PRODUCTION AND CHARACTERIZATION OF CUTINASE FROM TROPICAL FUNGI AND APPLICATION WITH DETERGENT FOR STAIN AND FUZZ REMOVAL FROM SPUN POLYESTER FABRICS

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การผลิตและลักษณะสมบัติของคิวติเนสจากราเขตร้อนและการประยุกต์ร่วมกับสารซักฟอกสำหรับการขจัดคราบและขนจากผ้าสปันพอลิเอสเทอร์

น.ส.ทวีพร สุกใส

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Thesis Title	PRODUCTION AND CHARACTERIZATION OF CUTINASE FROM TROPICAL FUNGI AND APPLICATION WITH DETERGENT FOR STAIN AND FUZZ REMOVAL FROM SPUN POLYESTER FABRICS
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การคัดแยกราจากดินปลูกพืชทางการเกษตรและผิวตัวอย่างพืช ได้ราทั้งสิ้น 44 ไอโซเลต และมี 23 ไอโซเลต ที่สามารถผลิตเอนไซม์คิวติเนสได้ เมื่อนำราที่สร้างวงใสจากการใช้เทคนิค PCL-plate clearing assay ทั้งหมดมาเลี้ยงในอาหารสูตรเกลือแร่ที่มี PCL หรือคิวตินสกัดจากเปลือกมะละกอเป็นแหล่งคาร์บอนมาทำการตรวจสอบแอคติวิตีของคิวติเนส โดยใช้พาราไนโตรฟินิล บิวทาเรต (*p*-NPB) เป็นสับสเตรท พบว่าไอโซเลต PBURU-T5 ให้ค่าแอคติวิตีสูงสุด จึงคัดเลือกไอโซเลตนี้มาจำแนกรานี้ตามลักษณะสัณฐานวิทยา รวมทั้งการศึกษาลำดับนิวคลีโอไทด์ของยีนบริเวณ ITS rDNA สามารถระบุได้ว่าราชนิดนี้คือ *Fusarium falciforme* เมื่อศึกษาถึงภาวะที่เหมาะสมในการผลิตเอนไซม์คิวติเนสจาก *F. falciforme* PBURU-T5 พบว่า pH เริ่มต้นของอาหารเลี้ยงเชื้อคือ 7.0 โดยบ่มที่อุณหภูมิ 30 องศาเซลเซียส ที่ความเร็ว 150 รอบต่อนาที โดยในอาหารเลี้ยงเชื้อใส่คิวตินสกัดจากเปลือกมะละกอความเข้มข้น 2.5 กรัมต่อลิตร เป็นแหล่งคาร์บอนและมีเปปโทนความเข้มข้น 4 กรัมต่อลิตร เป็นแหล่งไนโตรเจน ทำการเก็บเกี่ยวผลผลิตวันที่ 4 ให้แอคติวิตีของเอนไซม์คิวติเนสสูงที่สุดคือ 4±0.15 ยูนิตต่อมิลลิลิตร เมื่อศึกษาสมบัติการทำงานของเอนไซม์คิวติเนสหยาบที่ผลิตได้ พบว่าสามารถทำงานได้ดีที่พีเอช 9.0 อุณหภูมิ 35 องศาเซลเซียส ผลิตเอนไซม์คิวติเนสได้สูงสุด ในการนำคิวติเนสหยาบรูปแบบผงมาประยุกต์ใช้ร่วมกับสารซักฟอกแบบผงสำหรับการขจัดคราบและขนบนผิวผ้าสปันพอลิเอสเทอร์ โดยมีการวัดระดับขุยขน ค่าน้ำหนักของผ้าที่หายไปและการสูญเสียความแข็งแรงด้านแรงดันทะลุ พบว่าผงซักฟอกทางการค้าที่ผสมคิวติเนส สามารถนำมาใช้ซักเพื่อช่วยลดคราบและกำจัดขนผ้าพอลิเอสเทอร์ที่ถักด้วยเส้นด้ายใยสั้นจากขนผ้าระดับ 1 เมื่อผ่านการซักปกติ 8 รอบ ผ้าที่ผ่านการซักมีการลดลงของขนผ้าเป็นระดับ 3-4 นอกจากนี้พบว่าน้ำหนักผ้าที่หายไปลดลงเท่ากับร้อยละ 2.68 และมีค่าการสูญเสียความแข็งแรงของผ้าจากค่าแรงดันทะลุร้อยละ 8 ซึ่งไม่มีผลสำคัญต่อความแข็งแรงของผ้าต่อผู้สวมใส่

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: Fusarium sp., Cutinase, Fabric, Laundry detergent, Pill/Fuzz/Hairs, Spun polyester

Taweeporn Sooksai : PRODUCTION AND CHARACTERIZATION OF CUTINASE FROM TROPICAL FUNGI AND APPLICATION WITH DETERGENT FOR STAIN AND FUZZ REMOVAL FROM SPUN POLYESTER FABRICS. Advisor: Prof. HUNSA PUNNAPAYAK, Ph.D. Co-advisor: Assoc. Prof. SEHANAT PRASONGSUK, Ph.D.,Asst. Prof. USA SANGWATANAROJ, Ph.D.

Fungi were isolated from agricultural soil and plant specimens. Twenty three isolates from 44 fungal isolates were found to produce cutinase by using PCL-plate clearing assay and their cutinase activity were investigated. All the cutinase-producing fungi were cultured in liquid mineral medium (LMM) containing PCL or crude cutin extracted from papaya peels, which was a carbon source for cutinase production. Cutinase activity was assayed base on the hydrolysis of paranitrophenyl butarate (*p*-NPB). It was found that PBURU-T5 gave the highest cutinase activity. The isolate PBURU-T5 was selected for identification based on morphology together with comparative nucleotide sequencing from internal transcribed spacer region of the ribosomal RNA gene (rDNA-ITS) and it was identified as *Fusarium falciforme*. The optimum conditions for the production of cutinase from *F. falciforme* PBURU-T5, showed that the culture medium with initial pH at 7.0, incubation temperature 30 °C at 150 rpm with the optimal medium composition consisting of 2.5 g/L cutin extract from papaya peels as a carbon source, 4 g/L peptone as nitrogen source by harvesting on 4 days of incubation provided the highest cutinase activity of 4±0.15 U/ml. The crude cutinase showed the highest enzyme activity at pH 9.0 and temperature 35 °C. For the detergent application, cutinase enzyme (powder form) from *F. falciforme* PBURU-T5 was mixed with a commercial detergent and was used for oily stain and pill/fuzz removal from spun-polyester fabrics through laundry. Then, the fabric was measured for color values, analyzed for pilling rate, and tested for weight loss and bursting strength loss. When the weight ratio of detergent: enzyme at 1:0.1 was used for fabric washing, oily stain could be completely removed in one washing cycle while fuzzy hair and pill was removed to the acceptable pilling rate of 3-4 (from pilling rate of 1 before washing) in eight washing cycles. After washing, fabric lost 2.68 % of its weight and 8 % of its bursting strength which was not a big concern for polyester wearers.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Rationale and assumptions

Polyethylene terephthalate (PET) fiber is the synthetic fiber that is often used in textile industry (approximately 70%) because of its outstanding properties including high strength, light weight, durability, resistance to wrinkle and abrasion and easy to wash. This polymer is synthesized from terephthalic acid and ethylene glycol monomers as shown in **Figure 1.1**. The market share of this polymer is 31.3% in textile industry and tends to gain more percentage comparing with natural fibers (Yoon et al., 2002).

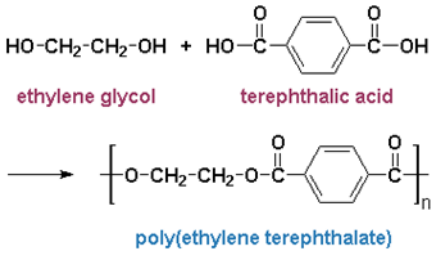


Figure 1.1 Production of Polyethylene terephthalate (PET)

However, the problem from using spun polyester knitted fabrics (made of stable or short fibers) is the pilling or fuzzing on fabric surface which occurs during wear or laundry. These fuzzy hair and pill cause unpleasant appearance. The removal of pilling and fuzzing can be done by physical, chemical and biological techniques. The use of enzymes is one part of biotechnological applications. Several enzymes have been reported to catalyze the removal of polyester fiber on textile surface that resulting to pilling reduction. In 2002, Yoon et al. reported the modification of PET fiber surface with serine esterase resulting in the enhancement of hydrophilicity, wettability and cationic dyeability on polyester fibers. PET treated with a commercial cutinase was found to improve the surface properties and increase hydrophilicity, wettability and dyeability of the PET materials (Donelli et al., 2009). The application of *Arthrobacter* and

Trichosporon PET-hydrolyzing enzymes for the modification of PET fabrics could reduce the pilling and improve oil stain removal that found to change in the physical properties of PET fibers including their tensile strength, viscoelasticity, and extension behavior (Oda and Kimura., 1998; Sato 1983). Cutinase from *Fusarium solani pisi*, *Fusarium oxysporum* and *Thermobifida fusca* are the most frequently studied in enzymatic reaction, structure, properties and its application in PET modification (Carvalho et al., 1988, 1999).

Nowadays, a number of cutinase-producing strains are still being screened and selected for industrial applications, especially in detergent industry, which mostly focus in alkaline ranges and/or the stability pH at the alkaline ranges. The alkali-active cutinase has been reported to produce by several strains of plant pathogens including *F. solini* f. sp. *pisi* (Purdy and Kolattukudy, 1975), *Fusarium roseum* f. sp. *culmorum* (Soliday and Kolattukudy, 1976) and *Rhizoctonia solini* which presented the pH optimum at 8.5 (Trail and Koller, 1990). Nimchua et al. (2008) found the high cutinolytic esterase produced from *Fusarium solani* that was obtained after cultivation in a medium with initial pH 11.0, at 25 °C for 4 days**.** In addition, Lee et al. (2009) established the optimum cutinase treatment conditions for cotton fabrics based on the relative enzyme activities at different pH levels, temperatures, enzyme concentrations and treatment time. The optimal conditions for cotton cuticle hydrolysis by cutinase treatment were found at pH 9.0, 50 °C with 100% on the weight of fiber of cutinase concentration, and treatment duration time of 60 min.

The aims of this study were to isolate alkali-active cutinase-producing fungi in Thailand and determine the properties of the obtained cutinase. The best selected isolate was optimized a culture condition. Finally, this cutinase was added into a commercial powder detergent and the mixed detergent was used for oily stain and fuzz removal from spun polyester knitted fabrics through laundering.

1.2 Objectives of this study

1. To screen and identify the selected isolate fungal that produce alkaline cutinase from different habitats of Thailand.

2. To optimize the cutinase production from the selected isolate.

3. To characterize the produced cutinase.

4. To apply the detergent containing cutinase for oily stain releasing and fuzz removal of spun polyester fabrics.

1.3 Key words

Fusarium sp., cutinase, laundry detergent, spun polyester, fabric, pill/fuzz/hairs

1.4 Anticipated benefits

The new formulation of detergent containing cutinase for oily stain and fuzz removal from polyester fabric would be beneficial for detergent and textile industries.

CHAPTER II

LITERATURE REVIEWS

2.1 Cutin

2.1.1 Cutin/cutan structure

Cutin is a polymer insoluble in all common solvents, which greatly limits the analytical tools available for its characterization, unlike cuticular waxes, which are monomeric lipids soluble in a wide range of non-polar solvents. However, it can be depolymerized into the corresponding monomers by saponification, for example $NaOCH_3$ (Graça et al., 2002) or BF_3-catalyzed (Leide et al., 2011) methanolysis, and its chemical structure can be inferred thereof **(Table 2.1)**.

Table 2.1 Structures of common cutin monomers, typical ranges of composition values, and hypothetical monomer connectivity patterns (Pollard et al., 2008).

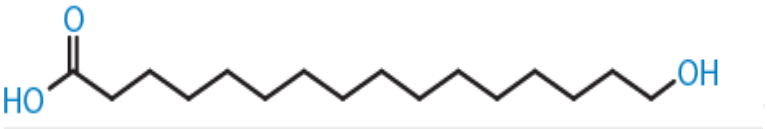
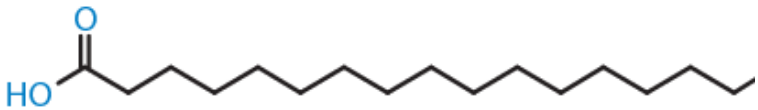
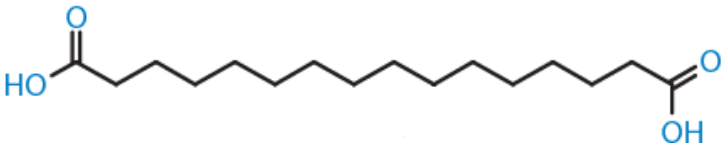
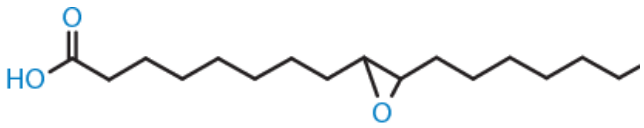
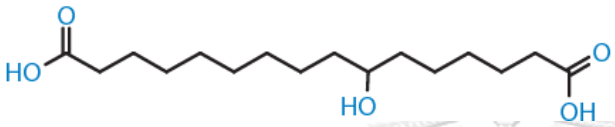
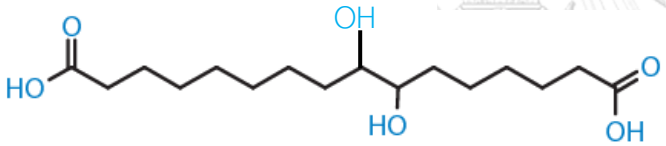
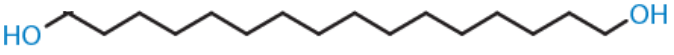
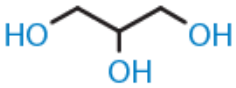
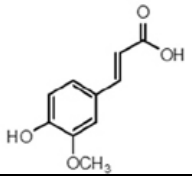
Monomer type	Abundance (%) and common monomers
Unsubstituted fatty acids 	1-25% C16:0 C18:0, C18:1, C18:2
ω- Hydroxy fatty acids 	1-32% C16:0 C18:1, C18:2
α, **ω**- Dycarboxylic acids 	Usually <5% but >50% in Arabidopsis C16:0 C18:0, C18:1, C18:2

Table 2.1 (continued)

Monomer type	Abundance (%) and common monomers
Mid-chain functionalized monomers Epoxy-fatty acids  Polyhydroxy-fatty acids 	0-34% C18:0 (9, 10-epoxy) C18:1 (9, 10-epoxy) 16-92% C16:0 (10, 16-idyhydroxy) C18:0 (9, 10, 18-trihydroxy)
Mid-chain functionalized monomers Polyhydroxy- **α**, **ω**- Dycarboxylic acids 	Traces
Fatty alcohols alken-1-ols and Alkan-1-ols  **α**, **ω**- Alkenediols and **α**, **ω**- Alkanediols 	0-8% C16:0, C18:1 0-5% C18:1
Glycerol 	1-14%
Phenolics 	0-1% Ferulate

Cutin is lipid polymers composing of majority epoxy- and hydroxy-substituted alkanoic acids (Kolattukudy, 1981; Holloway, 1982). Plant cuticle contain from 50% to 90% of cutin (Matzke, 1991; Martin, 1970).

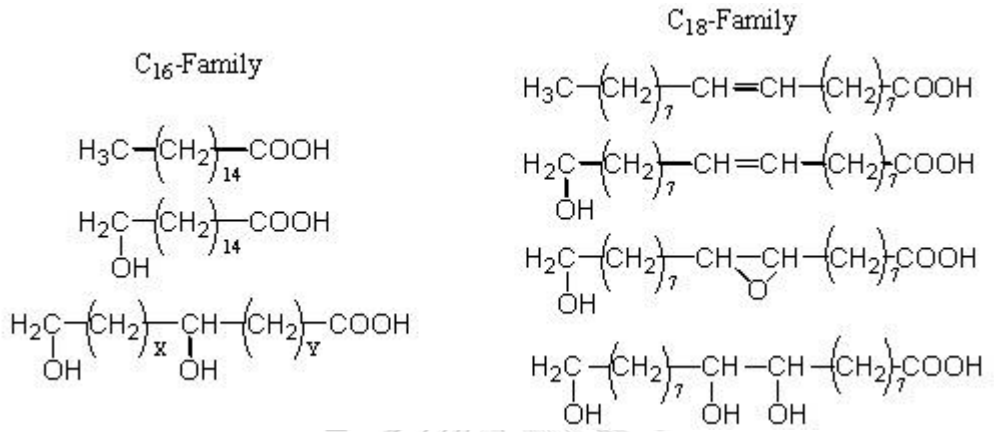


Figure 2.1 Major cutin monomer structures. X= 5, 6, 7, 8; Y= 5, 6, 7, 8, X+Y= 13. Minor monomers such as fatty acids, fatty alcohols, aldehydes, ketones, diacids as well as hydroxycinnamic acids can also be liberated.

The major hydrophobic constituents of the plant cuticle are the insoluble polymers, a mixture of chloroform-soluble intracuticular and epicuticular lipids, and cutan and cutin, collectively called waxes **(Figure 2.1)**. The cuticle is cross-linked to form a three-dimensional amorphous structure when noticed through an electron microscope, although it presents a lamellar ultrastructure in some plants,

Some bryophytes and vascular plants have advanced a cuticle, an outer hydrophobic skin to guards them from the loss of water and acts as a defense barrier toward insects, pathogens, and UV radiation (Baur, 1997; Kersteins, 1996; Buschhaus and Jetter, 2011). The cuticle capable to controls non-stomatal gas exchange. During organogenesis, certain cuticular mutants from the phenotypes suggest that it also acts as function to maintenances the separation of organs. The external surface of the epidermal cell walls of leaves, primary stems, flowers and fruits were covered by the cuticle (Martin and Juniper, 1970).

2.1.2 Isolation of cutin

Interruption glue of the pectinaceous that joins a cuticle in the epidermal layer using chemicals or enzymes is needing to release the cuticular layer. The most generally used, treatment with ammonium oxalate/oxalic acid or degrade pectin using enzymes that was gentle methods (Kolattukudy and Walton, 1973; Holloway, 1982). Physical separation can be releasing the cuticular layer and treatmented with carbohydrate-hydrolyzing enzymes on the subjected could additional to remove carbohydrates. Moreover, the crystalline waxes buried in the polymer matrix cause the diffraction able to reduce by soxhlet extractions for several days slowly. This problem is more significant in the case of the thicker cuticle found in fruits. The final product makes in powder form to easily study for physico-chemical and/or chemical.

2.1.3 Depolymerization of cutin

The chemical can be cleavage the ester bonds and release free monomers or their derivatives from cutin in depolymerize process, depending on the method in chemical cleavage used. furthermore, catalyzing by use the enzymes for the hydrolysis of ester bonds that able be depolymerize.

2.1.4 Degradation of cutin

(a) By Plants

An ultrastructural study raised the occasionally that enzymatic hydrolysis of cutin creates an changing in the cuticle located over the recently differentiated sunken stomata (Carr et al., 1978). Moreover, suspected case of cutin degradated using enzyme of a plant-generated is that catalyzed by a pollen enzyme during the penetration of stigmatic cuticle. The presence of such a cutinase was suggested by the finding that addition of cutin preparation to germinating pollen caused a delicate to enlarge capable to acid titration (Heinen et al., 1961; Linskens et al., 1962). Germinating pollen of nasturtium (*Tropaeolum majus*) was shown shown all types of monomers from labeled cutin was release defecated after cutinase catalyzed the hydrolysis (Shayk et al., 1977). It was a 40 Kda peptide consisting about 7% *N*-

glycosidically-attached carbohydrates and the hydrolysis catalyzing of *p*-nitrophenyl esters of C_2-C_{18} CIS fatty acids with similar V_{max} and Km. The pH optima for the *p*-nitrophenyl esters and the hydrolysis of cutin were 8.0 and 6.8, respectively. This enzyme showed a preference for primary alcohol esters, but triglycerides were not significant rates for hydrolysis. It was severely inhibited by thiol-directed reagents and it was completely oppressive to active serine-directed reagents (Babel and Steinbuchel, 2003).

(b) By Microbes

Many microorganisms, including pathogenic plant, able to grow on cutin as their sole carbon source and produce extracellular enzymes to hydrolyze cutin (Heinen et al., 1966; Shishiyama et al., 1970; Hankin et al., 1971; Purdy et al., 1973; Köller et al., 1991). Two isozymes of cutinase were isolated in homogeneous from the growth medium of *Fusarium solani pisi* (Purdy et al., 1975). The two isozymes were relating nearby in amino acid composition and immunological properties as well as catalytic properties (Purdy et al., 1975; Soliday et al., 1976). The only difference between the two proteins is that cutinase II contained what appeared to be a proteolytic nick near the middle of the polypeptide. More recently, some cutinase producing microorganisms studied clearly are given in the **Table 2.2**.

Cutin was Inducted to synthesis cutinase or cutin hydrolysate was accompanied all the time by the production of very small quantities of a nonspecific esterase which catalyzed hydrolysis of a variety of small esters, although the cutin hydrolysis or the cutin generate oligomers by cutinase was not effect (Purdy et al., 1975; Soliday et al., 1976). This hydrolysis of *p*-nitrophenyl esters of C_2 - C_{18} fatty acids was catalyzed by cutinase.

Table 2.2 Cutinase producing microorganisms

Source	Genus	Species	References
Bacteria	*Streptomyces*	*scabies*	Lin et al. (1980)
		acidiscabies	Fett et al. (1992)
		badius	Fett et al. (1992)
	Pseudomonas	*putida*	Sebastian et al. (1988)
		putida	Sebastian et al. (1987)
		mendocina	Kim et al. (2003)
		aeruginosa	Fett et al. (1992)
	Thermomonospora	*fusca*	Fett et al. (1999)
	Thermoactinomyces	*vulgaris*	Fett et al. (2000)
Fungi	*Fusarium*	*Solani pisi*	Purdy et al. (1975)
		oxysporium	Pio et al. (2007)
		roseum culmorum	Goncalves et al. (1996)
	Colletotrichum	*kahawae*	Chen et al. (2007)
		gloeosporioides	Chen et al. (2007)
	Monilinia	*fructicola*	Wang et al. (2002)
	Venturia	*inaequalis*	Trail et al. (1990)
	Alternaria	*brassicicola*	Trail et al. (1993)
	Phytopthora	*cactorum*	Egmond et al. (1997)

2.2 Enzyme Cutinase

2.2.1 Cutinases

Cutinase (EC 3.1.1.74) are inducible extracellular, many phytopathogenic bacteria and fungi which can hydrolyse ester bonds in the polymer of cutin able to produce this enzymes (Purdy and Kolattukudy, 1975). Cutinases also a member of a class of serine hydrolases that cleavage the most variety of synthetic esters and show activity towards long and short chains of emulsified triacylglycerols as well as lipases of pancreas (Martinez et al., 1989). Cutinases display little, or do not display, interfacial activation, the activity of which is mostly expanded in the presence of a lipid-water interface, being active on both emulsified soluble and triglycerides, Against to lipases, (Verger and Haas, 1976).

2.2.2 Structure of cutinase

Genes of cutinase were primarily identified by observing the effect of their elimination upon the virulence of the expressing organism. Confident microorganisms survive on their sole carbon source by degrade cutin, while producing extracellular cutinolytic enzymes, and several cutinases was done to isolate and characterize from different sources, from bacteria, like *Thermobifida fusca* (Moser et al., 2008) and from a variety of both nonpathogenic and pathogenic fungi, along with the yeast *Cryptococcus* (Masaki et al., 2005).

A 197-residue protein in a compact one domain molecule was define as cutinase. A molecular weight this cutinase enzyme have around 22,000 daltons with highly preserved stretches, including two disulfide bridges forming, four invariant cysteines.

Cutinase produce from *F. solani* f.sp. *pisi* has an isoelectric point of 7.8 (Sebastiao et al., 1997). Cutinase belong to the ε/β-hydrolase and has a central β-sheet surrounding with five parallel strands covered by two, three and four helices on either side of the sheet. The stretch Gly-Tyr-Ser-Gln-Gly consisting the active site Ser120, has more robust homology with sequence accordding Gly-(Tyr or His)-Ser-X-Gly normally found in lipases. The catalytic triad Ser120, Asp175 and His188, is accessible to the solvent. It is indicated the protein ellipsoid with one extremity and surrounded with the loop 80-87 and surrounded more hydrophobic loop 180-188 too (Jelsch et al., 1998). Until now, cutinase enzyme about 40 X-ray structures and inhibitor conjugates and its mutants have been solved there active site (Martinez et al., 1992; Martinez et al., 1993; Martinez et al., 1994; Longhi et al., 1996; Longhi et al., 1997a; Longhi et al., 1997b; Jelsch et al., 1998; Prangé et al., 1998).

