ลักษณะสมบัติของคิวติโนไลทิกเอสเทอเรสจาก *Fusarium solani* เพื่อการประยุกต์ในการดัดแปรเส้นใยพอลิเอสเทอร์

นางสาวธิดารัตน์ นิ่มเชื้อ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CHARACTERIZATION OF CUTINOLYTIC ESTERASE FROM *Fusarium solani* FOR APPLICATION IN POLYESTER FIBER MODIFICATION

Miss Thidarat Nimchua

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	CHARACTERIZATION OF CUTINOLYTIC ESTERASE FROM *Fusarium solani* FOR APPLICATION IN POLYESTER FIBER MODIFICATION
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ธิดารัตน์ นิ่มเชื้อ : ลักษณะสมบัติของคิวติโนไลทิกเอสเทอเรสจาก *Fusarium solani* เพื่อการประยุกต์ในการดัดแปรเส้นใยพอลิเอสเทอร์. (CHARACTERIZATION OF CUTINOLYTIC ESTERASE FROM *Fusarium solani* FOR APPLICATION IN POLYESTER FIBER MODIFICATION) อ. ที่ปรึกษา : รศ. ดร. หรรษา ปุณณะพยัคฆ์, อ. ที่ปรึกษาร่วม : PROF. DOUGLAS E. EVELEIGH, Ph.D., ผศ. ดร. อุษา แสงวัฒนาโรจน์, 154 หน้า.

การคัดแยกเชื้อราที่มีความสามารถในการผลิตเอนไซม์ย่อยสลายเส้นใยพอลิเอสเทอร์ (PET) โดยทำการคัดแยกเชื้อราจากผิวของตัวอย่างพืช และดินด้วยเทคนิค PCL-plate clearing assay เพื่อหาเชื้อราที่สามารถผลิตคิวติโนไลทิกเอสเทอเรส พบว่าเชื้อรา 22 ไอโซเลต สามารถทำให้เกิดวงใสบน PCL-plate เมื่อนำเชื้อทั้งหมดมาเลี้ยงในอาหารสูตรเกลือแร่ที่มีซูเบอรีน หรือ เส้นใย PET เป็นแหล่งคาร์บอนโดยทำการตรวจสอบแอคติวิตีของคิวติเนสที่ผลิตได้โดยใช้ พาราไนโตรฟีนิล บิวทาเรต (*p*-NPB) เป็นสับสเตรท พบว่าไอโซเลต PBURU-B5 ให้ค่าแอคติวิตีสูงสุดเมื่อเลี้ยงในอาหารที่มีเส้นใย PET เป็นแหล่งคาร์บอน การจัดจำแนกเชื้อราดังกล่าวด้วยลักษณะทางสัณฐานวิทยา ร่วมกับเทคนิคทางอณูชีววิทยาโดยการหาลำดับเบสของ internal transcribed spacer (ITS) ของยีน rDNA ลำดับเบสของ ergosterol biosynthesis gene (ERG) และลำดับเบสของ translation elongation factor 1-α gene (TEF) สามารถตรวจสอบได้ว่าเชื้อราชนิดนี้คือ *Fusarium solani* จากนั้นเมื่อทำการศึกษาภาวะที่เหมาะสมในการผลิตคิวติโนไลทิกเอสเทอเรสของเชื้อ พบว่าที่ภาวะการบ่มที่ 25 องศาเซลเซียสและค่าความเป็นกรดด่างเริ่มต้น 11.0 นั้นจะให้ค่าแอคติวิตีสูงสุดในวันที่ 4 ของการเลี้ยงเชื้อ เมื่อนำเอนไซม์มาทำให้บริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต และแยกโดยเทคนิคโครมาโทกราฟีด้วย คอลัมน์ Hitrap Q FF ion-exchange คอลัมน์ Hitrap Phenyl HP hydrophobic และ คอลัมน์ HiPrep 16/60 Sephacryl S-200 gel fitration พบว่า เอนไซม์มีความบริสุทธิ์เพิ่มขึ้น 69 เท่า มีผลผลิตร้อยละ 11 และมีแอคติวิตีจำเพาะ 137.5 ยูนิตต่อมิลลิกรัมโปรตีน โดยเอนไซม์บริสุทธิ์แสดงแถบโปรตีน 1 แถบบน SDS-PAGE จากการศึกษาโดย gel filtration และ SDS-PAGE พบว่าเอนไซม์ประกอบด้วย 1 หน่วยย่อยที่มีน้ำหนักโมเลกุล 19 กิโลดาลตัน pH และอุณหภูมิที่เหมาะสมในการทำงานของเอนไซม์คือ 9.0 และ 45 องศาเซลเซียส ตามลำดับ ค่า K_m สำหรับ *p*-Nitrophenyl butyrate เท่ากับ 0.53 โมลาร์$^{-1}$ สารละลายคอปเปอร์ซัลเฟตที่ความเข้มข้น 10 มิลลิโมลาร์ มีผลทำให้แอคติวิตีของเอนไซม์มีค่าลดลงร้อยละ 50 เมื่อนำเอนไซม์หยาบจากเชื้อราดังกล่าวไปดัดแปรสมบัติของผ้าพบว่า เอนไซม์สามารถย่อยพันธะเอสเทอร์ของเส้นใยพอลิเอสเทอร์ได้ นอกจากนั้นยังเพิ่มความสามารถในการดูดซับน้ำและความชื้นของผ้าซึ่งเป็นการบ่งชี้ถึงความชอบน้ำที่เพิ่มขึ้นของผ้าพอลิเอสเทอร์หลังจากย่อยด้วยเอนไซม์ การศึกษาการเปลี่ยนแปลงบนพื้นผิวของผ้าด้วยการย้อมผ้าด้วยสีเบสิค ศึกษาหมู่ฟังก์ชันบนผิวผ้าด้วยเครื่องเอทีอาร์ฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกโทสโกปี และภาพถ่ายจากกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด แสดงให้เห็นถึงความสามารถในการเปลี่ยนแปลงพื้นผิวของผ้าทั้งทางเคมีและกายภาพ

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THIDARAT NIMCHUA: CHARACTERIZATION OF CUTINOLYTIC ESTERASE FROM *Fusarium solani* FOR APPLICATION IN POLYESTER FIBER MODIFICATION. THESIS ADVISOR: ASSOC. PROF. HUNSA PUNNAPAYAK, Ph.D., THESIS COADVISORS: PROF. DOUGLAS E. EVELEIGH, Ph.D., ASST. PROF. USA SANGWATANAROJ, Ph.D., 154 pp.

Microfungi were selectively isolated for production of polyethylene terephthalate (PET) fiber-degrading enzymes potentially to be used to modify the surface of polyester fabric. Over one hundred fungi were isolated from plant surfaces and soil samples using a polycaprolactone (PCL) plate-clearing assay technique, and screened for cutinolytic esterase (cutinase) activity. Twenty-two isolates showed clearing indicating the production of cutinase. The ability of the fungi to produce cutinase in mineral medium (MM) using either potato suberin or PET fiber as substrates was assessed based on the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB). All isolates exhibited activity towards *p*-NPB, isolate PBURU-B5 giving the greatest activity with PET fiber as an inducer. PBURU-B5 was identified as *Fusarium solani* based on its conidial morphology and also comparative nucleotide sequencing from internal transcribed spacer region of the ribosomal RNA gene (rDNA-ITS), ergosterol biosynthesis gene (ERG) and translation elongation factor 1-α gene (TEF). The highest esterase yield was obtained in the supernatant from cultures grown at 25°C, initial pH 11.0 for 4 days. The enzyme was purified by sequential use of 50-80% ammonium sulfate precipitation, Hitrap Q FF, Hitrap Phenyl HP and HiPrep 16/60 Sephacryl S-200 High Resolution Chromatography. It was purified approximately 69 fold with overall 11% recovery to a specific activity of 137.5 U/mg. The enzyme was homogeneous by SDS-PAGE. The enzyme was a single polypeptide with a molecular weight of about 19 kDa as determined by gel filtration and SDS-PAGE. The optimum pH and temperature for activity of the purified enzyme were pH 9 and 45°C. The K_m value for *p*-NPB was 0.53 M^{-1}. Though enzyme activity was reduced to 50% by 10 mM $CuSO_4.5H_2O$, this was the exception as the enzyme was unaffected by a range of the other metals. Enzymatic modification of PET cloth material properties using crude enzyme from strain PBURU-B5 showed hydrolysis of ester bonds of the PET fiber. The modification of the PET fabric resulted in increase of water and moisture absorption, and general enhancement of hydrophilicity of the fabric. Analytical data on enzyme treated PET fabrics to characterize the surface modifications were obtained by dyeing with basic dye, and also ATR-FTIR and SEM studies. The overall changes resulted in improvements could facilitate processing of fabric ranging from easier dyeing while also yielding a softer feeling fabric for the user.

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CHAPTER I

INTRODUCTION

Biopolyesters occur in higher plants as the structural component of the cuticle that covers the outer surfaces of plants. This insoluble polymer, cutin, attached to the epidermal cell walls is composed of inter-esterified hydroxy and hydroxy epoxy fatty acids (Kolattukudy, 2001). Another type of plant polyester is suberin, a polymeric material deposited in cell wall when a plant needs to erect a barrier as a result of physical or biological stress from the environment, or during development. Suberin is composed of aromatic domains derived from cinnamic acid, and aliphatic polyester domains derived from C_{16} and C_{18} fatty acids and their elongation products (Bernards, 2002). The ester bond within these polyesters can be hydrolyzed by lipase (EC 3.1.1.3), and cutinase (EC 3.1.1.74), a polyesterase produced by bacteria and fungi (Kolattukudy, 1980; 2001). The major function of the polyester in plants is as a protective barrier against physical, chemical, and biological factors in the environment, including plant pathogens. These phytophatogens such as fungi from the genus *Fusarium* and bacteria in the genus *Streptomyces*, are capable of producing cutinolytic esterase enzymes which can degrade cutin and suberin (Fernando, Zimmermann and Kolattukudy, 1984; Fett *et al.*, 1992). A common interest in these microbes also arises because of their abilities to degrade many types of synthetic polyesters including aliphatic and aromatic polyesters (Kim and Rhee, 2003).

Synthetic polymers have been intensively developed during the last 50 years and are present nowadays in all areas of life. In general, most aliphatic polyesters including polyhydroxyalkanoates (PHAs), poly (ε-caprolactone) (PCL), and poly (L-lactide) (PLA) can easily be degraded by microorganisms that are widely distribute in nature (Murphy *et al.*, 1996). On the other hand, aromatic polyesters such as polyethylene terephthalate (PET) are more resistant to microbial attack (Kim and Rhee, 2003). Many of biodegradation polyester's products are based on aliphatic polyesters and exhibit only limited applications. Aromatic polyesters were more widely used because of their superior properties (Müller *et al.*, 2005). The polyester fibers made of

PET have very good strength properties. They are resistant to chemicals and abrasion, stretching, shrinking and wrinkling (Yoon, Kellis and Poulose, 2002). However, the great disadvantage of the PET fiber is its low hydrophilicity and inactive surface. These affect processing of the fibers such as lose a degree coloring and also bereave "coziness" of the fabric to the wearer. The conventional modification of PET fiber properties is through strong alkaline treatment at high temperature which concomitantly gives both weight loss and excessively hydrophilic fibers. Both alkaline wastes and the high processing temperature invoke environmental concern (Kim and Rhee, 2003; Vertommen *et al.*, 2005). An interesting alternative in the surface modification of PET fibers is the application of hydrolytic enzymes which can be accomplished under mild conditions. Hydrolases, esterases and lipases that act on biopolyesters such as cutin and suberin could have potential in surface modification of PET fiber (Carvalho, Aires-Barros and Cabral, 1998, 1999; Khoddami, Morshed and Tavani, 2001 and Vertommen *et al.*, 2005). Although PET-fabrics were initially considered quite inert, there has been accumulating evidence for enzymatic modification of PET fibers. Sato (1983) found changes in the physical properties of PET fibers including their tensile strength, viscoelasticity and extension behavior following incubation with an enzyme preparation from *Cladosporium cladosporioides*. Treatments of the polyester fabrics with several lipases improved their water wettability and absorbent properties indicating an increase in hydrophilicity due to enzymatic hydrolysis (Hsieh *et al.*, 1997; Hsieh and Cram, 1998). Polyester-hydrolyzing bacterial and fungal esterases reduce pilling of PET fabrics (Andersen *et al.*, 1999). Yoon *et al.* (2002) showed the modification of surface of PET fibers by a serine esterase resulted in enhanced hydrophilicity, wettability and cationic dye binding ability of the fibers. The treated PET fabrics showed less pilling and improved oil stain removal properties. The application of *Arthrobacter* and *Trichosporon* enzymes for the modification of PET fibers has also been described (Oda and Kimura, 1998). Cutinases (EC 3.1.1.74) from the phytopathogen, *Fusarium solani* f. sp. *pisi* and a thermophilic actinomycete, *Thermomonospora fusca*, have been successfully used for the modification of PET (Müller *et al.*, 2005; Vertommen *et al.*, 2005) including their ability to improve the hydrophilic properties and activation of the surface of PET fibers (Nimchua, Punnapayak and Zimmermann, 2007).

This range of studies illustrates the proof of concept for the modification of PET fibers, though new and more efficient enzymes need to be considered for industrial development. In this study, I isolated further fungi producing extracellular cutinolytic esterases from plant leaves, underground storage organs, fruits, and soil in different habitats of Thailand. This esterase production was assessed. Moreover, screening for the most efficient enzyme on PET fiber was carried out. The best selected isolate was identified as a *Fusarium* sp.. The purification and characterization of the most efficient enzyme was investigated. Finally, this esterase was shown for modifying the properties of polyester fabric.

Objectives of this study

1. To obtain *Fusarium* isolates for the production of cutinolytic esterase from different habitats in Thailand
2. To identify the selected fungus that produced the highest yield of cutinolytic esterase
3. To purify and characterize cutinolytic esterase obtained from the selected fungus
4. To detect the modification of polyester fabric surface by esterase treatment to improve their functional properties

CHAPTER II

LITERATURE REVIEW

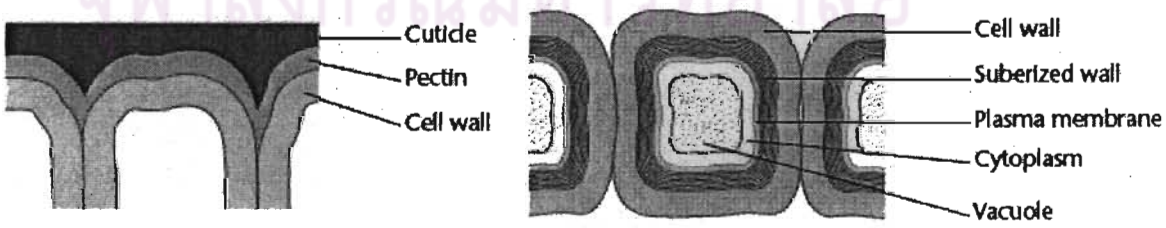
2.1 The plant biopolymers

2.1.1 Occurrence of cutin and suberin

Plants have polyester outerwear, as the outer parts of higher plants are covered with a cuticle layer whose structural component is a polyester, cutin. The term cutin came from the name cutose, this being used originally to describe the material that formed part of the epidermis of leaves and which resisted the action of strong acids (Martin and Junifer, 1970). Studies on the nature of plant cuticle began in the 19th century. In these early studies, the thin films which remained after treatment of the aerial parts of plants with strong acids were considered to be the true cuticles (Kolattukudy, 2001a). Cutin constitutes the structural component of the cuticles of the higher plants. Even the angiosperms that are submerged in the oceans, such as *Zoestra marina* or sea grass, are covered with cutin composed of the same type of monomers as those found on land plants (Kolattukudy, 2001a). There is evidence that cuticle occurs in lower plants including mosses, lycopods, ferns, and liverworts (Holloway, 1982). Cutin containing layers are found not only on the surfaces of all aerial part of plants including stems, petioles, leaves, flower parts, fruits, aerial roots, and some seed coats, but also on internal parts such as juice sacks of citrus (Kolattukudy, 2001a). The thickness of the polymer layer varies among species and among organs in the same plant. In higher plant leaves, the thickness ranges from 0.5 to 14 μm, with more than 20 to 600 μg cutin per cm^2 of the surface area. In some fruits with a well-developed cuticle, the cutin content may reach 1.5 mg/cm^2. In lower plants the cuticle in usually very thin (~0.1 μm) (Kolattukudy, 2001a). This lipid-derived polyester covering is unique to plants, as animals use carbohydrate or protein polymers as their outer covering. Cutin, the insoluble cuticular polymer of plants, is composed of interesterified hydroxy and hydroxy epoxy fatty acids derived from the common cellular fatty acids and is attached to the outer epidermal layer of cells by a pectinaceous layer (Figure 2.1a). The insoluble polymer is embedded in a complex mixture of soluble lipids collectively called waxes (Kolattukudy, 2001b).

The name suberin was first used almost 200 years ago by Chevruel to describe the insoluble material that formed the major portion of the cork used to seal bottles (Kolattukudy, 2001c). The first reports appeared in 1878, when nitric acid treatment of cork was shown to produce suberic acid – this being the same material obtained some 80 years previously from the oxidation of cork. Later, it was recognized that suberic acids were degradation products from suberin monomers. Towards the end of the 19th century, the alkaline hydrolysis products were isolated using classical chemical methods, and this further raised the possibility of suberin containing a polyester domain. Althought several of the major aliphatic components of cork had been identified by the mid-20th century, only in the latter stages of the century with the introduction of more definitive analytical techniques, was it that the general picture of the structure of suberin began to emerge (Kolattukudy, 2001c).

Cork cells, as well as the periderm, the outer barrier that covers barks and the underground organs such as tubers and roots, is formed by depositing on the walls of the outer one or two cells of the polymer suberin, which is composed of aromatic and aliphatic domains. Suberized walls (Figure 2.1b) are also found in a variety of other anatomical regions within plants such as epidermis and hypodermis of roots, endodermis (Casparian bands), the bundle sheaths of grasses, the sheaths around idioblasts, the boundary between the plant and its secretory organs such as glands and trichomes, the pigment strands of grains, the chalazal region connecting seed coats and vascular tissue, and certain cotton fibers (Kolattukudy, 1987; Kolattukudy and Espelie, 1989; Kolattukudy *et al.*, 1994). The aromatic domains of suberin are derived mainly from cinnamic acid while the esterified aliphatic components are derived from common cellular fatty acids (Kolattukudy, 2001c).



(a) (b)

Figure 2.1 Schematic representation of the cuticle (a) and suberized cell wall (b) (Kolattukudy, 2001a).

2.1.2 Isolation of plant polyesters

The cuticle, being attached to the epidermal cells via a pectinaceous layer, can be released by disruption of this layer by chemicals such as ammonium oxalate/oxalic acid or by pectin-degrading enzymes. After treatment of the recovered cuticular layer with carbohydrate-hydrolyzing enzymes to remove the remaining attached carbohydrates, the soluble waxes can be removed by exhaustive extraction with organic solvents such as chloroform. The cutin sheets can be powdered and subjected to chemical and/or enzymatic depolymerization (Holloway, 1982; Kolattukudy, 1980).

Suberin, being an adcrustation on the cell wall, cannot be separated from cell walls. However, suberin-enriched wall preparations can be obtained by digesting away as much carbohydrate polymers as possible using pectinases and cellulases (Kollattukudy, 1981; Kolattukudy and Espelie, 1989).

2.1.3 Depolymerization of plant polyesters

Cutin can be depolymerized by cleavage of the ester bonds either by alkaline hydrolysis, transesterification with methanol containing boron trifluoride or sodium methoxide, reductive cleavage by exhaustive treatment with $LiAlH_4$ in tetrahydrofuran, or with trimethylsilyl iodide (TMSiI) in organic solvents to release free monomers or their derivatives (Holloway, 1982; Kolattukudy, 1980). Enzymatic depolymerization is also effected e.g. lipases such as pancreatic lipase or cutinases that catalyze the hydrolysis of ester bonds (Holloway, 1974; Kolattukudy and Walton, 1973).

The polyester domains of suberized walls can also be depolymerized using chemical and/or enzymatic approaches similar to those used for cutin. The aromatic domains are more difficult to depolymerize as C-C and C-O-C crosslinks are probably present in such domains. Therefore, more drastic degradation procedures such as nitrobenzene, CuO oxidation, or thioglycolic acid/HCl treatment are used to release aromatic fragments (Kolattukudy and Espelie, 1985; 1989).

2.1.4 Composition of cutin

The major components of cutin are derivatives of saturated C_{16} (palmitic) acid and unsaturated C_{18} acids (Table 2.1). The major component of the C_{16} family of acids is 9- or 10,16-dihydroxyhexadecanoic acid (and some midchain positional isomers), with

less 16-hydroxyhexadecanoic acid and much smaller amounts of hexadecanoic acid (Deas, Baker and Holloway, 1974; Espelie, David and Kolattukudy, 1980). The major components of the C_{18} family of monomers are 18-hydroxy-9,10-epoxy C_{18} acid and 9,10,18-trihydroxy C_{18} acid together with their monounsaturated homologues (Table 2.1). Lower amounts of 18-hydroxy C_{18} saturated, mono-, and diunsaturated fatty acids and still lower amounts of their unhydroxylated homologues occur. Fatty acids longer than C_{18}, their ω-hydroxylated derivatives, and the corresponding dicarboxylic acids are minor components of cutin (Walton and Kolattukudy, 1972). The major components of cutin have been described (Table 2.2).

Table 2.1 Structure of major monomers of cutin (Walton and Kolattukudy, 1972)

C_{16}-Family	C_{18}-Family
$CH_3-(CH_2)_{14}-COOH$	$CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH$
$CH_2(OH)-(CH_2)_{14}-COOH$	$CH_2(OH)-(CH_2)_7-CH=CH-(CH_2)_7-COOH$
$CH_2(OH)-(CH_2)_x-CH(OH)-(CH_2)_y-COOH$	$CH_2(OH)-(CH_2)_7-CH-CH-(CH_2)_7-COOH$ (O bridge)
* X=5, 6, 7, 8; y= 5, 6, 7, 8, x+y=13	$CH_2(OH)-(CH_2)_7-CH(OH)-CH(OH)-(CH_2)_7-COOH$

The composition of cutin shows species specificity although most plants contain different types of mixtures of the C_{16} and C_{18} family of acids. Composition of cutin differ with the anatomical location. For example, cutin preparations from fruit, leaf, stigma, and flower petal of *Malus pumila* contain 73%, 35%, 14%, and 12%, respectively, of hydroxy and hydroxy-epoxy C_{18} monomers (Espelie, Dean and Kolattukudy, 1979). In general, fast-growing plant organs have higher content of C_{16} family of monomers.

