NATURAL TEMPLATE-MEDIATED SYNTHESIS OF HIGH LOADING MICROCAPSULES FOR PERSONAL CARE PRODUCTS

BY

MS. BUNTHOEURN KHANN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF ENGINEERING (ENGINEERING TECHNOLOGY)

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ENTITLED

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was approved as partial fulfillment of the requirements for
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ABSTRACT

Essential oils have attracted increasing attention for applications in various fields, especially pharmaceutical products, cosmetic products, and food preservative applications. Owing to their sensitivity to environment including light, temperature, and oxygen, they are easily deteriorated, leading to low quality and shelf life of the desired products. The protection or encapsulation into polymeric microcapsule is required. However, the microcapsules are mainly produced from synthetic polymers, which are non-degradable, leading to waste accumulation and contamination of the ecosystem. The development of natural microcapsules or plant-based microspores for encapsulation of bioactive ingredients is of interest. Then, this work aims to utilize the natural microcapsule of *Lycopodium clavatum* spores for encapsulation of black pepper essential oils (BEOs) and Sacha inchi oil using a facile passive loading technique. This work is divided into two main parts.

Part one concerned the extraction of *L. clavatum* spore by chemical extraction and extraction of BEOs, including characterization of Sacha inchi oils. Chemical extraction was applied to remove the native biomolecules such as proteins and lipids from *L. clavatum* spore. Scanning electron micrographs and Fourier-transform infrared

spectroscopy analysis have shown the spore with clean, intact, and microcapsule (hollow) structure. At the same time, BEOs were obtained from the extraction of Thai commercial black pepper (*Piper nigrum)* by using the hydro-distillation method. The major compounds of the extracted BEOs analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) composed of caryophyllene (38.18%), D-limonene (33.5%), and followed by α-copaene (4.5%), and γ-elemene (2.6%). In parallel, commercial Sacha inchi oils compositions were characterization by GC-MS before used. Results showed that a high percentage of fatty acid such as linoleic (ω6:28.3%) and oleic acid (ω9: 10.68%), α-Tocopherol (1.08%), cholesta-3,5-diene (2.44%), γ-Tocopherol (9.39%), Tri-o-methyl-dopamine (1.88%), Stigmasterol (8.29%), γ-sitosterol (4.52%) was obtained.

Part II involved utilization of extracted *L. clavatum* microcapsule spores for encapsulation of BEOs and Sacha inchi oil by using a facile passive loading technique. The different ratios of oil: spores were investigated. For encapsulation of BEOs, with the ratio of BEOs: spores at 1:1, high encapsulation efficiency (%EE) and loading capacity (%LC) of 50.64$\pm$4.92 and 29.26$\pm$3.70% were obtained. Moreover, perfect morphologies of BEOs-encapsulated microcapsule spores were still observed. For the encapsulation of Sacha inchi oils, the high %EE and %LC of 42.17% and 30.46% were obtained using the ratio of oil: spores at 0.75:1. The release behaviours of the Sacha inchi oil from the microcapsule under friction techniques showed that 96.43$\pm$1.14%. Moreover, the as-prepared microcapsule spores had the potential for antioxidant properties of 71.58$\pm$0.50% at 40 mg/ml.

Keywords: Natural microcapsule, *Lycopodium clavatum* spores, Essential oils, Sacha inchi oils, Encapsulation, Passive loading

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Ms. Bunthoeurn Khann

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LIST OF SYMBOLS/ABBREVIATIONS

Symbols/Abbreviations	Terms
BHA	2-tert-Butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole
CLSM	Confocal laser scanning microscope
CO_2	Carbone dioxide
EOs	Essential oils
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	Hydro Performance Liquid Chromatography
H_3PO_4	Ortho Phosphoric Acid
KOH	Potassium Hydroxide
NaOH	Sodium Hydroxide
PBS	Phosphate-buffered saline
SEM	Spectroscopic Electron Microscopic
UV-vis	Ultraviolet-Visible Spectroscopy
%EE	% Encapsulation Efficiency
%LC	% Loading Capacity

CHAPTER 1
INTRODUCTION

1.1 Background of study

The development of polymeric microcapsules has attracted vast attention for applications in various fields, especially pharmaceutical products, drug delivery carriers, cosmetics products, insecticides, paints, and textile products. These materials are mainly produced from conventional polymers, which impose adverse effects on the environment due to their non-degradable nature, leading to waste accumulation and contamination of the ecosystem. The development of "green" products from renewable raw materials instead of fossil fuels has attracted vast attention in countries worldwide (Kozlowska, Prus, & Stachowiak, 2019).

Plant-based spores, i.e., *Lycopodium* (*L.*) clavatum present one form of robust natural microcapsules for drug delivery carriers. The *L. clavatum* spore provides a readymade capsule scaffold with high structural uniformity and a large internal cavity porous shell. It is a rigid outer shell structure that can stay intact in severe environments. It resists strong acids and strong bases and hardly dissolves in organic solvents. It is crucial to prevent the allergy problem because allergy would cause by native biomolecules. Proteins are removed before encapsulation of active ingredients in natural *L. clavatum* spores. The natural *L. clavatum* spores were chemically treated by the conventional treatment method, by using acetone, potassium hydroxide, and phosphoric acid followed by a series of washing steps to produce intact clean spores by modified chemical cleaning process (Mundargi, Potroz, Park, Park, et al., 2016). Functional groups, e.g., hydroxyl groups, phenolic groups, and carboxylic acid groups, were observed. For example, Dyab et al. (2014) demonstrated the effective encapsulation of erythromycin and bacitracin antibiotics into the natural spores by using a passive diffusion followed by a vacuum loading technique. These two antibiotics were successfully encapsulated, leading to a 16.2% loading capacity and a 32.4% entrapping efficiency.

Sacha inchi (*Plukenetia volubilis*), also known as Maní Inca, is a wild oleaginous plant of the Euphorbiaceae family distributed in Central America and the

Amazon. Different studies have shown interest in this oil since its composition accounts for around 93% of fatty acids, from which approximately 45.2% correspond to linolenic acid (omega-3) and 36.8% to linoleic acid (omega-6). Sacha inchi essential oil has aroused interest in the food and cosmetic industries because of the high quantity and quality of the edible oils contained in its seeds. Different studies have shown interest in this oil since its composition accounts for around 93% fatty acids, from which approximately 40-50% correspond to linolenic acid (omega-3), 30-40% to linoleic acid (omega-6), and ~10% oleic acid (Fanali et al., 2011; Wendel & Heller, 2009). For use in cosmetics, the omega-6 fatty acid, linoleic acid plays a role in the regeneration of the lipid barrier structure in the stratum corneum skin. Moreover, omega-3 linolenic acids are precursors of other polyunsaturated fatty acids and arachidonic acid, which are involved in the differentiation of keratinocytes (Chirinos et al., 2013). In addition, natural antioxidants, including tocopherols and polyphenolics, have also been found in Sacha inchi oil. Recently, a clinical study to assess the moisturizing efficiency and skin irritation potential of Sacha inchi oil was demonstrated. Results indicated that Sacha inchi oil is safe to use and provide evidence supporting the performance of the oil as an active moisturizing ingredient. However, its use is always confronted by several factors including their high volatility and high risk of deterioration upon direct exposure to heat, humidity, light, or oxygen. To overcome these problems, polymeric nano/micro-particles have been introduced to encapsulate essential oil, shielding them with good stability, controlled delivery, enhanced bioavailability, and improved efficacy.

In this work, *L. clavatum* natural spores are utilized to encapsulate bioactive compounds, i.e., black pepper essential oils, Sacha inchi oil, by using a facile passive method. To prevent the allergy problem, allergy-causing native biomolecules, i.e., proteins, are removed before encapsulation of active ingredients in natural spores by chemical treatment, using acetone, potassium hydroxide, and phosphoric acid by a series of washing steps. The clean, intact, and hollow spores are determined by Fourier-transform infrared spectroscopy (FTIR) and field emission-scanning electron microscopy (FE-SEM). Sacha inchi essential oil is quickly loaded into the treated spore, the encapsulation efficiency and capacity loading of encapsulated spores are evaluated

by UV vis spectroscopy. The release behaviors of the Sacha inchi oil from the microcapsule were determined by UV vis spectroscopy as well.

1.2 Objectives

The objectives of the research work are:

1. To extract essential oil from commercial black pepper by a simply hydro distillation.
2. To clean *L. clavatum* spore or remove native biomolecules, e.g., proteins, by chemical treatment techniques.
3. To encapsulate essential oils, e.g., black pepper oil and Sacha inchi oil, into treated microspores by passive loading and vacuum techniques.
4. To investigate the properties of encapsulated microspores, e.g., encapsulation efficiency, loading capacity, release behavior, and antioxidant activity.

1.3 Scope of study

This work is divided into two main parts:

Part 1 Extraction of essential oils and *L. clavatum* microspores.

1. Black pepper essential oils were extracted by a simply hydro distillation. The obtained black pepper essential oils were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) to determine the compositions.
2. Moreover, commercial oils (Sacha inchi oils) were used as the active ingredient for encapsulation. Before use, Sacha inchi oils were extracted from clod press, and its composition was also determined by Gas Chromatography-Mass Spectrometry (GC-MS).
3. *L. clavatum* was extracted to remove the native biomolecules by using acetone, KOH, and phosphoric acid under the desired conditions. After, it was cleaned with hot water and consequently by ethanol to remove some residues on the surface of spores. The treated microspores were characterized by FTIR, FE-SEM analysis for each extracted condition to

observe the change of functional groups on the spore's surface and morphology, respectively.

Part 2 Encapsulation of BEOs and Sacha inchi oils into microcapsule spores.

1. BEOs oils were encapsulated into native *L. clavatum* spores via a passive loading technology.
2. Sacha inchi oils were encapsulated into the treated spores by passive loading and vacuum loading techniques.
3. The encapsulation efficiency and capacity loading of the encapsulated microspores were evaluated by using UV-vis spectroscopy. Moreover, the encapsulated material was tested with confocal laser scanning microscopy to ensure essential oil was located in the capsule.
4. The release behavior of oils from the microcapsules was conducted by using the pressure technique. Then, the release sample was analyzed with UV-vis spectroscopy.
5. The antioxidant activity of the Sacha inchi oil encapsulated microcapsules were determined to evaluate the potential compounds that can prevent or delay oxidation.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Microencapsulation

Recently, the development of microencapsulated materials is increasing with requirements of the usage in the global market. Microencapsulation is the process to form the microparticle that will be applied in different applications according to the various materials (Khan, Gauttam, Chandel, Ali, & Tariq, 2016). Encapsulation is the process that would formulate the particle into different diameters and shapes. Thus, the active agent inside the microcapsule will be the bioactive and microcapsule, which is the shell that could protect the bioactive from unfavorable environments. The primary purpose of encapsulation is to control the target release profile with a specific time and place (Donhowe & Kong, 2014). For many decades, select bioactive and capsule research will still interest all the research and industries. There are various individualized morphologies such as particle, sphere, or specific geometry properties (Martín, Varona, Navarrete, & Cocero, 2010). Most encapsulation products with size nanometers are called nanoencapsulation, whereas microencapsulation technology refers to sizes ranging from 1μm to 1mm (M. J. Uddin et al., 2019).

Microencapsulation has been extensively used in various areas, such as the medical, pharmaceutical, agricultural, and food industries (Jyothi et al., 2010). As research on the food chain, microencapsulation can enhance the product quality of coloring, sweeteners, flavoring, and microorganisms (Aghbashlo, Mobli, Rafiee, & Madadlou, 2012). At the same time, most of the drugs are formulated with bioactive compounds. In this case, they use antioxidants such as Vitamin E, C, A, mineral (Zn, Cu), ascorbic acid, tocopherols, propyl gallate (PG), tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA)n are the compounds to prevent skin from the harsh environment (Ravetti et al., 2019). Issue of the synthesis material concerns to the environment which was not degraded on the earth such as polyethylene terephthalate (PET), polyvinylchloride (PVC), polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyamide (PA), which would form the microplastic or microbead in the environment. Therefore, the alternative solution of the microcapsule

is to use natural and biopolymer. Several biopolymer materials were selected to fabricate microcapsules such as polylactic acid (PLA), chitosan, natural capsule (spores). In addition, bioactive compounds are the main functional property used to cooperate with microcapsules, which are essential oils, lipids, protein, fatty acid, and etc.

2.2 Encapsulation techniques

Microencapsulation can apply in different industrial activities, which depend on the desired structure that they design. The structure of each material can be determined by different factors, namely encapsulation technology, the origin of wall or shell materials. Generally, the encapsulation methods are divided into two main parts; chemical and physical methods, as displayed in Table 2.1. In addition, the details of several methods are described as following:

Table 2.1 Three techniques of microencapsulation.

Chemical	Physical	
	Physico-chemical	Physico-mechanical
▪ Polymerization in situ Emulsion, ▪ suspension, dispersion ▪ Interfacial polycondensation	▪ Coacervation ▪ Solvent evaporation, ▪ Layer by layer adsorption ▪ Complex precipitation ▪ Ionic gelation ▪ Supercritical fluid precipitation	▪ Spray-drying congealing ▪ Electrostatic encapsulation ▪ Pan coating ▪ Vacuum encapsulation ▪ Extrusion ▪ Air suspension ▪ Multiorifice-centrifuge

2.2.1 Chemical methods

First, polymerization is a method of the interfacial process; however, it was used to encapsulate water-immiscible solid and liquid. Shell walls make from the polymerization of the monomers then it was added into the batch encapsulation. Polymerization happened in the continuous phase and on the continuous phase side of the interface formed by the disperse bioactive core and continues phase. Formation of copolymer's ready to form in growing size with low molecular weight interacts into the surface of the disperse bioactive core being encapsulated. Recent research studied the different phase change materials (PCMs) polystyrene shells prepared by suspension polymerization. Industrial researchers have prepared the different core materials such as Paraffin wax PRS, tetradecane, Rubitherm RT27, and nonadecane, which were encapsulated with styrene as a shell material by suspension polymerization. Firstly, microcapsules synthesis was carried out in a 1-1 glass reactor equipped with digital control of stirring rate, oil thermostat bath, a reflux condenser, and a nitrogen gas inlet tube. The synthesis process involves two phases: a continuous phase containing water and polyvinylpyrrolidone and a discontinuous phase containing styrene, PCM, and benzoyl peroxide. There are various properties of commercial PCMs (Paraffin wax, tetradecane FLUKA, PEG 800 CLARIANT, PEG 1000 PANREAC, Rubitherm RT27, Nonadecane) were investigated. The continuous phase was added to a glass reactor with mild agitation. After mixing with the monomer and PCM, the efficient operation was needed to minimize the idle time. Next, the disperse phase was added into the continuous phase keep under vigorous agitation. The reaction dispersion was bubbled with nitrogen and held temperature at 110℃ in the thermostat bath. The polymerization process was processed for 6 h under a nitrogen controlling (Sánchez, Sánchez, de Lucas, Carmona, & Rodríguez, 2007). Finally, average diameters of PCMs were obtained such as PRS paraffin wax (237.81μm), Tetradecane (254.59 μm), PEG 800 (013 μm), PEG 1000 (212.554 μm), Rubitherm 27 (212.54 μm), Rubitherm 20(311.42 μm), and Nonadecane (442.03 μm).

Emulsion polymerization is one of an excellent method to produce nanoparticles (10 to 1000 nm). It is relative to the emulsification of hydrophobic monomer in an aqueous phase with the monomer; this method needs surfactant (emulsifier), and it is

pretty challenging to remove from polymer. The study of encapsulating Oral Insulin into poly-(Alkyl cyanoacrylate) nanospheres were produced with dispersion them together. This work was conducted by polymerizing isobutyl cyanoacrylate (IBCA) in an acidic medium, insulin (15U/ml) added to the polymerization medium for 60 min. Then, nanospheres(364MW) displayed a mean size of 145 nm and a contribution rate of 1U of insulin/mg of polymer (Damge, Vranckx, Balschmidt, & Couvreur, 1997).

Interfacial polycondensation (Figure 2.1) is the complementary monomers at the interface of a two phases system. The microcapsule preparation required carefully controlled conditions to make tiny droplets of one phase (disperse phase) in the other phase (continue phase/suspension medium). Interfacial polycondensation was used to form both matrix type and mono core microcapsules based on the solubility of the polycondensate in the droplet phase. Many polymer materials, namely polyurea, polyamide, polysulfanamide, polyester, or polyurethane, are applied to prepare microcapsules by this technique. There are two mechanisms of organic solvent dispersion to form microcapsules (Tech; Tuncay et al., 2000). In addition, the type of microcapsule was observed with two phenomena; first, if the polymer is not soluble, it precipitates around the droplets and leads to the formation of mono core types of microcapsules. If the polymer soluble in the droplets matrix type microcapsules are formed as shown in Figure 2.1 (Dubey, 2009).

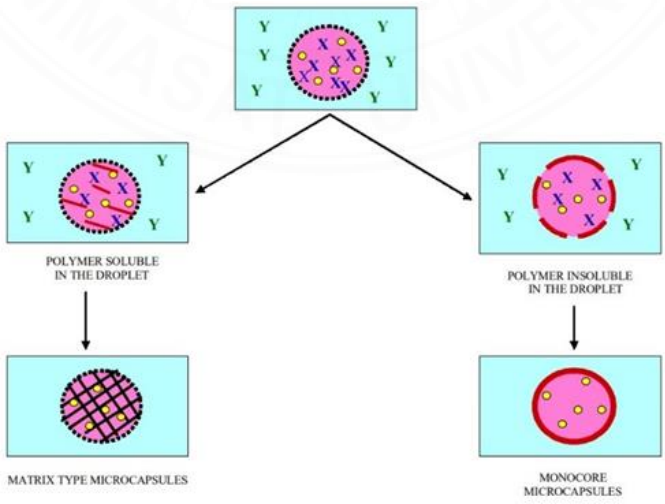

Figure 2.1 Mechanism of matrix type or monocore type microcapsule by interfacial polymerization (X and Y are bifunctional monomers).

2.2.2 Physico-chemical

The process of physico-chemical is involved both chemical material and external physical conditions in producing microcapsules. As the following process:

Coacervation is a term of microencapsulation to form microcapsules by continuous agitation to encapsulate liquids and solids. There are two types of this process which are simple and complex coacervation. Generally, a simple process was involved with a single colloidal solute (one phase). Simple coacervation occurs the separation of the binary or ternary systems induced by changing the polymer solution's temperature. All these solutions (gelatin or ethylcellulose or organic media) were used the coacervation technique to encapsulate by adding sodium sulphate or ammonium sulphate or alcohol to those solutions and form two phases. However, complex coacervation relevant to two charged polymeric materials, such as gelatin and acacia. The process of different microencapsulation is preparing an aqueous polymer under suitable temperature, pH, and concentrations to separate the polymer-rich complex coacervate phase. At pH values below its isoelectric, the gelatin becomes positively charged and keeps the gum acacia natural anionic water-soluble polymer (Tech; B. Wang, Akanbi, Agyei, Holland, & Barrow, 2018).

Solvent evaporation: it is the noticeable process to form microcapsules, both solid and liquid core. First, the polymer materials are dispersed in the liquid manufacturing vehicle phase with continuous agitation to obtain the desired size, the stirring rate is reduced, and evaporation of the solvent for the polymer is performed under atmospheric or reduced pressure at an appropriate temperature. Second, the core material is dissolved in the coating solution to make microcapsule polymer. Third, those prepared microcapsules are collected by centrifuge, filtration then uses freeze-dried. A few contribute factors that affect the size of microcapsule solubility shell material, type core material, type and concentration discharge agent, and agitation. The equipment propeller-style blade with a specific speed was used to stir and control microcapsule size (Maa & Hsu, 1996). However, solvent evaporation mainly uses toxic organic, which is very concerned with health regulation because of some residuals of an organic solvent such as methylene chloride, dichloromethane, chloroform, and ethyl acetate contaminant the environment final products.

2.2.3 Physico-mechanical

Spray-drying: it is a low-cost process that they usually use to encapsulate fragrance, oils, and flavors. This process encapsulates and produces the Active pharmaceutical Ingredient (API) or oral administration (Torrado & Augsburger, 2008). An experiment of this technique prepares by dispersed core and coating solution while the core is insoluble, and polymer dissolved. Next, the hot air stream was processing by atomizing the mixture to remove solvent from the polymer coating solution, and thus shell coating solidification is achieved. Generally, the spray drying technique produces coated aggregates rather than coated single particles. It is helpful for encapsulation of heat-sensitive drugs such as protein and peptides because it requires mild temperature. Spray drying contains different components, as shown in Figure 2.2, such as an air heater, atomizer, spray chamber, or blower, cyclone, and product collector.

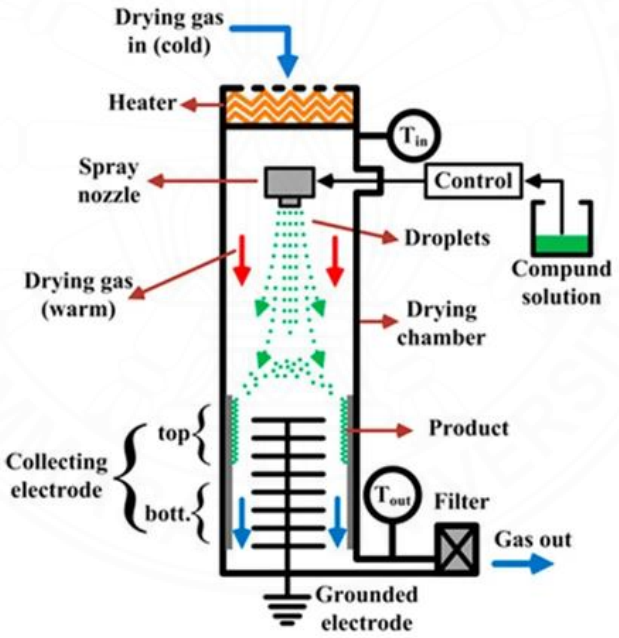

Figure 2.2 Spray drying process.

Air suspension: this process contributes to a machine that consists of the control panel, nozzle, air distribution plate, and coating chamber. The coating chamber particles were put and suspended on the upward moving air stream. And in coating zone was applied by spraying to the moving core particles. Thus, the core material repeatedly flows through the coating zone until desired coating thickness is received. There are

several points to consider of the effective encapsulated technique: density, surface area particle size/shape, melting point, solubility, wettability, volatility, and flowability of the core material. Secondly, considering on concentration and amount of coating material. Thirdly, the air stream volume needs the contribute fluidized the core material, and the last part to consider is inlet and outlet operating temperature. However, this process can encapsulate only solid particles, which range 74-250μm of the inspect equipment (Ichikawa, Fujioka, Adeyeye, & Fukumori, 2001).

Electrostatic encapsulation: This process focuses on the liquid core material that liquid can be encapsulated by atomizing the core material and molten shell material and charging the two kinds of droplets with opposite polarity. Those droplets are mixed in the collision chamber. The particles are allowed to keep as a suspension in a thermostatic space, and then the capsules are incredible in the powder form (Sliwka, 1975).

Vacuum Encapsulation: The principle of this process appropriate to the nonvolatile core material under applying high vacuum. During the process, the wall material is moved around in a vacuum and condensed on the colder particles; commonly, paraffin, waxes, metals, oxides were used as shell material. Interestingly, various apparatus can be used for this process. The core material particles are cooled in a container vessel and fall onto a vibrating chute under a vacuum. Those cores will be struck by a jet of vaporized wall material and coated (Luzzi, 1970). Similar to vacuum loading, passive loading generally used to encapsulate with the pre-formed microcapsule. The shaking technique could produce microencapsulate materials.

2.2.4 Factors controlling sizes of capsule

Various factors affect capsule properties, such as the composition of wall materials, degree of cross-linking, capsule thickness, emulsified agents, and technique to encapsulate. According to the study of forming microcapsules from sodium alginate, which applies spray drying technique and desirable microcapsule, some parameters were considered. The solvent of calcium chloride solution is put under a nozzle, the parameters of the encapsulated such as pressure, liquid flow, vibration frequency, and electrostatic voltage. A procedure was used to obtain alginates-starch microparticles,

and the condition a mixture of 1.5% sodium alginate and 1.5% starch solution was prepared in ratio 1:1(g) to receive microparticles (768 μm). Several parameters were set up as follows electrode(2500V), vibration frequency(100Hz), amplitude(6), pressure(500mbar), and diameter of a nozzle(570μm) (Kozlowska et al., 2019). Table 2.2 shows the recent studies that used different techniques to set up the various emulsification parameters to obtain the desired size.