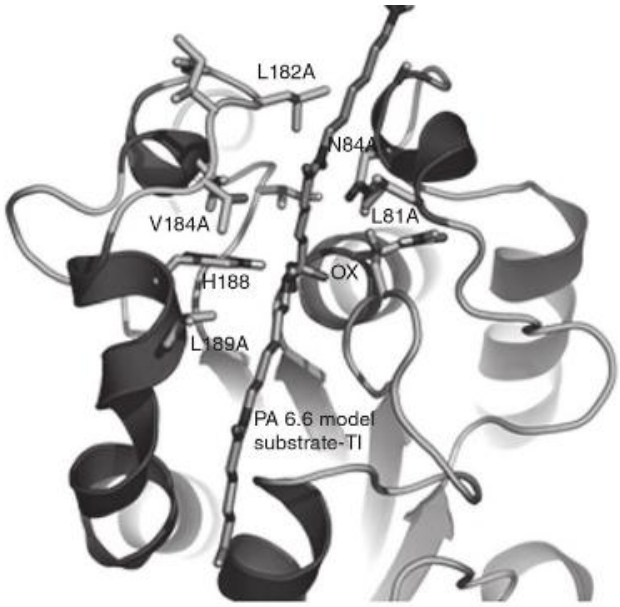


Figure 2.2 Detail of the active site x-ray structure of cutinase with the energy minimized structure of the tetrahedral intermediate (TI) PA 6.6 model substrate.The catalytic histidine (H188) and oxyanion-hole (OX) are shown. Residues mutated in this study are labelled as: L81A, N84A, L182A, V184A and L189A (Araujo et al., 2007).

The structure and function of cutinase are pro studied and previously to improve their properties for some applications like remove fat stain by detergents by using genetic engineering (carvalho et al., 1999; Longhi and Cambillau, 1999; Egmond and de Vlieg, 2000). Site-directed mutagenesis of cutinase produce from wild-type *Fusarium solani pisi* was carried out to expanse the active site in order to better adjust the synthetic substrate. Some cutinase mutants obtain by an enlarged active site were receiving present higher activity nears polyamide substrate than cutinase produce from wild-type (Araujo et al., 2007) in **Figure 2.2** it shows the single exchanges in amino acids of a *Fusarium solani* cutinase.

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2.2.3 Cutinases and their potential applications

Cutinases are a group of versatile enzymes, showing some useful properties for application in products and industrial processes. In recent years, the stereolytic activity of cutinase has been wide explored. An enzymatic preparation consisting

cutinase was developed in order to improve the pharmacological effect of agricultural chemicals, taking advantage of its *in vitro* cutinase activity. These enzymes also present great potential in the management of residues from fruits and vegetables. Apple and tomato skin and orange peel are used, through cutin-catalyzed hydrolysis, in the production of important industrial chemicals, for example ricinoleic acid, whose main source is mamone oil (Carvalho et al., 1997). Cutinases hydrolyze, *in vitro*, the wide array of esters, ranging from soluble synthetic (like *p*-nitrophenyl ester), insoluble long-chain triglycerides (like triolein and tricaprilin), to emulsified triacylglycerols (Egmond, 2000).

Cutinases can also be used in the synthesis of structured polymers, triglycerides and surfactants, in many creations of personal care products, and several processes in agrochemicals and pharmaceutical chemistry compounds consisting one or more chiral centers (Macedo and Pio, 2005). The ingredient of dishwashing and laundry detergents, cutinase has been used as well as a lipolytic enzyme.

2.3 Textile

2.3.1 Polymer formation

PET (polyethylene terephthalate) is industrially produced and is a condensation polymer from either dimethyl terephthalate or terephthalic acid and ethylene glycol (**Figure 2.3**) shown as follows:

(a) Terephthalic acid (TPA), direct created from p-xylene with bromide-controlled oxidation.

(b) Dimethyl terephthalate (DMT), produce from primary stages by esterification of methyl alcohol and terephthalic acid. nevertheless, a different method including two oxidation and esterification stages now considers for many DMT.

(c) Ethylene glycol (EG) the early generated from ethylene oxide as an intermediate product through oxidation reaction at high temperature. Additionally, ethylene glycol is received by reaction of ethylene oxide with water.

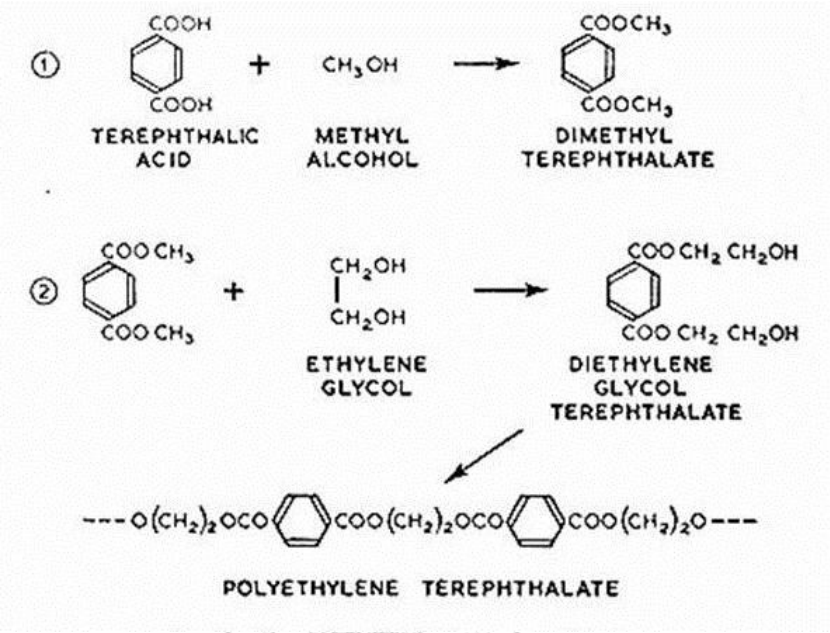


Figure 2.3 Production of polyethylene terephthalate (Rosalind, 1989)

2.3.2 Types of polyester

The polyester fibers are generally available in two varieties PCDT (poly-1, 4-cyclohexylene-dimethylene terephthalate) and PET (polyethylene terephthalate). PET is the popular general production. Although PCDT has more elasticity and resilience, but PET stronger. PET can be used blended or alone with several fibers to producing stain resistant and wrinkle free clothing that can keep its form. PCDT is more appropriate for heavier applications, like furniture coverings and draperies.

2.3.3 Microscopic properties

Polyester fibers are produced in a diversity of cross section including trilobal, pentalobal and round shape. Observe through the microscope, round fibers appear as long, smooth rods with spots of pigment (**Figure 2.4**).

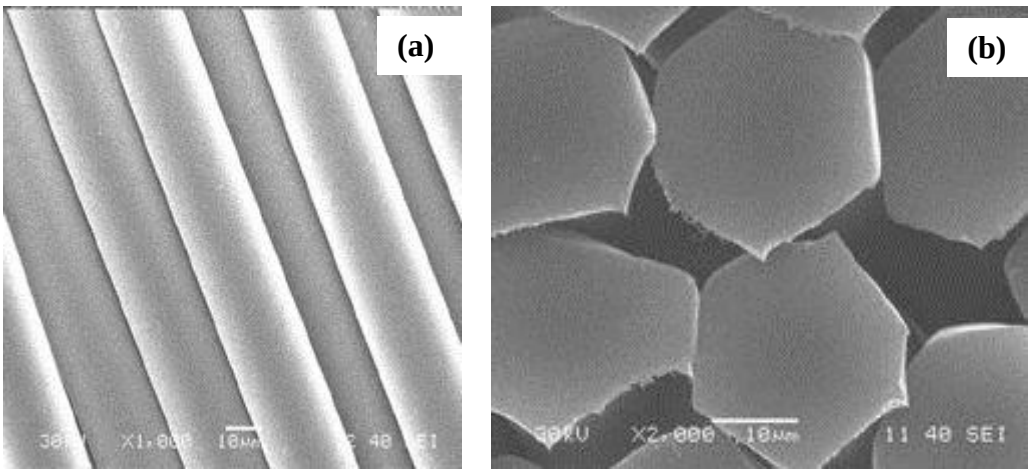


Figure 2.4 Photomicrographs of regular polyester fiber **(a)** longitudinal view and **(b)** cross-section (Wang et al.a, 2013).

2.3.4 Man-made fibers production worldwide

In 1980, The requirement of polyester was only 5.2 million tons around the world and it had up to 19.2 million tons, by 2000. In 2014, importune increased at 46.1 million tons. During 2004-2011, the PET production in the global reached up from 11.3 million tons to around 18.6 million tons, over 7% registering an average annual growth. In 2012, it exceeded the 19.8 million tons mark. In the same year, Asia accounted for the largest share of the world's total PET production volume, harvesting more than 9.5 million tons of the polyester material. Top five countries of the globe's PET manufacturing were USA, China, Mexico, Taiwan and South Korea, with a combined share of closely 55% of the overall production; their production volume was approximately at over 10.7 million tons in 2012 (Alasdair, 2014).

The greatest widely used polymers in textile industry are polyamide (PA), polyacrylonitrile (PAN) and Polyethylene terephthalate (PET). To suppose that the filament fiber utilization is 50% of that of staple, the total PET consumption only in the US nonwovens industry would be over 450 million pounds. To date, polyester has become the most widely used polymer in the nonwovens industry since 1995. Secondary, the widely used was polypropylene (Harrison, 1997). Synthetic

fibers in common have a market share of 54.4% in textile industry and lean to obtain even more distribute compared to natural fibers (Gashti et al., 2011).

Synthetic fibers of polyester are hydrophobic, which is acceptable for lightweight facing fabrics used in the inducible industry. The cost of polyester is low compare to rayon. China Resources Packaging, Far Eastern Textile Ltd., JBF Industries Ltd, DAK Americas, M&G Group, Indorama Ventures PCL, Jiangsu Sanfangxiang Group Co, Nan Ya Plastics Corp, SINOPEC, Octal Holding & Co, and Shahid Tondgooyan Petrochemical Co, Lotte Chemical, are among the major companies employed in the world PET industry (Elamri et al., 2017).

2.4 Problems of polyester fabric

2.4.1 Pilling

Pilling is a property of any synthetic fiber. Fabrics consisting fibers example acrylic, nylon, and polyester have a trend to pill. Process from frequently wear and wash causes being abraded surface of the fibers to unwind and the loose ends ball up on the fabric. A ball of fuzz/hairs is so called “pill” in the textile trade. Pilling is a characteristic which depends on some factors example fiber dimensions, fiber blends or types, type of yarn and their characteristics, chemical processing and fabric construction. These pretend clothe quality since they result in an undesirable fabric form. The pilling rate of fabric propensity is evaluated by comparing the tested specimens with usual standards with range of pill ratings. The observed resistance to pilling is reported on a foresight scale from rates 5 (no pilling) to 1 (very severe pilling).

Fabric made from wool, cotton, and rayon rarely show fuzz/hairs problems since the anchor fibers are easily split and fuzz fall from the fabric soon after they are occurred. But polyester fabric, however, the stronger anchor fibers are hardly split and the hairs that are formed are not easy to release from the surface fabric, leading to feature problems. Fabric and yarn expression a main role play for pilling. Tighter constructions show fewer problems than do looser constructions. Preventing fuzz/pills on a fabric surface is important to keep up customer fulfillment (Daniel and Mead, 1959).

2.4.2 Remove pilling from clothes

2.4.2.1 Physical process technic

a) Shearing/Cropping

This machine used to reduce the length of the fibers. It gives a much superior handle to singeing (Krishnaveni et al., 2011).

b) Singeing

Singeing with shearing/cropping treatment with heat setting or burn off it to minimize pill/fuzz. The protruding fibers from the surface of fabric or yarn was burn off. After fuzz removal the results show the smooth surface of fabric and improve the reflects light. (Krishnaveni et al., 2011).

c) Brushing

Highly twisted yarn was made from tighter-woven fabric, the surface of fabric easy loose fibers where it can be removed by a close shearing, brought to by means of brushing. That a procedure is impossible on loosely spun yarns with a downy surface (Krishnaveni et al., 2011).

d) Anti-pilling finish

Pilling/fuzz problem, an undesirable characteristic companioned with spun yarn fabrics, particularly when they consist synthetics. Manmade fibers are more easily lead to the surface of a fabric owing to their smooth surface and cause more higher abrasion resistance and tensile strength. In case knit "picking" able be occurs: by abrasion, unique fibers work themselves out of yarn loops onto the surface, and the garment hooks on a objected or rough point (Krishnaveni et al., 2011).

e) Thermosetting

Heat-setting give to produce smooth fibers or yarn laying down the individual surface fibers. Additionally, it able to improve the fabric body and surface of polyester fibers provide slightly stiffer to benefits of smooth fabric surface ad flat (Krishnaveni et al., 2011).

f) Heat Setting

Synthetic fabrics take out the primary tensions with the generation of the fiber during produce by heat setting process and the new state was fastened with rapidly cooling. This heat setting fixes the fabrics in the relaxed state and thus avoids subsequent creasing or shrinkage of fabric. Heat setting can be apply using hot air, on a pin stenter amount 210 ℃ for 15 - 25 sec for polyester goods and at a lower temperature range of 180 – 230 ℃ for 20 - 25 sec for polyamides. Acrylics may be heat set partially at 165 – 185 ℃ for 20 - 65 sec to decrease evalution of running creases, should be avoided to yellow occurring when use higher temperature. It can be included with optical or dyeing brightening (Krishnaveni et al., 2011).

g) Steam setting

Saturated or super-heated steam using for steam setting. Processing of steam, to ensure uniform treatment by starting the sequence of alternate short steaming and apply with vacuum for 25 - 35 min temperature range of 125-135 ℃ under pressure. Stenters and setting time is 26% faster than for hot air because of quicker heating up rate was used in super-heated steam. Heat set the material before start, to remove lubricants, sizing agents, spin preparations and impurities as these are probably to be burned in drying heat setting making their difficult to remove should be washed. Steaming at temperatures more than 100 ℃ (normally 125 ℃ or 135 ℃) able to decrease the hairs tendency because of the coefficient of friction was increased (Krishnaveni et al., 2011).

2.4.2.2 Chemical process technic

The several chemical finishing accesses have been made to prevent fuzz/pills from storing on surface of the fabric by the following:

a) Padding and coating techniques by polymers apply

Treatment by amine capable to combined with other operations of textile finishing, like Softeners, dyeing. internal lubrication was decrease fiber to fiber friction and receive smoothness in fabric, like amino functional polysiloxanes and non-ionic organo-modified silicone micro emulsions.

b) Anti-static finish

The fabric sticking with anti-static finish able to prevents dust. Anti-static effective chemicals are largely chemically inert and require heat treatment or thermasol for attaching on fabrics of polyester. Anti-static finishes could be adapted in fabrics of polyamide, being treatable at average temperatures.

2.4.2.3 Special treatments technic

Controlling the fuzz/hairs in fabrics and fibers could be treatments using the special machine.

a) Fabric shaver or electric piller

The benefit of electric piller or fabric shavers are easy to pill removal on a flat knit, but they may not appropriate applied on knits with textures, naps or highly surfaces.

b) Knit picker

In snags or loose ends of yarn could be eliminate using knit picker. It is a very small latch hook that grabs the snag, therefore pulls it through to the opposite side and it's fastened.

c) Sanforising

Sanforising can be improve the rate of pill. There are two possible demonstrations for improve the rate of pill of the fabric after passed this process. Firstly, during sanforising, friction between the steel drum and the rubber blanket, make an effort the fabric yarns to close-knit, possibly causes some weakening of the fabric surface fibres. Secondly, after fabric shrinkage as a result of sanforising, the number of fibres and yarns per unit area enlarge, expand the possibility of the formation and presence of hairs per unit area of the fabric, leading to a poor the rate of pill.

d) Treatment with UV

UV irradiation is a modern method, it applies to reduce pill from knitted fabrics using a mild wet oxidation treatment, such as salts of permonosulphuric acid or hydrogen peroxide. This method used radiation to create anti-hairs fabric significantly affect the loss of strength cause using hydrogen peroxide as a photo initiator to absorb UV radiation. After treatment, the surface of fibers is much weaker, no anchor fibers are capable to fix the hairs on surface of the fabric. The treatment which a highly effective using method of preventing pilling in wool knitwear by chlorine-free.

2.4.2.4 Application of enzymes (bio finish)

Process of bio-polishing object to remove the small fiber ends protruding from surface of the yarn, then rid of the fuzz or hairs from the fabrics. The enzyme hydrolyses to weakens the protruding fibers to the extent that a little force of physical abrasion that enough to remove and break them. During wet processing, bio-polishing can be reduced at any time but is great convenient showed after bleaching. This bio-polish able to apply in both batch or continuous processes. Although, continuous processes have to several incubation time to take place for degraded by enzyme. The pilling removal makes the color brighter, more obvious, and reduces pilling of the fabric texture.

2.5 Detergent

Detergents are mixture of compounds, most of compound is a surfactant that possess both a hydrophobic and a hydrophilic group that used to break up and removal all stain from surfaces (salager, 2002; jonhson, 2007).

2.5.1 Ingredient in laundry detergent

(a) Surfactant

Surfactants are likely the largest contributors to the efficiency of laundry detergent. Surfactants of anionic are separated in water in an amphiphilic anion, and a general an alkaline metal (Na^+, K^+) which is a cation, and a quaternary ammonium.

They are the most normally surfactants using. Including with alkylbenzene sulfonates (detergents), di-alkyl sulfosuccinate (wetting agent), lauryl sulfate (foaming agent), lignosulfonates (dispersants Non-ionic Detergents) and soaps (fatty acid).

Non-ionic detergents characteristic uncharged cause a hydrophilic head group and They are appropriate broken lipid-protein and lipid-lipid interactions. They are frequently mean to as non-denaturing detergents and are used to separate membrane proteins of active biological and have commonly do not break protein-protein interactions.

Cationic Surfactants are separated in water into an anion, the positive charge makes them useful in anti-static products, like fabric softeners and an amphiphilic cation.

Surfactants of amphoteric or zwitterionic have a both positive and negative on their hydrophilic end, called a twin charge. The positive charge can be variable and sulfonates including , as in the sultaines CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate). The negative charge is based on primary, secondary, or tertiary amines or quaternary ammonium cations. Amphoteric surfactants are often used in personal care products.

Surfactants of zwitterionic are frequent respond to pH and do behave as positive charge or negative charge based on pH. A phosphate anion with an amine or ammonium was the normal use for biological zwitterionic surfactants.

(b) Bleach

Bleach which imply a chemical agent used to whiten or remove color from textiles, to make whiter or lighter in color. A famous common bleaching agent is the system of hydrogen peroxide.

(c) Peracids

The efficient to use formula space consists of paracids including in formulations show a benefit, bleaching carry out at lower temperatures and productive bleaching on pH of washing arrangement.

(d) Bleach activators

The form of peracids with reaction of hydrogen peroxide which are molecules of bleach activators, mean to as perhydrolysis, *in situ* during washing process.

(e) Metal catalysis

To date, a many research publication which objects at the improvement of metal-based catalysts for hydrogen peroxide. Normal formulation deliberations the bleach element must be sufficiency constant in the formulation, comparable with other detergent components, stable enough through washing, have to additives presenting for example the chelators, to washing machines and non-damaging to garment.

(f) Enzymes

The enzymes mixed detergents used to help in cleaning textiles and depends on many factors, such as, arrange of wash pH, temperature of washing and the weight ratio of detergent to enzyme, differences source of stains to remove and water hardness.

(g) Builders

The advantage of formulation with builders can still in rule be moderately wide in phosphate-free detergent wash pH, temperature, development of stain removal belonging to type of stains and development on maintenance of whiteness.

(f) Chelating agent

Chelators is a chemical component which mostly typically polyvalent molecules, most commonly have two or more binding sites, usually either aminocarboxylates or aminophosphonates.

(h) Polymers

The performance of polymers to be ingredient in detergency can still is a popular research part, every year sustainable increased because of polymers have a many type that provide functional used.

(i) Others

-fluorescents agents whitening

Fluorescent agents could be adsorbing UV light after that emit visible (blue-violet) light result to optical brighteners.

-suds suppressors

Suds suppressors great normal to be granular detergent particles which consist of silicone oil and silica particles including and other polymeric/ hydrophobic components. The surfactant film at the air/liquid interface was disrupted by which collapse of the air bubbles.

- Buffer, stabilizers and solvents

Detergent in liquid form, solvents of organic are most frequently had to complement water as backbone of the formula, and to confirm that procedure is homogeneous, fluid and stable cross a range of storage conditions.

-Perfume

Perfume are most often need to add in formulation of detergent, thereby make a pleasant to customers from the odor of the laundry detergents.

- Dyes, Electrolytes and Salts

Ingredients of detergent products may mix with other compositions for example water-soluble salts, such as sodium lauryl sulphate or other electrolytes.

2.6 Enzymes in detergents

The use of enzymes in detergents has been the biggest of all enzyme applications. Enzymes are now well accepted as compositions in laundry detergents, for propose to stain removers or laundry pre-spotters. Detergent enzymes amount 20% of total worldwide enzyme production and represent one of the largest and most successful applications of modern industrial biotechnology (Valls et al., 2011).

2.6.1 Proteases

Proteases are the common popularity used enzymes in the detergent industry. They stain removal in group of protein such as grass, blood, mucus, faces, egg and human sweat. These organic stains have a trendy to adhere strongly to textile fibers. The proteins act as glues, preventing the waterborne detergent systems from removing several of the other components of the soiling, such as pigments and street dirt.

The incapability of detergents no added enzymes for proteins removal can result in permanent stains due to denaturing and oxidation caused by drying and bleaching. Like, if not remove blood stain before bleaching will leaved a rustcolored spot.

Proteases degrade proteins and cut them out into free amino acids or more soluble polypeptides (Vojcic et al., 2015).

2.6.2 Lipases

Lipases can hydrolyse triglycerides stains into more hydrophilic and thus easier to remove substances. This applies particularly to materials made up of a blend of cotton and polyester. The lipase is capable of removing fatty stains such as fats, butter, salad dressing, lead, chocolate, sauces, lipstick and the tough stains on collars and cuffs (Hasan et al., 2010). Some lipases combined detergents have been developed and being a main market share for enzyme producers (Novozymes, Genencor, Gist-Brocades, Amano, Showa Denko, etc.).

2.6.3 Amylases

A type of stains to remove by amylase come from residues of starch-based foods, like, baby food, custards, gravies, potatoes, spaghetti, pasta and chocolate. Amylases enzyme capable used in liquid or powder detergent as well as in dishwashing detergent (Souza, 2010).

2.6.4 Cellulases

Improvement of enzymes mixed detergent has mainly emphasized on enzymes able to remove the stains (Sukumaran et al., 2005). Although, cellulase has properties empowering structure modifying on cellulose fiber on cotton blends and cotton. After added cellulase in a detergent, it presents the following effect:

Color brightening-When cotton blends or cotton cloths have been washed more many times, they tend to get a 'fluffy' look and the colors become duller. This effect is owing to the formation of microfibrils that become partly detached from the main fibers. The light falling on the garment is reflected back to a greater extent giving the impression that the color is duller. Although, the cellulase capable degraded these fibrils, the surface of the fiber return smoothness and its original color return to the cloth.

2.7 Cutinases modifying polyester fabric

Cutinase is a group enzyme of hydrolase which able of deducting the cutin component of the cuticle. It is known that cutinases can catalyze the hydrolysis of ester bonds in cutin and attack ester bonds in some aromatic polyesters such as polyethylene terephthalate (PET) (Yoon et al., 2002). as well as aliphatic polyester which is polycaprolactone (PCL) (Murphy et al., 1996)

Some reports showed that the cutinase produced from *Fusarium solani pisi* has potential use for cotton cuticle degradation and exhibits a good synergistic effect with pectinase, an enzyme utilized to degrade pectin, in the scouring of cotton fiber (Carvahal et al., 1999; Degani et al., 2002; Gupta et al., 2008; Zhang et al., 2011). Furthermore, the specific amino acid residues near the active site of cutinase replace by perform the site-directed mutagenesis (Araujo et al., 2007) close to polyesters for its hydrolytic activity improvement. More research, a bacteria cutinase from the *Thermobifida fusca*, which a thermophilic has been identified and overexpressed in *Escherichia coli* in these laboratory (Moser et al., 2008). Because of it has temperature stability and alkali resistance from recombinant *T. fusca* cutinase bring it potentially capable of being treat textile bioscouring.