2.1.5 Structure of the polymer cutin

Being an insoluble, amorphous polymer, only a limited number of methods can be applied to investigate the structure of this polymer. Early study of the chemical structure of isolated cutin was investigated using Transmission Fourier transform infra-

red (FTIR) spectra (Villena *et al.*, 2000). One method used oxidation of the free hydroxyl group with CrO_3-pyridine followed by depolymerization with $NaOCH_3$ in anhydrous methanol and analysis of the carbonyl containing monomers (Dea and Holloway, 1977). The other involved mesylation of the free hydroxyl groups in the polymer by treatment with methanesulfonyl chloride followed by depolymerization with $LiAlD_4$ (Kolattukudy, 1977). All methods led to similar conclusions. Most of the primary alcohol groups are in ester linkage indicating that the polyester is held together predominantly by primary alcohol ester linkages. About half of the secondary alcohols were also found to be in ester linkages, indicating branching and/or cross-link (Kolattukudy, 1977).

Table 2.2 Fatty acids with one or more additional functional groups as components of cutin (Kolattukudy and Espelie, 1985).

Monomer	Source	Percentage of total aliphatics
Monohydroxy acids		
8-Hydroxy C_8	*Psilotum nudum* stem	0.7
9-Hydroxy C_9	*Solanum tuberosum* leaf	0.5
9-Hydroxy $C_{14:1}$	*Coffee Arabica* leaf	4.5
15-Hydroxy C_{16}	*Astarella lindenbergiana* leaf	72
2-Hydroxy C_{18}	*Conocephalum conicum* leaf	3.3
20-Hydroxy C_{23}	*Conocephalum conicum* leaf	2
Dihydroxy acids		
7,16-Dihydroxy C_{16}	*Pisum sativum* seed coat	4.1
9,16-Dihydroxy C_{16}	*Malabar papaiarnarum* fruit	73
10,16-Dihydroxy C_{16}	*Ribes grossularia* fruit	83
10,18-Dihydroxy $C_{18:1}$	*Vaccinium macrocarpon* fruit	1.1
Trihydroxy acids		
6,7,16-Trihydroxy C_{16}	*Rosmarinus officinalis* leaf	17
9,10,16-Trihydroxy C_{17}	*Citrus paradisi* fruit	1.9
9,10,18-Trihydroxy $C_{18:1}$	*Citrus paradisi* seed coat	23
Epoxy and oxo acids		
16-Hydroxy-10-oxo C_{16}	*Citrus limon* fruit	34
9,10-Epoxy-18-hydroxy C_{18}	*Citrus paradisi* seed coat	37
Dicaboxylic acids		
C_9 Diacid	*Solanum tuberosum* leaf	1.7
$C_{16:1}$ Diacid	*Vaccinium macrocarpon* fruit	0.1
7-Hydroxy C_{16} diacid	*Welwitchia mirabilis* leaf	15
$C_{19:1}$ Diacid	*Pinus radiata* stem	8

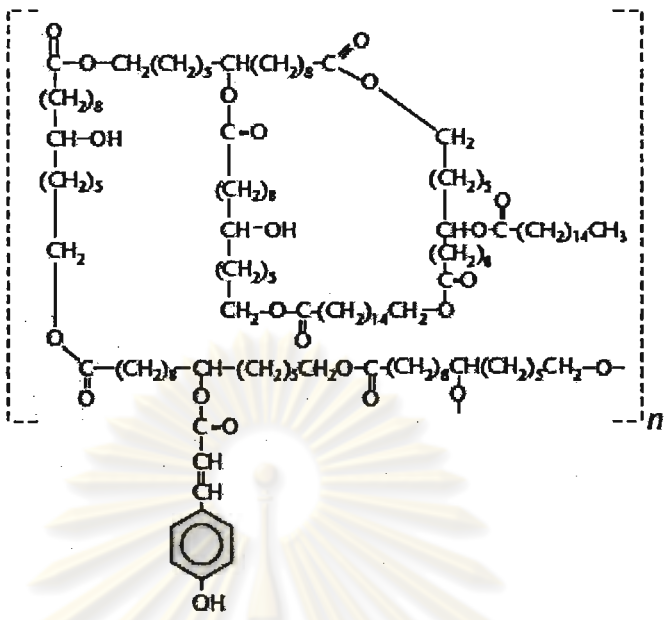


Figure 2.2 A models illustrating structures in cutin containing mainly the C_{16} family of monomers (Kolattukudy, 2001a).

More recently, more direct structural studies have been carried out using Nuclear Magnetic Resonance (NMR) (Fang *et al.*, 2001; Round *et al.*, 2000). Cutin is shown to be moderately flexible netting with motional constraints probably at cross-link sites. In cutin containing primarily mid-chain hydroxyl groups, with a higher potential for cross-linking, even higher portions of the methylenes may be in the rigid category. Structural studies on enzymatically released oligomers using solution state NMR and liquid secondary ion mass spectrometry (LISMS) gave direct proof for the predicted presence of secondary alcohol esters involving the 10-hydroxyl group of the major cutin monomer, 10,16-dihydroxyl group C_{16} acid (Ray and Stark, 1998). On the basis of these studies, it was concluded that the cutin structure consists of an amorphous three dimensional network of polymethylene molecules containing double bonds and free carboxylic functions (Figure 2.2).

2.1.6 Composition of suberin

The aliphatic monomers of suberin constitute 5–30% of the suberin-enriched cell wall preparations (Cordeiro *et al.*, 1998; Kolattukudy and Espelie, 1985). The main

aliphatic structural components of suberin are illustrated in Figure 2.3, including 1-alkanols (trace amounts to 11% of monomers), ω-hydroxyalkanoic acids (13–61% of monomers), α,ω-dioic acids (2–33% of monomers), mid- chain epoxide- and di- and tri-hydroxy-substituted octadecanoates (trace amounts to 58% of monomers), ferulates and glycerol (10–26% of monomers) (Bernards, 2002; Holloway 1983; Graça and Pereira 1997). According to Matzke and Riederer (1991), the α,ω-dioic acids represent aliphatics unique to suberized tissue, and can be used as diagnostic markers to differentiate between suberized and cutinized tissues. Homologues containing more than 20 carbons with an even number of carbon atoms are often significant components of ω-hydroxy acid and dicarboxylic acid, unlike those found in cutin (Kolattukudy and Espelie, 1985). The more polar acids which contain epoxy, hydroxy, and dihydroxy functions similar to those found in cutin are usually minor components in suberin.

The compositional distinction between cutin and suberin (Table 2.3) originally formulated in 1974 (Kolattukudy and Agrawal, 1974) and based on a limited number of samples has been essentially confirmed by recent extensive analyses of such polymers from a large number of plant species (Kolattukudy and Espelie, 1985; Matzke and Riederer, 1991).

The more characteristic feature of the aliphatic components of suberin is the presence of very long chain (>C18) components and dicarboxylic acids, mostly unsubstituted dicarboxylic acids with small amounts mid-chain hydroxy or epoxy acids. The major polyfunctional aliphatic components found in suberin are listed In Table 2.4.

Table 2.3 Compositional difference between cutin and suberin (Kolattukudy and Espelie, 1985)

Monomer	Cutin	Suberin
Dicarboxylic acids	Minor	Major
In-chain-substituted acids	Major	Minor[a]
Phenolics	Low	High
Very long-chain (C20–C26) acids	Rare and minor	Common and substantial
Very long-chain alcohols	Rare and minor	Common and substantial

[a] In some cases substantial

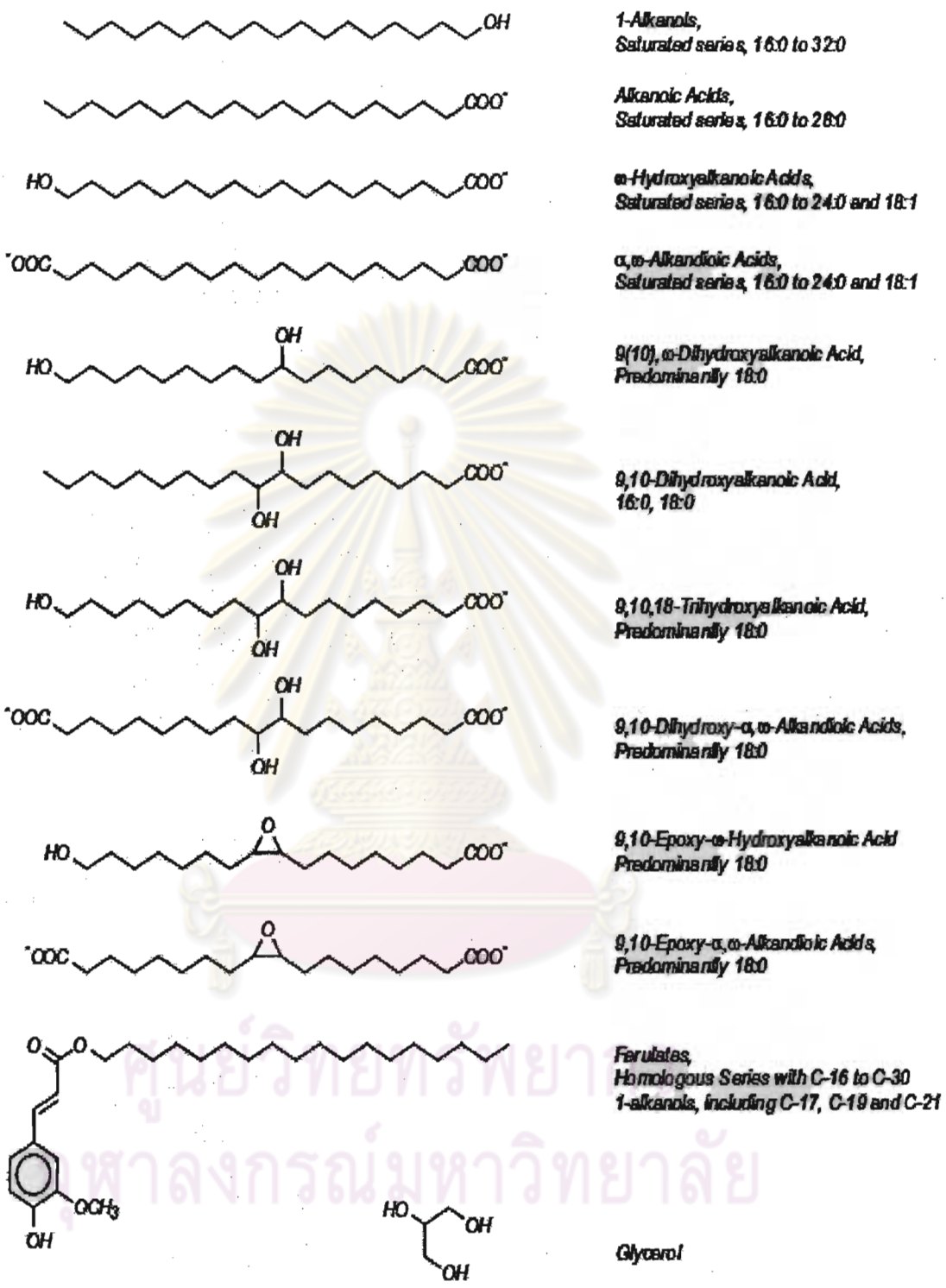


Figure 2.3 Aliphatic precursors of suberized tissues (Bernards, 2002)

Table 2.4 Fatty acids with one or more additional functional groups of suberin (Kolattukudy and Espelie, 1985).

Monomer	Source	Percentage of total aliphatics
Monohydroxy acids		
16-Hydroxy C_{16}	*Populus tremula* bark	22
18-Hydroxy $C_{18:1}$	*Solanum tuberosum* storage organ	31
20-Hydroxy C_{20}	*Beta vulgaris* tuber	2.9
26-Hydroxy C_{26}	*Quercus ilex* bark	2
Dicarboxylic acids		
C_{16} Diacid	*Citrus paradisi* seed coat	13
C_{18} Diacid	*Ribes nigrum* bark	2.8
$C_{18:1}$ Diacid	*Solanum tuberosum* tuber	31
9,10-Dihydroxy C_{18} diacid	*Acer griseum* bark	17
9,10-Epoxy C_{18} diacid	*Quercus suber* bark	16
C_{22} Diacid	*Gossypium hirsutum* green fiber	25

Aromatic components from a polymer matrix that are attached to cell walls on one side, and aliphatic polyester domains on the other sides, as indicated below. In addition, aromatic components are attached to the polymer matrix by ester and amide linkages. Esterified ferulic acid was found to be a constituent of suberized cell walls of the periderm of potato, sweet potato, carrot and red beet (Riley and Kolattukudy, 1975). Other phenylpropanoic acids may also be esterified to suberin. Tyramine is found in amide linkage with phenylpropanoic acids and possibly with other acids in suberin (Negrel *et al.*, 1996). Structures of phenolic precursors of suberized tissue are shown in Figure 2.4.

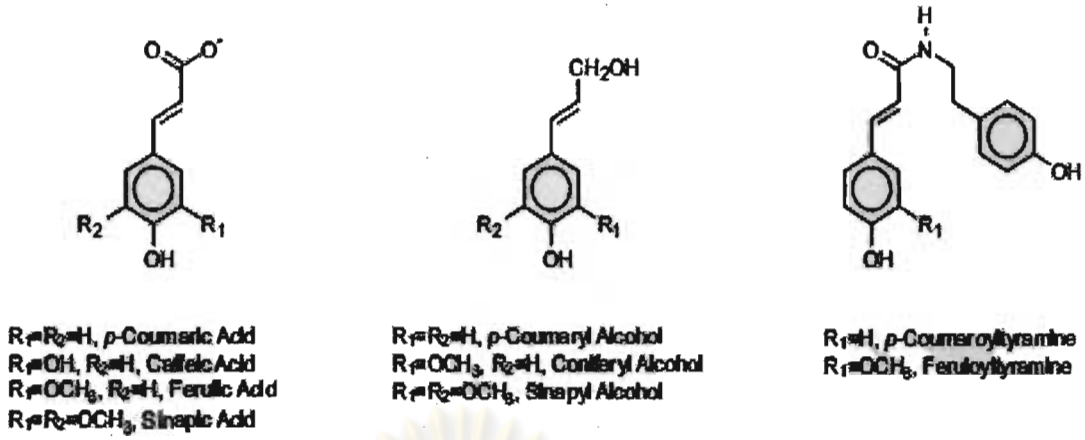


Figure 2.4 Phenolic precursors of suberized tissues (Bernards, 2002)

2.1.7 Structure of polymer suberin

Since suberin is attached to the cell wall, only suberin-enriched cell wall preparations can be obtained for analysis. The soluble waxes extracted from such walls with organic solvents can be analyzed using standard analytical methods. The composition of suberin-associated waxes is somewhat similar to that of cuticular waxes but with some characteristic differences (Kolattukudy and Espelie, 1989).

Depolymerization methods to cleave ester bonds release a variety of the aliphatic components while more drastic cleavage methods such as nitrobenzene or CuO oxidation, or thioglycollate treatment, release fragments of the aromatic matrix. According to the current working hypothesis concerning the structure and organization of suberized walls (Figure 2.5) (Kolattukudy, 2001c), aliphatic domains are attached to the phenolic matrix and soluble waxes are associated with the hydrophobic accrustation giving rise to the characteristic lamellar appearance in the electron micrographs of suberin. ^{13}C CPMAS NMR studies show the presence of polymethylenic aliphatic polyester domains and aromatic domains derived from phenylpropenoic acids. Both ultrastructural and chemical studies have identified suberization not only in the natural periderm of tubers, roots and barks and wound periderms in tubers, leaves and fruits, but also in the internal barrier layers deposited to respond to stress or during development: casparian band of endodermis, mestome sheet and bundle sheath cell walls of monocots, chalazal region of seed coat and crystal idioblast walls (Kolattukudy, 1980; Kolattukudy and Espelie, 1989).

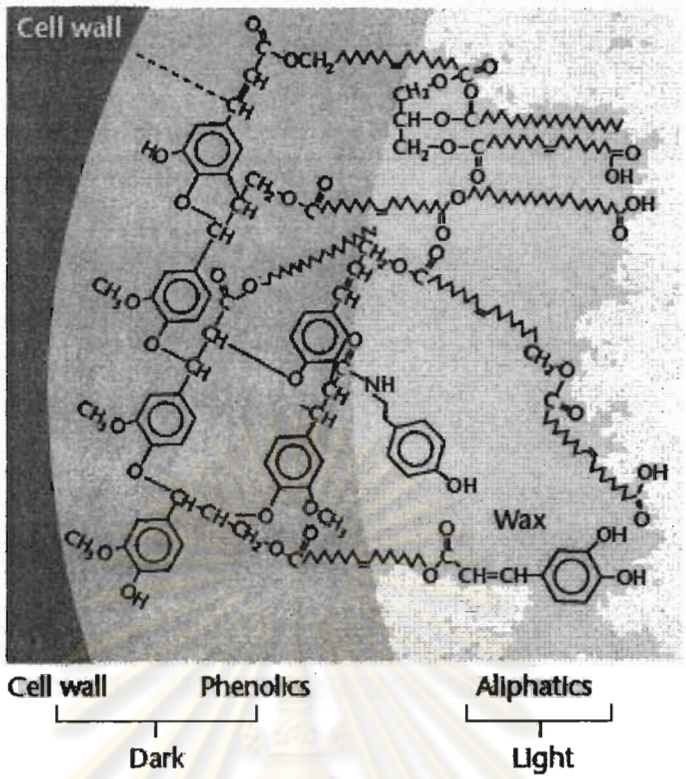


Figure 2.5 A model showing the type of structures present in the polymers suberin (Kolattukudy, 2001c).

2.1.8 Function of cutin

2.1.8.1 Material Exchange with the environment

Cutin as the structural component of the outer barrier of plants plays a major role in the interaction of the plant with its environment. Development of the cuticle is thought to be responsible for the ability of plants to move onto land where the cuticle limits diffusion of moisture and thus prevents desiccation (Edwards, Abbott and Raven, 1996). The plant cuticle controls the exchange between leaf and atmosphere. The transport properties of the cuticle strongly influence the loss of water and solutes from the leaf interior as well as uptake of nonvolatile chemicals from the atmosphere to the leaf surface. In the absence of stomata the cuticle controls gas exchange. The cuticle as a transport-limiting barrier is important in both its physiological and ecological functions. The diffusion across plant cuticle follows basic laws of passive diffusion across lipophylic membranes (Schreiber, Kirsch and Riederer, 1996).

2.1.8.2 Low temperature adaptation

Electron microscopic examination of winter rye (*Secale cereale*) grown at 5 °C showed the thickening of the epidermal cell walls which were four-fold grater than in plant grown at 20 °C (Griffith *et al.*, 1985). This increase of polyester occurs as 18-hydroxy-9,10-epoxy-C_{18} acid that is probably the major factor in the increased freeze resistance of cold-hardened plants, in part by decreasing ice nucleation from the environment as described recently in the bud axes of *Vitis vinifera* (Jones and McKersie, 2000)

2.1.8.3 Role of cutin in the interaction with microbes

The cuticle being a first contact point with environmental microbes plays a significant role in the interaction of the plant with microorganisms (Figure 2.6). It can provide the carbon source for the growth of microbes that occupy the aerial plant surfaces. Fungal conidia have chemicals that prevent them from germinating and differentiating until they reach a favorable ecological niche such as a plant surface. These chemicals, called self-inhibitors, are most often lipophilic molecules (Macko, 1981). Upon contact with the host surface, these inhibitors can diffuse into the lipophilic cuticle and thus relieve the self-inhibition (Hegde and Kolattukudy, 1997) permitting transcription of genes – such as calmodulin gene – that play important roles in pathogenesis (Liu and Kolattukudy, 1999). Contact of fungal conidia with the host surface itself can induce the expression of fungal genes involved in the early events that are crucial for the germination and differentiation of the infection structures required for successful penetration into the plant host. As the conidia of pathogens carry small amounts of cutinase (Köller, Allan and Kolattukudy, 1982), small amounts of cutin monomers are likely to be generated during the early phases of fungal interaction with the plant surface. Such cutin monomers, as well as certain soluble cuticular components (Podila, Rogers and Kolattukudy, 1993), were found to trigger the differentiation of the infection structure (appressorium) in some fungi (Francis, Dewey and Gurr, 1996; Gilbert, Johnson and Dean, 1996). The next step is actual penetration into the host. Since cutin is the major structural component of the cuticle, it is a major physical barrier to penetration by the invading pathogens, particularly fungal pathogens.

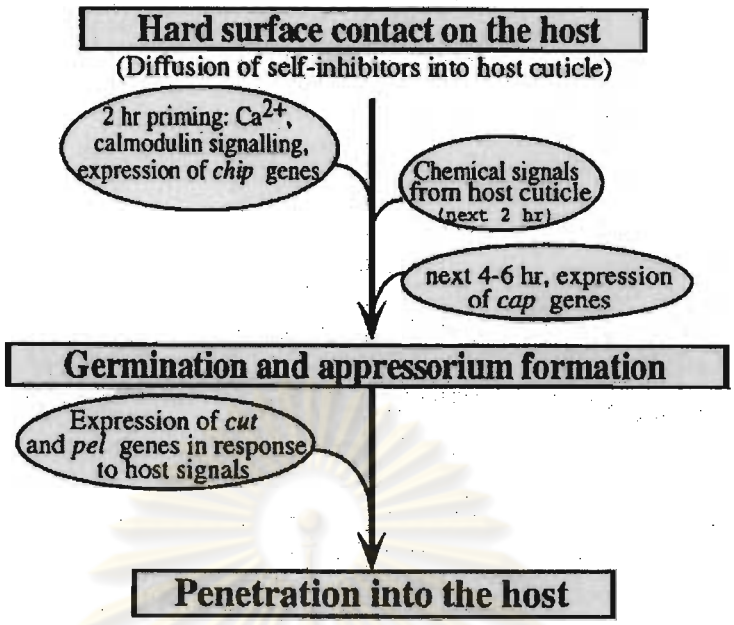


Figure 2.6 Hypothetical scheme of the molecular events in the early stages of fungal interactions with plant cuticle. *chip=Colletotrichum* hard surface induced protein; *cap=Colletotrichum* appressorium genes (Kollatukudy, 2001a)

As cutin is the first barrier, one of the key enzymes involved in invasion was postulated to be cutinase, latter summarized as follows (Kolattukudy, 1996):

(a) Pathogenic fungi produce and secrete cutinase targeted at the penetration point and during actual infection of the host. Such an enzyme has been detected immunocytochemically.

(b) Specific inhibition of cutinase by chemicals or antibodies including monoclonal antibodies prevents infection.

(c) Cutinase-deficient mutants have significantly reduced virulence but infection can be restored with exogenous cutinase.

(d) Pathogens that cannot infect a host without a breached cuticle (wound) can be genetically engineered to provide cutinase-producing capability and such engineered organisms can infect intact hosts, without requiring a breached cuticle.

(e) Knocking out cutinase gene decreases virulence of organisms that have a single cutinase gene.

The cutin monomers as a result of fungal enzyme action may act as early alarm signals of fungal attack and trigger defense reactions in the host. Cutin monomers could induce alkalanization of the medium when plant cell cultures were treated with cutin monomers, possibly indicative of the early phase of the defense reaction (Schweizer *et al.*, 1996). Hypocotyl segments produced an oxidative burst (H_2O_2) when treated with cutin hydrolysate, indicative of the defense reaction triggered by cutin monomers (Fauth *et al.*, 1998). Thus, the polymer may act not only as a physical barrier against fungal infection but also serve as the sentry that sends early signals to alert the host.