As the flowing Table 2.2, they are the recent studies that used a different technique to set up the various parameter of emulsification and other techniques:

Table 2.2 Encapsulation technique to control capsule size (Maes, Bouquillon, & Fauconnier, 2019)

Encapsulation method	Encapsulation preparation	Capsule size	Solvent and encapsulated materials	Reference
Emulsification	▪ Simple emulsion ▪ Spontaneous emulsification method ▪ The organic phase is added to an aqueous phase then stirring at a temperature (25) for 15 min.	75nm (droplet)	▪ Organic phase: oils carrier (medium-chain triglyceride), EOs, and surfactant (tween), ▪ aqueous phase: citrate buffer	(Lou et al., 2017) (Y. Chang, McLandsborough, & McClements, 2013)
	▪ High-pressure homogenization (HPH) technique, primary emulsions obtained by stirring at 24000rpm for 5 min are then put into HPH ten times at 350MPa.	57-175nm	▪ Organic phase: oils carrier (sunflower or palm oils), EO, and surfactant (soy lecithin, tween, glycerol mono-oleate) ▪ Aqueous phase: water	(Donsì, Annunziata, Sessa, & Ferrari, 2011)

	▪ Final step: crystallization by rapid cooling in an ice bath			
Multiple emulsion	▪ Emulsion of aqueous phase 1 in organic for 10 min at 800rpm, then second emulsion in the aqueous phase (2) at 500 rpm(W1/0/W2). Stirring is continued for 3 hours to allow solvent evaporation. ▪ Finally, microcapsules are filtered, wash and air-dried overnight at room temperature.	200-400 μm	▪ Carbohydrate polymer-protein blends: ▪ Aqueous phase1: sodium alginate ▪ Organic phase: EO, methylene chloride solution of ethylcellulose, and surfactant(tween) ▪ Aqueous phase 2: gelatin and surfactant(tween)	(Banerjee et al., 2013)
	▪ Liquid atomization into tiny droplets:	0.2-40 μm	▪ Arabic gum and maltodextrin	(López et al., 2014),(Pudziuvelyte et al., 2019).

Spray drying	▪ Emulsion oils/water(O/W), dissolve matrices in water at 50 C for 2 hours. Then, add oil phases by stirring at 24000 rpm for 30 min. ▪ Spray-dried, A hot air stream atomizes emulsions in the drying chamber of a spray dryer.	3-4.5 μm	▪ Inulin solution to make Raftalin microparticles.	(Beirão-da-Costa et al., 2013)
		223-399nm	▪ Alginate and cashew gum	(de Oliveira, Paula, & de Paula, 2014)
		9-15 μm	▪ Chitosan, chitosan, and alginate, and chitosan and inulin	(Dima, Pătraşcu, Cantaragiu, Alexe, & Dima, 2016)
		12-13 μm	▪ Modified starch and Arabic gum	(de Barros Fernandes, Borges, & Botrel, 2014)

2.2.5 Control release of microcapsule system

The trigger releases have various techniques, such as biological, magnetic, electrical, thermal, chemical, and physical approaches. In addition, various factors contribute to the release of capsule materials. For example, the chemical changes of microcapsules cause by chemical reactions with shell material, and physical changes relate to phase interaction with external factors such as mechanical, heat, and pressure (Casanova & Santos, 2016).

2.2.5.1 Release by chemical changes

Chemical changes mean the approach to control the reaction on the wall materials, which provide many benefits to drug carriers and cosmetics systems. Three mechanisms based on chemical changes in the shell wall were studied: shell wall switching reaction, the disintegration of the shell wall by chemical cross-links, and triggered depolymerization of the shell wall. Each mechanism can be essential for specific applications with regards to the desired synthetic and applied materials. The details of the concept behind each approach will be addressed with a few examples as following Figure 2.3:

Switching: the process is not entirely related to the reaction involving covalent bond formation, but it was controlled the porosity of microcapsule. Moreover, applied energy stimulant causes the change in conformation. Thus, light, electricity, and chemical stimulants are used to adjust or modify a shell wall's porosity. The benefit of this process includes the ease of the trigger incorporation into the shell wall, and the capsule’s ability of the opening pore and the bioactive will come out directly.

Cross-link removal: the shell wall cross-link polymer was disintegrated reaction by chemically separated. The shell wall material incorporates cross-link polymer chains. Many different chemical triggers have been used to reduce disulfide bonds, the separation of acetals group by acid, the hydrolysis of carbonate esters by a base, and the separation of peptides by enzymes. This technique's usefulness is the control release time with burst release and attention to the drug delivery system (Esser-Kahn, Odom, Sottos, White, & Moore, 2011). The disadvantage of this process is a requirement of

cross-links to protect bioactive, then the formation of complex microcapsule synthesis is obtained with higher loading in the shell wall.

Shell wall depolymerization: This technique uses the triggered depolymerization of a wall polymer to remove a protecting headgroup. The headgroup could be carbonate esters or carbamates. The recent work was published in 2010, which studied the self-immolation capsule and its release contents when triggered by light, acid, and base. The process is like cross-link removal; however, self-immolation (wall depolymerization) provides hundreds of carbonate ester and needs a lower trigger. The mechanism of self-immolated polymers (SIPs) forms a new class that can promptly degrade by the sequence of polymerization reactions to their monomers based on cut-out end-group functionality.

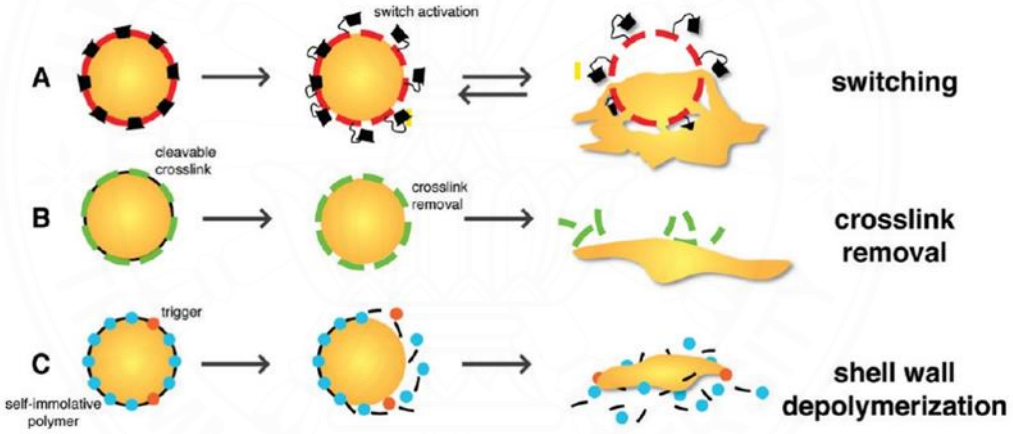

Figure 2.3 Release studies by (A)switching, (B) crosslink removal, (C) shell wall depolymerization.

2.2.5.2 Release by bulk changes

The variety changes of this technique refer to the bioactive release from encapsulated materials which capsules rapture by applying mechanical force to the shell. There are four main sections to discuss: pressure-induced raptures, shell wall melting, changes in porosity, and thermomechanical degradation of the shell materials. Those techniques are highlighted in Figure 2.4.In addition, physical force is a crucial

factor trigger for wall materials rapture and capsule opening and brought much attention to the research and industrial materials (Keller & Sottos, 2006).

Pressure-induced rupture: Pressure-induced rupture is a method of releasing bioactive core cause by internal pressure within a microcapsule material. Two ways are relevant to increase pressure on the inside of the microcapsule wall. In the first approach, the cores liquid is vaporized by heating the capsule materials and mastication and pressing (Silva et al., 2014; Wong, Yu, Curran, & Zhou, 2009). The second approach is to cause the wall material to contract or smaller than the previous structure by external force factor and thermal The internal pressure capsule reaches a critical pressure value (J. Hu, Chen, & Zhang, 2009).

Shell wall melting: It is another technique that releasing core materials based on the temperature goes up. For wall materials melting, consider the melting point polymer. The melting of wall polymer must be enough that melting occurs before core liquid vaporization. The polymer chemistry adjustment was significantly improved by advancing in polymer chemistry to receive well-defined melting points that can be finely tuned for specific usages.

Changes in porosity: the overall structure of shell material open-pore through one polymer chain was shrunk by thermal then the core materials were released. The shell polymer generates from a polymer or a mixture of two polymers (W.-C. Yang, Xie, Pang, Ju, & Chu, 2008).

Thermomechanical degradation of the shell wall: In this method, they try to use external impeach such as mechanical force, magnetic, and electric field to degrade the shell materials or cracking and allowed the core release (S.-H. Hu, Tsai, Liao, Liu, & Chen, 2008).

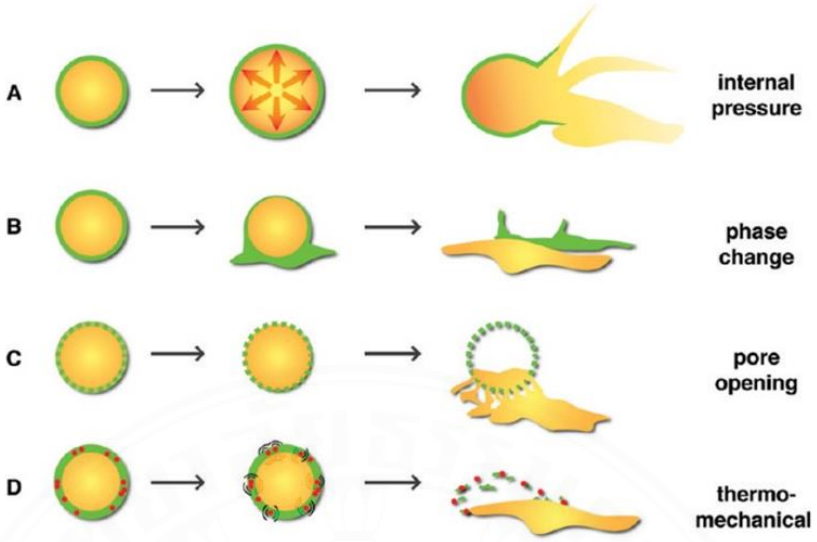

Figure 2.4 Release studies by bulk changes (A) Internal pressure, (B) Phase change, (C) open change, (D) thermo-mechanical.

2.3 Biopolymer microcapsules

There are various encapsulation applications on the market, mainly related to food and feed, agricultural and environment, home and personal care, chemistry, human and animal health products. According to the products on the market nowadays, there are many different types of cosmetic, drugs, and personal cares that use microcapsule to carrier the bioactive compounds not only of that but also encapsulate of favoring, self-healing materials and Palliative Care Management (PCM) (Elgegren et al., 2019). Some previous research has reported using polymer or synthesis polymers that are affected to the environment and the human's health. By the way, an alternative solution has been fixed by using natural materials for microcapsules preparation (Ghayempour, Montazer, & Rad, 2015). Biopolymer microcapsules can derive from alginate, cellulose, chitosan, and sporopollenin, forming beads or particles (Mahdavi, Jafari, Ghorbani, & Assadpoor, 2014).The formation of the biopolymer microcapsules can be prepared by physicochemical methods such as the coacervation method the spray drying, including chemical methods such as interfacial polymerization, miniemulsion polymerization, and gelling in the solvent (Kozlowska et al., 2019).According to Basto et al., a current study stated on microencapsulation that black pepper (*Piper nigrum L.)*

essential oils were encapsulated with gelatin and sodium alginate by complex coacervation (Physico-chemical).

There have several parameters for set up and control the size and thickness of the microcapsule and bioactive between gelatin (GE) and sodium alginate (NaAlg). The variation of ratio GE: NaAlg, gelatin: sodium alginate (1:1, 1:2, 1:3, 1:4, 1:6, 2:1, 4:1, and 6:1(w/w)) were tested since the ratio factor can affect the preparation of microcapsule. Moreover, the pH is another factor that affects the formation of the GE/NaAlg complex, and it was analyzed by potential zeta and turbidity in the range of pH (2.0-7.0). Next, the capsule considers the concentration of GE/NaAlg capsule (0.7,1.4, or 2.1%(w/w) and biopolymer mixing ratio 6:1. Black pepper EO was mixed with 40%(w/w) of Tween 20 and then added to GE/NaAlg solution then the solution was emulsified using Ulta-Turrax at the stirring rate of 10 rpm for 3 min; it takes around 30 min at 25℃ to homogenize with adjusting of pH(4.0) using acetic acid(20%v/v). Further, solution 25ml of calcium chloride(30mg/ml) was dropped and dissolved by magnetic stirring. Finally, the ratio of 6:1(gelatin/sodium alginate) at pH 4.0 was the best condition to form a capsule with efficiency from 49.13 to 82.36% (Bastos, Vicente, dos Santos, de Carvalho, & Garcia-Rojas, 2020)

Another work reported by da Silva Barbosa, Yudice, Mitra, and dos Santos Rosa (2021)that they encapsulate EOs of Rosewood and cinnamon using Tween 80 and poly (butylene adipate-co-terephthalate (PBAT) as wall material and they apply solvent evaporation (Physico-chemical method) to encapsulate the bioactive in the application of food package. The methodology of the capsule will prepare a solution of polysorbate 80 in (0.1%wt), then essential oils were added in 1:3 ratio (polysorbate: EO), those disperse stirring for 2h. However, PBAT was dissolved in chloroform for 24h in a magnetic stirrer to form the polymeric solution; then, the polymeric solution was added to an aqueous solution using a syringe; the mixture was stirred for 12h, waiting until the chloroform evaporates. Encapsulated material was separated and clean with ethanol from the surface capsule absorb EOs then, drying at room temperature for 12h. Finally, the encapsulated material has been characterized with SEM, which shows the spherical shape with a diameter (3.9 μm and 3.4 μm) for Rosewood and Cinnamon capsules, respectively. The encapsulated materials of loading efficiency is 11% and 29% for

Rosewood and Cinnamon show significant differences that may affect EO's nature and interaction of physical and chemical properties of EO main compounds (da Silva Barbosa et al., 2021).

Saito, Taguchi, and Tanaka (2018) demonstrated the preparation of chitosan microcapsule encapsulated with essential oils, e.g., camila oils and oleic acid, and applied as hair care products. The technique to prepare microcapsules was heterocoagulation in the continuous water phase at 40 ℃. The oleic acid (0.2g) and camelia oils solution(2g) were dispersed in a water phase(W) and kept stirring (2000-10000 rpm for 5min) with the rotor-stator homogenizer to form (O/W) emulsion. Then chitosan aqueous solution (0.2g) and water (20g) were added to the (O/W) emulsion with stirring 60 rpm for 4h and holding temperature at 40℃. The detailed information of substance and experiment set up conditions to perform the hairdressing as the Figure 2.5. Last, the diameter of the microcapsule in the range from 1.5 to 4.5 μm could be prepared with this condition of the experiment (Saito et al., 2018),

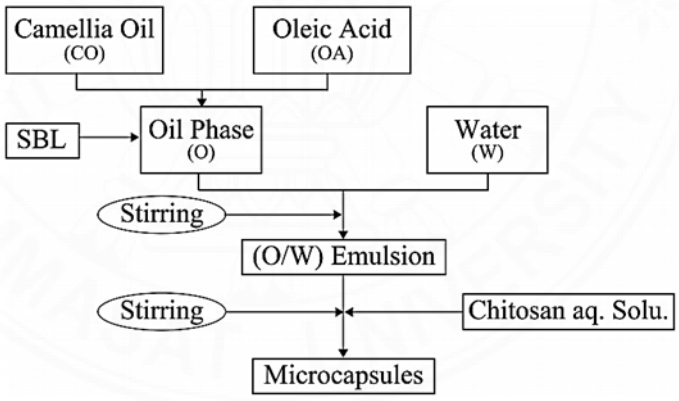

Figure 2.5 Flow sheet for preparing microcapsules.

2.4 *Lycopodium (L.)* clavatum microcapsule spores

Lycopodium (L.) clavatum spore is a natural flower source and grows as pollen with a specific uniform structure. The structure of sporopollenin is shown in Figure 2.6 Structure of sporopollenin; (a) sporopollenin in state powder; (b) Structure of constituents in the cavity of sporopollenin.

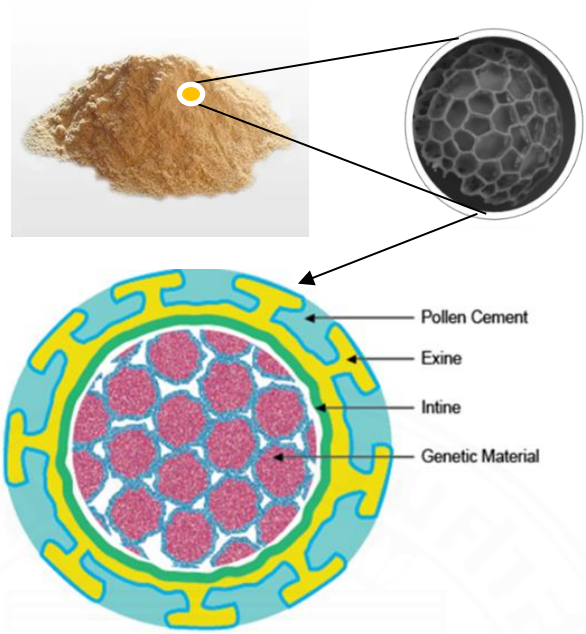

Figure 2.6 (a) Sporopollenin in state powder; (b) structure and constituents in the cavity of sporopellenin.

The spores are uniform and round particle shape with a diameter of around 25 μm. The particle surface is composed of a nano-channel of between 15 to 20 nm. The spore structure comprises of two wall layers to protect protein or bioactive inside. The external structure of spores was composed of exine and pollen cement and hydrocarbon component, and oxygen that is characteristic of polycarotenoid. The exine of the spore is elastic, which can resist intense chemical and high temperatures and a non-toxic environment. Furthermore, the internal spore structure composed of intine inner layer and the cavity, which is cellulose (Dyab, Mohamed, Meligi, & Mohamed, 2018).

Inside the cavity, there are biomolecules, e.g., proteins. The biomolecules or unwanted materials can be extracted or removed through the solvent base or acid to obtain the hollow spore, as shown in Figure 2.7. It has been reported that natural *L. clavatum* spores were hydrophobicity. Acetone treatment partially solubilizes unsaturated phospholipids from the spores while native proteins are retained. The acetone-treated spores were still hydrophobicity. Following KOH treatment, a significant number of proteins were removed.

Moreover, this step partially hydrolyzed esters to carboxylic acid salts and resulted in a hydrophilicity spore. After that, the spores were treated with the acid H_3PO_4. The remaining lipid was removed (Diego-Taboada et al., 2012). To protect the shell of sporopollenin from applying different applications, the optimization of the treated steps was considered at different conditions.

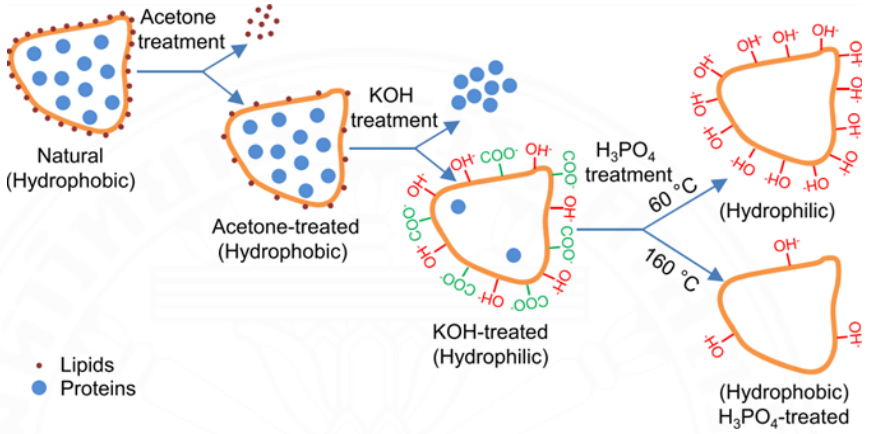

Figure 2.7 The cytoplasm in cavity of spores and wettability after different solvents treatment.

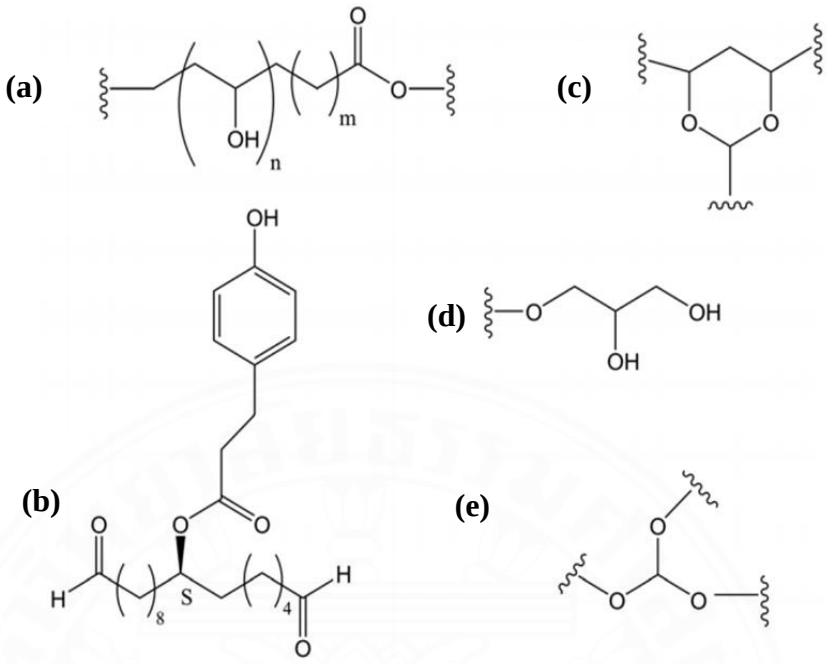

Figure 2.8 Sporopollenin chemical structure; (a). polyester polymers of unit; (b) connecting of an m-dioxane ring; (c) remaining hydroxyl groups are cover by glycerol group;(d) Long chain unit by carboxylic groups at the outer surface;(e) Cross linking structure (c) and (d).

The recent work proposed that the spore comprises two-unit structures with different chemical structures, as shown in Figure 2.8 (C. S. Pomelli, F. D'Andrea, A. Mezzetta, & L. Guazzelli, 2020). The first structure comprises of long saturated fatty acid chains with some hydroxyl groups that make the polyester chains (Figure 2.8(a)). The second (Figure 2.8(b)) was triangle-shaped; the second structure carries by connecting the aldehyde functional group at the outer surface and an internal phenolic functional group. The two-unit functional groups (a & b) have a cross-link reaction to form m-dioxane group presented in Figure 2.8(c). Glycerol units attach the remaining free hydroxyl groups in Figure 2.8(d). Last, formation structure (e) is the interaction between structure (c) and (d) (F.-S. Li, Phyo, Jacobowitz, Hong, & Weng, 2019). Those robust and composed structures make the biopolymeric material that can protect from UV and microbial attack. However, some paper has not mentioned the exact structure, and they stated that there is not presence of the phenolic compound but contain lactones (ester group) as a part of the ring (Wu, Liang, Pei, Li, & Liang, 2020).

2.4.1 Morphology of treated *L.clavatum* spores

Mundargi, Potroz, et al. (2016a) studied the structure of treated spores compared with native spores by using scanning electron microscopy (SEM), as shown in Figure 2.9.

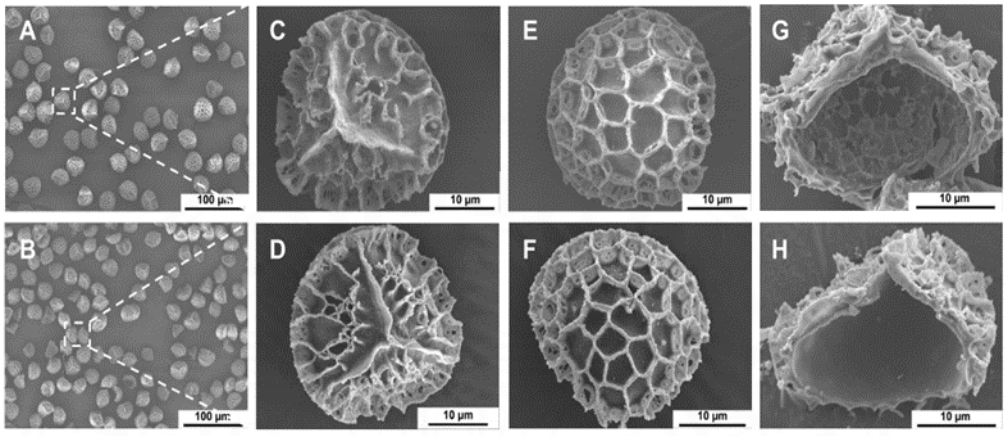

Figure 2.9 Scanning electron micrograph of *L. clavatum* spores, (A) spores before chemical treatment. (B)Spore after chemical treatment. (C) Y shaped of natural spores before chemical. (D) Y shaped after treatment showing thin Y shaped. (E) The exine of natural spores show reticulate the intact spores shell. (F) The exine of treated spores show thinness and clean. (G) Cavity of natural spores show the cytoplasmic contents. (H) Cavity of treated spores show a clean core of cytoplasmic materials.

