetic, semi-synthetic or natural origin.

The choice of preparation method of polymer particles and the constituents depend on the physicochemical properties of the active ingredient, the desired characteristics of drug release, purpose of therapy, and route of administration.[127] The method chosen should allow the proper encapsulation with excellent performance and should be potentially applicable to an industrial point of view.

8.1.1 Preparation of nanoparticles by polymerization of monomers

Emulsion polymerization has been developed to limit the use of toxic organic solvents that are used during production of the polymer. The method is to disperse monomer in an aqueous medium containing a surfactant with a high shear. The monomer droplets are formed by hydrophilic initiator and stabilized by the surfactant.

Birrenbach and Speiser in 1976 used this method to prepare nanoparticles of crosslinked polyacrylamide from acrylamide and N, N'-methylene bis acrylamide.[121] Then, Kreuter and Speiser[144] have developed polymethylmethacrylate (PMMA) particles, based on the protocol developed by Birrenbach and Speiser. However, this type of nanoparticle is restricted for use as a vector, because the polymers are not (bio) degradable so, they will be accumulated in the body. To solve this problem, nanoparticles bioeliminable poly(alkylcyanoacrylate) (PACA) were developed by Couvreur and colleague since 1979.[122]

8.1.2 Preparation of nanoparticles from preformed polymers

The use of preformed polymers has some advantages over the method using the polymerization of monomers method. Indeed, preformed polymers have well-defined physicochemical properties and the process does not require a step of removing residual monomers and reagents (initiator, catalyst, etc.). In addition, it may not have any reaction with the active ingredient during the preparation of nanoparticles. The example methods using preformed polymers are emulsion / solvent evaporation and
nanoprecipitation method.

8.1.2.1 Emulsion/solvent evaporation

In 1981, the emulsion/solvent evaporation method was begun by Gurny et al.[145] by preparing of PLA nanospheres. At first, the chosen polymer to form the nanoparticles is dissolved in an organic solvent immiscible in water (dichloromethane, chloroform, or ethyl acetate).[129] Then, this mixture is emulsified in an aqueous phase containing a stabilizer (sodium cholate, poly (vinyl alcohol) (PVA)) by strong shearing or sonication to form a stable O/W emulsion.[146-147] The organic solvent is

then evaporated by raising the temperature or pressure, or simply shaking at room temperature. This method allows the encapsulation of hydrophobic active ingredient by simple addition of the compound in the polymer solution. After evaporation of organic solvent, the drug is encapsulated within the polymer matrix.[148]

In the case of a hydrophilic active ingredient, this active compound must be dispersed in an aqueous solution which is inside the organic phase containing the polymer (W/O). Moreover, this emulsion is itself dispersed in an external aqueous phase to form a double emulsion W/O/W.[147] After evaporation of the organic solvent, nanospheres containing the hydrosoluble active ingredient are formed. This technique has been used to encapsulate bovine serum albumin (BSA)[147] or erythropoietin.[149]

8.1.2.2. Nanoprecipitation

The process of nanoprecipitation was first described by Fessi et al. in 1986.[150] This requires the use of a polar organic phase containing the polymer and the organosoluble active ingredient. The organic phase must be an organic solvent miscible with water such as acetone.[151] This polymer solution is emulsified in an aqueous phase (the surfactant is optional) to form droplets. The spontaneous diffusion of the solvent in the aqueous phase leads to precipitation of the polymer and the formation of solid nanoparticles.

This process allows an excellent incorporation of a large quantity of medication, however, it is limited to the hydrophobic active ingredients, which are soluble in polar solvents but insoluble in water, for example, isradipine (antihypertensive drug).[151]

8.2 Materials used in nanoparticle preparation

The materials used for preparing the nanoparticle are made from hydrophobic polymers such as poly (lactic acid) (PLA), poly (ε-caprolactone) (PCL), poly (alkyl cyanoacrylate) (PACA). They are biodegradable and/or bioeliminable to avoid accumulation in the body.[152-153,122]

Polylactic acid (PLA) has been widely applied as carriers for drug delivery by emulsion evaporation method.[154] The evaluation of PLA nanoparticles by stabilizing with water-soluble α-methoxy-ω-hydroxyl polyethylene (MPEO) was reported.[155] However, these hydrophobic nanoparticles can be easily caught and eliminated by the organs of the mononuclear phagocyte system (MPS). So, it is necessary to achieve a hydrophilisation the surface of nanoparticles to reduce catching from MPS by modification of nanoparticle surface with hydrophilic layer and neutral.[130] This modified nanoparticle consists of amphiphilic polymers, i.e. dextran-based or hydrophilic polymers such as poly (ethylene oxide) (PEO), a linear polysaccharide as pullulan and pullulan acetate (PA).

At the beginning of the 1980s, the first study of polymer surfactants based on polysaccharides started. Many reports studied on emulsan; a kind of biosurfactant, which is a polymer of polysaccharide backbone carrying esterified fatty acids and proteins. Their properties in emulsification and characteristics of hydrocarbon-in-water emulsions were investigated. However, the identification of emulsan structure is lacking, especially, the protein part which is potentially surface-

active molecules. So, it is difficult to deduce the clear information about the effect of polymer structure. In 1982, Landoll characterized viscometric, solubility and surface-active properties of cellulose modified with alkyl chains. They used different hydrophilic backbones and varied bearing hydrophobic groups.[156] Subsequently, there were many researchers studied on hydrophobically modified polysaccharides, but their surface-active properties were not mentioned. The first preparation of hydrophobic derivative dextran in aqueous phase system by partial esterification with benzoic acid was investigated.[157]

For several years, Durand et al in LCPM laboratory, Nancy-France, have been studying the properties of “modified dextran”; the neutral polymeric surfactants which consisted of the polysaccharide backbone consisting mainly of α-1,6-glucosidic linkages (dextran) and hydrophobic groups (aromatic rings, aliphatic or cyclic hydrocarbons) attached to dextran by covalent bonding. They varied the number of grafted hydrophobic group to define the best conditions for using this polymer as the stabilizer of nanoparticle. The method used to prepare the nanoparticle was miniemulsion polymerization.[158]

In 2003, Rouzes et al, synthesized the dextran derivative containing various grafted hydrophobic groups (phenoxy, P; decyl, C_{10}) and used this product to be the stabilizer for preparing of poly(lactic acid) (PLA) nanospheres by an oil in water with emulsion evaporation technique. The effect of dextran modification on particle size and stability was evaluated. Moreover, they examined encapsulation of lidocaine into the PLA nanosphere.[159]

In order to modify the polarity of the nanoparticle core, two conjugated compounds such as modified dextran and PLA were used to prepare nanoparticle for encapsulating various polarity active molecule.[159] The examples of conjugated nanoparticle are PLA and poly (ethyleneglycol) conjugated nanoparticles encapsulating with lactoferrin. There was the novel brain delivery system reported in 2009.[160] Methoxy poly (ethylene glycol) (MPEG) and poly (lactide) (PLA) were used to prepare the nanoparticle to deliver anticancer drug.[161] Chitosan and cyclodextrin were used in nasal delivery.[162] For chemotherapy drug delivery, Poly(DL-lactide-co-glycolide)-grafted pullulan nanospheres was used to control release of Adriamycin[163] and hydrogel nanoparticles of pullulan acetate/oligo-sulfonamide was used with doxorubixin, anticancer drug.[164]

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Soil sample

The soil samples used in this study were oil contaminated soils collected from oil mill wastes and biodiesel plant in Songkhla province, the southern part of Thailand; Chiangmai province, the northern part of Thailand and Pudong, a district of Shanghai, China.

2. Media

2.1 basal salt medium (see Appendix)

2.2 nutrient agar (NA) medium, Himedia®, India

2.3 nutrient broth (NB) medium, Himedia®, India

2.4 YM agar

2.5 YM broth

2.6 PDA agar, Himedia®, India

3. Materials

3.1 $DexP_{20}$ (see Methods and Appendix).

3.2 $DexC10_{164}$ obtained from Laboratoire de Chimie Physique Macromoléculair, Nancy, France; preparations followed as Rouzes et al., 2003 (see Appendix).[159]

3.2 polylactide (PLA), Aldrich®, France (see Appendix).

3.3 silica gel 60 for column chromatography (0.0036-0.200 nm) Merck, Germany

3.4 TLC aluminum sheets of silica gel 60 F_{254}, pre-coated, 20x20 cm, Merck, Germany

3.5 glass columns with inner diameter 2, 2.5, 3, 3.5, and 10 cm

4. Chemicals

4.1 glycerol, Merck®, Germany

4.2 glucose, Merck®, Germany

4.3 n-hexadecane, Fluka®, Switzerland

4.4 soy bean cooking oil, Morakot Co., Ltd

4.5 corn oil, Golden Drop®, USA.

4.6 palm oil, Morakot Co., Ltd,

4.7 olive oil, Bertolli®, UK.

4.8 ammonium chloride (NH_4Cl) AR grade Merck®, Germany

4.9 nitric acid (HNO_3), Merck®, Germany

4.10 urea, Ajax Finechem®, New Zealand

4.11 ammonium sulfate ($(NH_4)_2SO_4$) AR grade Merck®, Germany

4.12 ammonium nitrate (NH_4NO_3) AR grade Fluka®, Switzerland

4.13 tween20, Sigma®, USA.

4.14 kerosene, Carco®, Thailand

4.15 ethyl acetate ($CH_3COOC_2H_5$) AR grade Merck®, Germany

4.16 hexane (C_6H_{14}) AR grade Mallinckrodt®, USA

4.17 methanol (CH_3OH) AR grade Merck®, Germany

4.18 tetrahydrofuran: THF (C_4H_8O) HPLC grade VWR Prolabo®, France

4.19 diethyl ether ($CH_3CH_2OCH_2CH_3$) HPLC grade Merck®, Germany

4.20 trifluoroacetic acid, 99% (CF_3CO_2H) AR grade Merck®, Germany

4.21 dichloromethane (CH_2CL_2) HPLC grade Merck®, Germany

4.22 ρ-anisaldehyde, Fluka®, Switzerland

4.23 phosphomolybdic acid, Aldrich®, France

4.24 solketal, 97%, Alfa Aesar®, USA

4.25 myristol chloride, Aldrich®, France

4.26 dextran T40 Pharmacia®, Sweden

4.27 1,2-epoxy-3-phenoxypropane, Aldrich®, France

4.28 sodium hydroxide (NaOH), Aldrich®, France

4.29 ethanol (C_2H_5OH), Aldrich®, France

4.30 sodium dodecyl sulphate (SDS), Bio-Rad®, USA

5. Reagent

5.1 ρ-anisaldehyde reagent (see Appendix)

5.2 phosphomolybdic acid (PMA) reagent (see Appendix)

METHODS

1. Screening of biosurfactant producing microorganisms

1.1 Isolation of microorganisms

10 grams of the oil contaminated soils were suspended in 100 ml distilled water and diluted 10 -fold dilution from 10^{-1} to 10^{-6}. Aliquots of 0.1 ml of the soil suspension were added on basal medium agar containing 1% palm oil, 0.1% tween20 and 1.5% agar and then spreaded plate. The agar plates were incubated in an incubator (Sheldon manufacturing INC., USA.) at 30ºC until microorganisms grew. Isolated colonies of microorganisms were picked and subcultured to a new nutrient agar plate. The pure colony was selected and grown in liquid basal medium with 4% of palm oil, shaking 200 rpm 30°C (yeast) or 37°C (bacteria) for 2 days. The supernatant of culture broths was tested for surfactant activity and emulsion activity. A microorganism which could produce biosurfactant with high surfactant and emulsion activities would be chosen for further studied.

1.2 Surfactant activity and emulsion activity assay

1.2.1 Surfactant activity[165]

1.2.1.1 Drop collapsing test

HPLC syringe (Hamilton Company, USA.) was used to drop 2 µl mineral oil to wells of a 96 well-plate lid. After 1 hour of settling, 5 µl of cell free-microorganism culture broth was added on three oil-coated wells, waited for 1-2 min and measured the scale of culture broth drop diameter under stereomicroscope (Kyowa®, Japan) at 150 magnifications. The drop diameter scale would be calculated to millimeter (mm). The test was done in three independent experiments.

1.2.1.2 Modified drop collapsing test

HPLC syringe was used to drop 2 µl mineral oil to wells of a 96 well-plate lid. After 1 hour of settling, 2 µl of the 10 mg/ml tested surfactant dissolved in methanol or ethanol was added on three oil coated wells, waited 2 min for methanol and 5 min for ethanol. Then 5 µl of distilled water was dropped to each well, waited for 1-2 min and measured the scale drop diameter of distilled water under stereomicroscope (Kyowa®, Japan) at 150 magnifications. The drop diameter scale would be calculated to millimeter (mm). The test was done in three independent experiments.

1.2.2 Emulsion activity assay

Emulsification activity and emulsification stability were measured using a modified method of Patel and Desai, 1997.[78] One ml of kerosene was added to 1 ml of culture medium and mix vigorously (Vortex genie®2, USA.) at high speed for 2 min. After 24 h, the height of the interface emulsion layer and the total height were measured. The emulsion activity was calculated as the formula below. The emulsifying stability will be determined with the same procedure after 48 h.

Emulsion activity/stability (%) = height of emulsion layer/ total height x 100

2. Identification of microorganism

The isolate which produced the highest surfactant and emulsion activities was selected and identified by macroscopic morphology, microscopic morphology and genetic analysis of 28S ribosomal RNA gene.

2.1 Macroscopic morphology

The isolate of selected microorganisms was cultured on YM agar plate (yeast and mold strains) for 7 days. The characteristics of colonies on the agar plate were evaluated everyday.

2.2 Microscopic morphology

The isolate of selected microorganisms was cultured on YM agar plate (yeast and mold strains) for 7 days. The pure colony was picked up, smeared on a glass slide and stained with Gram's stain and observed under light microscope, x 400 magnification.

2.3 Identification by DNA sequencing

The isolate of the selected microorganisms was cultured on YM agar plate (yeast and mold strains) for 3-5 days. The cultured colony was used for isolation of genomic DNA in order to

perform PCR of 28S rRNA gene. The nucleotide divergence in the 5'-end of the large subunit (28S) ribosomal DNA (rDNA region D1/D2) was amplified using the primer NL-1(5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG). The PCR products were sequenced by the automate DNA sequencer and DNA homology analyzed by "Gapped BLAST and PSI-BLAST".[166] The genetic was analysis was performed by Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology (MU-OU: CRC), Thailand.

3. Optimization of culture conditions

3.1 Preparation of spore stock

The selected microorganism was cultured on PDA agar at 30ºC about 5-7 days. The spore was scraped from the culture plate and put into distilled water, washed and finally adjusted the concentration of spores to 10^6 spore/ml with water by counting with hemacytometer (Reichert®, USA.).

3.2 Cultivation method

The basal salt medium was used as the culture media for cultivation with the carbon and nitrogen sources supplementation for biosurfactant production. Media and culture conditions were varied in order to increase the biosurfactant production. The general cultivation method for mold strains was as follows. 0.4 ml of the spore stock was added into 20 ml of the tested medium prepared in 100 ml flask, shaked at 200 rpm in shaking incubator at 30°C (Thermo Fisher Scientific Inc., USA.), for 16 days. The culture broth was taken everyday to determine the surfactant activity.

3.3 The tested media or conditions

The basal salt medium was used as a basic culture medium, supplementation of carbon and nitrogen sources were varied to determine the suitable sources by measuring the surfactant and emulsion activity/stability. The supernatant of culture broths was taken everyday and tested for surfactant activity.

3.3.1 Carbon source effect

3.3.1.1Carbon source type

The basal salt medium either with oil free or supplemented with 4% of glycerol, glucose, n-hexadecane, soybean oil, corn oil, palm oil or olive oil, at pH 5.3 was prepared and used for cultivation.

3.3.1.2Carbon source concentration

The basal salt medium with varying concentration at 0.5, 1, 2, 4, 5, 6 and 8% of the suitable type of carbon source, at pH 5.3 was prepared and used for cultivation.

3.3.2 Nitrogen source effect

3.3.2.1 Nitrogen source type

The basal salt medium with suitable type and concentration of carbon source was prepared. Nitrogen source at 0.3% concentration of either NH_4Cl, HNO_3, urea, $(NH_4)_2SO_4$ or NH_4NO_3 were supplemented, adjusted pH to 5.3 and used for cultivation.

3.3.2.2 Nitrogen source concentration

The basal salt medium with varying the concentration at 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6% of the suitable type of nitrogen source and carbon source, at pH 5.3 was prepared and used for cultivation.

3.3.3 Effect of pH in medium

The basal salt medium with the suitable type and concentration of carbon and nitrogen sources were prepared and adjusted to either pH 4.5, 5, 5.3, 6, 6.5 or 7 and used for cultivation.

3.3.4 Effect of temperature

The basal salt medium with the suitable type and concentration of carbon and nitrogen sources and optimum pH were used for cultivation of tested strain in a shaker incubator at the temperature 28, 30, 37 and 40°C for 16 days.

3.3.5 Time course of cell growth and biosurfactant production

The basal salt medium with the suitable type and concentration of carbon and nitrogen sources and optimum pH and temperature were used for cultivation of tested strain in a shaker incubator. The culture broths were taken every 24 h to determine growth and biosurfactant production. The microbial growth was measured by the optical density at 600 nm (OD_{600}) using spectrophotometer (Shimadzu UV-160A®, Japan). The biosurfactant production was determined by surfactant activity as previously describe.

3.4 Statistical analysis

The cultivation and all the activity assays were done in three independent experiments. The data were calculated from triplicate determinations of surfactant activity. Statistical significant difference of tested media and culture conditions were evaluated by one way ANOVA ($P<0.05$) using SPSS 10 software.

4. Extraction and purification of biosurfactant

The microorganism which could produce the biosurfactant with high surfactant activity and emulsion activity/stability was selected for further study. The selected strain was cultivated in 6 liters of basal salt medium with optimum culture conditions (in section 3). The total broth was extracted twice for biosurfactant with ethyl acetate (ethyl acetate/culture medium, 2:1). Ethyl acetate fraction was collected and evaporated by rotary evaporator at 40ºC. The extract was dissolved with methanol and hexane by ratio 1:2.[53] The methanol fraction was collected and evaporated by rotary evaporator at 40ºC and kept as a crude extract.

The crude extract was purified by solvent fractionation using silica gel column chromatography. All fractions was collected, dried and tested for surfactant activity (drop collapsing

test). The highest surfactant activity fraction was collected, dried and checking the purity on silica gel TLC plate which was visualized by ρ-anisaldehyde reagent spray and heated at 110ºC for a few minutes. The step of silica gel column chromatography was repeated using various ratio of eluting solvent system until the biosurfactant pure compound was obtained.

5. Structure determination of the biosurfactant

The purified compound has been identified by ^{1}H-NMR, ^{13}C-NMR, HSQC, COSY (BRUKER spectrometer Avance 300) in deuterated chloroform ($CDCl_3$) and Atmospheric Pressure Chemical Ionization Mass Spectrometer (APCI MS).

6. Synthesis of monoacylglycerol

For the study on the encapsulation of the biosurfactant in nanoparticles, the synthetic monomyristin, myristic acid (a saturated long-chain fatty acid (C_{14})) esterified with glycerol, a kind of monoacylglycerol, was used. The structure of monomyristin was shown in Figure 6.

The structure of synthetic monoacylglycerol was based on myristic acid (which is a saturated long-chain fatty acid (C_{14})) esterified with glycerol. The molecular formula was $C_{17}H_{34}O_4$. For the study of the encapsulation of the biosurfactant in nanoparticles, this model monoacylglycerol was synthesized. The schematic of synthesis process was shown in Figure 7.

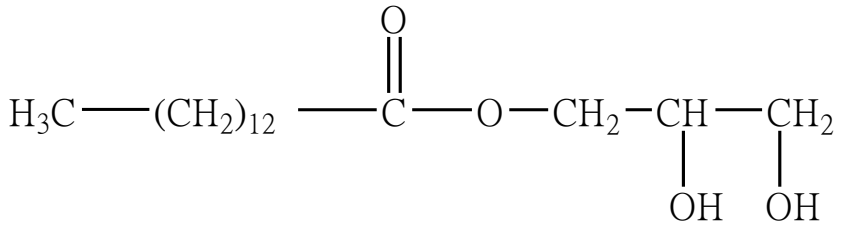


Figure 6 Structure of monomyristin (glycerol ester of myristic acid)

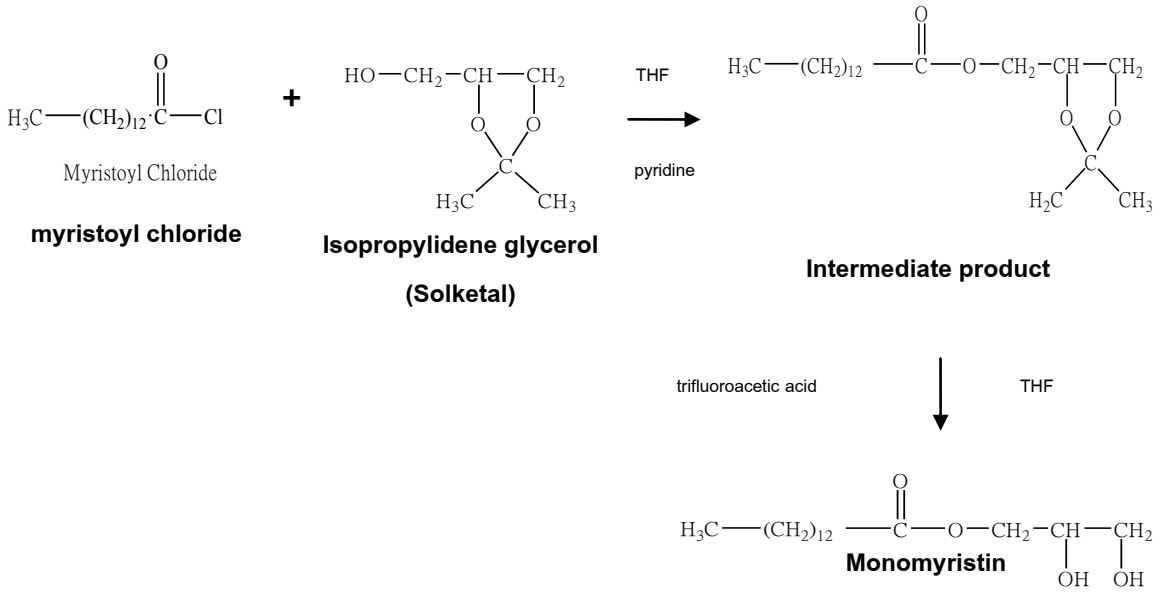


Figure 7 The synthetic process of monomyristin

6.1 The synthetic process method

6.1.1 1.32 g solketal was mixed with 10 ml THF (tetrahydrofuran) and 4.94 g myristyl chloride was added dropwise. Then 1.6 g pyridine was added into the reaction as a catalyst.

6.1.2 The mixture was extracted by diethyl ether and MilliQ water by ratio 1:1, three times. The organic phase which dissolved in diethyl ether was collected and dried over $MgSO_4$ to absorb water. $MgSO_4$ was removed by filtration. The intermediate solution was evaporated and the obtained product was purified by silica gel adsorption chromatography, checking the purity by thin layer chromatography, sprayed with a phosphomolybdic acid (PMA) reagent (see appendix). The chemical structure of the intermediate product was analyzed by ^1H NMR in $CDCl_3$.

6.1.3 The intermediate product was dissolved in 8ml THF and 4 ml trifluoroacetic acid was added dropwised. The reaction was neutralized to pH7 with $NaHCO_3$.

6.1.4 The mixture was extracted by diethyl ether and MilliQ water by ratio 1:1, three times. The organic phase which dissolved in diethyl ether was collected and evaporated. The product was purified further by silica gel column chromatography. The product pattern was checked by thin layer chromatography and sprayed with PMA reagent. The structure was analyzed by ^1H NMR in $CDCl_3$.

7. Synthesis of the hydrophobic derivatives of dextran, $DexP_{20}$

The hydrophobic derivatives of dextran (DexP) were prepared from dextran T40 (manufacturer data: *M*n =13,750 g/mol, *M*w =37,500 g/mol). 10 g of dextran T40 in 1M aqueous NaOH was added with 1,2-epoxy-3-phenoxypropane and stirring at ambient temperature for 48 h. Then, the crude product was precipitated twice with ethanol and dialyzed with deionized water for 24 h and then freeze-dried. The chemical structure of the hydrophobically modified dextran was presented in Appendix.

8. Surfactant properties determination

8.1 Interfacial tension measurements

Interfacial tension measurements were carried out at 25ºC between chloroform and water by using a K8 surface tensiometer (Krüss, Germany) with a platinum ring.

Monoacylglycerol was diluted in chloroform at various concentration; 1, 0.1, 0.01, 0.001, 0.0001 g/L. All samples were equilibrated for a sufficient time (15 min to 1 h) to reach constant readings.

8.2 Hydrophobic-lipophilic balance (HLB)

HLB was calculated according to the chemical groups composition of the molecule by Davies's group contribution method[79] as the formula below.

$$HLB = \sum (\text{Hydrophilic group contribution}) - \sum (\text{Hydrophobic group contribution}) + 7$$

9. Preparation of nanoparticles

Two methods were used for preparing nanoparticles: emulsion/solvent evaporation and nanoprecipitation methods.