2.1.8.4 Regulation of cutinase gene transcription

Conidia from pathogenic fungi that constitute low levels of cutinase to sense the contact with the host, and the cutin monomers generated upon contact. These can be the inducers that allow the fungus to produce enough quantities of cutinase to gain access into the host (Figure 2.7) (Kollatukudy, 2001a; Köller *et al.*, 1982).

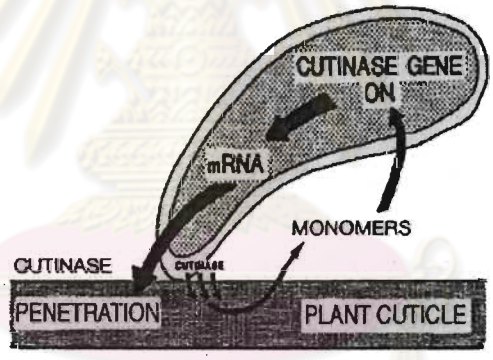


Figure 2.7 Schematic representation of how the plant cuticle induces cutinase in a fungal spore (Kollatukudy, 2001a)

CTF1ß is the transcription factor involved in the constitutive expression of *cut 2/3* genes (the sensor genes) that are expressed, albeit at low levels. *Cut 2/3* genes differ from the highly inducible *cut1* gene in that palindrome 1 of these genes have two nucleotide substitutions that prevent a palindrome 1 binding protein (PBP) binding to the promoter of these genes. In the absence of PBP binding, CTF1ß can tranactivate these genes by binding to palindrome 2 in these genes. PBP binding to palindrome 1 would sterically prevent binding of CTF1α to palindrome 2 of *cut1* gene as the two palindromes is overlap. These results strongly suggest that PBP binding precludes CTF1α binding of *cut1* promoter, and thus PBP probably acts as a repressor of the

inducible *cut1* gene. On the other hand, the repressor PBP cannot bind the overlapping palindrome 2 in these promoters (Figure 2.8) (Kämper *et al.*, 1994; Kollatukudy, 2001a).

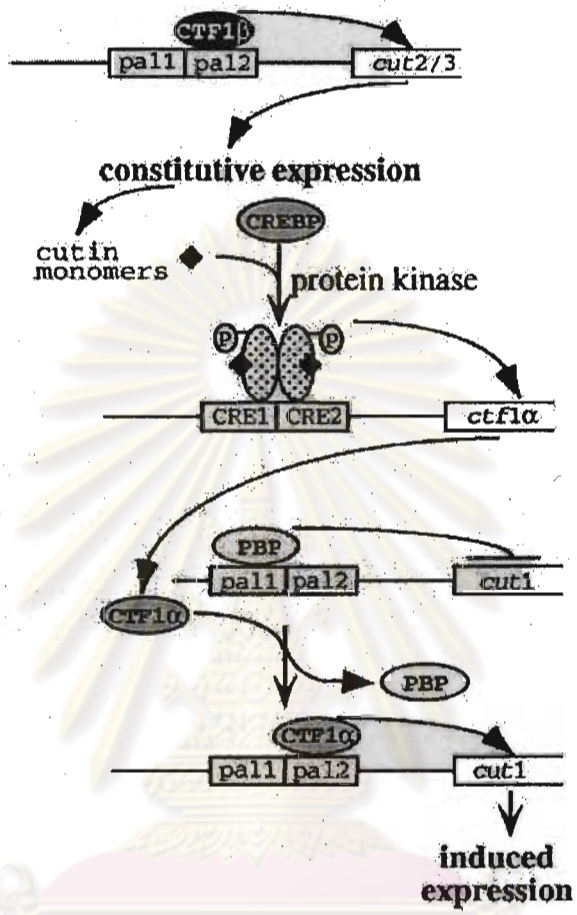


Figure 2.8 Postulated mechanism by which cutin monomers are produced by constitutive expression of *cut*2/3 induce *cut*1 (Kollatukudy, 2001a)

The promoter segment of cutinase transcription factor *ctf1α* gene shows a palindrome sequence resembling steroid hormone receptor element or cutin responsive element (CRE) (Kollatukudy, 2001a). In microorganisms producing cutinase, CRE binding protein (CREBP) was expressed and this protein was found selectively to bind the CRE. CREBP was phosphorylated by the fungal extract and this phosphorylation was stimulated by cutin hydrolysate. Thus, cutin hydrolysate-induced phosphorylation of CREBP enhances its binding to CTF1α promoter and causes up-regulation of *ctf1α* gene. The increased CTF1α levels allow CTF1α to effectively compete with PBP for

binding to the palindrome 2 in *cut1* promoter, thus causing transcriptional activation of cut1 gene (Figure 2.8) (Kollatukudy, 2001a; Kolattukudy *et al.*, 1995).

2.1.9 Function of suberin

One of the major functions of suberin is as a diffusion barrier this function being best illustrated with wound-healing tissue. Wound-healing in potato tuber discs deposit suberin in the walls of one or two oultermost layers of cells within 7 days after cutting (Kolattukudy, 1987). Suberization can be influenced by physical stress (e.g., mineral deficiency), and both chemical and ultrastructural examinations have shown that magnesium deficiency causes the hypodermis and endodermis of *Zea mays* roots to have more heavily suberized cell walls (Pozuelo *et al.*, 1984). Plants may also resort to suberization in seeking protection against freezing damage, as demonstrated by suberization of the bark of *Vitis vinifera* (Meiering *et al.*, 1980; Patoshy *et al.*, 1980). Suberization can also be induced by biological stress, and many reports have suggested that fungal or vital attack can elicit the deposition of suberin in cell walls, thereby limiting infections (Kolattukudy and Espelie, 1989). Many reports indicate that older plant organs are resistant to pathogens because of a heavily suberized periderm, whereas very young organs without such protection are susceptible to pathogen attack (Lulai and Corsini, 1998).

2.1.10 Cutin degradation

2.1.10.1 Cutin degradation by animals

Large amounts of cutin are ingested by animals as part of the vegetable and fruits in their diet. Pancreatic lipase involves in the hydrolysis of this polyester (Brown and Kolattukudy, 1978). Bile salts stabilize the pancreatic lipase and co-lipase reversed the inhibition caused by bile salt with cutin as a substrate. Thus the interaction of the enzyme with the cutin surface involving co-lipase and bile salt is similar to that observed for triglycerides. This lipase releases oligomers and monomers in similar manner to fungal cutinase.

2.1.10.2 Cutin degradation by plants

Since the insoluble cutin polymer covers expanding plant organs, this polymer may undergo a "make and break" type of expansion during the growth of the organ. However, how the polymer structure adjusts to the expansion of the organ it covers is not known. No enzymatic degradation of cutin in the plant organ has been detected except for pollen cutinase that may be involved in fertilization (Kollatukudy, 2001a; Linskens, 1975). A pollen cutinase is thought to be involved in gaining access through the stigmatic cuticle. When cutin was added to germinated pollen, a slight increase in acidity was noted, suggesting hydrolytic release of cutin monomers by pollen enzyme(s) (Heinen and Linskens, 1961; Linskens and Heinen, 1962). The amino acid composition of pollen cutinase was quite different from that of fungal cutinases as it contained a much higher content of acidic amino acids and Cys residues (Heinen and Linskens, 1961; Linskens and Heinen, 1962).

Catalytic properties of the pollen enzyme were drastically different from those of bacterial and fungal cutinases. The pH optimum for the pollen enzyme was 6.8, similar to that observed for a few fungal cutinases, whereas most of the microbial enzymes usually show much higher alkaline pH optima. In contrast to the fungal enzyme that showed stability at both acidic and basic conditions, the pollen enzyme was unstable except under neutral conditions (Maiti, Kolattukudy and Shaykh, 1979). The catalytic mechanism of this pollen enzyme does not involve the active serine catalytic triad (Kollatukudy, 2001a), as this enzyme was totally insensitive to active serine-directed reagents. On the other hand, the pollen enzyme was extremely sensitive to inhibition by thiol-directed reagents that have no effect on fungal cutinases. Thus, this pollen enzyme seems to a thiol polyesterase. In spite of such contrasting molecular and catalytic properties, the substrate specificity of the pollen enzyme resembled that of the microbial cutinase. Pollen cutinase showed a high degree of preference for hydrolysis of primary alcohol esters. It also hydrolyzed *p*-nitrophenyl esters of C_2–C_{18} fatty acids (Kolattukudy 1980; Kollatukudy, 2001a).

2.1.10.3 Cutin degradation by bacteria

Bacteria were isolated from soil and surface of aerial plant organs were found to degrade cutin (Hankin and Kolattukudy, 1971; Sebastian, Chandra and Kolattukudy,

1987). First bacterial cutinase was purified and characterized from the extracellular fluid of *Pseudomonas mendocina* (Sebastian and Kolattukudy, 1988). This bacterial cutinase is a 30-kDa protein with an amino acid composition distinctly different from that of fungal cutinases, and it does not show an immunological relationship with fungal cutinases (Sebastian and Kolattukudy, 1988). It hydrolyzes *p*-nitrophenyl esters of C_4 to C_{16} fatty acids and short chain triacylglycerols such as tributyrin, although long chain esters are less readily hydrolyzed. This bacterial cutinase uses a catalytic triad involving active serine for catalysis (Sebastian and Kolattukudy, 1988). Cutinase from *Streptomyces scabies* has also been purified and characterized (Lin and Kolattukudy, 1980). More recently other bacteria, especially the thermophiles such as *Thermomonospora*, have been found to produce cutinase activity (Fett *et al.*, 1999). Such species are being examined for potential commercial applications such textile industry (Gouda *et al.*, 2002).

2.1.10.4 Cutin degradation by fungi

Fungal cutinase degradation has been studied extensively, not only because of its potential importance in fungal infection of plants that cause extensive loss in food and fiber production, but also because of it potential industrial applications. It has been postulated for almost a century that fungal invasion of the aerial parts of plants involved enzymatic cutin degradation in order to gain entry through the cuticle (Purdy and Kolattukudy, 1975).

2.1.11 Suberin degradation

Ability to degrade suberin has been reported for *Rosellinia desmazieresii* (Ofong, AU and Pearce, 1994), *Armillaria mellea* (Zimmerman and Seemuller, 1984), and *Mycena meliigena* (Schultz, Chamuris and Dallabrida, 1996), while a variety of fungi were found to grow on potato suberin as the sole source of carbon (Kolattukudy and Espelie, 1989). *Fusarium solani* f. sp. *pisi* was found to grow more rapidly than the other fungi. The culture filtrate was tested for enzymes that could release labeled components from potato suberin biosynthetically labeled by incorporation of labeled cinnamic acid and labeled oleic acid. The enzyme that released the labeled esterified aliphatic components was purified to homogeneity and characterized (Fernando, Zimmermann

and Kolattukudy, 1984). This enzyme was found to be identical to the cutinase produced by *F. solani* f. *pisi* grown on cutin. When the periderm of *Rubus idaeus* was incubated with this purified enzyme, a variety of aliphatic monomers of this suberin were released (Espelie, Köller and Kolattukudy, 1983). It is not surprising that the fungus produces the same polyesterase to grow on the polyester containing suberin as that produced upon growth on cutin.

2.1.12 Cutinase

Cutinases (EC 3.1.1.74) are inducible extracellular enzymes produced by several phytopathogenic fungi, bacteria and pollen which are able to hydrolyse ester bonds in the cutin polymer (Ettinger, Thukral and Kolattukudy, 1987). Cutinases belong to a class of serine hydrolases that hydrolyse a large variety of synthetic esters and show activity towards short and long chains of emulsifed triacylglycerols as effciently as pancreatic lipases (Geus, Lauwereys and Matthyssens, 1989). Contrary to lipases, the activity of which is greatly enhanced in the presence of a lipid-water interface, cutinases do not display, or display little, interfacial activation, being active on both soluble and emulsifed triglycerides (Verger and Haas, 1976).

2.1.12.1 Catalysis properties of cutinase

Fungal cutinase catalyzes hydrolysis of model substrates and in particular *p*-nitrophenyl esters of short chain fatty acids, providing a convenient spectrophotometric assay for this enzyme activity (Kolattukudy, 1984; Kolattukudy, Purdy and Maiti, 1981). Hydrolysis of model esters by this cutinase showed the high degree of preference of this enzyme for primary alcohol ester hydrolysis. Wax esters and methyl esters of fatty acids were hydrolyzed at low rates. Alkane-2-ol esters were hydrolyzed much more slowly than wax esters and esters of mid-chain secondary alcohols were not hydrolyzed at significant rates. Triglycerides were hydrolyzed by the purified fungal cutinases at slow rates and this activity was as sensitive as cutinase to active site-directed reagents, showing that both activities involve the same catalytic site. Trioleyl glycerol and tributyryl glycerol were hydrolyzed 5–30 times as rapidly as tripalmitoyl glycerol by several fungal cutinases. Cinnamoyl esters of alcohols and cholesterol esters were not hydrolyzed at measurable rates, whereas cyclohexyl esters were readily hydrolyzed. With cutin as the

substrate fungal cutinase showed both *exo*- and *endo*-esterase activity. Thus, short-term incubation of biosynthetically labeled cutin with purified cutinase released oligomeric and monomeric labeled products (Purdy and Kolattukudy, 1975). Cutinase also catalyzed hydrolysis of the oligomers to monomers.

Cutinase is a serine esterase that catalyzes hydrolysis of ester bonds using the catalytic triad involving histidine, aspartic acid and "active" serine (Figure 2.9) (Köller, Kolattukudy, 1982). A variety of organic phosphates and other reagents that are known to react with active serine irreversibly inhibited fungal cutinases. Some of them showed 50% inhibition at lower than nmol/l concentrations, strongly suggesting that the enzymes contain extremely reactive serine (Kolattukudy, 1984).

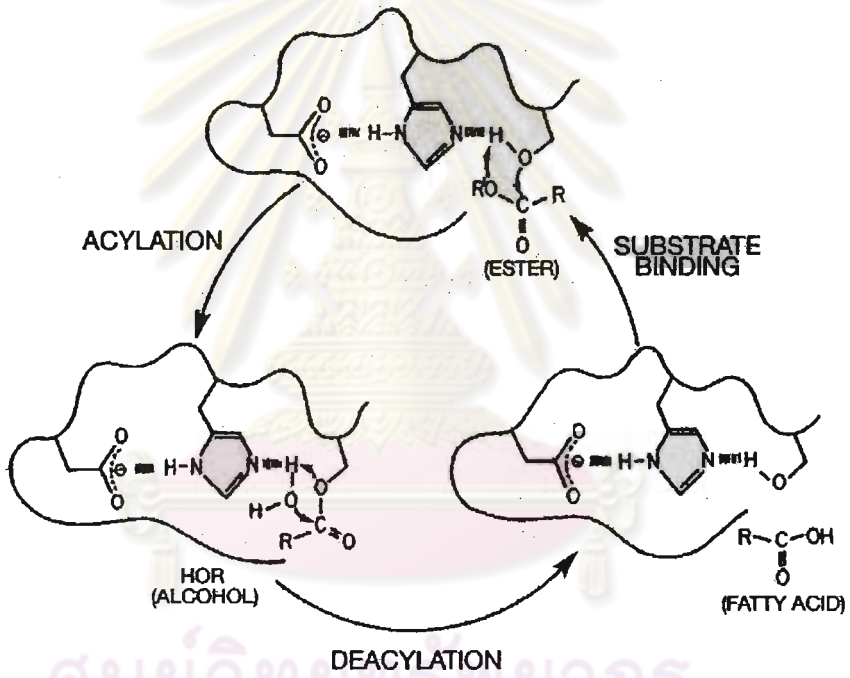


Figure 2.9 Mechanism of catalysis by cutinase via the active serine catalytic triad (Köller, Kolattukudy, 1982)

2.1.12.2 Purification and molecular characterization

Several cutinases from pathogenic fungi including *Alternaria brassicicola* (Trail and Köller, 1992.), *Botrytis cinerea* (conidial cutinase) (Gindroo and Pezet, 1999), *Colletotrichum gloeosporioides* (Dickman Patil and Kolattukudy, 1982), *F. solani* f. sp. *pisi* (Purdy and Kolattukudy, 1975), *Monilinia fructicola* (Wang *et al.*, 2000), *Venturia inaequalis* (Köller and Parker, 1989.) and pathogenic bacterium *Streptomyces scabies*

(McQueen and Schottel, 1987) have been purified and characterized. Among these is the cutinase from the pea pathogen, *F. solani* f. sp. *pisi,* the first cutinase purified and characterized and the only one that has been extensively studied structurally and biochemically (Kolattukudy, 1984; Martinez *et al.,* 1992). QAE-Sephadex chromatrography is used to remove all of the colored material from the enzyme. The intense color of the concentrated culture filtrates was caused from presence of covalently attached phenolics in the cutin preparation (Purdy and Kolattukudy, 1975). The colorless enzyme preparation was subjected to hydrophobic chromatrography on Octyl-Sepharose, followed by SP-Sephadex cation-exchange chromatrography to yield a highly purified enzyme that shows a single band in SDS-PAGE. This general procedure yields purified cutinases from a variety of fungi (Kolattukudy *et al.*, 1981). The majority of fungal cutinases purified to date are single polypeptides in the range of 18-60 kDa (Kolattukudy, 1984). The pH optimum for the conidial cutinase is 6.0 (Gindroo and Pezet, 1999) while most of the microbial enzymes usually show much higher alkaline pH optima. The fungal enzyme also showed stability at both acidic and basic conditions (McQueen and Schottel, 1987; Purdy and Kolattukudy, 1975)

2.1.12.3 **Structure of cutinase**

The three dimensional structure of *F. solani* f. sp. *pisi* cutinase (Figure 2.10) that had been cloned and expressed in *E. coli*, was solved (Jelsch *et al.*,1998; Longhi *et al.*, 1997). Cutinase is a 197-residue protein in a compact one domain molecule. This cutinases have a molecular weight around 22,000 daltons with highly conserved stretches, which include four invariant cysteines, forming two disulfide bridges. *F. solani* f.sp. *pisi* cutinase has an isoelectric point of 7.8 (Petersen *et al.*, 1997). This enzyme is also an α/ß-hydrolase and has a central ß-sheet consisting of five parallel strands covered by four helices on either side of the sheet. It belongs to the class of the serine esterases. The stretch Gly-Tyr-Ser-Gln-Gly containing the active site Ser120, has even stronger homology with the consensus sequence Gly-(Tyr or His)-Ser-X-Gly commonly present in lipases. The catalytic triad Ser120, Asp175 and His188, is accessible to the solvent. It is located at one extremity of the protein ellipsoid, and is surrounded by the loop 80-87 and by the more hydrophobic loop 180-188 (Jelsch *et al.*, 1998). To date,

around 40 X-ray structures of cutinase and its mutants and inhibitor conjugates have been solved (Martinez *et al.*, 1992; Longhi *et al.*, 1997; Jelsch *et al.*, 1998).

Figure 2.10 Ribbon representation of cutinase. α-Helices (red), ß-strands (blue) and coils (yellow) are shown. The residues of the catalytic triad (Ser120, green; His188, blue; Asp175, red), are represented. Residues of the loops delimiting the catalytic crevice (residues 80-88 and 180-188) have been labeled (Longhi and Cambillau, 1999).

2.1.13 Cutinases and their potential applications

As a lipolytic enzyme, cutinase has been presented as a versatile enzyme showing several interesting properties for applications with industrial products and processes. Hydrolytic and synthetic reactions catalyzed by cutinase have potential use in the dairy industry for the hydrolysis of milk fat, in household detergents, in the oleochemical industry, in the synthesis of structured triglycerides, polymers and surfactants, in the synthesis of ingredients for personal-care products, and the synthesis of pharmaceuticals and agrochemicals containing one or more chiral centers (Murphy *et al.*, 1996). At low water activities transesterification of fats and oils or (stereo) selective esterification of alcohols can be achieved. Some of these processes are already applied in industry, while others are still under evaluation at a research level. In recent years, the

esterasic activity of cutinase has been largely exploited and several applications in different industrial fields have been presented. Taking profit from its *in vivo* cutinolytic activity, an enzymatic preparation containing cutinase has been developed for increasing the pharmacological effect of agricultural chemicals (Genencor, 1988).

In vitro, cutinases display hydrolytic activity towards a broad variety of esters, from soluble synthetic esters (e.g. p-nitrophenyl esters) to insoluble long-chain triglycerides. Moreover, cutinases can be considered as a link between esterases and lipases as they efficiently hydrolyze soluble esters and emulsified triacylglycerols (Egmond and van Bemmel, 1997). Therefore cutinase has been evaluated as a lipolytic enzyme in laundry and dishwashing detergent formulations (Okkels *et al.*, 1997; Egmond and van Bemmel, 1997; Unilever, 1994). Some benefits were achieved with cutinase, when compared with a commercial lipase, LipolaseTM, on the removal of triacylglycerols in a single wash process, as cutinase was able to hydrolyze the fats in the absence of calcium (Egmond and van Bemmel, 1997).

An interesting application of cutinase is the degradation of synthetic polyester. It is known that cutinases are able to catalyze the hydrolysis of ester bonds in cutin or suberin and also attack ester bonds in some aliphatic polyester such as polycaprolactone (PCL) (Murphy *et al.*, 1996) as well as aromatic polyesters which is polyethylene terephthalate (PET) (Yoon *et al.*, 2002).

Polyester fibers made of PET have useful properties. They are resistant to a wide range of chemicals and also withstand abrasion, stretching, shrinking and wrinking. However, the fibers also exhibit a number of negative features affecting the wearing comfort of PET textiles. These include a low flexibility and high hydrophobicity of the fibers, the tendency to pilling, static charges and resistance to the removal of oil stains, currently, chemical treatments with sodium hydroxide are used in industry under harsh condition to improve the flexibility of fibers and to increase their hydrophilicity. However, this treatment is not environmentally benign. It reduces the quality of the fibers and causes a loss of fiber material in the process (Shukla *et al.*, 1997; Zeronian and Collins, 1989). Enzymatic modification for the textile finishing process of PET fibers would have the advantage that it can be performed under mild and environmentally friendly process conditions. The reaction is selective and only involves the surface of the fibers. The

potential of enzymes to modify synthetic polyester has recently been reported (Yoon *et al.*, 2002). Cutinase from *F. solani* f.sp. *pisi* can hydrolyze ester bonds of PET in polymer chains, resulting in the generation of hydroxyl and carboxyl groups at the surface and in the formation of terephthalic acid and ethylene glycols as reaction products (Carvalho *et al.*, 1999; O' Neill *et al.*, 2007). Pilling properties of polyester fabrics were found to be improved by treatment with enzyme preparations from *Humicola* sp., *Candida* sp. and *Pseudomonas* sp. (Andersen *et al.*, 1999). The application of *Arthrobacter* and *Trichosporon* for the modification of PET fibers has also been described (Oda and Kimura, 1998). Improved stain resistance, wettability and/or dyeability of PET fabrics treated was reported with so-called polyesterases (lipases, esterases or cutinases) (Yoon *et al.*, 2002). A thermostable enzyme from *Thermomonospora fusca* strain with catalytic properties similar to a fungal cutinase has been described which efficiently hydrolysed aliphatic–aromatic co-polyesters (Kleeberg *et al.*, 2005)

2.2 Synthetic polyester: Polyethylene terephthalate (PET)

2.2.1 Introduction

Polyethylene terephthalate (PET) is the most widely used polymers in textile industry. PET, manmade fiber, only has a market share of 31.3% in textile industry and tends to gain even more share compared to natural fibers (Yoon *et al.*, 2002). The primary drive for this growth is demand for fiber and container resin. Seventy five percent of the entire PET production is directed toward fiber manufacturing. Hoechst, Dupont and Eastman are the three world largest polyester producers. Additional current U.S. Polyester Fiber Producers are: Acordis Industrial Fibers, Inc.; Allied Signal Inc; Cookson Fibers, Inc.; KoSa; Intercontinental Polymers, Inc., Martin Color-Fi. Nan Ya Plastics Corp., Wellman, Inc. (Harris, 1996). Dramatic growth in PET fiber production is foreseen in Asia in the near future (Harris, 1996). Forty nine percent of the total nonwovens market share in the USA belongs to polyesters, reaching 291 million pounds in 1996 and ranking number one among all kinds of fiber supplies. Moreover, the filament fiber consumption is half of that of staple, the total PET consumption in the USA nonwovens industry alone would be over 450 million pounds. Thus, polyester has become the most widely used polymer in the nonwovens industry since 1995 (Harrison, 1997).