They reported that, after chemical treatments (acid and base), spores' overall morphologies did not change from the original form. Except for the internal structure of the spore, the clean and empty cavity of the spore was presented (Mundargi, Potroz, Park, Park, et al., 2016). The structures of natural spores are shown in the upper line (Figure 2.9(A, C, E, and G)), whereas the structure of treated spores is displayed in the lower line (Figure 2.9(B, D, F, and H)). Figures 2.9(A) and Figure 2.9(B) show the overall structure of spores before and after treatment. The proximal face of both a natural and a treated spore showed the trilete similar (Y-shaped) scar (Figure 2.9C and D). While the exine of both natural and treated *L. clavatum* spores showed non-damage architecture and spore size slightly decreased from $31.1 \pm 1.3 \mu m$ to $28.7 \pm 1.6 \mu m$ after

chemical treatment (Figure 2.9E and F). The manually broken natural spore showed cytoplasmic contents residing inside the core (Figure 2.9G). In contrast, the manually broken treated spore showed a clean core or devoid of the cytoplasmic material (Figure 2.9H).

Moreover, they also investigated internal morphologies of spores by using transmission electron microscopy (TEM), as shown in Figure 2.10. TEM images of natural spores demonstrate a natural lycopodium spore showed double-layered wall (exine and intine walls) and cytoplasmic material inside a natural spore (Figure 2.10A). The wavy gray material in the enclosed space is the resin. Figure 2.10(B) showed two distinct layers (exine and intine) of the natural spore wall. After an acid (H_3PO_4)-treated spore (Figure 2.10C), removing cytoplasm from cavity occurred while the exine layer of the H3PO4-treated spore remained. The average exine wall thickness was 1.62 ± 0.95μm (Figure 2.10D) (Mundargi, Potroz, et al., 2016a).

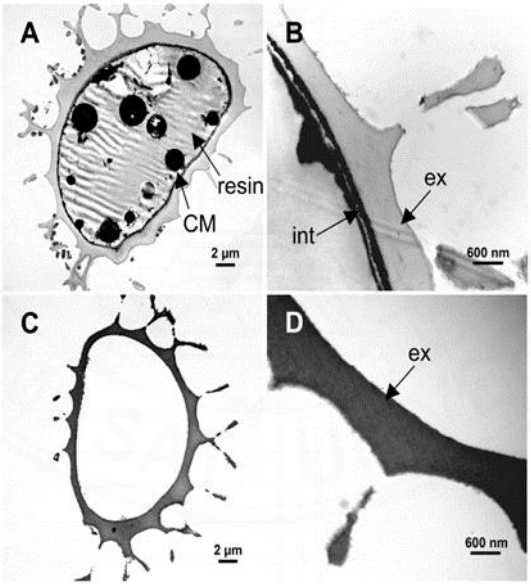

Figure 2.10 TEM micrographs of ultrathin sections of *L. clavatum* spores. (A) A natural spore showing its double layered wall and cytoplasmic material inside spores. (B) Two distinct layers (exine and intine). (C) Acid treated spores. (D) The wall of acid treated spores showing the exine layer remain after chemical treatment.

Confocal laser scanning microscopy is one tool that can characterize the morphology of samples by excitation and emission of photon materials. Moreover, the

different cytoplasmic materials and lipids will absorb or emit a different level of light. The formation of lipid is a cellular process that is the neutral droplets cholesterol esters or fatty acid class. The flow process of accumulation of the cytoplasmic lipid happened in every condition if it was improperly holding. Fluorescent microscopy is potentially used to analyze the flow lipid or oils condition (Greenspan, Mayer, & Fowler, 1985). Significantly, the researcher found that lipids, oils are easy to detect, different stain dye was used to interact with red, blue, and oils. According to Barrier et al. (2011) the different dyes were selected to encapsulate into the microcapsule spores to confirm of the dye can diffuse into the capsule spores, namely malachite green (b), Evans blue (c), Nile red (d) and Evans blue-stained a-amylase (e) in capsule spores as shown in Figure 2.11.

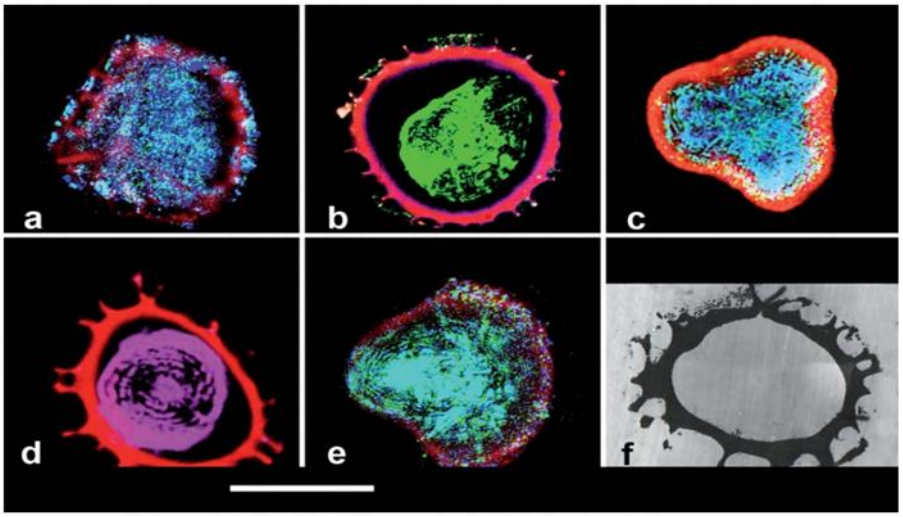

Figure 2.11 Morphology of L. clavatum spores investigated using confocal laser scanning microscopy.

2.4.2 Methodology of encapsulated spores

The recent work microencapsulation spores are increasing to obtain degradable material spores and toughness of composition spores. Many applications have been taken the spore material as a capsule to protect the bioactive or ingredient and to perform the specific application. Several techniques could encapsulate the ingredients into the spores, such as vacuum loading, passive loading, precipitation, compression

method. As the recent work, they encapsulated various bioactive such as macromolecule (BSA), protein, fatty acid, biotics, and Fe_2O_3 core nanoparticles into microcapsule spores (Thomasson et al., 2020). A simple passive loading was applied to fabricate BSA-encapsulated microcapsule spore. BSA was suspended into spores by using 0.6ml of purified water and use vortex for 3-5 min after that suspended solution was transferred to thermoshaker at 4 ℃ (500rpm). In the case of compression loading, a compressed tablet was prepared by using a hydraulic press at 5ton pressure for 20 s (die diameter 13mm, area 123.75mm^2,370 MPa). The spore tablet was soaked in the bioactive solution to allow bioactive or protein uptake by spore particles. However, vacuum loading was used a freeze-drying machine to encapsulate bioactive (BSA). The suspended solution was placed into a freeze-drier with a 1mbar vacuum. All the process of encapsulating the suspension was incubated for 2h, and the spores-loaded macromolecule was placed in a freezer at -70℃ for 30 min to freeze the sample, then freeze-dried for 24 h (Mundargi, Potroz, Park, Park, et al., 2016).

The other work of antibiotics (erythromycin (EM) and bacitracin (BAC)) encapsulate into *Lycopodium clavatum* sporopollenin (LCS). The unwanted materials were removed from the spores before encapsulation. They have stated that those antibiotics can encapsulate by vacuum loading. A 125mg of either EM or BAC antibiotics powder was dissolved in 5ml of (1:1) v/v water-ethanol solution and stirred for 10 min for complete dissolution. Next, 125 mg of dry LCS were suspended in the antibiotics solution and stirred using vortex for 15 min at 500 rpm for passive loading, then continue to put in the vacuum desiccator to allow the vacuum loading technique for another 2 hours. The EM or BAC loaded LCS microparticle was collected by filtering Whatman paper 11μm and washing properly with ethanol and water to remove the adhered substances to spores surface (Dyab et al., 2018).

Another work involved the fabrication of magnetic nanoparticles-encapsulated microcapsule spores by applying a passive loading technique. The formation of magnetic nanoparticles has occurred inside the microcapsules via the co-precipitation method. Firstly, the nanoparticle of humic acid-metal (HA-mental) ion complexes (Zn^{2+}, Cd^{+2}, Ni^{2+}, Pb^{2+}) with a sample of HA-sodium salt (20mg), and the form suspensions were stirring for 24 h at room temperature. Magnetic encapsulated

microcapsule spores were formed with dispersing of $FeCl_3$, $FeSO_4$ in HCl (5M) and heat up to 50℃ until complete dissolution, then 0.5 g of either the powder or a compressed tablet spore was redispersed for 2 h in the 20 ml of this solution. Finally, the magnetic spores successfully entrapped magnetic nanoparticles, as shown in Figure 2.12 (Dyab, Abdallah, Ahmed, & Rabee, 2016).

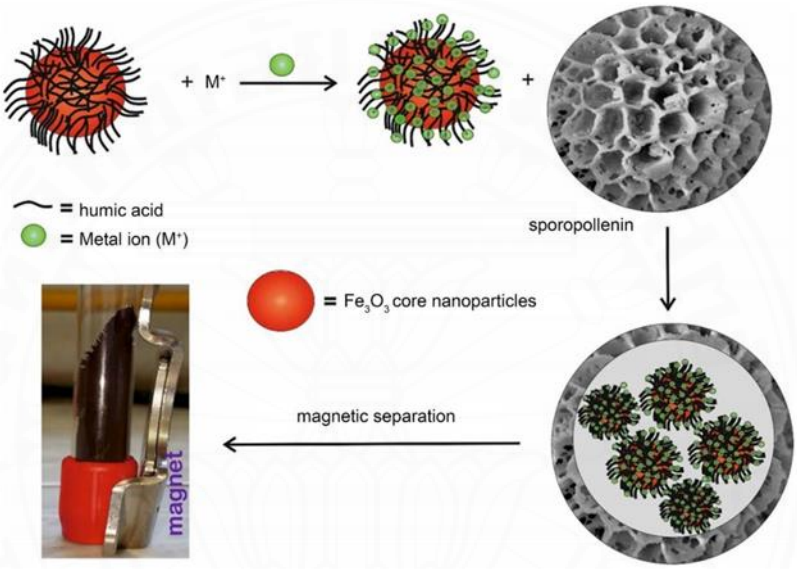

Figure 2.12 Encapsulation of magnetic HA-mental complexes inside sporopollenin exines.

2.5 Applications of microcapsule spores

The advantages of the microcapsule spore have a uniform size and monodisperse in as solvent. Moreover, the capsule can protect bioactive and use it in the specific target site (Ma et al., 2014). According to these properties, spores were used to encapsulate many different bioactive such as encapsulate biotics to cure the bacteria infection. This work focuses on erythromycin (EM) and bacitracin (BAC) to function as macrolide and polypeptide antibiotics. Erythromycin made from saccharopolyspora erythraea strains is helpful for the treatment of several bacterial infections, and sometimes it uses as an alternative for patients allergic to penicillin. However, EM was counted as a labile antibiotic since it degrades in the acidic phase of the stomach via intramolecular dehydration. Therefore, to protect and delay drug release in the stomach,

the coating of this drug was applied. Especially, spore is the microcapsule known as the degradable material and resistant to the strong acid and alkali in the gastrointestinal. So, it was taken to encapsulate the antibiotic to preserve pharmacokinetic properties (Elgegren et al., 2019).

In addition, recent work has known that drug delivery is vital to target the site of an organ. BSA is an example of albumin protein (hydrophilic macromolecule), which encapsulates into spores and obtain encapsulation efficiency of around 59.2% by vacuum loading technique (Mundargi, Potroz, Park, Park, et al., 2016). The control release study was performed in both in vitro and in vivo, according to vitro profile of BSA in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4). The release of the BSA from in SGF indicates 90% macromolecules released in the first 5 min and slowly release until 30-60 min. Moreover, the release BSA in both SGF and SIF have burst releases (Deng et al., 2019; Fan et al., 2020; Sudareva et al., 2017).

There are many ways to treat wastewater, such as filter osmotic, biological treatment, organic material, etc. Another application they have done with encapsulation complex metal ions with humic acid to be nanoparticles then entrapment through nanochannel into the cavity of spore. They also use microspores to entrap humic acid as the absorbent to remove metals and pollution from different aqueous media. However, some condition of wastewater that contains Humic acid which its high solubility, so that is very difficult to separate from suspension (Gezici, Kara, Ersöz, & Abali, 2005). Humic acid originated from the decaying of plants and has a multifunctional structure containing carboxyl, hydroxyl, carbonyl, amino, and ether groups (K. Yang, Zhu, Lou, & Chen, 2005). So, it was essential to immobilize HA to a specific solid material (Microcapsule spore) to perform Ion exchange with heavy metal in the wastewater that heavily affects the human health and environment (Dyab et al., 2016; Hatay, Gup, & Ersöz, 2008).

2.6 Essential oils

Essential oil is a concentrated hydrophobic liquid containing volatile chemical compounds from plants. Essential oils have become an integral part of human needs.

They were used in various ways such as aromatherapists, cooking flavor, perfume, drug, insecticide, and agriculture which are entirely safe while applied the essential oil on these applications (Hummelbrunner & Isman, 2001).The available essential oils were noticed in over 90 plants and applied for medical applications or formulation to drug or personal care. Mostly bacteria, fungi, or virus has been treated by those essential oils. Currently, there has been developed to nanomaterial that cooperates with a special kind of essential oil to kill the virus that can get efficiency around 98% (Buckle, 2014).

Since essential oils are mainly composed of bioactive substances, short-chain molecules, that quickly degrades or oxidizes with room temperature, sunlight, and various environments, so, encapsulation is one of the important process to keep the essential oils in an excellent property by considering between shell material and essential oil (Donsì et al., 2011). Meanwhile, it is vital to pick up the appropriate encapsulation technique based on the actual size, physicochemical properties, nature of the core material, and wall material. Nowadays, essential oils encapsulation is widely applied in food, cosmetics, and packaging (Kozlowska et al., 2019).

2.6.1 Extraction of essential oils

Essential oils are aromatic compounds extracted from various plants (Moghrovyan et al., 2019). Moreover, essential oils' properties can be expected as a new level of ecological products for killing household insects. So that broad range of essential oils activities is regarding applying for both industrial and household uses. There are two main extraction methods which are conventional and modern. Examples of the first method are hydro distillation and streaming. The modern method consists of microwave extraction, supercritical CO_2 extraction, solvent extraction, solid-phase extraction (SPEM), and supercritical fluid extraction (Tavakolpour et al., 2017). According to the research, works show the complex composition of essential oils depends on volatile compounds, and it is essential to keep those volatile compositions in suitable properties. Thus, the extraction techniques contribute to the effect of both chemical composition and essential oils yield. However, conventional extraction techniques were considered high consume energy by research and industry, so the alternative technique to cost-effective, sustainable, and beneficial to protect would

improve essential oils' characteristics (Cassel, Vargas, Martinez, Lorenzo, & Dellacassa, 2009).

2.6.2 Conventional essential oils techniques

Expression or cold pressing is the conventional extraction method, and it uses to extract the citrus essential oils from citrus peel. This technique applied the physical process during the peel pressing or any samples in the liquid suspension, continuously centrifuged to separate the essential oils (Mejri, Aydi, Abderrabba, & Mejri, 2018).

Solvent extraction is the process of suspending the mixture between solvents and natural plants into temperature conditions. This method separates compounds based on the solubility in two immiscible liquids. The solvent that commonly uses in these methods is hexane, alcohol, petroleum ether, and methanol. This process use temperature and essential oils may contaminate with an organic solvent which needs to carefully consider especially when the final products are supplied to food and pharmaceutical- industries. Mainly, this solvent extraction can produce the essential oils to perform as perfume products.

The steam distillation extraction could be steam distillation or water distillation. This process can take time between 1 to 10 h, and the yield of the actual oil production depends on the duration of distillation time, pressure, temperature, and type of plant materials. First, heat is applied to essential oil using a heating mantle to converts a liquid phase of the oils into vapors phases and flows into a steam tube. Vapors flow continually into steam, and another container, mantle steam, naturally captures the volatile molecules contained in the plants. Water vapors and volatile molecules then travel through a cold-water refrigerate tube and condense themselves into a liquid condense in the condenser (Figure 2.13). Finally, essential oils or a mixture of the volatile molecule were obtained with some liquid. Water or organic liquid residues were removed by sodium hydroxide. The disadvantage of using this method is that the essential oils were exposed to boiling water for an extended period and affected the volatile oil fraction composition.

Extraction with supercritical fluid gas method has been developed from the solvent and steam distillation to reduce the effect of organic solvent residues on the

fraction of essential oils. The industry applied supercritical fluid extraction (SFE) to produce large of essential oils. The solid plant materials are subject to the vessel added under a reasonable flow rate until they reach the extraction condition. This process was performed with:

- Low temperature, adjust thermal to sensitive compounds.
- The interaction of solute (plant material) and solvent can be controlled by changing the pressure and temperature, leading to high selectivity. But high pressure can cause the extraction of unwanted high molecular weight compounds(wax).
- The slow input of solvent and pressures leads to lowering viscosity and increasing diffusivity that can flow into porous solid materials more effectively than solvent and steam distillation methods.
- Generally, SFE is conducted using CO_2 because of its low critical temperature(short time), low cost, nontoxicity, ease of removal (Lang & Wai, 2001). Figure 2.13 shows the steps of supercritical extraction.

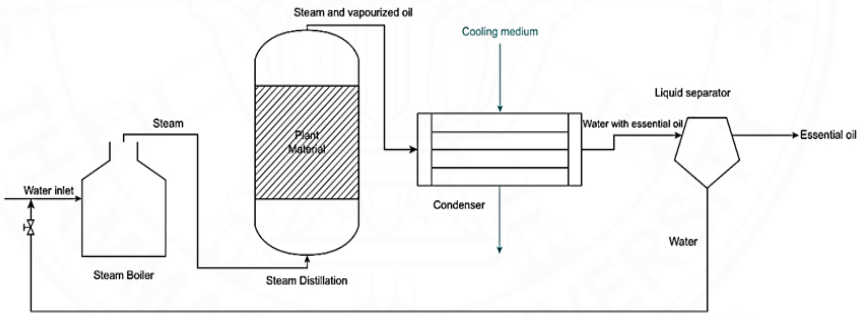

Figure 2.13 Typical industrial steam distillation unit for extraction of essential oils

Hydrodistillation is also a traditional extraction method, but it is a simple way to collect the essential oils. First, prepare the sample mix in a water balloon and subject it to the distillation chamber. The mixture is directly heated until boiling and vaporize, steam carrying volatile compounds along the tube; the steam is then directed to the cooling condenser chamber. Finally, some distillate (water and volatile compounds) will be dropped into the flask. The essential oils (volatile compounds) may be lighter or denser than water resulted in the phase separation between water and essential oils.

2.6.3 Novel or green extraction methods

Microwave-assisted extraction is a new development method since it has a unique heating mechanism, reasonable cost, short time, and good performance than solvent extraction, steam extraction (Zhang, Yang, & Wang, 2011). Figure 2.14 shows the parameter to control the microwave:

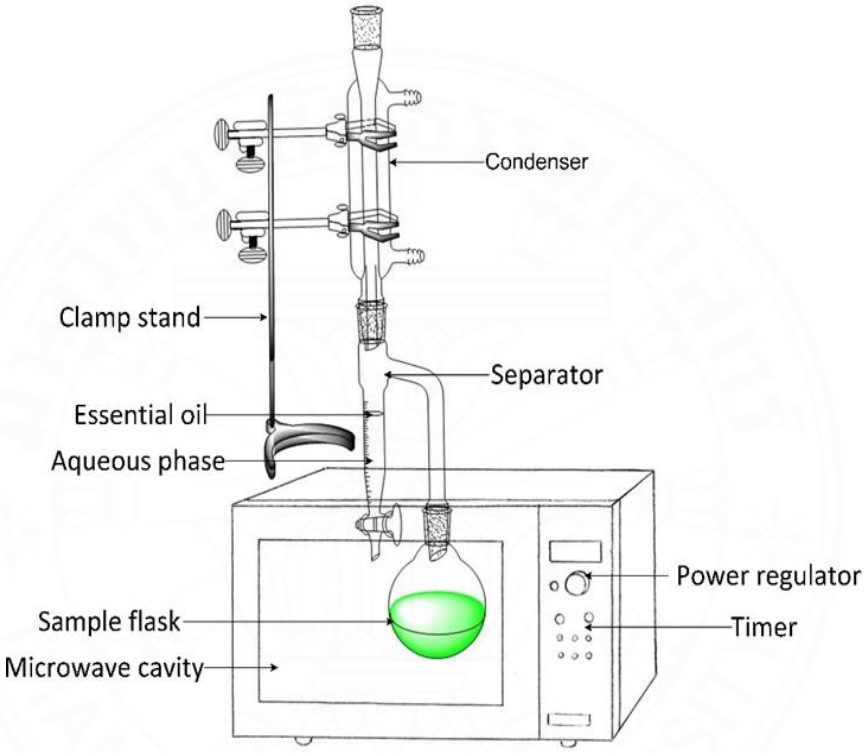

Figure 2.14 Microwave-assisted extraction process.

Ultrasonic-assisted extraction is a good technique for food processing because it can preserve the oils, protein, and polysaccharides properties. The main factor contributing to this process is cavitation phenomena, increasing size while they collapse violently. This process produced higher yield oils and a shorter time than supercritical fluid, around 30% (Riera et al., 2004).

2.7 Effect of extraction method on characteristic of essential oils

The extraction method is a leading factor that influences the chemical composition of essential oils. So, the method optimization differs essential oils profile

to perform the antimicrobial property exceptionally. A current study by Feng, Jiang, Wang, and Li (2010) studied the chemical profile and yield of essential oils by hydrodistillation (HD) and microwave-assisted hydrodistillation (MHD) of *piper nigrum L.* (black pepper) plant. The yield of essential oils were 1.24% and 1.45%, respectively. In addition, they have compared the chemical composition of the essential oils by analyzing GC-MS. The hydrodistillation step was prepared with 25g of pepper powder grinding and put into Clevenger type apparatus in 400 mL water for 1.5 h; the yield of oils(v/w) was calculated based on the dry weight of the pepper. The essential oils were dried with anhydrous sodium sulfate. Another process of microwave-assisted hydrodistillation was subjected to a Clevenger-type device with Dean-Stark distillation using microwave radiation MS-23F301EFS model, and operating conditions were 2450MHz, 7000W. 50 mL of distilling water were added to 25 g of pepper for 30 min. The result comes out with a high yield of MHD, and in this process, the volume of used solvent (50mL) was less than HD (400mL). So, the solvent or water ratio to extract the essential oils influences the essential oils to yield and the chemical composition of essential oils as following Figure 2.15 and Figure 2.16. Another experimental chemical characterization from HD of black pepper essential oils caryophyllene (47.17-50.88%), copaene (7.79-8.02%), sabinene (5.52-6.92%) andcubenol (3.97-5.20%). The obtained main essential oils from MHD were caryophyllene (8.25-52.68%), caryophyllene oxide (4.79-63.13%), sabinene (2.024-11.73%), copaene (5.95-9.28%), and cubenol (3.85-52.68). However, both chemical profile results are not significantly different (Rmili, Ramdani, Ghazi, Saidi, & El Mahi, 2014).

The recent work from Jeleń and Gracka (2015) reported the similarity percentage composition of monoterpenes and sesquiterpene compounds from black pepper that extracted from solid-phase microextraction(SPME) and hydrodistillation (HD) (Jeleń & Gracka, 2015). They have compared the volatile compound by two different methods, which are SPME and HD.

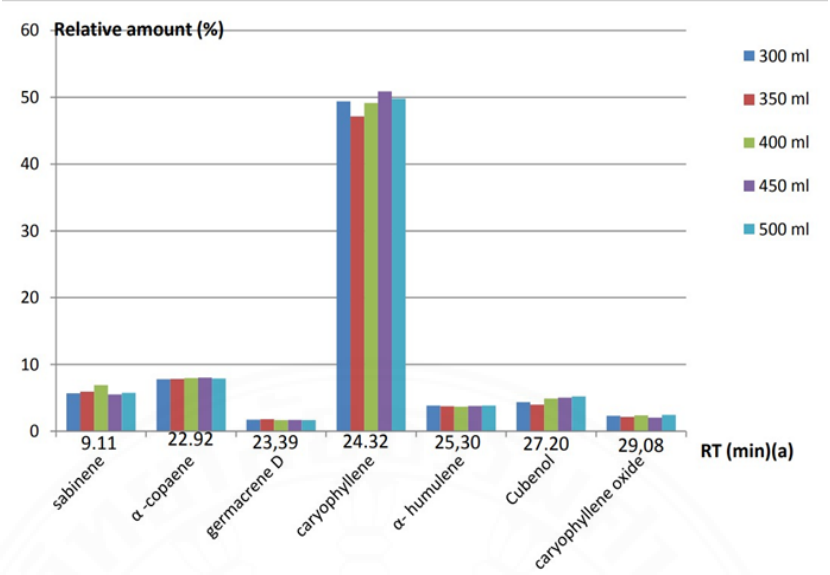

Figure 2.15 Influence of the solvent volume on the relative amount of CHD essential oils compounds as a function of retention

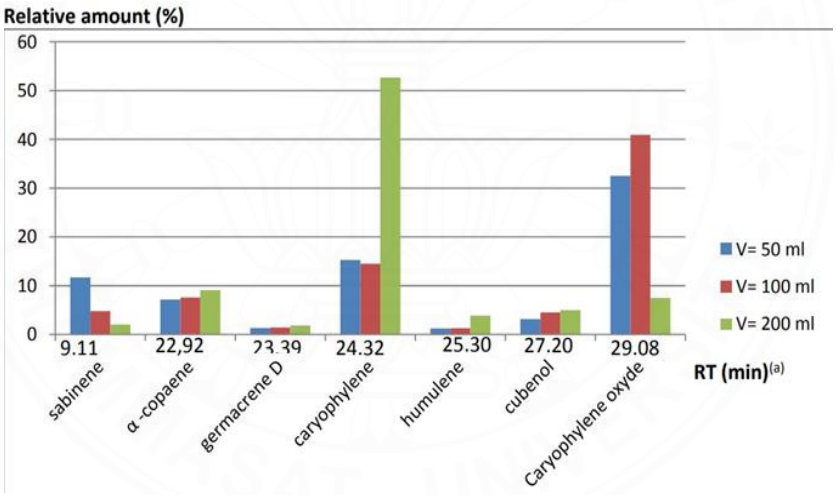

Figure 2.16 Influence of the solvent volume on the relative amount of MHD Eos compounds as a function retention time in GC analysis.