The size, stability of nanoparticles, the amount of coagulum and ^{1}H-NMR of coagulum were used to analyze the best conditions for nanoparticles preparation.

9.1 Emulsion/solvent evaporation method[159]

Types of material and solvent used for preparing the nanoparticles by emulsion/solvent evaporation method were shown in Table 5 and the methods of preparation were described in section 9.1.1-9.1.5.

Table 5 Types of material and solvent used in nanoparticles preparation by emulsion/solvent evaporation method

Aqueous phase	Organic phase
$DexP_{20}$ in milliQ water	PLA in dichloromethane
$DexP_{20}$ in milliQ water	MAG in dichloromethane
$DexP_{20}$ in milliQ water	$DexC10_{164}$ in dichloromethane
$DexP_{20}$ in milliQ water	PLA and MAG in dichloromethane
$DexP_{20}$ in milliQ water	$DexC10_{164}$ and MAG in dichloromethane

9.1.1 Preparation of aqueous phase: 10 ml of a solution of the modified dextran ($DexP_{20}$) in milliQ water at various concentration; 0, 0.5, 1, 2, 5, 8, 10 g/L was prepared the day before.

9.1.2. Preparation of organic phase: 0.5, 1, 1.5, 2 ml of dichloromethane solution containing 0.5, 1, 1.5, 2 mg/ml of Polylactide (PLA) and/or monoacylglycerol (MAG) and/or modified dextran ($DexC10_{164}$) was prepared. The proportion of monoacylglycerol, PLA and $DexC10_{164}$ was optimized by varying the ratio of monoacylglycerol and PLA or $DexC10_{164}$ by 0:100, 75:25, 50:50, 25:75, 100:0.

9.1.3 The organic and aqueous phases were mixed vigorously with vortex for 1 minute and the mixture was quickly sonicated with a Vibracell model 600 W (Sonics & Materials Inc., Danbury,CT) with pulsed mode, 10 W, 5 power, 50% active cycle, 1 min in an ice bath.

9.1.4 The mixture was left at room temperature to allow the solvent to evaporate for 24 h.

9.1.5 The size of nanoparticles was measured by High Performance Particle Sizer (Malvern Instrument Ltd., Worcestershire, UK). After 24 h, nanoparticles size was determined again to check the stability of the nanoparticles.

In case of the macroscopic aggregation occured, the large particles were separated by centrifugation at 1500xg for 15 min. The upper suspension was taken and measured for the particle size by High Performance Particle Sizer and removed water using freeze-drying process. The amount

of dried polymer in upper suspension was measured by balance (ABT 220-5DM, KERN®, Germany) and the structure of the polymer was determined by ^{1}H-NMR.

In the aggregated part, the water was removed by freeze-drying process. The amount of dried coagulum was measured and the structure was determined by ^{1}H-NMR.

9.2 Nanoprecipitation method[167]

Types of material and solvent used for preparing the nanoparticles by nanoprecipitation method were shown in Table 6 and the methods of preparation were described in section 9.2.1-9.2.5.

Table 6 Types of material and solvent used in nanoparticles preparation by nanoprecipitation method

Aqueous phase	Organic phase
$DexP_{20}$ in milliQ water	PLA in THF
$DexP_{20}$ in milliQ water	MAG in THF
$DexP_{20}$ in milliQ water	$DexC10_{164}$ in THF
$DexP_{20}$ in milliQ water	$DexC10_{164}$ and MAG in THF
$DexP_{20}$ in milliQ water	PLA in Acetone
$DexP_{20}$ in milliQ water	MAG in Acetone

9.2.1 Preparation of aqueous phase: 9 ml of a solution of the modified dextran ($DexP_{20}$) in milliQ water at various concentrations; 0.5, 1, 2, 4, 5, 8, 10 g/L was prepared the day before.

9.2.2 Preparation of organic phase: 4.5 ml of acetone or THF solution containing 10, 25 mg/ml of PLA I and/or monoacylglycero and/or modified dextran ($DexC10_{164}$) was prepared. The proportion of monoacylglycerol, PLA and $DexC10_{164}$ was optimized by varying the ratio of monoacylglycerol and PLA or $DexC10_{164}$ by 0:100, 75:25, 50:50, 25:75, 0:100.

9.2.3 4.5 ml of the organic phase was added dropwise to the aqueous dextran derivative solution. The suspension was stirred with magnetic bar, 300 rotations per minute during the addition of the organic phase and 10 minutes further.

9.2.4 The mixture was left at room temperature to allow the solvent to evaporate for 24 h.

9.2.5 The size of nanoparticles was measured by High performance particle sizer (Malvern Instrument Ltd., Worcestershire, UK). After 24 h the size of nanoparticles was determined again to check the stability of the nanoparticles.

In case of the macroscopic aggregation occured, the large particles were separated by centrifugation at 1500xg for 15 min. The upper suspension was taken and measured for the particle size by High Performance Particle Sizer and removed water using freeze-drying process. The amount

of dried polymer in upper suspension was measured by balance (ABT 220-5DM, KERN®, Germany) and the structure of the polymer was determined by ^{1}H-NMR.

In the aggregated part, the water was removed by freeze-drying process. The amount of dried coagulum was measured and the structure was determined by ^{1}H-NMR.

CHAPTER IV

RESULTS

1. Screening of biosurfactant producing microorganisms

A total of 102 microorganisms were isolated from oil contaminated soils of three areas: Shanghai, China (4 isolates); Chiangmai, Thailand (9 isolates) and Songkhla, Thailand (89 isolates). After testing the surfactant activity, 6 microbial culture broth showed high surfactant activity. The culture broth of SK80 showed the highest in surfactant activity, emulsion activity and emulsion stability. The result was summarized in Table 7.

Table 7 The surfactant activity, emulsion activity and emulsion stability of 6 culture broth which showed high activity

Microorganisms	Drop collapsing test (mm)	Emulsion activity (%)	Emulsion stability (%)
SK4	3.55±0.08	3.45	3.45
SK19	3.38±0.04	10.34	6.90
SK26	3.62±0.04	6.90	3.45
SK47	3.60±0.04	14.81	14.81
SK48	3.55±0.11	25.00	17.86
SK80	**3.62±0.11**	**41.38**	**41.38**
basal medium + 4% palm oil (medium control)	2.90±0.04	00.00	00.00
Negative control (water)	2.81±0.04*	00.00**	00.00**

*Negative control of drop collapsing test is distilled water.

**Negative control of emulsion activity/stability is the microorganism-free culture medium.

2. Identification of microorganism

2.1 Macroscopic morphology of SK80

The SK80 colonies were slow growing. On the first 4 days, the colonies were brownish-black and yeast-like appearance as shown in Figure 8A. On day 5-7), they became suede-like, olivaceous grey and mold-like as shown in Figure 8B and 8C.

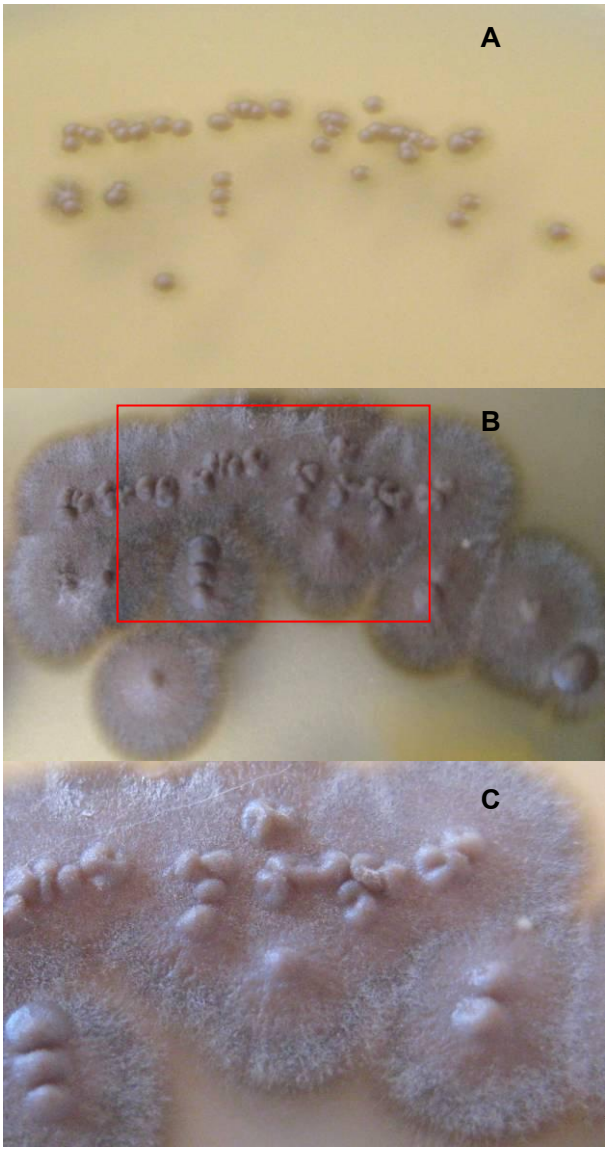


Figure 8 The macroscopic morphology of SK80 grown on YM media agar plate: **(A)** day 1-4; brownish-black and yeast-like appearance, **(B)** day 5-7; suede-like, olivaceous grey and mold-like appearance, **(C)** enlarged picture of Figure B (in square).

2.2 Microscopic morphology of SK80

The microscopic study of SK80 with Gram's staining at the initial growth, it appeared as unicellular, ovoid to elliptical, budding yeast-like cells which were hyaline and thin-walled as shown in Figure 9A. On day 5-7, it became darkly pigmented (dematiaceous) and thick-walled. At mold stage, the flask-shaped to cylindrical phialides without distinctive collarettes were produced. Conidia were hyaline to pale brown, round to ovoidal cell. The size of conidia was about 2.0-4.0 x 2.5-6.0 μm with smooth-walled and accumulated in slimy balls (glioconidia) as shown in Figure 9B.

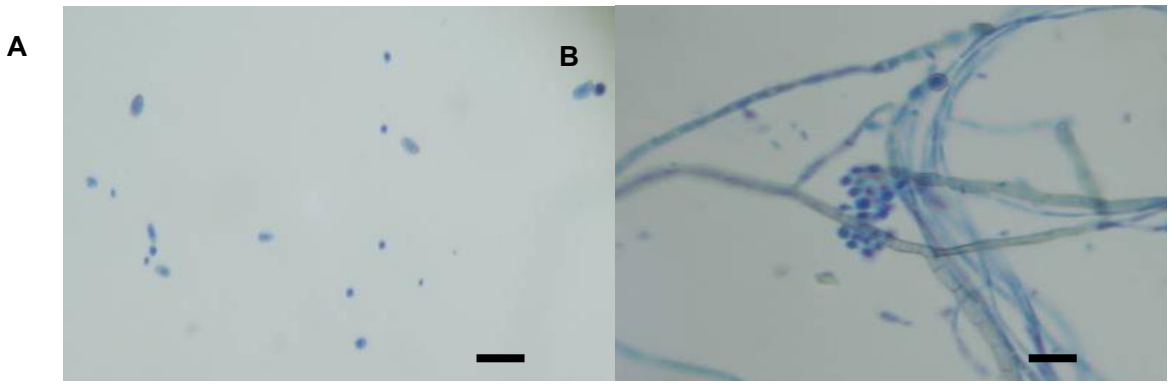


Figure 9 The microscopic morphology of SK80 (Gram stain, light microscopic at 400 magnifications) **(A)** day 1-4; yeast-like cells appearance, unicellular, ovoid to elliptical, budding, **(B)** day 5-7; mold-like appearance, flask-shaped to cylindrical phialides without distinctive collarettes; hyaline to pale brown conidia. Bar is 10 microns.

2.3 Identification of SK80 by DNA sequencing

The 600bp of 28S rDNA sequence D1/D2 was amplified using primer of NL-1 and NL-4. The 28S rDNA sequence was determined[165] and performed similarity search (Appendix). The result showed that the amplified 28S rDNA have 99% similarity to *Exophiala dermatitidis*.

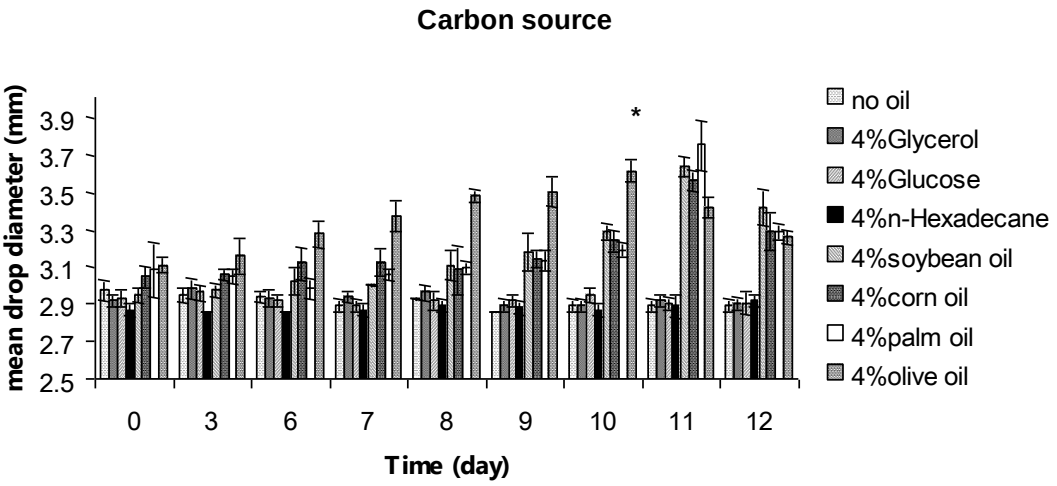
From macroscopic and microscopic morphological study including 28S rDNA sequence results, it indicated that SK80 is *Exophiala dermatitidis*.

3. Optimization of culture conditions

3.1 Carbon source effect

3.1.1 Carbon source type

The results of drop collapse test (Figure 10) suggested that biosurfactant production was increased with time of cultivation. The surfactant activity was detected in vegetable oils supplemented group, i.e. soybean oil, corn oil, palm oil and olive oil. Basal culture medium without carbon source (no oil) as well as supplementation with glycerol, glucose or n-hexadecane could not induce the biosurfactant production. The surfactant activity values obtained from all days of C-source cultivation were statistically tested and palm oil was shown to induce highest surfactant activity on day 11 which gave the significant difference at P-value < 0.05.



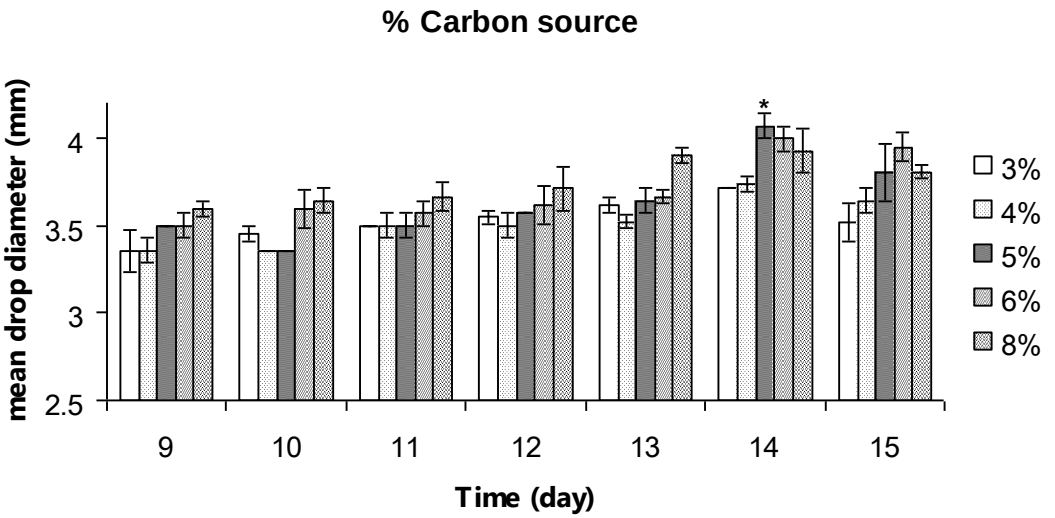
* Significant differences, *P*-value < 0.05

Figure 10 Effect of type of carbon source used for cultivation on surfactant activity. The condition used for cultivation was basal medium (0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract) supplemented with various types of carbon source, pH 5.3 shaking at 200 rpm at 30°C.

As the result of surfactant activity suggested the highest activities obtained with palm oil-basal medium, and palm oil substrate give the lowest production cost, so palm oil was used for further study.

3.1.2 Carbon source concentration

To evaluate the optimum concentration of palm oil used for supplementation into the basal medium, various concentrations of palm oil from 3 to 8% were used and the surfactant activity was measured. The results (Figure 11) showed that the highest activities obtained with 5% palm oil-basal medium at day 14 and this concentration of palm oil gave the significant difference to other concentrations of palm oil in all days tested at *P*-value < 0.05. Therefore this medium was used for cultivation in further experiment.



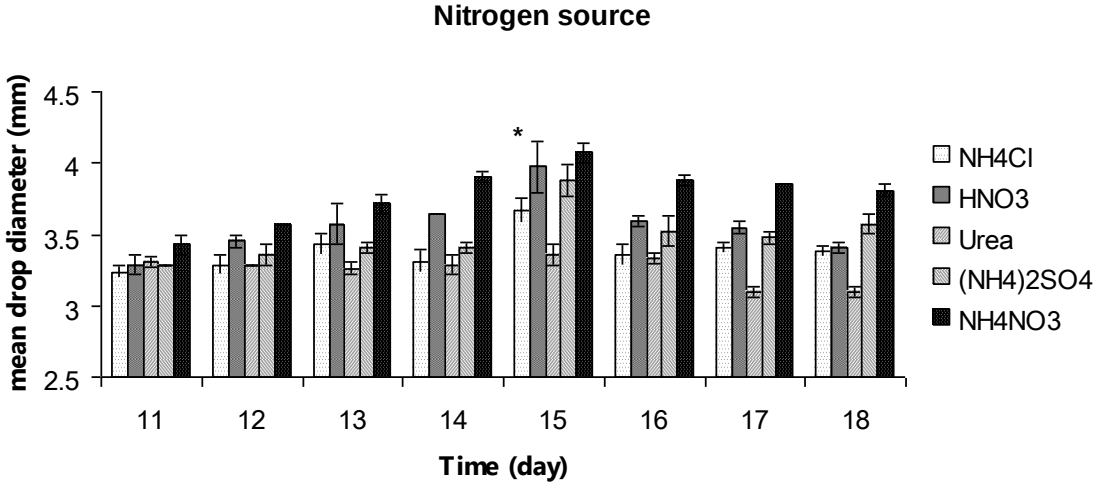
* Significant differences, *P*-value < 0.05

Figure 11 Effect of concentration of palm oil supplemented in the basal culture media on surfactant activity. The condition used for cultivation was basal medium with varying concentration of palm oil, pH 5.3 shaking at 200 rpm at 30°C.

3.2 Nitrogen source effect

3.2.1 Nitrogen source type

The drop collapsing results suggested that biosurfactant production was highest on day 15 of cultivation when using NH_4Cl, HNO_3, $(NH_4)_2SO_4$, NH_4NO_3 as the nitrogen source except urea which could not induce the biosurfactant production (Figure 12). NH_4NO_3 was shown to induce highest surfac alue < 0.05.



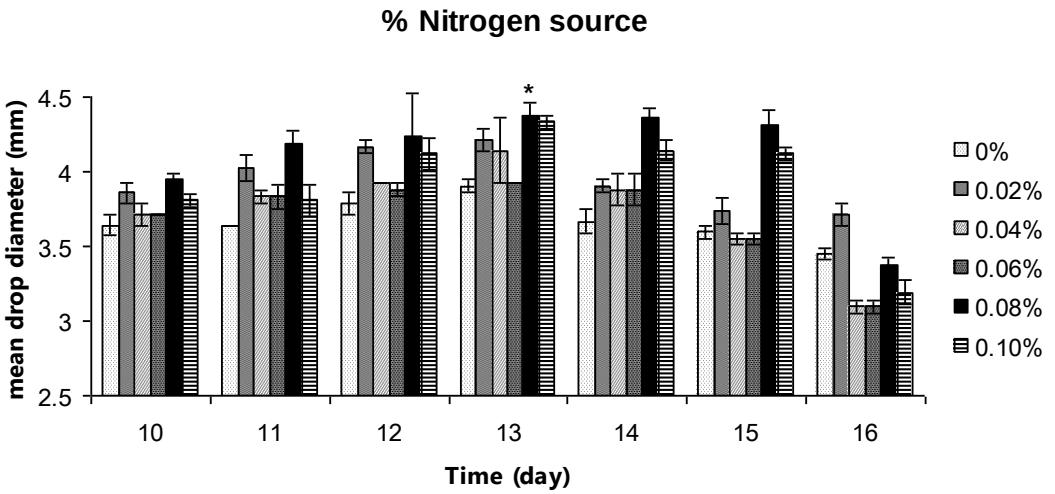
* Significant differences, *P*-value < 0.05

Figure 12 Effect of type of nitrogen sources on surfactant activity. The condition used for cultivation was basal medium supplemented with 5% palm oil and 0.3% of varying type of nitrogen source, pH 5.3 shaking at 200 rpm at 30°C.

The result of surfactant activity suggested the highest activities obtained with NH_4NO_3 supplementation, so NH_4NO_3 was used for further study.

3.2.2 Nitrogen source concentration

To evaluate the optimum concentration of NH_4NO_3 used in the basal medium, various concentration of NH_4NO_3 varying from 0 to 0.10% were tested and the surfactant activity was measured. The results (Figure 13) showed that the highest activities obtained when 0.08% NH_4NO_3 supplemented medium was used at day 13 to day 15 and this concentration gave the significant difference to other concentrations of NH_4NO_3 in all days tested at *P*-value < 0.05. Therefore, this medium was used for cultivation in further experiment.

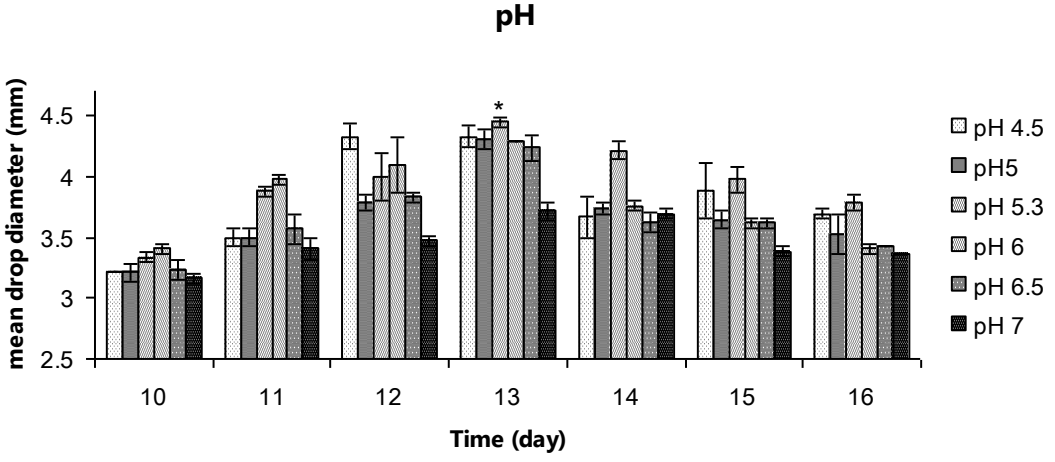


* Significant differences, *P*-value < 0.05

Figure 13 Effect of concentration of nitrogen sources on surfactant activity. The condition used for cultivation was basal medium containing 5% palm oil with varying concentration of NH_4NO_3, pH 5.3 shaking at 200 rpm at 30°C.

3.3 Effect of pH in medium

To optimize pH of the culture medium pH varying from 4.5 to 7 were adjusted and the surfactant activity of the cultivation broth was measured. The results (Figure 14) showed that the highest activities obtained at pH 5.3 at day 13 and this pH gave the significant difference to other pH in all days tested at *P*-value < 0.05. So, this pH was used for cultivation in further experiment.



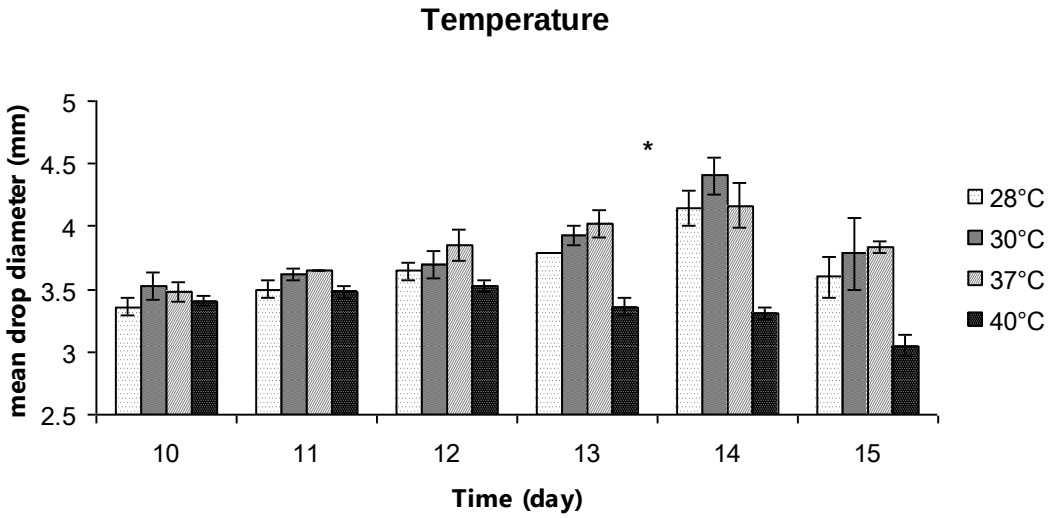
* Significant differences, *P*-value < 0.05

Figure 14 Effect of pH on surfactant activity. The condition used for cultivation was basal medium containing 5% palm oil, 0.08% NH_4NO_3 with varying pH, shaking at 200 rpm at 30°C.