2.2.2 PET fibers

Polyester fiber is a manufactured fiber in which the fiber forming substance is any long chain synthetic polymer composed at least 85% by weight of an ester of a dihydric alcohol (HOROH) and terephthalic acid (p-HOOC-C_6H_4COOH) (Figure 2.11). The most widely used polyester fiber is made from the linear polymer poly (ethylene terephthalate), and this polyester class is generally referred to simply as PET.

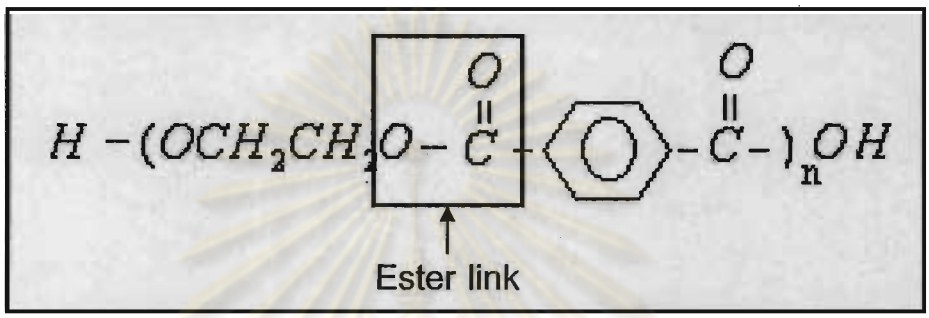


Figure 2.11 The repeating unit of PET

(http//www.rjclarkson.demon.co.uk/candrands/polymerization.htm)

2.2.3 Polymer formation

PET is a condensation polymer and is industrially produced from either terephthalic acid or dimethyl terephthalate with ethylene glycol (Figure 2.12) (Cook, 1968). Other polyester fibers of interest include:

(a) Terephthalic acid (PTA), produced directly from p-xylene with bromide-controlled oxidation.

(b) Dimethyl terephthalate (DMT), made in the early stages by esterification of terephthalic acid with methyl alcohol. However, a different process involving two oxidation and esterification stages now accounts for most DMT.

(c) Ethylene glycol (EG) initially generated as an intermediate product by oxidation of ethylene. Further ethylene glycol is obtained by reaction of ethylene oxide with water.

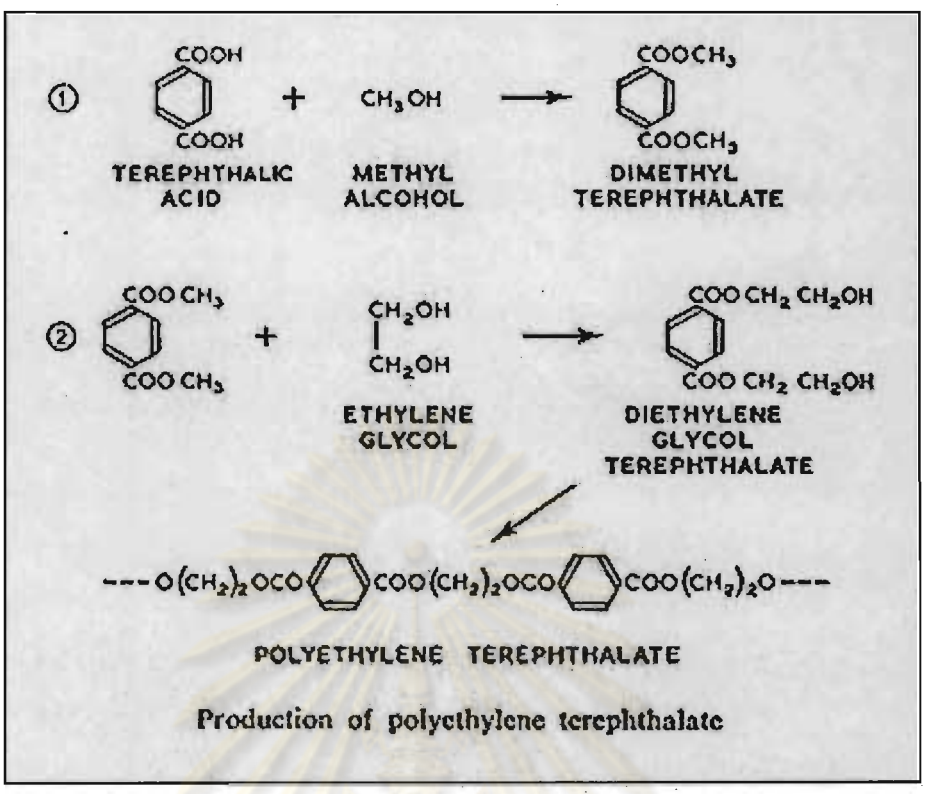


Figure 2.12 Production of polyethylene terephthalate (Rosalind, 1989)

2.2.4 Microscopic properties

Polyester fibers are manufactured in a variety of cross section including round, trilobal and pentalobal. Under the microscope, round fibers appear as long, smooth rods with spots of pigment (Figure 2.13). This pigmented appearance decreases the luster or brightness of polyesters. Longitudinally, multilobal fibers appear striated (Phyllis, 1978).

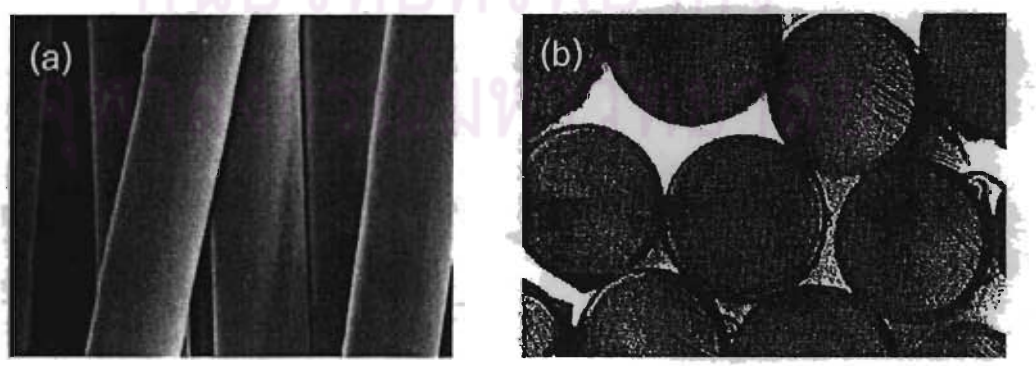


Figure 2.13 Photomicrographs of regular polyester fiber, longitudinal view (a) and cross-section (b) (Phyllis, 1978)

2.2.5 Physical properties

The physical properties of PET fibers include (East, 2005):

(a) Shape and appearance

Except for the multilobal varieties, polyesters are generally round and uniform. They can be of any length or diameter as required by fiber producers and yarn and fabric manufacturers. The fiber is partially transparent and white of slightly off-white in color. Optical brighteners are frequently added to produce clear, bright white polyester fibers.

(b) Strength

The strength or tenacity of polyester varies with the type of fiber; however, as a general category, polyester would be considered a relatively strong fiber. Regular filaments have a breaking tenacity of 4 to 6 g/d; high tenacity filaments are rated at 6.3 to 9.5 g/d.

(c) Elasticity and resilience

Polyester fibers do not have a high degree of elasticity. In general, polyester fiber is characterized as having a high degree of stretch resistance, which means that polyester fabrics are not likely to stretch out of shape too easily. Polyester fiber has a high degree of resilience. Not only does a polyester fabric resist wrinkling when dry, it also resists wrinkling when wet.

(d) Density and specific gravity

The density or specific gravity (1.38 or 1.22 depending on type) is moderate. Fabrics made from polyesters are medium in weight.

(e) Absorbency and moisture regain

Polyester is the one of the least absorbent fibers. Absorbency is quite low for polyester, ranging from 0.4 to 0.8 percent of moisture regain. This low absorbency has two important advantages. Polyester fabrics will dry very rapidly since almost all the moisture will lie on the surface rather than penetrate the yarns. Therefore they are well suited for the water-repellent purposes such as rainwear. Furthermore this low absorbency means that polyester fabrics will not stain easily. Fabrics of low absorbency generally have the disadvantages of being clammy and uncomfortable in humid weather because they will not absorb perspiration or atmospheric moisture.

(f) Dimensional stability

Polyesters that have been given heat-setting treatments have excellent dimensional stability, so long as the hest-setting temperature is not exceeded. If polyester fabrics have not been heat-set, they may shrink at high temperature.

Typical physical and mechanical properties of PET fibers are reviewed in Table 2.5.

Table 2.5 Physical properties of polyester fibers (Hearle and Miraftab, 1995)

Property	Filament yarn		Staple and tow	
	Regular tenacity[a]	High tenacity[b]	Regular tenacity[c]	High tenacity[d]
Breaking tenacity (N/tex)[e]	0.35-0.50	0.62-0.85	0.35-0.47	0.48-0.61
Breaking elongation	24-50	10-20	35-60	17-40
Elastic recovery at 5% elongation (%)	88-93	90	75-85	75-85
Initial modulus (N/tex)[f]	6.6-8.8	10.2-10.6	2.2-3.5	4.0-4.9
Specific gravity	1.38	1.39	1.38	1.38
Moisture regain (%)[g]	0.4	0.4	0.4	0.4
Melting temperature (^{o}C)	258-263	258-263	258-263	258-263

[a]Textile-filament yarns for woven and knit fabrics. [b]Tire cord and high strength, high modulus industrial yarns. [c]Regular staple for 100% polyester fabrics, carpet yarn, fiberfill, and blends with cellulosic blends or wool. [d]High strength, high modulus staple for industrial applications, sewing thread, and cellulosic blends. [e]Standard measurements are conducted in air at 65% rh and 22^{o}C.[f]To convert N/tex to g/d, multiply by 11.33. [g]The equilibrium moisture content of the fibers at 21^{o}C and 65% rh.

2.2.6 Thermal properties

The thermal properties of PET fibers depend on the method of manufacture. The thermal desorption analysis (TDA) and thermomechanical analysis (TMA) (Figure 2.14) data for fibers spun at different speeds show peaks corresponding to glass transition,

crystallization, and melting regions. Their contours depend on the amorphous and crystalline content. The curves shown for 600 m/min and above are characteristic of drawn fiber. The glass transition range is usually in the range of 75°C; crystallization and melting ranges are around 130°C and 260°C, respectively (Morton and Hearle, 1975).

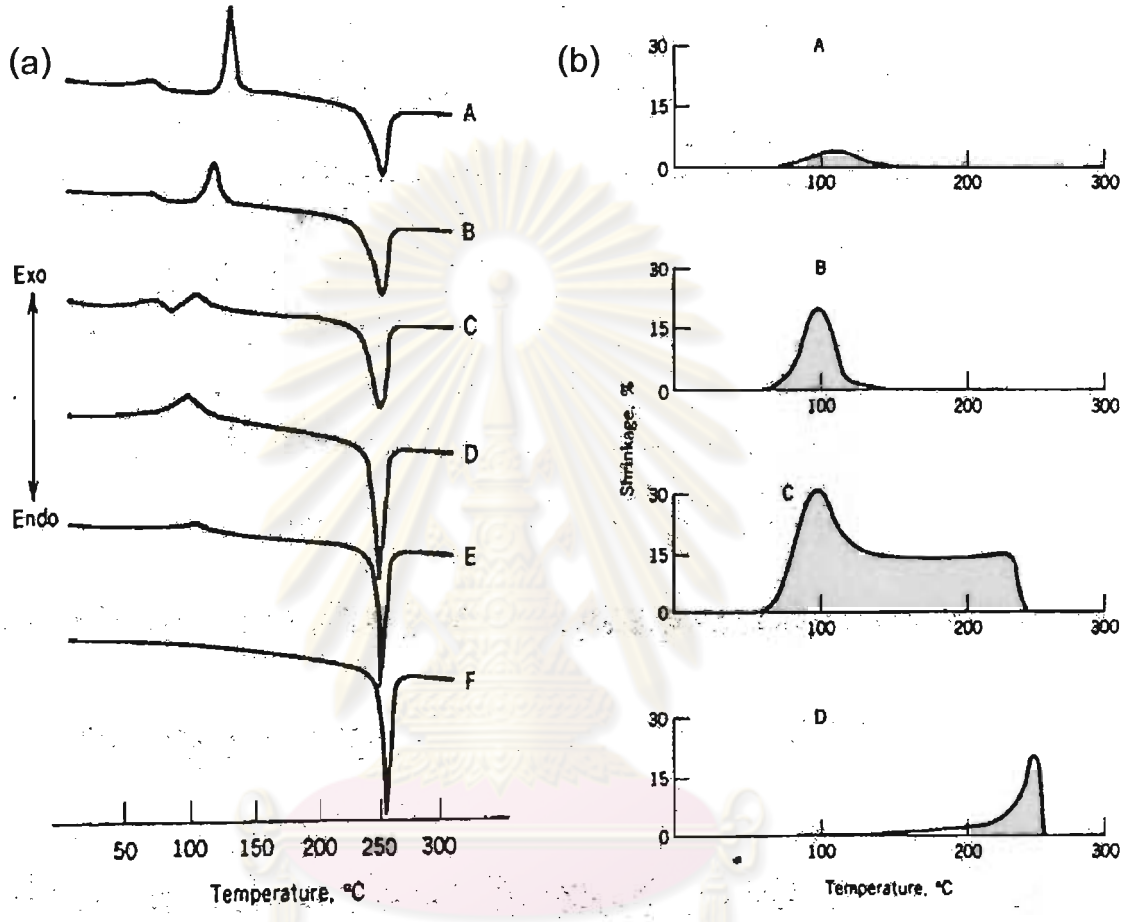


Figure 2.14 TDA curves for PET yarn spun at different spinning speed (m/min) (a): A=2,000, B=3,000, C=4,000, D=4,500, E=5,000 and F=6,000; TMA (shrinkage) curves of spun yarn at different spinning speeds (m/min) (b): A=<1,000, B=1,000-2,000, C=2,000-5,000 and D=>6,000 (Morton and Hearle, 1975).

The thermal degradation of PET proceeds by a molecular mechanism with random chain scission at ester linkages, although a radical mechanism has also been proposed. A chain-scission scheme is shown as figure 2.15.

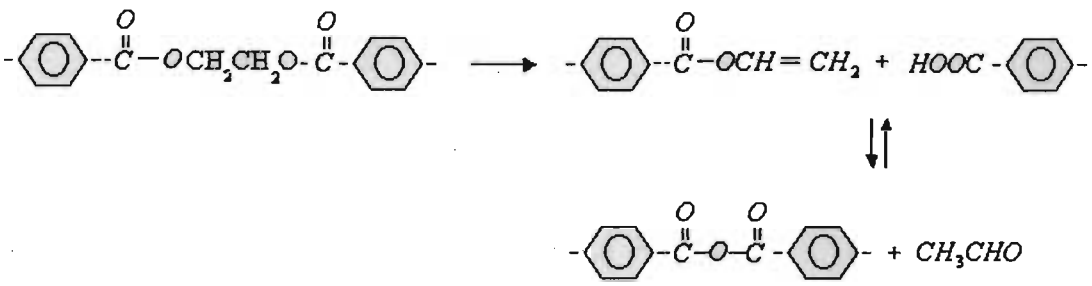


Figure 2.15 A chain-scission scheme of PET polymer proceeded by the thermal degradation (Morton and Hearle, 1975).

The degradation products can undergo further changes, but at ordinary processing temperatures a certain proportion of carboxyl groups is introduced into the polymer structure. Color formation upon degradation has been attributed to the formation of polyenaldehydes from acetaldehyde and from a further breakdown of poly(vinyl ester)s. As the fiber melts, it forms a gray or tawny-colored bead that is hard and non-crushable. Polyester will burn and procedure a dark smoke and an aromatic odor. However, they do not burn as rapidly as many other fabrics (Moncrieff, 1963).

2.2.7 Chemical properties

Polyester fibers have good resistance to weak mineral acids even at boiling temperature, and to most strong acids at room temperature, but are dissolved with partial decomposition by concentrated sulfuric acid. Hydrolysis is highly dependent on temperature. Thus conventional PET fibers soaked in water at 70°C for several weeks do not show a measurable loss in strength, but after one week at 100°C, the strength is reduced by approximately 20%.

Polyesters are highly sensitive to bases such as sodium hydroxide and methylamine, which serve as catalysts in the hydrolysis reaction. These alkalies penetrate the structure initially through non-crystalline regions, causing the degradation of the ester linkages. The fiber loses weight as the reaction occurs. The hydroxyl ions attack the electron-deficient carbonyl carbons of the polyester to form an intermediate anion. Chain scission follows and results in the production of hydroxyl and carboxylate end-groups (Figure 2.16) (Zeronian and Collins, 1989). This susceptibility to alkaline attack is sometimes used to modify the fabric aesthetics during the finishing process.

The porous structures produced on the fiber surface by this technique contribute to higher wettability and better wear properties (Hsieh, 1996).

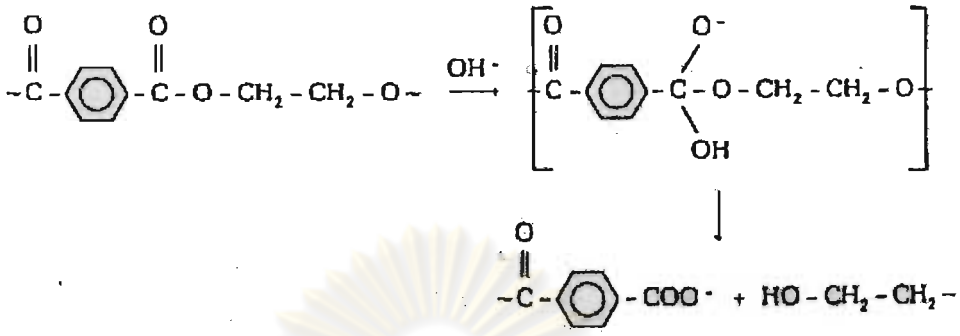


Figure 2.16 A chain-scission scheme of PET polymer produced by the alkaline degradation (Zeronian and Collins, 1989).

Polyester displays excellent resistance to oxidizing agents, such as conventional textile bleaches, and is resistant to cleaning solvents and surfactants. Also, PET is insoluble in most solvents except for some polyhalogenated acetic acids and phenols. Concentrated solutions of benzoic acid and o-phenylphenol have a swelling effect.

PET is both hydrophobic and oleophilic. The hydrophobic nature imparts water repellency and rapid drying. But because of the oleophilic property, removal of oil stains is difficult. Under normal conditions, polyester fibers have a low moisture regain of around 0.4%, which contributes to good electrical insulating properties even at high temperatures. The tensile properties of the wet fiber are similar to those of dry fiber. The low moisture content, however, can lead to static problems that affect fabric processing and soiling (Lewin and Pearce, 1985).

2.2.8 Dyeing properties

Polyester fibers can be dyed to useful depths only by dyes with low solubility in water. Disperse dyes are the most important dyes used in dyeing polyester fibers. Because of their high crystallinity, hydrophobic nature, the absence of dye sites, very low swelling, low diffusion of the dye molecules, high glass-transition temperature and the absence of chemically reactive groups in the molecular chain, these fibers can be

dyed with a satisfactory rate of dyeing, either at temperatures in the region of 130°C or at lower temperatures in the presence of a carrier (Georgiadou *et al.*, 2002).

2.2.9 Biological properties

Insects will not destroy polyesters if there is other food available. However, if trapped, beetles and other insects will cut their way through the fabric as a means of escape. Although PET was classified as non-biodegradable polymer, some pathogenic microbes produced cutinase can hydrolyze ester bond of this polymer. Usually, any discoloration is easily removed, since it does not penetrated the fiber.

2.2.10 Main problems

Of all the man-made fibers, polyester fiber is widely used because of its outstanding properties such as high strength, high wear resistance, pleasant appearance and ease of washing. However, the inherent poor absorbency of polyester, 0.2-0.8 %, lowers the comfort factor of skin-contact apparel and upholstery. Low moisture absorption is also associated with the high build-up of static charges that can add to discomfort and cause garments to drape improperly (Lewin and Pearce, 1985).

The conventional modification of woven, knitted and non-woven textiles can be modify some quality of polyester fiber or fabric that is need to enhance its aesthetic, performance properties and surface characteristics. The modification results in either physical or chemical changes of the fiber or fabric which can be achieved through chemicals such as sodium hydroxide (Zeronian and Collins, 1989).

In addition to alterations in the aesthetics and mechanical properties of chemically modified textiles, there are disadvantages to this form of processing. These disadvantages include the use of large amounts of chemicals, high temperature treatments, increased cost, and environmental concerns. Environmental problems arise from both airborne particulate emission during processing, and water pollution caused by the discharge of untreated effluents. These concerns and limitations have led to the further development of alternative modification processing methods. An enzymatic modification of PET and other polyester fibers has been investigated, since it could be performed without damaging the fibers using environmentally friendly and energy saving

process conditions (Guebitz and Cavaco-Paulo 2003). There are many applications of using the enzymes including cutinase and lipase to improve polyester fabric properties as described previously (Hsieh and Cram 1998; Yoon *et al.* 2002). Thus, the potential use of the enzymes will help to improve of many undesirable properties of polyester and opens new possibilities to improve product quality with the environmental friendliness of related industrial processes.