2.8 Chemical composition of essential oils

Essential oils have properties of hydrophobic liquid containing volatile chemical compounds from plants (Soković et al., 2009). Essential oils have various physical properties which different from water, such as optically active, high refractive index, and insoluble with water but soluble with ethanol or ether, chloroform. Especially, the oils would be stored in a cool place and tightly with vial container (Hosseini, Razavi, & Mousavi, 2009). Volatile oil usually formulates with two

segments like hydrocarbon-terpene; interestingly, the oxygenated part produces the odor and taste of volatile oils. The classification of major compounds in essential oil was divided as Table 2.3

Table 2.3 The classification of major volatile compounds of essential oils.

Formulate	Name
$C_{10}H_{16}$	Monoterpenoid
$C_{15}H_{24}$	Sesquiterpenoid
$C_{20}H_{32}$	Diterpenoid
$C_{25}H_{40}$	Sesterpenoid
$C_{30}H_{48}$	Triterpenoid
$C_{40}H_{64}$	Tetraterpenoid
$C_{40}H_{64} > C_xH_y$	Polyterpenoid

Generally, essential oils are a very complex natural mixture consisting of 20 to 60 components at different concentrations. Synthesis and reaction of terpene produce various components, e.g., phenolic, thymol, sabinene, alcohol caryophyllene. Therefore, essential oils demonstrate a great variety of structures, and each compound responsible for the different aroma, as shown in Table 2.4.

Table 2.4 Major chemical compounds responsible for the aroma, pungency, and medicinal property (Shaaban, El-Ghorab, & Shibamoto, 2012).

Chemical Compound	Type of odor
α–Terpineol	Floral
Acetophenone	Irritant, sharp
Hexonal	Green apple
Nerol	Fresh, Floral, Herbal
Nerolidol	Mild spicy, Rooty
1, 8 – cineol	Camphory
Dihydrocarveol	Warm, Woody
Citral	Citrussy
α–pinene	Terperic, Oxidised
Piperonol	Sweet, Floral

It has been reported that the chemical composition of essential oils could be affected by two factors, such as the agriculture process and the production process of the plant. The agricultural factors or environmentally-condition factors, such as light, precipitation, growing site, and soil, may affect the qualitative/quantitative yield of the volatiles in the essential oils. On the other hand, the production factors are mostly related to the anatomical and physiological characteristics of the plants connected to chemical variation between different parts of the plant (Figueiredo, Barroso, Pedro, & Scheffer, 2008). Moreover, some work has been stated that few factors have been involved, such as variation among different plant parts, developmental stages, and soil type. Lastly, extraction condition is also central for changing the essential oils' chemical compounds.

The different plants may provide various chemical compositions in essential oils (Swamy, Akhtar, & Sinniah, 2016). Essential oils and their major chemical composition from various plants have inhibited the growth of bacteria(bacteriostatic) or kill bacterial cells(bactericidal) in Table 2.5.

Table 2.5 Chemical composition of various essential oils and their antimicrobial activity (Ali et al., 2015).

Name Plants	Major chemical compounds	Inhibited microorganism	References
Achilla clavanae	Camphor, myrcene,1,8-cineole, caryophyllene, linalool, geranyl acetate	Klebsiella-pneumoniae, Streptococcus-pneumonia, Haemophilus-influenzae, Pseudomonas-aeruginosa	(Skočibušić, Bezić, Dunkić, & Radonić, 2004)
Aetemisia dracunculus	Methylechaicol, methyl eugenol, beta-phellandrene, terpinolene	E.*coli, S. aureus, S.epidermidis, L. monocytogenes,* Shewanella putrefaciens	(Teixeira et al., 2013)
Artemisa cana	Santolina treine, alpha-pinene, camphene	*E.coli, S. aureus, S.epidermidis,*	(Lopes-Lutz, Alviano, Alviano, & Kolodziejczyk, 2008)
Salvia lavandulifolia	Camphor, alfa-thujene, beta-thujene, Camphene, alfa-pinene, terpineol	*P.vulgaris, P.aeruginosa, K.pneumonia, E.faecalis*	(Jirovetz et al., 2007)
Ocimum species(Ocimum basilicum, O.kilimandscharicum)	Eugenol, Methyl eugenol, cis-ocimene,trans-ocimene, alfa-pine camphor	*C.albicanres, C.tropicalis, C.glabrata, P. notanum, R.*	(Matasyoh, Maiyo, Ngure, & Chepkorir, 2009)

		stolonfier, M.mucedo	
Piper nigrum L.	Limonene, 3-carene, alfa-pinene, beta caryophyllene, bata-pinene, sabinene, terpinene, humlene, copaene, eugenol, terpine-4-ol, camphene, safrole	*S.aureus, E.coli*	(Murbach Teles Andrade, Nunes Barbosa, da Silva Probst, & Fernandes Júnior, 2014)
Eucalyptus *(E. globulus)*	Cineole (70-80%), aromadendrene limonene terpinene, cymene, phellandrene, and pinene	Throat infection, catarrh, bronchitis, asthma,	(Hillis, 1967; Mulyaningsih, Sporer, Reichling, & Wink, 2011)
Peppermint (*Mentha piperita Linn)*	44% menthol, carvacrol, carvone, methyl acetate, limonene, and menthone	Relieve pain spasms, arthritic, irritable bowel syndrome	(Tassou, Drosinos, & Nychas, 1995)

2.8.1 Essential fatty acid oils

The difference between essential oil and fatty acid oils is the properties of molecular chemical compounds. Essential oil is mainly obtained from the leave and flower parts of a plant. Essential oils exist as a tiny molecule, and each molecule is built from rings or short-chain, which is easy to volatile. Moreover, it can circulate in plants and human bodies, and it also cannot spoil or turn rancid (Bartels, 2020). In contrast, fatty acid oil comes from the seed of a plant or vegetable. However, fatty acid oils are large molecules produced from the long chain that are not volatile compounds. Moreover, fatty acid oils may spoil and quickly return to rancid by heat (Elgegren et al., 2019).

The fatty acid source may be from natural plants such as Sacha seed, soybean seed, and Fabaceae seeds (*Glycine soja, Vigna angularis, Phaseolus coccineus*), which contribute to the bioactivity (Doan et al., 2019). Among those seeds contain the wild distribution compounds such as mineral, protein, amino acid, and essential fatty acid (Soković et al., 2009). Interestingly, fatty acids constitute unsaturated and saturate fatty acids, which provide beneficial health. The importance of unsaturated fatty acids is two polyunsaturated fatty acids, linoleic and linolenic acids, essential and unobtainable in the human body, except dietary. Linoleic acid, an unsaturated omega-6 fatty acid is found in some specific plants, including pumpkin seeds, canola oil, soybeans, and flaxseeds. They play an essential role for a wide range of human biological organs, including the nervous, skeletal, and reproductive systems, and allows them to function healthily (USDA, 2007). The previous research works reported that linoleic acid is converted into docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), responsible for reducing cholesterol and inflammation, improving brain functions. Furthermore, it would prevent cancers and autoimmune conditions (Campos, Baylin, & Willett, 2008). Besides the health benefit, recent works have mentioned that linoleic acid can protect irritated skin, build a block of the healthy membrane, cover moisture, keep out irritation, and protect from biological attach (Soimee et al., 2020).

2.8.2 Sacha inchi oil

Sacha inchi oil (Plukenetia volubilis L.) is an edible oils product obtained by a cold-pressed oil from the Sacha inchi seed. The oil contains a large amount of omega-3 of 40 - 50% and omega-6 of 30 - 40%, and oleic acid is 10%. The omega-6 fatty acid (linoleic acid or 9,12-octadecadienoic) are the essential compounds of the lipid barrier structure in the Sacha inchi (Elgegren et al., 2019; S. Wang, Zhu, & Kakuda, 2018). Moreover, linoleic acid and omega-3 fatty acid (linolenic acids or 9,12,15-octadecadienoic) are the leading functional group of other polyunsaturated fatty acids arachidonic acid. Tocopherols and polyphenolics are a natural antioxidant, have also been existed in Sacha inchi oil. Since the present amount of fatty acid and other physical chemistry in oils, some research works interested and tried to use in the cosmetic to

preserve the moisture on the skin and food nutrient (Hanssen & Schmitz-Hübsch, 2011).

2.8.3 Antimicrobial of essential oils

Antimicrobial activity is an inhibition of microbial growtth. There has observation of antimicrobial activity on various bioactive compounds such as essential oils, fatty acid, and chitosan which present some functional groups and could bacteriostatic. While chemical compounds of essential oils are different, they will cause different antimicrobial effects with microorganisms and synergistic effects (Mahboubi & Kazempour, 2014). Recent works have evaluated essential oils' potential properties in various fields such as cosmetics aromatherapy, medicinal aromatherapy, psycho-aromatherapy, and massage aromatherapy. The research work of potential use black pepper has attracted to apply in the pharmaceutical and cosmetics applications, encapsulation process between black pepper essential oils and hydroxypropyl-beta-cyclodetrin (HPβCD). In addition, the antimicrobial activity of free and black pepper oils was tested against *S. aureus* and *E. coli* by microdilution method. Table 2.6 shows the minimum inhibitory and bactericidal concentration of black pepper essential oils (Rakmai, Cheirsilp, Mejuto, Torrado-Agrasar, & Simal-Gándara, 2017).

Table 2.6 Antimicrobial activity of black pepper essential oils tested using gram-positive and gram-negative bacteria.

Antimicrobial compounds	*S.aureus*		*E. coli*	
	MIC ($\mu g/ml$)	MBC ($\mu g/ml$)	MI ($\mu g/ml$)	MBC ($\mu g/ml$)
Black pepper oils	1000	2000	2000	> 2000
Black pepper oils-HPβCD[a]	250	500	500	1000

[a] Values are based on the actual concentrations of black pepper oils encapsulated in the HPβCD (calculated from encapsulation efficiency)

Table 2.6 illustrates the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of black pepper oils and encapsulated black pepper oils. Black pepper oils effect to gram-positive bacteria *(S.aureus*) around 1000 $\mu g/ml$ for MIC and 2000 $\mu g/ml$ for MBC and effect to gram negative bacteria (*E. coli*) 2000 $\mu g/ml$ and less than 2000 $\mu g/ml$. The result test of *E. coli* has less efficiency than *S.aureus* because the gram-negative bacteria has an external of outer membrane that compose of lipopolysaccharides and proteins better tolerates the test sample. And the formation of the gram-negative bacteria builds from a hydrophilic surface which protects the hydrophobic (black pepper oils) molecules into the bilayer (Zengin & Baysal, 2014). However, encapsulated black pepper oils in HPβCD, the tested results provide the improvement of antimicrobial activity 4 times against S.aureus and E. coli. The complex encapsulated black pepper oils would increase the solubility and improve antimicrobial efficacy at lower concentration of encapsulated compounds (Hill, Gomes, & Taylor, 2013). As the target sits for antimicrobial activity were found at the cell membrane and inside of cytoplasm, the HPβCD may enhance the properties of essential oils to these targets by improving water solubility of the oils (T. Wang, Li, Si, Lin, & Chen, 2011). Regarding to study of Y.-x. Li et al. (2020) report that 3-Carene is bicyclic monoterpene, which has antifungal activity. Other research they state that monoterpene such as D-Limonene and caryophyllene show inhibitory to the antimicrobial and antioxidant (D. Yang, Michel, Chaumont, & Millet-Clerc, 2000).

As we know, Sacha inchi oils were applied in various applications such as pharmaceuticals, skincare to soften the skin, heal a wound, kill insects, and skin infection (Moser, Freis, Gillon, & Danoux, 2007). Significantly, the development of cosmetics and pharmaceuticals with Sacha inchi oils is increasingly to perform antibacterial, skin tightening, anti-inflammatory, and anti-aging effects (Hanssen & Schmitz-Hübsch, 2011; Soimee et al., 2020). The recent study of Sacha inchi oils (*Plukenetia volubilis L.*) on the skin has the ability to protect bacteria *S.aureus* adheres on human skin and keratinocyte. The human keratinocyte cell line was prepared with trypsin to obtain cells around $2x10^5$ viable cells/ml. Then cell suspensions were put on the adherence microtiter plates. Human skin explants were cut into 12 mm in diameter disc that prepares by Biopredic International France. Then skin explants in 24-well plate

with 300μl were incubated for 24 h at 32℃ (Yasuoka, Larregina, Yamaguchi, & Feghali-Bostwick, 2008).

Antibacterial testing of Sacha inchi oil and Coconut oil (CocO) did not show any antibacterial activity with the control group. However, Sacha inchi oils have studied the removal of bacteria from the skin instead. Table 2.7 present result of Sacha inchi oils (SIO) and CocO on the viability of *S. aureus*, keratinocytes cell, and human skin explants (model skin). After Sacha inchi oils expose to the bacteria, they observe 90% of the *S. aureus* still alive. More than 90% of both keratinocytes and human skin explants are non-toxic. These results suggested that those oils are non-toxic to the skin cell. In another study on the pre-adherence inhibition (% prevention), Sacha inchi oils were more effective than 40% of CocO 25.5 ±2.2 in inhibiting *S. aureus* attachment to keratinocytes. Post-adherence inhibition of Sacha inchi oils and CocO could remove *S. aureus* from keratinocytes with 33.92±2.21 and 29.1±1.9, respectively. However, on the human skin explants shown that CocO was ineffective on the detachment process with explants which Sacha inchi oils have the same capacity to remove *S. aureus* from both keratinocytes and human skin explants around 33.62±2.20 (Gonzalez-Aspajo, Belkhelfa, Haddioui-Hbabi, Bourdy, & Deharo, 2015).

Table 2.7 Activity of Sacha inchi oils on keratinocytes, human skin explant, and S.aureus.

Oil	Viability %			Antiadhesive effect (prevention)%	Detachment effect	Curative (%)
	S. aureus	Keratinocytes	Explant	Keratinocytes	Keratinocytes	Explant
SIO	88.1 ± 5.7	94.2±2.6	96.9± 3.0	39.2 ±2.4	33.92±2.2	33.62±2.2
CocO	88.3 ±7.5	80.8 ±3.0	ND	25.5 ±2.2	29.1±1.9	7.12±2.25

S.aureus: Staphylococcus aureus.

Phenol killed 100% of keratinocytes and *S.aureus* at 0.5% (data not shown).

ND: not determined, experiment repeated 3 times.

2.8.4 Antioxidant activity of fatty acid and essential oils

The antioxidant activity has described the prevention of the oxidation by the substance such as vitamin E or C to remove the potentially damaging oxidizing agents in the material or plant. Many different factors impact level antioxidants, such as processing, time, container, and chemical composition properties in oils (S. Wang et al., 2018). Mainly, the evaluation of antioxidant has conducted with DPPH assay and 2,2'-azino-bis (3-ethylbenzothiazoline6-sulphonic acid (ABTS) radical cation discoloration assay.

Sacha inchi oils are rich in essential and non-essential fatty acids and contain other compounds such as tocopherols (α, β, γ tocopherol) and polyphenol compounds which perform the antioxidant and antimicrobial activity. Elgegren and Macrat (2019) worked on the encapsulation of Sacha inch oil into alginate(AL) and chitosan (CS) nanoparticles which were obtained by assistance of high-intensity ultrasound (Elgegren et al., 2019). They have studied the antioxidant Sacha inchi oils compared with vegetable oils, olive, and soybean. They compared the antioxidant activity of two samples, which are the type of vegetable oils (Sacha inchi oils, olives, and soybeans) and AL-CS nanoemulsions encapsulated the oils.

The evaluation of antioxidant activity was studied using, ABTS radical cation discoloration assay (Kim et al., 2017). $ABTS^+$ free radical cation was obtained between reaction 7nM ABTS solution and 2.45 mM potassium persulfate; that solution was kept for 12h at room temperature. Ethanol was diluted in $ABTS^+$ free radical solution to reach absorbance 0.7 at 734 nm, and the absorbance of 3ml of this solution was set as a control ($A_{control}$). A total of 1 ml of the encapsulated Sacha inchi oils AL-CS emulsion after lyophilization was added to 3 ml of diluted $ABTS^+$ free radical solution, and absorbance was measured for 1 h (A_{sample}). Equation (2.1) was used to calculate the radical scavenger effect (%):

$$radical\ scavenging\ effect(\%) = \frac{A_{control} - A_{Sample}}{A_{control}} \times 100 \qquad (2.1)$$

The antioxidant time is1 h, and the percentage of inhibition (%) is shown in Figure 2.17 Among those bioactive compounds, Sacha inchi achieved higher antioxidant activity (95% of the radical reduction in 15 min) because it contains β, γ-tocopherols (Wagner, Isnardy, & Elmadfa, 2004). Generally, the effectiveness of α, β, γ tocopherol from Sacha inchi oils are efficient to trap reactive nitrogen oxide (toxic in body) (e Silva, da Silva Carvalho, Rabelo, & Hubinger, 2019; Mathur, Ding, Saldeen, & Mehta, 2015). In addition, the Sacha inchi oil encapsulated in AL-CS nanoparticles revealed the reduction in a short period, 95% in 15min because the well-known chitosan has its scavenging ability against 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH•), hydroxyl radical (•OH), and superoxide radical ($\bullet O_2^-$) (S.-H. Chang, Wu, & Tsai, 2018). The radical scavenging capacity of CS perform around 30% in 60 min without incorporate of oils.

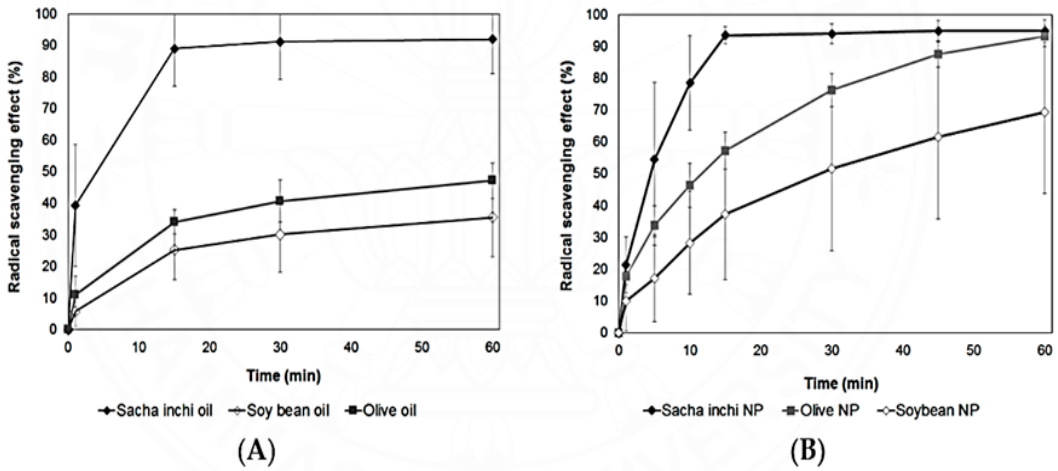

Figure 2.17 Evaluation of the antioxidant activity of (A) free vegetable oils (from sacha inchi oils, olive, and soybean) and (B) oils encapsulated AL-CS nanoemulsions. NP: Nanoparticles.

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CHAPTER 3
MATERIAL AND METHODOLOGY

3.1 Reagents

Table 3.1 shows the different instrument to implement with this work, and deionized (DI) water was applied throughout the work.

Table 3.1 Reagents used in this study.

Reagent	Supplier	Grade
Acetone	Carlo Erba Chemical	Analytical reagent grade
Ethanol	Carlo Erba Chemical	Analytical reagent grade
Sodium hydroxide	Carlo Erba Chemical	Analytical reagent grade
Potassium hydroxide	Carlo Erba Chemical	Analytical reagent grade
Ortho-phosphoric acid 85 %	Carlo Erba Chemical	Analytical reagent grade
Hydrochloric acid 37%	Carlo Erba Chemical	Analytical reagent grade
Sacha inch oil	Myskin company in Thailand	Cosmetics grade
Lycopodium spores	Sigma Aldrich	Analysis grade
Black peppercorn	Arichit International Pepperand Spice Co., Ltd	-
Nile Red	FUJIFILM Wako Pure chemical cooperation, Japan	Pathology research
Fluorescein isothiocyanate, isomer (FITC 95%)	Alfa Aesar	Analysis grade

3.2 Apparatus

The apparatus used in the present study is listed in Table 3.2.

Table 3.2 List of apparatus used in the present study.

Apparatus	Trademark /Supplier
Centrifuge	HERMLE-z 206 A
Fourier Transform Infrared	Thermo Scientific Nicolet iS5
Scanning Electron Microscopy	Malvern, Zetasizer Nano ZS
Magnetic Stirrer	Ika, Scilgex
Shaking Machine	Heidolph Rotamax 120, D-91126 Schwabach, Germany
UV-vis Spectroscopy	Thermo Scientific, GENESYS 180 180
Hot plant	IKA^{R} C-MAG HS 7
Vortex	Scientific Industries, INC; VORTEX-GENE 2
Shacking Incubator (Shacking water bath)	SWB-110x12, Boibase Biodustry (Shandong) CO, Ltd
Freeze dryer	CHRIST, LC Plus
Hot air oven	Binder, SPC RT CO.,
Vacuum Pump	Millipore^{R}, WP51222050
Confocal Microscopy	FLUOVIEW, FV10i; OLYMPUS
GC-MS	Agilent Technologies, USA
Refrigerator	TOSHIBA
Sonication machine	Elma Elmasonic S, Gottlieb-Dalmler-Str.17|D-78224 Singen, Germany
Ultrasonic machine	BRANSON 550, Asia Branson Ultrasonic Co, Ltd.

3.3 Extraction of commercial black pepper essential oils

3.3.1 Hydrodistillation of black pepper essential oils

BEOs (Black Pepper Essential Oils) were extracted by the hydrodistillation method. Commercial black pepper (*Piper nigrum L)* (50 g) was put into a round bottom flask, which was connected to a "Clevenger type apparatus". Then, DI water (500 mL) was added, and the temperature was increased to 180°C. The extraction time was varied e.g., 30, 60, 90, 120, 150, 180 and 210 min (Jeleń & Gracka, 2015; Y. Wang, Jiang, & Li, 2009). The extracted white liquid products were stored in the refrigeration at 4 °C for the following experiments. The experiment for each sample was duplicated.

After moisture was removed, the yield of the extracted BEOs was determined by using the equation (3.1) (Elyemni et al., 2019):

$$\%\text{BEOs yield} = \frac{M_f - M_i}{M \times \frac{100-H}{100}} \times 100 \quad (3.1)$$

Where M_f is the weight of vial containing BEOs, M_i is the weight of vial, M is the weight of commercial black pepper powder (50g), and H is the percentage moisture content of black pepper.

3.3.2 Determination of moisture content

Before each extraction process, dried samples were prepared to determine the moisture content to calculate the dry mass used for extraction. 2g of dried pepper were weighed then placed on the aluminum plates, which had been dried in an air oven at around 105℃ for 3 h or 4 h until stable mass was obtained. The following equation (3.2)was used to calculate the moisture content:

$$\text{Moisture content} = \frac{M_S - (M_1 - M_0)}{M_S} \quad (3.2)$$

Where M_0 is weight in a gram of plate, M_1 is weight in a gram of plate and sample after drying, M_s is the weight of the sample.

3.3.3 Characterization of essential oils by GC-MS

To identify the chemical compositions of the extracted BEOs, gas chromatography-mass spectrometry (GC-MS; GC model 7890B, and MS model 5977A

MSD, Agilent Technologies, USA) was applied. The extracted sample (10 to 20 mg) in 1 mL dichloromethane was prepared. GC-MS was set up with a nonpolar column (HP-5ms, 30 m × 0.25 m ; 0.25 μm). The injection condition was set up by this method. Helium gas was used as carrier gas at a flow rate of 1 mL/min. 1 μL of the sample was injected with a split ratio of 80:1 with the initial column temperature was 70 ℃, gradient temperature of 5 ℃/min to final temperature of 280 ℃ and then hold at 280 ℃ for 20 min. All data were obtained by collecting the full-scan mass spectra within the scan range 40 to 500 amu (scan time = 0.2 sec). The MS provided molecule weight which compared to the library NIST11 and NIST11s.