3.4 Effect of temperature

The basal medium with 5% palm oil, 0.08% NH_4NO_3, at pH 5.3 was used as culture medium for testing the optimum temperature. The result of surfactant activity showed in Figure 15

suggested the optimum temperature for cultivation was 30°C and this temperature gave the significant difference of surfactant activity to other temperatures in all days tested at *P*-value < 0.05 level.



* Significant differences, *P*-value < 0.05

Figure 15 Effect of temperature of cultivation on surfactant activity. The condition used for cultivation was basal medium containing 5% palm oil, 0.08% NH_4NO_3 pH 5.3 with varying temperatures shaking at 200 rpm.

The result showed that the best condition for cultivation of *Exophiala dermatitidis* SK 80 for the production of biosurfactant to gain the maximum of surfactant activity is the basal medium (0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract) supplemented with 5% palm oil, 0.08% NH_4NO_3, pH 5.3 and shaking 200 rpm at 30°C.

3.5 Time course of cell growth and biosurfactant production

The production of biosurfactant and growth of *Exophiala dermatitidis* SK 80 were consistency (Figure 16). The cell growth was increasing on day 10-14 and decreasing at day 15 which was correlated to the biosurfactant production. They showed the highest both in surfactant activity and cell growth when the cultivation time was 14 days.

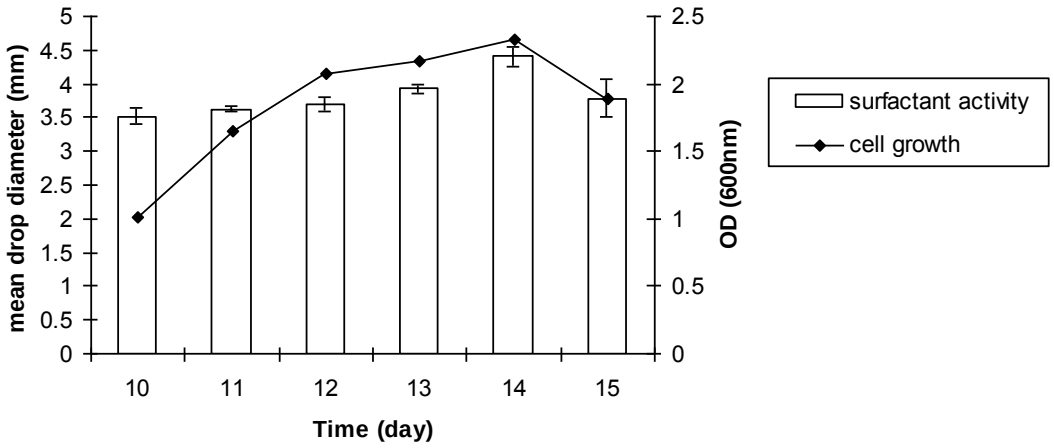


Figure 16 Time course of cell growth and biosurfactant production of *Exophiala dermatitidis* SK 80 in the optimal condition which was basal medium (0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract) supplemented with 5% palm oil, 0.08% NH_4NO_3, pH 5.3 and shaking 200 rpm at 30°C.

4. Extraction and purification of biosurfactant

After cultivation of *Exophiala dermatitidis* in 6 liters basal salt medium with optimum culture conditions (5% palm oil, 0.08% NH_4NO_3, pH 5.3 and 30°C) the culture medium was extracted with ethyl acetate and evaporated. 239.86 g of crude extract obtained was dissolved in methanol/hexane by ratio 1:2 and 24 g of yellowish-brown crude extract was obtained from methanol fraction.

4.1 First column chromatography

The 24 g of crude extract was purified by silica gel column chromatography. The mixtures of organic solvent used in the first column as eluting solvents and number of fractions obtained were shown in Appendix (Table 23).

The purity of the fractions obtained was visualized on silica gel TLC plate by ρ-anisaldehyde (Figure 17). The fractions which showed similar R_f values were pooled, dried and tested for surfactant activity using modified drop collapsing test (Table 8). The pooled fraction named F6 showed the highest surfactant activity and was used for the further purification.

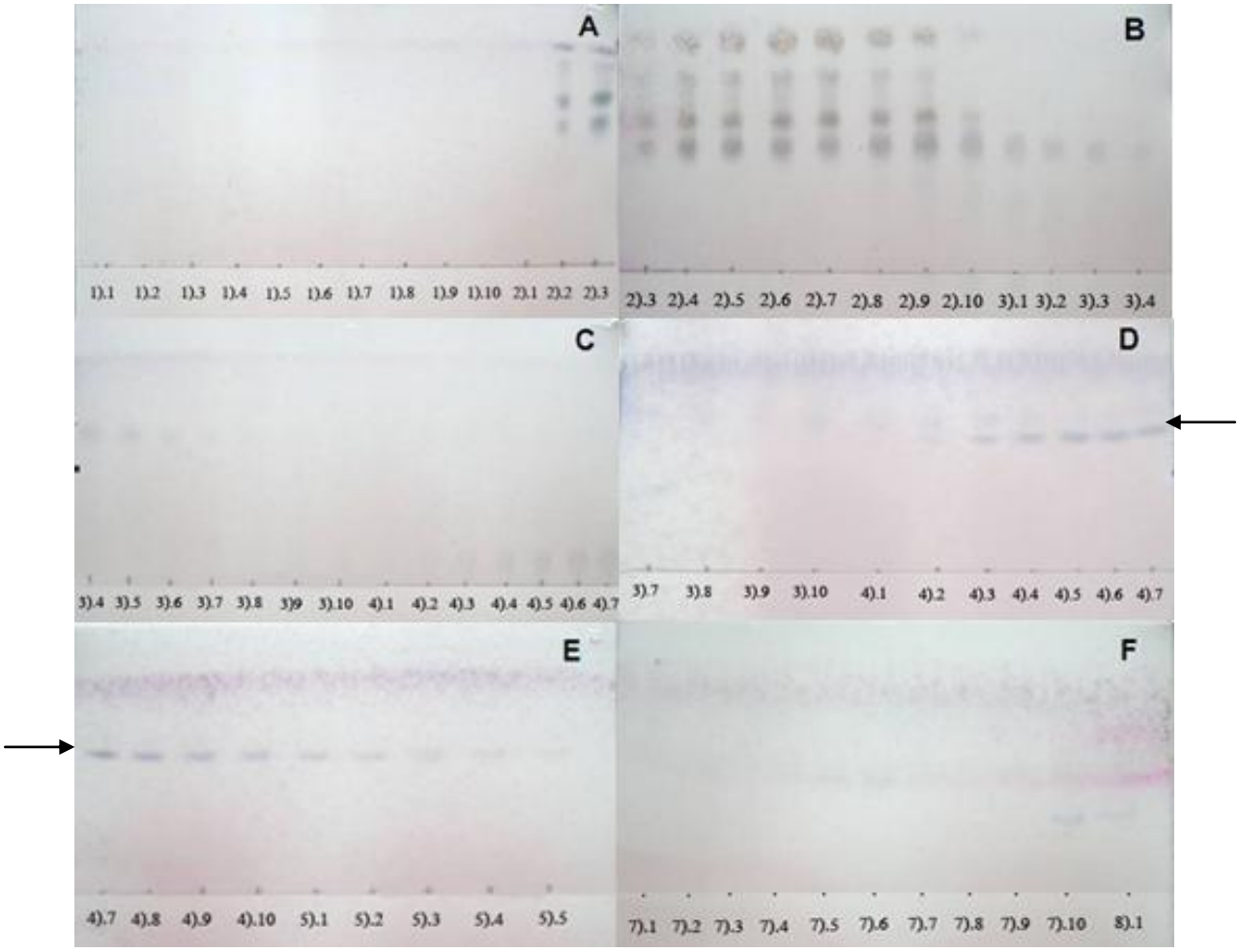


Figure 17 Thin layer chromatography of some fractions eluting from first silica gel chromatography. One μl of all fractions collected was spotted on silica gel thin layer chromatography and spraying with ρ-anisaldehyde reagent. Mobile phase: plate **A-C** hexane:ethyl acetate, 7/3(v/v), plate **D-F,** chloroform:methanol:H_2O, 65/15/2(v/v); plate fractions 1).1-2).3; plate **B.** fractions 2).3-3).4; plate **C.** fractions 3).4-4).7; plate **D.** fractions 3).7-4).7; plate **E.** fractions 4).7-5).5; plate **F.** fractions 7).1-8).1; **Arrow** showed the position of highest surfactant activity fractions (fractions 4.3-5.4).

Table 8 Surfactant activity of the pooled fractions obtained from the first column chromatography

Solvents used as eluent	Proportion	Pooled fraction No.	Mean drop diameter (mm) (surfactant activity)
hexane:ethyl acetate	(100:0)-(70:30)	F1	3.07±0.07
	(70:30)	F2	3.21±0.00
	(50:50)	F3	3.57±0.07
	(50:50)	F4	3.79±0.17
	(30:70)	F5	3.43±0.04
	(30:70)-(10:90)	F6**	5.71±0.07

ethyl acetate:methanol	(90:10)-(50:50)	F7	3.79±0.00
methanol			2.86±0.04
(negative control)			

** The fraction which gave the highest surfactant activity was used for further purification.

4.2 The second column chromatography

The 0.3 g of pooled (F6) fraction was subjected to purify in the second silica gel column chromatography. The eluting solvent system and number of fraction obtained was shown in Appendix (Table 24).

The purity of the fractions obtained was visualized on silica gel TLC plate by ρ-anisaldehyde reagent as shown in Figure 18 The fractions which showed similar R_f values were pooled, dried and tested for surfactant activity (modified drop collapsing test) (Table 9). The pooled fraction named F6.(6) exhibited the highest surfactant activity was used for the further purification.

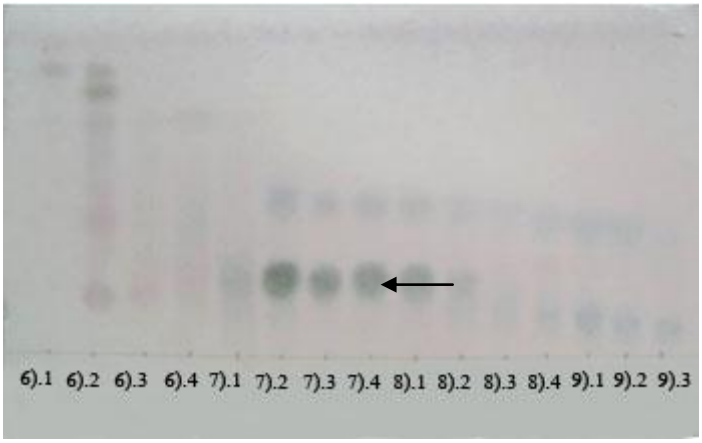


Figure 18 Thin layer chromatography of the fractions eluting from the second silica gel chromatography. One μl of the collected fractions no. 6).1-9).3. was spotted on silica gel chromatography and spraying with ρ-anisaldehyde reagent; Mobile phase; hexane:ethyl acetate, 1/1(v/v); **Arrow** shows the position of highest surfactant activity fractions (fractions 7.2-8.2).

Table 9 Surfactant activity of the pooled fractions obtained from second column chromatography

Solvents used as eluent	Proportion	Pooled fraction No.	Mean drop diameter (mm) (surfactant activity)
chloroform:ethyl acetate	(70:30)	F6.(1)	3.26±0.04
	(70:30)	F6.(2)	3.45±0.04
	(70:30)	F6.(3)	3.81±0.11
	(70:30)	F6.(4)	3.98±0.04

	(70:30)	F6.(5)	4.21±0.00
	(50:50)-(30:70)	F6.(6)**	5.71±0.00
	(50:50)-(0:100)	F6.(7)	4.14±0.07
methanol			2.86±0.04
(negative control)			

** The fraction which gave the highest surfactant activity was used for further purification.

4.3 The third column chromatography

The 0.23 g of pooled (F6.(6)) fraction was used to purify in the third silica gel column chromatography. The eluting solvent system was shown in Appendix (Table 25).

The purity of the fractions obtained was visualized on silica gel TLC plate by ρ-anisaldehyde spray as shown in Figure 19 The fractions which showed similar R_f values were pooled, dried and tested for surfactant activity (modified drop collapsing test) (Table 10). The pooled fraction named F6.(6).2 exhibited the highest surfactant activity.

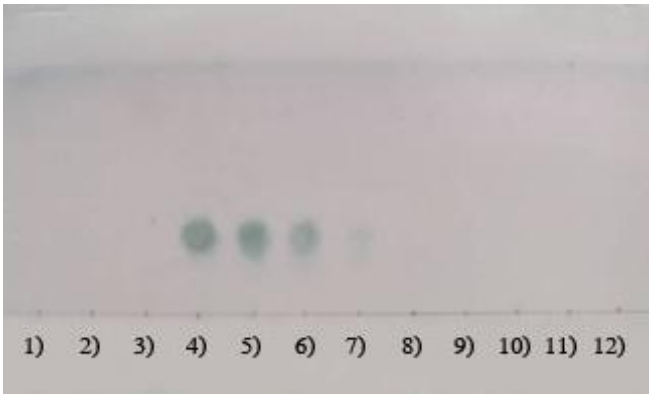


Figure 19 Thin layer chromatography of fractions eluting from third silica gel column chromatography. One μl of all fractions no. 1)-12) were spotted on silica gel chromatography and spraying with ρ-anisaldehyde reagent. Mobile phase; hexane:ethyl acetate, 1/1(v/v); the highest surfactant activity were obtained from the fractions 4-6.

Table 10 Surfactant activity of the pooled fractions obtained from the third column chromatography

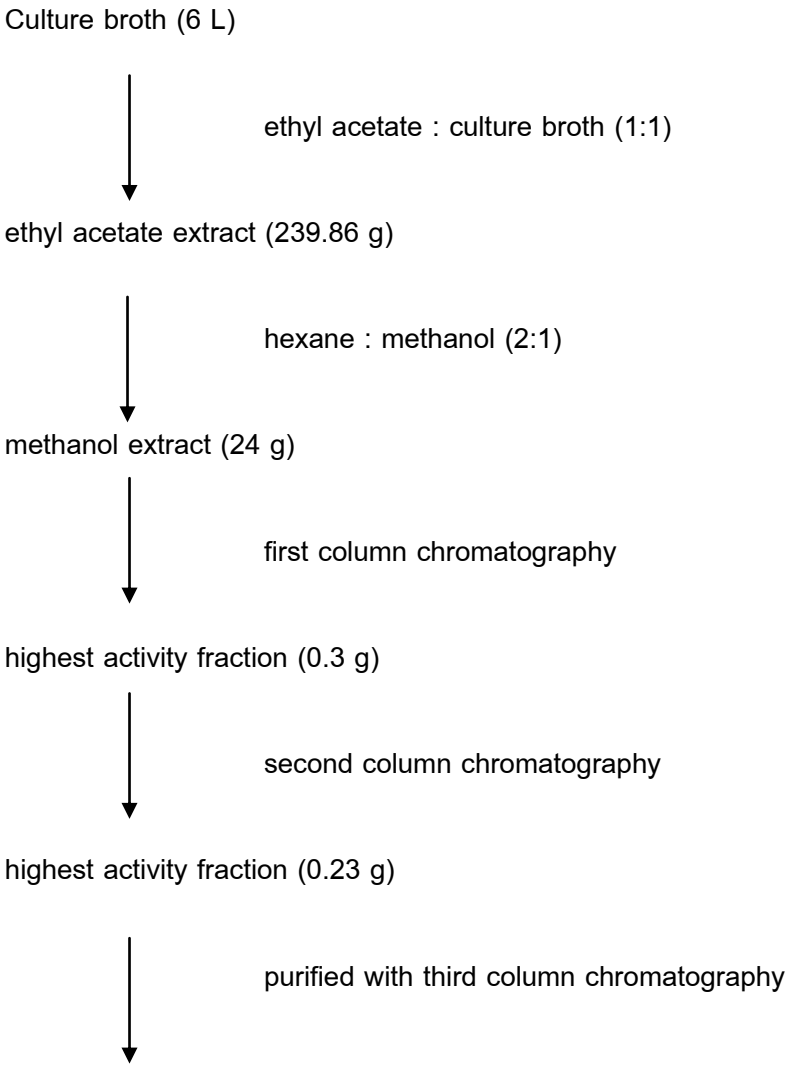
Solvents used as eluent	Proportion	Pooled fraction No.	Mean drop diameter (mm) (surfactant activity)
chloroform:ethyl acetate	(90:10)	F6.(6).1	4.25±0.04
	(80:20)-(60:40)	F6.(6).2**	5.79±0.07
methanol			2.86±0.04
(negative control)			

** The fraction which gave the highest surfactant activity was used for further purification.

The 0.2 g of yellow liquid biosurfactant obtained from the third column chromatography (F6.(6).2) gave one band at R_f value 0.34 (hexane:ethyl acetate, 1/1(v/v)) as shown in Figure 20. Yield of the extracts obtained at each purification steps was shown in Figure 21.



Figure 20 Silica gel thin layer chromatography of the purified fractions F6.(6).2 eluting from third silica gel column chromatography. Mobile phase; hexane:ethyl acetate, 1/1(v/v), detection with ρ-anisaldehyde spray.



highest activity purified fraction (0.2 g)

Figure 21 Yield of the extracts obtained at each purification steps

5. Structure determination of the biosurfactant

The purified biosurfactant (SK80) was analyzed the chemical composition by ^{1}H NMR (Figure 22), ^{13}C NMR (Figure 23), HSQC (Figure 24) and COSY (Figure 25).

NMR spectra of SK80 were taken in $CDCl_3$. The ^{1}H NMR spectrum showed that the signals of $–CH_3$ group were at 0.9 ppm, $–CH_2$ group at 1.3-2.4 ppm and –CH= group at 5.4 ppm of the alkyl chain. The signals of $–CH_2$ group were at 3.6 ppm, –CH group at 3.9 ppm and $–CH_2$ group at 4.1 ppm for the glycerol moiety.

^{13}C NMR spectrum of SK80 showed the signals at 14.8 ppm for $–CH_3$, at 25.6, 27.8, 30.0 and 35.0 ppm for $–CH_2$, at 130.7 for –CH= of the alkyl chain. The signals of $–CH_2$ were at 64.0 and 65.8, –CH group at 71.0 of the glycerol moiety and at 175.0 for C=O of ester.

HSQC and COSY experiment confirmed these attributions.

Apart from that main product, NMR spectra revealed the presence of a minor product which could not be identified.

The result was established that SK80 was a *sn*-1-monoacylglycerol which was proposed by the following formula:

$$CH_3–(CH_2)_6–CH_2–CH=CH–CH_2–(CH_2)_4–CH_2–CH_2–COO–CH_2–CH(OH)–CH_2–OH$$

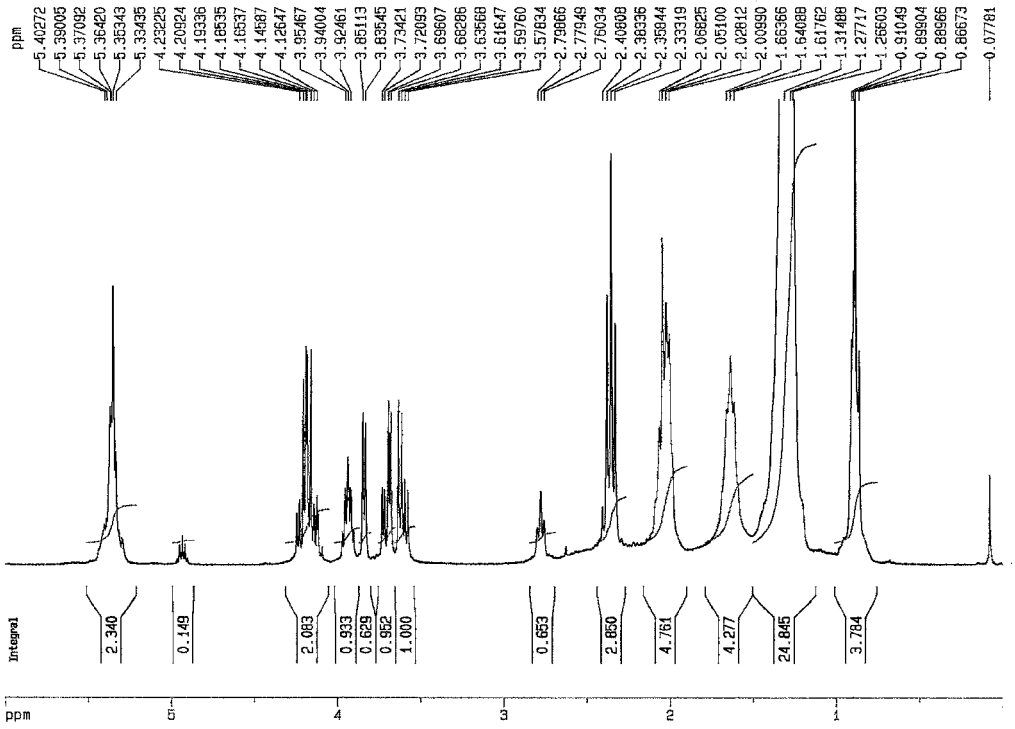


Figure 22 ^{1}H NMR spectrum of purified biosurfactant, in $CDCl_3$, 300 MHz

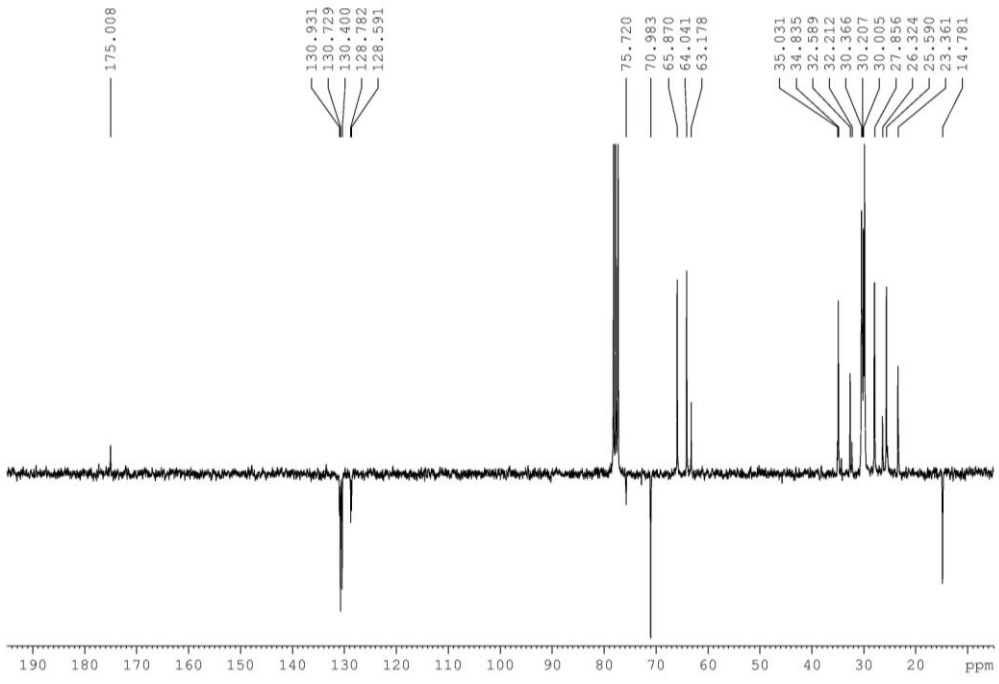


Figure 23 ^{13}C NMR spectrum of purified biosurfactant, in $CDCl_3$, 300 MHz

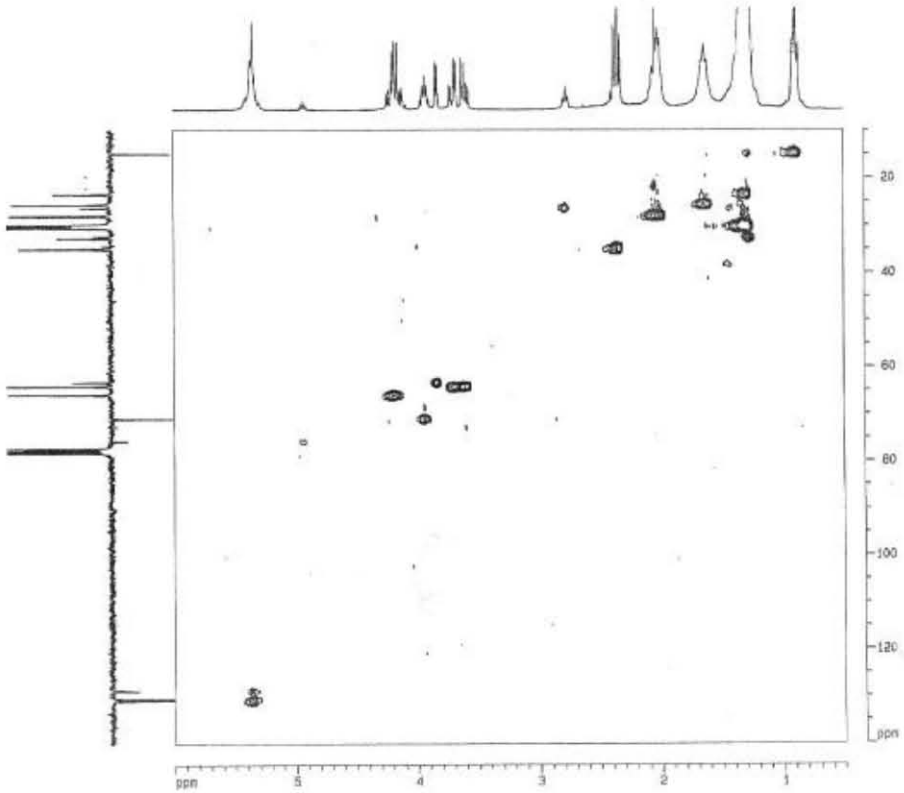


Figure 24 2D spectrum (HSQC) of purified biosurfactant, in $CDCl_3$, 300 MHz

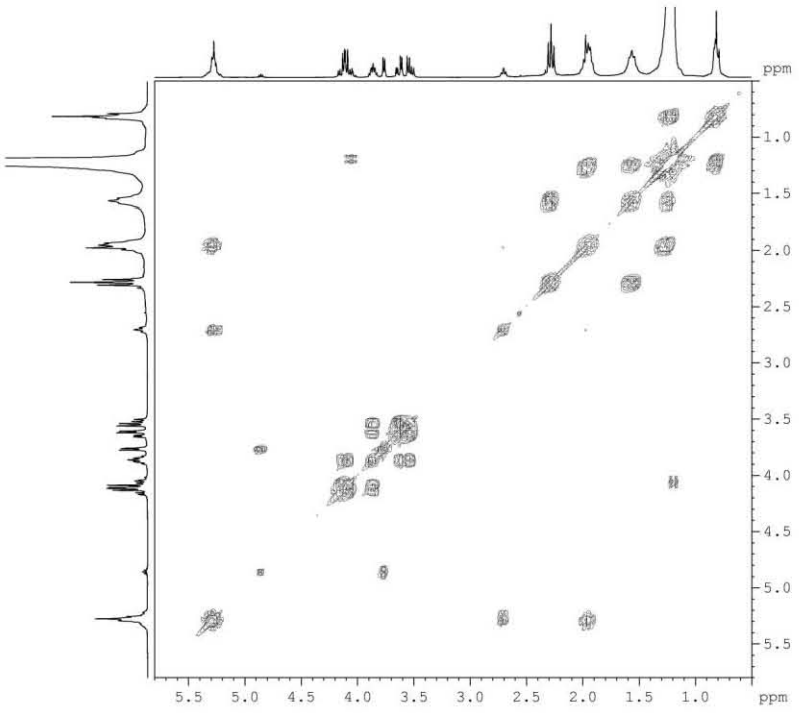


Figure 25 2D spectrum (COSY) of purified biosurfactant, in $CDCl_3$, 300 MHz

APCI MS experiments gave a molar mass of 356 g/mol as shown in Figure 26 for the main product. This value is completely consistent with the previous formula. In addition, several peaks in

APCI MS were consistent with fragmentation schemes proposed for monoacylglycerols[168] and confirmed the presence of a long aliphatic hydrocarbon chain within the molecule.