Cutinases capable to hydrolyse a broad type of synthetic esters and triacylglycerols, as well as lipases, without performing interfacial activation (Martinez et al., 1992; Egmond and Bemmel, 1997; Baker et al., 2012). Therefore, cutinases were appropriate for adaptation in the laundry detergents, dishwashing industry for fats and oils stain removal, in the synthesis of structured agrochemicals and polymers, plastics degradation and triglycerides (Flipsen et al., 1998; Carvalho et al., 1999; Dutta and Dasu, 2011; Chen et al., 2013; Yang et al., 2013; Ping et al., 2017).

Cutinases which may be esterases or lipases also called polyesterases, , have been reported by Yoon et al. (2002), they apply enzyme to modify surface of PET and polytrimethylene terephthalate (PTT). The resulted shown that enzymatic treatment have significant for depilling, efficient desizing, increased hydrophilicity and reactivity with cationic dyes and also helped oily stain removal.

The advantage of using cutinase make a new chance for emphasis on enzyme improve functionalization of PA and PET surface, polymers formerly determined as being resistant to biodegradation. Nechwatal et al. (2006) have been tested several commercial lipases and esterases for their ability to hydrolyze oligomers formed during manufacture of PET. These low-molecular-weight molecules are insoluble in water and can deposit themselves onto the dye apparatus, resulting in damage. The authors found that *Triticum aestivum* lipase removed amount 80 %wt of oligomers from the liquor bath treatment, but the noticed reduced appears to be more related to adsorption of oligomers on the enzyme than with catalytic hydrolysis of ester groups (Nechwatal et al., 2006).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and Equipment

- Autoclave: Ta Chang Medical Instrument Factory, Taipei, Taiwan
- Autopipette: Pipetman, Gilson, Villiers, France
- Bushing strength tester, Yasuda, Mullen type
- Centrifuge, refrigerated centrifuge: Model universal 32R, Hettich, Burladingen, Germany
- Centrifuge, microcentrifuge: Model Denville 260D, Denville Scientific Inc., Metuchen, NJ, USA
- Color Assessment Cabinet (VeriVide CAC 60)
- Colorimeter Macbeth Color-Eye 7000
- Electrophoresis unit: Model Mini-Protean II Cell, Bio-Rad, CA, USA
- E.Z.N.A. ® Gel Extraction Kit D2500-01, Omega Bio-tek Inc., USA
- Fluorescence spectrophotometer: Model LS 55, Perkin Elmer Inc., Vienna, Austria
- Fourier-Transform Infrared spectrometer (FT-IR)
- Glasswares
- Hot plate boiler and stirrer: Model C-MAC HS10, IKA, NC, USA
- ICI Pilling/Snagging box tester, Union TSL Limited
- Incubator: Model KT115, Binder, Tuttlingen, Germany
- Incubator Shaker: New Brunswick Scientific Co., Edison, NJ, USA
- Infrared Moisture determination balance, And-4715, Goettingen, Germany
- Laminar flow: Model BV 123, ISSaC, Bangkok, Thailand
- Light microscope: Model BX51. E for L international Co. Ltd.
- Laundering machine, Gyrowash (James H. Heal& Co., Ltd, England)
- Macbeth reflectance spectrophotometer, COLOR-EYE® 7000
- Microcentrifuge: Model Denville 260D, Denville Scientific, NJ, USA
- Microplate reader, Opsysmr, Bio-Active Co. Ltd.

- Peristaltic pump: Model EP-1 Econo Pump, Bio-Rad, CA, USA
- pH meter: Model Tolado S220, Mettler Tolado (Thailand) Ltd.
- PCR machine: PTC-100^{TM} Peltier Thermal Controller cycler MJ Research Inc., USA
- Spectrophotometer: Model UV-2800, Unico, DaytDn, NJ, USA
- Stainless steel balls
- Ultracentrifuge: Model Optima XL-100K, Beckman, CA, USA
- Vortex mixture: Model Genie 2, Scientific industries, NY, USA
- Water bath: Model Clifton NE5-28D, Fisher Scientific, Leicestershire, UK
- Weigh balance, 2 digits: Model Bl610, Sartorius, Goettingen, Germany
- Weigh balance, 4 digits: Model TC-205, Denver Instrument Company, Denver, CO, USA

3.2 Chemicals

- Acetic acid: Ajex Finechem, Auckland, New Zealand
- Alpha-naphthyl butyrate: Sigma-Aldrich Inc., Milwaukee, WI, USA
- Alpha-naphthyl acetate: Sigma-Aldrich Inc., Milwaukee, WI, USA
- Ammonium sulfate: Ajex Finechem, Auckland, New Zealand
- Bacto peptone: Ajex Finechem, Auckland, New Zealand
- Bovine serum albumin-(BSA): Ajex Finechem, Auckland, New Zealand
- Calcium chloride dihydrate: Ajex Finechem, Auckland, New Zealand
- Chloroform: Carlo Erba, Milano, Italy
- Copper sulfate: Carlo Erba, Milano, Italy
- Ethanol absolute: Merck, Darmstadt, Germany
- Ethylene diamine tetra-acetic acid (EDTA): Ajex Finechem, Auckland, New Zealand
- Ferrous sulfate: Fluka, Buchs SG, Switzerland
- Gel filtration LMW Calibration Kit: GE Healthcare, Uppsala, Sweden
- Hexadecyltrimethyl ammonium bromide (CTAB): Sigma-Aldrich Inc., Milwaukee, WI, USA
- Hydrochloric acid: Fisher Scientific, Leicestershire, UK
- Isopropanol: Fisher Scientific, LE, UK
- Lactophenol cotton blue: Fluka, Buchs SG, Switzerland

- Peptone: Himedia, Mumbai, India
- Phenol: Merck, Darmstadt, Germany
- Phenolpnthalein: Fluka, Buchs SG, Switzerland
- *p*-Nitrophenyl butyrate (*p*-NPB): Sigma-Aldrich Inc., Milwaukee, WI, USA
- Polycaprolactone: Sigma-Aldrich Inc., Milwaukee, WI, USA
- Potassium chloride: Ajex Finechem, Auckland, New Zealand
- Potassium hydrogen sulphate: Ajex Finechem, Auckland, New Zealand
- Magnesium chloride: Ajex Finechem, Auckland, New Zealand
- Magnesium sulfate heptahydrate: Scharlau, Barcelona, Spain
- Manganese sulfate heptahydrate: Scharlau, Barcelona, Spain
- Mercuric chloride: Scharlau, Barcelona, Spain
- Methanol: Merck, Darmstadt, Germany
- Rose Bengal: Sigma-Aldrich Inc., MO, USA
- Silver nitrate: Scharlau, Barcelona, Spain
- Sodium carbonate: Scharlau, Barcelona, Spain
- Sodium chloride: Schar1au, Barcelona, Spain
- Sodium dodecyl sulfate: Scharlau, Barcelona, Spain
- Sodium thiosulfate: Merck, Darmstadt, Germany
- Sodium hydrosulfite: Carlo Erba, Milano, Italy
- Sodium hydroxide: Ajex Finechem, Auckland, New Zealand
- Terephthalic acid: Merck, Darmstadt, Germany
- Triton X-100: Sigma-Aldrich Inc., Milwaukee, WI, USA
- Tris-base: Sigma-Aldrich Inc., Milwaukee, WI, USA
- Zinc sulfate heptahydrate: Scharlau, Barcelona, Spain

3.3 Microbial cultures and nucleotide sequence accession numbers

The DNA sequences of Internal transcribed spacer (ITS) rRNA genes of *Fusarium falciforme* PBURU-B5 was deposited in the GenBank database under accession numbers MF373584.1

3.4 Procedures

3.4.1. Sample collection

Samples of plant including leaves, fruits collected from fresh market in central of Thailand and Samples of soil nearby the roots of these plants were collected from several habitats in Thai provinces **(Figure 3.1)**; the sampling collections in the rainy and dry season.

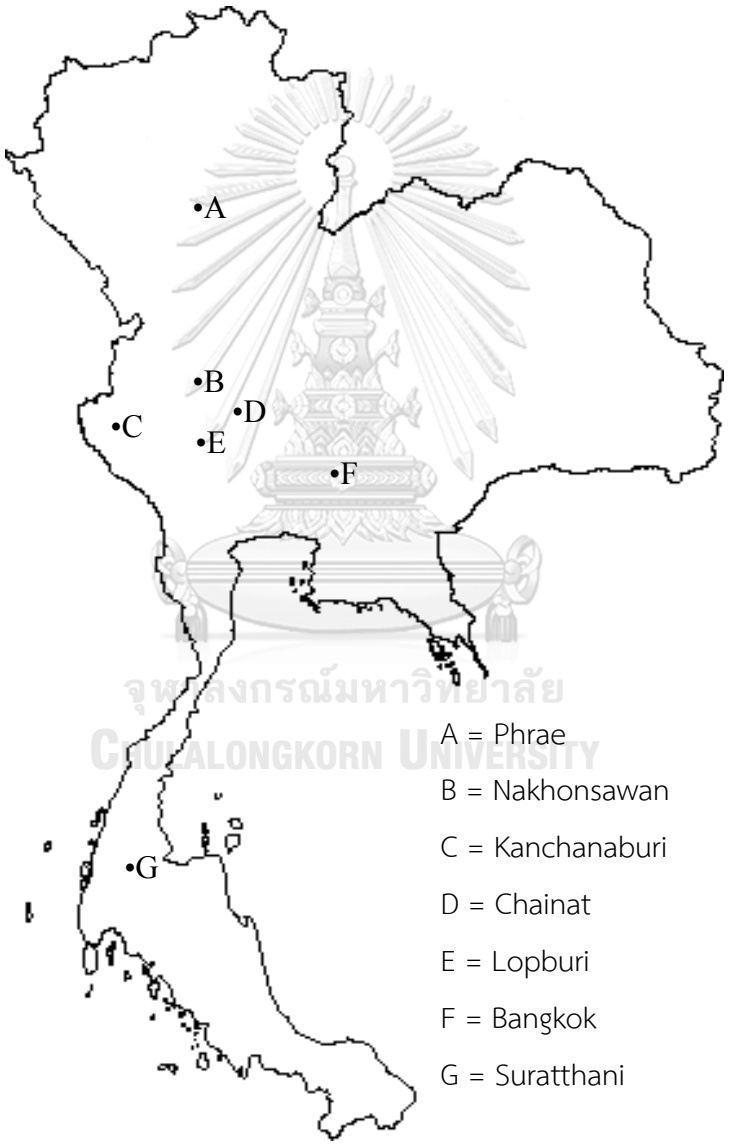


Figure 3.1 Map of collection sites of plant and soil samples from seven provinces in Thailand

The samples were collected from low altitudes (0.4 m. Suratthani Province) to high altitudes (1.10 m. Phrae Province) at ranges from 24.0 ℃ (Phrae Province) to 32.0 ℃ (Bangkok Province) and from five provinces (Nakhonsawan, Lopburi Suratthani, Kanchanaburi and Chainat).

3.4.2 Screening for alkaline cutinase enzymes

Sample of tropical fungi were collected from aerial surface parts of plants, fruits, and young stems (decaying/fungal infected fruits), alkaline soil samples from several vicinities in Thailand.

Plant specimens, for the source of fungi, were cut into small pieces (0.5 cm x 0.5 cm) and placed on rose bengal chloramphenicol (RBC) agar plate before the selection of isolate cut and move to a mineral agar medium (MM; $(NH_4)_2SO_4$ 2 g, KH_2PO_4 4 g, Na_2HPO_4 6 g, $MgSO_4$ 0.2 g, $FeSO_4.7H_2O$ 1 mg, $CaCl_2$ 1 mg, H_3BO_3 10 mg, $MnSO_4$ 10 mg, $ZnSO_4$ 70 mg, $CuSO_4$ 50 mg, MoO_3 10 mg) with a suspension of polycaprolactone (PCL) 0.5 g/L according to the method of Murphy (1996). The fungal colonies showing clear zones with in 2 to 7 days at 30℃ was selected for further studies.

For fungal isolation from soil, 1 g of soil sample was added in 9 mL sterilized water. After serial dilution (10^{-2} to 10^{-3}), 0.1 mL of each dilution was spreaded on rose bengal chloramphenicol (RBC) agar plate which a selective medium for the enumeration of fungi before punching the spore area with cork borer on a selective medium, as described above.

3.4.3 Determination of growth and cutinase activity

Growth pattern and cutinase production of the selected isolate in production medium was studied. The cutinase activity was determined with a growth curve pattern daily up to the point which cutinase reduces activity or mass cell was in death phase. The Bradford method using for determining protein concentration (Bradford 1976). The protein standard to construct the calibration curve using bovine serum albumin.

3.4.3.1 Growth determination

a mineral agar medium (MM; $(NH_4)_2SO_4$ 2 g, KH_2PO_4 4 g, Na_2HPO_4 6 g, $MgSO_4$ 0.2 g, $FeSO_4.7H_2O$ 1 mg, $CaCl_2$ 1 mg, H_3BO_3 10 mg, $MnSO_4$ 10 mg, $ZnSO_4$ 70 mg, $CuSO_4$ 50 mg, MoO_3 10 mg) with a suspension of polycaprolactone (PCL) 0.5 g/L and peptone 2 g/L used for the production of cutinolytic esterase. The cultivation was performed at 30 ℃, agitation at speed 150 rpm for 7 days. The supernatant was taken every 24 h for determining the total proteins and dry cell weight. Each samples were carried out in triplicate.

3.4.3.2 Cutinase assay

Cutinase was determined by spectrophotometric assay using para-nitrophenyl butyrate *(p*-NPB) as the substrate. Reaction mixture, containing 100 µl 0.3% Triton X-100, 800 µl Tris-HCl buffer (0.1 M, pH = 9.0), 500 µl *p*-nitrophenyl butyrate (*p*-NPB, Sigma, 0.1 mM) and 100 µl of crude enzyme. The mixture was incubated at 35 ℃ for 10 min and the reaction was followed by measuring the absorbance at 405 nm with a Microplate reader (Opsysmr, Bio-Active Co. Ltd.). One unit (U) of cutinase activity was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per minute at 35 ℃. Each samples were carried out in triplicate.

3.4.4 Enzyme production

Fungal isolate was cultivated according to the method of Pio and Macedo (2009). Cutinase was produced from the selected isolate by using papaya cutin, which was extracted by applied method of Macedo and Pio (2005), cutin (extraction from papaya peels) used as a sole carbon source and peptone used as a nitrogen source in concentration of 2.5 g/L and 4 g/L, respectively, in liquid mineral medium (LMM) cultivated at 30 ℃ with shaking condition on a rotary shaker speed at 150 rpm for 4 day. The supernatants (centrifuge culture medium with 12,000 xg, 10 min) were used as the crude enzyme to determine the activity.

3.4.5 Identification of the fungal isolate

The selected fungal isolate with the highest cutinase activity was identified using morphological examination. Microscopic morphology of the isolate was studied using bright-field microscopy and the identification was conducted using keys given by Sharma (2006) and Webster and Weber (2007).

Molecular identification was used to support the identification. Genomic DNA of the selected isolates was extracted by using modifed method of Saghai-Maroof (1984). The fungal cell wall was disrupted by grinding with glass beads in liquid nitrogen. The powdered mycelium was then transferred to an extraction buffer that contains detergent cetyl tri-methyl ammonium bromide (CTAB) and Sodium dodesyl sulphate and EDTA. DNA was adjusted to 20 ng/µl used for working concentration and stored at 4 ℃.

Part of Internal transcribed spacer (ITS) rRNA universal primers (forward primer: ITS1_5'- TCCGTAGGTGAACCTGCGG-3' and reverse primer: ITS4_5'-TCCTCCGCTTATTGATATGC-3'), were used to amplify about 500 bp fragment from the ITS rRNA gene. All amplification reactions were carried out in a DNA thermal cycler (PCR machine, Thermal cycler model PTC-100 MJ Research Inc., USA) and the program was set as follows: 1 cycle of 5 min denaturation at 94 ℃ and further 35 cycles consisting of (i) 30 sec denaturation at 98 ℃, (ii) 1 min primer annealing at 60 ℃ and (iii) 2 min primer extension at 72 ℃. After the 35th cycle, the extension reaction was continued for another 20 min at 72 ℃ to ensure the completion of the final extension step. The PCR product was purified using the QIAquick PCR Purification kit and was subsequently sequenced by Macrogen Inc., Seoul, Korea. The obtained sequences was assembled and edited using program BLAST and ClustalW.

3.4.6 Optimization of culture conditions for enzyme production

3.4.6.1 Nitrogen source

Various nitrogen sources including beef extract, yeast extract, peptone, tryptone, urea, ammonium sulfate, and potassium nitrate were examined for their effect on enzyme production by replacing of nitrogen sources in the production medium. Each samples were carried out in triplicate.

3.4.6.2 An experimental analysis and optimal production using RSM

3.4.6.2.1 Effects of concentrations of carbon and nitrogen sources on cutinase production

A central composite design (CCD) containing three levels for each independent variable was employed to evaluate the effects of two major parameters, including carbon and nitrogen sources, with cutinase activity. These variables and their levels were determined with the cutin (carbon source) concentration in the ranges to 2.0, 2.5 and 3.0 g/L and peptone (nitrogen source) concentration in the ranges to 3.0, 4.0 and 5.0 g/L. The response obtained or cutinase activity was fitted to a second-order polynomial model and was statistically analyzed using the design Expert software (Stat–Ease, Inc.) for regression coefficients, variable of the model (ANOVA) and the three-dimensional response surface plot (RSM).

3.4.6.2.2 Effects of incubation of temperature and pH on cutinase production

A central composite design (CCD) containing three levels for each independent variable was employed to evaluate the effects of two main parameters, including pH and temperature sources, with cutinase activity. These variables and their levels were determined with the pH in the ranges to 6.0, 7.0 and 8.0 and temperature in the ranges to 25, 30 and 35 °C. The response obtained or cutinase activity was fitted to a second-order polynomial model and was statistically analyzed using the design Expert software (Stat–Ease, Inc.) for regression coefficients, variable of the model (ANOVA) and the three-dimensional response surface plot (RSM).

3.4.7 Characterization of the crude enzyme obtained from selected fungal isolate

3.4.7.1 Optimal pH of cutinase

To fine the optimal pH for enzyme activity, 0.1 M of potassium phosphate, sodium acetate, Tris-HCl and borate was used as reaction buffers for pH 4.0-6.0, 6.0-

8.0, 8.0-9.0 and 9.0-10.0, respectively. The relative activity was calculated as the percentage of enzyme activity in comparison to the maximum activity.

3.4.7.2 pH stability of cutinase

For pH stability test, the enzyme at alkaline condition, crude enzyme was incubated at optimal pH (pH 9.0) and incubated at 35 °C, aliquots of the enzyme different amounts of time (0, 30, 60, 90 and 120 min). The result was shown relative to the activity. Residual activity was calculated as a percentage of initial activity before incubation.

3.4.7.3 Optimal temperature of cutinase

The fine optimal temperature of the cutinase activity was be determined by incubating the reaction mixture of crude enzyme with para-nitrophenyl butyrate (*p*-NPB) at various temperatures ranging from 25 ℃ to 55 ℃. The relative activity was calculated as the percentage of enzyme activity in comparison to the maximum activity.

3.4.7.4 Thermostability of cutinase

To test the temperature stability of the enzyme, crude enzyme was measured after incubating the cutinase for 0, 30, 60, 90 and 120 min at optimal temperature. After incubation, the relative activity was calculated as the percentage of enzyme activity in comparison to the maximum activity. The residual activity was measured at standard condition.

3.4.7.5 Effect of metal ions on cutinase activity

To determine the effect of metal ions on the enzyme activity, metal ions including $CaCl_2.2H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $HgCl_2$, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $ZnSO_4$ and a metal chelator EDTA was separately added to the reaction mixture at two different final concentrations of 1.0 and 10 mM, respectively, prior to performing the enzyme assay under the optimum conditions. The residual activity was

compared with the activity of enzyme without metal ions and reported as a percentage of the relative activity. Each samples were carried out in triplicate.

3.4.7.6 Effect of surfactants on cutinase activity

The effect of several surfactants (Triton X-100, Tween 20, Sodium dodesyl sulphate (SDS)) on enzyme stability was studied, The residual activity were determined with reaction mixtures containing with surfactants in difference concentration (10 mM and 50 mM of Tween 20, 10 mM and 50 mM of Triton X-100, 1 M and 5 M of SDS), 900 µl pH buffer, 500 µl *p*-nitrophenyl butyrate (*p*-NPB, Sigma, 0.1 mM) and 100 µl of crude enzyme. The residual activity was measured. The activity of the enzyme without any surfactants was taken as 100%.

3.5 Application of detergent containing cutinase for oily stain and fuzz removal from spun polyester fabric

In this study, various amounts of cutinase powder from 3.5.3 were added into the selected commercial powder detergent and the prepared detergents were used for washing spun polyester knitted fabric in order to remove oily stain and fuzzy hairs on fabric surface.

3.5.1 Fabric

Two types of fabrics were used for the study as follows;

- White knitted polyester fabric (spun yarn) was used to determine the lowest amount of the selected commercial detergent that could completely remove oily stain from fabric through one washing cycle. It was called “white spun polyester fabric or white fabric” for the whole document. The reason for using white fabric for oily stain test because it was easier to determine for the presence or the absence of oily stain on this fabric than on dark color fabric.
- Red knitted polyester fabric (single jersey construction, spun yarn) was used to determine the fuzz removability of the prepared detergent (selected commercial detergent containing cutinase). It was called “red spun polyester fabric or red fabric” for the whole document. The reason for using red fabric for fuzz removal test

because the amounts of fuzz/hairs and pills were easier to determine on this fabric than on light color fabric.

3.5.2 Detergent and pH of detergent

This test was conducted in order to determine the most suitable detergent to be used or mixed with cutinase enzyme (detergent should have the similar pH as the enzyme). In this work, seven commercial powder detergents were tested for their pH (approximately 1 g of detergent in 50 g of distilled water) and the detergent with the closest pH to the optimal pH of cutinase was selected for further detergent/enzyme preparation.

3.5.3 Cutinase enzyme powder

To increase the enzyme activity for detergent application, crude extracellular enzyme from the selected isolate PBURU-T5 was concentrated to 10 times of its initial volume using the ultrafiltration (hollow fiber 10 kDa conjugated two membranes together). Then, the concentrated crude enzyme was frozen at -20 °C for 24 hours, freeze dried into powder, and finally checked for activity. An enzyme unit (1 U/mg) was defined as the amount of enzyme required for the release of 1 μmol *p*-nitrophenol from the substrate per minute at pH 9 and 35 °C. The enzyme was then mixed with the selected detergent at various ratios and used for oily stain and fuzz removal studies.

3.5.4 Preparation for oily stain fabric

The oily stain fabric was prepared according to the standard test method AATCC 130 Soil Release: Oily Stain Release Method. Approximately 10 cm x 10 cm of white spun polyester fabric was placed on a plastic plate and 0.5 mL of palm oil was dropped on fabric surface. Then a glassine paper was laid on top of the stain area before leaving a 2.22 kg weight on the paper for 1 min. After that, the paper and the weight were removed, and the fabric was left dry for 1 hour.

3.5.5 Preparation for fuzzy fabric

The fuzzy fabric was prepared according to the standard test method ISO 12945: Determination of fabric propensity to surface fuzzing and to pilling - Part 1: Pilling box method. Approximately 10 cm x10 cm or 1.3 g of red spun polyester fabric was mounted on polyurethane tube and placed in a pilling box (cork liners). Sample was spun in pilling tester for 4,500 revolutions at a constant speed of 30 rpm to obtain fabric with pilling rate 1 (rate 1 = maximum pill and hairs, rate 5 = minimum pill and hairs) (**Figure 3.2**).