For Sacha inchi oil characterization, the oil (20 mg) was dissolved in hexane (2000 ppm). GC-MS was set up with a nonpolar column (DB-5ht, 30 m × 250 μm; 0.1 μm). Helium gas was used as carrier gas at a flow rate of 1 mL/min, 1 μL of the sample was injected with a split ratio of 25:1 with split-flow 25ml/min, the initial oven temperature was 80 ℃, gradient temperature of 10 ℃/min to final temperature of 280 ℃ and then hold at 280 ℃ for 10 min. Columns were set up with 9.32 psi, flow rate 1mL/min, and average velocity 36.96cm/sec. All data were obtained by collecting the full-scan mass spectra within the scan range 30 to 550 amu (scan time = 0.2 sec). The MS provided molecule weight which compared to the library NIST11 and NIST11s.

3.4 Treatment of *L. clavatum* spores

Chemical treatment was applied to remove all native bio-compounds from the cavity of the spores (Md Jasim Uddin, Noureddine Abidi, Juliusz Warzywoda, & Harvinder Singh Gill, 2019).

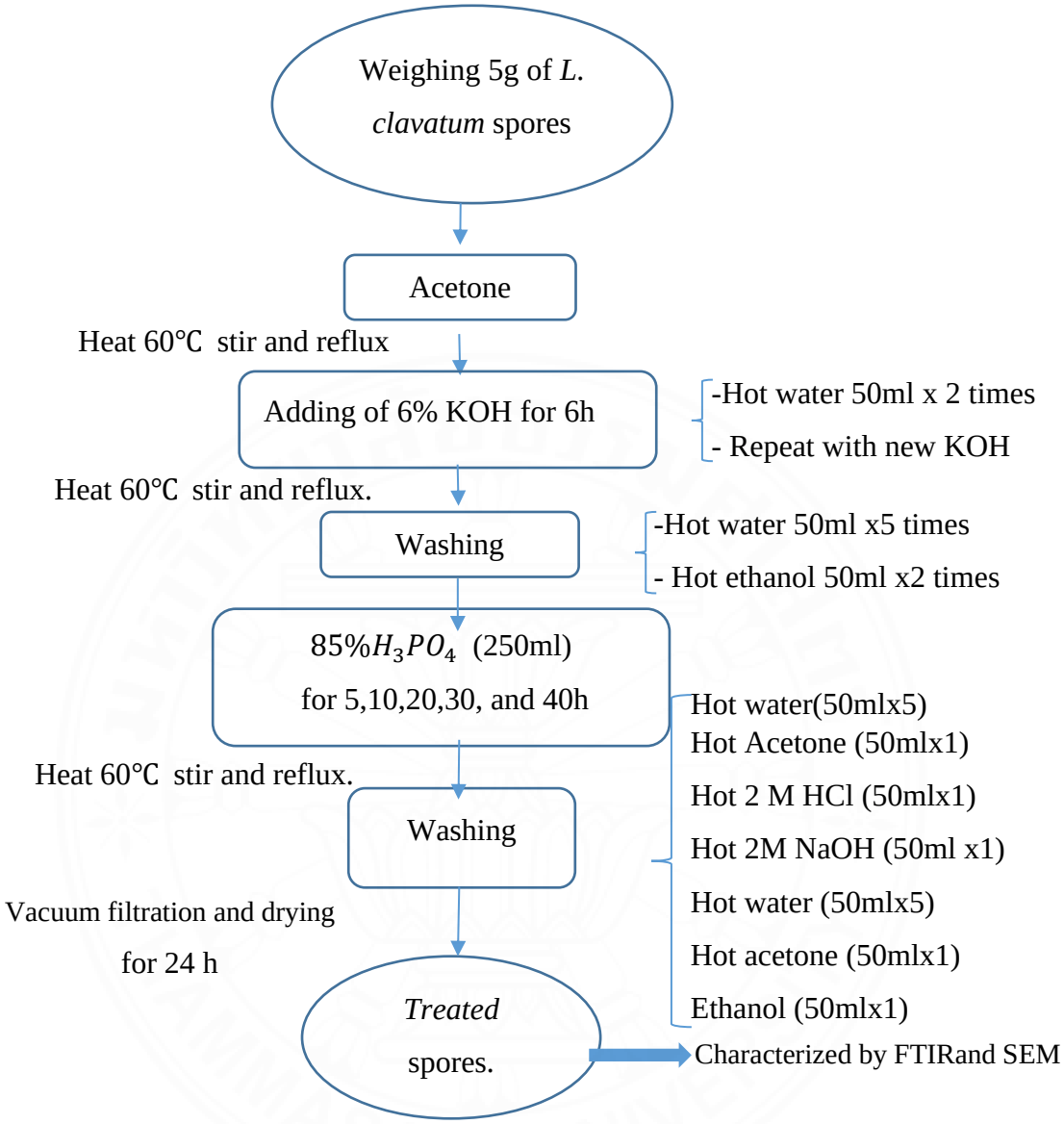

Figure 3.1 Extraction step of microcapsule of *Lycopodium clavatum* L.(M. J. Uddin, N. Abidi, J. Warzywoda, & H. S. Gill, 2019).

The step of extraction of L. clavatum is shown Figure 3.1. Briefly, 5 g of native *L. clavatum* spores were firstly refluxed in 250 mL of acetone at 60 ℃ for 6h. The dispersion was cooled down and filtered by using a vacuum filtration unit. After drying, the acetone-treated spores were refluxed in 250 mL of 1M (6% w/v) KOH for 12 h at 60 ℃. The dispersion of KOH-treated spores was kept warm, diluted with DI water, filtered, and substantially washed with hot water and ethanol. Finally, KOH-treated

spores were refluxed with 250 mL H_3PO_4 at 60 ℃ for various times, e.g., 5, 10, 20, 30, and 40 h. After that, H_3PO_4-treated spores solution was sequentially washed with the hot water, acetone, 2M HCl, 2M NaOH, water, acetone, and 90 % ethanol and dried at 60 ℃ in an oven for 24 h. All samples of spores were kept at room temperature before further characterizations.

3.5 Preparation of microcapsules

3.5.1 Encapsulation of BEOs into *L. clavatum* microcapsules

The extracted BEOs were encapsulated into L.clavatum microcapsules by using the passive loading technique. The method was conducted with a shaking machine. Firstly, the spore and BEOs with different weight ratios, e.g., 2:1, 1:1, and 0.75:1, were dispersed in 98% hexane using vortex for 2 min. The mixture was then transferred to a shaking machine and homogenized for 24 h at room temperature. After that, the encapsulated spores were centrifuged and washed with 98% hexane to remove free BEOs. Finally, the loaded spore was filtrated by a syringes filter ($0.2\mu m$) and dried with a freeze dryer.

3.5.2 Characterizations of encapsulated BEOs-microcapsule spores

UV-vis spectrophotometer (Thermo Scientific, GENESYS^{TM} 180) was applied to determine the encapsulation efficiency (%EE) and loading capacity (%LC) of the obtained products. The supernatant after filtration by syringe filter was determined by UV-vis spectrophotometer at a wavelength of 290 nm. The %EE and %LC are calculated using the equation (3.3) and (3.4), respectively (Bastos et al., 2020).

$$\%EE = \frac{\text{Mass of BEOs encapsulated in spores} \times 100}{\text{Mass of BEOs}} \quad (3.3)$$

$$\%LC = \frac{\text{Mass of BEOs ecapsulated in spores} \times 100}{\text{Mass of encapsulated spores}} \quad (3.4)$$

Fourier transform infrared (FTIR) spectrometer (Thermo Scientific Nicolet iS5) was carried out to recognize the presence of BEOs in the encapsulated spores. The spectra were collected at a spectral resolution of 500-3500 cm^{-1} with 32 scans. Before characterizations, the encapsulated spores were crushed to reveal inside area.

Field emission scanning electron microscopy (FE-SEM: JEOL JSM7800F, JAPAN) was used to characterize the morphology of microcapsule. Samples were coated with platinum at the thickness of 10 nm. The image was recorded with an acceleration voltage of 5.00 kV.

3.5.3 Encapsulation of Sacha inchi oil into *L. clavatum* microcapsules

Vacuum and passive loading techniques were applied for encapsulation of Sacha inchi oil into the treated spores. For vacuum loading, Sacha inchi oils and the treated spores were weighted with a different ratio of 0.5:1 and 0.75:1. Then, the suspension between Sacha inchi oils and treated spores was vortexed for 2 min and placed in freeze-drier at 1 mbar for 2 h. The encapsulated spores were centrifuged and cleaned with 9% of ethanol in water to rinse some oils that penetrate the surface of the treated spore. The encapsulated microcapsule spores were collected by filtering and washed with 9% ethanol to remove non-encapsulated oils. The supernatant was measured by UV-vis spectrometer for finding the %EE and %LC.

For passive loading, Sacha inchi oil with a specific amount of 50 mg, 75 mg, 125 mg was firstly dissolved in 2 ml of 9% ethanol and stirred for 5 min. Then, the treated spores were suspended in the Sacha inchi oils solution to prepare the mixture with various ratios, e.g., 0.5:1, 0.75:1, and vortexed at 1000 rpm for 5 min. After that, the mixture was shaken at room temperature for 24 h. Next, the encapsulated microcapsule spores were collected by filtering and washed with 9% ethanol to remove non-encapsulated oils. Similarly, the supernatant was measured by a UV-vis spectrometer for finding the %EE and %LC. And the sacha inchi oil-loaded microcapsule spores were dried by a vacuum freeze dryer at 0.75 mmHg until constant weight and stored at 4 ℃ before use in the further process.

3.5.4 Determination of encapsulation efficiency (EE) and loading capacity (LC)

Different approaches to find the encapsulation efficiency (%EE) of bioactive encapsulated are reported in the literature (Schuch, Tonay, Köhler, & Schuchmann, 2014). They can be divided into indirect and direct measurement techniques. First, an indirect method is found by giving the total amount of bioactive used by measuring the supernatant (containing free active compounds). At the same time, the direct method is determined the actual mass of bioactive compounds in a certain mass microcapsule.

For the indirect method, the %EE and %LC of the microcapsule as described in the equation (3.3) and (3.4) were adopted as shown in the equation (3.5) and (3.6).

To determine %EE and %LC by the direct method, the encapsulated microcapsule spores (15 g) were suspended in 98% ethanol (50 ml) and shake for 60 min, followed by sonicated at 24 %amp for 5 min with three cycles. Then, the encapsulated microcapsule spores were removed out by nylon syringes (0.2μm) filtration. The supernatant was measured via UV-Vis spectrometer at λ= 238 nm. The %EE and %LC were calculated using the following formula that practical loading is the Sacha inchi oils in spores and theoretical loading in the equation (3.7), (3.8), and (3.9) (Timilsena, Adhikari, Barrow, & Adhikari, 2016):

Indirect method

$$\%EE = \frac{(\text{Total mass of sacha inchi oils} - \text{Mass free sacha inchi oils}) \times 100}{\text{Total mass of sacha inchi oils(mg)}} \quad (3.5)$$

$$\%LC = \frac{\text{Mass of sacha inchi oils ecapsulated in spores(mg)} \times 100}{\text{Total mass of encapsulated spores(mg)}} \quad (3.6)$$

Direct method

$$\text{Theorical loading (\%)} = \frac{\text{Initial mass sacha inchi oils add to system(mg)} \times 100}{\text{Inititial mass capsule spores (mg)}} \quad (3.7)$$

$$\%LC = \frac{\text{Mass of sacha inchi oils release from spores(mg)} \times 100}{\text{Total mass of encapsulated spores(mg)}} \quad (3.8)$$

$$\%EE = \frac{\text{Loading sacha inchi oils}(\%LC) \times 100}{\text{Theoretical loading }(\%)} \quad (3.9)$$

3.6 Characterizations

3.6.1 Field emission -scanning electron microscopy

Field emission-scanning electron microscopy (FE-SEM) was used to characterize the structure of microcapsule spores. The spores were ground before characterizations. Then, the samples were coated with platinum at a thickness of 10 nm. The image was recorded with an acceleration voltage of 5.00 kV at different magnifications to observe the morphological changes before and after each treated spore's process.

3.6.2 Fourier transform infrared spectrometry

Fourier transform infrared spectrometer (FTIR) in an attenuated total reflection mode (Thermo Scientific Nicolet iS5) were carried out to recognize the functional groups of spores. All FTIR spectra were collected at a spectral resolution of 400 – 4000 cm^{-1} with 32 scans.

3.6.3 Confocal laser scanning microscopy (CLSM)

For the preparation of samples, two dyes were added to the Sacha inchi oil solution. FITC (100ppm) and Nile red (100ppm) are selected dyes and were dissolved into Sacha inchi oil (200μl of each dye) by vortex for 2 min then this dispersion was mixed with treated spores and followed the passive encapsulation at the section 3.5.3. Natural and Sacha inchi oils loaded spores were mounted on sticky slides (Ibidi, Germany), and spore particles were covered with another sticky slide.

A confocal laser scanning microscope was used to analyze the oils loaded microcapsule spores. The following conditions were set up with laser excitation line 405 nm (60%), 473 nm (50%), and 600 nm (50%) with a plan-Abochromat 63 x ¼ oil differential interference contrast (CID) M27 objective lens. Fluorescence from natural and entrapped Sacha inchi oils into spores was collected in a photomultiplier tube

equipped with the following emission filters: 416-477nm, 498-550 572-620nm. The laser scan speed was set at scanning with a 3.00 μs pixel dwell. All the images were recorded focusing on the inside section of spores connecting thin glass slides and bind within vectashield at 60X. The autofluorescence of the spore will interfere with the observation of FITC-Sacha inchi oils and Nile red-Sacha inchi oils. So, we fixed CLSM settings with the same condition then use the same setting for FITC-Sacha inchi oils loaded spores, and Nile red-Sacha inchi oils loaded spores (Mundargi, Potroz, Park, Park, et al., 2016).

3.7 Control release study

Sacha inchi oil-loaded microcapsule spores at a 0.75:1 (300 mg) ratio were placed on natural rubber film, which in this study, to represent skin human. Then, it was gently pressed under an aluminum plate by using spatula for 1, 2, 3, 5, 7,9,11min (Diego-Taboada et al., 2012). After that, the sample stuck on the surface of the rubber film was sonicated with 9% of ethanol (40ml) for 5 min. Then, the mixture was filtrated by using a nylon syringes filter (0.2 μm). Ethanol (9%) was added into the supernatant to keep the sample volume of 15 ml. The absorbance of the supernatant was measured by UV-vis spectrometer to calculate % Sacha inchi oils release by using the following equation (3.10).

$$\% \text{ Release of Sacha inchi oils} = \frac{Release\ sacha\ inchi\ oils(mg)}{Total\ sacha\ inchi\ oils(mg)} \times 100 \qquad (3.10)$$

Where release Sacha inchi oils: the amount of Sacha inchi oils loaded in microcapsule spores obtained from LC measurement (direct method).

3.8 Antioxidant activity

The evaluation of antioxidant activity was carried out by using 2,2-diphenyl-1-picryl-hydrazyl-hydrate radical (DPPH●+) as free radical reagent. DPPH+ free radical solution was diluted with dichloromethane (DCM) to reach absorbance of 0.7 at 517 nm, and the absorbance of 3 ml of this solution was set as a control ($A_{control}$). The concentrations of Sacha inchi oils were prepared from 100, 80, 60, 40, 20,10, 7.5, 5,

and 2.5 mg/ml and dissolve in 100% of DCM as a solvent. A total of 1 ml of the Sacha inchi oils was added to 3 ml of diluted DPPH+ free radical solution, and absorbance was measured for 30 min (A_{sample}). % Inhibition was determined by equation (3.11).

$$\%\ inhibition = \frac{A_{Control} - A_{sample}}{A_{Sample}} \times 100 \quad (3.11)$$

CHAPTER 4
RESULTS AND DISCUSSIONS

4.1 Extraction of black pepper essential oils and characterization

4.1.1 The yield of black pepper essential oils

Yield and moisture content of the extracted BEOs were determined (Y. Wang et al., 2009), as shown in Table 4.1.

Table 4.1 The Yield and moisture content of the extracted BEOs

Sample	Moisture content (%)	BEOs yield (%)
Commercial dried black pepper	13.05±0.11	2.85±0.35

It was found that the extracted BEOs yield is quite low of 2.85± 0.35%. Several reasons possibly contribute to a low percentage of EOs yields, such as age, genetic, stage maturity, geographic origin, climate, and extraction technique, grinding size, and the ratio of black pepper to water (Elyemni et al., 2019; Y.-x. Li et al., 2020; Y. Wang et al., 2018). The moisture content of 13.05±0.35% was obtained. It was reported that the value in between 10 to 14% was well preserved the bioactive in dried black pepper and not allowed the fungi growth during storage (Sreedharan, Symon, & Narayanan, 1985).

4.1.2 Determination of active compounds of BEOs

The active compounds of the extracted BEOs were determined under GC-MS analysis, and data are shown in Table 4.2.

Table 4.2 Chemical composition of extracted BEOs analyzed by GC-MS.

No	Compounds name	RT^1(min)	RC^2 EOs(%)
1	Benzene, methyl(1-methylethyl)-	4.19	5.45
2	D-Limonene	4.27	33.15
3	Pinene	4.73	0.08
4	γ-Terpinene	4.77	0.21
5	Cyclohexene,3-methyl-6-(1-methylethylidene)-	5.29	0.74
6	α-Terpinene	5.34	0.98
7	1,6-Octadien-3-ol, 3,7-dimethyl-	5.52	0.89
8	p-Mentha-1,5-dien-8-ol	6.83	0.11
9	Terpinen-4-ol	7.23	0.29
10	1,3-Cyclohexadiene, 2-methyl-5-(1-methylethyl)-, monoepoxide	7.78	0.25
11	1,6-Octadien-3-ol, 3,7-dimethyl-	8.05	0.15
12	Terpineol	8.95	0.34
13	4,7,7Trimethylbicyclo[4.1.0] hept-3-en-2-one	10.39	0.18
14	γ Elemene	11.04	2.62
15	α-Copaene	11.98	4.52
16	Bicyclo[5.3.0]decane,2-methylene-5-(1-methylvinyl)-8-methyl-	12.36	1.19
17	Caryophyllene	13.07	38.18
19	α-Humulene	13.84	2.42
20	β-Selinene	14.61	0.89
21	β-Bisabolene	15.14	0.11
22	δ Cadinene	15.48	1.5
22	Caryophyllene oxide	16.84	2.49
23	Isospathulenol	17.86	0.92

where RT^1: Retention time, RC^2 EOs: Relative content essential oils.

The major compounds of the extracted BEOs composed of caryophyllene (38.18%), D-limonene (33.5%), and followed by α-copaene (4.5%), γ-elemene (2.6%), caryophyllene oxide (2.49%), α-Humulene (2.42%), and cadinene (1.5%). The other chemical compositions were less than 1 %. The result agreed well with other previous works (Rouatbi, Duquenoy, & Giampaoli, 2007), (Myszka, Schmidt, Majcher, Juzwa, & Czaczyk, 2017), (Amalraj, Haponiuk, Thomas, & Gopi, 2020). These compounds have been claimed to have the potential for antimicrobial, antioxidant, and anti-inflammatory properties (Heckert Bastos, Vicente, Corrêa dos Santos, Geraldo de Carvalho, & Garcia-Rojas, 2020; Rakmai et al., 2017)

4.2 Determination of active compounds of commercial Sacha inchi oil

Similarly, the active compounds of the commercial Sacha inchi oils were determined under GC-MS, and data are shown in Table 4.3

Table 4.3 Chemical compositions of Sacha inchi oils determined under GC-MS.

No	Compound name	RT (min)	RC sacha inchi oils (%)
1	Hexadecanoic acid	14.50	19.96
2	9,12-Octadecadienoic acid	16.08	28.3
3	9-Octadecenoic acid	16.23	10.68
4	5-Hydroxypent decanoic acid	17.22	3.15
5	Alfa-Tocopherol	20.77	1.08
6	Cholesta-3,5-diene	21.65	2.44
7	unknown	22.121	6.53
8	Gamma-Tocopherol	22.56	9.39
9	Tri-O-methyl-dopamine	23.56	1.88
10	Stigmasterol	24.70	8.29
11	Gamma-sitosterol	25.37	4.52
12	unknown	25.63	2.63
13	unknown	28.73	1.34

The significant compounds of Sacha inchi oils composed of fatty acid such as hexadecanoic acid (palmitic acid (19.96%)), 12-octadecadienoic acid (Linoleic acid (28.3%)), 9-octadecenoic acid (Oleic acid (10.68%)), 5-hydroxypent-2-enoic acid (3.15%), α -tocopherol (1.08%), cholesta-3,5-diene (2.44%), γ-tocopherol (9.39%), tri-o-methyl-dopamine (1.88%), stigmasterol (8.29%), γ-a-sitosterol (4.52%), unknown (2.63%), and unknown (1.34%). This finding was not agreed with the study reported

by Liu et al. (2014), which contain a high amount of linoleic($\omega 6$) and oleic acid($\omega 9$) but for this study they found the major of fatty acid in Sacha inchi oils are linoleic acid($\omega 6$) and linolenic acid($\omega 3$) (Liu et al., 2014; Ramos-Escudero et al., 2021). It may cause the origin of the Sacha inchi seed, which grows in different places, and the extraction process of Sacha inchi oils. We have known that linoleic ($\omega 6$) and oleic acid ($\omega 9$) are the essential fatty acid and can keep the skin in the moisture condition (S. Wang et al., 2018). Tocopherols, one of an important composition in Sacha inchi oils, are the antioxidant agent and can slow down the process of oxidation (Kinen, Kamal-Eldin, Lampi, & Hopia, 2000). Both of α -Tocopherol and γ-Tocopherol have antioxidant properties as escribed from Kinen et *al*., (2000).

4.3 Treatment of *L. clavatum* spores

4.3.1 Morphologies of treated spores

Several chemical solvents were used to remove unwanted molecules from the native spores, e.g., fat, cellulose, and proteins (cytoplasm) (Mundargi, Potroz, et al., 2016a). The acetone and KOH were used to remove fat, lipid, and protein. However, fat, protein, lipid are not entirely removed from the *L.clavatum* spores by alkali and acetone (Barrier, 2008; Barrier et al., 2011). So, the acid-treatment with stir reflux from 5 to 40h was added to optimize the structure spores change and removed unwanted material from *L.clavatum* spores (Blackmore & Knox, 2016; Mundargi, Potroz, et al., 2016a). Scanning electron microscopy (SEM) was employed to examine the morphology and structure of the treated spores. The data is shown in Figure 4.1.

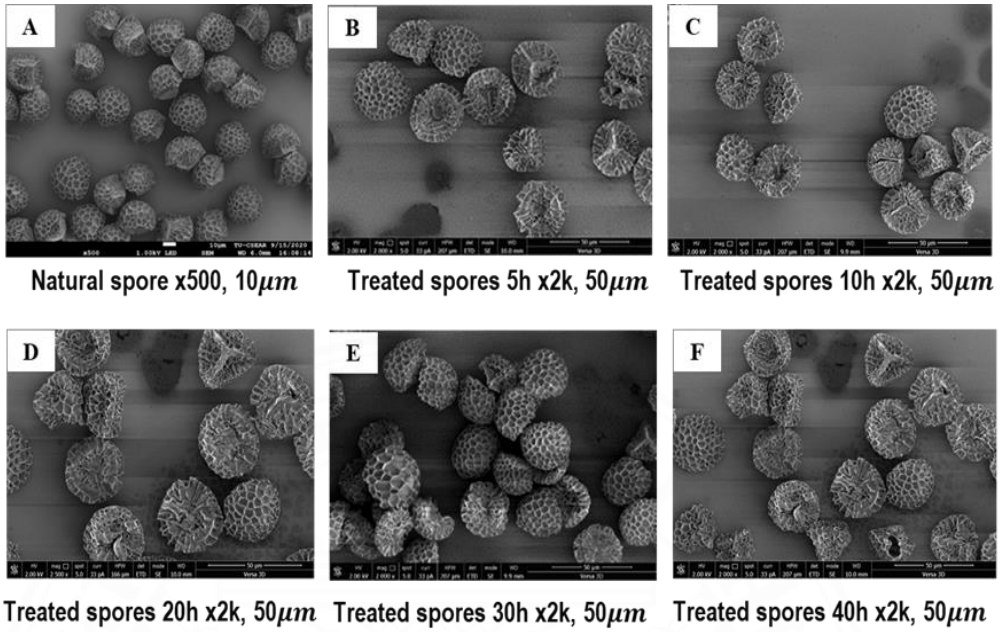

Figure 4.1 SEM images of *L. clavatum* spores during different time of acid-treatment(H_3PO_4). Natural *L. clavatum* spores (A), and acid-treatment of *L. clavatum* spores for 5h (B), 10h (C), 20h (D), 30h (E), and 40h (F).