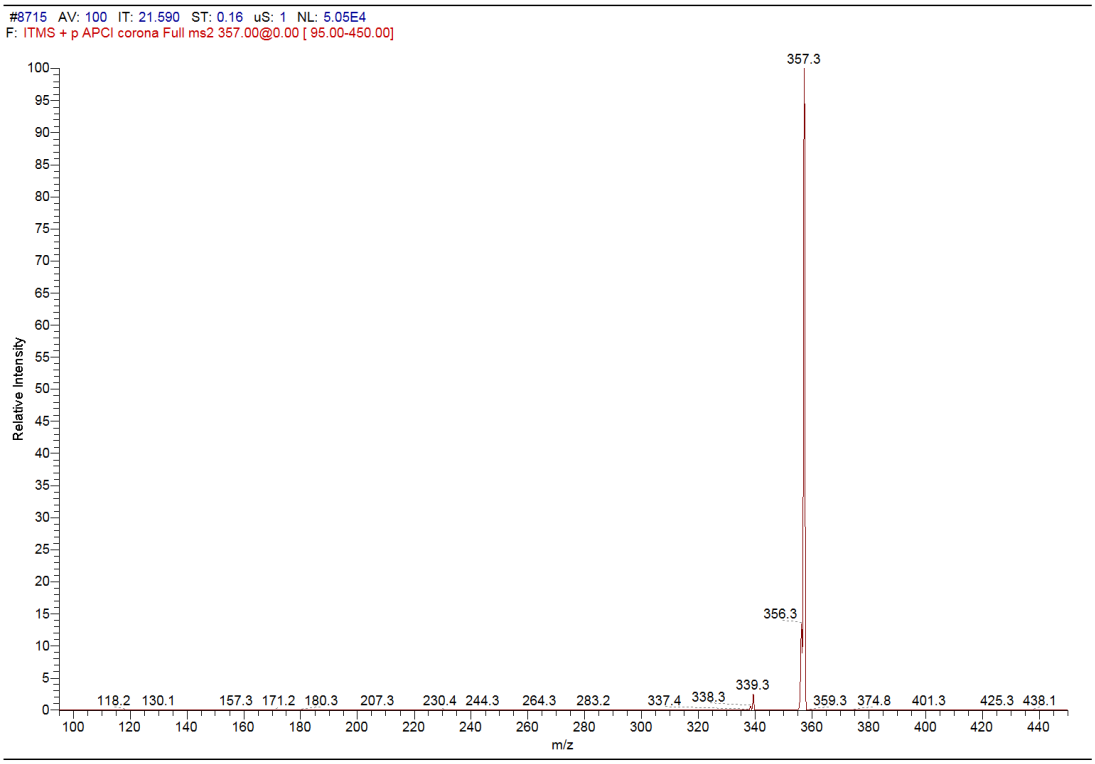


Figure 26 Mass spectrometry of purified biosurfactant

The results were concluded that *E. dermatitidis* can produce a biosurfactant based on glycerol ester of oleic acid which oleoyl glycerol, glyceryl oleate, glycerol monooleate and monoolein were synonymous.

6. Synthesis of monoacylglycerol

The monoacylglycerol (monomyristin) was synthesized by mixture of solketal and myristyl chloride using pyridine as a catalyst and adding trifluoroacetic acid to continue the second synthetic process.

The intermediate product which was obtained after mixing myristoyl chloride and solketal was evaporated and purified by adsorption chromatography on silica gel column. The solvent system for eluting of this intermediate product by column chromatography was ethyl acetate:petroleum ether (5:95).

The purity was checked with thin layer chromatography (Figure 27). The purified intermediate product was collected from fraction no. 11-14 and the structure was analyzed by ^{1}H NMR (Figure 28).

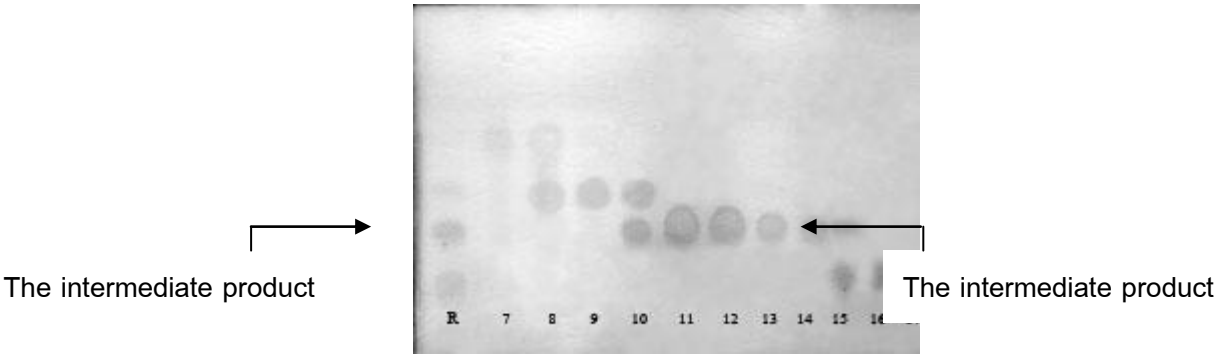


Figure 27 Thin layer chromatography of product from the first synthetic reaction of monomyristin, spraying with phosphomolybdic acid (PMA) reagent. Mobile phase; ethyl acetate:petroleum ether, 1/9(v/v).

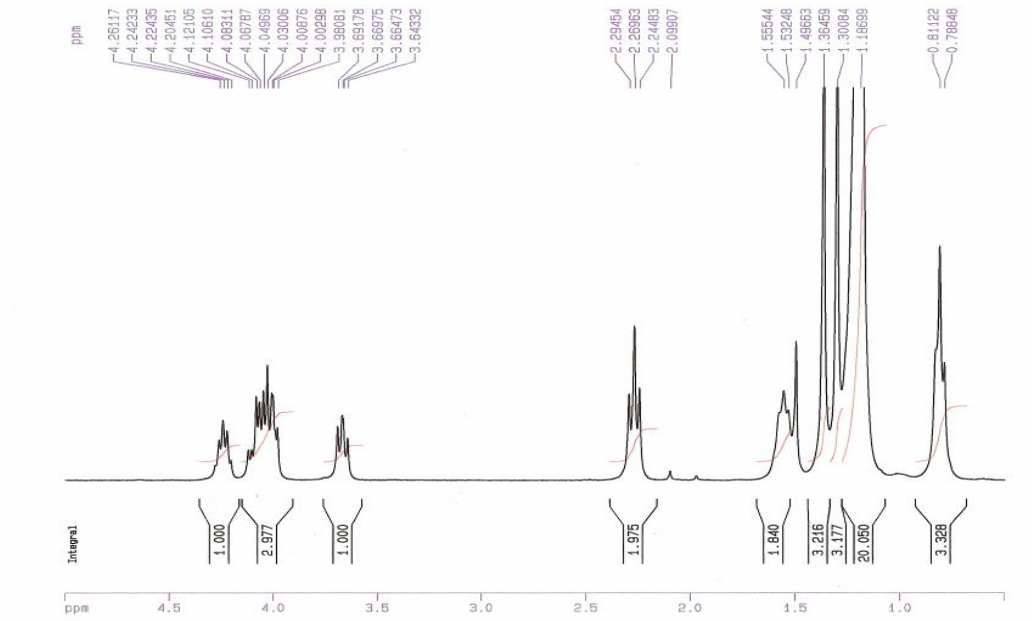


Figure 28 ^{1}H NMR spectrum of purified intermediate product of monomyristin, in $CDCl_3$, 300 MHz

Monomyristin was the end product after the last step of the synthesis which is a deprotection process. This product was purified by silica gel column chromatography. The solvent system for eluting this product was detailed in Table 11.

Table 11 The solvent system used as eluting solvent in the column chromatography

No. of eluting solvent	Mixture of organic solvent	Proportion	No. of fractions obtained
1	chloroform:ethyl acetate	90:10	1).1 - 1).10
2	chloroform:ethyl acetate	80:20	2).1 - 2).10
3	chloroform:ethyl acetate	70:30	3).1 - 3).10
4	chloroform:ethyl acetate	60:40	4).1 - 4).10
5	chloroform:ethyl acetate	50:50	5).1 - 5).10

The purity of monomyristin was checked by thin layer chromatography (Figure 29) and the structure was analyzed by ^{1}H NMR (Figure 30).

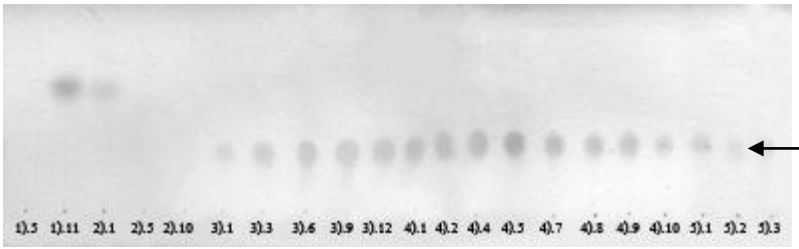


Figure 29 Thin layer chromatography of product from the second synthetic reaction of monomyristin, spraying with phosphomolybdic acid (PMA) reagent. Mobile phase; ethyl acetate:petroleum ether, 1/9(v/v).

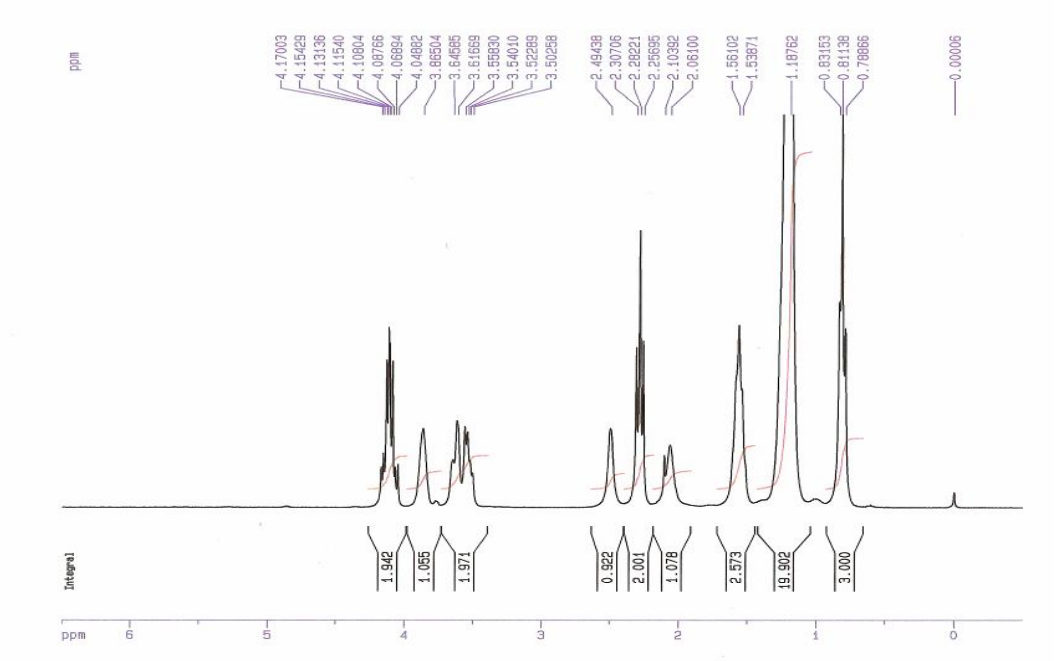


Figure 30 ^{1}H NMR spectrum of purified monomyristin, in $CDCl_3$, 300 MHz

7. Synthesis of the hydrophobic derivatives of dextran, $DexP_{20}$

The result of ^{1}H NMR spectrum in DMSO (Figure 30) was relevant to the structure of modified dextran as described in Appendix. The percentage of the modified molecule compared with non modified molecule was τ, calculated from the surface area of the peak ($\mathbf{A_1}$, $\mathbf{A_2}$) from ^{1}H NMR spectrum result (Figure 31) as the formula below. The polymer obtained is called as $DexP_{\tau}$.

$$\tau = 100 \times \frac{\text{number of attached phenoxy groups}}{\text{number of glucose units in the chains}}$$

$$\tau = \frac{4 \times \mathbf{A}_2/5}{\mathbf{A}_1 + (\mathbf{A}_2/5)} \times 100$$

$\mathbf{A}_1$ = surface area of the peak between 4.2 and 5.2

$\mathbf{A}_2$ = surface area of the peak between 6.5 and 7.5

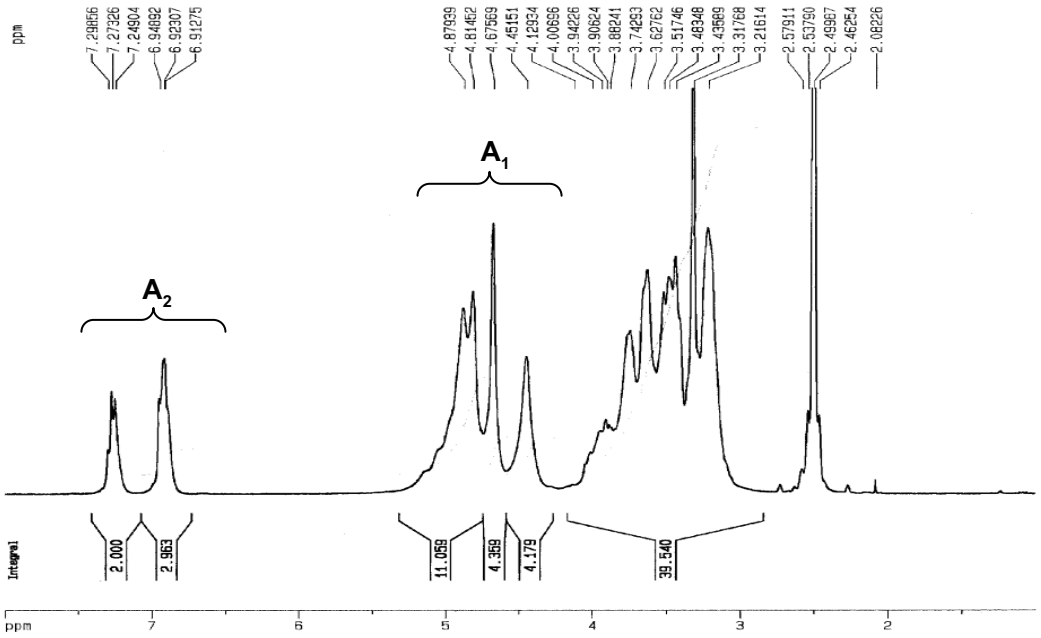


Figure 31 ^{1}H NMR spectrum of synthesized $DexP_{20}$, in DMSO, 300 MHz

$$\tau = 4 \times \frac{(11.059+4.359+4.179)/5}{(11.059+4.359+4.179)+(2.000+2.963)/5} \times 100$$

$$= 19.29\%$$

The result showed the percentage of the modified molecules compared with non modified molecules approximately is 20%.

8. Surfactant properties determination

8.1 Interfacial tension measurements

By using a K8 surface tensiometer (Krüss, Germany), interfacial tension was measured between chloroform and water of three conditions.

- Between chloroform and water containing diluted $DexP_{20}$.
- Between chloroform containing diluted Glycerol ester of oleic acid (SK80) and water.
- Between chloroform containing diluted monoacylglycerol (monomyristin) and water.

The result of interfacial tension value of $DexP_{20}$, Glycerol ester of oleic acid (SK80) and r

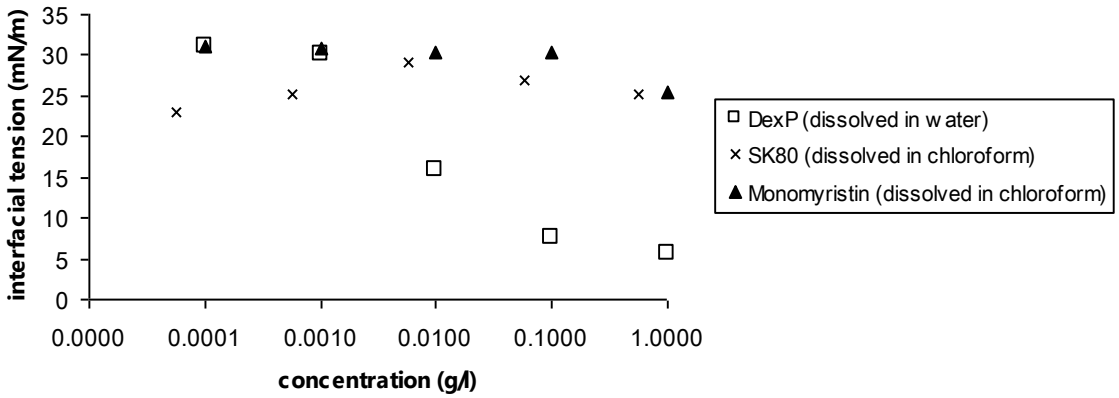


Figure 32 Chloroform to water interfacial tension of solution of $DexP_{20}$, Glycerol ester of oleic acid (SK80) and monomyristin

8.2 Hydrophobic-lipophilic balance (HLB)

Davies's group contribution method[79], Formulation:

$$HLB = \sum \text{(Hydrophilic group contribution)} - \sum \text{(Hydrophobic group contribution)} + 7$$

8.2.1 Glycerol ester of oleic acid (SK80)

Glycerol ester of oleic acid has the following structure:

$$CH_3–(CH_2)_6–CH_2–CH=CH–CH_2–(CH_2)_4–CH_2–CH_2–COO–CH_2–CH(OH)–CH_2–OH$$

Group contribution of the hydrophobic groups:

Structure	value	amount	Total value
CH_3–	0.475	1	0.475
–CH_2–	0.475	16	7.6
-CH=	0.475	1	0.475
-CH-	0.475	2	0.95

Group contribution of the hydrophilic groups:

Structure	value	amount	Total value
-CO-	0.2	1	0.2
-O-	1.3	1	1.3
–OH	1.9	2	3.8

HLB = Σ (0.2+1.3+3.8) - Σ (0.475+7.6+0.475+0.95) + 7

= 2.8

8.2.1 Monomyristin

Monomyristin has the following structure:

$$CH_3–(CH_2)_{12}–COO–CH_2–CH(OH)–CH_2–OH$$

Group contribution of the hydrophobic groups:

Structure	value	amount	Total value	
CH_3–	0.475	1		0.475
–CH_2–		0.475	14	6.65
-CH-		0.475	1	0.475

Group contribution of the hydrophophilic groups:

Structure	value	amount	Total value	
-CO-		0.2	1	0.2
-O-		1.3	1	1.3
–OH		1.9	2	3.8

HLB = Σ (0.2+1.3+3.8) - Σ (0.475+6.65+0.475) + 7

= 4.7

9. Preparation of nanoparticles

9.1 Emulsion/solvent evaporation method

The nanoparticles were prepared by emulsion/solvent evaporation method in varying conditions. The compositions used for nanoparticles preparation in each condition were described in 9.1.1-9.1.7.

9.1.1 aqueous phase: 10 ml of a solution of the modified dextran ($DexP_{20}$) in milliQ water at various concentration; 0.5, 1, 2, 3, 4, 6, 8 g/L

organic phase: 1 ml of dichloromethane solution containing 25 mg/ml Polylactide (PLA)

The first experiment was displayed by using only PLA as a material in organic phase. The result of nanoparticles size in various concentration of $DexP_{20}$, measuring at 0 and 24 h was shown in Figure 33. 2 g/L $DexP_{20}$ led to the smallest PLA nanoparticles. Between 0 and 24 h, only 8 g/L $DexP_{20}$ showed small difference of nanoparicles size.

2 g/L $DexP_{20}$ was defined as good concentration for using in further preparation of

F

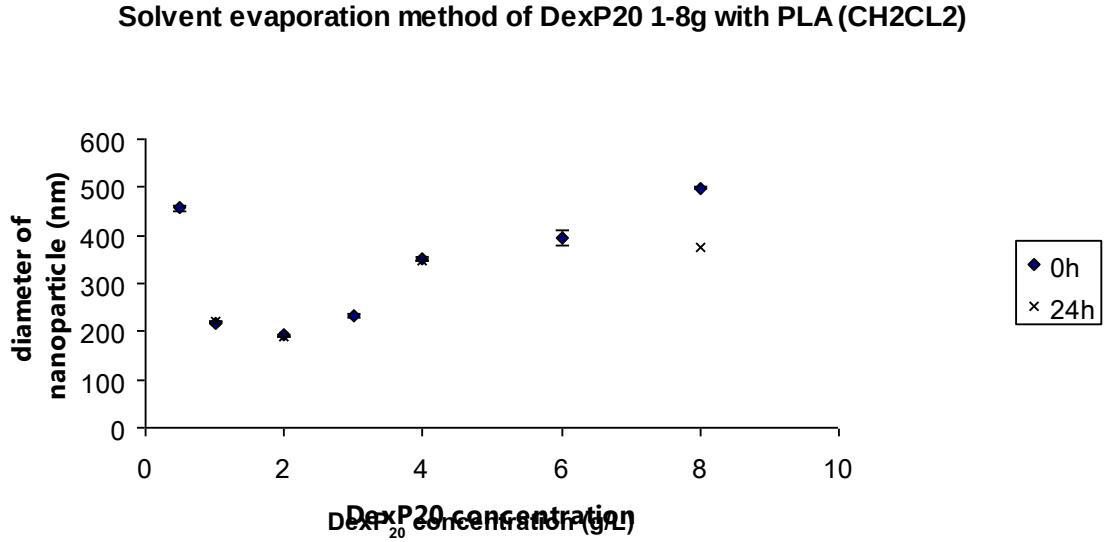


Figure 33 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of $DexP_{20}$ concentration in the aqueous phase at 0 h (♦), 24 h (✕). The PLA concentration in the organic phase was 25 mg/ml.