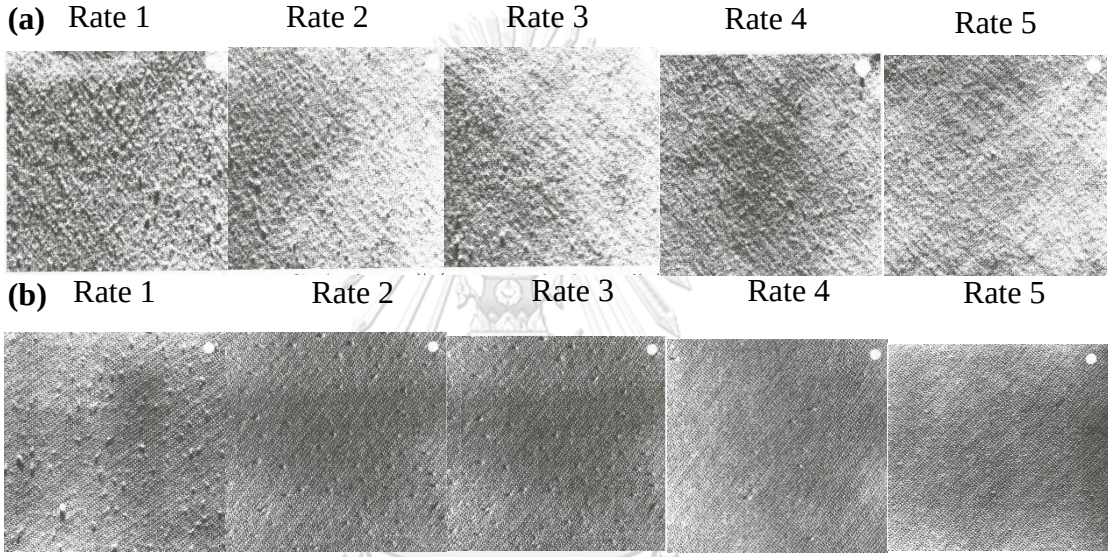


Figure 3.2 (a) Standard pilling images of knitted single jersey fabric and **(b)** Standard pilling images of knitted double jersey fabric, from pilling rates 1 to 5.

3.5.6 Ratio of detergent to fabric for oily stain removal

After the most suitable powder detergent was selected, various amounts of it ranging from 0.02-0.20 g in 10 mL of water were used for washing 1.3 g of white spun polyester fabric containing oily stain. The required amount of detergent in this work was set at the lowest amount that could completely remove oily stain from fabric through one washing cycle. It was found that the required ratio of detergent to fabric to water was 1 g: 6.5 g: 50 mL, respectively. This ratio was further used for the fuzz removal study.

3.5.7 Ratio of detergent to cutinase for fuzz removal

The selected powder detergent and cutinase were mixed well at various weight ratios of powder detergent to cutinase at 1:0, 1:0.05, 1:0.10, 1:0.15 and 1:0.20, and they were used for washing red spun polyester fabric with pilling rate of 1.

3.5.8 Washing procedure

Solutions of detergent and detergent containing cutinase were prepared according to the ratios previously mentioned. Fabric was washed in the prepared solution at 35 °C for 1 hour per cycle using a launder-o-meter (Gyrowash, James H. Heal& Co., Ltd, England) as a washing tester. After each washing cycle, fabric was water rinsed for 30 min and then air dried. Both normal washing test (without stainless steel ball) and accelerated washing test (with stainless steel balls) were conducted. The accelerated washing was performed by adding 10, 20, 30 and 40 stainless steel balls to the test.

It was found that the washing condition using 20 stainless steel balls showed the best pill removal efficiency (pilling rate of after washed fabric was rated 3-4, before washing was rated 1). Additional experiment was also done to determine the relationship between numbers of washing cycle when washing with and without stainless steel ball, by washing fabric (pilling rate 1) without stainless steel ball at 35 °C for several cycles (1 hour per cycle) until the fabric pilling rate improved to 3-4. It was found that 8 washing cycles at 35 °C (1 hour per cycle) without stainless steel ball was comparable to 1 washing cycle using 20 stainless steel balls at same temperature and time per cycle.

3.5.9 Fabric testing

3.5.9.1 Pilling rate

Red fabric was evaluated for pilling rate by eye-comparing its surface appearance with 5 standard photographs of fabric (pilling rates 1 to 5) and grading to the nearest rate (i.e.1,2,3,4,5) or between two rates (i.e.1-2, 3-4), according to the standard test method ISO 12945-1. Fabric with pilling rate 1 was determined to have

the highest number of pills and hairs while fabric with pilling rate 5 had a smooth surface with the lowest amount of pills and hairs. Based on the requirement for exporting apparel to major markets, the acceptable pilling rate should be at least 3-4.

3.5.9.2 Surface appearance

Red fabric was observed under an optical microscope to analyze for the amount of pill and hair contents on its surface.

3.5.9.3 Color measurement

Red fabric before and after pilling as well as before and after washing with detergent was measured for color values using a colorimeter Macbeth Color-Eye 7000 according to the AATCC Evaluation Procedure 6. Fabric color strength (K/S) or color depth was measured at fabric surface at wavelength 510 nm (red color) in which fabric with higher K/S showed darker color than that with lower K/S. Each color strength value was converted from the reflectance value according to the Kubelka-Munk equation (1) as follows.

$$K/S = (1-R^2)/2R \qquad (1)$$

Where K = the absorption coefficient

S = the scattering coefficient

R = the reflectance factor of material at the wavelength of maximum absorption (λ_{max})

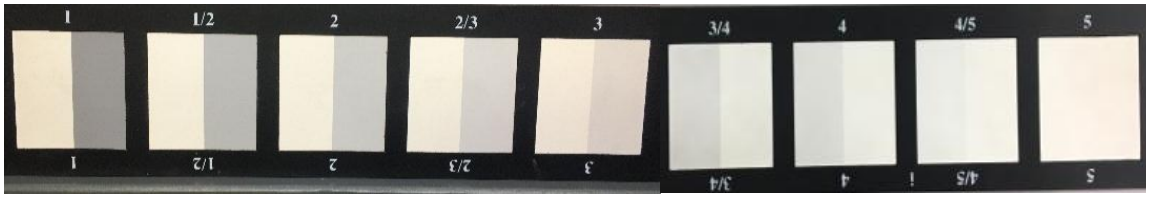
In addition, fabric color difference (ΔE*), lightness (L*) and color shade (a*, b*) were also measured. Fabric after pilling showed some color difference from fabric before pilling because pills and fuzzy hairs on fabric surface were capable of changing the color appearance of fabric. On the contrary, when pills and hairs were removed, the color difference between fabrics should be minimized. In his work, fabric after pilling (both before and after washing) was color evaluated to see how

much its color was different from that of fabric before pilling (pilling rate 5, $\Delta E^* = 0$). The washing process that could produce fabric with ΔE^* closed to 0 would be the optimal washing process for pill removal in this work. Measurement of fabric lightness and color shade was also done in order to respectively evaluate the brightest white ($L^* = 100$) or darkest black ($L^* = 0$) of fabric color as well as to see the color shade of fabric ($a^* > 0$ is red, $a^* < 0$ is green, $b^* > 0$ is yellow, $b^* < 0$ is blue, $a^* = 0$ and $b^* = 0$ is true neutral gray).

White fabric before and after palm oil staining as well as before and after washing with detergent was measured for its whiteness index using a colorimeter Macbeth Color-Eye 7000 according to the AATCC Evaluation Procedure 6, in order to determine for the stain removability of the prepared detergent. In this work, it was set that an acceptable detergent should be able to completely remove oily stain from fabric through washing, and the fabric's whiteness index after staining and washing should be similar to that before staining.

Grey scales (gray scales) were also used for assessing color change and staining of textile materials (**Figure 3.3**). Both scales were used for visual assessment in the standard lighting cabinet to specify a rating from 1 to 5, with 5 being 'good' and 1 being 'poor'. The color change scale was composed of nine pairs of grey colored chips, from grades 1 to 5 (with four half steps). Grade 5 was defined as no color change or no color difference between two fabrics and grade 1 as severe color change or significant color difference between two fabrics, according to the AATCC Evaluation procedure 1: Grey scale for color change. For staining scale, it was composed of nine pairs of grey and white colored chips from grades 1 to 5 (with four half steps). Grade 5 was defined as no staining and grade 1 as severe staining, according to the AATCC Evaluation procedure 2: Grey scale for staining. In this work, red fabric was rated for grey scale for color change and white fabric was rated for grey scale for staining.

(a)



(b)

Figure 3.3 (a) Gray scale for Assessment color change (ISO 105-A02-1993) and **(b)** Gray scale for Assessment staining (ISO 105-A03-1993**)**

3.5.9.4 Weight loss

Red fabric dry weight (at 105 °C) before and after washing was determined using an infrared balance to analyze for %fabric weight loss after pill removal through washing. Each samples were carried out in triplicate.

3.5.9.5 Strength loss

Red fabric was tested for bursting strength (kPa) using a hydraulic-type bursting strength tester, according to the standard test method ASTM D 3786. Its strength before and after washing was measured and results were used for calculation of %fabric strength loss after pill removal through washing. Each samples were carried out in triplicate.

CHAPTER IV

RESULTS

4.1 Screening for alkaline cutinase enzymes

4.1.1 Sample collection

Forty-four samples of soil, including agriculture soil, forest soil and other pathogen of plant were collected between 2015 and 2016 from seven provinces throughout region of Thailand (**Figure 3.1**).

4.1.2 PCL degradation by fungi

A diverse variety of soil and plant surfaces provided fungi 23 out of 44 isolates that were able to degrade PCL, was indicated by clearing zone on MM-PCL agar plates (**Figure 4.1**). Clearing zones wide range between 1-3 mm. around colony grown could be observed after 3-4 days of incubation.

4.1.3 Source and collection sites of fungi attacking PCL

The isolates were dominated by *Fusarium*, 20 of 23 isolates. A few of *Aspergillus* species isolated from soil were found to degrade PCL, the cutin analog. These cutin degraders were generally isolated from different aerial parts of plants (fruit peel) and also from soil nearby root, and all produced cutinase (**Table 4.1**). The sampling times included the rainy season (June, July, August and September) and the dry season (March, April), and from low altitude (0.5 m, Suratthani Province) and high altitude (1,000 m, Phrae Province), at temperature ranges from 25 °C (Phrae) to 35 °C (Bangkok) isolates were from seven provinces. Twenty-three fungal isolates of 44 isolated were capable of exhibiting cutinase activity after cultivation in liquid medium supplement with PCL with the clear zone diameter ranging from 1-3 mm. The fungi with the high cutinase activity were in isolates of PBURU-T5 (3.57±0.12 U/mL), PBURU-T6 (3.2±0.14 U/mL) and PBURU-T7 (3.07±0.33 U/mL). This is found to be higher than the crude enzyme activity from *Fusarium solani* PBURU-B5 (0.3 U/mL)

(Nimchua et al., 2008). From this result, the best cutinase producer, PBURU-T5 was selected for the strain identification.

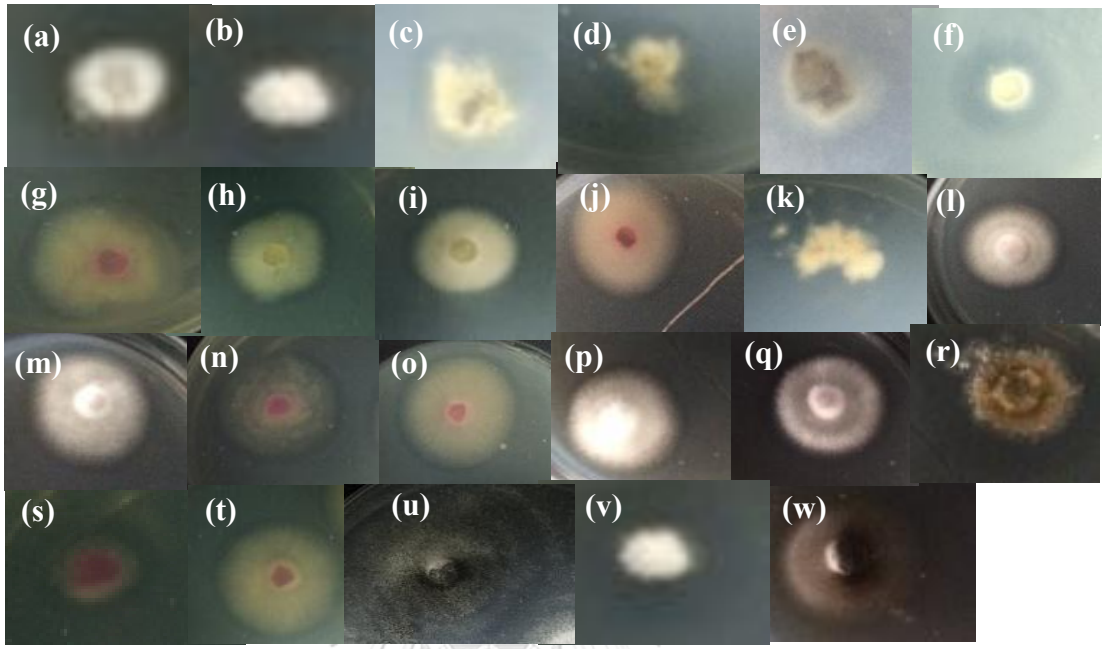


Figure 4.1 The cutinase activity of the fungal isolates towards PCL as indicated by observe clearing zones on polycaprolactone plates. (Most isolate was *Fusarium* spp. except *Aspergillus* spp. in PBURU-T18, PBURU-T21, PBURU-T23) clearance zone on PCL plate **a** PBURU-T1**, b** PBURU-T2, **c** PBURU-T3, **d** PBURU-T4, **e** PBURU-T5, **f** PBURU-T61, **g** PBURU-T7, **h** PBURU-T8, **I** PBURU-T9, **j** PBURU-T10, **k** PBURU-T11, **l** PBURU-T12, **m** PBURU-T13, **n** PBURU-T14, **o** PBURU-T15, **p** PBURU-T16, **q** PBURU-T17, **r** PBURU-T18, **s** PBURU-T19, **t** PBURU-T20, **u** PBURU-T21, **v** PBURU-T22**,** **w** PBURU-T23

4.2 Identification of the fungal isolate

The morphology of isolate PBURU-T5 grown on PDA showed typical Fusaria characteristics. The colony grew to 2-3 cm diameter in 3 days and the color shown was whitish (**Figure 4.2(a)**). The shape of microconidia and macroconidia were used as the primary criteria for the identification of *Fusarium* species. It produced both of microconidia and macroconidia in sized of 25 µm and 50 µm, respectively (**Figure**

4.2(c, d)). **Figure 4.2(c)** Microconidia (mostly 1 cell) were ellipsoidal-shape, produced from long monophialides in aerial mycelium. **Figure 4.2(d)** Macroconidia (multicellular) was hyaline and canoe-shape in side view.

The shape of microconidia and macroconidia were used as the primary criteria for the identification of *Fusarium* species. It produced both of microconidia and macroconidia in sized of 25 µm and 50 µm, respectively (**Figure 4.2(c, d)**). **Figure 4.2(c)** Microconidia (mostly 1 cell) were ellipsoidal-shape, produced from long monophialides in aerial mycelium. **Figure 4.2(d)** Macroconidia (multicellular) was hyaline and canoe-shape in side view.

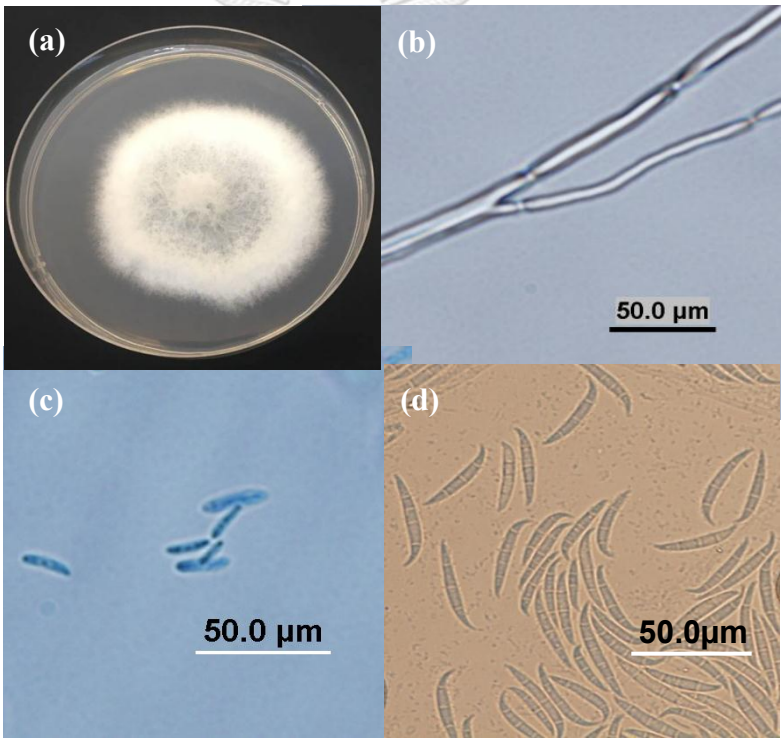


Figure 4.2 Colony and morphology of *Fusarium* sp. PBURU-T5 (**a**) Colony (4 cm in diameter) grown on PDA for 3 days at 30±2 °C, (**b**) Septate hyphae (monophialides), (**c**) Ellipsoidal-shape microconidia, (**d**) Curve "banana" or "canoe" shape macroconidia

Table 4.1 Esterase activity of fungi from Thailand and cutinase assay in Tris-HCl buffer pH 9.0

Location	Source	Isolate	Enzyme activity (U/mL)
Bangkok	*Solanum melongena*	*Fusarium* sp. PBURU-T1	3.03±0.01^{l}
Bangkok	*Solanum melongena*	*Fusarium* sp. PBURU-T2	3.05±0.14^{l}
Bangkok	*Cucumis sativus*	*Fusarium* sp. PBURU-T3	1.41±0.19^{cd}
Bangkok	*Cucumis sativus*	*Fusarium* sp. PBURU-T4	2.24±0.01^{j}
Phrae	*Carica papaya*	*Fusarium* sp. PBURU-T5	3.57±0.12^{a}
Phrae	*Carica papaya*	*Fusarium* sp. PBURU-T6	3.20±0.14^{m}
Nakhonsawan	Cane field soil	*Fusarium* sp. PBURU-T7	3.07±0.33^{l}
Nakhonsawan	Cane field soil	*Fusarium* sp. PBURU-T8	2.74±0.02^{k}
Nakhonsawan	Cane field soil	*Fusarium* sp. PBURU-T9	2.78±0.15^{k}
Lopburi	Green beans field soil	*Fusarium* sp. PBURU-	1.70±0.13^{g}
Lopburi	Green beans field soil	*Fusarium* sp. PBURU-	1.38±0.31^{c}
Lopburi	Green beans field soil	*Fusarium* sp. PBURU-	1.66±0.10^{fg}
Lopburi	Green beans field soil	*Fusarium* sp. PBURU-	1.63±0.24^{f}
Lopburi	Green beans field soil	*Fusarium* sp. PBURU-	1.09±0.27^{b}
Lopburi	Green beans field soil	*Fusarium* sp. PBURU-	1.07±0.04^{b}
Lopburi	Asparagus field soil	*Fusarium* sp. PBURU-	1.83±0.13^{h}
Lopburi	Asparagus field soil	*Fusarium* sp. PBURU-	2.15±0.24^{ij}
Kanchanaburi	Green beans field soil	*Aspergillus* sp. PBURU-	2.14±0.04^{i}
Chainat	Pomelo field soil	*Fusarium* sp. PBURU-	1.92±0.20^{h}
Nakhonsawan	Green beans field soil	*Fusarium* sp. PBURU-	0.96±0.16^{n}
Suratthani	forest soil	*Aspergillus* sp. PBURU-	1.30±0.18^{c}
Suratthani	forest soil	*Fusarium* sp. PBURU-	1.51±0.08^{d}
Suratthani	Bamboo field soil	*Aspergillus* sp. PBURU-	1.35±0.06^{c}

Genomic DNA of the isolates PBURU-T5 were extracted and analyzed by agarose gel electrophoresis (Appendix F). The genomic DNA were used as DNA templates to ITS rRNA gene using the primers ITS4 and ITS1. The PCR products were purified and visualized by agarose gel electrophoresis (**Figure 4.3**). The amplified product sizes were about 550 bp.

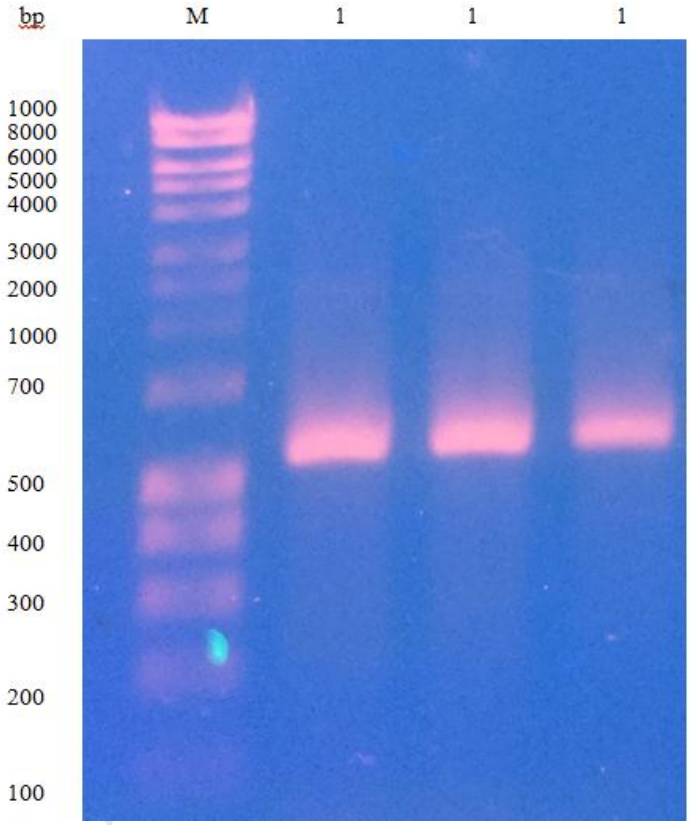


Figure 4.3 Purified amplification product of ITS gene from cutinase-producing isolates. Lanes: M = Molecular marker; 1 Kb ladder, 1 = PCR product of the isolate PBURU-T5.

The gene encoding ITS rRNA region was amplified by polymerase chain reaction (PCR). After purification of the amplified products by agarose gel DNA purification kit, ITS rDNA gene was sequenced in both directions with ITS1 and ITS4 primers at Solgent Co., Ltd., Korea. The total size of the ITS1 and ITS4 regions was ~550 base pair (bp). The alignment of this sequence was blasted with the ITS sequences submitted in the GenBank databases. The ITS sequencing data indicated that the isolate was *Fusarium falciforme*.

4.3 Determination of growth and cutinase production

Growth pattern and cutinase production of the selected isolate in production medium after optimization was studied and the result was shown in **Figure 4.4**. The incubation period is directly associated with the production of enzyme. The *F. falciforme* PBURU-T5 was used for enzyme production in liquid minimal medium consisting 2.5 g/L cutin as a sole carbon source and 4 g/L peptone as a nitrogen source, initial pH 7.0. Shaking with rotary shaker at speed 150 rpm at temperature 30 ℃ and 50 mL erlenmeyer flask adding with 20 mL of the medium. Each flask was inoculated with 1% v/v from culture medium of *F. falciforme* PBURU-T5 from 2-day-old culture of the isolate actively growing on PDA plates. Measuring enzyme activity and protein daily from 0 to 7 days, cutinase and protein showed optimum numbers on 4th day (96 h) with maximum cutinase activity 4.0 ± 0.15 U/mL.

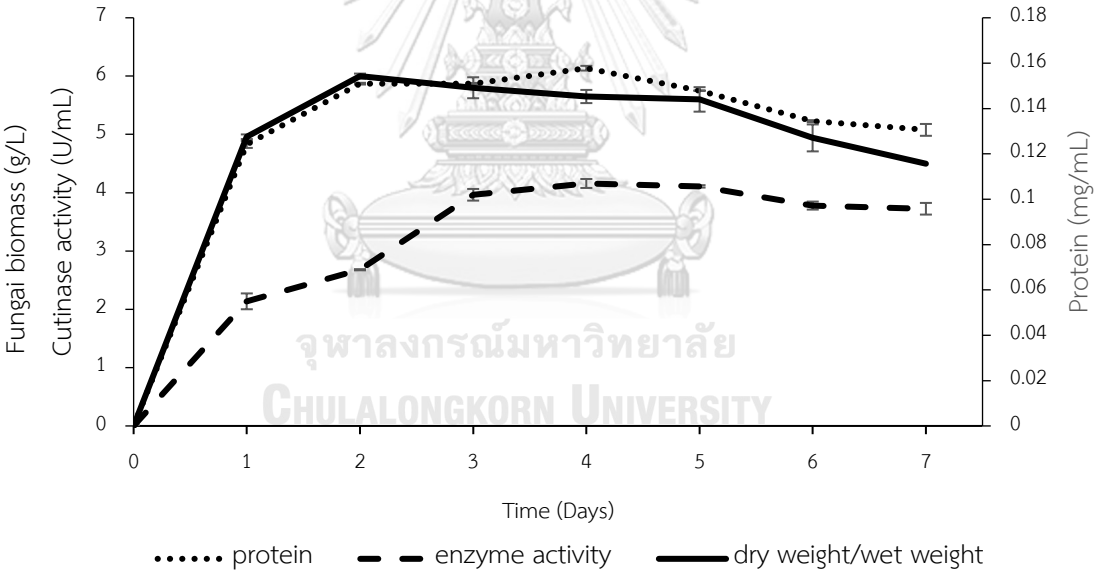


Figure 4.4 Time course of cutinase production of *F. falciforme* PBURU-T5

4.4 Nitrogen source on enzyme production

The effect of difference nitrogen sources on the production of enzyme by PBURU-T5 was investigated. Complex inorganic and organic nitrogen sources, such as beef extract, peptone, tryptone, Urea, KNO_3, $(NH_4)_2SO_4$ and yeast extract on constant concentration 4 g/L. The maximum cutinase activity (3.61±0.01 U/mL) was

found when using peptone as sole nitrogen source while tryptone (1.95±0.06 U/mL), yeast extract (1.90±0.04 U/mL), potassium nitrate (0.95±0.1 U/mL) have the weakly effect to cutinase production (**Figure 4.5)**.