The natural spore (Figure 4.1A) shows the roughness pattern surface with a round shape and diameter around 28.90 ±1.45. After acid (H_3PO_4) treatment for 5 to 40h (Figure 4.1(B-F), there are no obvious changes in the external structure of *L.clavatum* spores that agree well with the study of Mundargi, Potroz, et al. (2016a). Another observation, even after prolonged acid treatment, the external structural spores still no significant changes. This confirmed the shell or exine of the spore is high stability, which can resist strong chemical and high-temperature environments.

To determine the inside structure of spores, the samples were broken by gridding before characterization and data are shown in Figure 4.2.

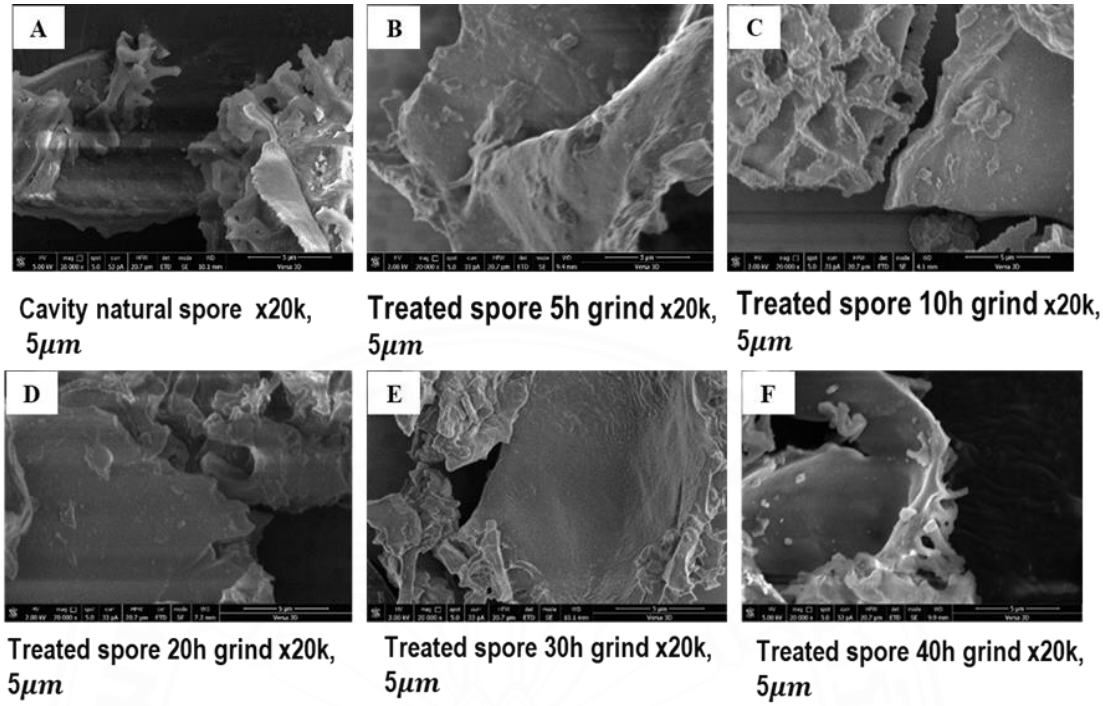

Figure 4.2 SEM images of inner structure of *L. clavatum* spores during different time of acid-treatment(H_3PO_4). Inner cavity of natural *L. clavatum* spores(A), and acid-treatment of *L.clavatum* spores for 5h(B), 10h(C), 20h(D), 30h(E), and 40h(F).

From Figure 4.2(A), the inside structure of the native spore shows a cavity with some small particles. It has been reported that the core of the natural spore contains different genetic materials such as cytoplasm, lipid, and protein (Gaonkar, Vasisht, Khare, & Sobel, 2014). Figure 4.2(B-F) shows the inner cavity at different acid-treatments times (5– 40h). The inner wrinkle and roughness of *L.clavatum* spores from small particles was detected during 5h of acid-treatment (Figure 4.2(B)). With increasing time of refluxing (10 to 20h) (Figure 4.2 (C and D)), it was found that the small particles were partially removed with the smoother surface of the inside shell wall than that of 5h. After keeping prolonged acid treatment until 30 and 40h, the clean cavity of capsule spores was observed (Figure 4.2(E and F)). This result agrees well with Mundargi, Potroz, et al. (2016a) 's previous study, which produced the cleaned spore within 30 h of the acid treatment. (Archibald et al., 2014; Md Jasim Uddin et al.,

2019). This treatment could be implied that the unwanted molecules were utterly removed from the spore, and the ready microcapsule spores for encapsulation of active compounds were produced.

4.3.2 FTIR analysis

FTIR analysis was used to determine the change of functional groups of the treated spores during the chemical treatment, and data are shown in Figure 4.3.

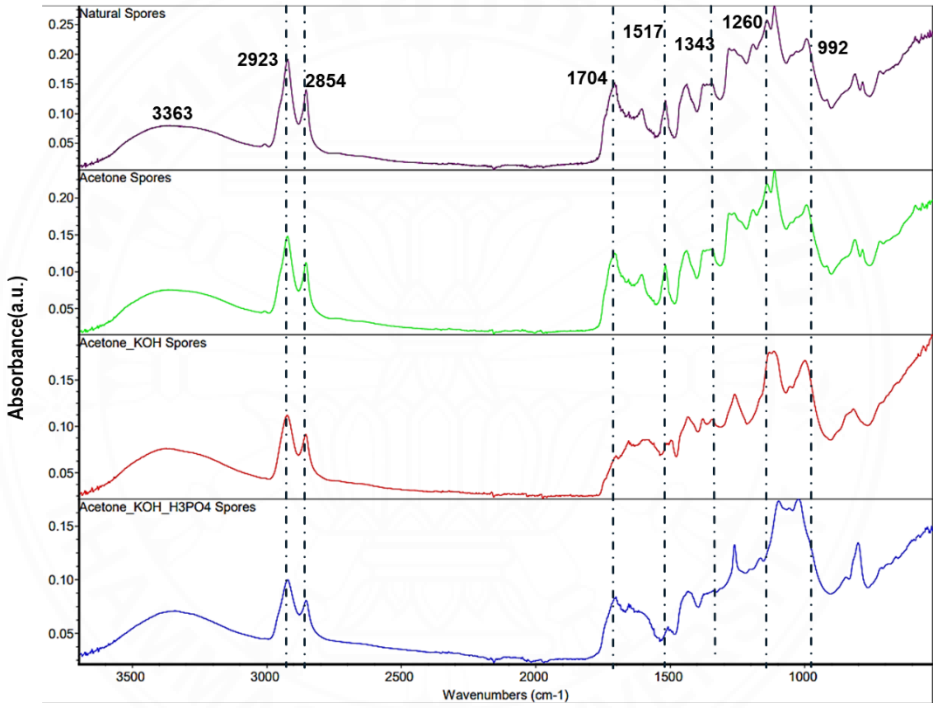

Figure 4.3 FTIR spectra of native *L. Clavatum* spores and the treated spores with acetone, KOH, H_3PO_4 with different time e.g.,5,10,20,30, and 40h.

FTIR spectra showed the functional groups changed with different solvent treatments. The natural spore showed the broad vibration at 3363 cm^{-1} which is O-H stretching of spore structure, at 2923cm^{-1} and 2854 cm^{-1} which is =C-H, and CH_2-stretching vibration aliphatic that can present from unsaturated fatty acid (carboxylic) ester (lipid). Another peak at 1704 cm^{-1} is the carbonyl group (C=O) stretching vibration relate to esters or carboxylic acids. Peak 1517 cm^{-1} is assigned to C=C of phenolic compound and 1343 cm^{-1} is attributed to the C−H bending vibration in the methyl ($-CH_3$) group of lipids. Moreover, the peak at 1260 cm^{-1} is C−O stretching in carbohydrates which was seen. The peak at 847-814 cm^{-1} of the spectrum is a vibration of C-H of aromatics ring spores.

According to the previous work, acetone treatment was used to solubilize the unsaturated phospholipid from the spores. So, the amount of protein still sticks on the surface of the spore, resulting in the spectrum of acetone-treated spores showed the same peaks as the original spore. KOH treatment could remove an amount of protein, and it also hydrolyzed ester to carboxylic acid salts. However, the KOH-treated spore spectrum was still similar to the original one due to the incomplete removal of protein molecules. For H_3PO_4 treatment, the residual protein was removed entirely, and the remaining esters were hydrolyzed to carboxylic acid. Then, the peak at 1260cm^{-1} corresponding to the C−O stretching of carbohydrates could not be detected for 30 and 40h (Md Jasim Uddin et al., 2019).

However, the obtained results from this study show that all spectra from 5 to 30 h still showed the same characteristic peaks of the treated spores without significant changes. If we compare FTIR with SEM results during different acid-treatment times, FTIR spectra are supposed to appear at different peaks of the treated spores. In comparison, SEM observation indicates some residue inside the cavity of spores. It was not detected from FTIR; it may come from the shallow depth of penetration of IR beam is minor than 1.2μm at 1600 cm^1 (Mojet, Ebbesen, & Lefferts, 2010). In addition, sample spores have an average thickness of 1.6μm, it causes that the thick of spores wall limits the IR beam interaction with lipid, protein, cellulose inside the cavity (Zimmermann & Kohler, 2014). Therefore, the acid-reflux of spore for 30h remove

cellulose, protein, and lipid as evident of our SEM morphology (Figure 4.2) and FTIR (Figure 4.3) that agree with an earlier study (Mundargi, Potroz, et al., 2016a).

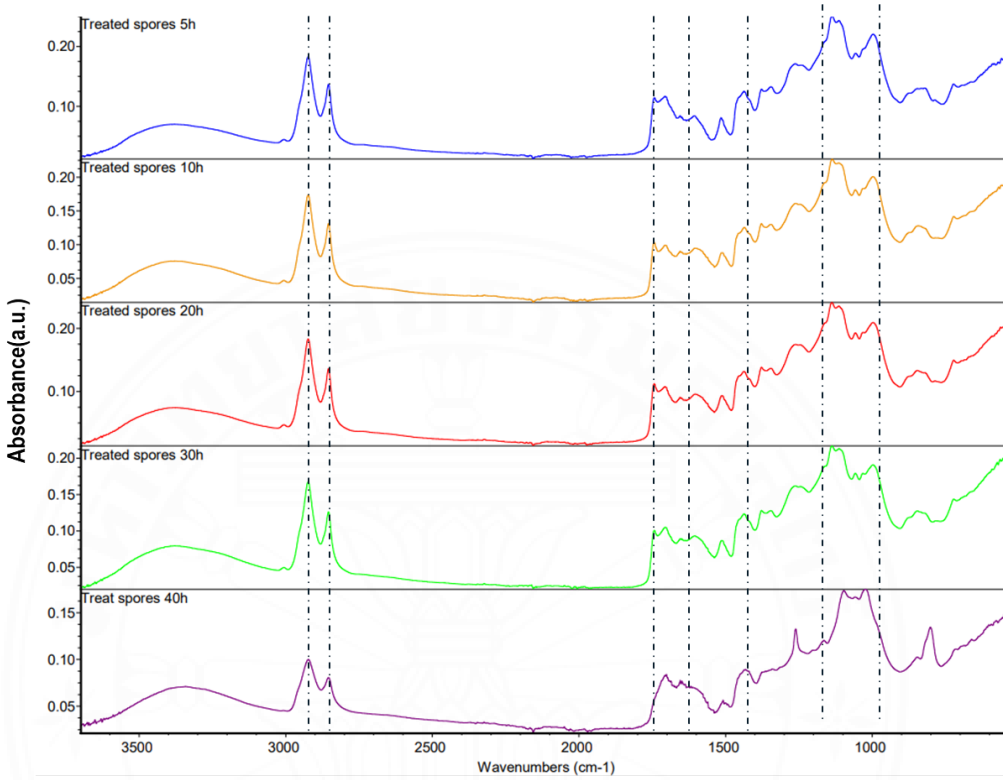

Figure 4.4 FTIR spectra of spore treated in acetone-KOH-H_3PO_4 at 60℃ in different time from 5 to 40h.

Another result from the acid-treatment H_3PO_4 from 20 h to 120 h (3days) at 60℃ provides no change of functional groups of *L.clavatum* spores APPENDIX A.

4.3.3 FTIR analysis of residues from acetone and KOH treatment

It was not easy to analyze the functional group of treated spores since the spore structure has a bonding complex with O-H, C=O bonding. Therefore, the residues of acetone and the KOH treatment step were analyzed to confirm the partial removal of lipids and proteins from the exine and inside the natural spore, as shown in Figure 4.5. However, acid treatment(H_3PO_4) residue was not analyzed since it is difficult to separate between acid and lipid-protein by filtering and drying (oven).

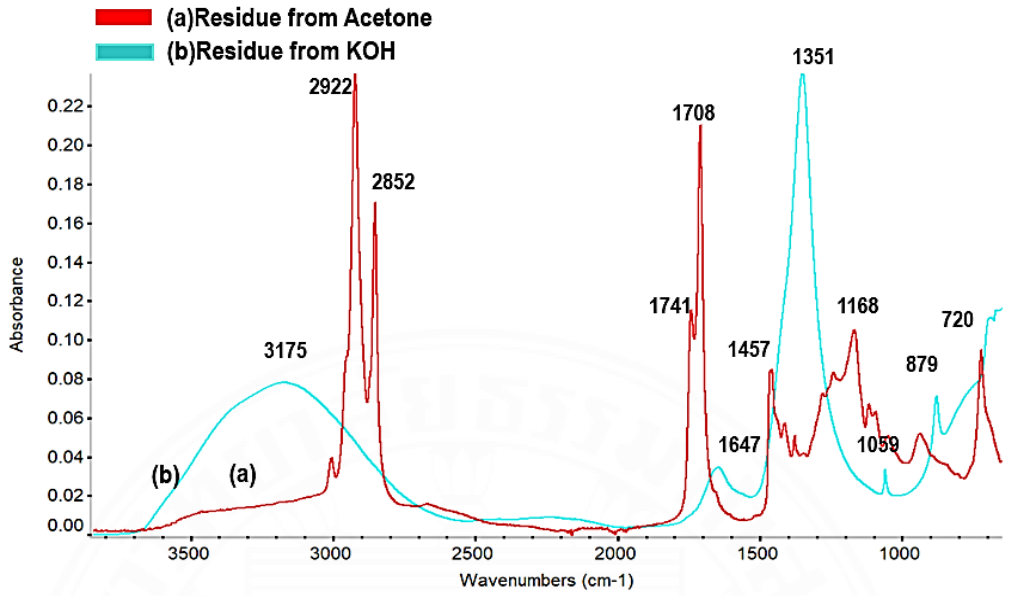

Figure 4.5 FTIR spectra of residue from (a) acetone and(b) KOH treatment**.**

The residue sample from acetone and KOH treatments of spores was filtered and dried at 60℃ until constant weight. For residues sample of acetone, treatment was obtained in the form of lipid (liquid), and residues sample of KOH treatment was in the form of paste (liquid and powder). Moreover, KCl and KOH were measured to subtract with the residue sample as the APPENDIX E. The paste of residue KOH treatment may cause by salt (KOH). For acetone treatment (red spectrum, Figure 4.5a), the peaks at 3006, 2922, 2852, 1741, 1708, 1457, 1377, 1168, 1117, and 722 cm^{-1} were detected which response to the bonding C-H scissoring in the methylene group of lipid (Jardine, Abernethy, Lomax, Gosling, & Fraser, 2017). Peak at 879 cm^{-1} and 720 cm^{-1} are usually N-H out of plane bending sharp peak and secondary aliphatic amines, respectively (Socrates, 2004). According to the previous work, acetone could remove lipids and dissolve some protein in the grain of the spore. They mentioned that the vibration at 1239 cm^{-1}, 1168 cm^{-1}, and 938 cm^{-1} was the asymmetric C-O-PO^{-}_{2} stretching and PO^{-}_{2} asymmetric stretching in phospholipids. The spectrum from residue KOH (Figure 4.5b) shows the broad vibration at 3175 cm^{-1} which refers to O-H from water or residues from hydrate KOH in the sample (Socrates, 2004). After the subtract with KOH, the peak at 1647 cm^{-1} assigned to OH of water and peak at 1059 cm^{-1} is from acidic salt (KOH)

(Md Jasim Uddin et al., 2019). Another high peak at 1351cm^{-1} may come from carboxylic acid that respond to symmetric aliphatic CH bending -CH_3 group of the spores (Mayo, Miller, & Hannah, 2004). Generally, carboxylic acid salts have a strong, characteristic band in the region of 1695-1540 cm^{-1} due to the asymmetric stretching vibration of COO^- (Max & Chapados, 2004). The asymmetric stretching vibration of this group gives rise to a band in the range 1440-1335cm^{-1} and is of medium intensity broad and has two or three peaks (OR, 2016). Unfortunately, water may be presented in the sample, and it has adsorption at around 1640 cm^{-1}. So, it may difficult in identifying bonding due to their amide II band also occurring in this region (Socrates, 2004).

4.4 Analysis of encapsulated microcapsule spores

Bioactive stability, targeting site, and behavior release are the main characteristics of microencapsulation. Some microcapsules give the complex structure and lengthy process to form capsules. Then, sporopollenin shell material extracted from plant spores demonstrated excellent properties over the formulated microcapsules. The advantages of these microcapsules are monodispersed, uniform size and completely degradable in the typical environment.

4.4.1 Black pepper essential oils loaded microcapsule spores

4.4.1.1 Morphology of black pepper essential oils encapsulated microcapsules

The morphologies of BEOs-encapsulated microcapsules and pristine spores were determined under FE-SEM, and data is shown in Figure 4.6.

The pristine spores showed uniformity of size and microstructure with a diameter of spores around 25-30μm (Figure 4.6A). They contained a ready cavity for encapsulation of bioactive compounds, which enter through nanochannels (15-20 nm) (Figure 4.6B) (Diego-Taboada et al., 2012). After encapsulation with BEOs, their morphologies' change could not be observed compared to Figure 4.6C and Figure 4.6D for pristine spore and encapsulated spore, respectively. It is essential to note that the passive loading method did not affect microcapsules' morphology, which agreed well with Mundargi, Potroz, and Park (Mundargi, Potroz, Park, Park, et al., 2016). It has

been reported that *L. clavatum* spores provided a tough outer shell structure that could stay intact in severe environments. They resisted strong acids and strong bases and hardly dissolved organic solvents (Barrier et al., 2011; Heslop-Harrison, Grant, Muir, van Gijzel, & Shaw, 1971). Then, the BEOs-encapsulated (spores) microcapsules are highly used in various applications such as cosmetic applications.

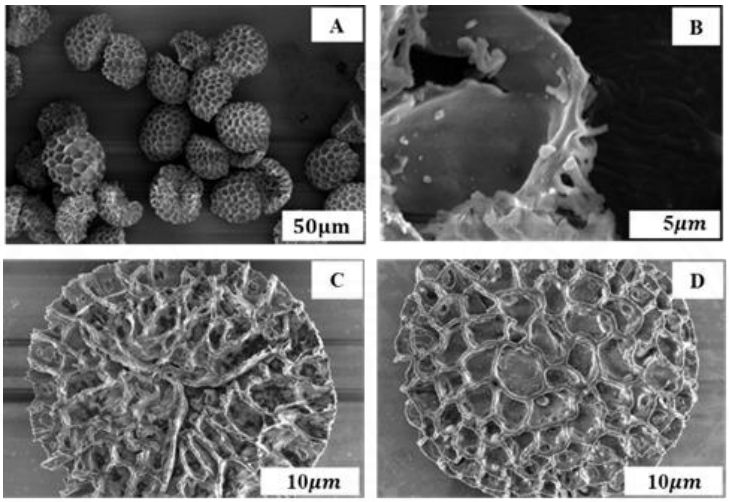

Figure 4.6 SEM images of pristine spores(A), cavity of pristine spores (B), surface structure of pristine.

4.4.1.2 FTIR of free and encapsulated black pepper essential oils

The chemical compositions or the presence of BEOs trapped in the microcapsule spore were determined by FTIR analysis. The FTIR spectra of BEOs, spores, and BEOs-encapsulated microcapsules are shown in Figure 4.7.

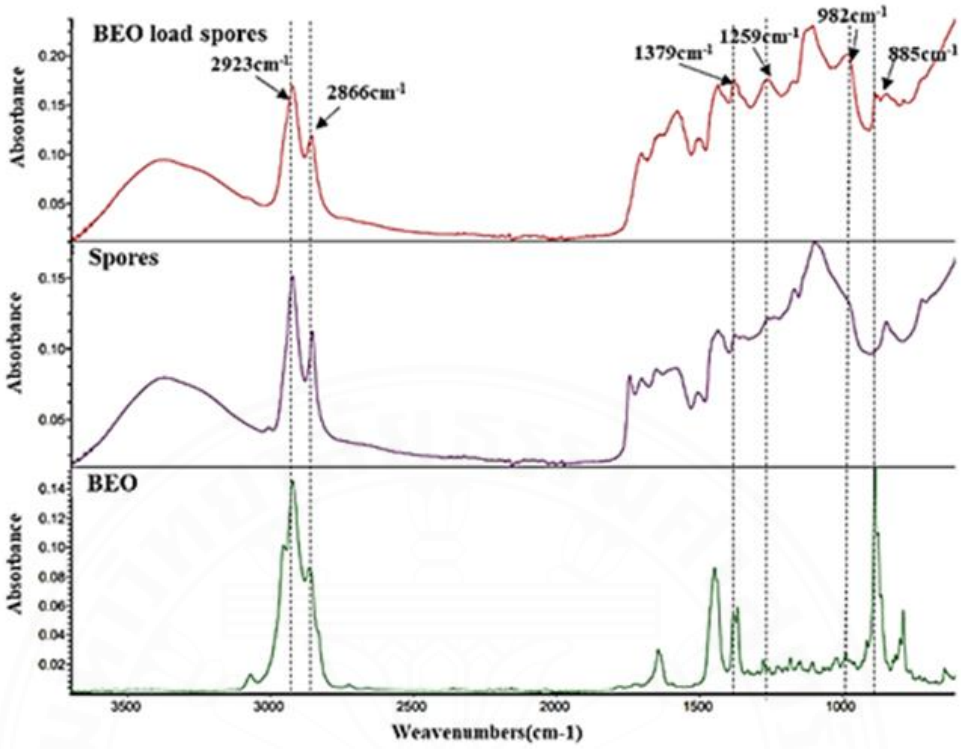

Figure 4.7 FTIR spectra of free BEOs, microcapsule before and after encapsulated with BEOs.

The characteristic peaks of the spores are observed as follow: broad peak at 3363 cm^{-1} correspond to O-H stretching of alcohol, phenol, carbohydrate. The peaks at 2923 $cm^{-1}$and 2866cm^{-1} correspond to asymmetric C-H and -CH_2- of ester. Another peak at 1704 cm^{-1} is the carbonyl group (C=O) which comes from esters or carboxylic acid. The peaks at 1517 cm^{-1} and 1343 cm^{-1} attribute to carbon double bond (C=C) and the C−H bending vibration of methyl (−CH_3) group of lipids. And, the peak at 1096 cm^{-1} is C−O stretching in carbohydrates (Mundargi, Potroz, et al., 2016b; C. S. Pomelli, F. D'Andrea, A. Mezzetta, & L. J. N. J. o. C. Guazzelli, 2020). The appearance of characteristic peaks of BEOs at 1379, 1259, 982, and 885 cm^{-1} in the spectrum of BEOs-loaded microcapsules confirmed the successful loading of BEOs into the cavity of natural spores (Wu et al., 2020).

4.4.1.3 BEOs-encapsulated microcapsules

For encapsulation of BEOs into the natural cavity spores, the one-step passive loading technique was applied. This technique provides attractive features, including both versatility and simplicity, with the potential to allow the encapsulation of a variety of small or large biomolecules under ambient processing conditions. The results from the quantitative determination of spore formulation yield after the encapsulation process in terms of encapsulation efficiency (%EE) and loading capacity (%LC) in natural spores are shown in Table 4.4.

Table 4.4 BEOs-loaded spores encapsulated.

BEOs: spores ratios	Mass ratios [mg]	EE [%]	LC [%]
0.75:1	115:150	36.84± 4.04	18.26 ± 2.62
1:1	150:150	50.64± 4.92	29.26± 3.70
2:1	300:150	51.13±5.10	28.04±1.93

It was observed that the %EE and %LC of the encapsulated-spores increased when increased the ratio of BEOs: spores. However, using the ratio of BEOs: spores of 1:1 and 2:1, it was found that both %EE and %LC did not show significantly different values. The highest values of %EE and %LC of 51.1% and 29.2% were obtained.

4.4.2 Sacha inchi oils loaded-microcapsule spores

The bioactive substances of Sacha inchi oils were loaded into the treated microcapsule spores using the passive loading method. The possible way for loading is the diffusion through nanochannel of the wall surface of *L. clavatum* spore. The properties of the obtained microcapsule spores, e.g., morphology, encapsulation efficiency, loading capacity, and release behavior, were characterized by SEM, FTIR, UV-vis spectroscopy, and confocal microscope.