9.1.2 aqueous phase: 10 ml of a solution at 2 g/L of modified dextran ($DexP_{20}$) in milliQ water

organic phase: 0.5, 1, 1.5, 2 ml of Polylactide (PLA) in dichloromethane at various concentration; 5, 10, 25, 50 mg/ml

This experiment was conducted for determination of the conditions which influenced the nanoparticles diameter. The nanoparticles size were determined at 0 h with various concentration of PLA and volume of organic phase (Figure 34) and weight% of (PLA)/(PLA+$DexP_{20}$) (Figure 35). The result showed that the ratio between $DexP_{20}$ and PLA had a great influence on the nanoparticles size but the volume of organic phase did not affect the size of nanoparticles

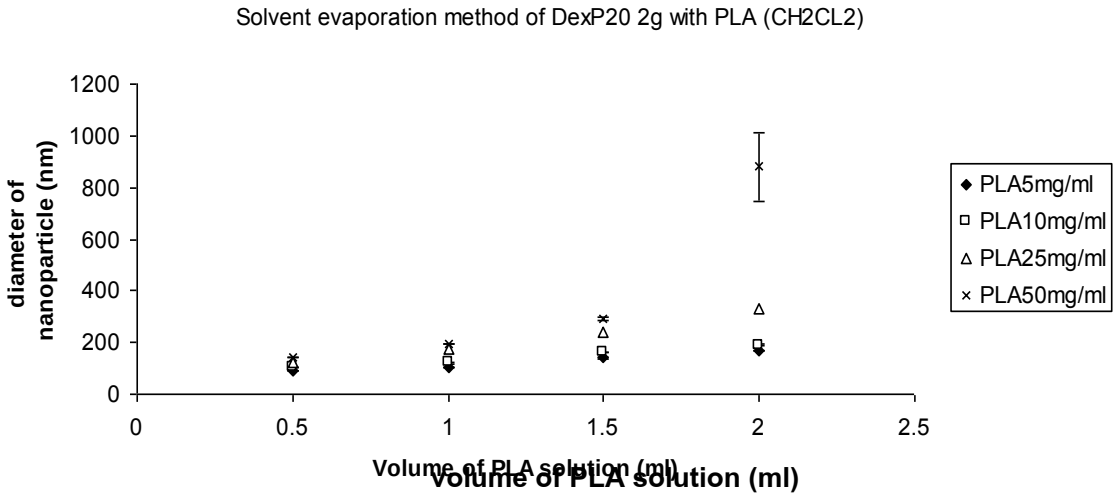


Figure 34 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function c ntration was v

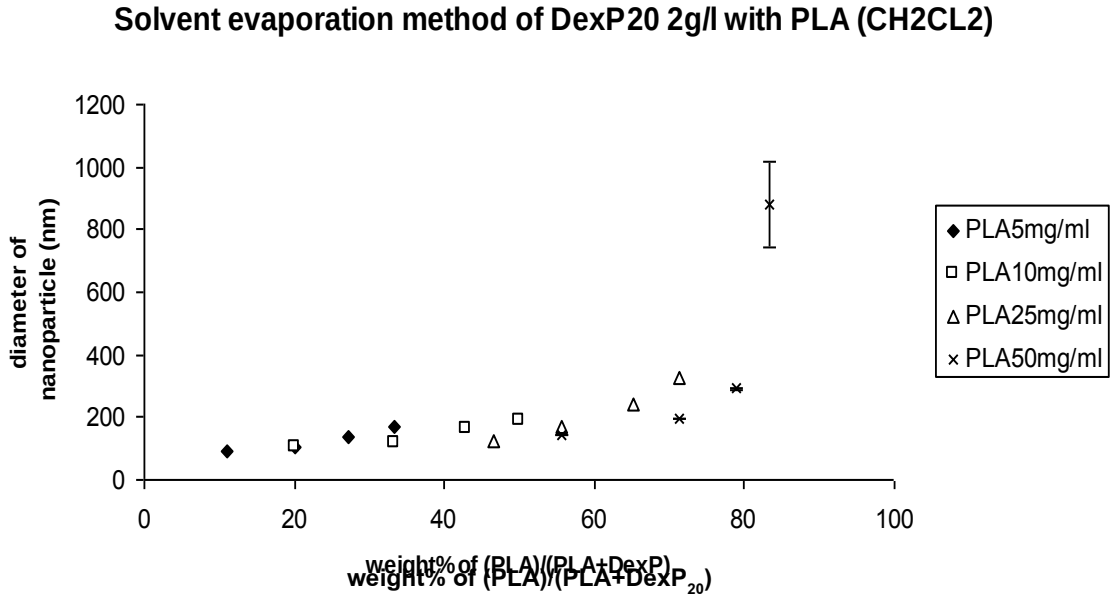


Figure 35 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of weight% of (PLA)/(PLA+$DexP_{20}$). The $DexP_{20}$ concentration in the aqueous phase was 2 g/L. The PLA concentration was varied: 5 mg/ml (♦), 10 mg/ml (□), 25 mg/ml (Δ), 50 mg/ml (✕).

9.1.3 aqueous phase: 10 ml of a solution of the modified dextran ($DexP_{20}$) in milliQ water at various concentration; 0, 1, 2, 5, 10 g/L

organic phase: 1 ml of dichloromethane solution containing 25 mg/mL $DexC10_{164}$

Only $DexC10_{164}$ was used as a material in organic phase in this experiment. The size of $DexC10_{164}$ nanoparticles in various concentration of $DexP_{20}$, measuring at 0h was shown in Figure 36. The diameter of particles was decreased when increasing of $DexP_{20}$ concentration. The submicronic particles were obtained when $DexP_{20}$ at concentrations 5 g/L and higher were used. The nanoparticles showed the smallest size at 10 g/L $DexP_{20}$. So, this concentration of $DexP_{20}$ was used f

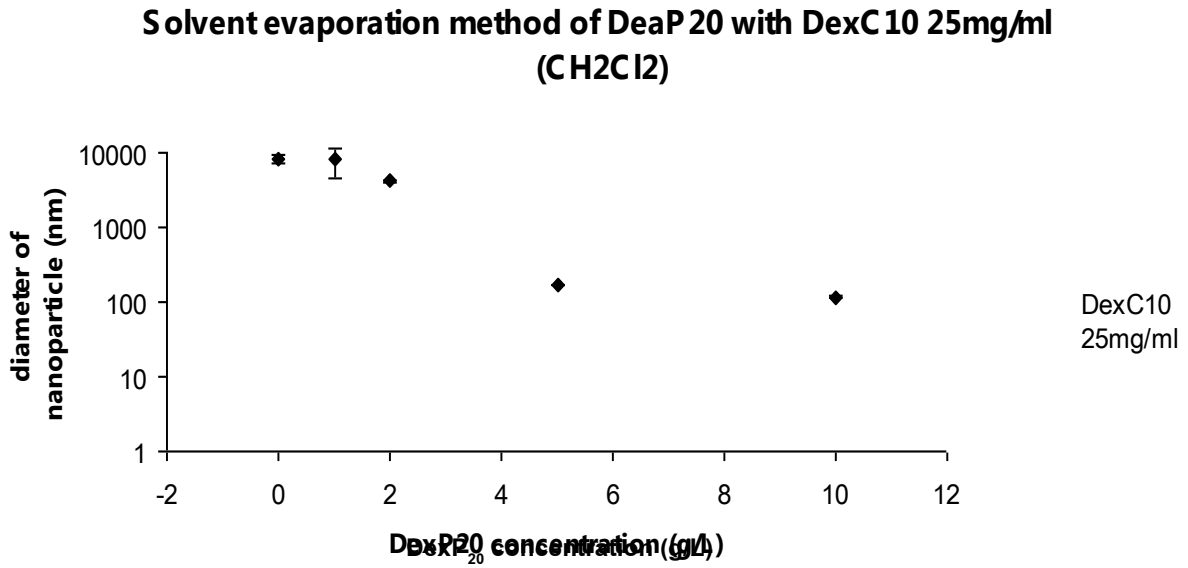


Figure 36 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of $DexP_{20}$ concentration in the aqueous phase. The $DexC10_{164}$ concentration in the organic phase was 25 mg/ml.

9.1.4 aqueous phase: 10 ml of a solution at 2 g/L of modified dextran ($DexP_{20}$) in milliQ water

organic phase: 0.5, 1, 1.5, 2 ml of a solution of monomyristin in dichloromethane at various concentration; 5, 25 mg/ml monomyristin

This experiment was the preparation of nanoparticles by using only monomyristin as a material in organic phase. The result of nanoparticles size in various concentration of monomyristin and volume of organic phase when measuring at 0 h was shown in Figure 37. The results showed t

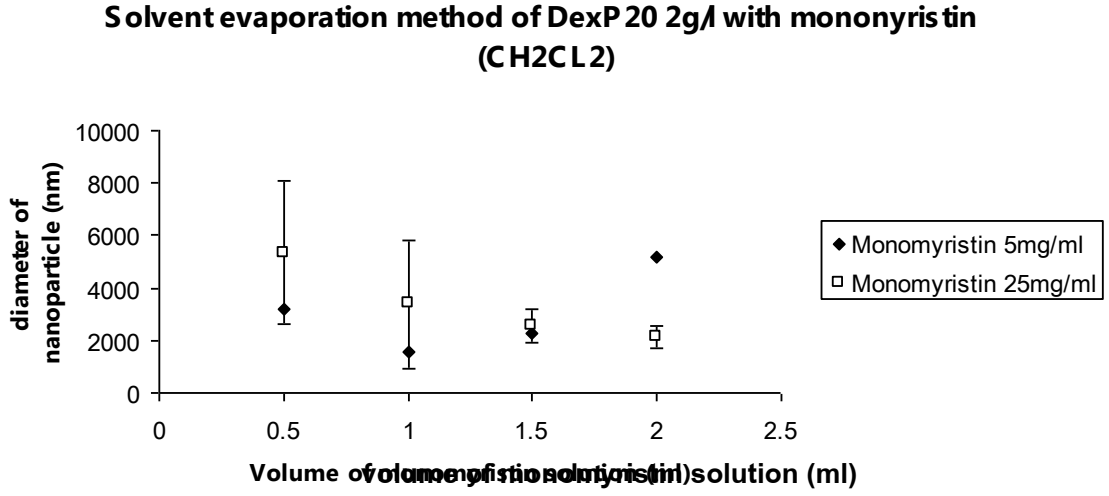


Figure 37 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of volume of monomyristin solution. The $DexP_{20}$ concentration in the aqueous phase was 2 g/L. The monomyristin concentration was varied: 5 mg/ml (♦), 25 mg/ml (□).

9.1.5 aqueous phase: 10 ml of a solution at 2 g/L of the modified dextran ($DexP_{20}$) in milliQ water

organic phase: 1 ml of a solution in dichloromethane at 25 mg/ml of a mixture of monomyristin/PLA in various proportions

In this experiment, PLA and monomyristin was used the organic phase. The proportion of both materials was varied. The particle size according to weight% of (monomyristin)/(monomyristin+PLA), measuring at 0 and 24 h was showed in Figure 38. Either micronic or macroscopic aggregation was occurred in the suspension of all condition. At proportion of monomyristin higher than 50 weight%, the diameter of nanoparticles at 0 and 24 h was consistent.

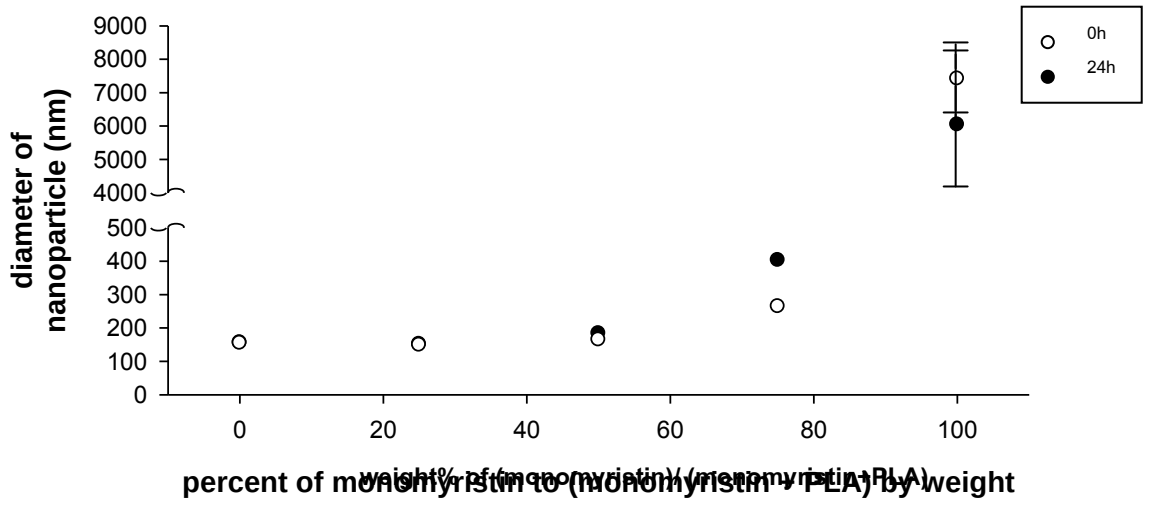


Figure 38 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of weight% of (monomyristin)/(monomyristin+PLA) with a total concentration kept at 25 mg/ml, 0 h (●) and 24 h (○). The $DexP_{20}$ concentration in the aqueous phase was 2 g/L.

After centrifugation, the diameter of particle in supernatant was measured comparing with the condition before centrifugation as shown in Figure 39. At higher than 50 weight% of monomyristin, before and after centrifugation gave the different size of nanoparticles by the diameter obtained after centrifugation decreased.

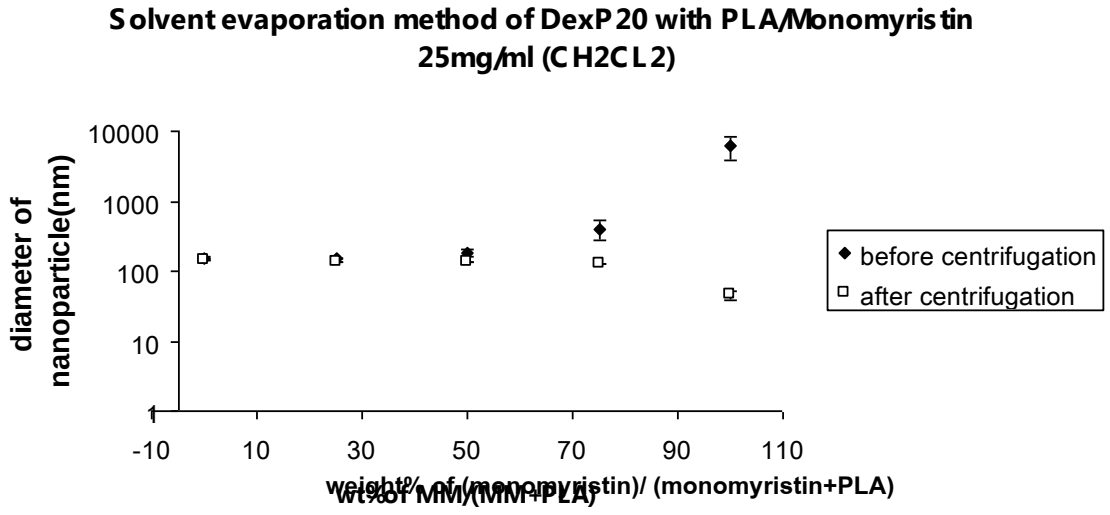


Figure 39 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of weight% of (monomyristin)/monomyristin+PLA) with a total concentration kept at 25 mg/ml, (♦) before centrifugation and (□) after centrifugation.

The $DexP_{20}$ concentration in the aqueous phase was 2 g/L.

The suspension was centrifuged to separate the large aggregation from submicronic nanoparticles. After freeze-drying, the amount of large aggregated polymer was measured (Table 12)

and defined the structure by ^{1}H-NMR (Figure 40) for calculating the ratio of monomyristin/PLA in all conditions (Table 13). The dried polymer in upper solution was also defined the structure by ^{1}H-NMR and calculated at 50 weight% of monomyristin (Table 123).

When the weight% of monomyristin raising, the diameter of nanoparticles after centrifugation was decreased (Figure 39) but the large aggregation was increased (Table 12). It was correlated with ^{1}H NMR result of the large aggregate which showed the enhancement of monomyristin when increasing of weight% of monomyristin.

Table 12 The weight of large aggregated polymer at 2 g/L $DexP_{20}$ concentrations used

Weight% of (monomyristin)/ (monomyristin+PLA)	Weight (mg)
0	4.4
25	5.8
50	7.4
75	8.2
100	9.0

-The total amount of hydrophobic polymer was 25 mg.

The ratio of monomyristin/PLA was χ, calculated from the surface area of the peak ($\mathbf{B_1}$, $\mathbf{B_2}$) from ^{1}H NMR spectrum result (Figure 40) as the formula below.

$$\chi = \frac{(\mathbf{B_2}/3) \times 356}{(\mathbf{B_1} \times 72) + [(\mathbf{B_2}/3) \times 356]}$$

$\mathbf{B_1}$ = surface area of the peak between 5.0 and 5.5

$\mathbf{B_2}$ = surface area of the peak between 0.5 and 1.0

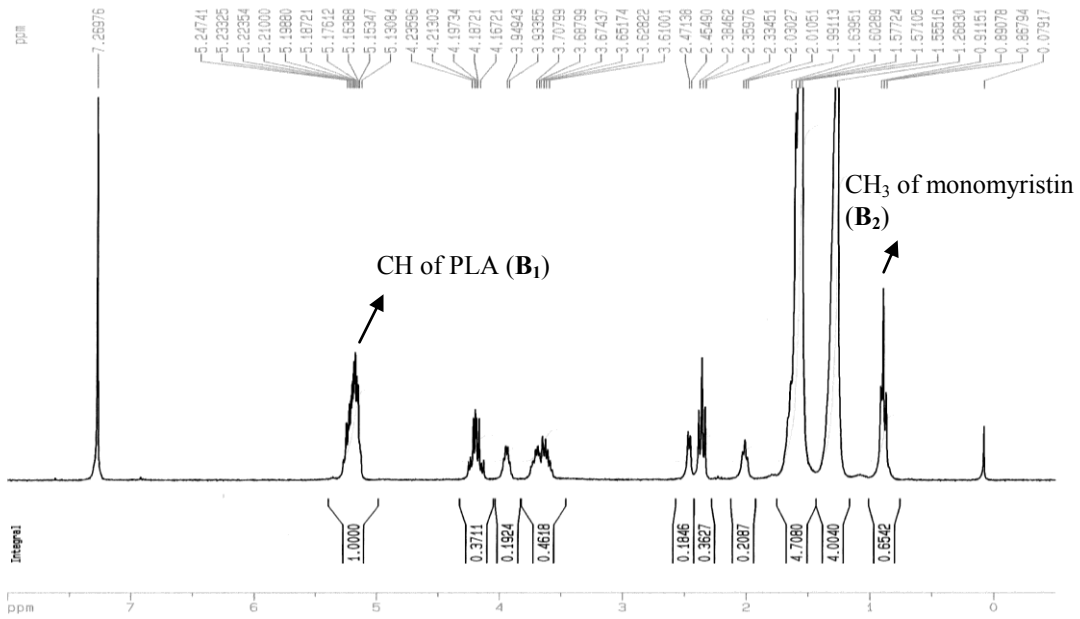


Figure 40 ^{1}H NMR in $CDCl_3$ of coagulum at 25 weight% of (monomyristin)/(monomyristin+PLA). The $DexP_{20}$ concentration in the aqueous phase was 2 g/L.

Table 13 The ratio of monomyristin/PLA at 2 g/L $DexP_{20}$ concentrations used

Weight% of (monomyristin)/ (monomyristin+PLA)	Ratio of monomyristin/PLA (χ)	
	Aggregated part	Upper solution part
0	0	ND*
25	0.51	ND
50	0.71	0.23
75	0.85	ND
100	0.84	ND

* ND = Not determined

9.1.6 aqueous phase: 10 ml of a solution of the modified dextran ($DexP_{20}$) in milliQ water at various concentration; 2, 5, 10 g/L

organic phase: 1 ml of a solution in dichloromethane at 25 mg/ml of a mixture of monomyristin/PLA, 50/50 (w/w)

When increasing of $DexP_{20}$ concentration, the tendency of particles size was decreased either before or after centrifugation (Table 14) but the large aggregation was increased (Table 15). The $DexP_{20}$ concentration also effected to the chemical composition of aggregation which proving by ^{1}H NMR. They showed enrichment of monomyristin in the aggregation (Table 16) while nanoparticles in supernatant had low amount of monomyristin (Table 16). The PLA content in the

aggregation part was deducted when high concentration of $DexP_{20}$ which was showed by ^{1}H NMR (Table 16).

Table 14 Average diameter of nanoparticles obtained by emulsion/solvent evaporation process, as a function of $DexP_{20}$concentration, before centrifugation and after centrifugation

$DexP_{20}$ concentration (g/L)	Average particle diameter (nm)	
	Before centrifugation	After centrifugation
2	220	134
5	281	106
10	167	102

Table 15 The weight of large aggregated polymer at various $DexP_{20}$ concentrations used

$DexP_{20}$ concentration (g/L)	Weight (mg)
2	1.0
5	8.6
10	13.5

-The total amount of hydrophobic polymer was 25 mg.

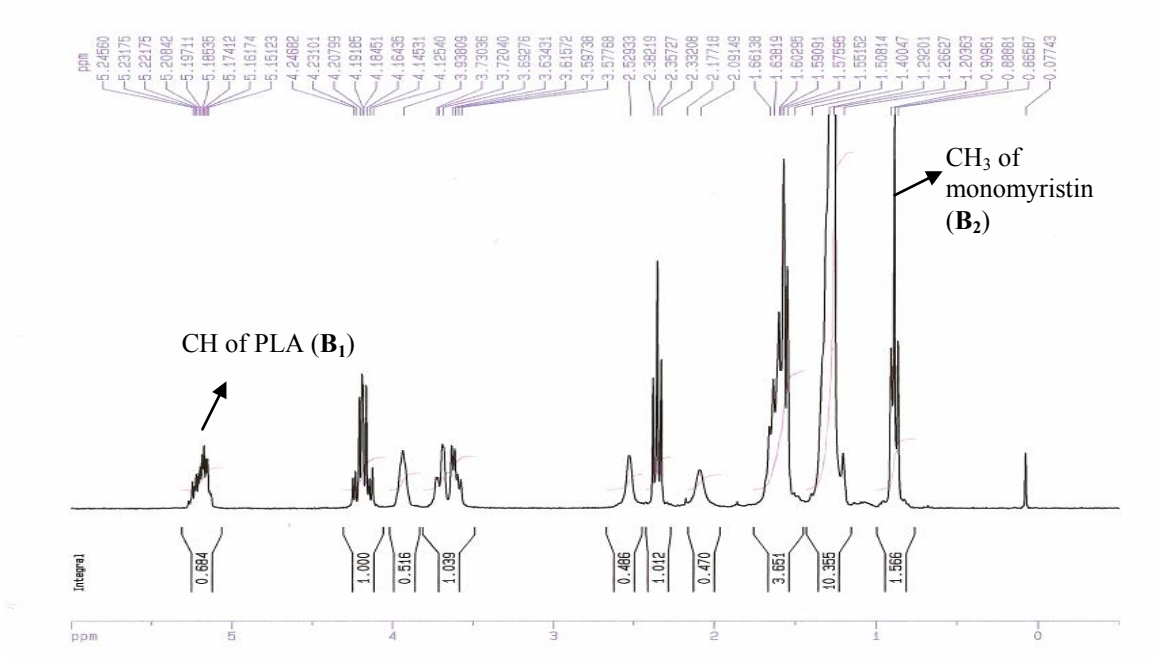


Figure 41 ^{1}H NMR in $CDCl_3$ of coagulum at 50 weight% of (monomyristin)/(monomyristin+PLA). The $DexP_{20}$ concentration in the aqueous phase was 2 g/L.