2.2.11 Applications

DuPont Company produced the first U.S. commercial polyester fiber in 1953. Since polyester fiber has a lot of special characteristics, most of them are used in the following three major areas (Cook, 1968):

(a) Apparel: Every form of clothing

(b) Home Furnishings: Carpets, curtains, draperies, sheets and pillowcases, wall coverings, and upholstery

(c) Other Uses: Hoses, power belting, ropes and nets, thread, tire cord, auto upholstery, sails, floppy disk liners, and fiberfill for various products including pillows and furniture

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Equipment

ABI PRIAM® 3100 Automated DNA Sequencer: Applied Biosystems, Foster City, CA, USA

AD 4715 infrared moisture determination balance: A&D weight, Thebarton, South Australia

ABI PRISM® 3100 BigDye Terminators v3.0 Cycle Sequencing reactions: Applied Biosystems, Foster City, CA, USA

Autoclave: Ta Chang Medical Instrument Factory, Taipei, Taiwan

Autopipette: Pipetman, Gilson, Villiers, France

Bright-field microscopy: Model CH30, Olympus, Tokyo, Japan

Centrifuge, refrigerated centrifuge: Hettich, Burladingen, Germany

Centrifuge, microcentrifuge: Model Denville 260D, Denville Scientific Inc., Metuchen, NJ, USA

Electrophoresis unit : Model mini-protein cell, Bio-Rad Applied Biosystem Company, Foster City, CA, USA

Fluorescence spectrophotometer : Model LS 55, Perkin Elmer Inc., Vienna, Austria

Gel documentation: Bio-Rad Labolatories, Hercules, CA, USA

HiPrep 16/60 Sephacryl S-200 High Resolution Column: GE Healthcare Bio-Sciences, Uppsala, Sweden

Hitrap Q FF column: GE Healthcare Bio-Sciences, Uppsala, Sweden

Hitrap phenyl HP column: GE Healthcare Bio- Sciences, Uppsala, Sweden

Hounsfield universal testing machine: Model H10KM, Brisbane, Australia

Incubator Shaker: New Brunswick Scientific Co., Edison, NJ, USA

Laminar flow: Model BV 123, ISSOC, Bangkok, Thailand

Luminescence spectrophotometer : Model LS 50 B, Perkin Elmer Inc., Vienna, Austria

Membrane filter: Whatman No.1, Tokyo, Japan

Peltier PTC-100™ Thermal Cycler: MJ ResearchTM, Inc., MA, USA

pH meter: Model PP-50, Sartorius, Goettingen, Germany

Power supply: Model Power PAC1000, Bio-Rad Laboratories, Hercules, CA, USA

Reflectance spectrophotometer: Macbeth, Newburgh, NY, USA

Regenerated cellulose tubular membrane: Membrane Filtration Products Inc., Seguin, TX, USA

Scanning electron microscope: JSM-6400, Tokyo, Japan

Spectrophotometer: Model UV-2800, Unico, Dayton, NJ, USA

Thermo Nicolet Nexus 670 spectrophotometer: GMI, Inc., Ramsey, MN, USA

UV transluminater: Model ECX-26-M, Vilber Lourmat, France

Vivaspin 15 Polyethersulfone membrane, MWCO 5,000, Sartorius, Goettingen, Germany

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

Agarose: GenePure, Minneapolis, MN, USA

Ammonium sulfate: Ajex Finechem, Auckland, New Zealand

Avolan IW: DyStar Thai Ltd., Bangkok, Thailand

Bacto peptone: Ajex Finechem, Auckland, New Zealand

Blankit® IN (BASF): DyStar Thai Ltd., Bangkok, Thailand

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

Deoxyribonucleotide triphosphate (dNTP): Vivantis, Shah Alam Selangor DE, Malaysia

Dianix Red CC dye a kind gift from DyStar Thai Ltd., Bangkok, Thailand

Ethylene diamine tetra-acetic acid (EDTA): Ajex Finechem, Auckland, New Zealand

Fast blue RR salt: Sigma-Aldrich Inc., Milwaukee, WI, USA

Ferrous sulfate: Fluka, Buchs SG, Switzerland

Formaldehyde 37%: Ajex Finechem, Auckland, New Zealand

Gel filtration LMW Calibration Kit: GE Healthcare, Uppsala, Sweden

Hexadecyltrimethyl ammonium bromide(CTAB): Sigma-Aldrich Inc., Milwaukee, WI, USA

Hydrochloric acid: Carlo Erba, Milano, Italy

Isopropanol: Fisher Scientific, LE, UK

Lactophenol-cotton blue: Fluka, Buchs SG, Switzerland

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

QIAquick PCR Purification Kit: QIAGEN, Inc., Valencia, CA, USA)

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

Silver nitrate: Scharlau, Barcelona, Spain

Sodium carbonate: Scharlau, Barcelona, Spain

Sodium chloride : Scharlau, 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

Trizma base: Sigma-Aldrich Inc., Milwaukee, WI, USA

Zinc sulfate heptahydrate: Scharlau, Barcelona, Spain

3.3 Enzymes and DNA primers

Taq polymerase: Vivantis, Malaysia

ITS5 (GGAAGTAAAAGTCGTAACAAGG): Vivantis, Shah Alam Selangor DE, Malaysia

ITS4 (TCCTCCGCTTATTGATATGC): Vivantis, Shah Alam Selangor DE, Malaysia

ERG3-1 (CAGCATGGCTACGAGTTC): Vivantis, Shah Alam Selangor DE, Malaysia

ERG3-4 (CTCCTTGTTTCCCGGCTT): Vivantis, Shah Alam Selangor DE, Malaysia

EF-1 (ATGGGTAAGGARGACAAGAC): Vivantis, Shah Alam Selangor DE, Malaysia

EF-2 (GGAR*GTACCAGTS*ATCATGTT): Vivantis, Shah Alam Selangor DE, Malaysia

3.4 Polyester samples

Prewashed 100% PET fibers: Germany

Woven 100 % PET fabric: Asia Fiber Public Company, Thailand

3.5 Procedures

3.5.1. Sample collection

Twenty three samples of plant surfaces including leaves, flowers, underground storage organs, barks and fruits and thirty two samples of soil nearby the roots of these plants were collected from several habitats in nine Thai provinces (Figure 3.1). The sampling collections included the rainy season (June, July, August and September) and the dry season (March). The samples were collected from low (0.5 m, Samutsakorn Province) to high (1,100 m, Chiangmai Province) altitudes, at ranges from 20.2 °C (Chaingmai Province) to 32.0 °C (Bangkok Province), and from seven provinces.

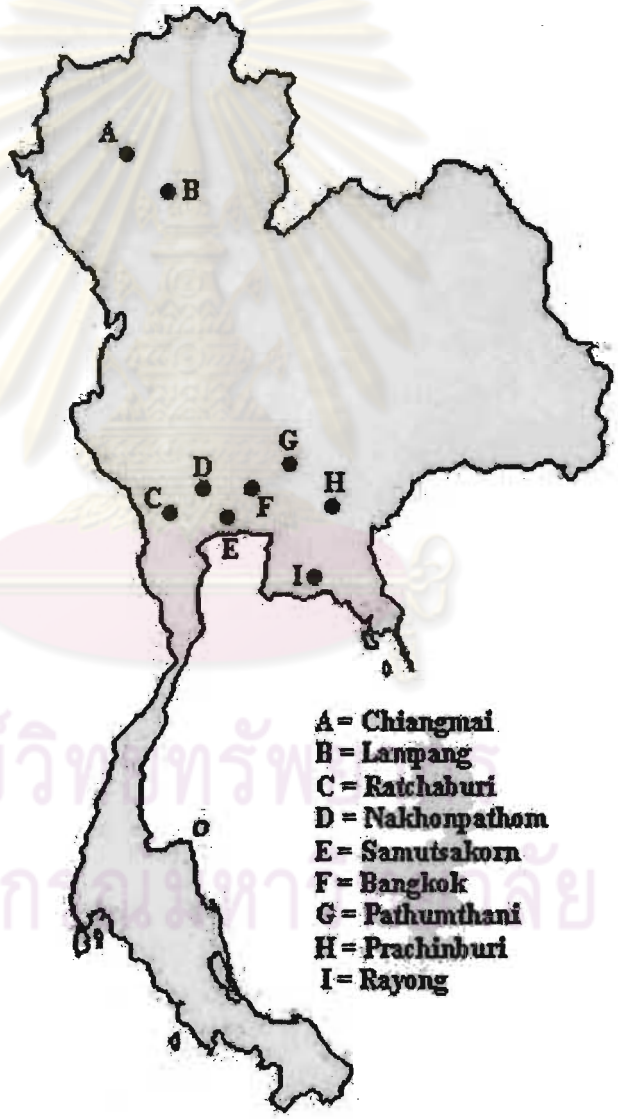


Figure 3.1 Map of collection sites of plant and soil samples from nine provinces in Thailand

3.5.2 Screening for PET-hydrolyzing enzymes

The surface of leaves, flowers, underground storage organs, barks and fruits were aseptically cut into small pieces (0.5 x 0.5 cm) and placed on a selective agar of mineral medium (pH 7.0) supplemented with a suspension of polycaprolactone (MM-PCL) 0.5 g/l, (see Appendix A) (Murphy *et al.*, 1996). This cutin analog, polycaprolactone (PCL - mol. wt. 14,000), was purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. For selective isolation of soil fungi, soil samples near by roots (10 g) were mixed into sterile water (100 ml). Soil suspensions were vigorously shaken until thoroughly mixed and the suspensions (10 ml) were mixed with 90 ml of sterile distilled water. Ten-ml samples were transferred until appropriate dilution serial was obtained (Barron, 1968). One-ml aliquots of the selected dilutions were spread plated onto MM-PCL agar plates. All cultures were incubated at 30 °C for 7 days and examined daily for growth and capability to clear the MM-PCL agar plates. The fungi producing clearing zones were isolated and transferred to fresh medium.

The active isolates were cultivated in liquid mineral medium (LMM-see Appendix A) (Murphy *et al.*, 1996) containing either potato skin suberin (see Appendix A) 2 g/l, isolated from *Solanum tuberosum* Linn. (Walton and Kolattukudy, 1972) or PET fibers 2 g/l (1 cm, untreated prewashed 100% PET filament fibers, the kind gift of Dr. Th. Böhme KG, Geretsried, Germany). Inocula were prepared in LMM containing glucose 0.1% (w/v) and incubated at 30 °C for 4 days. These inocula were transferred to the LMM containing suberin or PET fiber, and the cultures incubated at the same temperature up to 21 days. The culture supernatants were assessed for esterase activity (see appendix D) daily by spectrophotometric assay at 405 nm using p-nitrophenyl butyrate (p-NPB) as substrate (Sebastian *et al.*, 1987). An enzyme unit (1 U) was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol per minute at pH 7.0 and 25 °C.

3.5.3 Fungal identification

3.5.3.1 Morphological observation

All active fungal isolates on PCL degradation were grown on potato dextrose agar (PDA-see Appendix A) pH 7.0 at 30 °C for several days and the colony

morphology (color and texture) were examined. Slide cultures were prepared using banana leaf agar (BLA-see Appendix A) (Seifert, 1996) stained with lactophenol-cotton blue, and examined by bright-field microscopy (mycelium, mycelium color, septa and conidia). Isolates were identified to genus level using Barnett & Hunter (1998) and Fusaria by use of Seifert (1996).

3.5.3.2 Molecular identification of *F. solani* PBURU-B5

3.5.3.2.1 DNA extraction

PBURU-B5 produced the most promising enzyme and was focused on. Fresh mycelia of PBURU-B5 grown in potato dextrose broth (PDB-see Appendix A) for 5 days (pH 7.0, 30 °C, 150 rpm) were collected by filtration on paper filter under suction and then washed with Tris-HCl buffer (50 mM, pH 7.5) (see Appendix B). The mycelium was disrupted by grinding in liquid nitrogen in mortar, and placed into microcentrifuge tubes. Genomic DNA was extracted using the hexadecyltrimethyl ammonium bromide (CTAB) method (Saghai-Maroof *et al.* 1984). CTAB buffer (2x, see Appendix B) 700 µl was added to the microfuge tube containing broken mycelium. The contents were stirred with a pipet tip, and mixed to resuspend. Chloroform 700 µl was added into this tube and mixed to emulsify. The microfuge contents were centrifuged at maximum speed for 10 min to separate the emulsion. The upper aqueous phase was removed to a fresh 1.5 ml microfuge tube. Precipitation of the DNA was performed by adding isopropanol (-20°C) 500 µl and the tube was inverted several times to mixing. Visible DNA strands were often seen. The DNA pellet was collected by centrifugation at maximum speed for 5 min. The supernatant was discarded and the pellet was washed with 70 % ethanol 1,000 µl. Recentrifuging (maximum speed for 3 min) allowed collection of the DNA. The DNA pellet was dissolved in TE buffer (see Appendix B) by vortexing. This preparation was the stock genomic DNA used for further experimentation.

3.5.3.2.2 Nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing

The ITS region of the ribosomal DNA (rDNA) was amplified using primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.*, 1990). Amplification reactions were performed using Peltier PTC-100™ Thermal

Cycler (MJ ResearchTM, Inc., MA, USA) in a total volume of 100 µl containing 10 mM Tris-HCl buffer, 50 mM KCl, 2.5 mM $MgCl_2$, 12.5 pmol each dNTP, 50 pmol each primer, 2 U Taq polymerase, 4 µl of stock genomic DNA , and 55.5 µl of sterile distilled water. The amplification conditions included a hot start for 6 min at 80°C and a denaturation step for 2 min at 95 °C, followed by primer annealing for 30 s at 56 °C and primer extension for 1 min at 72 °C. After 35 cycles, the extension step was followed at 72 °C for 10 min. Successful PCR products were cleaned of primers and salts, using the QIAquick PCR Purification Kit (QIAGEN, Inc., Valencia, CA, USA). ABI PRISM® 3100 BigDye Terminators v3.0 Cycle Sequencing reactions (Applied Biosystems, Foster City, CA, USA) were prepared according to the manufacturer's protocol, using primers ITS5 and ITS4 and the PCR product as templates (White *et al.*, 1990). Sequences were analyzed using an ABI PRIAM® 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

3.5.3.2.3 Sequencing of the ergosterol biosynthesis gene (ERG)

A portion of ERG was amplified with ERG3-1 (CAGCATGGCTACGAGTTC) and ERG3-4 (CTCCTTGTTTCCCGGCTT) primers (Zhang *et al.*, 2006). PCR amplifications were carried out in a total volume of 100 µl reaction mixtures as described in ITS sequencing session. The amplification conditions were performed in a Peltier PTC-100™ Thermal Cycler (MJ ResearchTM, Inc., MA, USA). The Program included an initial denaturation step for 3 min at 95 °C, followed by primer annealing for 30 s at 55 °C and primer extension for 1 min at 72 °C. After 35 cycles, an extension step was followed at 72 °C for 10 min. The PCR products were purified and then sequenced using primers ERG3-1 and ERG3-4 by the same protocol as described in ITS sequencing session.

3.5.3.2.4 Sequencing of the translation elongation factor 1-α gene (TEF)

A portion of TEF was amplified with the primer EF-1 (ATGGGTAAGGARGACAAGAC) and EF-2 (GGAR*GTACCAGTS*ATCATGTT) (Zhang *et al.*, 2006). Amplification of PCR products was performed using the same ratio of reaction mixtures and PCR conditions as carried out with ERG. Purification of the PCR products was performed using the QIAquick PCR Purification Kit (QIAGEN, Inc., Valencia, CA,

USA). The sequence of TEF was investigated by the same method as described previously.

*Remarks: R = A, G; S = C, G

3.5.3.2.5 Analysis of the sequences

Sequences obtained from section 3.5.3.2.2 (ITS), 3.5.3.2.3 (ERG) and 3.5.3.2.4 (TEF) were subjected to blast analysis based on the with nucleotide collection in GenBank using BLASTN programs.

3.5.4 Optimization of culture conditions for cutinolytic esterase production from PBURU-B5

3.5.4.1 Inoculum preparation

PBURU-B5 was grown on PDA at 30 °C for 4 days. Suspensions of mycelia in sterile distilled water were used to inoculate in LMM containing glucose 0.1% (w/v) and incubated at 30 °C for 4 days. These inocula were transferred to the LMM containing suberin (2 g/l) and peptone (5 g/l) as a production medium.

3.5.4.2 Effects of initial pH

To determine pH optimum for cutinolytic esterase production of PBURU-B5, samples (2 ml) of active inocula were transferred to production media (100 ml) which were adjusted to different initial pH including 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0. The cultures were incubated at constant temperature (30 °C) in rotary incubating shaker (150 rpm) for 10 days. The culture supernatants of each pH were collected and measured for the esterase activity as described above everyday. Duplicate assay per pH were run and the experiment was repeated once.

3.5.4.3 Effects of incubation temperature

The production media were adjusted pH to 11 using 6 M NaOH. Active inocula (2 ml) were added to each flask of this medium (100 ml). The cultures were incubated at each different temperature including 15 °C, 20 °C, 25 °C, 30 °C and 35 °C on a rotary incubation shaker at 150 rpm for 10 days. Daily esterase activity was determined from a culture supernatants as described previously. Duplicate assays per temperature were run and the experiment was repeated once.

3.5.4.4 Statistical analysis

Results of the effect of pH, temperature, enzyme activity, and incubation time were statistically analyzed using a multiple regression.

3.5.5 Estimation of PBURU-B5 growth in solid state culture

3.5.5.1 Culture conditions and sample preparation

Production media were prepared in 250 ml Erlenmenyer flasks with a working volume 100 ml. pH was adjusted to 11. Active inocula (2 ml) were used to inoculate in each flask of production medium. These cultures were incubated at optimum temperature that gave the greatest esterase yield at 25 °C on rotary shaker at 150 rpm at 10 days. Culture supernatants were assessed for esterase activity everyday as well as pH value. After that, the residual materials, suberin and mycelia were collected by filtration through filter paper under suction. Samples were dried at 105 °C to constant weight, and then finely ground to powder. Daily triplicate assays were done.

3.5.5.2 Conversion of chitin to glucosamine

. Samples (100 mg) of dried residual materials, suberin and mycelia was mixed with 2 N HCl (5 ml) in a hard glass tube (capacity 15 ml) which was sealed and placed into a boiling water bath for 2 h and then cooled under tap water. An aliquot sample was transferred into a 5 ml volumetric flask and a drop of 0.5% phenolpnthalein solution (see Appendix B) added followed by careful addition of 1 N NaOH until the solution turned pink. The solution was back-titrated drop-wise with 1% potassium hydrogen sulfate (see Appendix B) until the color just disappeared and the resulting colorless mixture made up to 5.0 ml with distilled water (Tsuji *et al.*,1969). This glucosamine solution was further used to determine the glucosamine content in sample.

3.5.5.3 Glucosamine assay

Glucosamine solution (1.0 ml) (resulting from the extractions described above) was mixed with acetylacetone reagent (1.0 ml, see Appendix B) in an ampoule which was sealed and heated in a vigorously boiling water bath for 20 min. After cooling to room temperature the ampoule was opened and 6 ml of absolute ethanol added followed by 1 ml of Erhlich's reagent (see Appendix B). The mixture was incubated at 65

°C for 10 min, cooled to room temperature and optical density read at 530 nm against a reagent blank (Swift, 1973). A calibration curve (see Appendix C) was determined based on a glucosamine hydrochloride standard (0, 20, 40, 60, 80, 100,120, 140, 160, 180 and 200 μg/ml). A linear correlation obtained was found [intensity (530 nm) = 0.0048 x conc. (μg/ml) with R^2 = 0.9969] and was linear between 0 and 200 μg/ml glucosamine solution.

3.5.6 Purification of cutinolytic esterase from PBURU-B5

3.5.6.1 Preparation of crude enzyme

Crude extracellular polyethylene terephthalate-hydrolyzing enzyme was prepared by culturing isolate PBURU-B5 in production medium as described previously at pH 11 in 2 liter shake flasks (500 ml working volume, 10 flasks) with incubation on a rotary shaker at 150 rpm (25 °C). After 4 days, culture supernatant was recovered following filtration through filter paper under suction. The residual materials, suberin and mycelia (352 g wet weight), were also extracted by stirring in 0.1 % Triton X-100 in one liter of 50 mM Tris-HCl buffer (pH 8.5) at 4 °C overnight (Fernando *et al.*, 1984). The insoluble residues were again removed by filtration. These culture supernatant (4,640 ml) and Triton X-100 wash (974 ml) were pooled and then concentrated by ultrafiltration (Vivaspin 15 polyethersulfone membrane, MWCO 5,000, Sartorius AG, Goettingen, Germany). The enzyme activity was determined using p-nitrophenyl butyrate as described above. Protein concentration was determined using the Bradford assay (Bradford, 1976) with Bovine Serum Albumin (BSA) as standard (see Appendix C). The optical density was measured at 595 nm. The correlation obtained was absorbance at 595 nm = 0.0095 x conc. (μg/ml) with R^2 = 0.9957 and was linear between 0 and 100 μg/ml BSA solution.

3.5.6.2 Optimization of ammonium sulfate precipitation

Precipitation of protein was carried out using ammonium sulfate fractionation method (Scopes, 1994). The culture extract from the section 3.5.6.1 (578 ml) was placed in beaker and pre-chilled to 4°C. The required amount of ammonium sulfate was calculated to give the sequential fractionations of saturations (0-10 %, 10-20%, 20-30%,

30-40%, 40-50%, 50-60%, 60-70%, 70-80% and 80-90%) from the equation given in Appendix D (Scopes, 1994). Ammonium sulfate was slowly added to crude enzyme until 10% saturation was achieved with mild stirring. The suspension was stirred for 2 h at 4°C. The precipitate was recovered by centrifugation at 9,000 rpm for 30 min and it was totally dissolved in 50 mM Tris-HCl buffer, pH 8.5 in a minimal volume. The suspension was dialyzed against the same buffer using regenerated cellulose tubular membrane (Cello Sep, MWCO 5,000, Membrane Filtration Products, Inc., Texas, USA) in 1 liter, with 3 changes for overnight at 4 °C (Fernando *et al.*, 1984). Assay of esterase activity and protein concentration (0-10% saturation) was determined as described previously. This procedure was repeated by adding ammonium sulfate to 10 % saturation culture supernatant until 20% saturation was obtained and precipitate was recovered as described above. This procedure was repeated for the other fractions. Purification factors of each fraction were calculated from the following equation (Harris and Angel, 1989).

$$\text{Purification factor} = \frac{\%\ \text{enzyme precipitation}}{\%\ \text{protein precipitation}}$$

The higher purification fractions were pooled and concentrated by ultrafiltration (Vivaspin 15 Polyethersulfone membrane, MWCO 5,000, Sartorius AG, Goettingen, Germany).