4.4.2.1 Morphology of Sacha inchi oils encapsulated spores

SEM analysis was employed to determine the stability of the loaded-microcapsule spores via morphological change, and data is shown in Figure 4.8

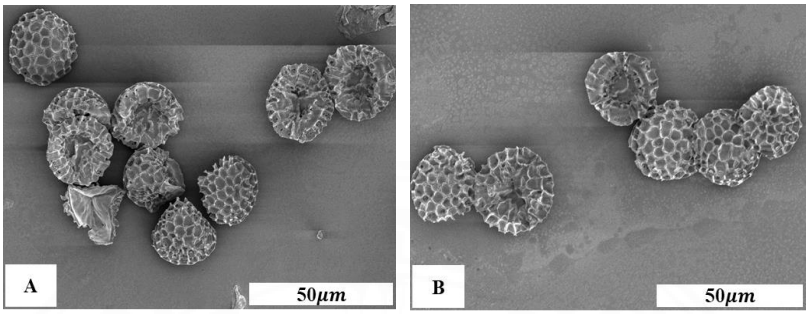

Figure 4.8 SEM images of treated *L. clavatum* spores before and after Sacha oils loading (A–B) respectively.

It was found that the loaded-microcapsule spores maintained well morphology without broken as seen in Figure 4.8(B). Moreover, non some residual on the surface was shown on the particle's surface compared with the treated microcapsule spores Figure 4.8(A). As described above, using a facile passive loading method, the oil was diffused into the microcapsule spore's cavity through a nanochannel that naturally presents on their surface (Diego-Taboada et al., 2012). It has been reported that the nanochannel has a diameter of around 15-20nm.

4.4.2.2 Confocal laser scanning microscopy analysis

To observe the presence of bioactive Sacha inchi oils located in the cavity of microcapsule spore, confocal laser scanning microscopy (CLSM) was applied. Before loading into the microcapsule spores, the bioactive Sacha inchi oil solution was stained with fluorescent dyes, e.g., FTIC and Nile red. All the images were recorded focusing on the middle cavity of spores fixed in between thin glass slides and insert within vectashield at 60X.

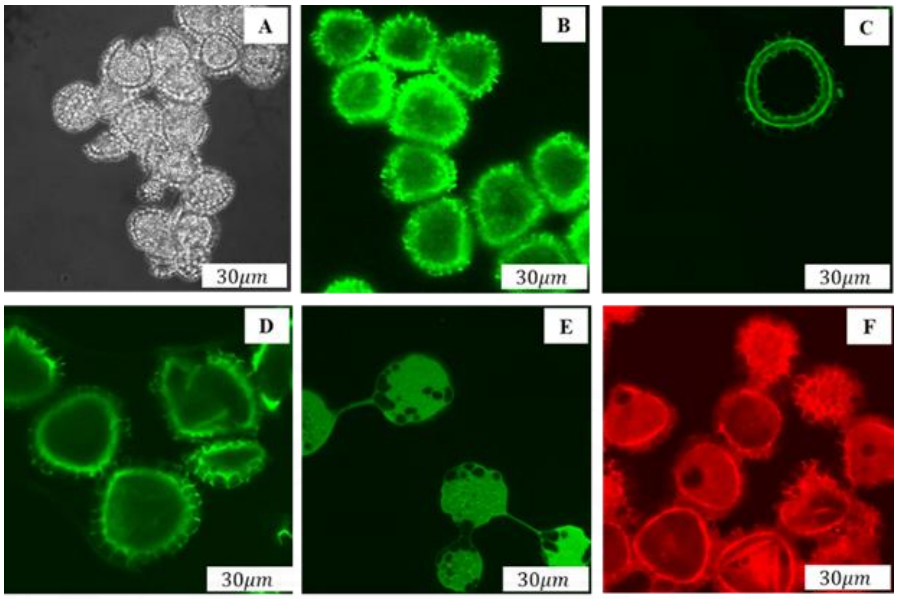

Figure 4.9 (A) DIC (differential interference contrast) and CLSM images of (B) natural *L. clavatum* spores, (C) the treated spores, (D) Sacha inchi oil- loaded microcapsule spores, (E) FITC@oil- loaded microcapsule spores, and (F) Nile red@oil- loaded microcapsule spores.

Under DIC (Differential Interference Contrast), natural *L. Clavatum* spores **(**Figure 4.9A) displayed the phase contrast of the sample and glass slides. For confocal fluorescent condition (Figure 4.9B), the natural spores showed autofluorescence with green brightness color due to the presence of some molecules, e.g., terpenoid, phenolic, and carotenoid in the side cavity of the spores (Atwe, Ma, & Gill, 2014; Mitsumoto, Yabusaki, & Aoyagi, 2009; Roshchina, 2012). However, when treating the spores to remove these molecules, the hollow structure appeared (Figure 4.9C). After loading bioactive Sacha inchi oil into the treated spores (Figure 4.9D), the obtained microcapsule spores with obscure green color inside the particle were presented. After adding fluorescent dye, i.e., FITC (Figure 4.9E), the green brightness color was seen throughout the particles. Including the particles loaded with Nile red (Figure 4.9 F), the red brightness color in the spores' cavity was displayed. Interestingly, the red color of each capsule spores illustrates different bright, and some capsule spores show a slightly bright red color, which affects the concentration of Nile red dye and Sacha inchi oil.

The recent work has studied the FITC-conjugated BSA into natural spores using confocal scanning microscopy within vectashield at 100x. The sample presented the bright color of FITC-BSA inside the cavity. If we compare this work with our study, it is shown the same result of terpenoid presented of natural spores. But it quite clears of color terpenoid and FITC-BSA because they used the vectashield at 100x (Mundargi, Potroz, Park, Park, et al., 2016). Moreover, the encapsulated biotics into cavity spores were observed by CLSM, which form a complex of biotics-eosin Y dye. They have captured the image on a slice of depth with z-stacked of around 12.5μm, of all the samples that the same as our observation. This study indicates well-extracted capsule spores which no autofluorescence from the cytoplasm in spores. They also mentioned the surface after loading, which needs to clean carefully with water/ethanol to remove any residual dye stick on the surface (Dyab et al., 2018). However, an earlier study has worked on the encapsulation of fish oils and liver oils and observed the florescent signal of oils loading by CLSM. The results of CLSM showed an intense color of dye conjugate oils inside because these works use such a high number of oils into cavity spores (Barrier et al., 2011; Diego-Taboada et al., 2012)

4.4.2.3 FTIR analysis of encapsulated microcapsule spores

To confirm the loading of Sacha inchi oil into the microcapsule spore, FTIR analysis was applied. Before characterization, the samples were broken by gridding to reveal the inside of particles.

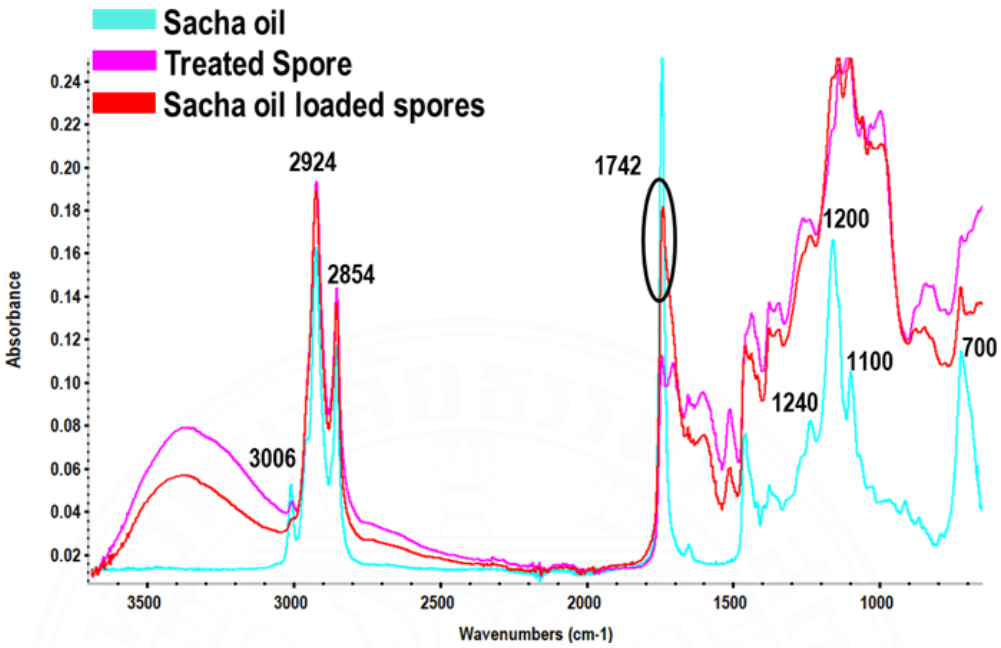

Figure 4.10 FTIR spectra of Sacha inchi oil (turquoise line), treated spore (purple line), and encapsulated microcapsule spore (red line).

Spectrum of Sacha inchi oils (turquoise line) shows the intensity of peak at 1742 cm^{-1} which corresponds to the stretching vibration of the carbonyl group (C=O). Moreover, the two peaks at 1100 and 1240 cm^{-1} corresponding to the stretching vibrations of the C–O bonding in the ester group are observed. And the band at 1200 cm^{-1} is characteristic of the bending vibration of the methylene group (= CH_2). The higher band of 700 cm^{-1} corresponds to the isolated trans alkene group (–HC=CH–). The spectra of encapsulated spores (red line) show the peaks at 3006, 2024, and 2854 cm^{-1} which are =C-H stretching vibration of the cis alkene group (-HC=CH) and asymmetric and symmetric C−H stretching of a methylene group (=CH_2). The high-intensity band at 1742 cm^{-1} results from the stretching vibration of the carbonyl group (C=O), which is characteristic of Sacha inchi oils. This result can confirm the presence of Sacha inchi oils loaded in microcapsule spores.

4.4.2.4 Encapsulation efficiency and loading capacity

%EE and %LC of the prepared Sacha inchi oil-loaded microcapsule spores were determined by indirect and direct methods. For indirect method, UV-vis spectroscopy was utilized for the indirect method to determine the mass of Sacha inchi oil-free dissolved in the supernatant after encapsulation. Firstly, the standard curve of different concentrations of Sacha inchi oil was created as shown in Figure 4.11(a-b), and the mass of oils-free was calculated by absorbance of Sacha inchi oils dissolved in the supernatant as shown in Figure 4.12.

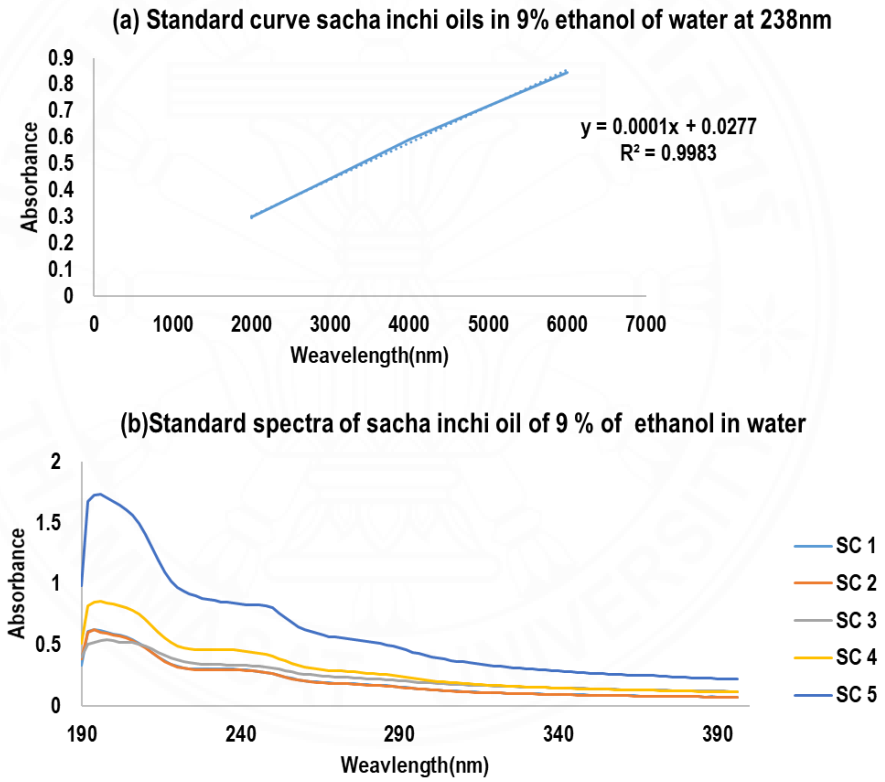

Figure 4.11 (a) Standard curve Sacha inchi oil dissolved in 9% of ethanol in water, (b) UV-vis spectra of different concentrations of Sacha inchi oil 9% of ethanol in water (SC1: 10^3 ppm, SC2: $2x10^3$ ppm, SC3: $3x10^3$ ppm, SC4: $4x10^3$ ppm, SC5: $6x10^3$ ppm).

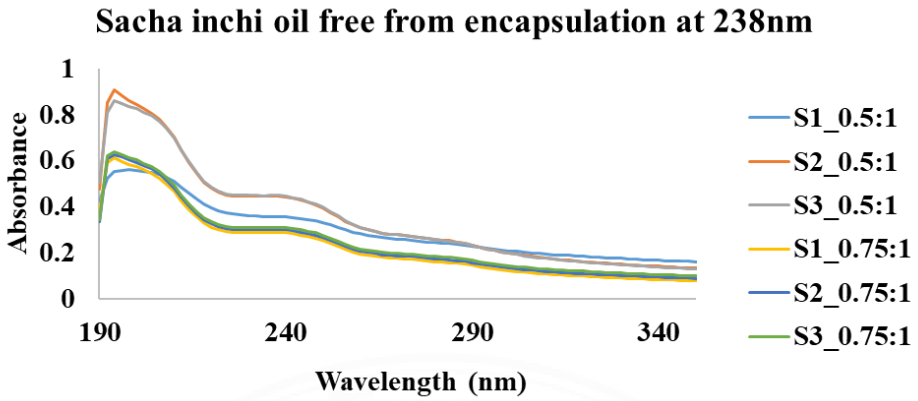

Figure 4.12 UV-vis Spectra of Sacha inchi oil free from supernatant in 9% ethanol (S1, S2, S3: Sacha inchi oils with triplicate measurement).

At the same time, the direct method was also used to determine the %EE and %LC of the encapsulated microcapsule spores. Similarly, the standard curve of sacha inchi oil with different concentrations was conducted under UV-vis spectroscopy analysis at a wavelength of 238 nm (Sahi, Varshney, Poddar, Vajanthri, & Mahto, 2019)

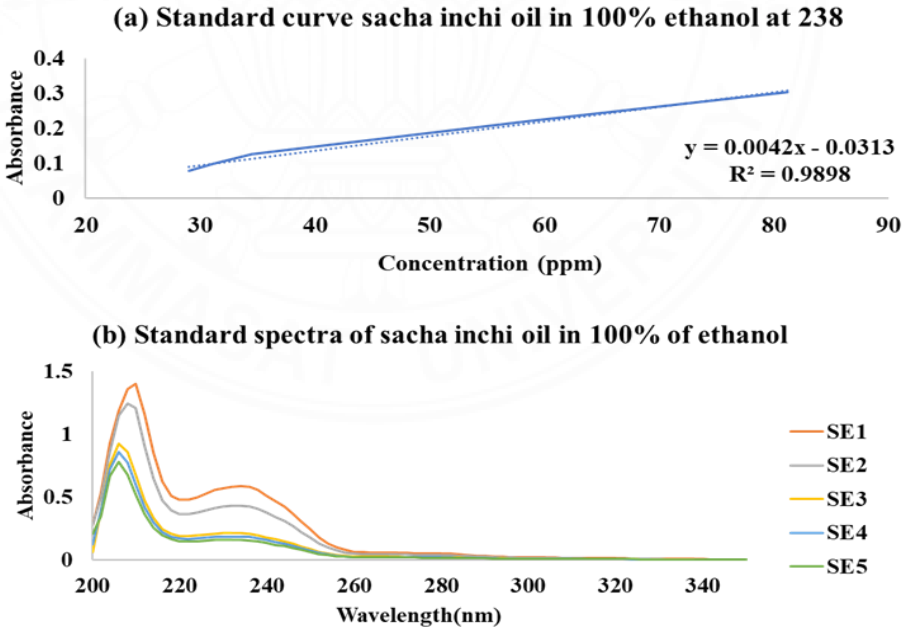

Figure 4.13 (a) Standard curve Sacha inchi oil with different concentration dissolved in 100% of ethanol, (b) UV-vis spectra of Sacha inchi oil dissolved in 100% of ethanol(SE1: 29ppm; SE2: 31.2ppm; SE3: 34.54ppm; SE4: 58.87ppm; SE5: 81.19ppm).

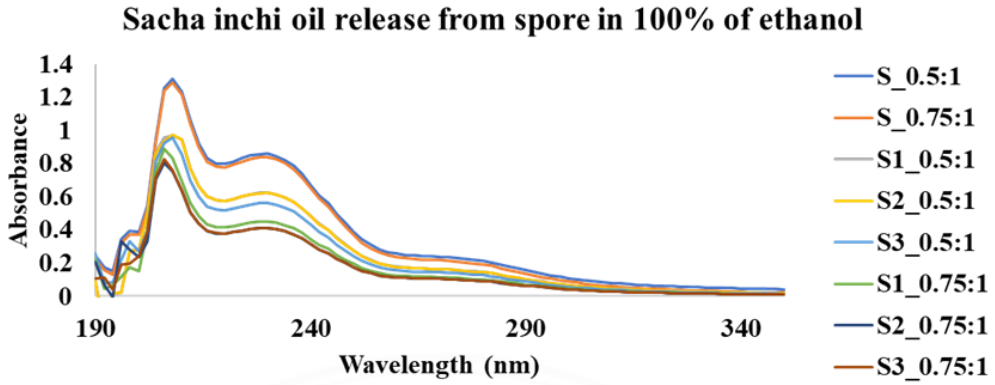

Figure 4.14 UV-vis spectra of Sacha icnhi oil released from microcapsule and dissolved in 100% ethanol (S: initial concentration Sacha inchi oils; S1, S2, S3: Sacha inchi oils with triplicate measurement).

The standard curve of Sacha inchi oils in 100% ethanol were stated in Figure 4.13(a-b). Figure 4.14 presented Sacha inchi oil entrapment in capsule releasing from capsule spore in 100% ethanol at 238 nm.

The microcapsule spores have a diameter $\approx 28\mu m$ and the nanosize ≈15-20nm, which might allow diffusion of the oils into the cavity of the spore by passive and vacuum loading techniques.

Moreover, 9% ethanol in water is a water-based vehicle or carrier solution by suspension between Sacha inchi oils and microcapsule spores. The dispersion of the microcapsule spore absorbs Sacha inchi oil solution through nano-size pore and diffusion by isotonicity condition. The microcapsule spores have the free volume to fill the Sacha inchi oils. Isotonicity occurs in this passive loading while a high concentration of Sacha inchi oil-ethanol flows into the low concentration into microcapsule spores. During shacking, the Sacha inchi oil-ethanol concentration outside of the microcapsule spores diffuse into inside of microcapsule spores until the concentration inside is equal to the concentration of Sacha inchi oil-ethanol outside of the microcapsule spores. The collision of oils and microcapsule spores was stirred with suitable speed to make the uniform suspension to avoid the hard cake of the spores during shaking.

The %EE of the sacha inchi oils loaded-microcapsule spores was determined by using UV-vis spectroscopy. There are two ways (indirect and direct methods) to determine the %EE of the encapsulated sacha inchi oils. The results are shown in Figure 4.15. The bar chart with blue color represents %EE obtained from indirect method whereas green color bar chart represents %EE which obtained from direct method. The orange bar chart mention about %LC from indirect method whereas the yellow bar chart refers to %LC that obtained from a direct method.

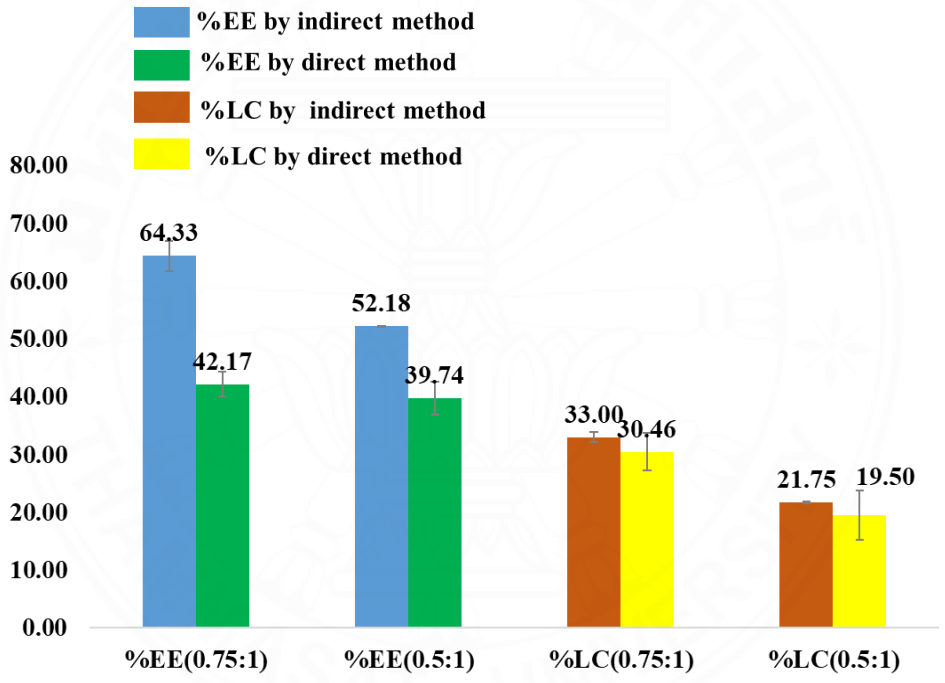

Figure 4.15 %EE and %LC of the Sacha inchi oil-loaded microcapsule spores prepared via passive loading technique.

For the results obtained by an indirect method, at ratio Sacha inchi oil: spore of 0.75:1 and 0.5:1, the encapsulated microcapsule spores showed %EE from an indirect method of 64.33±3.82 % and 52.18±0.05 %, respectively. While %EE (green bar chart) of the prepared microcapsule spores obtained from the direct method at ratio oil: spore of 0.75:1 and 0.5:1 is 42.17±2.17% and 39.74±2.80%, respectively. It was found that %EE obtained from a direct method is lower than that of from indirect method

because it was calculated from the percentage of the actual Sacha inchi oils content loaded in microcapsule divided by the percentage of the theoretical content of sacha inchi oils (Bastos et al., 2020; Timilsena et al., 2016). Then, the preferable result of %EE and %LC were obtained from the direct method. Similarly, several recent works used the direct method to define the %EE and %LC of the active ingredient loaded in the encapsulated particles (Bastos et al., 2020; Mundargi, Potroz, Park, Park, et al., 2016). When considering the %LC of the encapsulated microcapsule spores, it was found that the maximum value of %LC (≈30%) was obtained from both indirect and direct methods of the microcapsule spore prepared by using oil: spores ratio of 0.75:1.

For encapsulated microcapsule spores prepared via a vacuum loading technique, %EE and % LC were investigated by the direct method. The result of %EE and %LC were shown in Figure 4.16. The bar chart with blue color represents %EE while the yellow bar chart mentions about %LC of microcapsules prepared with ratios of oil: spore of 0.75:1 and 0.5:1.

Results show that the encapsulated microcapsule spores showed %EE around 62.47±2.38%, and 51.63±6.39%, for a ratio of 0.75:1 and 0.5:1, respectively. At the same time, %LC around 32.85±4.25% and 22.35± 2.21% of these two ratios were obtained. As comparing the results of %EE and %LC (direct method) from passive and vacuum loading techniques, it was found that the high %EE including %LC was obtained when using vacuum loading technique. So, the vacuum loading technique might be more capable of encapsulating sacha inchi oils into the treated microcapsule spores. The vacuum loading has the external pressure to push the sacha inchi oils into the spores' cavity. However, the vacuum loading needs multi-steps and requires the specific machine high pressure for the freeze-dried step. Then, it is not convenient for this study. Therefore, the simple passive loading technique with acceptable %EE and %LC (at ratio oil: spore of 0.75:1) is selected for further experiments.