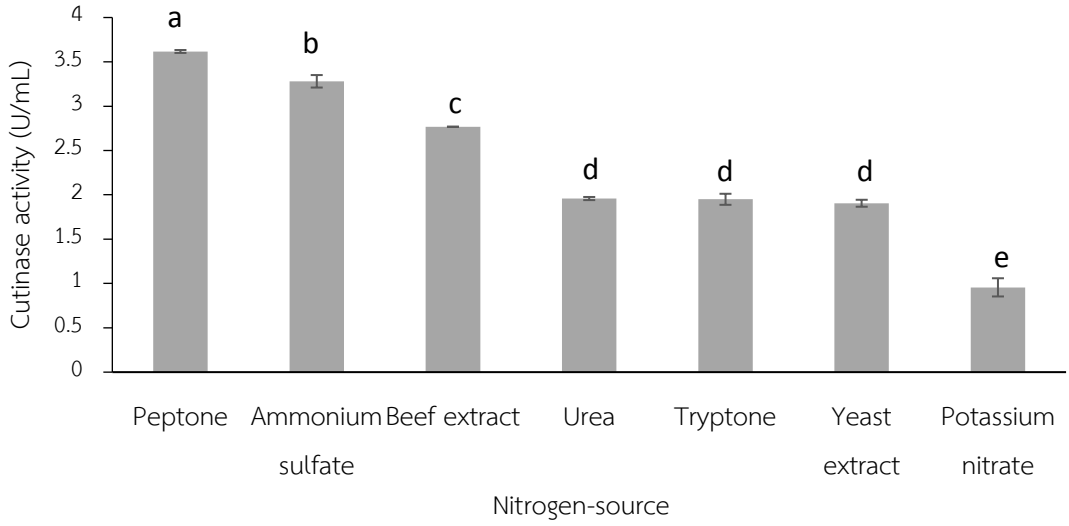


Figure 4.5 Effect of nitrogen source on cutinase production by *F. falciforme* PBURU-T5 in LMM added 2 g/L of cutin and 4 g/L peptone when incubation 4 days in 30 °C, shaking on 150 rpm

4.5 Optimization of cutinase production

4.5.1 Effect of carbon source and nitrogen source on cutinase activity

3D response surface plot and contour plot from the analysis of the experimental data of CCD showed a relationship between two variables was obtained. The three-dimensional surface plots show the combined effect of two independent variables for cutinase activity produced by *F. falciforme* PBURU-T5, the interaction of cutin concentration and peptone concentration indicated the importance of those factors for cutinase activity. The maximum cutinase activity was received (3.90 U/mL) at cutin concentration of 2.5 g/L and peptone concentration of 4 g/L in liquid minimal medium.

Figure 4.6(a) showed that RSM plots illustrating the effects of cutin and peptone concentration on cutinase activity. Using RSM based on CCD model,

optimum conditions for cutinase production by the *F. falciforme* PBURU-T5 was: cutin of 2.5 g/L, peptone of 4.0 g/L at incubation time of 96 h. The model for cutinase production was regressed by considering the significant terms ($P< 0.05$) as shown in following equation (2) Regression equitation was manipulated to fit the experimental data shown in **Table 4.2** and was obtained after analysis of variance using design expert (**Table 4.3**).

$$Y = -14.583 + 9.72X_1 + 2.6X_2 - 1.94X_1^2 - 0.35X_2^2 + 0.11X_1X_2 \quad (2)$$

Where Y is the predicted cutiase activity (U/mL), X_1 is cutin concentration (g/L), X_2 is peptone concentration (g/L).

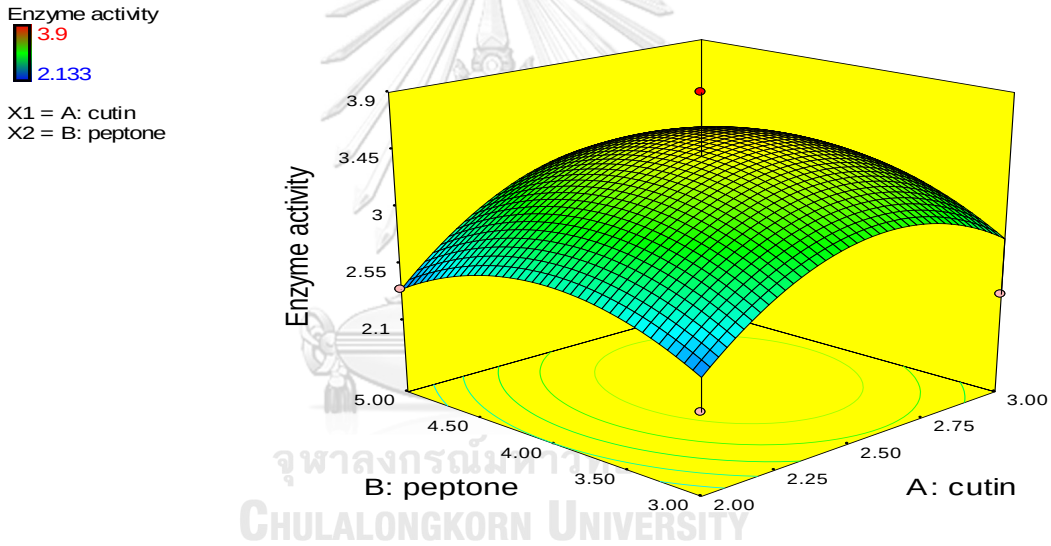


Figure 4.6 (a) 3D response surface plot for the effects of cutin and peptone, incubation at pH 7.0, temperature 30 ℃ on the cutinase activity of *F. falciforme* PBURU-T5 after 4 days of incubations.

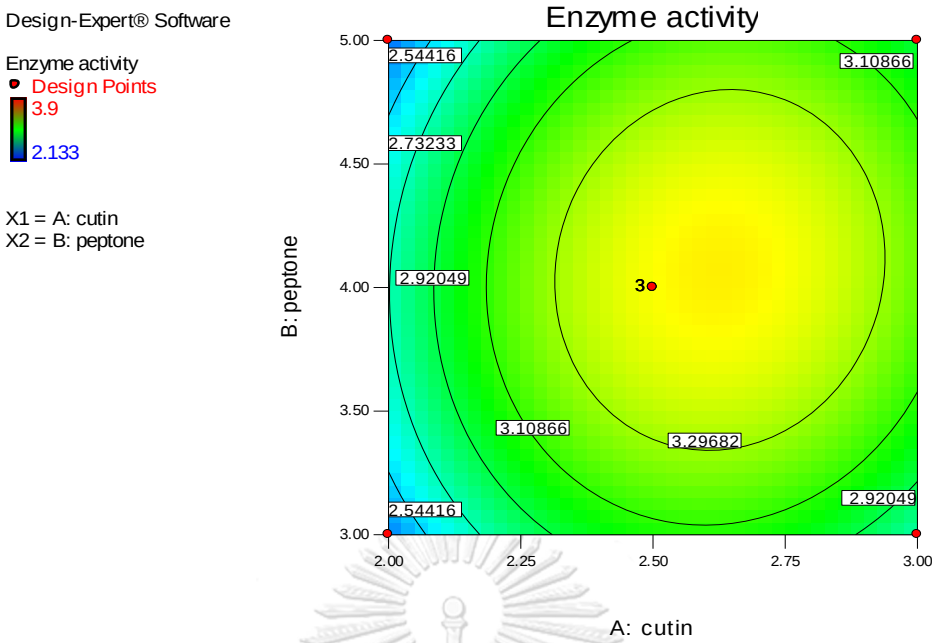


Figure 4.6 (b) Contour plot for the effect of cutin and peptone on the cutinase activity of *F. falciforme* PBURU-T5 after 4 days of incubation. Number indicated the levels of cutinase activity (U/mL).

Table **4.2**. Optimization of cutinase activity using central composite design with corresponding observed and predicted values. Cutin vs Peptone.

Run	X1: cutin (g/L)	X2: peptone (g/L)	cutinase activity (U /mL)	
			Observed	Predicted
1	2.00 (-1)	3.00 (-1)	2.13	2.39
2	3.00 (1)	3.00 (-1)	2.31	2.76
3	2.00 (-1)	5.00 (1)	2.35	2.36
4	3.00 (1)	5.00 (1)	2.75	2.95
5	1.79 (-1.414)	4.00 (0)	2.23	2.13
6	3.21 (1.414)	4.00 (0)	3.19	2.82
7	2.50 (0)	2.59 (-1.414)	3.1	2.70
8	2.50 (0)	5.41 (1.414)	2.84	2.80
9	2.50 (0)	4.00 (0)	3.13	3.45
10	2.50 (0)	4.00 (0)	3.33	3.45
11	2.50 (0)	4.00 (0)	3.90	3.45

Values in bracket are coded variable level.

Table 4.3. ANOVA results for response surface for the quadratic equation of Design-Expert. Cutin vs Peptone

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	Probability
Model	2.09	5	0.42	2.23	0.1999 not significant
Residual	0.94	5	0.19		
Lac of fit	0.62	3	0.21	0.24	0.4645 not significant
Pure error	0.32	2	0.16		

S.D.: 0.43, R^2: 0.69. R_{adj}-squared: 0.3805

The maximum activity obtained by performing experiment was 3.45 ± 0.4 U/mL, which closely related to the predicted value. Under this condition, the model at the suggested optimum condition predicted enzymatic activity of 3.45 U/mL at a confidence level of 95%. The experimental enzymatic activity of 3.90 U/mL confirmed the accuracy of the model.

4.5.2 Effect of incubation temperature and initial pH on cutinase activity

The three-dimensional surface plot shown the combine effect of two independent variables for cutinase activity produced by PBURU-T5, the interaction of temperature and pH indicated the importance of those factors for cutinase activity. The maimum cutinase activity was received (4.15 U/mL) incubation in temperature 30 °C with initial pH 7.0, shake at 150 rpm (**Figure 4.7(a)**).

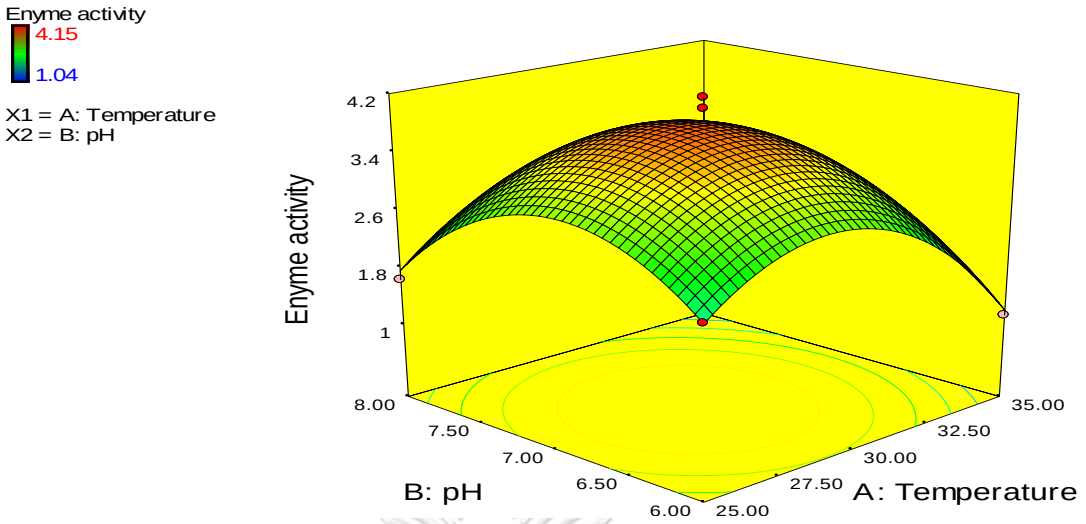


Figure 4.7 (a) 3D response surface plot for the effects of Temperature (°C) and pH, at concentration of cutin 2 g/L and peptone 4 g/L in liquid minimal medium, on the cutinase activity of *F. falciforme* PBURU-T5 after 4 days of incubation.

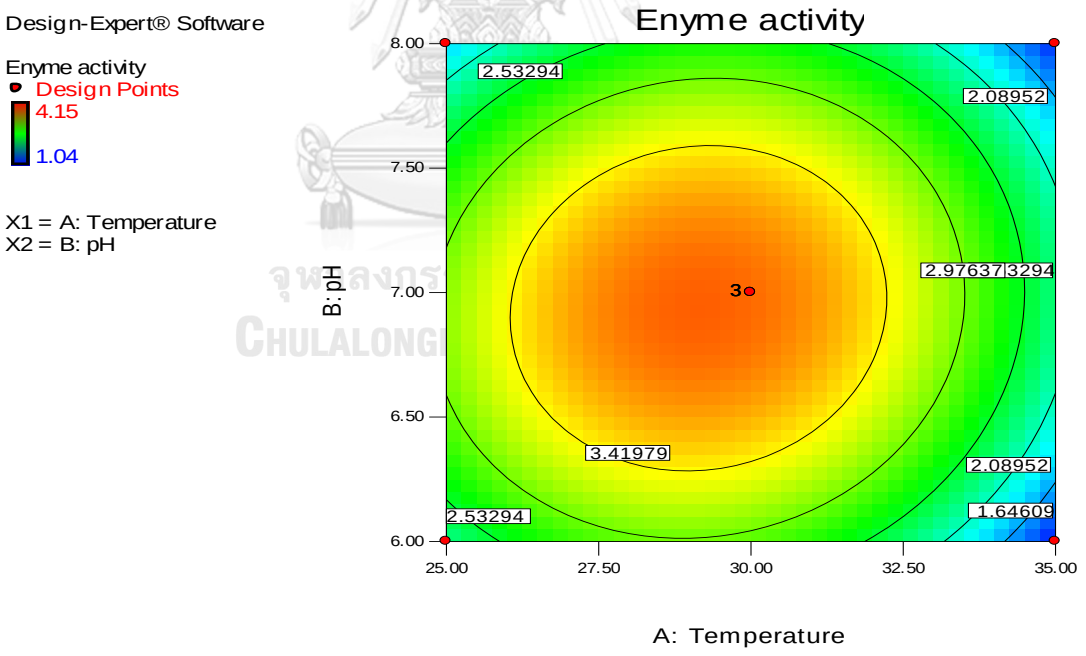


Figure 4.7 (b) Contour plot for the effect of Temperature (°C) and pH on the cutinase activity of *F. falciforme* PBURU-T5 after 4 days of incubation. Number indicated the levels of cutinase activity (U/mL).

The interaction of pH and temperature of cutinase activity by *F. falciforme* PBURU-T5 was also analyzed by CCD. The regression model of these two independent factors considering the significant terms (P< 0.05) was shown in the equation (3). Regression equitation was employed to fit the experimental data presented in **Table 4.4** and was obtained after analysis of variance using design expert (**Table 4.5**).

$$Y = -80.47 + 2.53X_1 + 13.68X_2 - 0.046X_1^2 - 1.04X_2^2 + 0.026X_1X_2 \quad (3)$$

Where Y is the predicted cutinase activity (U/mL), X_1 is Tempeature (°C), X_2 is initail pH.

Table 4.**4** Optimization of cutinase activity using central composite design with corresponding observed and predicted values. Temperature vs pH

Run	X1: Temperature (°C)	X2: pH	Enzyme activity (U /mL)	
			Observed	Predicted
1	25.00 (-1)	6.00 (-1)	2.26	2.25
2	35.00 (1)	6.00 (-1)	1.13	1.20
3	25.00 (-1)	8.00 (1)	1.64	1.77
4	35.00 (1)	8.00 (1)	1.04	1.25
5	22.93 (-1.414)	7.00 (0)	2.10	2.05
6	37.07 (1.414)	7.00 (0)	1.10	0.94
7	30.00 (0)	5.59 (-1.414)	1.90	1.90
8	30.00 (0)	8.41 (1.414)	1.79	1.60
9	30.00 (0)	7.00 (0)	3.33	3.82
10	30.00 (0)	7.00 (0)	4.00	3.82
11	30.00 (0)	7.00 (0)	4.15	3.82

Values in bracket are coded variable level**.**

The maximum activity obtained by performing experiment was 4.0 ± 0.15 U/mL, which closely related to the predicted value. Under this condition, the model

at the suggested optimum condition predicted enzymatic activity of 3.82 U/mL at a confidence level of 95%. The experimental enzymatic activity of 4 U/mL confirm the accuracy of the model.

Table 4.5. ANOVA results for response surface for the quadratic equation of Design-Expert. Temperature vs pH

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	Probability
Model	12.08	5	2.42	23.41	0.0018 significant
Residual	0.52	5	0.10		
Lac of fit	0.13	3	0.045	0.24	0.8663 not significant
Pure error	0.38	2	0.19		

S.D.: 0.32, R^2: 0.959. R_{adj}-squared: 0.918

4.6 Characterization of cutinase

4.6.1 Optimal pH for the cutinase activity of the isolate

Cutinase activity was highest in slightly alkaline pH, enzymes exhibited a pH optimum of about 9.0 for the hydrolysis of *p*-NPB (**Figure 4.8**). The effect of pH on enzyme activity using para-nitrophenyl butyrate (*p*-NPB) assay was determined by measuring cutinase activity in different buffers (0.1 mM) from pH 3.0 to 11.0. The optimal pH of crude cutinase activity was at pH 9.0. At lower pH 3.0 and higher pH 10.0, the relative enzyme activities were 26.17% and 4.24%, respectively. The crude enzyme assays with *p*-NPB as the substrate at pH-values lower than 3.0 is too low and higher than 11.0 are not feasible.

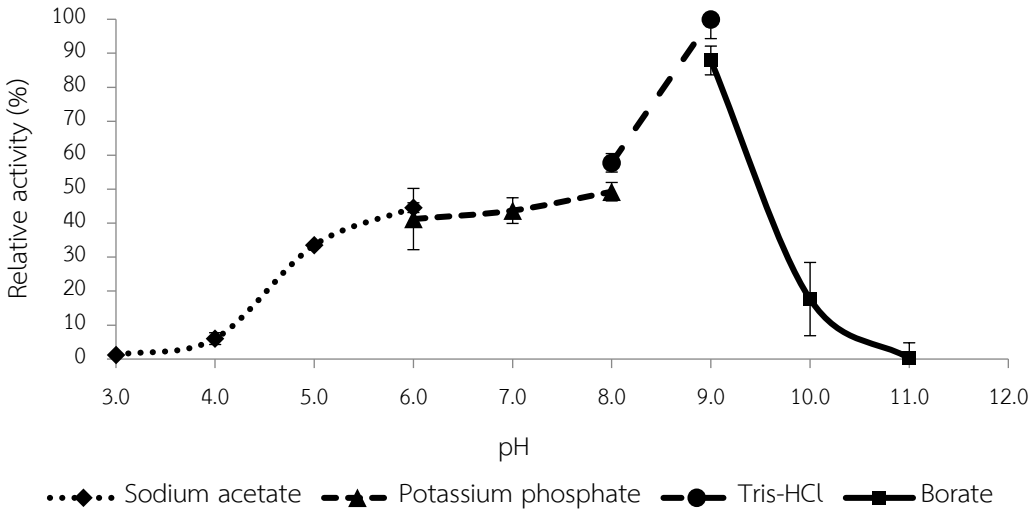


Figure 4.8 Effect of pH on cutinase activity

4.6.2 pH stability of cutinase

F. falciforme PBURU-T5 cutinase was stable at pH range of 8.0-9.0 retaining more than 50% activity after preincubation at 35 °C for 60 min. However, at pH 8.0 and 9.0, the relative activities were decreased to 89% and 95%, respectively so the highest stability was observed with alkaline pH (**Figure 4.9**).

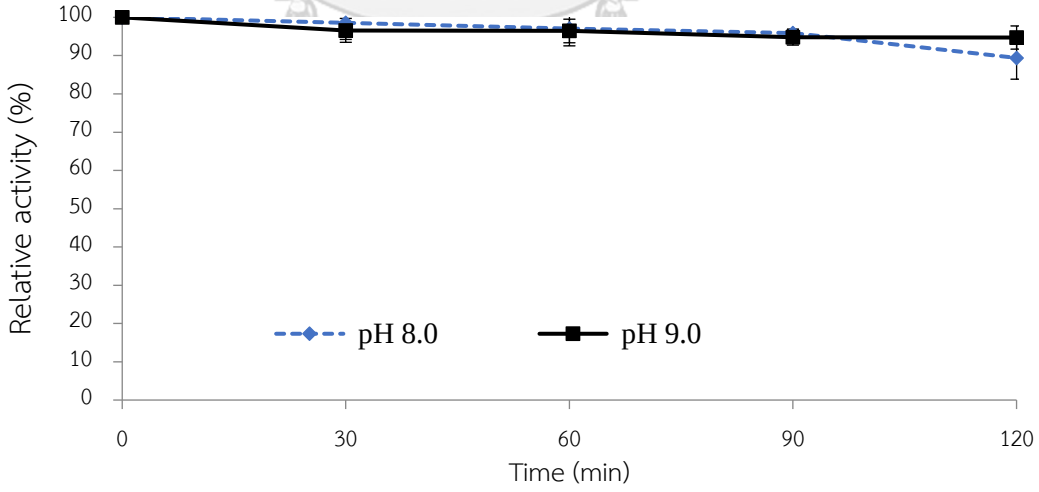


Figure 4.9 pH stability of cutinase activity from *F. falciforme* PBURU-T5, at 35 °C

4.6.3 Optimal Temperature for the cutinase activity of the isolate

The temperature optimum of enzyme activity was determined at 25, 30, 35, 40, 45 and 50 ℃. Because the pH of Tris buffer was temperature dependent, the buffers was adjusted to pH 9.0 at the desired temperature. At temperature between 45 to 55 ℃ the enzyme activity was rapidly decreased by the enzyme. The enzyme was optimum temperature at 30 ℃ to 40 ℃. Enzyme activity rapidly decreased to 80% of relative activity at higher temperatures at 45 , 50 and 55 ℃ and decreased to 75% at low temperature at 25 ℃ (**Figure 4.10**).

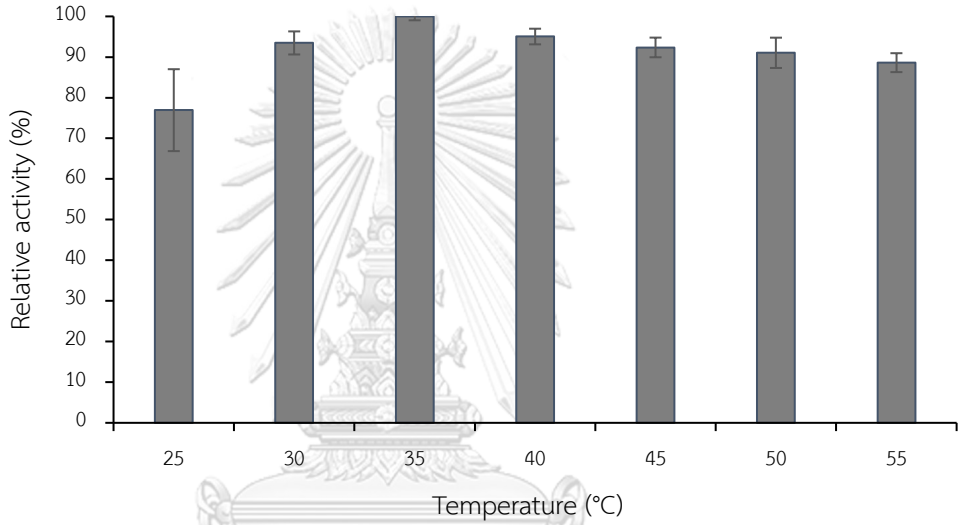


Figure 4.10 Effect of temperature on cutinase activity

4.6.4 Thermostability of cutinase

The thermal stability of cutinase was investigated by incubating the enzyme in 0.1 M Tris-HCl (pH 9.0). The enzyme was stable up to 35 ℃ and more efficiently than 85% when incubated temperature between 30 ℃ to 40 ℃ for 120 min (**Figure 4.11**).