3.5.6.3 Purification of cutinolytic esterase by column chromatography

3.5.6.3.1 Hitrap Q FF column (QAE sepharose, anion exhanger)

The enzyme was further purified using anion exchange chromatography. The Hitrap Q FF column (1.6 x 7.5 cm, 15 ml; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was pre-equilibrated with 50 mM Tris-HCl, pH 8.5 (150 ml, see Appendix B). The concentrated enzyme (5 ml) was applied to this column by using flow rate of 5 ml/min. After the application of protein, the column was washed with the same buffer with at least 5 column volumes (75 ml) or until no material appeared in the effluent. Proteins were eluted with linear gradient (100 ml) of 50 mM Tris-HCl, pH 8.5 and 50 mM

Tris-HCl (pH 8.5) + 1 M NaCl (see Appendix B). The 2 ml fractions were collected and determined for the esterase activity as described above. The proteins were detected using spectrophotometric assay at 280 nm. The column effluent fractions containing esterase activity were pooled and dialyzed against the excess volume of 20 mM sodium phosphate buffer (pH 7.5) for overnight. This enzyme solution was concentrated by ultrafiltration. The protein concentration using the Bradford method as described in section 3.5.6.1 (Bradford, 1976) and esterase activity as described previously. This enzyme preparation was further purified by hydrophobic interaction column.

3.5.6.3.2 Hitrap Phenyl HP Column (Hydrophobic interaction Chromatography)

The concentrated enzyme (3 ml) obtained from the Hitrap Q FF column was added with $(NH_4)_2SO_4$ to the final concentration of 1.3 M and then loaded at 1 ml/min to Hitrap phenyl HP column (1.6 x 5.0 ml, 10 ml; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated with 20 mM sodium phosphate buffer (pH 7.5) + 1.3 M $(NH_4)_2SO_4$ (Start buffer-see Appendix B) 70 ml. The column was washed with the start buffer for at least 5 column volumes or until the OD at 280 nm of the effluent returned to near baseline. A gradient elution of mixture between start buffer and elution buffer (20 mM sodium phosphate buffer, pH 7.5) in total volume of 125 ml was applied. The OD_{280} was measured for each fraction (2 ml) as well as esterase activity. The active fractions was pooled and then dialyzed against excess 50 mM Tris-HCl, pH 8.5 with 0.15 M NaCl for overnight. This enzyme was concentrated using ultrafiltration as described in procedure 3.5.6.3.1 and then quantified the esterase activity and protein concentration using Bradford's method (Bradford, 1976) as described previously. The enzyme was further purified on gel permeation chromatography.

3.5.6.3.3 HiPrep 16/60 Sephacryl S-200 High Resolution Column (Gel filtration chromatography)

The concentrated enzyme (5 ml) from section 3.5.6.3.2 was applied to HiPrep 16/60 Sephacryl S-200 High Resolution Column (1.6 x 60 ml,120 ml; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) pre-equilibrated with 50 mM Tris-HCl, pH 8.5 + 0.15 M NaCl (240 ml, see Appendix B). The protein was eluted with the same buffer at the flow

rate 0.4 ml/min. Fractions of 2 ml were collected. The fractions were monitored for protein and enzyme activity as previously described. The active fractions were pooled and then concentrated by ultrafiltration. The purified enzyme was determined the esterase activity and protein concentration using Bradford's method (Bradford, 1976) as described previously.

3.5.6.4 Determination of enzyme purity and protein pattern

3.5.6.4.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The denaturing gel electrophoresis was performed according to Weber and Osborn (1957). The gel was contained with 0.01% (W/V) SDS in 12% (W/V) separating (see Appendix B) and 5.0% stacking gels (see Appendix B). Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as electrophoretic buffer (see Appendix B). Samples to be analyzed and stained with silver staining were treated with SDS reducing buffer (see Appendix B) and boiled for 10 min prior application to the gel. However, samples to be stained with esterase activity staining were added with SDS reducing buffer without 2-mercaptoethanol and it was not denaturated by heating.

3.5.6.4.2 Silver staining

Protein was fixed by incubating the gel in a fixing solution (see Appendix B) 200 ml for 1 hour to overnight, at room temperature with gentle shaking. The fixing solution was discarded and the gel was washed by with 50 ml of 50% Methanol for 20 minutes 3 times. The gel was then pretreated with 0.02% thiosulfate solutions (freshly preparation, see Appendix B) and incubated for 1 minute exactly. Deionized water (50 ml) was used to wash the gel for 20 seconds 3 times. This gel was impregnated with 0.2% $AgNO_3$ (see Appendix B) 200 ml and 37% formaldehyde 0.15 ml for 20 min with mild shaking. The gel was washed with deionized water (50 ml) for 30 seconds twice. The gel was incubated in developing solution (200 ml - see Appendix B) for 10 min at room temperature with gentle agitation. Stained bands of protein appeared within a few minutes. With continued incubation until the desired contrast was obtained. The gel was rewashed with deionized water (50 ml) for 2 min twice. The reaction was stopped by

incubating the gel in 100 ml of 50% Methanol and 12% Acetic acid for 10 min, and it was then washed with 50 % Methanol for 20 minutes at 4°C. The gel was incubated with 30 % Methanol for 30 minutes at 4°C and then 3% glycerol for 30 minutes at 4°C. Finally, the gel was dried for overnight at room temperature (Blum *et al.*,1987).

3.5.6.4.3 Esterase activity staining

For SDS gels analysis, proteins were renatured by incubating the gel in 50 mM Tris-HCl buffer, pH 8.5, containing 0.1% Triton X-100 for 2 hour at room temperature with mild shaking. The gel was briefly washed with 50 mM Tris-HCl buffer, pH 8.5, twice prior to esterase activity staining. Esterase activity staining was performed by incubating the gel in solution containing α-naphthyl acetate (50 μg) and α-naphthyl butyrate (50 μg), and Fast blue RR salt (10 mg) in 100 ml of 50 mM Tris-HCl buffer, pH 8.5 for 1 hour at room temperature or until the desired color intensity appeared (Ibrahim and Rowe, 1995).

3.5.7 Characterization of purified cutinolytic esterase from PBURU-B5

3.5.7.1 Effect of pH on cutinolytic esterase activity

The purified cutinolytic esterase was incubated with p-NPB at different pHs and the enzyme activity was assayed as previously described in section 3.5.2. The 0.1 M of sodium acetate, potassium phosphate, Tris-HCl and borate were used as reaction buffers for pH 4.0-6.0, 6.0-8.0, 8.0-9.0 and 9.0-11.0, respectively. The result was monitored as a percentage of the relative activity. The pH which gave the maximum activity was set as 100%.

3.5.7.2 Effect of temperature on cutinolytic esterase activity

The activity of purified enzyme was determined as described in procedure 3.5.2 at various temperatures (15, 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 °C). The result was displayed as a percentage of the relative activity. The temperature which gave maximum activity was set as 100%.

3.5.7.3 Effect of pH on cutinolytic esterase stability

pH stability of the enzyme was measured, after incubating the enzyme in 50 mM buffers at various pHs (section 3.5.7.1) for 30 min at 4 °C. Residual esterase activities were assay at 25°C and pH 9.0 as described in section 3.5.2. The result was shown relative to the activity. Esterase activity of enzyme determined at 25°C and pH 9.0 without incubation was set as 100% activity.

3.5.7.4 Effect of temperature on cutinolytic esterase stability

Thermostability of the enzyme was measured, after incubating the enzyme for 30 min at various temperatures (10°C-65°C) at pH 9.0. Residual esterase activities were assay at 25°C and pH 9.0 as described in section 3.5.2. The result was shown relative to the activity. Esterase activity of enzyme determined at 25°C and pH 9.0 without incubation was set as 100% activity.

3.5.7.5 Effect of metal ions on cutinolytic esterase activity

The same amounts of purified enzyme were applied for the remaining esterase activity measurement as described in section 3.5.2 when each metal ion was added with the reaction mixture at three concentrations (0.1 mM, 1.0 mM and 10 mM). The metal ions included $CaCl_2.2H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $HgCl_2$, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$ and $ZnSO_4.7H_2O$. The residual activity was compared with the activity of enzyme without metal ions and reported as a percentage of the relative activity.

3.5.7.6 Effect of NaCl on cutinolytic esterase activity

The effect of NaCl (0-700 mM) on enzyme activity (50 mM Tris-HCl, pH 9.0 at 4°C for 30 min) was tested. The residual activity was assayed as described in section 3.5.2. The control was activity of the enzyme without NaCl. The result was reported in the relative activity.

3.5.7.7 Effect of ethylene diamine tetra-acetic acid (EDTA) cutinolytic esterase activity

The effect of EDTA on enzyme was evaluated based on esterase activity (section 3.5.2). The different concentrations of EDTA (0-3.0 mM) were added into the reaction

mixture. The residual activity was compared with the activity of enzyme without EDTA and reported as a percentage of the relative activity.

3.5.7.8 Kinetic study of cutinolytic esterase

p-NPB was used as a substrate for the kinetic studies. The assay was performed using purified enzyme with concentrations of p-NPB ranging from 0.1-1.0 mM. The activity was determined as described in section 3.5.2. The kinetic constants, K_m and V_{max}, was determined from Lineweaver-Burk plot (Rudolph, 1979)

3.5.7.9 Molecular weight determination of purified cutinolytic esterase by gel filtration on HiPrep 16/60 Sephacryl S-200 High Resolution Column

HiPrep 16/60 Sephacryl S-200 High Resolution Column (1.6 x 60 ml,120 ml; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was equilibrated with 50 mM Tris-HCl, pH 8.5 containing 0.15 M NaCl (240 ml, see Appendix B) at the flow rate of 0.4 ml/min to allow stabilization of bed volume of the column. An aliquot of the concentrated enzyme solution was applied to the column and then eluted with the same buffer. Fractions of 2 ml were collected. The elution profile was monitored for protein and enzyme activity as described previously.

The elution volume (V_e) of enzyme was compared with the standard protein molecular weight markers including ribonuclease A (13,700 Da), chymotrypsinogen (25,000 Da), ovalbumin (43,000 Da) and albumin (67,000 Da). Blue dextran 2000 and potassium dichromate were used to determine the position of the void volume (V_0) and the total bed volume (V_t), respectively. The partition coefficient (K_{av}) values for each standard protein marker calculated from $(V_e-V_0)/(V_t-V_0)$ were plotted against log molecular weight of each protein to obtain a calibration curve. The K_{av} of the enzyme was calculated and used to determine its native molecular weight from the calibration curve.

3.5.8 Applications of cutinolytic esterase produced from PBURU-B5 on surface modification of PET

3.5.8.1 Enzyme preparation

Crude extracellular polyethylene terephthalate-hydrolyzing enzyme was prepared as described in section 3.5.6.1. This crude enzyme was further precipitated by ammonium sulfate precipitation (50-80% saturation), followed by removal of the salt by dialysis as described in procedure 3.5.6.2. Ultrafiltration was used to concentrate the enzyme as previously described. The enzyme activity was determined using p-nitrophenyl butyrate as described above. Protein concentration was determined using the Bradford assay (Bradford, 1976). This crude enzyme was used for further study in PET modification.

3.5.8.2 Assay for enzymatic hydrolysis of PET fibers based on ultraviolet light absorbance (UV assay)

The terephthalic acids (TPA), PET monomers, released from PET fibers were measured following incubation of PET yarn (0.2 g) in 100 ml 50 mM Tris-HCl buffer, pH 9.0 with the different enzyme concentrations (0.5 U/ml, 1.0 U/ml and 2.0 U/ml). Reaction mixtures were incubated at 45 ^{o}C and sampled periodically up to 48 hours, followed by analysis via absorbency at 240 nm (Yoon *et al.*, 2002). Controls included mixtures lacking enzyme and also inactivated autoclaved enzyme. All assays were minimally carried out in duplicate. A calibration curve was carried out using standard solutions with different concentrations of TPA concentration (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg/ml) dissolved in a 50 mM NaOH solution. The correlation obtained was Intensity (240 nm) = 0.0255 x Conc. (μg/ml) with R^2 = 0.9995 and was linear between 0 and 100 μg/ml of TPA (see Appendix C).

3.5.8.3 Assay for enzymatic hydrolysis of PET fibers based on fluorescence response

PET fibers were treated with the enzyme as described in section 3.5.8.2, but TPA released from PET fibers was analyzed as fluorescence absorbency using a luminescence spectrophotometer (Model: LS 50 B, Perkin Elmer Inc., MO, USA) at 425 nm (O'Neill and Paulo, 2004). Controls included mixtures lacking enzyme and also

inactivated autoclaved enzyme. All assays were minimally carried out in duplicate. A standard curve was performed using standard solutions with different concentrations of TPA concentration (0, 10, 20, 30, 40, 50 and 60 μg/ml) dissolved in a 50 mM NaOH solution. The correlation obtained was Intensity (425 nm) = 5.7113 x Conc. (μg/ml) with R^2 = 0.9988 and was linear between 0 and 60 μg/ml of TPA (see Appendix C).

3.5.8.4 Enzymatic and alkaline treatment of fabrics

Woven PET fabrics were a kind gift from Asia Fiber Public Company, Bangkok, Thailand. These prewashed fabrics were prewashed in boiling water for 10 min, then water rinsed and dried. The swatches were incubated with the different concentrations of enzyme (0.5 U/ml, 1.0 U/ml and 1.5 U/ml) in 50 mM Tris-HCl buffer, pH 9.0 at 45 °C, liquor to fabric ratio of 20:1 on a rotary shaker at 150 rpm and sampled periodically up to 48 h. Controls were carried out without enzymes and treated identically with the corresponding buffers. After the treatment, the swatches were washed thoroughly several times with tap water, then with a 2 g/l sodium carbonate solution for 30 min at 70°C (to remove the remaining protein from the surface of the fabrics) and finally with distilled water at 70°C for 1h (2 times) (Kellis *et al.*, 2001; O'Neill and Paulo, 2004). The samples were dried at room temperature.

For alkaline treatments, the swatches were treated with different concentrations of NaOH (1.5 M and 3.0 M) in the same way of enzymatic treatment as described above. After the reaction, the samples were washed thoroughly several times with tap water to neutralization, followed by washing the swatches with distilled water at 70 °C for 1h (2 times) and dried at room temperature. All experiments were carried out in triplicate.

3.5.8.5 Measurement of wettability of the enzyme and alkaline treated PET fabrics

In order to investigate the wettability (or hydrophilicity) of untreated and treated fabrics from the section 3.5.8.4, a water droplet absorption time measurement was applied according to AATCC standard test method 79 (Absorbency of Bleached Textiles). A distilled droplet was allowed to fall from a burette held 10-mm height from the stretched fabric surface. The time for the disappearance of water-mirror on the

surface was measured as the wetting time. The averages of wetting time at different positions on the sample surface were recorded. The studied area of fabric was divided (Figure 3.2) and the wetting time of each area was determined. The area horizontal to the chamber was divided into 9 parts, including I, II, III, IV, middle, V, VI, VII, VIII as illustrated in Figure 3.2. The minimum or zero of wetting time obtained from the measurement showed the most of enhancing hydrophilicity or ability to adsorb water of fabrics treated by enzyme or alkali.

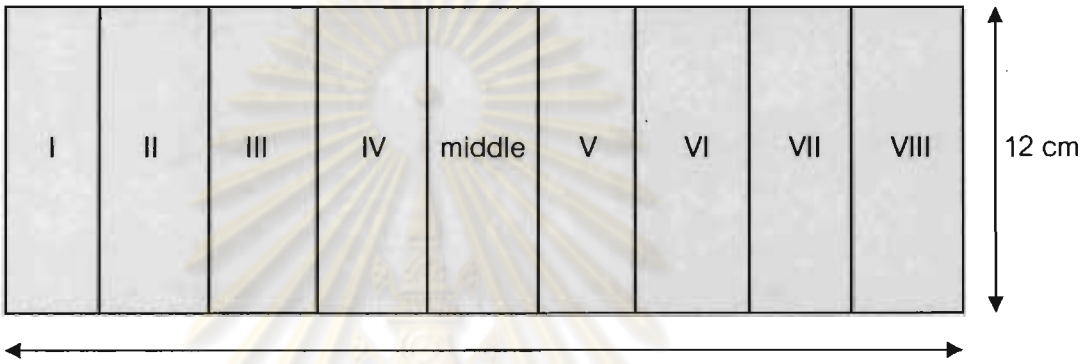


Figure 3.2 Horizontally divided areas of the fabric

3.5.8.6 Measurement of water absorption of the enzyme and alkaline treated PET fabrics

The effect of the enzyme and of alkali on modification in the water adsorption ability of PET fabrics (section 3.5.8.4) was determined using the wicking test following a standardized method (DIN 53924). All test strips of a series had the threads running the same way. The test strip was immersed the edge of the fabric (0.5 cm) into the beaker charged with the corresponding amount of distilled water up to 10 min. The rising height of water in treated and untreated PET fabrics was measured. All treatments were performed in triplicate. The higher water height absorbed by test strips displayed the higher increase of hydrophilicity of the fabrics.

3.5.8.7 Measurement of moisture content of enzyme and alkaline treated PET fabrics

Enhanced hydrophilicity of treated PET fabrics from section 3.5.8.4 was demonstrated by measuring the changes of moisture content of fabrics before and after treatment with the enzyme and alkali. Fabric pieces were 10 x 10 cm. These fabrics were incubated at The moisture content of untreated and treated PET fabrics was determined using an AD 4715 infrared moisture determination balance (A&D weight, Bradford, MA, USA) (Figure 3.3) at 105°C for 5 min. All analyses were performed 5 times.

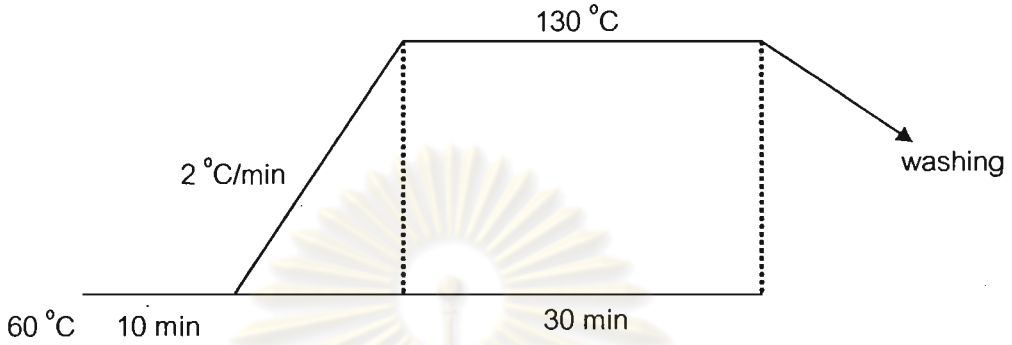
Figure 3.3 Infrared moisture determination balance (Model: AD 4715, A&D weight, Bradford, MA, USA)

3.5.8.8 Dyeability testing of enzyme and alkaline treated PET fabrics

3.5.8.8.1 Disperse dye (Dianix Red CC)

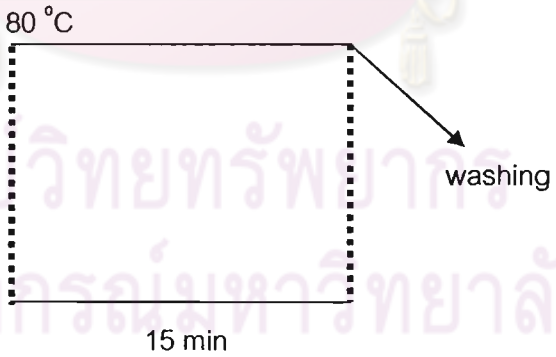
Treated and untreated PET fabrics from section 3.5.8.4 were dyed in the solutions containing disperse dye (Dianix Red CC), a kind gift from DyStar Thai Ltd., Bangkok, Thailand. The solutions contained the disperse dye (20 g/l) and wetting agent (1g/l) at a liquor ratio (L:R) of 20:1 at pH 4-5. The initial temperature of dyeing process was 60 °C for 10 min, then raised to 130 °C (2 °C/min) and maintained at this temperature for 30 min (Figure 3.4). After dyeing, the fabrics were washed in water containing 2 g/l wetting agent at 60 °C for 15 min. The unfixed dye was removed using

the reduction clear process shown in Figure 3.5. This process required 2 g/l sodium hydrosulfite, 2 g/l sodium hydroxide and 1 g/l wetting agent, and it was conducted at 80 °C for 15 min. Then the fabrics were washed, air dried, and measured the color intensity as described in section 3.5.8.8.3.



(L:R =20:1) Polyester fabric
pH 4-5
Wetting agent 1 g/l
Dianix Red CC 20 g/l

Figure 3.4 The dyeing process of disperse dye for polyester fabric

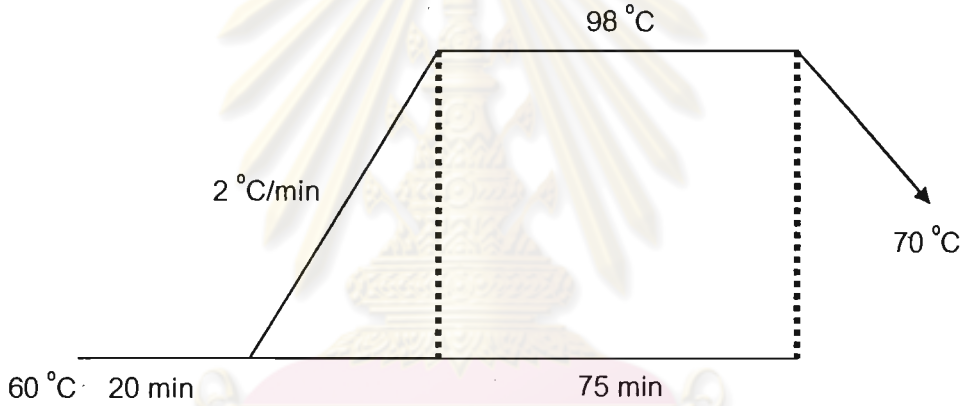


(L:R =20:1) $Na_2S_2O_4$ 2 g/l
NaOH 2 g/l
Wetting agent 1 g/l

Figure 3.5 The reduction clear process for PET fabric after disperse dyeing

3.5.8.8.2 Basic dye (Astrazon Red FBL 200% 03)

Treated and untreated PET fabrics from section 3.5.8.4 were dyed in the solutions containing basic dye (Astrazon Red FBL 200% 03), a kind gift from DyStar Thai Ltd., Bangkok, Thailand, in solutions containing dye (20 g/l) and Avolan IW (0.5 %) at a liquor ratio (L:R) of 20:1. The initial temperature of dyeing process was 60 °C for 20 min, then raised to 98 °C (2 °C/min) and maintained at this temperature for 75 min (Figure 3.6). The unfixed basic dye was removed from the fabric using the reduction clear process shown in Figure 3.7. This process required Blankit® IN (BASF) 1 g/l and this solution was adjusted the pH to 8.5-9.0 using Na_2CO_3. It was held at 50-70 °C for 20 min, and then the fabrics were washed several times with distilled water to neutralize the fabrics, air dried and measured the color intensity as described in section 3.5.8.8.3.