Table 16 The ratio of monomyristin/PLA at 50 weight% of (monomyristin)/(monomyristin+PLA) used

$DexP_{20}$ concentration (g/L)	Ratio of monomyristin/PLA (χ)	
	Aggregated part	Upper solution part
2	0.79	0.23
5	0.93	ND*
10	0.98	ND

* ND = Not determined

9.1.7 aqueous phase: 10 ml of a solution at 10 g/L of the modified dextran ($DexP_{20}$) in milliQ water

organic phase: 1 ml of a solution in dichloromethane at 25 g/L of a mixture of $DexC10_{164}$/monomyristin in various proportions

The organic phase was composed of $DexC10_{164}$ and monomyristin in varying proportion. The effect of weight% of (monomyristin)/(monomyristin+ $DexC10_{164}$) on particle size was shown in Figure 42. There were no visually large particles inside the suspension at the proportion of monomyristin below 40 weight%. Between 10-20 weight% of monomyristin, the diameter of particle in suspension before centrifugation (initial suspension) and after centrifugation (supernatant) were similar (

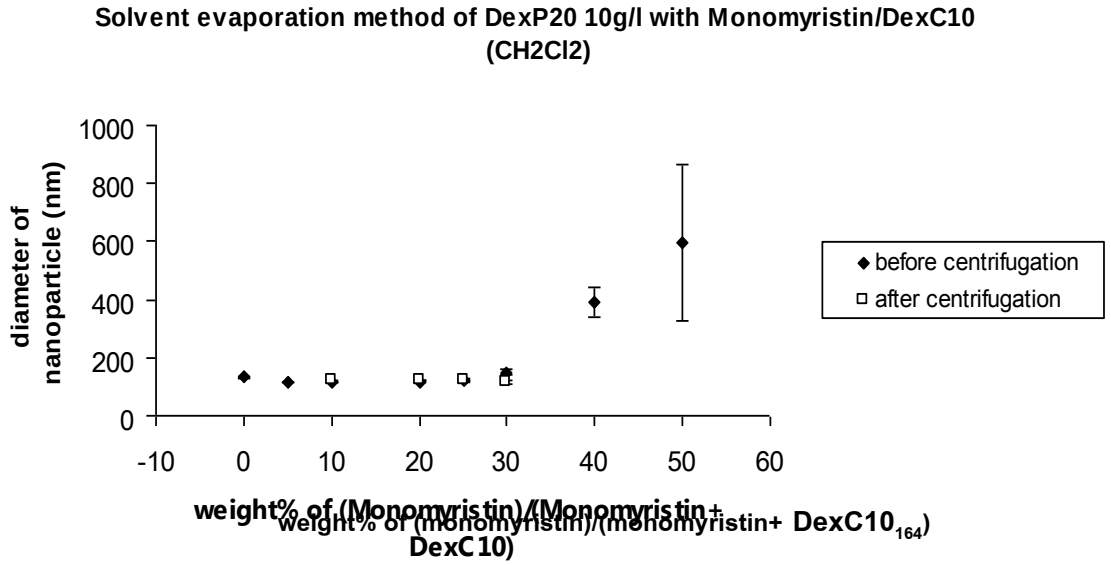


Figure 42 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of weight% of (monomyristin)/(monomyristin+ $DexC10_{164}$) with a total concentration kept at 25 mg/ml, (♦) before centrifugation and (□) after centrifugation. The $DexP_{20}$ concentration in the aqueous phase was 10 g/L.

Table 17 Average particle diameter for nanoparticles suspensions prepared by emulsion/solvent evaporation using mixtures of $DexC10_{164}$ and monomyristin (MM): The suspension samples were taken for particle size determination from initial suspension, supernatant recovered after centrifugation and suspension obtained after re-dispersion of the centrifuged solid in water.

$DexC10_{164}$:MM (wt:wt)	Average particle diameter (nm)		
	Initial suspension	Supernatant	Re-dispersed solid
90:10	116	114	150
80:20	114	116	247
70:30	116	115	253

The tendency of large aggregated polymer weight was increased when weight% of monomyristin was higher (Table 18). ^{1}H NMR results of the composition of the aggregates obtained from centrifugation showed mainly monomyristin (Figure 43). There was less or no $DexC10_{164}$ in the aggregates although the concentration of $DexC10_{164}$ increased from 60-95 weight%. From ^{1}H NMR results, $DexP_{20}$ was found in both aggregates and supernatant (Figure 44).

Table 18 The weight of large aggregated polymer when increasing of weight% of (monomyristin)/(monomyristin+ $DexC10_{164}$).

Weight% of (monomyristin)/(monomyristin+ $DexC10_{164}$)	Weight (mg)
5	0.7
10	2.1
20	2.3
30	7.8
40	7.6

-The total amount of hydrophobic polymer was 25 mg.

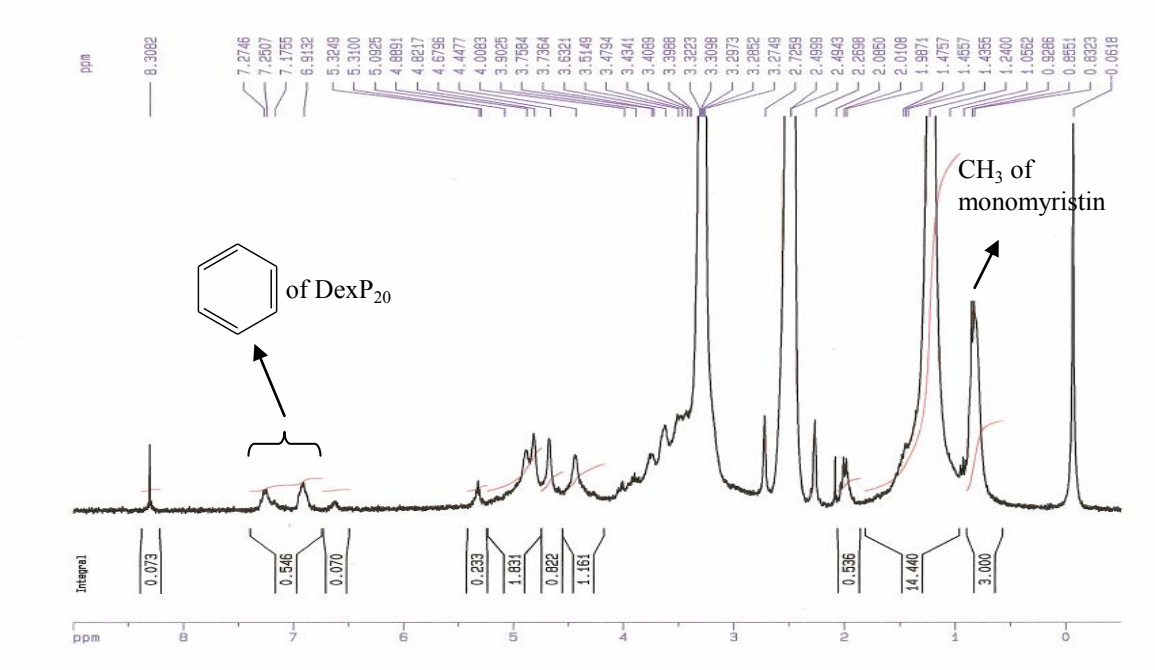


Figure 43 ^{1}H NMR in DMSO of coagulum at 5 weight% of (monomyristin)/(monomyristin+DexC10$_{164}$). The DexP$_{20}$ concentration in the aqueous phase was 10 g/L.

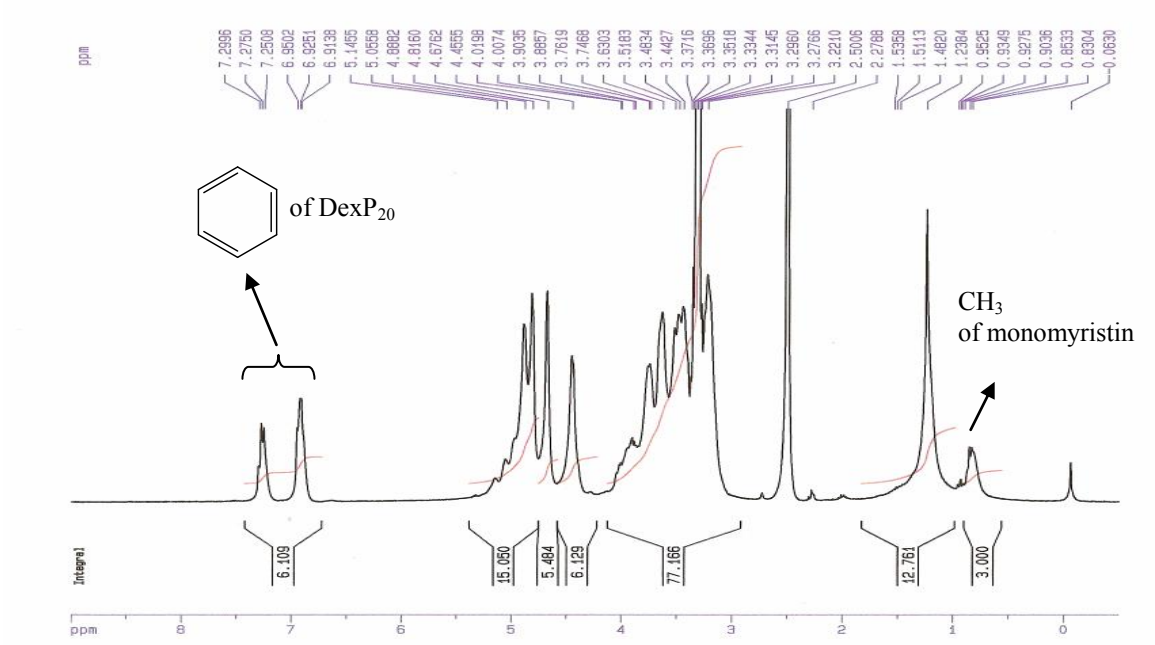


Figure 44 ^{1}H NMR in DMSO of the polymer in upper solution at 5 weight% of (monomyristin)/(monomyristin+ DexC10$_{164}$). The DexP$_{20}$ concentration in the aqueous phase was 10 g/L.

9.2 Nanoprecipitation method

The nanoparticles were prepared by nanoprecipitaion method in varying conditions. The compositions used for nanoparticles preparation in each condition were described in 9.2.1-9.2.7.

9.2.1 aqueous phase: 9 ml of a solution of the modified dextran (DexP$_{20}$) in milliQ water at various concentration; 1, 2, 4, 8 g/L

organic phase: 4.5 ml of THF solution containing 25 mg/ml Polylactide (PLA)

This experiment used only PLA as a material in organic phase. The Figure 45 showed the nanoparticles size, measuring before and after centrifugation when various concentration of $DexP_{20}$ were used. 1 g/L $DexP_{20}$ led to the smallest PLA nanoparticles. The large particles (macroscopic aggregates) were in the all suspension. The large aggregated particle weight obtained after centrifugation was shown in Table 19. There was 70-80 weight% of PLA comparing with the initial PLA.

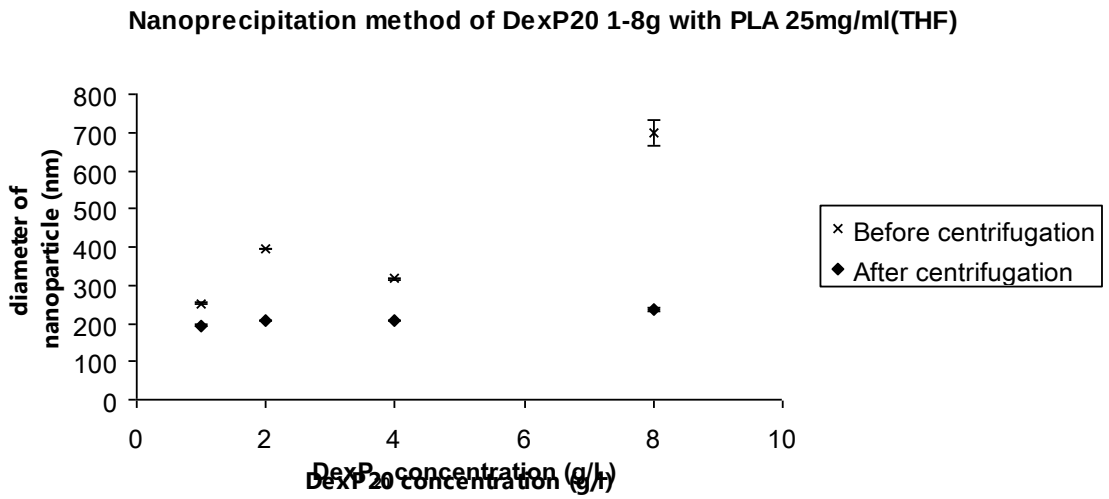


Figure 45 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of $DexP_{20}$ concentration, (✕) before centrifugation and (♦) after centrifugation. The PLA concentration in the organic phase was 25 mg/ml.

Table 19 The weight of large aggregated polymer when increasing concentration of $DexP_{20}$

Concentration of $DexP_{20}$ (g/L)	Weight (mg)
1	91.83
2	93.97
4	88.63
8	81.29

-The total amount of hydrophobic polymer is 112.5 mg.

The aggregated polymer occurred by nanoprecipitation method, therefore, the suspension was centrifuged and the size of particle was measured after centrifugation. All of ^{1}H NMR results showed that the composition of the aggregated part was only PLA as shown in Figure 46-49.

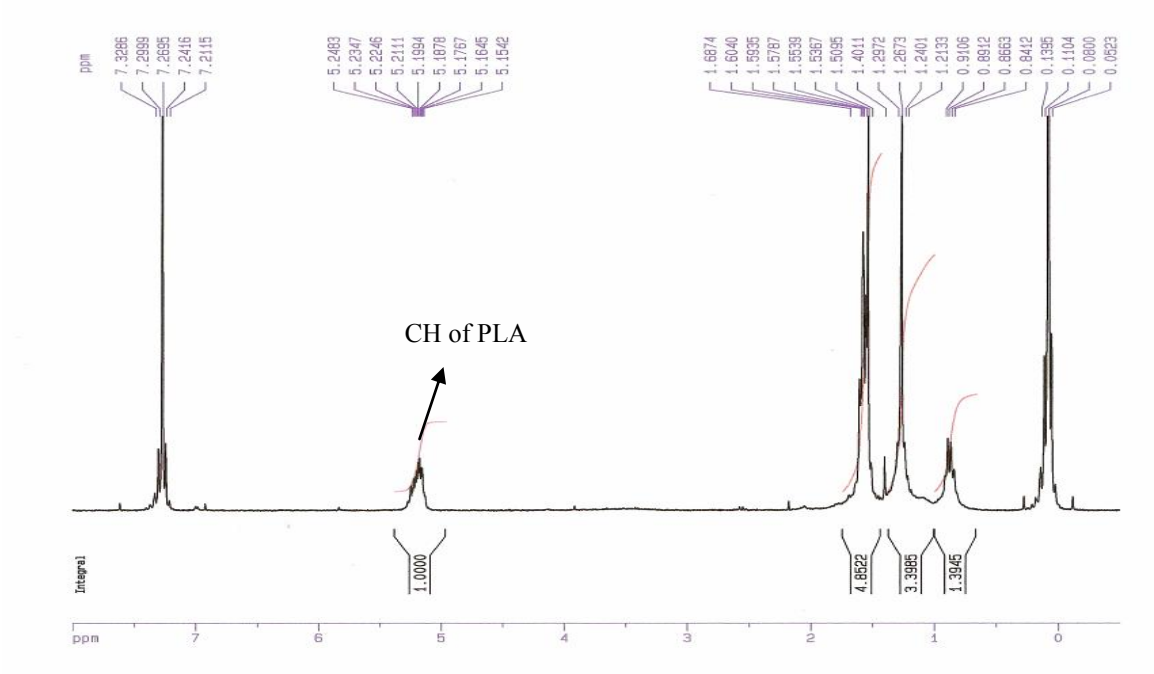


Figure 46 ^{1}H NMR in $CDCl_3$ of coagulum at $DexP_{20}$ concentration was 1 g/L. The PLA concentration in the organic phase was 25 mg/ml.

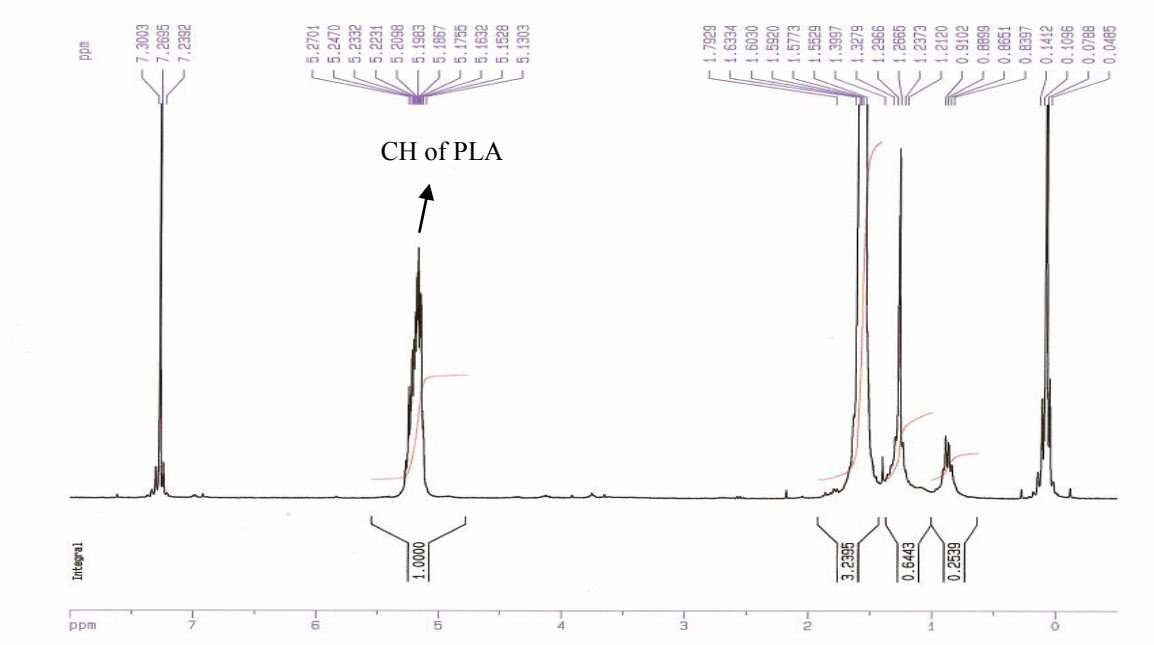


Figure 47 ^{1}H NMR in $CDCl_3$ of coagulum at $DexP_{20}$ concentration was 2 g/L. The PLA concentration in the organic phase was 25 mg/ml.

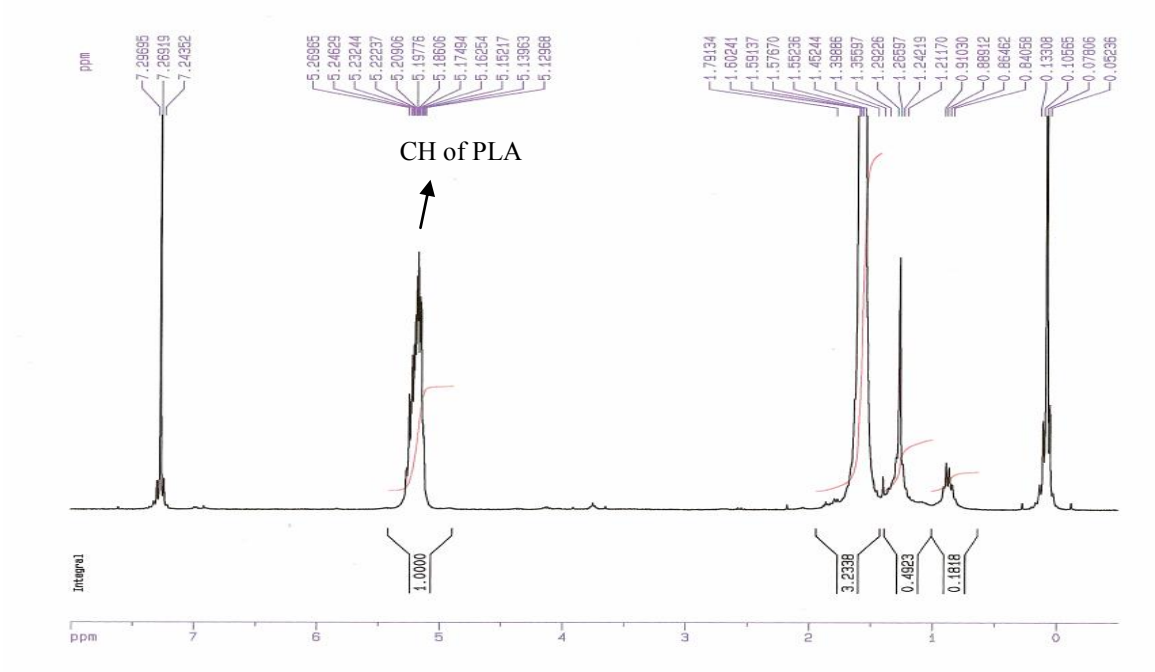


Figure 48 ^{1}H NMR in $CDCl_3$ of coagulum at $DexP_{20}$ concentration was 4 g/L. The PLA concentration in the organic phase was 25 mg/ml.

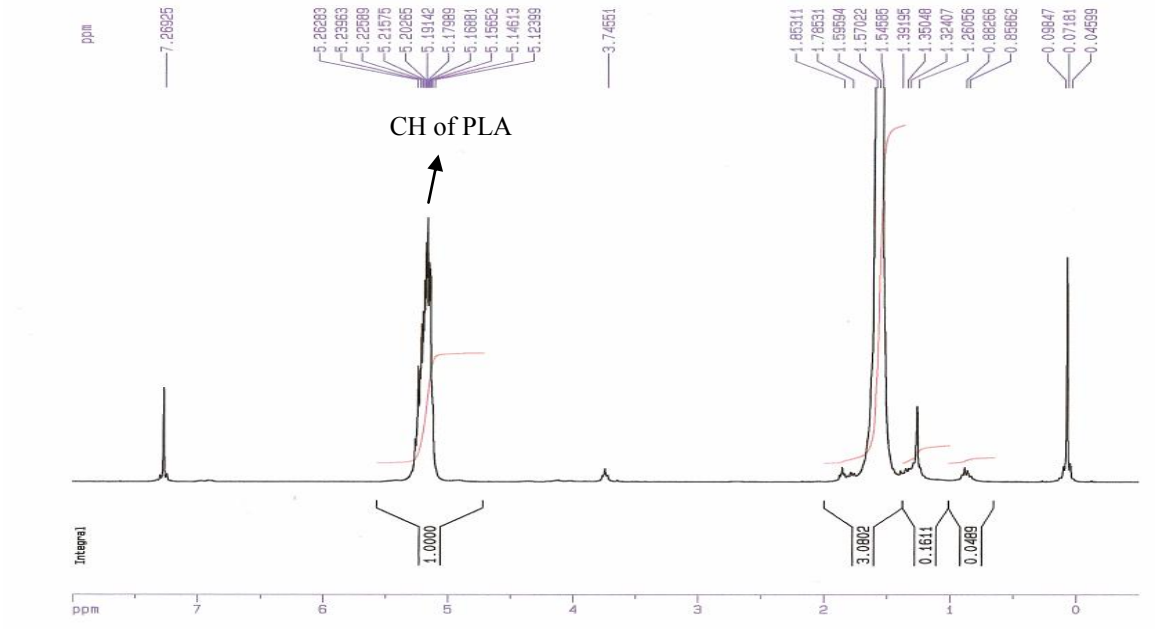


Figure 49 ^{1}H NMR in $CDCl_3$ of coagulum at $DexP_{20}$ concentration was 8 g/L. The PLA concentration in the organic phase was 25 mg/ml.

9.2.2 aqueous phase: 9 ml of a solution of the modified dextran ($DexP_{20}$) in milliQ water at various concentration; 0.5, 1, 2, 4, 8, 10 g/L

organic phase: 4.5 ml of THF solution containing 10 mg/ml Polylactide (PLA)

The condition used in this experiment was the same as describe in 9.2.1 but decreasing the concentration of PLA as 10 mg/ml to prepare the nanoparticles. The particle size smaller (Figure 50) and the amount of aggregates (Table 20) were lower than the condition of 25 mg/ml but there were still some large particles inside the suspension. The suspension was centrifuged and the size of particle was measured after centrifugation. The weight% of PLA aggregates was decreased as 6-20 weight% when comparing with the initial PLA. The size of nanoparticles before and

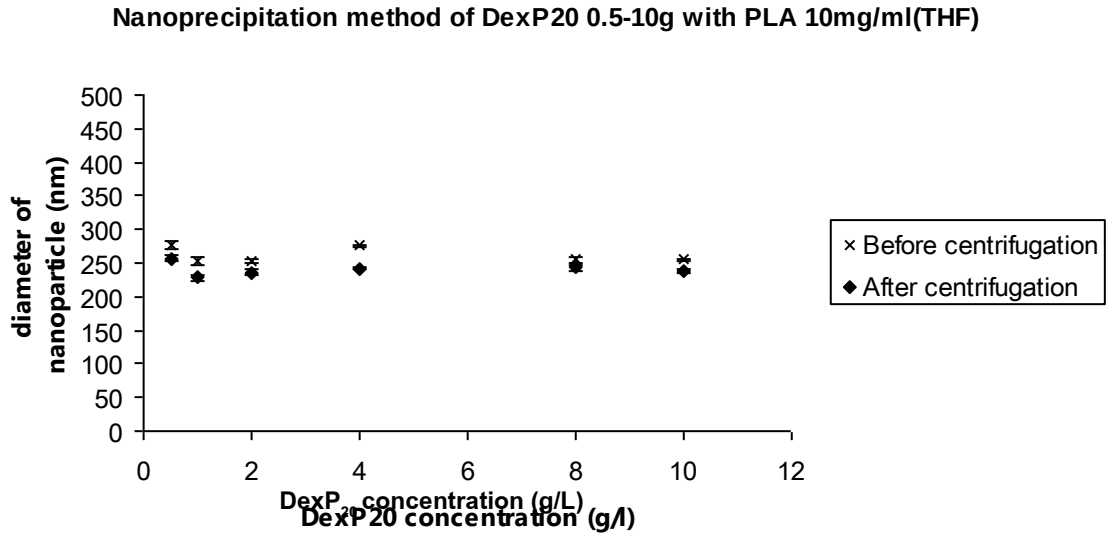


Figure 50 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of $DexP_{20}$ concentration, (✕) before centrifugation and (♦) after centrifugation. The PLA concentration in the organic phase was 10 mg/ml.

Table 20 The weight of large aggregated polymer when using concentration of PLA as 10 mg/ml in THF

Concentration of $DexP_{20}$ (g/L)	Weight (mg)
0.5	2.7
1	3.4
2	5.5
4	7.2
8	7.7
10	9.2

-The total amount of hydrophobic polymer is 45 mg.