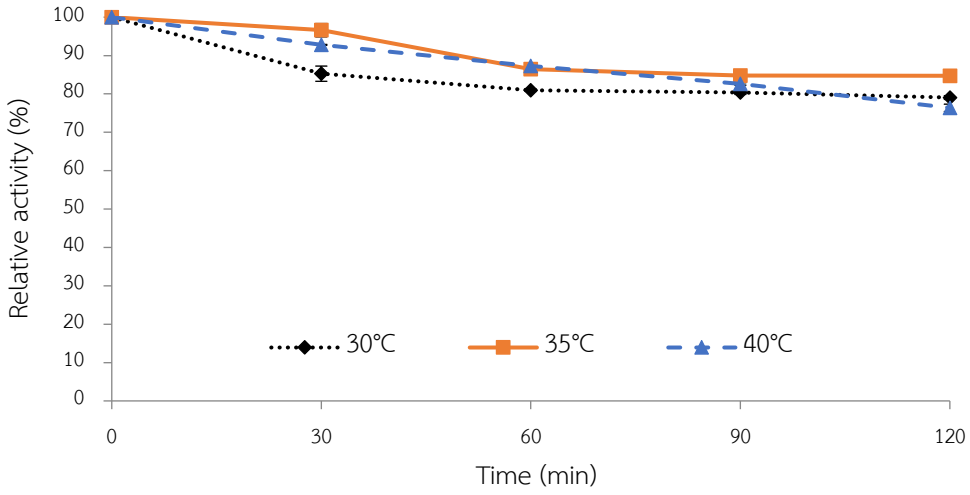


Figure 4.11 Temperature stability of cutinase activity from *F. falciforme* PBURU-T5 at pH 9.0

4.6.5 Effect of metal ions on cutinase activity

The effects of different metal ions on cutinase activity were studied. Metal ions like Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} inhibited the enzyme activity by 30–50 % with high concentration at 10 mM. Metal ions like Hg^{2+}, Fe^{2+} and Mg^{2+} did not have effect on cutinase activity at concentration 1 mM, whereas cutinase were a medium inhibitory effect by Ca^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} ions. (**Figure 4.12**).

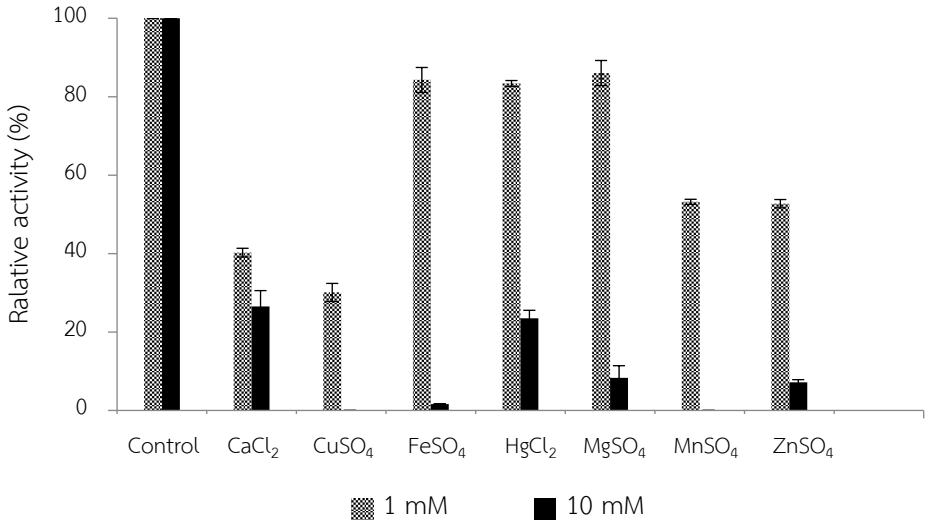


Figure 4.12 Effect of metal ions on cutinase activity from *F. falciforme* PBURU-T5

4.6.6 Effect of surfactant on cutinase activity

Stability was observed for cutinase toward various surfactant (**Table 4.6**). Upon incubation with 10 mM of Tween 20, Triton X-100 and 5 mM of SDS at temperature 35 °C for 10 min, cutinase exhibited residual activities 52.28, 57.61 and 54.49%, respectively. Cutinase retained 76.63% of activity after incubation with 1 mM SDS, cutinase retained only 12.43% and 22.05% after incubation with 50 mM Tween 20 and 50 mM Triton X-100, respectively. Cutinase was inhibited after incubation with chelating agent at concentration 10 mM of EDTA its was found that inhibited cutinase activity more than 70% and 50 mM of EDTA it was found that completely inhibited cutinase activity.

Table 4.6 Stability of *F. falciforme* PBURU-T5 cutinase in the presence of various surfactants and chelating agent (EDTA).

Additives	Concentration (mM)	Residual activity (%)
None	-	100 (4 U/mL)
EDTA	10	24.92
EDTA	50	0
Tween 20 (nonionic)	10	52.28
Tween 20 (nonionic)	50	12.43
Triton X-100 (nonionic)	10	57.61
Triton X-100 (nonionic)	50	22.05
SDS (anionic)	1	76.63
SDS (anionic)	5	54.49

4.7 Application of detergent containing cutinase for oily stain and fuzz removal from spun polyester fabric

4.7.1 pH of detergents

Seven commercial powder detergents (detergents A to G) were tested for their pH and the detergent with its pH closest to pH 9 was selected to be used with cutinase enzyme (its highest activity at pH 9). Results in **Table 4.7** show that these 7

detergents had pH values ranging from 9.75 (detergent A) to 10.60 (detergent G) and therefore the detergent A was selected for the study.

Table 4.7 pH of seven commercial powder detergents in water (1 g detergent/50 mL water).

Detergent	pH
A	9.75
B	9.80
C	10.04
D	10.06
E	10.34
F	10.51
G	10.60

4.7.2 Ratios of detergent to fabric to water for oily stain removal in terms of fabric's whiteness index and grey scale for staining

This test was conducted in order to determine for the lowest amount of detergent required for oily stain removal from fabric through one washing cycle. After 1.3 g of white fabric was stained with 0.5 mL of palm oil, it was then washed in water (10 mL) containing various amounts (0.02-0.20 g) of detergent (A) and its whiteness index and gray scale for staining rate were determined shown in **Table 4.8**. Prior to oil staining, white fabric had its whiteness index at 160 and grey scale for staining rate at 5. After oil staining, the fabric's whiteness index decreased to 129 and grey scale decreased to 4.0. When the oily stained fabric was one-cycle washed with various amounts of detergent, its whiteness index and grey scale were improved to 147-160 and 4.5-5.0 respectively, in which both values of fabric regained to its original values (whiteness index at 160 and grey scale at 5, before staining) when the oily stained fabric was washed with 0.2 g detergent. Therefore, the lowest amount of detergent for removing 0.5 mL oily stain from 1.3 g white fabric through one washing cycle was 0.2 g detergent in 10 mL water, or the ratio of detergent to fabric to water was 1 g: 6.5 g: 50 mL, respectively. This ratio was further used for the fuzz removal study.

Table 4.8 Whiteness index and grey scale for staining of white fabric before and after oil staining and washing 1 cycle with detergent (1.3 g fabric and 10 mL water).

Fabric	Whiteness index	Grey scale for staining
No stain	160	5.0
Staining	129	4.0
Staining/Washing 0.02 g det	147	4.5
Staining/Washing 0.05 g det	155	5.0
Staining/Washing 0.10 g det	158	5.0
Staining/Washing 0.15 g det	157	5.0
Staining/Washing 0.20 g det	160	5.0

det = detergent

4.7.3 Ratio of detergent to cutinase for fuzz removal from red fabric according to fabric's pilling rate, % weight loss, surface appearance, color values and strength loss

4.7.3.1 Pilling rate, % weight loss and surface appearance

Samples of detergent and cutinase mixed powder at various weight ratios of 1:0, 1:0.05, 1:0.10, 1:0.15 and 1:0.20 were prepared and used for hairs and pills removal from red fabric (with pilling rate of 1 or maximum hairs and pills) through washing. Results in **Table 4.9** indicated that the original fabric had a pilling rate of 5 with minimum hairs and pills. Once the fabric was spun in a standard pilling box for 4,500 revolutions at a constant speed of 30 rpm, significant amounts of hairs and pills took place on fabric surface which indicated the fabric's pilling rate of 1. After the fabric was washed with detergent only (detergent:cutinase at 1:0), fabric lost 0.73% of its weight before washing due to the removal of small amounts of hairs and pills through washing with detergent only. However, the pilling rate of fabric was unimproved and remained at 1. When the fabric was washed with detergent and cutinase mixed powder at the ratios of 1:0.05, 1:0.10, 1:0.15 and 1:0.20, the pilling rate of fabric was improved (from 1 of fabric washed with only detergent) to 1-2, 2 and 2-3, and the fabric weight loss increased (from 0.73% of fabric washed with only detergent) to 2.09-2.60%. At this moment, it could be concluded that the suitable

ratio of detergent to cutinase being used for hairs and pills removal from fabric through one washing cycle was at 1:0.10 (after washing, fabric showed pilling rate of 2-3 and weight loss of 2.60% or the highest hairs and pills removal from fabric). However, it was stated earlier that the acceptable pilling rate of fabric should be at least 3-4 for exporting apparel (see section 3.5.9.1). Therefore, more hairs and pills should be removed from fabric surface during washing with detergent and cutinase mixed powder at the ratio of 1:0.10, and this led to the conclusion that more mechanical action (abrasion) in washing should be added or more washing cycles should be used.

To add more mechanical action to accelerate the washing process, 10-40 stainless steel balls were added to the test. Results in **Table 4.9** showed that when fabric was washed with 1:0.10 detergent:cutinase and 20 stainless steel balls, the pilling rate of fabric was improved to an acceptable rate of 3-4 due to higher hairs and pills removal or higher % fabric weight loss of 2.78%. For a comparison, fabric was also washed with only detergent (no cutinase) and 10-40 stainless steel balls and found that the pilling rate of fabric was improved from 1 to 2 and 2-3, having not yet reached an acceptable rate of 3-4. In addition, % fabric weight loss was also low at the range of 1.36-2.60%. Therefore, it could be concluded that the optimal condition for hairs and pills removal from red fabric (pilling rate of 1) was to one-cycle wash fabric with detergent:cutinase 1:0.10 (weight ratio) and 20 stainless steel balls at room temperature (35 ^{o}C) for 1 hour. However, for a practical application of this washing condition, it was necessary to find out that one washing cycle with 20 stainless steel balls was comparable to how many normal washing cycles without the use of 20 stainless steel balls (there was no stainless steel ball in normal/typical home washing). **Table 4.9** showed that the pilling rate and the % weight loss of fabric after washing with detergent:cutinase 1:0.10 in the absence (8 washing cycles) and the presence of 20 stainless steel balls (1 washing cycle) were similar at the pilling rate of 3-4 and the % weight loss of 2.68% (absence of ball) and 2.78% (presence of 20 balls), respectively. Results from this study indicated that 8 washing cycles with no stainless steel ball (typical washing) were equaled to 1 washing cycle

with 20 stainless steel balls. Therefore, it could be concluded that the best practical washing condition for hairs and pills removal from this red fabric (pilling rate of 1, highest amount of hairs and pills) was to wash fabric with detergent:cutinase 1:0.10 (weight ratio) for at least 8 cycles (1 hour per cycle) at room temperature (35 °C). The after washed fabric would appear a smoother surface (image (d) in **Figure 4.13**) with acceptable pilling rate of 3-4 and small % weight loss of 2.78% (**Table 4.9**).

Table 4.9 Pilling rate and %weight loss of red fabric before and after washing 1 cycle.

Fabric washing conditions	**Pilling rate**	**% Weight loss**
Before pilling, no wash	**5**	-
After pilling, no wash	**1**	**0**
Washing with detergent	1	0.73
Washing with detergent : cutinase 1:0.05	1-2	2.09
Washing with detergent : cutinase 1:0.10	**2-3**	**2.60**
Washing with detergent : cutinase 1:0.15	2	2.52
Washing with detergent : cutinase 1:0.20	2	2.42
Washing with detergent : cutinase 1:0.10 and 10 SS balls	2	2.44
Washing with detergent : cutinase 1:0.10 and 20 SS balls (see image (d) in **Figure 4.13**)	**3-4**	**2.78**
Washing with detergent : cutinase 1:0.10 and 30 SS balls	2-3	2.16
Washing with detergent : cutinase 1:0.10 and 40 SS balls	2-3	2.59
Washing with detergent and 10 SS balls	2	1.89
Washing with detergent and 20 SS balls	2-3	1.36
Washing with detergent and 30 SS balls	2-3	2.37
Washing with detergent and 40 SS balls	2-3	2.60
Washing with detergent : cutinase 1:0.10 for 8 cycles	**3-4**	**2.68**

SS = stainless steel

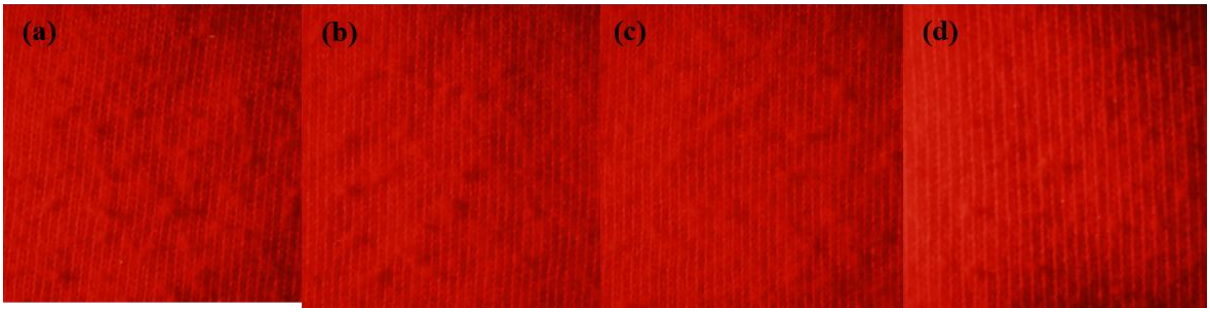


Figure 4.13 Optical microscope images of red-fabric's pills (a) after pilling/no wash, pilling rate 1, (b) after pilling/washing fabric 1 cycle with detergent, pilling rate 1, (c) after pilling/washing fabric 1 cycle with detergent and 20 stainless steel balls, pilling rate 2-3, (d) after pilling/washing fabric 1 cycle with detergent: cutinase 1:0.10 and 20 stainless steel balls, pilling rate 3-4.

4.7.3.2 Color values

The after washed red-fabric was also measured for color values and results were shown in **Table 4.10**. It showed color values of fabric after pilling and after some washing conditions using detergent and detergent containing cutinase, compared to color values of fabric before pilling. All fabrics in this table showed similar yellowish red color (L*, a* and b* $>$ 0). Pilling and washing actions did not change much of the lightness (L*) and color shade (a* and b*) of fabrics. It was found that fabric before pilling had the lowest color strength (K/S = 24.4) or the lightest color. Once the fabric was pilled to rating 1, its color strength significantly increased 3 units (K/S = 27.4) to a darker shade and showed a noticeable color difference of 1.82 (ΔE* $\geq$ 1, eyes detectable) when compared with the color of fabric before pilling (ΔE* = 0). As fabric with pilling rate 1 was washed with detergent as well as with detergent containing cutinase, the color strength (K/S) and color difference (ΔE*) decreased and the values were closer to those values of fabric before pilling (pilling rate 5, K/S = 24.4, ΔE* = 0) than to those values of fabric after pilling (no wash). This could be explained that washing fabric with detergent in the presence or absence of cutinase could promote the fabric pill removal and decrease the change of color appearance, with a higher efficiency of pill removal when detergent containing cutinase was used. It was clear to say that fabric washed with detergent: cutinase

1:0.10 (with and without 20 stainless steel balls) showed the lowest color strength and unnoticeable color difference ($\Delta E^* < 1$) and the values were the closest to those values of fabric before pilling, especially when washing fabric with detergent: cutinase 1:0.10 and 20 stainless steel balls. This could mean that an addition of cutinase enzyme into the commercial detergent could assist the pill removal efficiency of the detergent itself.

Table 4.10 Color values of red fabric before and after washing 1 cycle: lightness (L*), red+/green- (a*), yellow+/blue- (b*), color difference (ΔE^*) and color strength (K/S).

Fabric washing condition	L^*	a^*	b*	ΔE^*	K/S
Before pilling, no wash (pilling rate 5)	36.6	55.5	29.4	0	24.4
After pilling, no wash (pilling rate 1)	36.8	55.8	30.3	1.82	27.4
Washing with detergent (pilling rate 1)	37.1	56.4	30.5	1.51	26.8
Washing with detergent : cutinase 1:0.10 (pilling rate 2-3)	37.1	56.1	30.0	0.96	25.6
Washing with detergent : cutinase 1:0.10 and 20 SS balls (pilling rate 3-4)	37.0	55.6	29.8	0.59	25.2
Washing with detergent and 20 SS balls (pilling rate 2-3)	37.2	55.7	29.7	1.10	25.9

SS = stainless steel

4.7.3.3 Strength loss

The after washed red-fabric was tested for its bursting strength shown in **Table 4.11**. It was found that after washing fabric with only detergent, the fabric lost 3.5% of its strength due to the fiber loss (0.73% weight loss) upon washing. Also the fabric's strength loss of 8.3% was observed when detergent:cutinase 1:0.10 and 20 stainless steel balls were used for washing. However, polyester knitted fabric was considered to be one of the strongest fabrics in the world textile market. Its bursting strength was reported to be several times higher than fabrics made of viscose,

acrylic, as well as cotton fibers (Degirmenci and Coruh, 2017). Therefore, in this work the 8% bursting strength loss of polyester fabric after 8 normal washing cycles using detergent:cutinase 1:0.10 (equivalent to one accelerated washing cycle using detergent:cutinase 1:0.10 and 20 stainless steel balls) was not a big concern for textile users.

Unfortunately, there might be some errors taking place in bursting strength testing on some fabric samples such as the original fabric before pilling (no wash) and the fabric after washing with detergent and 20 stainless steel balls in which the former's bursting strength should be higher than the fabric after pilling (no wash) due to no fiber loss and the latter's bursting strength should be lower than the fabric after pilling (no wash) due to fiber loss during washing. One explanation to these errors might be because these two samples were tested at the different time and some different conditions (of testing equipment) from other fabrics which could have made some errors on the test results.

Table 4.11 Bursting strength and %strength loss of red fabric before and after washing 1 cycle.

Fabric washing condition	**Bursting strength (kPa)**	**Strength loss (%)**
Before pilling, no wash (pilling rate 5)	991	-
After pilling, no wash (pilling rate 1)	1010	0
Washing with detergent (pilling rate 1)	975	3.5
Washing with detergent and 20 SS balls (pilling rate 2-3)	1025	-1.5
Washing with detergent: cutinase 1:0.10 and 20 SS balls (pilling rate 3-4)	926	8.3

4.7.4 Number of washing cycle for oily stain removal from red fabric using detergent:cutinase 1:0.10 according to fabric's color values and grey scale for color change

From results in 4.6.3, detergent:cutinase 1:0.10 (weight ratio) could be used to remove hairs and pills from red fabric perfectly through washing at temperature 35 ℃ for 8 typical washing cycles (1 hour per cycle). However, the oily stain removability of the detergent:cutinase 1:0.10 had not been answered. To approach for answer, red fabric containing palm oil was washed with detergent and with detergent:cutinase 1:0.10 for various cycles until stain was completely removed from fabric and fabric's color values and grey scale for color change regained to its original values (fabric before pilling and before oily staining or fabric with no oil and its pilling rate at 5).

Tables 4.12 and **4.13** showed color values of red fabric after staining and washing with detergent alone and with detergent:cutinase 1:0.10 for several cycles, respectively. **Table 4.12** indicated that 3 washing cycles with detergent were required in order to completely remove oily stain from red fabric because the lightness (L*), red+/green- (a*), yellow+/blue- (b*), color difference (ΔE*), color strength (K/S), and grey scale for color change of the after washed fabric were the most similar or the nearest to the original values of the fabric before pilling and before oily staining. Similarly, **Table 4.13** showed that 3 washing cycles with detergent:cutinase 1:0.10 were also needed to perfectly remove oily stain from red fabric. Therefore, in terms of palm oil removal from red fabric, it was required to wash fabric with either detergent alone or detergent:cutinase 1:0.10 for at least 3 typical washing cycles. This had proved that only detergent played a major role on oil removal from fabric, while cutinase did play a significant role on hairs and pills removal as shown in 4.6.3.

Results from 4.6.3 and 4.6.4 suggested that detergent:cutinase 1:0.10 could be used to completely remove palm oil from red fabric via 3 washing cycles, while it could be used for hairs and pills removal via 8 washing cycles.

Table 4.12 Color values of red fabric after staining and washing with detergent: lightness (L*), red+/green- (a*), yellow+/blue- (b*), color difference (ΔE*), color strength (K/S), and grey scale for color change.

Fabric washing condition	L^*	a^*	b*	ΔE^*	K/S	Grey scale for color
Before staining	**36.6**	**56.4**	**29.6**	**0**	**26.6**	**5**
After staining	33.2	51.2	25.4	6.85	23.0	2.5
Washing with detergent 1 cycle	36.3	56.5	29.9	0.94	24.1	4.5
Washing with detergent 2 cycles	36.2	56.2	28.8	0.66	24.1	4.5
Washing with detergent 3 cycles	**36.8**	**56.4**	**29.6**	**0.27**	**25.9**	**5**

Table 4.13 Color values of red fabric after staining and washing with detergent:cutinase 1:0.10: lightness (L*), red+/green- (a*), yellow+/blue- (b*), color difference (ΔE*), color strength (K/S), and grey scale for color change.

Fabric washing condition	L^*	a^*	b*	ΔE^*	K/S	Grey scale for color
Before staining	**36.6**	**56.4**	**29.6**	**0**	**26.6**	**5**
After staining	33.2	50.2	24.6	8.03	23.0	2.5
Washing with detergent:cutinase 1:0.10 for 1 cycle	36.2	56.6	30.3	1.33	24.0	4.5
Washing with detergent:cutinase 1:0.10 for 2 cycles	36.4	56.6	29.8	0.92	25.9	5
Washing with detergent:cutinase 1:0.10 for 3 cycles	**36.2**	**56.3**	**29.6**	**0.14**	**27.6**	**5**

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Screening and identification of cutinase enzymes

Since chloramphenicol inhibited the growth of bacteria, while Rose Bengal reduced fungal growth, the screen culture using PCL as a selected substrate, as observed by clearing zone on LMM-PCL agar plates. This clear zone could be noticed in fast after 3 days of incubation. But each side of clear zone of isolates were no obvious correlation with enzyme activity production by the fungi.

However, the isolation in this study was based on the cutinase activity at alkaline range (pH 9.0), and many species in the genus *fusarium* do not display this property. Most of the funga considered in this study were isolated from crops field soil in Thailand. The finding of cutinase enzyme producing fungi in these areas was consort with the report by Nimchua et al. (2007) that *Fusarium* is found frequenctly in the selective isolation technique. A few of the more generally occurring *Penicillium* and *Aspergillus* species were isolated from soil and found to attack polycaprolactone.

The use of polycaprolactone (PCL) as a screening isolation for that the simple assays used to find those cutinase activity. PCL has been found to be degraded by many microorganisms, including fungi, bacteria and yeasts. The chemical structure of a PCL trimer, which are inducers of cutinase activity, was shown to be similar to that of two cutin monomers (Masayuki, 2001).

Cutinase secreted by several phytopathogenic fungi including *Fusarium* sp. can have low substrate specificity. Cutinase was induced by cutin or suberin (Pollard et al., 2008), and was also significantly influenced by nitrogen source (peptone) concentration in the medium (Dutta et al., 2013). On these general bases, PCL degraded was used as an primary screen to find fungal isolates producing enzyme that may be active on cutin.