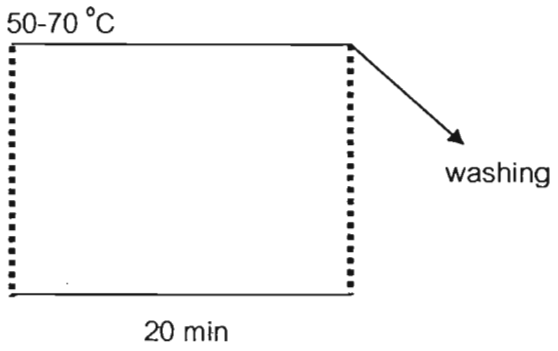


(L:R =20:1) Polyester fabric

0.5 % Avolan IW

Basic dye 20 g/l

Figure 3.6 The dyeing process of basic dye for polyester fabric



(L:R =20:1) pH 8.5-9.0 with Na_2CO_3

Blankit® IN (BASF) (stabilized hydrosulphite) 1 g/l

Figure 3.7 The reduction clear process for PET fabric after basic dyeing

3.5.8.8.3 Color measurement

The reflectance values of dyed fabric samples were evaluated using an Instrument Color System (I.C.S) Macbeth reflectance spectrophotometer (Figure 3.8) connected to a digital personal computer, to measure the reflectance of the employed sample and wavelength corresponding to the maximum absorbance of the employed dyes (disperse and basic). Each fabric sample was folded once leading to a total of two thicknesses of fabric. Each reflectance value was determined as the average of four measurements. Percent reflectance values (R) were converted into K/S values, the color strength of the fabric, according to the Kubelka-Munk equation:

$$K/S^{*} = \frac{(1-R^2)}{2R}$$

Remark: K = the absorption coefficient

S = the scatting coefficient

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

* The higher K/S values measured from dyed fabrics indicated the higher increase of color intensity of samples.

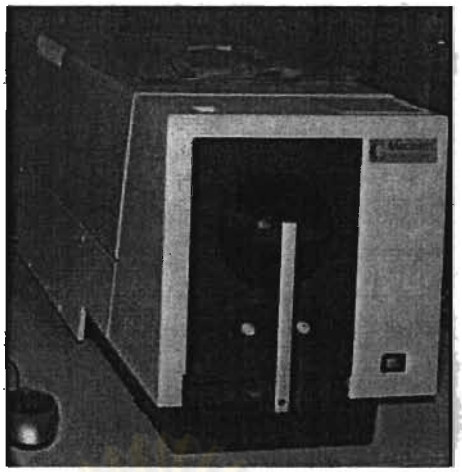


Figure 3.8 Instrument Color System (I.C.S) Macbeth reflectance spectrophotometer

3.5.8.9 Tensile property analysis of enzyme and alkaline treated PET fabrics

The tensile properties of treated and untreated PET fabrics from section 3.5.8.4 were determined using a Hounsfield universal testing machine (Model: H10KM, NSW, Australia) (Figure 3.9) with a 100 kN load cell, according to ASTM D5034. The testing was performed at room temperature. The speed of testing was 300 mm/min and the gauge length was 75 mm. The sample size was 4" x 6". The tensile values of fabric samples were measured.

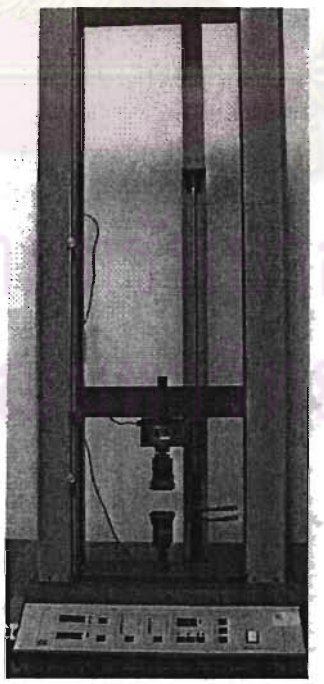


Figure 3.9 A Hounsfield universal testing machine model H10KM

3.5.8.10 Surface chemical analysis of enzyme and alkaline treated PET fabrics

The changes in surface chemical structure of the selected fabric samples were characterized using attenuated total reflection/ Fourier transform infrared spectroscopy (ATR-FTIR) (Thermo Nicolet Nexus 670 spectrophotometer, GMI, Inc., Minnesota, USA) (Figure 3.10). The sample from section 3.5.8.4 were scanned at the frequency range of 4,000 – 6,000 cm^{-1} 300 consecutive scans and 4 cm^{-1} resolution.

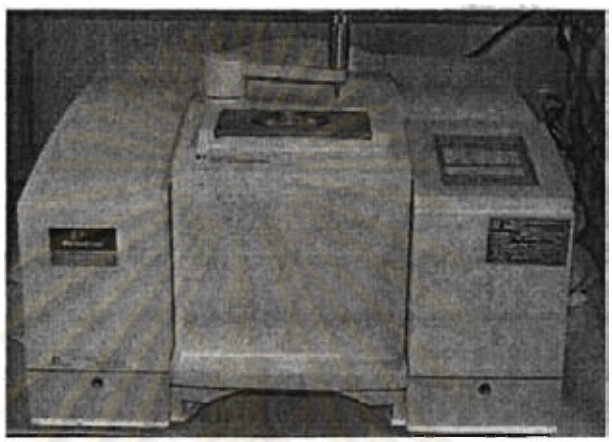


Figure 3.10 Attenuated total reflection/ Fourier transform infrared spectroscopy (ATR-FTIR) (Thermo Nicolet Nexus 670 spectrophotometer, GMI, Inc., Minnesota, USA).

3.5.8.11 Surface morphological analysis of enzyme and alkaline treated PET fabrics

In order to observe the influence of enzyme and alkaline treatment on surface morphology of untreated and treated PET fabrics, the morphology was observed by scanning electron microscope (SEM) (JSM-6400, MA, USA) (Figure 3.11). The samples were coated with thin evaporated layer of gold in order to improve conductivity and prevent electron charging on the surface before SEM analysis. The operation was at 15 keV or 10 keV acceleration voltages. SEM photographs were taken at different angles of view with magnification of 100X and 1,500X.

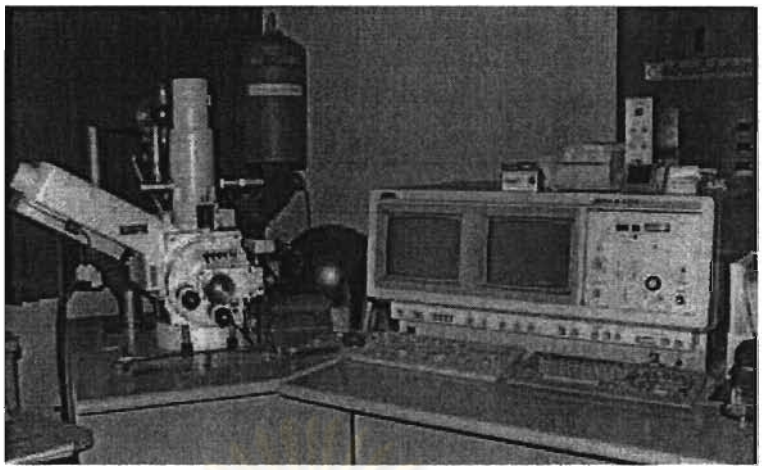


Figure 3.11 Scanning electron microscope (SEM) (JSM-6400, MA, USA)

CHAPTER 4

RESULTS

4.1 Screening for PET-hydrolyzing enzymes

4.1.1 PCL degradation by fungi

A diverse variety of plant surfaces and soil from a range of environmental niches yielded 22 fungi out of 115 isolates that were capable of degrading PCL, as indicated by zone of clearing on MM-PCL agar plates (Figure 4.1). Such zones could be easily observed after 3-5 days of incubation.

4.1.2 Source and collection sites of fungi attacked PCL

The isolates were dominanted by *Fusarium* cultures, 16 of 22 isolates. A few of the more generally occurring *Aspergillus* and *Penicillium* species isolated from soil were found to attack PCL, the cutin analog. These cutin degraders were generally isolated from different aerial parts of plants (fruit peel, leaf and bark) and also from soil nearby roots, and all produced cutinolytic esterase (Table 4.1). The sampling times included the rainy season (June, July, August and September) and the dry season (March), and from low (0.5 m, Samutsakorn Province) and high (1,100 m, Chiangmai Province) altitudes, at ranges from 20.2 °C (Chaingmai) to 32.0 °C (Bangkok) isolates were from seven provinces.

4.1.3 Screening of the most efficient enzyme for suberin and PET degradation

All isolates when cultivated on suberin produced esterases with maximum activities ranging from 11 (PBURU-B14) to 1,126 mU/ml (PBURU-B19) (Table 4.2). However it is noteworthy that only one fungus (*F. solani* PBURU-B5) was able to grow to any reasonable extent on PET fibers, and in doing so produced 61 mU/ml after 21 days growth (Table 4.2).

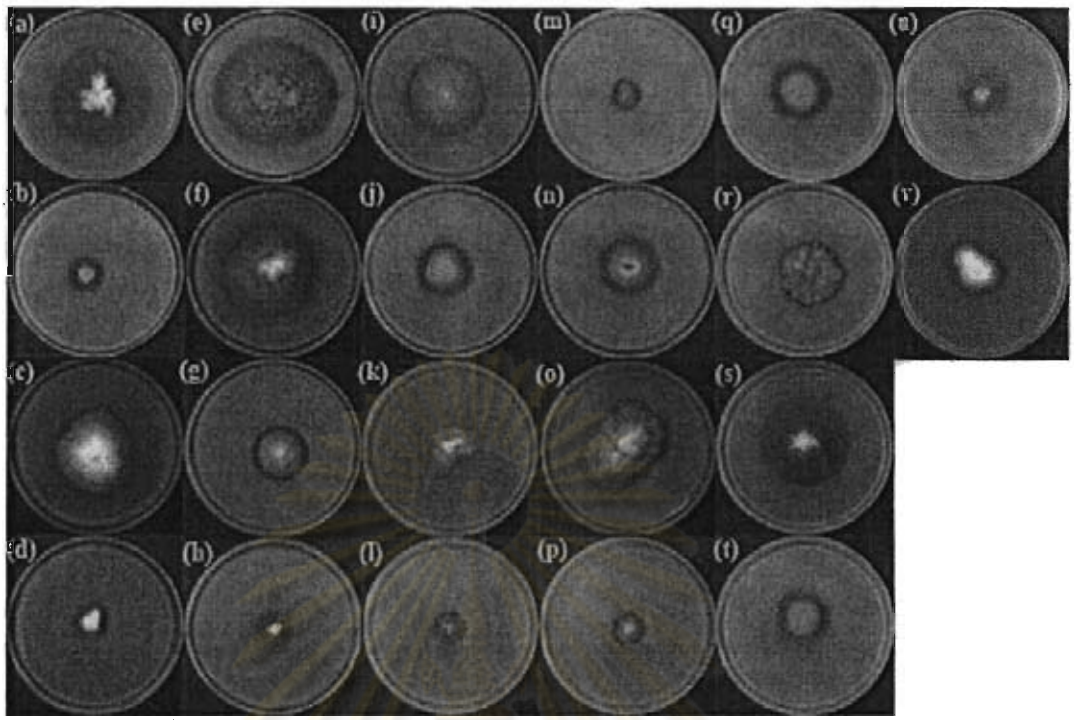


Figure 4.1 The esterase activity of the fungal isolates towards PCL as evidenced by clearing zones on polycaprolactone plates. (a) PBURU-B1, (b) PBURU-B2, (c) PBRU-B3, (d) PBURU-B4, (e) PBURU-B5, (f) PBURU-B6, (g) PBURU-B7, (h) PBURU-B8, (i) PBURU-B9, (j) PBURU-B10, (k) PBURU-B11, (l) PBURU-B12, (m) PBURU-B13, (n) PBURU-B14, (o) PBURU-B15, (p) PBURU-B16, (q) PBURU-B17, (r) PBURU-B18, (s) PBURU-B19, (t) PBURU-B20, (u) PBURU-B21, and (v) PBURU-B22

Table 4.1 Fungal isolates attacking polycaprolactone (PCL): sources and collection sites

Province	Description of collection site				Source	Fungal strain
	Mean Temp. (°C)	Elevation (m)	Collection Date	Area		
Bangkok	32.0	11	March 2005	Shady lowland	Banana (*Musa sapientum* Linn.) Leaf Potato (*Solanum tuberosum* Linn.) Peel Tomato (*Lycopersicon esculentum*) Peel Soil near by root of banana (*Musa sapientum* Linn.) Soil near by root of tomato (*Lycopersicon esculentum*)	*Fusarium* sp.PBURU-B1 *Fusarium* sp.PBURU-B2 *Fusarium* sp.PBURU-B19 *Penicillium* sp. PBURU-B13 *Fusarium* sp. PBURU-B7, *Fusarium* sp. PBURU-B8
Chiangmai	20.2	1,100	June 2005	Open highland	Soil near by root of fairy rose (*Rosa chinensis*) Water Lily (*Nymphaea stellata* Wild.) Leaf Soil near by root of lychee tree (*Litchi chinensis* Sonn.)	*Aspergillus* sp. PBURU-B10 *Fusarium solani* PBURU-B5 *Aspergillus* sp. PBURU-B3
Lampang	28.0	262	September 2005	Shady lowland	Soil near by root of mango tree (*Mangifera indica* Linn.)	*Fusarium* sp. PBURU-B16, *Fusarium* sp. PBURU-B22
Pathumthani	29.7	11	August 2005	Shady lowland	Burma Padauk (*Pterocarpus indicus*) Bark Bodhi tree (*Ficus religiosa* Linn.) Bark Soil near by root of Dwarf date palm (*Phoenix loureri* Kunth.)	*Fusarium sp.* PBURU-B11 *Fusarium sp.* PBURU-B15 *Fusarium sp.* PBURU-B20
Prachinburi	30.7	17	June 2005	Shady lowland	Soil near by root of jack fruit (*Artocarpus heterophylius* Lam.)	*Aspergillus* sp. PBURU-B9
Rayong	29.3	66	August 2005	Shady lowland	Soil near by root of Fan palm tree (*Licuala grandis* H. Wendl.) Soil near by root of mango tree (*Mangifera indica* Linn.) Soil near by root of lychee tree (*Litchi chinensis* Sonn.)	*Aspergillus sp. PBURU-B17*, *Fusarium* sp. PBURU-B4, *Fusarium* sp. PBURU-B12 *Fusarium sp.* PBURU-B18, *Penicillium* sp. PBURU-B6 *Fusarium sp.* PBURU-B21
Samutsakorn	27.0	0.5	August 2005	Muddy shore	Mangrove (*Rhizophora apiculata* Blume) Leaf	*Fusarium* sp. PBURU-B14

Table 4.2 Maximum esterase yields from the polycaprolactone attacking isolates

Isolate	Maximum esterase activity (mU/ml)[a]	
	Suberin powder	PET fiber
Fusarium sp. PBURU-B1	76	3
Fusarium sp. PBURU-B2	36	6
Aspergillus sp. PBURU-B3	20	5
Fusarium sp. PBURU-B4	20	7
Fusarium sp. PBURU-B5	308	61
Penicillium sp. PBURU-B6	21	8
Fusarium sp. PBURU-B7	23	8
Fusarium sp. PBURU-B8	494	6
Aspergillus sp. PBURU-B9	99	4
Aspergillus sp. PBURU-B10	132	5
Fusarium sp. PBURU-B11	552	5
Fusarium sp. PBURU-B12	29	9
Penicillium sp. PBURU-B13	84	5
Fusarium sp. PBURU-B14	11	11
Fusarium sp. PBURU-B15	527	3
Fusarium sp. PBURU-B16	353	3
Aspergillus sp. PBURU-B17	25	7
Fusarium sp. PBURU-B18	135	4
Fusarium sp. PBURU-B19	1,126	4
Fusarium sp. PBURU-B20	777	5
Fusarium sp. PBURU-B21	20	5
Fusarium sp. PBURU-B22	1,053	3

Enzymes from culture supernatants of isolates grown in liquid mineral medium containing either suberin powder or PET fiber as sole carbon source, [a] Determined with *p*-nitrophenyl butyrate as substrate – the extinction coefficient of p-nitrophenol = 1.84 x 10^4 $M^{-1}cm^{-1}$ (see Appendix D).

4.2 Identification of the fungal isolate PBURU-B5

4.2.1 Morphology

The colony grew to 5 cm diameter in 4 days and was whitish (Figure 4.2). Examination by bright field light microscopy of the morphology of PBURU-B5 grown on BLA for 5 days showed typical Fusaria characteristics (Barnett and Hunter, 1999; Seifert, 1993). It produced both macro- and microconidia (Figure 4.2). Macroconidia are hyaline and canoe-shaped in side view. Microconidia were ellipsoidal, produced from long monophialides in aerial mycelium. Chlamydospores usually occurred singly or in pairs. It was apparently *Fusarium solani*.

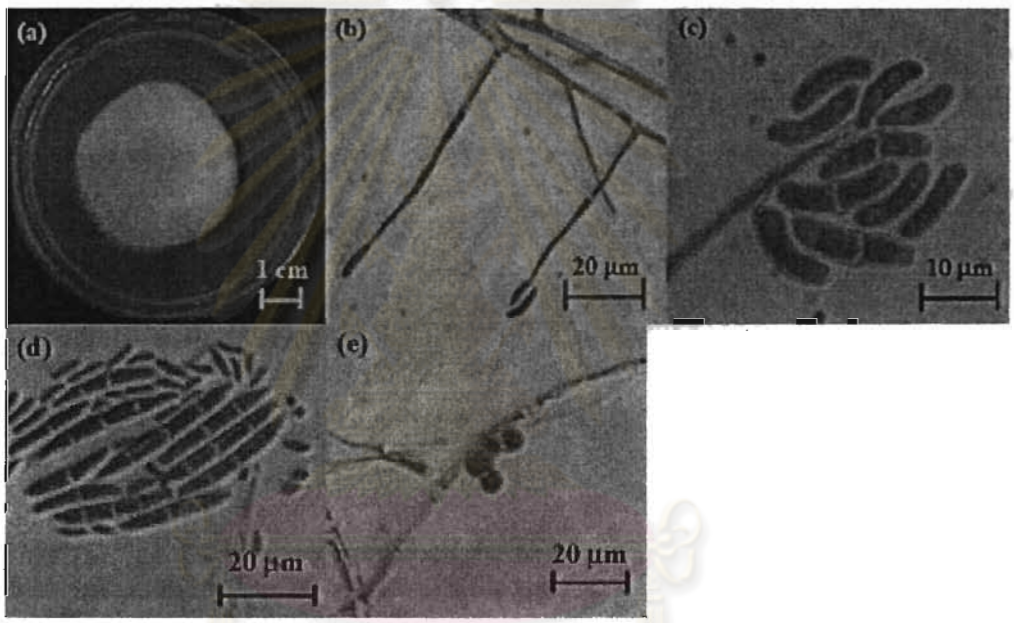


Figure 4.2 Colony and morphology of *Fusarium solani* PBURU-B5. (a) colony (5 cm in diameter) grown on PDA for 4 days at 30 °C, (b) septate hyphae and conidia, (c) ellipsoidal-shaped microconidia, (d) canoe-shaped macroconidia and (e) chlamydospores

4.2.2 Molecular biological identification of *F. solani* PBURU-B5

4.2.2.1 Sequence of Nuclear Ribosomal DNA Internal Transcribed Spacers (ITS)

Culture was grown in PDB, pH 6.5 for 7 days at 25 °C. The chromosomal DNA of PBURU-B5 was extracted and checked by agarose gel electrophoresis (see Appendix B). DNA obtained was larger than 10 kb (Figure 4.3) with an absorbance ratio ($A_{260/280}$) of 1.90. From the gel pattern and the absorbance ratio, the extracted DNA was clean enough for amplification (Kim and Hamada, 2005). After amplification using specific primers for ITS region of rDNA gene (section 3.5.3.2.2), a PCR product of about 500 bp was obtained (Figure 4.3).

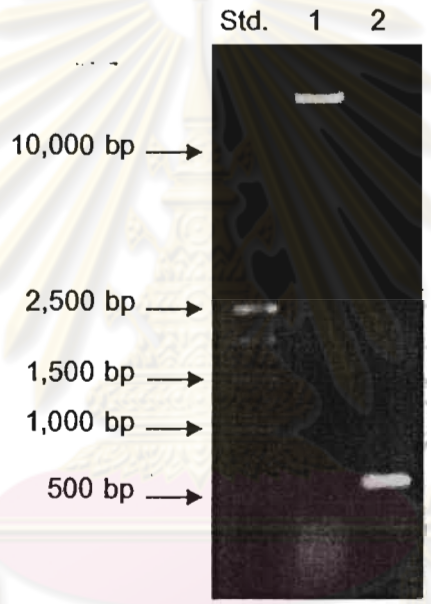


Figure 4.3 Amplification product of ITS region of rDNA from strain PBURU-B5
Lane Std. = DNA ladders (1 kb, Vivantis, Shah Alam Selangor, Malaysia)
Lane 1 = Genomic DNA of PBURU-B5
Lane 2 = ITS PCR product

The ITS sequence was isolated using primers mentioned in section 3.5.3.2.2. Each primer gave more than 500 bp sequences. Both sequences were extended and searched for the overlapping region. The resulting sequence was 525 bp (Figure 4.4). The alignment of this sequence was blasted with other ITS sequences deposited in the GenBank databases. The results indicated that PBURU-B5 showed 98% homology with

F. solani strain FRC#s1592 (DQ094746) and 98% homology with *F. solani* NRRL 22354 (AF178402).

GGATCATTACCGAGTTATACAACTCATCAACCCTGTGTTCCATACCTAAACGTT
GCTTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCC
CCTAACTCTGTTTCTATAATGTTTCTTCTGAGTAAAACAAGCAAATAAATTAAAACTTTCA
ACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG
TATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTG
GCGTTGGGGATCGGCGGAGCCCTCTGCGGGCACACGCCGTCCCCCAAATACAGTG
GCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACACCTCGCAACTGGAGAGCG
GCGCGGCCANCGCCGTAAAANCCCCCAACTTCTGAA

Figure 4.4 Nucleotide sequence of ITS region of rDNA

4.2.2.2 Amplification and sequencing of a portion of the ergosterol biosynthesis gene (ERG)

Amplification of ERG gene was performed using specific primers listed in section 3.5.3.2.3. A PCR product of about 500 bp was obtained (Figure 4.5). The ITS sequence observed using the same primers gave more than 500 bp. Analysis of both sequences resulted 532 bp (Figure 4.6). The alignment of this sequence was blasted with the ERG sequences deposited in the GenBank databases. The results indicated that PBURU-B5 showed 96% homology witth *F. solani* strain FRC#s1592 (DQ237259) and 96% homology with *F. solani* NRRL 22354 (DQ236829).