9.2.3 aqueous phase: 9 ml of a solution of the modified dextran ($DexP_{20}$) in milliQ water at various concentration; 0.5, 1, 2, 4, 8, 10 g/L

organic phase: 4.5 ml of acetone solution containing 10 mg/ml Polylactide (PLA)

This experiment focused on the solvent used in nanoparticle preparation THF was replaced by acetone. The particle size was not different (Figure 51) but the aggregates size obtained

after centrifugation was higher. The amount of aggregates was 70 weight% PLA when comparing the initial PLA (Table 21). ^{1}H NMR of aggregated polymer showed that this part was composed of PLA only (Figure 52-53).

Moreover, varying concentration of $DexP_{20}$ did not affect to size of nanoparticles.

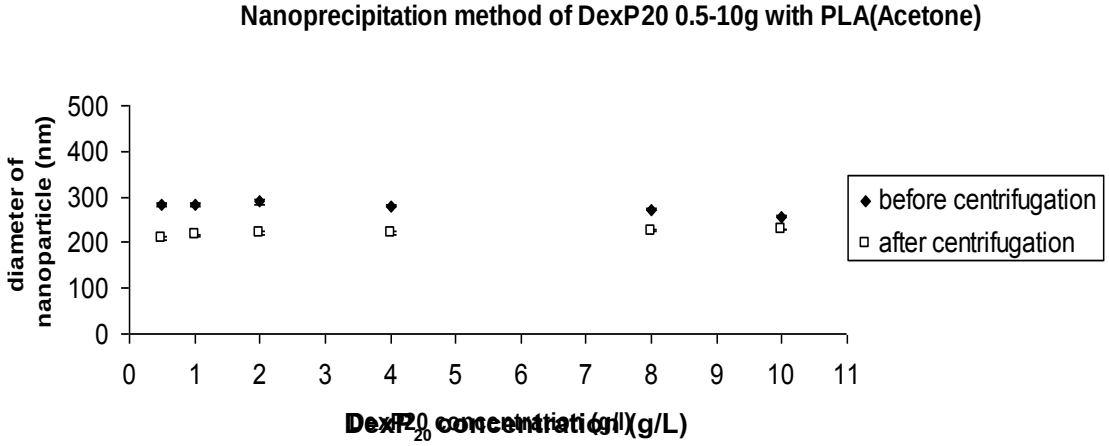


Figure 51 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of $DexP_{20}$ concentration, (♦) before centrifugation and (□) after centrifugation. The PLA concentration in the organic phase was 10 mg/ml.

Table 21 The weight of large aggregated polymer when using concentration of PLA as 10 mg/ml in acetone

Concentration of $DexP_{20}$ (g/L)	Weight (mg)
0.5	31.49
1	31.87
2	31.35
4	31.37
8	24.26
10	32.90

-The total amount of hydrophobic polymer is 45 mg.

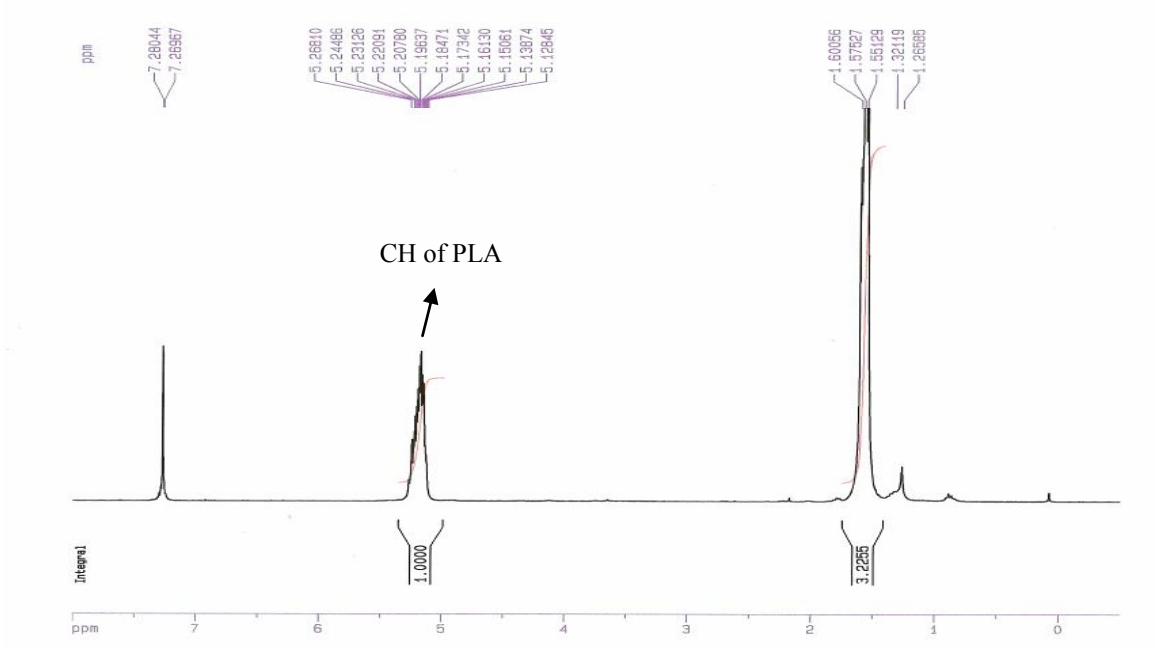


Figure 52 ^{1}H NMR in $CDCl_3$ of coagulum at $DexP_{20}$ concentration was 0.5 g/L. The PLA concentration in the organic phase was 10 mg/ml.

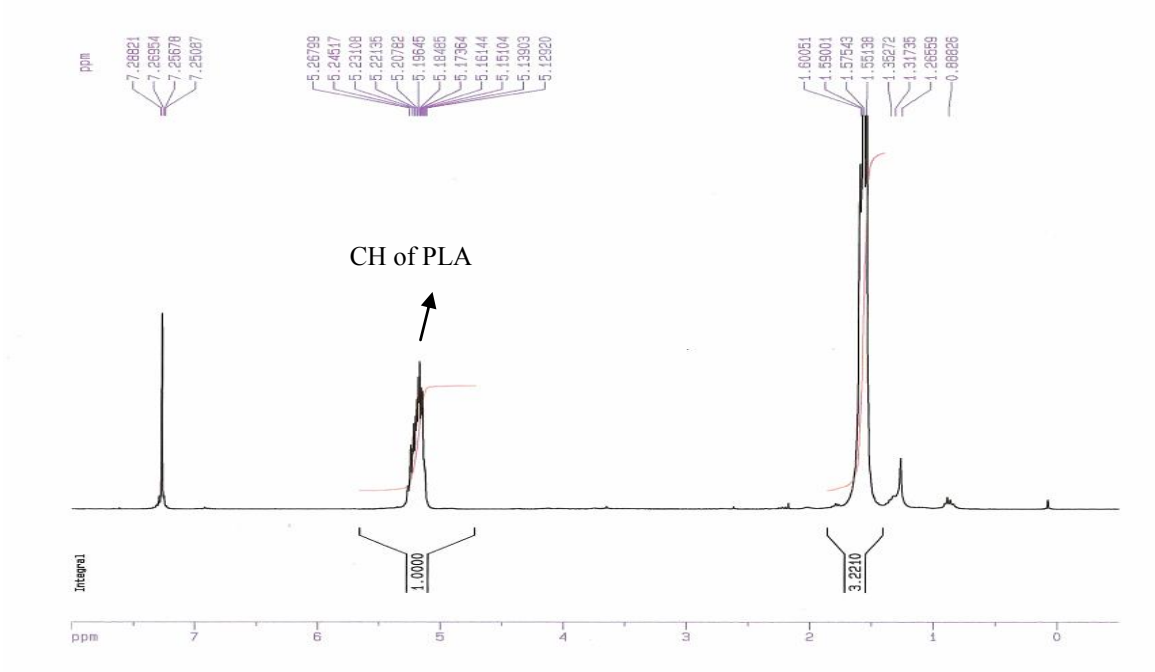


Figure 53 ^{1}H NMR in $CDCl_3$ of coagulum at $DexP_{20}$ concentration was 10 g/L. The PLA concentration in the organic phase was 10 mg/ml.

9.2.4 aqueous phase: 9 ml of a solution of the modified dextran ($DexP_{20}$) in milliQ water at various concentration; 1, 5, 10 g/L

organic phase: 4.5 ml of THF solution containing 10 mg/ml $DexC10_{164}$

$DexC10_{164}$ was used to prepare nanoparticles in this experiment by varying the concentration of $DexP_{20}$ and the result showed that 10g/L $DexP_{20}$ led to the smallest nanoparticles (Figure 54).

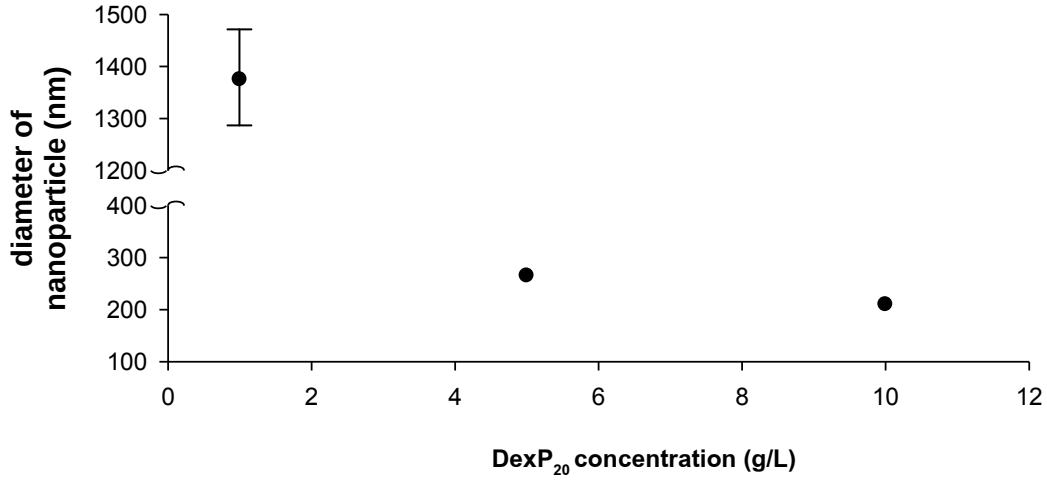


Figure 54 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of $DexP_{20}$ concentration. The $DexC10_{164}$ concentration in the organic phase was 10 mg/ml.

9.2.5 aqueous phase: 9 ml of a solution at 1 g/L of the modified dextran ($DexP_{20}$) in milliQ water

organic phase: 4.5 ml of a solution in THF at 10 mg/ml of a mixture $DexC10_{164}$/PLA in various proportions

The proportion of $DexC10_{164}$ and PLA in organic phase was varied but fixed concentration in organic phase as 10 mg/ml. The result shown in Figure 55 that the size obtained was around 200 nm and no macroscopic aggregation in the suspension.

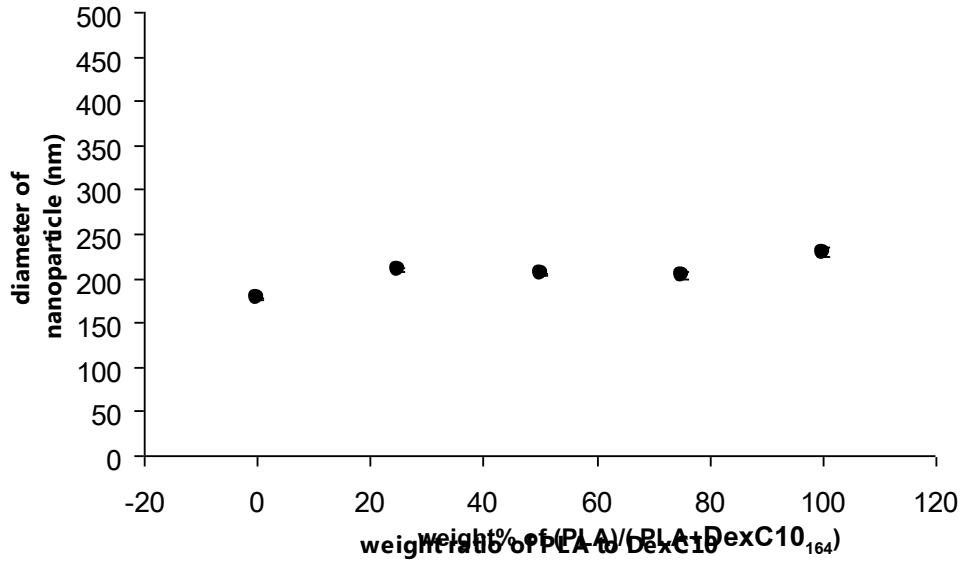


Figure 55 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of weight% of (PLA)/(PLA + $DexC10_{164}$) with a total concentration kept at 10 mg/ml. The $DexP_{20}$ concentration in the aqueous phase was 1 g/L.

9.2.6 aqueous phase: 9 ml of a solution at 1 g/L of the modified dextran ($DexP_{20}$) in milliQ water

organic phase: 4.5 ml of THF solution containing 10 mg/ml monomyristin : 1 ml of acetone solution containing 10 mg/ml monomyristin

Only monomyristin was used in organic phase (either THF or acetone) and 1 g/L $DexP_{20}$ was used as aqueous phase. At the optimum amount of organic phase and monomyristin, the supernatant gave the microscopic particle around 300 to 600 nm after centrifugation (Table 22).

Table 22 Average particle diameter for nanoparticles suspensions prepared by nanoprecipitation process using monomyristin in THF and acetone, supernatant recovered after centrifugation

Material	Average particle diameter (nm)
-4.5 ml of THF solution (containing 10 mg/ml monomyristin)	369.67
-1 ml of acetone solution (containing 10 mg/ml monomyristin)	669.37

9.2.7 aqueous phase: 9 ml of a solution at 10 g/L of the modified dextran ($DexP_{20}$) in milliQ water

organic phase: 4.5 ml of a solution in THF at 10 mg/ml of a mixture $DexC10_{164}$/monomyristin in various proportions

The mixture of $DexC10_{164}$/monomyristin in various proportions was used as an organic phase to prepare the nanoparticles. The result shown in the Figure 56 that using the monomyristin lower than 20 weight% gave the nanoparticles around 200 nm without the macroscopic aggregation.

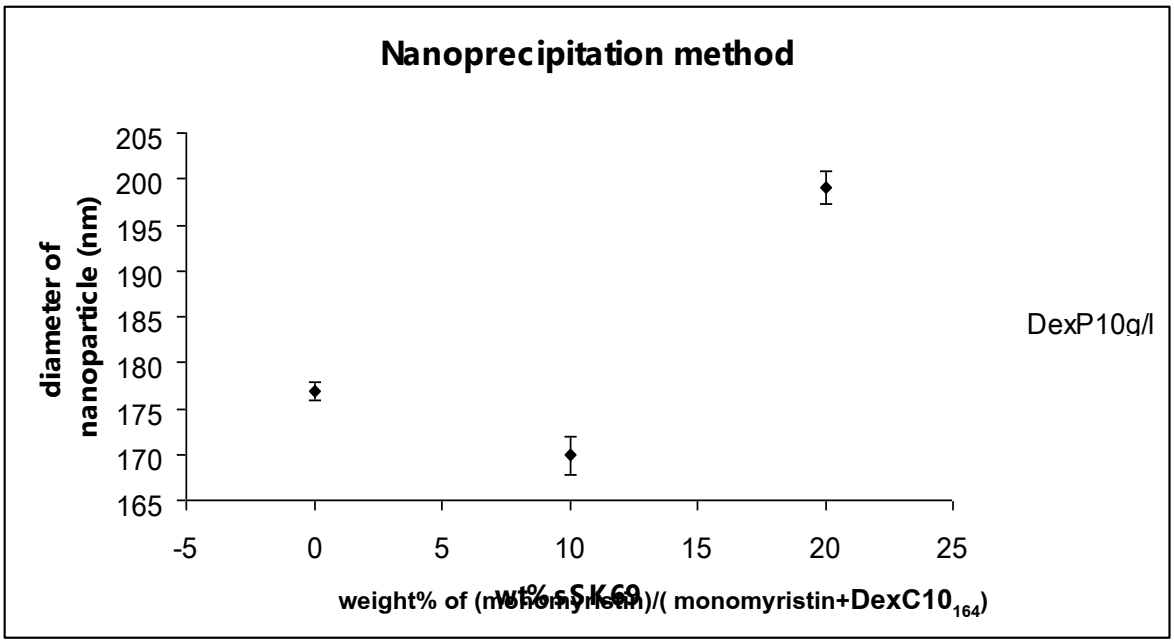


Figure 56 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of weight% of $DexC10_{164}$)/($DexC10_{164}$+monomyristin) with a total concentration kept at 10 mg/ml. The $DexP_{20}$ concentration in the aqueous phase was 1 g/L.

CHAPTER V
DISCUSSION

Biosurfactant is usually produced by many environmental microorganisms. The most common environmental sources for biosurfactant producer isolation are hydrocarbon contaminated soils or water from refinery wastes, petroleum plant, marine oil spill sites, etc..[169] There were reports of the isolation of microorganisms capable of biosurfactant production in many oil contaminated area worldwide such as Kuwait oil-contaminated soil, which resulted from the Gulf War,[170] oil contaminated Arid Southwestern soils,[171] Long Beach soil, California (USA), a diesel-contaminated soil in Hong Kong (China),[172] oil-contaminated sites in Vietnam[173] and in Thailand.[68] In this study, the biosurfactant producers were isolated from oil contaminated soils collected from oil mill wastes and biodiesel plant, Songkhla, Thailand.

Since the soil samples were collected from palm oil refinery waste, the media used for isolation microorganisms was basal medium containing basic mineral salts supplemented with nitrogen source and cooking oil as carbon source.

The surfactant activity and emulsion activity were used to screen the biosurfactant production of microorganism. Both activities describe the ability of the target biosurfactant in reducing surface tension between oil and water and its ability to emulsify these two phases, respectively. These properties are useful for assisting the oily drug to disperse in the water by assimilating it in the hydrophobic phase of nanoparticles. Drop collapsing test reported by Jain and colleagues in 1991[76] is a widely used technique to screen the biosurfactant producer because it is rapid, easy to perform, reproducible and no specialized equipment required.[165] Emulsion activity measures the efficiency of an emulsifier to mix two or more immiscible liquids. From this study, only surfactant activity (Drop collapsing test) was used to evaluate efficiency of biosurfactant production because this method gave high precision (as narrow range of standard deviation) so it provides good value for estimation of activity. In addition, drop collapsing test used only 5 µl of sample for assay so it could be assayed in triplicate sample while the emulsion activity assay used 1 ml of sample so large volume of the culture broths were required and lead to the difficulty of cultivation in each conditions tested.

Isolation and culture condition of biosurfactant producer

Among 102 microorganisms isolated from Shanghai (China), Songkhla province and Chiangmai province (Thailand), 6 microorganisms collected from oil mill wastes and biodiesel plant in Songkhla province exhibited high surfactant activity. Oil contaminated soil was used to screen biosurfactant producer since the microorganisms from this area are supposed to produce the biosurfactant for assimilating the oil into small droplets by which they can digest oil droplet as their

energy. The result showed that only one microorganism from Songkhla province showed the highest emulsion activity and stability. The best biosurfactant producer was identified as a fungus, *Exophiala dermatitidis*. So far, there is no report of biosurfactant produced from this microorganism therefore, it was selected for further study.

Exophiala dermatitidis (or *Wangiella dermatitidis*) can be found in plant debris and soil, and it was recognized as an opportunistic causative agent of mycetoma and phaeohyphomycosis in humans (immunocompromised patients),[174] like *P. aeruginasa*, a famous biosurfactant producer. Moreover, neurotropic and cerebral infections are frequently seen from total infection of *Exophiala dermatitidis*. The clinical manifestations of its infection include subcutaneous cystic lesions, endocarditis and brain abscesses.[175]

The yield and cost of the biosurfactant production cause a limited use of biosurfactant; so, the waste or cheap substrates and culture conditions were in concern in order to increase the yield and reduce the cost of production. Vegetable oil, a low cost substrate, is a popular used in glycolipid biosurfactant production such as rhamnolipid, mannosylerythritol lipid and sophorolipid.[176-178] However some microorganisms such as *Rhodococcus erythropolis* required n-decane and n-hexadecane as carbon sources.[179-180] According to the surfactant and emulsion activities, vegetable oils i.e. soybean oil, corn oil, palm oil and olive oil were effective carbon sources for *Exophiala dermatitidis* (Figure 11 and Table 8). The highest activity was obtained with palm oil which is the cheapest among various oils used; so, it was selected to be used as carbon source.

Nitrogen source is an important factor for production of biosurfactants. In this study, the best nitrogen source for biosurfactant production of *Exophiala dermatitidis* is ammonium nitrate which was similar to biosurfactant production of *B. subtilis*[181] and *Candida* sp..[177,8] At low concentration, 0.08% of ammonium nitrate, gave the highest surfactant activity. It was correlated to other reports that the limitation of nitrogen source lead to increase in biosurfactant production such as *Nocardia* sp. and *Candida tropicalis*.[182-183]

Culture conditions such as pH and temperature can determine the production of biosurfactants. From this study, *Exophiala dermatitidis* which was a fungus showed slightly different in biosurfactant production at pH and incubation temperature range from 4.5-6 and 28-37°C, respectively. At pH 5.3 and temperature at 30°C showed the best result but at pH 7 gave the remarkably decreasing of growth and biosurfactant activity. In contrast, *Bacillus licheniformis*, a gram positive bacteria showed the maximum yield of biosurfactant production at pH range from 7.0-9.0.[184] *Candida bombicola*, a yeast produced the highest sophorose lipid production at pH 3.5.[185] *Candida antarctica* showed the highest yield of MEL when culturing at 25°C.[31]

Biosurfactant isolation and structure elucidation

The crude extract was prepared from *Exophiala dermatitidis* total culture broth by ethyl acetate and methanol/hexane, respectively. Three steps of silica gel column chromatography were

used to isolate the biosurfactant. From the last column chromatography, 0.2 g of the yellow liquid biosurfactant was obtained and gave one band at R_f value 0.34 with silica gel TLC (hexane:ethyl acetate, 1/1(v/v)). The yield of purified biosurfactant (0.2 g) was 0.08% comparing to crude extract obtained from ethyl acetate (239.86 g).

There were many reports using organic solvents for biosurfactant extraction. The solvents used in the extraction depend on type of biosurfactant and the extracted material (cell or culture broth). The organic solvent commonly used in glycolipid extraction were chloroform and chloroform:methanol.[186-187] Moreover, ethyl acetate was also reported in glycolipid extraction produced by yeast.[53] In this study, ethyl acetate was used as a solvent for extraction because chloroform is toxic and more expensive when comparing with ethyl acetate and both solvent show similar in polarity. In the extraction process, the biosurfactants in culture broth were dissolved in ethyl acetate and therefore were separated from water phase of culture broth. Hexane was used in the second step to dissolve and remove oil substrate supplemented in the culture medium. The biosurfactants in methanol fraction was obtained by evaporation and used for further study.

The purified biosurfactant was investigated by ^{1}H-NMR, ^{13}C-NMR, COSY (BRUKER spectrometer Avance 300) in deuterated chloroform ($CDCl_3$) and Atmospheric Pressure Chemical Ionization Mass Spectrometer (APCI MS) and shown to be monoolein.

The monoolein is a compound found in composition of animal and human body such as a complex lipid mixture of human sebum.[188] It was also reported to be produced from microorganism, *Candida ishiwadae*.[53] The chemical synthesis of monoolein were reported by various techniques. The esterifying glycerin with food-grade oleic acid was used with suitable catalyst to form a monoglyceride ester.[189] Gupta in 1996 described the technique producing monoolein by tranesterification.[190] Some glycerol esters were prepared by reaction with epoxides such as propylene oxide and ethylene oxide.[191] In this study, the chemical synthesis, dehydrochlorination and deacetylation were used to synthesize the model structure monoacylglycerol, monomyristin.

The monoacylglycerol, either monoolein or monomyristin are widely used in many fields especially in pharmaceutic because not only the emulsifying property but also the biological activity.

Monoolein and monomyristin showed the antileukemic property by induction apoptosis of murine leukemic cell.[65] Moreover, monoolein was able to induce cell death in human leukemic cell lines.[66] Only monomyristin was reported to show anitimicrobial activity against *Helicobacter pylori*.[58,64]

Since the monoacylglycerols showed the advantage in biological activity, this compound was used as a drug model to be encapsulated in a nanoparticle. However, the yield of monoacylglycerol obtained from purification of *Exophiala dermatitidis* cultivation was very low for using in nanoparticle preparation study. Therefore, the monoacylglycerols was synthesized by chemical process. Comparing between monooelin and monomyristin, they show similarity of interfacial tension property, HLB value and exhibit the biological activity. However, the monomyristin has the simpler structure to be

synthesized; so, it was used as a model of monoacylglycerol biosurfactant to incorporate with the nanoparticle.

Monoacylglycerol synthesis and nanoparticle development

Monomyristin was synthesized by dehydrochlorination and deacetylation. After synthetic process, the silica gel column chromatography was used to separate the contaminants. Thin layer chromatography and ^{1}H NMR techniques were required to prove the purity of the synthesized product.

In this study, the emulsion/solvent evaporation process and nanoprecipitation processes were used for nanoparticle development. Either PLA or $DexC10_{164}$ was used as an inner core, $DexP_{20}$ as a stabilizer and monomyristin was encapsulated in the nanoparticle.