5.2 Identification of the fungal *F. falciforme* PBURU-B5

Using bright field light microscope to observe morphology of PBURU-T5 grown on PCL agar for 3 days it showed typical fusaria characteristics (John & Roland, 2007; Sharma et al., 2006). It produced both macroconidia and microconidia. Macroconidia are hyaline and canoe-shaped in side view. Microconidia were ellipsoidal, produced from long monophialides in aerial mycelium. The colony grew to 2-3 cm diameter in 3 days and was whitish. On morphological grounds it was *Fusarium* sp. The sequence of the ITS region of rDNA gene of PBURU-T5 yielded sequences highly similar to *F. falciforme* sequences in Genbank, which submitted to GenBank-accession number MF373584.

5.3 Determination of growth and cutinase production

F. falciforme PBURU-T5 was grown until the stationary phase was reached (48 h) on liquid minimal medium. Cutinase activity increased to maximum value in the middle of the stationary phase and then declined rapidly. This result is rather different from that of *Lactobacillus casei* CL96 (Choi and Lee, 2001), however, maximum cutinase activity shown during the late stationary phase, it exhibited may be similarities with that of *A. niger* I-1472 (Astheret al., 2002), *Bacillus* sp.4 (Asther et al., 2002), *Propionibacterium freudenreichii* ssp., *freudenreichii* ITG14 (Kakariari et al., 2000) which exhibiting maximum activity in the mid-stationary phase and a rapid fall was followed.

5.4 Optimization on cutinase production

5.4.1 Nitrogen source

The effect of various nitrogen sources to produce cutinase shown that peptone was found the most suitable. This result was similar to influence of nitrogen source in liquid medium those reported for fungal production of cutinase enzymes in previous works; yeast extract has strong positive influence on production the fungus *Colletotrichum lindemuthianum* cutinase (Rispoli and Shah., 2007). Most studies were needed to measure on the effect of several nitrogen sources on cutinase-producing, to get cheap price nitrogen source when up to large scale production.

There are not many reports on production of cutinase on effect of various nitrogen sources, indicating that cutinase-producing this fungus is not dependent on the concentration of the nitrogen source.

5.4.2 Experimental design for optimal production using RSM

For the experiment of optimum level and combined effect of different variables (cutin, peptone, temperature and pH), a central composite design was used, and a second-order polynomial equation was derived to explain the dependence of cutinase production on medium components.

The maximum cutinase activity obtained by showing experiment was 4.15 U/mL, which was closely related to the predicted value, i.e., 3.82 U/mL calculated by ANOVA analysis. The formuation of the culture medium using experimental planning, the best result was obtained for cutinase production (4.15 U/mL) in liquid minimal medium containing substrate papaya cutin 2.5 g/L and peptone 4 g/L, initail pH 7.0 and optimum incubation for temperature 30 °C. This was done similar to using response surface methodology (RSM) for influence by the concentration of carbon and nitrogen source, culture pH, temperature (Du et al., 2007; Kumari et al., 2016). Cutinase production on the microorganism was the result of difference operation variable interaction and influence combination the parameters in the culture, thus it is worthwhile optimization culture conditions to using response surface methodology. Previous studied the cutinase production have been applied to optimized by using a central composite design and response surface methodology, the cutinase from *P. cepacia* NRRL B 2320 which resulted in two-fold increase enzyme activity and also show two-fold from *Fusarium oxysporum* with flax oil used as a substrate (Dutta et al., 2013).

5.5 Characterization of crude cutinase

5.5.1 Optimal pH and temperature of cutinase activity

The crude cutinase from *F. falciforme* PBURU-T5 had the optimal pH 9.0. This optimal pH similar to that reported in *F. solani pisi* (Petersen et al., 2001) for cutinase was optimal at an alkaline pH 8.5. Furthermore, the optimum pH for the hydrolysis of

PNB was reported for cutinase at pH 7.2 from *Fusarium oxysporum* (Pio & Macedo, 2007) or pH 8.5 from *Fusarium solani pisi* cutinase (Carvalho et al., 1999).

The cutinase from *F. solani* f. sp. pisi and. *F. roseum* had the optimum pH at 10.0 (Soliday and Kolattukudy, 1976; Trail and Koller, 1993). The *Fusarium* cutin esterase thus fits between the ranges of 9.0 to 10.0 of other microbial cutinases. Tris-HCl buffer was the appropriate buffer for the PBURU-T5 enzyme. Previous studied, no typical pH optimum could be observed for the enzyme from *F. falciforme* reported before.

Cutinase from *F. falciforme* PBURU-T5 was stable in a pH short range of 8.0-10.0 upon incubation at 35 ºC for 60 min. This was similar to *P. putida* (Sebastian and Kolattukudy, 1988) and *F. roseum* (Soliday and Kolattukudy, 1976) which were stable in the pH ranges of 8.5-10.5 and 8.0-11.0, respectively. Though different from that of *F. solani* PBURU-B5 cutinase (Nimchua, 2007), was stable in a wide pH range of 6.0-10.0. The stability is highly dependent on the specific enzyme considered different cutinases showed different pH stability, such as cutinase stable in pH range 4.0 to 9.0 for *F. solani pisi* (Petersen et al., 2001).

The optimum temperature of cutinase from *F. falciforme* PBURU-T5 was 35 ºC. For previous studied cutinases was working in the range of optimum temperature from 40-50 ºC (Carvalho et al., 1998). Most fungal cutinases (*F. solani*, *F. solani pisi* and *F. oxysporum*) working in the range optimum temperature around 35-50 ºC.

5.5.2 Thermal stability

The *F. falciforme* PBURU-T5 was stable from 35-40 ºC for 2 hours when it was assayed for enzyme activity. This result was similar to the effect of those reported for other fungal cutinase enzymes in previous works (Peterson et al., 2001; Pio and Macedo, 2007; Chen et al., 2008; Kwon et al., 2009). Fungal cutinases (*F. roseum culmorum, Fusarium solani pisi, F. roseum sambucinum* and *Colletotricum gloeosporoides*) most optimum are unstable above 40 ºC and the relative activities were decreased to 20% in 1 h at 60 ºC (Sebastian et al., 1987).

5.5.3 pH stability

After incubation at temperature 35 °C, the *F. falciforme* PBURU-T5 cutinase was stable in a mildly alkaline condition (pH 8.0-9.0) when it was assayed. This was similar to the pH stability of enzymes from the following microorganisms; *Fusarium solani pisi* (pH 8.5) (Petersen et al., 2001), *Aspergillus oryzae* (pH 6.0-11.0) (Meada et al., 2005), *Fusarium solani* PBURU-B5 (pH 6.0-10.0) (Nimchua et al., 2008), The crude cutinase of soybean from *Fusarium oxysporum* (6.0-8.5) (Speranza et al., 2011), monomer of the fatty acid was easily released from the cutin substrate by alkaline degradation compared to the other pH lower than 11.0.

5.5.4 Effect of metal ions on cutinase activity

When cutinase from *F. solani* PBURU-B5 was tested for the effects of metal ions, 10 mM of divalent metal ions (Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+}) caused inhibition. The cutinase activity was reduced more 30% when the high concentration of these metal ions was presented. When they were incubated with 1 mM of these divalent metal ions, Mg^{2+}, Hg^{2+} or Fe^{2+} did not exhibit a significant effect on the enzyme activity, whereas Ca^{2+}, Cu^{2+}, Mn^{2+} or Zn^{2+} showed a medium inhibitory effect. Several divalent metal ions have been reported to have different effects on the cutinase activity in several microorganisms. Inhibitory effect by the presence of Hg^{2+} for cutinase activity produced by *Fusarium oxysporum* are consistent with previous report (Speranza et al., 2011), and by *F. solani pisi* cutinase (Chen et al., 2010). In the presence of 10 mM or 50 mM of EDTA, showed inhibitor effect by decrease the activity of cutinase. An inhibitory effect by presence of some metal ions, advising that the enzyme displayed metal ion dependence on its character of metallo-protein or its active site.

5.5.5 Effect of surfactants on cutinase activity

The activity of the cutinase was tested in the presence of the nonionic surfactants (Triton X-100 and Tween 20) and the anionic surfactants (SDS). SDS did not affected the enzyme activity at a concentration of 1 mM in the reaction mixture, whereas Tween 20 and Triton X-100 slightly decrease the cutinase activity at both 1

and 10 mM. A nearly observation have been reported by Chen (2010) for *Thermobifida fusca* cutinase. A novel cutinase from *F. falciforme* PBURU-T5 with high surfactant tolerance, that the enzyme is more suitable for sone kind of detergent formulations.

5.6 Application of detergent containing cutinase for oily stain and fuzz removal from spun polyester fabric

In this work, seven commercial powder detergents were tested for pH and detergent A was selected with its pH of 9.75. Various amounts of this detergent (0.02-0.20 g detergent/1.3 g fabric/10 mL water) were used for washing in order to determine for the lowest amount of detergent for removing oily stain from fabric through one washing cycle. It was found that 0.2 g detergent in 10 mL water was needed for washing 1.3 g fabric containing 0.5 mL palm oil, or the ratio of detergent to fabric to water was 1 g: 6.5 g: 50 mL, respectively. This ratio was further used for the fuzz removal study.

Samples of detergent and cutinase mixed powder at various weight ratios of 1:0, 1:0.05, 1:0.10, 1:0.15 and 1:0.20 were prepared and used for hairs and pills removal from fabric (with pilling rate of 1 or maximum hairs and pills) through washing. It was found that the suitable ratio of detergent to cutinase being used for hairs and pills removal from fabric should be at 1:0.10. However, to obtain the perfect removal of hairs and pills from fabric through washing with detergent:cutinase 1:0.10, it required 8 typical washing cycles. The after washed fabric had an acceptable pilling rate of 3-4, small % weight loss of 2.68%, smoother surface, similar color values to the original fabric, and 8.3% strength loss.

To study for the oily stain removability of the detergent:cutinase 1:0.10, fabric containing palm oil was washed with detergent and with detergent:cutinase 1:0.10 for various cycles until stain was completely removed from fabric. Results indicated that it was necessary to wash fabric with either detergent alone or with detergent:cutinase 1:0.10 for at least 3 typical washing cycles in order to remove oily stain. In addition, it was found that only detergent played a major role for oil removal from fabric, while cutinase did play a significant role on hairs and pills removal. The detergent:cutinase

1:0.10 could be used to completely remove palm oil from fabric via 3 washing cycles, while it could be used for hairs and pills removal via 8 washing cycles.

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APPENDICES

Appendix A

Culture Medium and Preparation

1. Liquid mineral medium (LMM)

$(NH_4)_2SO_4$	2 g
KH_2PO_4	4 g
Na_2HPO_4	6 g
$MgSO_4$	0.2 g
$FeSO_4.7H_2O$	1 mg
$CaCl_2$	1 mg
H_3BO_3	10 mg
$MnSO_4$	10 mg
$ZnSO_4$	70 mg
$CuSO_4$	50 mg
MoO_3	10 mg

Dissolved in distilled water to final volume 1 liter. pH was adjusted to 7.0.

2. Polycaprolactone (PCL) agar

Polycaprolactone	0.5 g
Agar	15 g

Dissolved PCL and agar in LMM, made pH to 7.0 and to 1 liter by LMM.

3. Cutin powder

Papayas purchased from the local market were used to extract cutin. The peel of papaya fruits was boiled in oxalate buffer solution (4 g of oxalic acid and 16 g of ammonium oxalate per liter and pH was adjusted to 3.2) for 3-4 hours or until these became fully devoid of pulp. The peels were filtered, washed and dried at 60 °C, extracted with chloroform–methanol (2:1, v/v) overnight with mild stirring and vacuum dried. A second extraction was done with chloroform in a Soxhlet apparatus for 24 h. Dried pellet was crushed and ground to powder form and treated with

cellulase and pectinase (Macedo and Pio, 2005) in pH 4.0 acetate buffer (50 mM) for 48 hours cutin was recovered by filtration. The fine powder was used as substrate (carbon source) in subsequent experiments. The Fourier transform infrared spectroscopy (FTIR) was carried out on Perkin Elmer's Table top Spectrum One FTIR spectrometer. Finely cutin powder was ground to avoid their interference in IR spectrum and dried prior to IR studies. Data were recorded with 4.0 cm^{-1} resolution in a transmittance mode at room temperature (28 ± 2 °C) within analytical range of 4000–700 cm^{-1}, and a spectral scan were collected. Bands were identified by comparison with earlier published assignments

Figure 1A. Soxhlet extraction devices

Table 1A Weight of crude cutin extraction from Papaya peels

Fruit:	Amount of peel (g)	A: Dried weight after treated oxalate buffer (g)	B: Dried weight after treated enzyme (g)	Ratio B: A	Percentage extraction of cutin (%)	Color of the resulting powder
Papaya peels	2000	86.12	33.05	0.3837	38.37	brown

*Cutin extraction ~0.02% of Papaya peels

Figure 2A. Crude cutin powder (papaya peels)

4. Potato dextrose agar (PDA)

glucose	20 g
Infusion from potato	1,000 mL
Agar	15 g

Potato infusion was prepared by boiling scrubbed and slice potatoes (200 g) in 1,000 mL distilled water for 1 h and then passing through fine sieve.

5. Potato dextrose broth (PDB)

Glucose	20 g
Infusion from potato	1,000 mL

6. Production medium

Cutin	2.5 g/L
Peptone	4 g/L

Dissolved in LMM 1,000 mL

Appendix B

Reagents

1. DNA Extraction (Saghai-Maroof et al., 1984)

A. Tris-HCl buffer (0.1 M, pH 7.5)

Tris-Base	6.1 g
Distilled water	800 mL

Set pH to 7.5 with conc. HCl and adjusted the volume to 1,000 mL by distilled water.

B. 2x Hexadecyltrimethy ammonium bromide (CTAB) buffer

1 M Tris-HCl, pH 8.4	10 mL
5 M NaCl	28 mL
0.5 M EDTA, pH 8.0	5 mL
CTAB	2 g
dH_2O to	100 mL

Dissolved by gentle heating

C. 10% Sodium dodesyl sulphate (SDS)

SDS	10.0 g
Distilled H_2O to	100 mL

D. TE buffer, pH 8.0

1 M Tris-HCl, pH 8.0	1000 µl
0.5 M EDTA, pH 8.0	200 µl
dI H_2O	100 mL

E. Polymerase chain reaction (PCR) condition

5x PCR buffer	10 µl
Forward primer (10 mM)	5.0 µl
Reward primer (10 mM)	5.0 µl

Taq DNA polymerase	0.5 µl
DNA template	2 µl
dI H_2O (to 50 µl)	27.5 µl

F. 5X Electrophoresis buffer, 1 litre

TrisBase	15.1 g
Glycine	71.3 g
SDS	2.5 g

Dissolve in distilled water to 1 liter. Do not adjust pH with acid or base (Final pH should be 8.3).

G. Buffer solution

Make up the following solutions

(1) 0.1 M acetic acid

(2) 0.1 M sodium acetate (tri-hydrate) (13.6 g/L)

Mix in the following proportions to get the required pH

pH	Vol. of 0.1 M acetic acid	Vol. of 0.1 M sodium acetate
3	982.3 mL	17.7 mL
4	847.0 mL	153.0 mL
5	357.0 mL	643.0 mL
6	52.2 mL	947.8 mL

Here, the primary salt is a solid and is weighed out in grams.

A measured amount of 0.1 M HCl or NaOH is added and made up to 1 liter to give the required pH.

pH	Salt mixture Dilute each mixture to 1-liter solution with distilled water
6	6.81 g potassium dihydrogen phosphate and 56 mL of 0.10 M NaOH
7	6.81 g potassium dihydrogen phosphate and 291 mL of 0.10 M NaOH
8	6.81 g potassium dihydrogen phosphate and 467 mL of 0.10 M NaOH
8	4.77 g sodium tetraborate and 26 mL of 0.10 M HCl
9	4.77 g sodium tetraborate and 46 mL of 0.10 M HCl
10	4.77 g sodium tetraborate and 183 mL of 0.10 M NaOH
11	2.10 g sodium bicarbonate and 227 mL of 0.10 M NaOH

Appendix C

Standard curve

Figure 1C. Stardard curve for protein determination by Bradford's assay

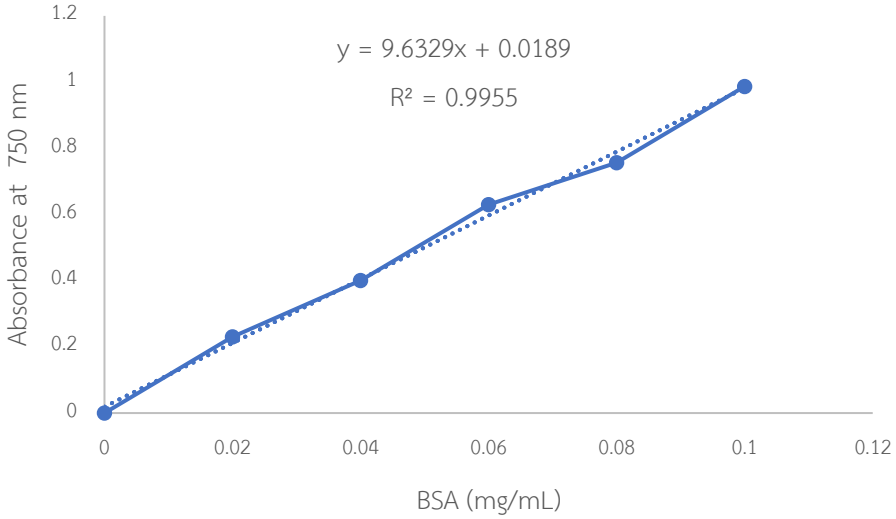
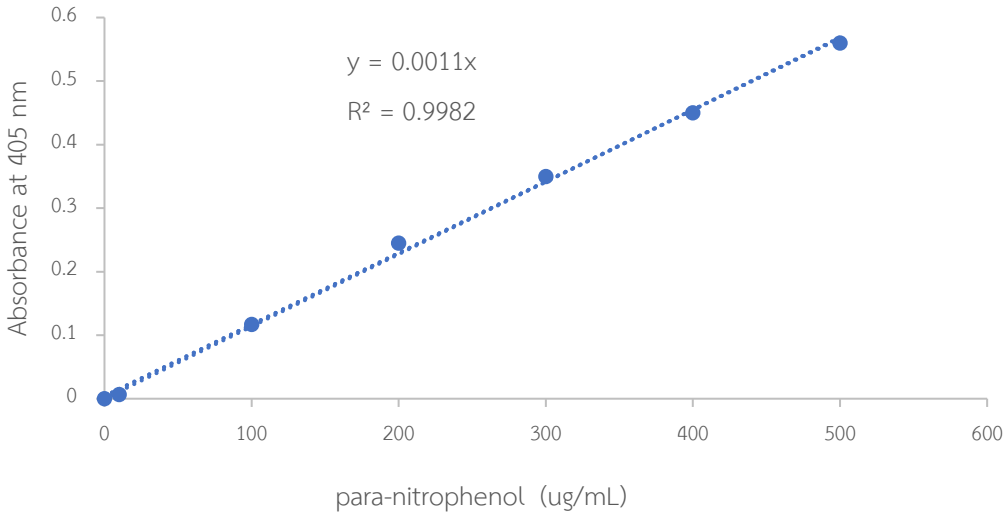


Figure 2C. Stardard curve for *p*-nitrophenol determination by cutinase assay using *p*-nitrophenylbutyrate



Appendix D

Calculation

1. Dry weight of the fungus and production of biomass

Mycelium of the fungal was cropped after every 1 day of incubation on growth, use a whatman No. 1 filter paper to separate the culture liquid by filtration on erlenmeyer flask. Wash the mycelial pellet with distilled water by repeatedly and overnight dried at 60 ℃ until weight of mass cell stable. The dry weight of the fungus was calculated by the following formula:

$$\text{Dry weight, (mg /L)} = \frac{(A - B) \times 1000}{\text{Volume of sample, mL}}$$

A = (weight of filter paper + dried mycelium)

B = (weight of filter paper)

The standard curve was prepared using the data collected above.

2. Esterase activity calculation

A solution of 0.1 mM *p*-nitrophenyl butyrate (*p*-NPB) dissolved in absolute ethanol was used as substrate. The reaction mixture consisted of 0.1 M Tris-HCl buffer (pH 9.0), 800 µl, 0.3% triton x-100, 100 µl, crude enzyme solution, 100 µl and 0.1 mM *p*-NPB, 500 µl. One-cm quartz cuvette was used for the measurements on a spectrophotometer.

$$\text{Unit of enzyme} = \frac{(\text{Abs of sample} - \text{Abs of blank})}{0.0011} \times \frac{1}{139.11(\text{mw}) \times 10\ (\text{T}) \times 0.1(\text{V})}$$

Remark: (Abs of sample – Abs of blank) = the being measured absorbance at 405 nm

Mw = molecular weight of *p*-Nitrophenylbutarate

T = the reaction time (min)

V = the volume of crude enzyme supernatant (mL), or the weight of crude enzyme powder (mg)

An enzyme unit (1 U) was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute at pH 9.0 and 35 ℃.

APPENDIX E

Supporting data

Figure 1E The Fourier transform infrared spectroscopy (FTIR) of crude cutin powder from papaya peels

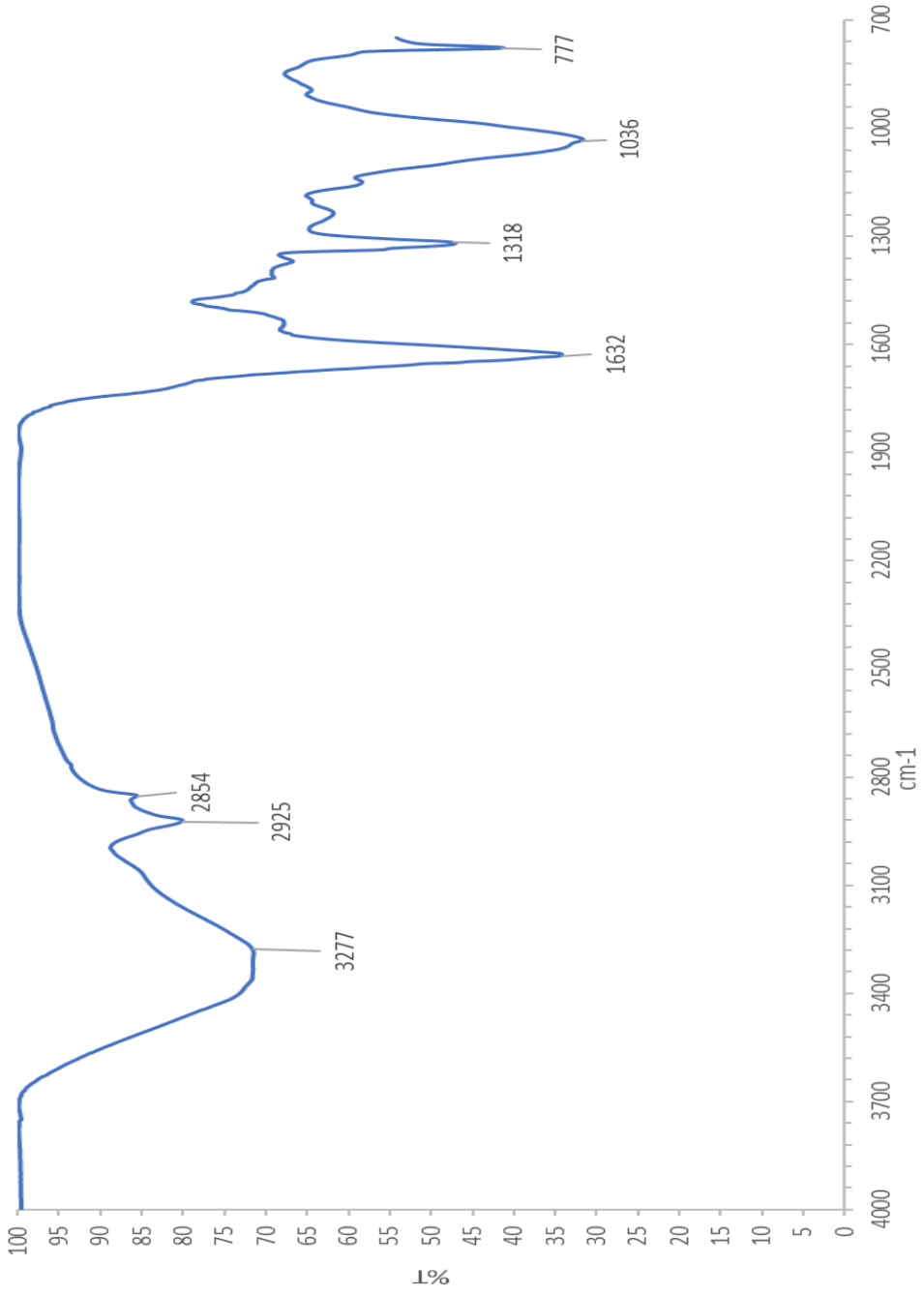


Table 1E Major FTIR spectra of crude cutin powder from papaya peels

Frequency (cm^{-1})	Assignment	Comments	Cuticle component
3277b	ν(O-H···O)	Retching vibration of hydrogen-bonded hydroxyl functional groups of alcohol and acids	Cutin
2925m	$\nu_{as}(CH_2)$	Asymmetric stretching vibration of methylene group, Aliphatic stretch, most repeated structural unit in cutin polymer	Cutin, waxes
2854sh	$\nu_{s}(CH_2)$	Symmetric stretching vibration of methylene groups, Aliphatic stretch, most repeated structural unit in cutin polymer	Cutin, waxes
1632w	ν(C=C)	Stretching of aromatic (conjugated (C=C))	Phenolic compounds
1318w	$\delta(CH_2)$	CH_2 scissoring of alkanes	Cutin, waxes
1036w	δ(O-H), ν(C-O-C)	Stretching of primary and secondary alcohols, glycosidic groups	Cutin
777w	$\delta(CH_2)$ $\gamma(CH_2)$	C-H bending and C-C out-of-plane bending vibration in the aromatic ring	Aromatics

ν: stretching, δ: bending, γ: out-of-plane bending, as: asymmetric, s: symmetric.

s: strong, m: medium, w: weak, vw: very weak, b: broad, sh: shoulder

1. Measuring pH of Soils

Air-dried the soil sample after that passed soil through a 2-mm sieve.