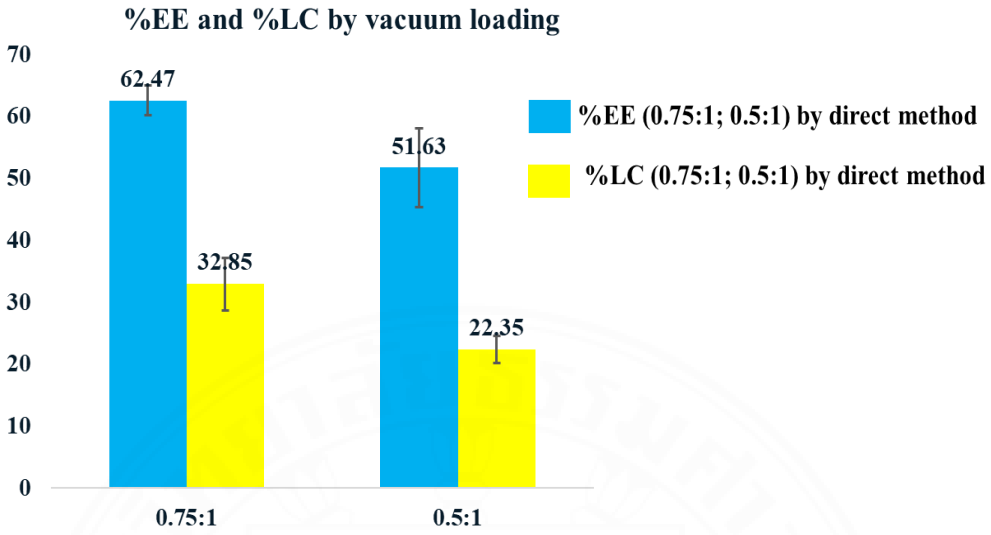

Figure 4.16 %EE and %LC of Sacha inchi oil-loaded microcapsule spores prepared by vacuum loading technique.

4.5 Stability of the encapsulated microcapsule spores in PBS media

As the discussion above, Sacha inchi oil was successfully loaded into the microcapsule spores. The as-prepared microcapsules will be used as one of the ingredients of personal care products. However, the existence of the loaded oil in the capsule must be stable before due time. Then, the stability of loaded Sacha inchi oil in the capsule or releasing oil to the unwanted environments was determined.

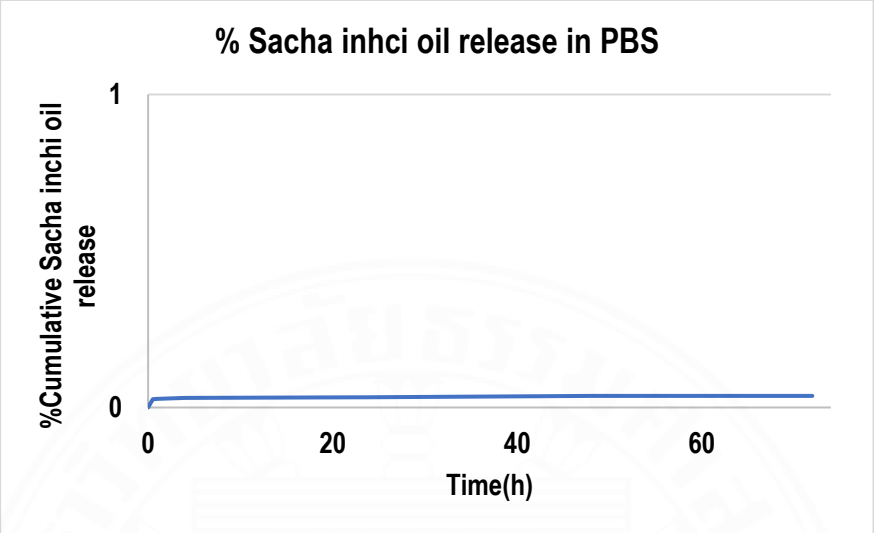

Figure 4.17 Cumulative release of Sacha inchi oil from microcapsule spores in PBS (pH=7.4).

The encapsulated microcapsule spores were suspended in a dialysis bag with PBS media at pH 7.4. Then, the release of oil was detected under UV-vis spectrometer analysis. Figure 4.17 shows the amount of releasing of Sacha inchi oils from microcapsule in PBS media. It indicates that no amount of Sacha inchi oils was released during storage for 72h. So, an encapsulated microcapsule is very stable in the solution without protecting or covering the particle with other molecules. As a comparison with other works, the BSA loaded spore was released in simulated gastric, and intestinal fluid to study the behavior of the drug release, and the encapsulated capsule were covered with alginate to close the diffusion of BSA through nanopores and to control the release of BSA mainly at the target site of the human body.

4.6 Control release study

Furthermore, to get a closure of personal care (active scrub bead) application, the release profile from the microcapsule by using pressure-stimuli (friction) was applied. Generally, the pressing time states for the duration of applying encapsulated microcapsule spores, and it could be a factor that affects to release of Sacha inchi oils.

In addition, the sonication time was considered an influencing factor on the separation of Sacha inchi oils and microcapsule spores after pressure stimuli. The as-prepared microcapsules at ratio oil: spore of 0.75:1 by a passive loading technique were used to control the release study.

The release profiles of Sacha inchi oil from the microcapsule spores with varying pressing times, e.g., 1, 2, 3, 5, 7, 9, and 11 min under pressure-stimuli are shown in Figure 4.18.

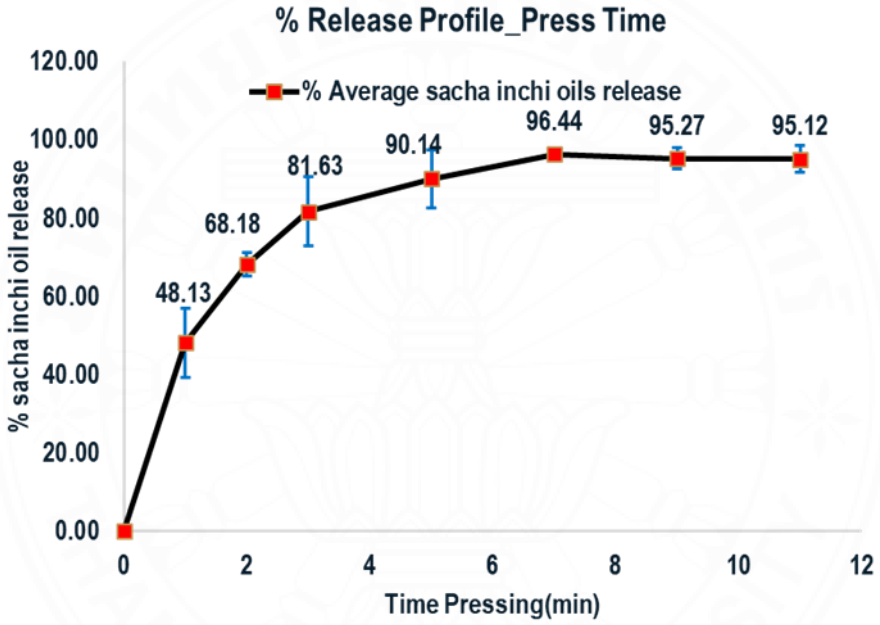

Figure 4.18 % release of sacha inchi oils from microcapsules by varying pressing times e.g., 1, 2, 3,5,7,9,11 min, and sonication time 5min.

The release profile indicates a systematic release trend based on time observed in the experiment in prolonged pressing time. There was a higher burst release with 1 min (48.12±7.45%), 2min (60.37± 5.09%), and 3 min (81.62±8.83%). Sustained-release up to 7 min (96.43±1.14%) and sacha inchi oils were consistently released in 9 min (95.26%±2.63%) and 11 min (95.11±3.36%). When considering pressing time in the application, it is vital to study release profiles in various pressing times and take a short time to release a high percentage of Sacha inchi oils. Therefore, the pressing

time to release a high percentage of Sacha inchi oils is 7 min with a force around 1990.8N.

To determine the force while pressing the microcapsule, the load cell with a Tensile machine was prepared. The random force of each press by handhold spatula was determined and obtain the average of each time pressing is 6.32 ± 1.01N and if we refer to pressure around 6.17MPa (area of spatula: 2.5cm x 1cm). There is 45 time of pressing in 1 min, then the force of investigating time t_i (1,2,3,5,7,9,11min) was calculated by following equation (4.1) and the force observation is in the (APPENDIX D):

$$\text{Force}(t_i) = 45 \times t_i \times 6.32\text{N} \tag{4.1}$$

4.7 Antioxidant activity of Sacha inchi oil

An antioxidant is a molecule stable enough to donate an electron to a group of free radicals and neutralize it, thus reducing its capacity to damage. These antioxidant delays or inhibit cellular damage mainly through their free radical scavenging property. Two principles of action have occurred for antioxidants: the chain-breaking mechanism of antioxidants and the removal of reactive oxygen species(ROS)/reactive nitrogen species initiators(Rice-Evans & Diplock, 1993). Sacha inchi oil has recently found a high level of omega 3 fatty acids in the form of α-linolenic fatty acid (~45%), various types of tocopherols ($\alpha, \gamma, \delta, and\ \beta$-tocopherol), Phyto steroids, and phenolic compounds that are known antioxidant substances (Cisneros, Paredes, Arana, & Cisneros-Zevallos, 2014). All those essential bioactivities are applied for medical and cosmetics applications.

According to the result characterization of Sacha inchi oil exists linoleic (ω6:28.3%) and oleic acid (ω9: 10.68%), α-tocopherol (1.08%), cholesta-3,5-diene (2.44%), γ-tocopherol (9.39%), tri-o-methyl-dopamine (1.88%), stigmasterol (8.29%), γ-sitosterol (4.52%). Interestingly, linoleic, oleic acid, stigmasterol, γ-tocopherol, γ-sitosterol, α-tocopherol are the functional compounds to reduce the oxidation.

In our experiment, we observed the various concentration of Sacha inchi oil with various concentrations e.g., 2.5, 5, 7.5, 10, 20, 40, 60, 80,100 mg/ml.

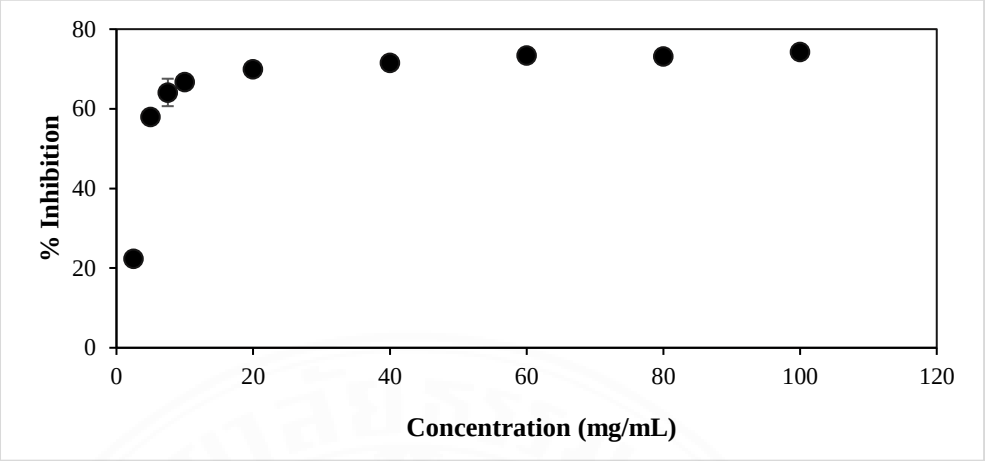

Figure 4.19 %inhibition of Sacha inchi oil.

The antioxidant activity of the sample was measured for 30 min, and the cumulative percentage of inhibition (%) is presented in Figure 4.19. Among nine different concentrations of Sacha inchi oils. The lower concentration (2.5 mg/ml) has a percentage inhibition of 22.38±0.52%, at 5mg/ml (57.94±0.30%), and the percentage inhibition rise while the concentration of Sacha inchi oils increases. However, 40 to 100mg/ml concentration presented the highest reduction of free radical from 71.58±0.50 to 74.28±0.25% for inhibition. Then concentration at 40 mg/ml (71.58±0.50) showed percentage reduction not significant different from concentration 100 mg/ml (74.28± 0.25%). This comparation result show the maximum of reduction at concentration 40mg/ml and even the concentration was increase, it only reaches to 74.28%. Overall, the result of reduction explains that the percentage inhibition (radical scavenging capacity) of Sacha inchi oils has been proven against 2,2-diphenyl-1-picryl-hydrazyl-hydrate radical (DPPH●$^{+}$). The first mechanism happens in a chain-breaking of compounds in the Sacha inchi oils such as γ-tocopherol, γ-sitosterol, and α-tocopherol. And the other compounds have the functional group of O-H bond to the ring that can donate an electron to free radical present in the systems.

According to a previous study on a different kind of tocopherol provide the efficiency antioxidant differently. Recent research evaluates the antioxidant of different oils such as olive, soybean, and Sacha inchi oil. Generally, those oils contain fatty acid and polyphenol, but the main difference is the type of significant tocopherols. Olive oil

exists major of α-tocopherol, while Sacha inchi oil contains mainly γ-tocopherol and δ-tocopherol. And the result of this research showed a high radical scavenging effect (%) on the Sacha inchi oil around 90% in 15 min while the olive, soybean show reduction of free radicals only 30%. This result concludes that the presence of γ-tocopherol in Sacha inchi oil can increase the reduction of the free radical in the system (Elgegren et al., 2019). Therefore, compared to our study with the presence of γ-tocopherol, α-tocopherol compounds from Sacha inchi are highly potent and very effective in donate electron, and trapping oxygen.

CHAPTER 5
CONCLUSIONS

Part 1 Extraction of essential oils and *L. clavatum* microspores:

In conclusion, black pepper essential oils (BEOs) were extracted from commercial black pepper (*Piper nigrum L)* using a green hydrodistillation method. For the chemical compositions of the extracted BEOs determined under GC-MS, the extracted BEO receives 23 compounds. The important bioactive compounds essential to biomedical and pharmaceuticals include caryophyllene (38.18%), D-limonene (33.5%), and followed by α-copaene (4.5%), γ-elemene (2.6%), caryophyllene oxide (2.49%), α-humulene (2.42%), and cadinene (1.5%). Sacha inchi oils characterization was obtained with a high percentage of fatty acid such as linoleic ($\omega 6$:28.3%) and oleic acid ($\omega 9$: 10.68%), α-tocopherol (1.08%), cholesta-3,5-diene (2.44%), γ-tocopherol (9.39%), tri-o-methyl-dopamine (1.88%), stigmasterol (8.29%), γ-sitosterol (4.52%) which are the main components for this present study for encapsulation. Secondly, the extraction of *L. clavatum* could remove the native biomolecule using acetone, KOH, and phosphoric acid (at 30 h), the native proteins were completely removed. Results from FTIR and FE-SEM indicated that the cleaned and ready microcapsule spores were obtained.

Part 2 Encapsulation of BEOs and Sacha inchi oils into microcapsule spores

The extracted BEOs and commercial Sacha inchi oil were successfully encapsulated into the natural and the treated microcapsule spores by a facile passive loading method. By using this method, the perfect morphology of encapsulated spores was still observed. Moreover, the successful encapsulation was confirmed by FTIR analysis. Using the ratio of BEOs: spores at 1:1, high %EE and %LC of 50.64$\pm$4.92%, and 29.26$\pm$ 3.70%, respectively, were obtained. Indeed, the future work will focus on the release profile and antimicrobial to employ the efficacy of the biomedical and pharmaceutical applications of the BEOs-loaded microcapsules.

To encapsulate commercial Sacha inchi oils, the high percentage of the encapsulation efficiency was received at ratio oil: spore of 0.75:1. Similarly, the as-prepared microcapsules with perfect morphology were obtained. The success of sacha

inchi oil encapsulated in the microcapsules was confirmed by FTIR and CLSM analysis. By using this system, the high stability of the existence of Sacha inchi oil in the microcapsule was produced without any covering of the encapsulated microcapsule's surface. Under the desired release condition (pressure-stimuli), a high percentage of Sacha inchi oils released from microcapsule (96.43±1.14%) is under 7 min.

Moreover, the as-prepared microcapsule spores had the potential for antioxidant properties at 40 to 100mg/ml concentration presented the highest reduction of free radical from 71.58±0.50 to 74.28± 0.25% for inhibition.

The structural greatness of the natural spores for entrapment the bioactive contributes encouragement for their further uses and investigate the carrier for various bioactive components. Noticeably, different bioactive compounds will perform various applications with a great property biomaterial that can be degradable in environmental conditions.

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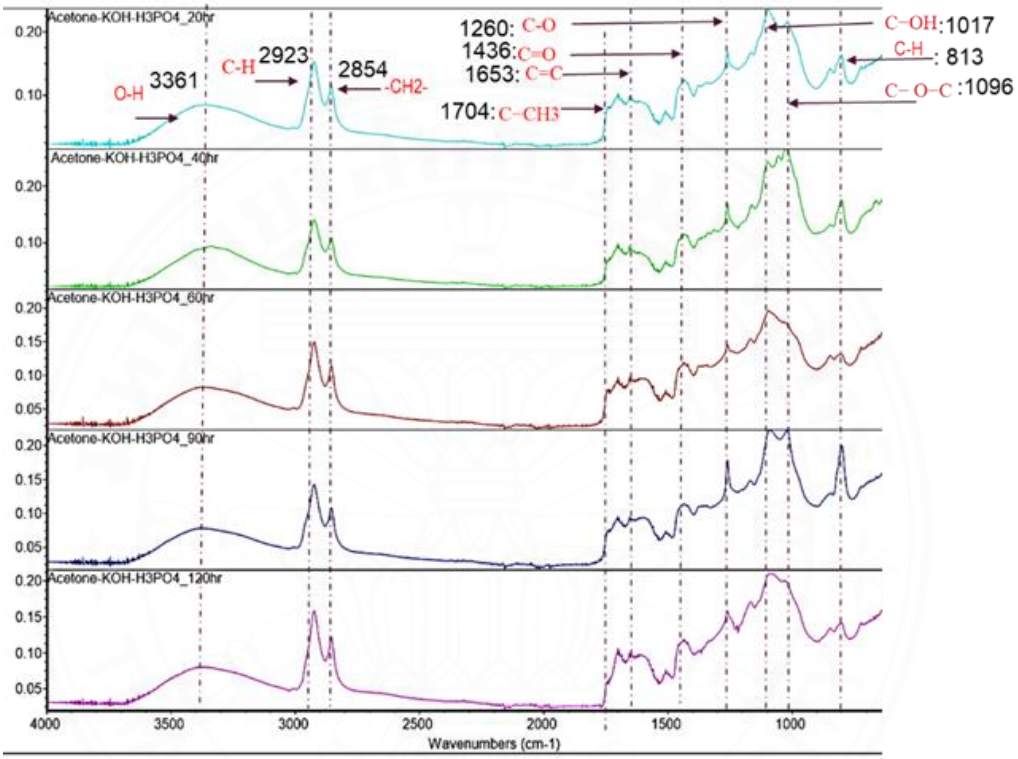
APPENDICES

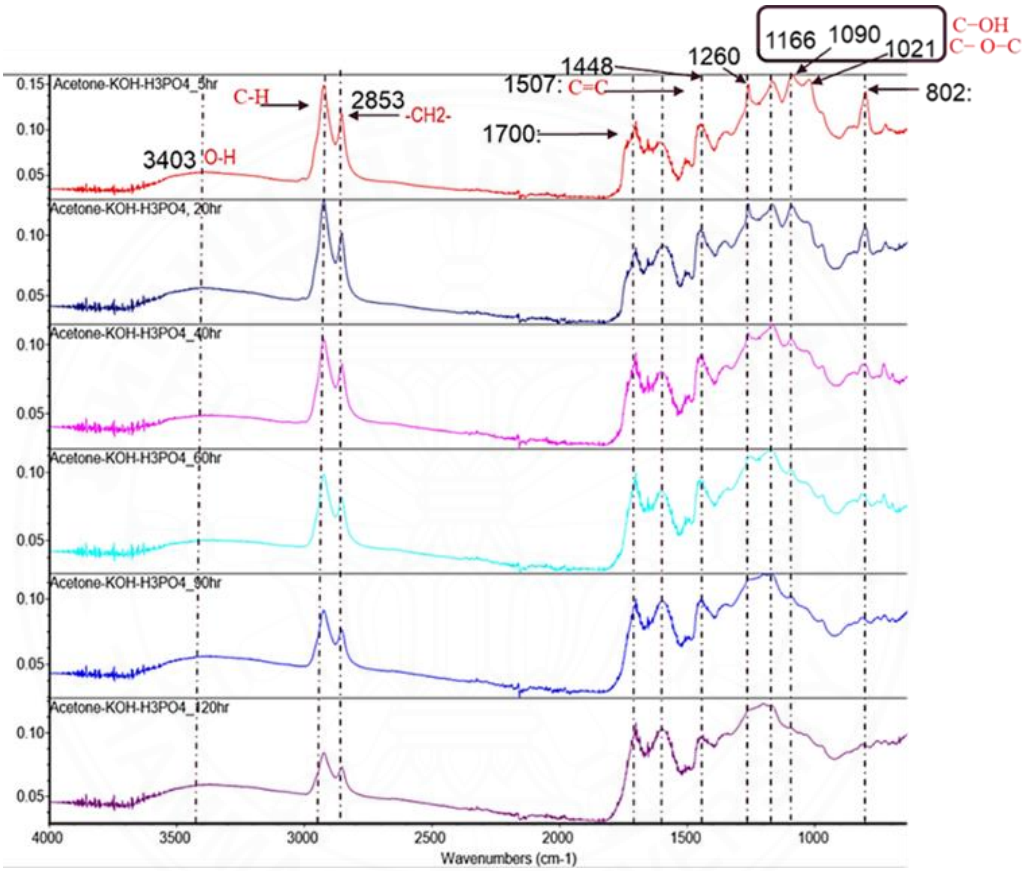
APPENDIX A

TREATMENT SPECTRA OF LYCOPODIUM SPORE IN ACETONE-KOH-H3PO4 AT 60°C IN DIFFERENT HOUR FROM 5H to 120H



APPENDIX B

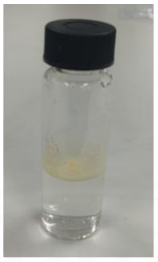
TREATMENT SPECTRA of LYCOPODIUM SPORE in ACETONE-KOH-H3PO4 at 160°C in DIFFERENT HOUR from 20h to 120h



APPENDIX C

WETTABILITY of TREATED SPORES

Natural spore



Acetone treated 6h at 60°C

KOH treated 6h at 60°C

Acid treated 30h at 60°C

Acid treated 40h at 60°C

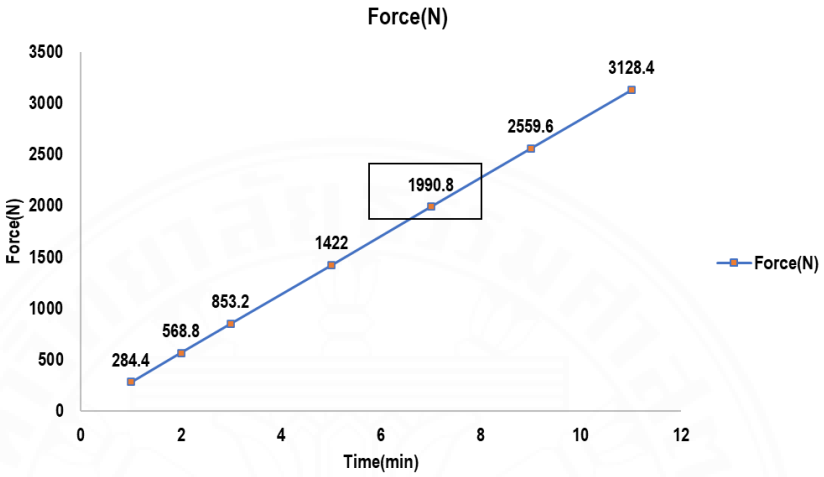
Acid treated 90h at 60°C

Acid treated 120h at 60°C

APPENDIX D

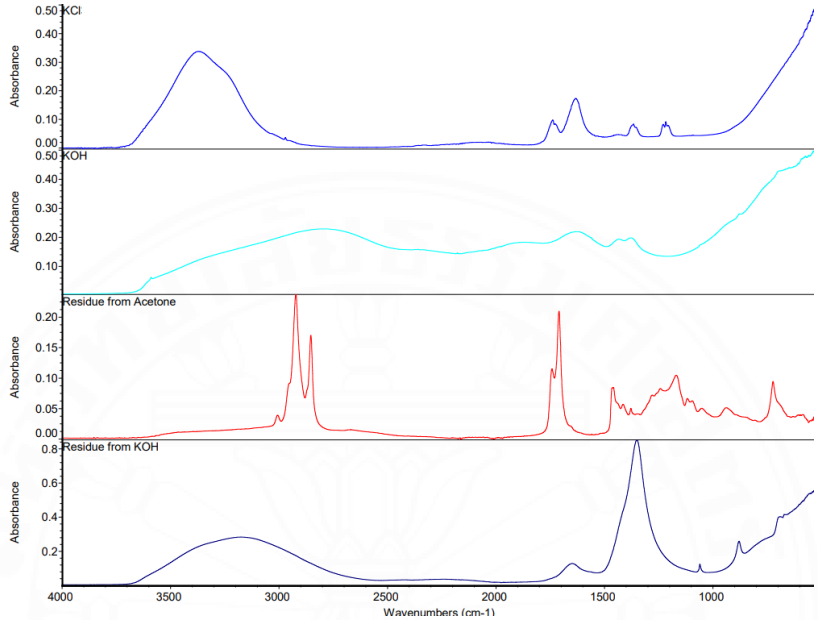
INVESTIGATION FORCE WITH THE TIME PRESSING

T_I (1,2,3,5,7,9,11MIN)



APPENDIX E

FTIR SPECTRA of KCL, KOH, RESIDUE of ACETONE and KOH.



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