In emulsion/solvent evaporation process, $DexP_{20}$ was used as a stabilizer in aqueous phase and dichloromethane is the solvent in organic phase. Firstly, preparation of PLA in dichloromethane as organic phase was determined (see experimental 9.1.1 and 9.1.3). The parameter changes were evaluated by the nanoparticle size. The concentrations of organic and aqueous phases were varied and the mean diameter of nanoparticles at 0 h and compared with 24 h was evaluated for the particle stability. The results were shown by plotted graphs to determine the best condition and the parameter which influenced the particle size. The graph plotted by function of weight% of (PLA)/(PLA+$DexP_{20}$) from all conditions gave a single curve. The size of particle was controlled by weight ratio of PLA to $DexP_{20}$ as shown in Figure 34. This similar result was reported by Durand and colleague in 2006 which studied the polymeric nanoparticles using modified dextrans as stabilizer. They reported that the weight ratio of initial materials (polymer and oil) influenced the nanoparticle preparation.[192,158]

The nanoparticles were stable at the concentration of $DexP_{20}$ as 6 g/L or lower and the best size of nanoparticle obtained when 2 g/L $DexP_{20}$ was used. So, this concentration of $DexP_{20}$ was used for further experiments.

When using $DexC10_{164}$ as organic phase instead of PLA and $DexP_{20}$ as the stabilizer at concentration from 5 g/L to higher (see experimental 9.1.3), the submicronic particles occurred. From the result showed that 10 g/L $DexP_{20}$ gave the best size of particles so, this concentration was proper for preparation of $DexC10_{164}$ nanoparticles and used for further study.

Monomyristin was used as encapsulated material in the polymeric nanoparticles by emulsion/solvent evaporation process. Only monomyristin was encapsulated in the nanoparticle (experimental 9.1.4). The experiment showed clearly that this material could not be encapsulated alone even using $DexP_{20}$ as a stabilizer. Moreover, the micronic and macroscopic aggregation was obtained when using monomyristin and PLA as organic phase (see experimental 9.1.5) so the centrifugation was used to separate the supernatant and aggregation parts. The proportion of monomyristin and PLA was varied. The average size of particle at 0 h and 24 h and before and after centrifugation was evaluated. The amount and chemical composition of aggregation part was compared with the initial material amount. When the weight% of monomyristin increased from 0 to 100, the average diameter of

particle was decreased while the amount of aggregation part was increased. ^{1}H NMR used to analyze the chemical composition of aggregation obtained after centrifugation showed large amount of monomyristin comparing with the initial. On the contrary, the supernatant showed the lower proportion of monomyrisitn than the initial material. This result indicated that monomyristin is involved in the creation of aggregation. When chloroform evaporated, monomyristin which is composed of polar group (hydroxyl groups) from the part of glycerol may contact to water immediately and form the aggregation. So, the surface of particles could not absorb $DexP_{20}$ compound due to interruption from this part of monomyristin (hydroxyl groups). Thus, the evaluation of the influence in concentration of $DexP_{20}$ stabilizer was established (see experimental 9.1.6). $DexP_{20}$ concentration was increased and average size of particle was measured either before or after centrifugation. The amount of aggregation part and chemical composition of aggregate and supernatant were compared with the initial material. The result showed that when increasing of $DexP_{20}$ concentration, average size of particle after centrifugation and the PLA in aggregation was decreased. This showed that increment of $DexP_{20}$ concentration induced to increase the stability of only PLA nanoparticle. It did not improve the encapsulation of monomyristin.

When PLA was replaced by $DexC10_{164}$ in experimental 9.1.7, organic phase were presented both monomyristin and $DexC10_{164}$. The result showed that at weight% of monomyristin lower than 40 gave no macroscopic aggregation. Then the centrifugation was used to separate the bigger particles which superimposed with nanoparticles. This aggregation part was re-dispersed in water and measured size of particles again. The result showed that the re-dispersed solid gave the biggest size of particles and the size of particles was higher when weight% of monomyristin increased. ^{1}H NMR result of aggregation part obtained after centrifugation showed that it mainly contained monomyristin, minority of $DexP_{20}$ and almost no $DexC10_{164}$ even the concentration of $DexC10_{164}$ increasing. All results contributed that monomyristin is involved in the formation of bigger particles because of during the formation of particles the surface lacking of coverage by $DexP_{20}$ stabilizer due to interruption of hydroxyl groups of monomyristin composition. This was the same mechanism as the report of ethyl cellulose nanoparticles.[193] Nevertheless, the increasing of $DexP_{20}$ and using $DexC10_{164}$ as the inner core of particles allowed suppressing the macroscopic and micronic aggregation.

The nanoprecipitation process was studied by using THF and acetone as an organic solvent, stabilizing with $DexP_{20}$. The first study using PLA alone in THF at concentration as 25 mg/ml (see experimental 9.2.1), the macroscopic and micronic aggregation was obtained in whatever $DexP_{20}$ concentration. Only PLA was shown in ^{1}H NMR result of the aggregation part. Therefore, decreasing concentration of PLA to 10 mg/ml was used in next experiment (see experimental 9.2.2). However, the aggregation still occurred, but was lower than the first experiment when comparing weight% with the initial amount. Preparing the nanopartlcles with acetone instead of THF at concentration of PLA as 10 mg/ml, the proportion of PLA in aggregation was higher than THF (see experimental 9.2.3). The average size of particles was similar to previous report by Legrand and colleagues. They prepared PLA nanoparticles by the same process with no surfactant.[167] The results showed that the

aggregation which occurring when prepared PLA nanoparticles by nanoprecipitation precess is depend on the solvent quality and polymer concentration, but not on $DexP_{20}$ stabilizer. That was contrary to emulsion/solvent evaporation process in this study which the concentration of stabilizer in aqueous phase had direct effect to the final particle size. Nevertheless, when the ionic strength is increased, this surfactant is very useful to stabilize the suspension.[194]

Changing the material from PLA to $DexC10_{164}$ (see experimental 9.2.4), only THF was studied because $DexC10_{164}$ was not soluble in acetone. The submicronic particles were obtained only at highest $DexP_{20}$ stabilizer concentration in aqueous phase (10 g/L). This result was relevant to the observation of solvent/evaporation precess. In experimental 9.2.5, the PLA and $DexC10_{164}$ were prepared as an inner core of particle by varying proportion. The concentration of organic phase was kept at 10 mg/ml. The nanoparticles were obtained without macroscopic formation aggregation.

Monomyristin was studied on encapsulation in the polymeric nanoparticles with nanoprecipitation process. Preparing particle with monomyristin alone, the micrometric particle was obtained from supernatant after centrifugation (see experimental 9.2.6). The experiment on encapsulation monomyristin in $DexC10_{164}$ particle showed in experimental 9.2.7 by which the proportion of monomyristin and $DexC10_{164}$ was varied. At weight% of monomyristin lower than 20, the particle size around 200 nm could be obtained without macroscopic aggregation, similarly to emulsion/solvent evaporation method. The submicronic particle was occured when using low weight% of monomyristin.

In summary, this study isolated new biosurfactant producer, *Exophiala dermatitidis* from oil contaminated soil by using drop collapse and emulsion activity tests. It showed the highest surfactant and emulsion activities comparing with others. The optimum condition to culture this biosurfactant producer for high biosurfactant production was basal salt medium (0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract) supplemented with 5% palm oil, 0.08% NH_4NO_3, pH 5.3 and shaking 200 rpm at 30°C. The culture broth obtained was extracted and purified by silica gel column chromatography. The chemical structure of biosurfactant was elucidated as monoolein, a kind of monoacylglycerol.

In addition to surfactant activity, monoacylglycerol showed good biological activities so it was used as a model in nanoparticle development. Monomyristin was chosen for synthesizing because of its simpler structure and similar physico-chemical properties with monoacylglycerol biosurfactant. The emulsion/solvent evaporation and nanoprecipitaion processes were used to prepare the dextran-cover nanoparticles for encapsulating monomyristin. Either PLA or $DexC10_{164}$ was prepared as a core of particle with variety of the polymers and monomyristin proportion. The results contributed that monomyristin induced the formation of macroscopic aggregation. However, the high concentration of stabilizer and/or using $DexC10_{164}$ as inner core of particle suppressed the macroscopic aggregation. This study is a model for encapsulation the raw biosurfactant in nanoparticles. The further study can be done to evaluate deeper the release of the encapsulated monomyristin/monoacylglycerol or raw

biosurfactant. This study was an interested starter of using biosurfactant as a drug in nanoparticle development.

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APPENDIX

APPENDIX

1. Culture medium

1.1 Basal salt medium[53]

NH_4NO_3	0.30%
KH_2PO_4	0.02%
$MgSO_4 \cdot 7H_2O$	0.02%
Yeast extract	0.05%

Adjust the volume to 1 liter with distilled water and sterilized by autoclaved at 121°C for 15 min (Sanyo labo autoclave®, Japan).

1.2 Nutrient agar (NA) medium, Himedia®, India

Beef extract	3.0 g
Peptone	5.0 g
Agar	12.0 g

Add distilled water to final volume 1 liter and adjust pH to 6.8 ± 0.2 then sterilized by autoclaved at 121°C for 15 min (Sanyo labo autoclave®, Japan).

1.3 Nutrient broth (NB) medium, Himedia®, India

The formula per liter was:

Beef extract	3.0 g
Peptone	5.0 g

Add distilled water to final volume 1 liter and adjust pH to 6.8 ± 0.2 then sterilized by autoclaved at 121°C for 15 min (Sanyo labo autoclave®, Japan).

1.4 YM agar

Malt extract	3.0 g
Yeast extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	15.0 g

Add distilled water to final volume 1 liter, then sterilized by autoclaved at 121°C for 15 min (Sanyo labo autoclave®, Japan).

1.5 YM broth

Malt extract	3.0 g
Yeast extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g

Add distilled water to final volume 1 liter, then sterilized by autoclaved at 121°C for 15 min (Sanyo labo autoclave®, Japan).

1.6 PDA agar, Himedia®, India

Potatoes	250.0 g
Dextrose	15.0 g
Yeast extract	1.5 g
Agar	15.0 g

Add distilled water to final volume 1 liter, then sterilized by autoclaved at 121°C for 15 min (Sanyo labo autoclave®, Japan).

2. Materials used in nanoparticle preparation

2.1 $DexP_{20}$ is dextran, bearing with phenoxy group which the percentage of the modified molecules compared with non modified molecules is 20% as the structure shown in Figure 69.

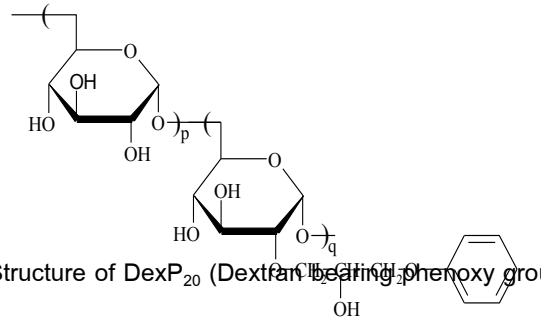


Figure 69 Structure of $DexP_{20}$ (Dextran bearing phenoxy group with q/(p+q) = 20%)

2.2 Dex $C10_{164}$ is dextran, bearing with hydrophobic group (*n*-$C_{10}H_{21}$-) which the percentage of the modified molecules compared with non modified molecules is 164% as the structure shown in Figure 70. The result of ^1H NMR spectrum in DMSO of $DexC10_{164}$ was shown in Figure 71.

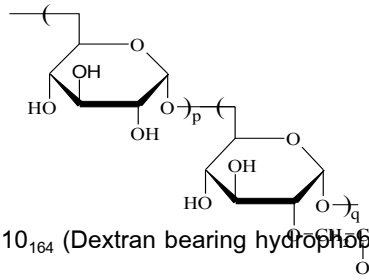


Figure 70 Structure of $DexC10_{164}$ (Dextran bearing hydrophobic group (*n*-$C_{10}H_{21}$-) with q/(p+q) = 164%)

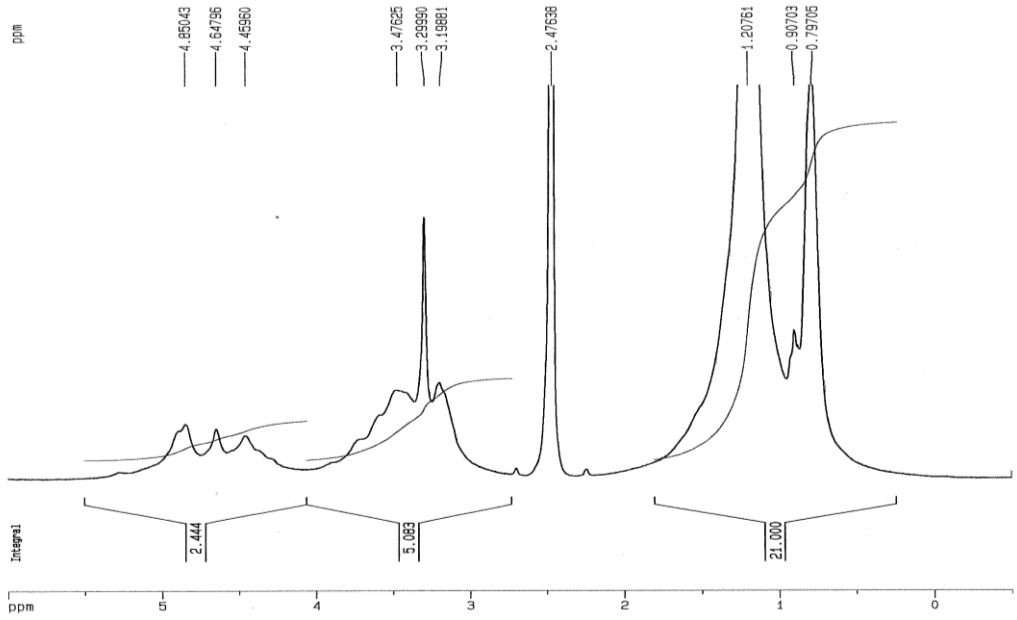


Figure 71 ^{1}H NMR spectrum of DexC10$_{164}$, in DMSO, 300 MHz

2.3 Polylactide (PLA) structure and ^{1}H NMR spectrum in $CDCl_3$ of PLA was shown in Figure 72 and 73, respectively.

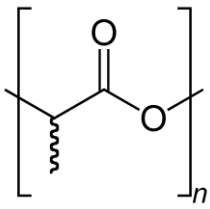


Figure 72 Structure of Polylactide (PLA)

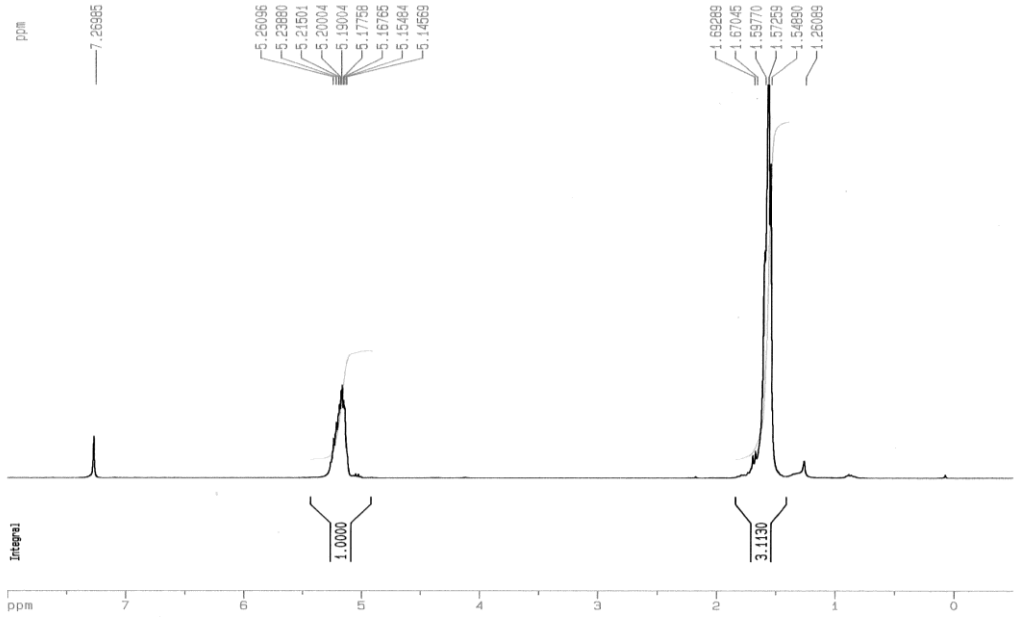


Figure 73 ^{1}H NMR spectrum of PLA, in $CDCl_3$, 300 MHz

3. Reagent

3.1 ρ-anisaldehyde reagent

ρ-anisaldehyde (4-methoxylbenzaldehyde)	0.5 ml
Ethanol	9.0 ml
Conc. H_2SO_4	0.5 ml

3.2 Phosphomolybdic acid (PMA) reagent

Phosphomolybdic acid	4 g
Ethanol	196 ml
H_2SO_4	4-5 drops

4. Blast result

RID: 1139388028-32467-69254653677.BLASTQ4

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
3,749,503 sequences; 16,556,997,203 total letters

```
gi|37786662|gb|AY213701.1|  Exophiala dermatitidis strain UWFP 874 28S ribosomal
RNA gene,
partial sequence
Length=617

 Score =  573 bits (289),  Expect = 2e-160
 Identities = 305/308 (99%), Gaps = 2/308 (0%)
 Strand=Plus/Plus

Query  5    ATTGCCTCAGTA-CGGCGAGTGCAAGCGGCAACAGCTCANATTTGAAATCTGGCCTCTTT  63
            ||||||||||||  |||||||||  ||||||||||||||||  ||||||||||||||||||||
Sbjct  37   ATTGCCTCAGTAACGGCGAGTG-AAGCGGCAACAGCTCAAATTTGAAATCTGGCCTCTTT  95

Query  64   GGGGTCCGAGTTGTAATTTGTAGAGGATGTTTCGGGCACCGCTCCGGTTTAAATTTCTTG  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  96   GGGGTCCGAGTTGTAATTTGTAGAGGATGTTTCGGGCACCGCTCCGGTTTAAATTTCTTG  155

Query  124  GAACAGAATGTCAAAGAGGGTGAGAACCCCGTCTTGGACCGGCAGTAGGGCCCATGTGAA  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  156  GAACAGAATGTCAAAGAGGGTGAGAACCCCGTCTTGGACCGGCAGTAGGGCCCATGTGAA  215

Query  184  ACTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAAATTTCATC  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  ACTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAAATTTCATC  275

Query  244  TAAAGCTAAATATTGGCCAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAA  303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  TAAAGCTAAATATTGGCCAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAA  335

Query  304  GCACTTTG  311
            ||||||||
Sbjct  336  GCACTTTG  343
```

5. The solvent systems used as eluting solvent in column chromatography

Table 23 The solvent system used as eluting solvent and fractions obtained in the first column chromatography

No. of eluting solvent	Mixture of organic solvent	Proportion	No. of fractions obtained
1	hexane:ethyl acetate	100:0	1).1 - 1).10
2	hexane:ethyl acetate	70:30	2).1 - 2).10
3	hexane:ethyl acetate	50:50	3).1 - 3).10
4	hexane:ethyl acetate	30:70	4).1 - 4).10
5	hexane:ethyl acetate	10:90	5).1 - 5).10
6	hexane:ethyl acetate	0:100	6).1 - 6).10
7	ethyl acetate:methanol	90:10	7).1 - 7).10
8	ethyl acetate:methanol	50:50	8).1 - 8).10
9	ethyl acetate:methanol	10:90	9).1 - 9).10

Table 24 The solvent system used as eluting solvent and fractions obtained in the second column chromatography

No. of eluting solvent	Mixture of organic solvent	Proportion	No. of fractions obtained
1	hexane chloroform	100:0	1).1 - 1).4
2	hexane:chloroform	70:30	2).1 - 2).4
3	hexane:chloroform	50:50	3).1 - 3).4
4	hexane:chloroform	30:70	4).1 - 4).4
5	hexane:chloroform	0:100	5).1 - 5).4
6	chloroform:ethyl acetate	70:30	6).1 - 6).4
7	chloroform:ethyl acetate	50:50	7).1 - 7).4
8	chloroform:ethyl acetate	30:70	8).1 - 8).4
9	chloroform:ethyl acetate	0:100	9).1 - 9).4
10	ethyl acetate:methanol	70:30	10).1 - 10).4
11	ethyl acetate:methanol	50:50	11).1 - 11).4
12	ethyl acetate:methanol	30:70	12).1 - 12).4
13	ethyl acetate:methanol	0:100	13).1 - 13).4

Table 25 The solvent system used as eluting solvent and fractions obtained in the third column chromatography

No. of eluting solvent	Mixture of organic solvent	Proportion	No. of fractions obtained
1	hexane:chloroform	30:70	1)
2	hexane:chloroform	0:100	2)
3	chloroform:ethyl acetate	90:100	3)
4	chloroform:ethyl acetate	80:20	4)
5	chloroform:ethyl acetate	70:30	5)
6	chloroform:ethyl acetate	60:40	6)
7	chloroform:ethyl acetate	50:50	7)
8	chloroform:ethyl acetate	40:60	8)
9	chloroform:ethyl acetate	30:70	9)
10	chloroform:ethyl acetate	20:80	10)
11	chloroform:ethyl acetate	10:90	11)
12	chloroform:ethyl acetate	0:100	12)

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AUTORISATION DE SOUTENANCE DE THESE
DU DOCTORAT DE L'INSTITUT NATIONAL
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VANDOEUVRE CEDEX

à soutenir devant un jury de l'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE, une thèse intitulée :

« Isolation and structure elucidation of biosurfactants from microorganisms and their applications in drug delivery systems »

en vue de l'obtention du titre de :

DOCTEUR DE L'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE

Spécialité : **« Génie des Procédés et des Produits »**

Fait à Vandoeuvre, le 09 février 2010
Le Président de l'I.N.P.L.,
F. LAURENT

TEL. 33/03.83.59.59.59
FAX. 33/03.83.59.59.55

Titre : Extraction et identification de la structure d'un tensioactif synthétisé par des microorganismes. Encapsulation dans des nanoparticules à libération contrôlée.

Résumé : Des microorganisms produisant des molécules tensioactives ont été isolés à partir d'échantillons de sols contaminés par des huiles, en provenance des provinces de Songkhla et Chiangmai (Thaïlande) et de Shianghai (Chine). Les différentes souches ont été sélectionnées de façon à obtenir les biosurfactants ayant les meilleures propriétés tensioactives et d'émulsification. Parmi 102 souches isolées, 6 microorganismes produisaient des biosurfactants. La souche SK80 a conduit aux meilleures propriétés tensioactives. Des observations morphologiques macroscopiques et microscopiques ont permis de caractériser la souche SK80. L'analyse de la séquence ARNr 28S indique que cette souche appartient à la famille *Exophiala Dermatitidis*. La composition du milieu de culture (source de carbone et d'azote) et les conditions de culture de ce microorganisme ont été adaptées de façon à obtenir des quantités importantes de biosurfactant. Des analyses spectroscopiques (RMN ^{1}H, RMN ^{13}C**,** COSY et de masse, APCI MS) ont révélé que ce biosurfactant était un monooléate de glycérol. La monomyristine a été choisie comme constituent synthétique modèle dans des etudes d'encapsulation. Deux méthodes de préparation, émulsion/évaporation de solvant, nanoprécipitation, ont été employées pour encapsuler la monomyristine dans des nanoparticules recouvertes de dextrane et dont le cœur était constitué de poly(acide lactique) ou de dextrane hydrophobisé. Les conditions d'encapsulation ont été variées afin de maximiser le rendement d'encapsulation et la stabilité colloïdale des particules.

Mots-clés : Microorganisme, Biosurfactant, Monomyristine, Nanoparticule, Encapsulation.

Title: Isolation and structure elucidation of biosurfactant from microorganism and its application model in drug delivery system.

Abstract: Biosurfactant producing microorganisms were isolated from oil contaminated soils collected from Songkhla and Chiangmai province, Thailand and Shianghai, China. Their culture broths were screened for obtaining biosurfactants with the highest surface activity and emulsification ability. Among 102 isolates, 6 microorganisms produced biosurfactants. The culture supernatant of SK80 strain exhibited the highest surface activity. SK80 was identified by macroscopic morphology, microscopic morphology and showed that it is a black mold. The 28S rRNA sequence homology analysis suggested that SK80 belongs to *Exophiala dermatitidis*. The composition of culture medium such as carbon source, nitrogen source, and culture condition of this microorganism was optimized to obtain high amounts of biosurfactant. ^{1}H NMR**,** ^{13}C NMR**,** COSY and Mass Spectrometer (APCI MS) results indicated that this biosurfactant was monoolein (oleoyl glycerol), a kind of monoacylglycerol. Monomyristin was chosen as a monoacylglycerol model to be synthesized and used as nanoparticle encapsulated drug. Two preparation methods, emulsion/solvent evaporation and nanoprecipitation, were used to encapsulate monomyristin in dextran-covered nanoparticles with poly(lactic acid) of hydrophobized dextran as the core material. Encapsulation conditions were optimized with regard to the yield encapsulation and the colloidal stability.

Keywords: Microorganism, Biosurfactant, Monomyristin, Nanoparticles, Encapsulation.