- Ratio of soil: liquid mixture usually in ratio 1: 1 or in ratio 1: 5 for clay soils with high organic matter.
- Add water (distilled or de-ionized water) to each beaker using a pipette or cylinder.
- Glass rod stirring in mix solution and let the soil solution sample sit for a half of hour.
- Calibrate the pH meter following to the work instructions. It is should to calibrate with at least two buffer solutions (pH 4.0, pH 7.0 and pH 9.0).
- Stir the sample again immediately before measuring the pH. Do not place the electrode(s) directly in the soil layer at the bottom of the cup. The electrode(s) should be measure in the solution that above the soil layer. Sometimes measurements triplicate in scientific experiment.
- Record pH.
- Properly triple rinse electrode(s) with distilled or de-ionized water after each use and before testing another sample.

Table 2E Samples collected several vicinities in Thailand

Sample	Location	Source	Collection Date	pH of soils
1	Bangkok	*Solanum melongena*	June 2016	-
2	Bangkok	*Solanum melongena*	June 2016	-
3	Bangkok	*Cucumis sativus*	June 2016	-
4	Bangkok	*Cucumis sativus*	June 2016	-
5	Phrae	*Carica papaya*	September	-
6	Phrae	*Carica papaya*	September	-
7	Nakhonsawan	Cane field Soil	January 2016	7.85
8	Nakhonsawan	Cane field Soil	January 2016	7.77
9	Nakhonsawan	Cane field Soil	January 2016	7.78
10	Lopburi	Green beans field Soil	March 2016	7.69
11	Lopburi	Green beans field Soil	March 2016	7.79
12	Lopburi	Green beans field Soil	March 2016	7.74
13	Lopburi	Green beans field Soil	March 2016	7.74
14	Lopburi	Green beans field Soil	March 2016	7.61
15	Lopburi	Green beans field Soil	March 2016	7.61
16	Lopburi	Asparagus field Soil	March 2016	7.81
17	Lopburi	Asparagus field Soil	March 2016	7.80
18	Kanchanaburi	Green beans field Soil	April 2016	7.67
19	Chainat	Pomelo field Soil	April 2016	7.76
20	Nakhonsawan	Green beans field Soil	April 2016	7.53
21	Suratthani	Soil in forest	July 2016	7.43
22	Suratthani	Soil in forest	July 2016	7.43

Table 2E Samples collected several vicinities in Thailand (continue)

Sample	Location	Source	Collection Date	pH of soils
23	Suratthani	Bamboo field Soil	July 2016	6.76
24	Bangkok	*Solanum lycopersicum*	August 2016	-
25	Bangkok	*Solanum lycopersicum*	August 2016	-
26	Chainat	Green beans field Soil	April 2016	7.54
27	Chainat	Green beans field Soil	April 2016	7.54
28	Chainat	Green beans field Soil	September 2016	7.53
29	Chainat	Palm field Soil	September 2016	7.7
30	Chainat	Palm field Soil	September 2016	7.7
31	Chainat	Cassava field Soil	September 2016	7.9
32	Chainat	Cassava field Soil	September 2016	7.9
33	Chainat	Green beans field Soil	September 2016	7.65
34	Chainat	Green beans field Soil	September 2016	7.69
35	Chainat	Green beans field Soil	September 2016	8.02
36	Kanchanaburi	Herb field Soil	March 2016	8.54
37	Kanchanaburi	Herb field Soil	March 2016	7.61
38	Kanchanaburi	Asparagus field Soil	September 2016	7.63
39	Kanchanaburi	Asparagus field Soil	September 2016	7.64
40	Lopburi	Green beans field Soil	July 2016	7.66
41	Lopburi	Green beans field Soil	July 2016	7.66
42	Lopburi	Green beans field Soil	July 2016	7.64
43	Nakhonsawan	Cane field Soil	June 2016	7.67
44	Nakhonsawan	Cane field Soil	June 2016	7.73

Table 3E Nucleotide sequence of ITS rRNA gene of *Fusarium* sp. PBURU-T5

Identical strain	Nucleotide sequence (5' to 3')
Fusarium falciforme PBURU-T5	CGTGGCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCTACTACGCAATGGA AGCTGCGGCGGGACCGCCACTGTATTTGGGGGACGGCGTTGTGCCCGCAG GGGGCTTCCGCCGATCCCCAACGCCAGGCCCGGGGGCCTGAGGGTTGTAA TGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCG TTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCA TTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAG TTTTAATTCACCTGCTTGTTTACTC

Figure 2E Sequence producing alignment

The sequence was submitted in genbank www.ncbi.com. The result of sequence alignment with accession number MF373584.1

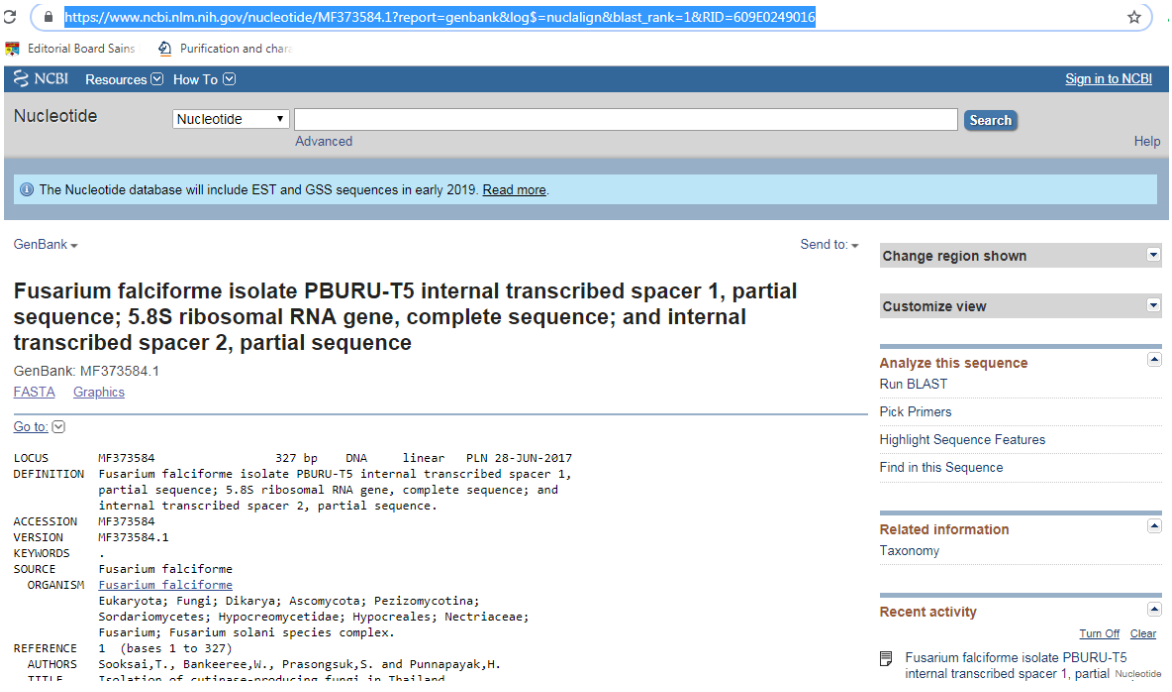


Figure 3E Test for bursting strength by THTI

(a) Report details test in spun polyester fabric

F-017E Rev.17, 1 เม.ย. 57, 1/1

THTI
THAILAND TEXTILE INSTITUTE
สถาบันพัฒนาอุตสาหกรรมสิ่งทอ

Foundation for Industrial Development
Thailand Textile Institute / Textile Testing Center
Soi Trimit, Rama 4 Road, Phrakanong, Klong-toey, Bangkok 10110, THAILAND.
Tel: (66) 2713 5492-9 Fax: (66) 2712 4527 www.thaitextile.org

TEST REPORT

CLIENT:	DEPARTMENT OF BOTANY FACULTY OF	REPORT NUMBER :	R 0071/60
	SCIENCE CHULALONGKORN UNIVERSITY	APPLICATION FORM No. :	29536
	254 PHAYATHAI ROAD, WANGMAI,	ISSUE DATE :	24/03/17
	PATHUMWAN BANGKOK 10330	PAGE :	1/2
DATE OF RECEIPT:	17/03/17		
DATE OF TEST:	21/03/17-24/03/17		

SAMPLE NUMBER	SAMPLE NAMES / DESCRIPTION (AS SPECIFIED BY THE CLIENT)
R 0071-1/60	SPUN POLYESTER
	FIBER CONTENT: POLYESTER

TEST RESULT(S): REFER TO THE FOLLOWING PAGE(S)

COMMENT(S): BASED ON THE SUBMITTED SAMPLE(S) AND THE TEST RESULT(S)

BURSTING STRENGTH : S

S = SEE THE ATTACHED RESULT(S)

AUTHORIZED BY

TIPAWAN P.

(MRS. TIPAWAN PANITCHAKARN)
(TEXTILE AND CHEMICAL ANALYSIS LABORATORY MANAGER)

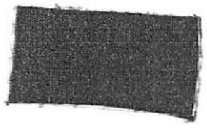
130148

(b) Report test bursting strength of spun polyester fabric non-pilling (5 replicate)

BURSTING STRENGTH TEST (BST)

Company: THAILAND TEXTILE INSTITUTE

Operator :



TEST RESULTS: Bursting Strength (Manual Mode)			Date: 01.01.2002	Time: 00:12
Specimen size: 30.5mm, 7.3cm2	Comment: R0071-1/60 ASTM D3786			
Specimen No.	Flowrate (cm3/min)	Time (Sec.)	Distension (mm)	Pressure (kPa)
1	95.0	12.2	12.1	993.7
2	95.0	14.2	16.6	1075.2
3	95.0	14.1	11.9	987.3
4	95.0	13.8	11.9	935.3
5	95.0	13.6	12.0	964.1
Mean after diaphragm correction	95.0	13.6	12.9	991.1
Sample Ref. : R0071/60	File: C:\Documents and Settings\Administrator\Desktop\23-3-2560\R0071-1-60.bst			

TEST RESULTS: Bursting Strength (Manual Mode)						Date: 01.01.2002	Time: 00:12
Specimen size: 30.5mm, 7.3cm2	Comment: R0071-1/60 ASTM D3786						
STATISTICS (No. of specimens = 5)							
	Mean	Min	Max	Range	Std. Dev.	C.V.%	95% Conf. Lim.
Time (sec)	13.6	12.2	14.2	2.0	0.8	5.9	12.6 - 14.6
Distension (mm)	12.9	11.9	16.6	4.7	2.1	16.0	10.3 - 15.4
Pressure (kPa)	991.1	935.3	1075.2	139.9	52.3	5.3	926.2 - 1056.1
Sample Ref. : R0071/60	File: C:\Documents and Settings\Administrator\Desktop\23-3-2560\R0071-1-60.bst						

(c) Report test bursting strength of spun polyester fabric after pilling rate 1 (5 replicate)

BURSTING STRENGTH TEST (BST)

Company: THAILAND TEXTILE INSTITUTE

Operator :

TEST RESULTS: Bursting Strength (Manual Mode)			Date: 16.05.2017	Time: 14:17
Specimen size: 30.5mm, 7.3cm2	Comment: R0084-1/60 ASTM D3786 Pill G 1			
Specimen No.	**Flowrate (cm3/min)**	**Time (Sec.)**	**Distension (mm)**	**Pressure (kPa)**
1	95.0	16.6	13.0	1011.3
2	95.0	16.8	13.1	934.5
3	95.0	19.2	13.7	1073.6
4	95.0	16.1	13.1	905.8
5	95.0	16.5	13.3	1126.4
Mean after diaphragm correction	**95.0**	**17.1**	**13.2**	**1010.3**
Sample Ref. : R0084-1/60	File: D:\Bursting 2560\16-5-2560\R0084-1-60.bst			

TEST RESULTS: Bursting Strength (Manual Mode)					Date: 16.05.2017	Time: 14:17
Specimen size: 30.5mm, 7.3cm2	Comment: R0084-1/60 ASTM D3786					
STATISTICS (No. of specimens = 5)						
	Mean	**Min**	**Max**	**Range**	**Std. Dev.**	**C.V.%**	**95% Conf. Lim.**
Time (sec)	**17.1**	**16.1**	**19.2**	**3.1**	**1.2**	**7.2**	**15.5 - 18.6**
Distension (mm)	**13.2**	**13.0**	**13.7**	**0.7**	**0.3**	**2.1**	**12.9 - 13.6**
Pressure (kPa)	**1010.3**	**905.8**	**1126.4**	**220.6**	**92.4**	**9.1**	**895.6 - 1125.1**
Sample Ref. : R0084-1/60	File: D:\Bursting 2560\16-5-2560\R0084-1-60.bst						

(d) Report test bursting strength of spun polyester fabric after washing with detergent A alone (5 replicate)

BURSTING STRENGTH TEST (BST)

Company: THAILAND TEXTILE INSTITUTE

Operator :

TEST RESULTS: Bursting Strength (Manual Mode)			Date: 16.05.2017	Time: 14:24
Specimen size: 30.5mm, 7.3cm2	Comment: R0084-2/60 ASTM D3786			
Specimen No.	**Flowrate (cm3/min)**	**Time (Sec.)**	**Distension (mm)**	**Pressure (kPa)**
1	95.0	15.4	13.3	1058.5
2	95.0	16.1	12.5	873.0
3	95.0	16.0	12.9	960.9
4	95.0	16.4	13.2	1023.3
5	95.0	16.5	13.3	958.5
Mean after diaphragm correction	**95.0**	**16.1**	**13.0**	**974.8**
Sample Ref. : R0084-2/60	File: D:\Bursting 2560\16-5-2560\R0084-2-60.bst			

TEST RESULTS: Bursting Strength (Manual Mode)					Date: 16.05.2017	Time: 14:24
Specimen size: 30.5mm, 7.3cm2	Comment: R0084-2/60 ASTM D3786					
STATISTICS (No. of specimens = 5)						
	Mean	**Min**	**Max**	**Range**	**Std. Dev.**	**C.V.%**	**95% Conf. Lim.**
Time (sec)	**16.1**	**15.4**	**16.5**	**1.1**	**0.4**	**2.8**	**15.5 - 16.6**
Distension (mm)	**13.0**	**12.5**	**13.3**	**0.8**	**0.4**	**2.7**	**12.6 - 13.5**
Pressure (kPa)	**974.8**	**873.0**	**1058.5**	**185.5**	**71.0**	**7.3**	**886.7 - 1063.0**

(e) Report test bursting strength of spun polyester fabric after washing with detergent A and added 20 stainless steel balls (5 replicate)

BURSTING STRENGTH TEST (BST)

Company: THAILAND TEXTILE INSTITUTE

Operator :

TEST RESULTS: Bursting Strength (Manual Mode)			Date: 16.05.2017	Time: 14:53
Specimen size: 30.5mm, 7.3cm2	Comment: R0084-3/60 ASTM D3786			
Specimen No.	**Flowrate (cm3/min)**	**Time (Sec.)**	**Distension (mm)**	**Pressure (kPa)**
1	95.0	15.9	14.6	1066.4
2	95.0	17.0	13.7	1017.7
3	95.0	17.3	14.5	1060.1
4	95.0	17.4	14.5	1077.6
5	95.0	16.9	14.0	904.2
Mean after diaphragm correction	**95.0**	**16.9**	**14.3**	**1025.2**
Sample Ref. : R0084-3/60	File: D:\Bursting 2560\16-5-2560\R0084-3-60.bst			

TEST RESULTS: Bursting Strength (Manual Mode)			Date: 16.05.2017	Time: 14:53
Specimen size: 30.5mm, 7.3cm2	Comment: R0084-3/60 ASTM D3786			
STATISTICS (No. of specimens = 5)				
	Mean	**Min**	**Max**	**Range**	**Std. Dev.**	**C.V.%**	**95% Conf. Lim.**
Time (sec)	**16.9**	**15.9**	**17.4**	**1.5**	**0.6**	**3.5**	**16.2 - 17.7**
Distension (mm)	**14.3**	**13.7**	**14.6**	**0.9**	**0.4**	**2.6**	**13.8 - 14.7**
Pressure (kPa)	**1025.2**	**904.2**	**1077.6**	**173.5**	**71.4**	**7.0**	**936.6 - 1113.8**

(f) Report test bursting strength of spun polyester fabric after washing with detergent A: cutinase at ratio of 1:0.1 and added 20 stainless steel balls (5 replicate)

BURSTING STRENGTH TEST (BST)

Company: THAILAND TEXTILE INSTITUTE

Operator : [redacted]

TEST RESULTS: Bursting Strength (Manual Mode)			Date: 16.05.2017	Time: 15:02
Specimen size: 30.5mm, 7.3cm2	Comment: R0084-4/60 ASTM D3786			
Specimen No.	Flowrate (cm3/min)	Time (Sec.)	Distension (mm)	Pressure (kPa)
1	95.0	15.8	13.8	900.2
2	95.0	17.5	14.4	988.1
3	95.0	17.3	13.9	925.7
4	95.0	17.1	13.6	892.2
5	95.0	26.4	14.1	921.7
Mean after diaphragm correction	95.0	18.8	14.0	925.6
Sample Ref. : R0084-4/60	File: D:\Bursting 2560\16-5-2560\R0084-4-60.bst			

TEST RESULTS: Bursting Strength (Manual Mode)			Date: 16.05.2017	Time: 15:02
Specimen size: 30.5mm, 7.3cm2	Comment: R0084-4/60 ASTM D3786			
STATISTICS (No. of specimens = 5)				

	Mean	Min	Max	Range	Std. Dev.	C.V.%	95% Conf. Lim.
Time (sec)	18.8	15.8	26.4	10.6	4.3	22.8	13.5 - 24.1
Distension (mm)	14.0	13.6	14.4	0.8	0.3	2.1	13.6 - 14.3
Pressure (kPa)	925.6	892.2	988.1	95.9	37.7	4.1	878.8 - 972.4

APPENDIX F

Stability of enzyme

Procedure

Samples of 0.02 g powder cutinase alone and 0.2 g powder detergent mixed with 0.02 g powder cutinase (1:0.1 optimal ratio of detergent to cutinase for washing and depilling 1.3 g fabric) were prepared and stored in a 1.5 mL eppendorf tube at 30 ℃ for 75 days. Every 15 days of storage, both samples were analyzed for enzyme activities and the remaining enzyme activity (%) was calculated. Each samples were carried out in triplicate.

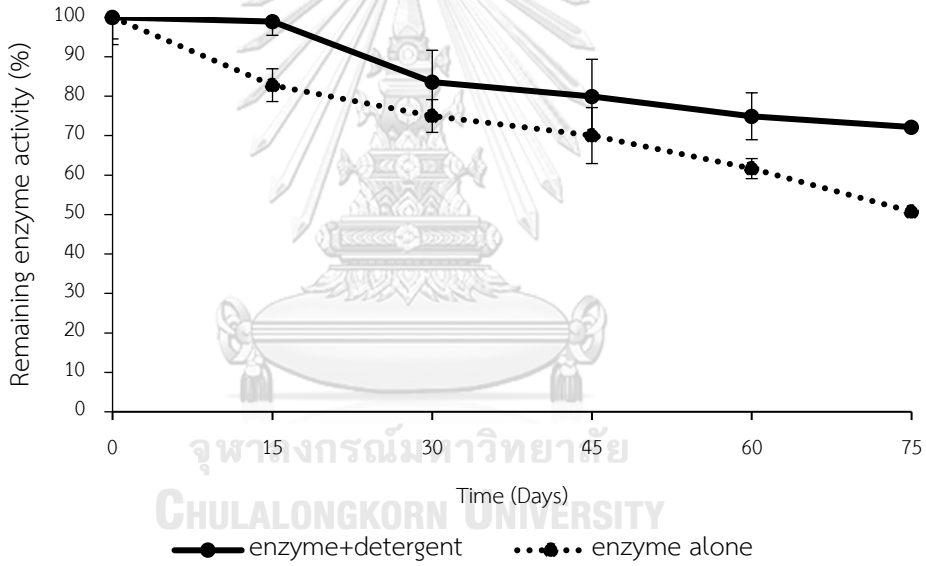


Figure 1E. Remaining enzyme activity (%) and time of storage (days) of 0.02 g cutinase and 0.2 g detergent containing 0.02 g cutinase.

Stability of 0.2 g detergent: 0.02 cutinase (1:0.1 ratio)

Detergent and detergent:cutinase 1:0.10 were stored at 30 ℃ for 75 days and their activities were analyzed as well as the remaining activities were calculated shown in **Figure 1E**. Before mixing with detergent, cutinase enzyme had its initial activity of 64.8±3.4 U/mg. **Figure 1E** indicated that after 75 days of storage, cutinase had the remaining activity of around 50% while the detergent:cutinase had 72% of

the remaining activity. These results suggested that mixing powder of cutinase and detergent could improve the stability of cutinase.

VITA

NAME	Taweeporn Sooksai
DATE OF BIRTH	7 October 1980
PLACE OF BIRTH	Phrae
INSTITUTIONS ATTENDED	She graduated from high school at Nareerat School Phrae in March 1999 and received the Bachelor of Science degree with a major in Biochemistry and biochemical technology from Chiangmai University in March 2003. The Master of Science degree with a major in Postharvest Technology from Chiangmai University in May 2006. In 2014, she study for the Degree of Doctor of Philosophy in Biotechnology Program, Faculty of Science, Chulalongkorn University.
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PUBLICATION	Taweeporn Sooksai, Hunsa Punnapayak, Sehanat Prasongsuk, and Usa Sangwatanaroj. 2017. Depilling of Spun Polyester Fabric through Laundering with Detergent Containing Alkaline Cutinolytic Esterase. IJCEA Vol.8 (6): 367-370 ISSN: 2010-0221.
AWARD RECEIVED	Oral and poster presentations: - Taweeporn Sooksai, Wichanee Bankeeree, Sehanat Prasongsuk, Pongtharin Lotrakul and Hunsa Punnapayak. 2014. Screening of laccase-producing strains from tropical isolates of Aureobasidium pullulans. The 8th Korea-ASEAN Joint Symposium 2014: Biomass Utilization and Renewable Energy, 18th-22nd August 2014, Korea University, Seoul, Republic of Korea. - Taweeporn Sooksai, Wichanee Bankeeree, Sehanat Prasongsuk, Pongtharin Lotrakul and Hunsa Punnapayak. 2015. Screening of cutinase production from tropical strains of Aureobasidium spp. The ASEAN Youth Exchange on Biotechnology of Biomass Utilisation for ASEAN Development, 25th -29th July 2015, Universitas Murawarman, Samarinda, Indonesia. - Taweeporn Sooksai, Wichanee Bankeeree, Sehanat Prasongsuk, Pongtharin Lotrakul and Hunsa Punnapayak. 2015. Laccase production by Aureobasidium pullulans using medium supplemented with black liquor and its application in biodegradation. The 9th Korea-ASEAN Joint Symposium 2015: Biotechnology Agriculture and Bioethanol for Future Word, 21st-24th November 2015, Insitut Teknologi Sepuluh, Malang, Indonesia