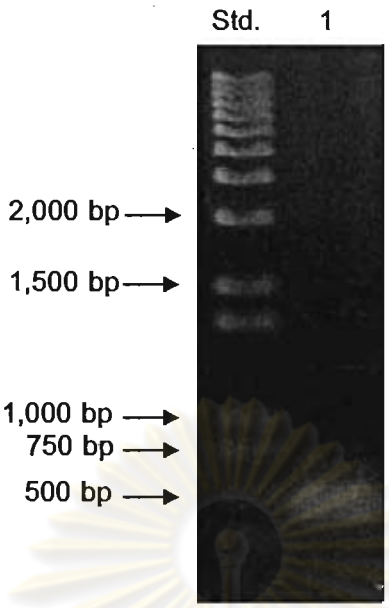


Figure 4.5 Amplification product of the portion of ergosterol biosynthesis gene (ERG)
Lane Std. = DNA marker (1 kb, Vivantis, Shah Alam Selangor, Malaysia)
Lane 1 = PCR product of ERG gene

GACGTAGACAAAGGTGGCCACGACGTACGAGAAGATGGTGTTGGCGGTAAG
GATCTGGATGAAGTTGTCCGAGATGAAGGTCCAGACGGGGAACTCGGCGCCCTGGG
CTGCGGTTCCGGCGGCGAGAATAGCAAGGGTGAACGTGCTGCTGTACAGAGCTAAG
ACAAGTCAGCACTTTATTCCTGCAAGGTCCGGGGATGTTGTAACACACTGTTGAGTCT
ATACTTGAGACGACCCCCAGAACGTAGCTCAGTGCCCTCCACCTCATAGGCTGGAAG
CACACGGTAGAGGATCAATGACAGCAGGAGGTAGCCAGCCGTAGCAGCAGAAGCCT
CCCAGCTAACAAGTCCAGCGAATCCATCCTGAGGCCACCCAACCTCTTGTTTAAGCTT
GTCGAGCGAGAGGGTCTTGGGACTCAGAAGAGACGGGGCGGGGCAGCCCGAGATG
TCGTTACAGACAAAGTTGAAGACGTATACGAGGACGGGGAGGAGGAAGGAGATGGC
GAATGCACCGGGACTAACACGTCAGTTAG

Figure 4.6 Nucleotide sequence of portion of the ergosterol biosynthesis gene (ERG)

4.2.2.3 Amplification and sequencing of a portion of the translation elongation factor 1-α gene (TEF)

A portion of TEF gene was amplified using specific primers as listed in section 3.5.3.2.4, and a PCR product of about 500 bp was obtained (Figure 4.7). The TEF sequence was obtained using the same primers and gave more than 600 bp. Analysis of both sequences resulted in 691 bp (Figure 4.8). The alignment of this sequence was blasted with the TEF sequences deposited in the GenBank databases. The results indicated that PBURU-B5 showed 99% homology with *F. solani* strain FRC#s1592 (DQ247446) and 99% homology with *F. solani* NRRL 22354 (AF178338).

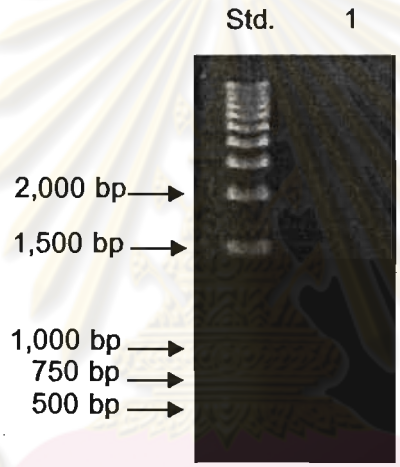


Figure 4.7 Amplification product of the portion of the translation elongation factor 1-α gene (TEF)

Lane Std. = DNA marker (1 kb, Vivantis, Shah Alam Selangor, Malaysia)

Lane 1 = PCR product of TEF gene

GACAAGACTCACCTCAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGT
CGACCACCGTAAGTCAAACCCTCCATCGCGATCTGCTTATCTCGGGTCGTGGAACCC
CGCCTGGCATCTCGGGCGGGGTATTCATCAGTCACTTCATGCTGACAATCATCTACAG
ACCGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCATCGAGAAGTTC
GAGAAGGTTGGTGACATCTCCCCCGATCGCGCCTTGATATCACACATCGAATTCCCTC
CCTCACGATCCGCGCTGCGCCCGCTTCTCCCGAGTCCCAAAATTTTTGCGGTGCGAC
CGTGAATTTTTTTGGTGGGGCATTTTACCCCGCCACTCGGGCGACGTTGGACAAAGC
CCTGATCCCTGCACACAAAAAACACCAAACCCTCTTGGCGCGCATCATCACGTGGTT
GACCACAGACGCTAACCGACTCAACAACAGGAAGCCGCTGAGCTCGGTAAGGGTTC
CTTCAAGTACGCCTGGGTCCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCAC
CATCGACATTGCCCTCTGGAAGTTCGAGACTCCCCGCTACTATGTCACCGTCATTGGT
ATGTTGCTGTCGTCTCTCTCAACCATGTCTCATCACTGACAATCAACAGACGCCCCCG
GCCACCGTGACTTCATCAAGAACATGATC

Figure 4.8 Nucleotide sequence of the portion of the translation elongation factor 1-α gene (TEF)

4.3 Optimization of culture conditions for cutinolytic esterase production from PBURU-B5

4.3.1 Effect of initial pH on esterase yield

The results of the effect of initial pH and incubation time on esterase production are illustrated in Figure 4.9 and were statistically analyzed by a multiple regression analysis. The ANOVA results indicated that there were significant differences in yields (Table 4.3). The regression is significant at the 1% significance level. The largest amount esterases were obtained from extracellular fluid of initial pH 11.0 (incubated at 30 °C for 4 days). This pH was used for further analysis of the effect of incubation temperature on enzyme production when pH was a constant variable.

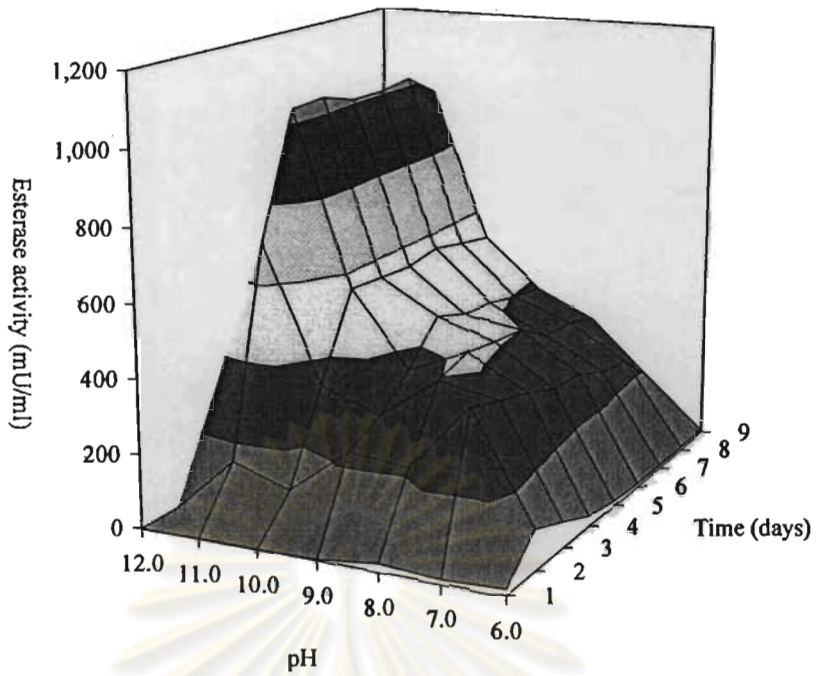


Figure 4.9 Interaction-effect of pH and incubation time on esterase activity at 30 °C

Table 4.3 The effect of initial pH on enzyme production: analysis of variance for regression*

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1250924	2	625461.856	8.320	.001[a]
	Residual	5036652	67	75173.903		
	Total	6287575	69			

a. Predictors: (Constant), Day, pH

b. Dependent Variable: Activity

*r^2= 0.2

4.3.2 Effect of incubation temperature

A multiple regression analysis was also applied for analysis of the results obtained from the effect of incubation temperature and culture time on esterase production. The ANOVA results indicated that there was a significant different amount treatment yields (Table 4.4). The regression is significant at the 1% significance level. The response surface graph showed in Figure 4.10. The optimal temperature given the most esterase yield was found at 25 °C on day 4 of incubation period when pH 11.0 was applied.

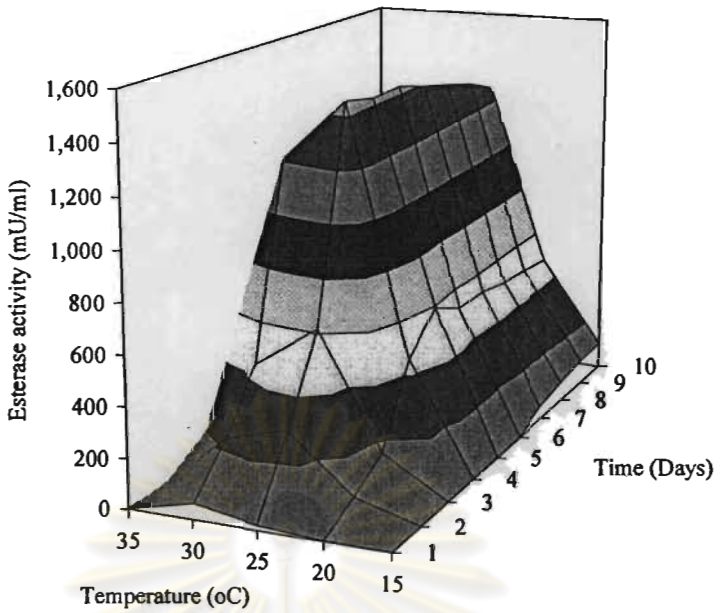


Figure 4.10 Interaction-effect of temperature and incubation time on esterase activity at pH 11.0.

Table 4.4 Analysis of variance for regression* of effect of incubation temperature on enzyme production experiment

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	2580997	2	1290498.294	5.515	.007[a]
	Residual	12168670	52	234012.891		
	Total	14749667	54			

a. Predictors: (Constant), Day, Temperature

b. Dependent Variable: Activity

*r^2= 0.18

4.4 Estimation of PBURU-B5 growth in a solid state culture system

PBURU-B5 was grown on production medium at 25°C, initial pH 11.0 for 10 days. Figure 4.11 shows the relationship between glucosamine content, esterase activity and culture pH measured from the culture of PBURU-B5 for a period of 10 days. The fungal growth indicated by the content of glucosamine showed rapid growth until day 4, followed by a slow down. It should be noted that mycelial yields as estimated by glucosamine analysis were quiet low. The enzyme production curve followed the same pattern as the growth determination in the first four days, and then was constant until day 8. The esterase activity was slightly decrease by the following day. The pH dropped from the initial pH 11.0 to 9.0 within 2 days.

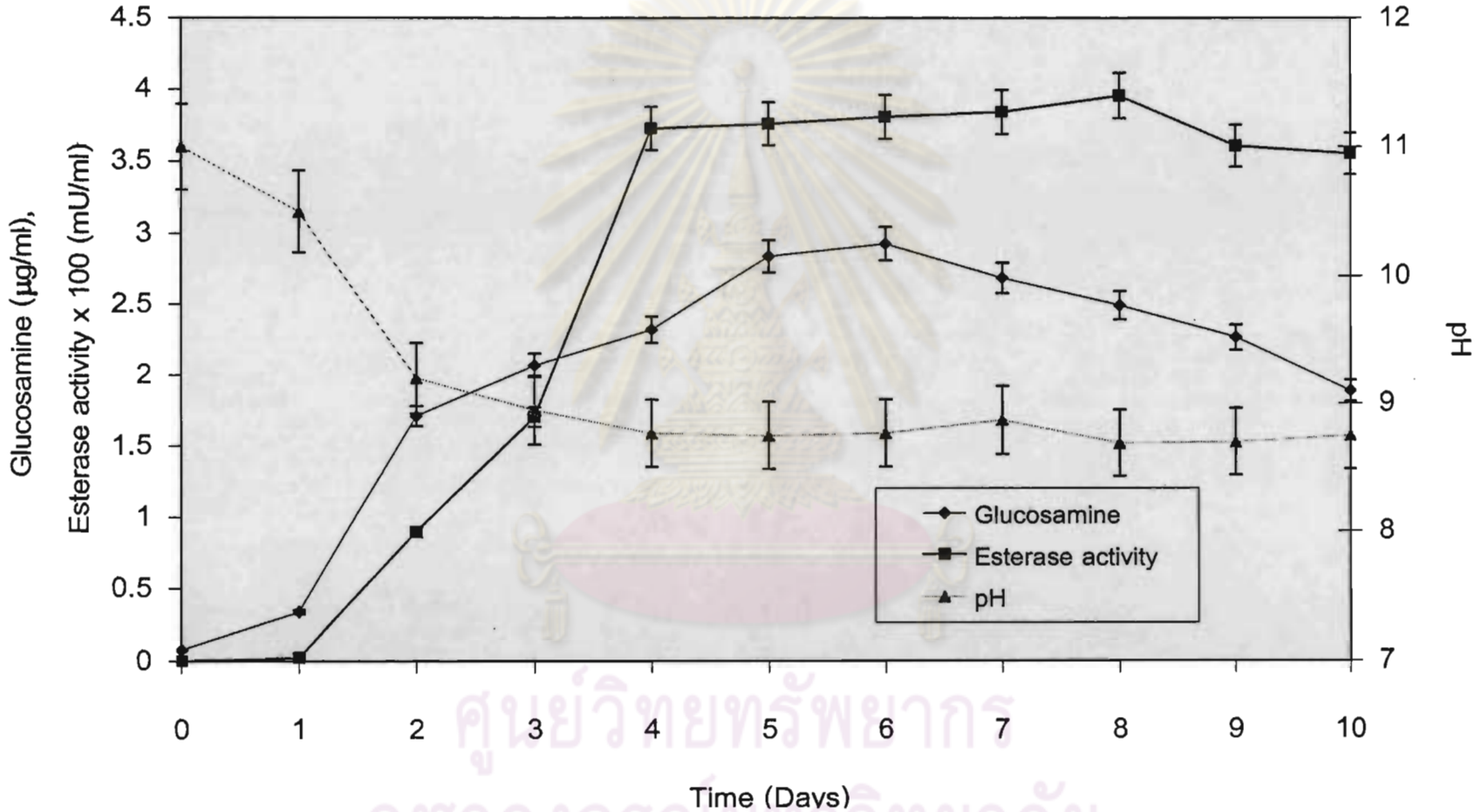


Figure 4.11 Esterase production by strain PBURU-B5: 25 °C, initial pH 11.0

4.5 Purification of cutinolytic esterase from PBURU-B5

4.5.1 Preparation of crude enzyme

Crude enzyme was prepared from 5 liters of the production medium containing 0.2% suberin as described in section 3.5.6.1. After washing the mycelium with Triton X-100, extracellular fluid and this Triton X-100 wash were pooled and yielded 5,614 ml of crude extract with 1,488 units of esterase activity and 752 mg protein. Thus, the specific activity of the enzyme in the crude preparation was 1.98 U/mg protein (Table 4.5). The crude enzyme was concentrated by ultrafiltration (Section 3.5.6.1) and yielded 578 ml of crude extract with 1,386 units, 653 mg protein and 2.12 U/mg of specificity was obtained.

4.5.2 Optimization of ammonium sulfate precipitation

Crude enzyme solution was further purified by ammonium sulfate precipitation as mentioned in section 3.5.6.2. To determine the suitable ammonium sulfate concentration for precipitation of cutinolytic esterase, preliminary experiment was performed by a stepwise increase at 10% increment from 0-90%. Most of enzyme activity was detected in the 50-60%, 60-70% and 70-80% fractions with the most activity in 60-70% fractions (Table 4.6). Therefore, to harvest the most of enzyme, protein fractionation was carried out in the range of 50-80% saturated ammonium sulfate precipitation. The protein remained 175 mg with enzyme activity yielded at 1,091 (about 73% yields from crude enzyme). The specific activity of the enzyme from this step was 6.23 U/mg protein (Table 4.5).

Table 4.5 Purification of cutinolytic esterase from *F. solani* PBURU-B5

Purification step	Total Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
Extracellular fluid	4,640	742	501	1.48	-	49.9
Triton X-100 wash	974	738	269	2.75	-	49.63
Crude extract	5,614	1,488	752	1.98	1.00	100
Ammonium sulfate	59	1,091	175	6.23	3.15	73
QAE Sepharose	15	756	23	32.87	16.61	51
Hitrap Phenyl HP	8	378	5.3	71.32	36.04	25
Sephacryl S-200	5	165	1.2	137.50	69.49	11

Table 4.6 Ammonium sulfate precipitation of cutinolytic esterase from *F. solani* PBURU-B5

Percent saturation range of ammonium sulfate	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Percent protein precipitated	Percent enzyme precipitated	Purification factor
Crude extract	200	209	429	2.05	100.00	100.00	1.00
0-10	11	30	0.17	0.01	14.35	0.04	0.00
10-20	11.5	24	0.11	0.00	9.80	0.03	0.00
20-30	18	32	0.93	0.03	13.06	0.22	0.02
30-40	16.5	26	0.34	0.01	10.61	0.08	0.01
40-50	10.5	17	0.39	0.02	6.94	0.09	0.01
50-60	10.5	17	105.54	6.21	6.94	24.62	3.55
60-70	12	25	225.12	9.00	10.20	52.52	5.15
70-80	11.5	20	80.56	4.03	8.16	18.79	2.30
80-90	12	10	1.09	0.11	4.08	0.25	0.06
90% saturation supernatant	205	16	10.32	0.65	6.53	2.41	0.37

4.5.3 Hitrap Q FF column (QAE sepharose, anion exhanger)

The enzyme obtained from 50-80% ammonium sulfate was applied onto Hitrap Q FF column (Section 3.5.6.3.1), unbound protein was washed out with 50 mM Tris-HCl, pH 8.5 and bound proteins were then eluted with a linear salt gradient (0 to 1.0 M NaCl) with the same buffer (Figure 4.12). The fractions containing esterase activity were pooled and concentrated, and then dialyzed against 1 liter of 20 mM sodium phosphate buffer (pH 7.5). There was 23 mg of protein with 756 activity units of enzyme (specific activity 32.87 U/mg) (Table 4.5). There was 17-fold purification with a 51% yield.

4.5.4 Hitrap Phenyl HP Column (Hydrophobic interaction chromatography)

The pooled esterase from Hitrap Q FF column was further purified using a Hitrap Phenyl HP column (Section 3.5.6.3.2). The chromatographic profile is shown in Figure 4.13. The unbound protein was eluted from the column with 20 mM sodium phosphate buffer (pH 7.5) containing 1.3 M ammonium sulfate. Bound proteins were eluted with a decreasing concentration of ammonium sulfate linear gradient (1.3 to 0 M) in the same buffer (Figure 4.13). Active fractions were pooled, concentrated and dialyzed against 50 mM Tris-HCl, pH 8.5. This yielded 5 mg esterase (378 units; specific activity 71 U/mg). The enzyme was purified 36 fold with about 25% yield (Table 4.5).

4.5.5 HiPrep 16/60 Sephacryl S-200 High Resolution Column (Gel filtration chromatography)

The enzyme was subsequent purified using HiPrep 16/60 Sephacryl S-200 High Resolution Column. The chromatographic profile is shown in Figure 4.14. The protein was eluted from the column with 50 mM Tris-HCl, pH 8.5 containing 0.15 M NaCl. The active fraction was pooled, concentrated and dialyzed against 50 mM Tris-HCl, pH 8.5. This protein remained in this step about 1.2 mg with 165 activity units and 138 U/mg of specific activity. The enzyme was purified to 69 fold with about 11% yield. The enzyme from this step was kept as aliquot at 4 °C for further characterization experiments.

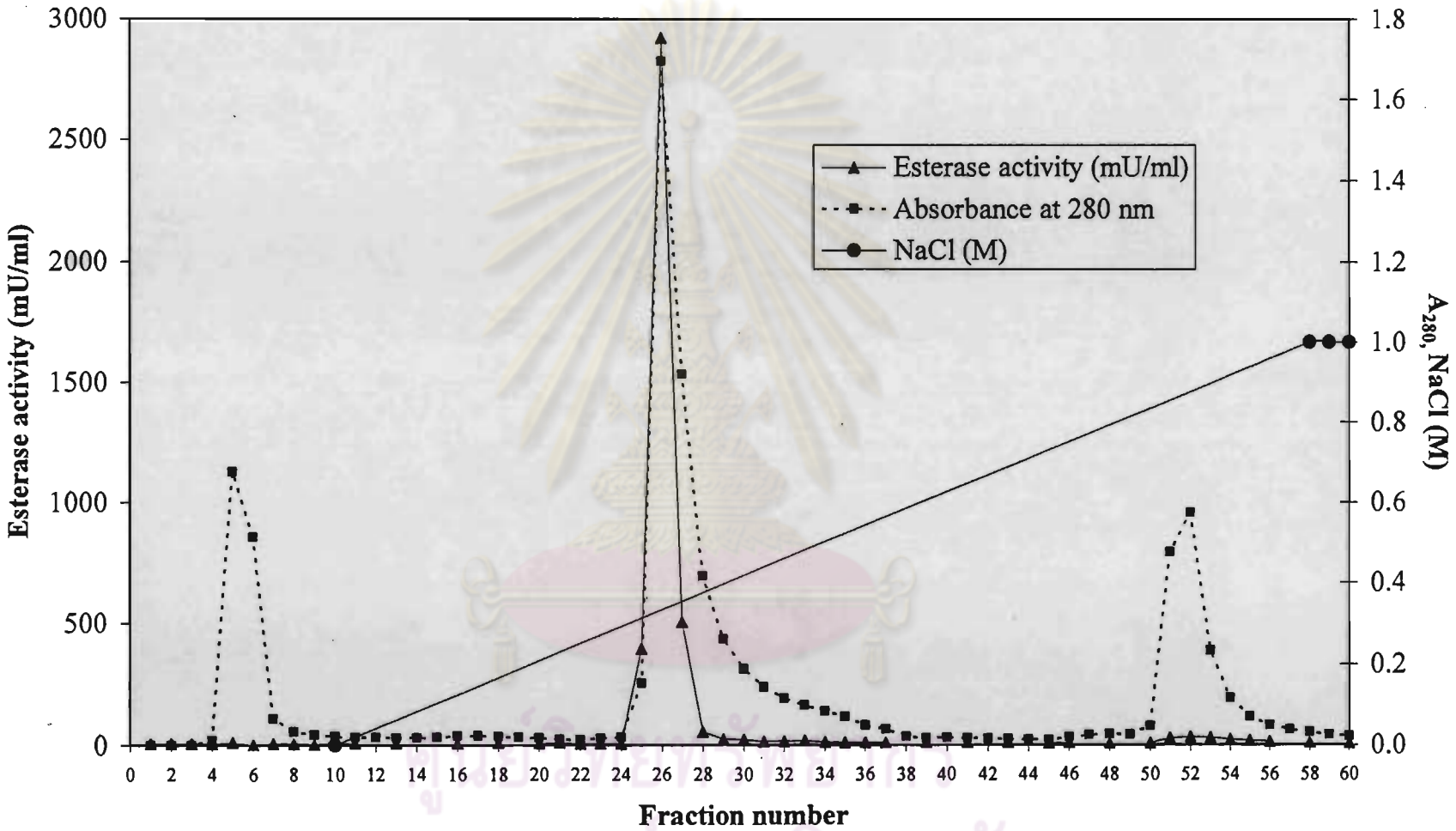


Figure 4.12 Purification of cutinolytic esterase from *F. solani* PBURU-B5 by Hitrap Q FF column

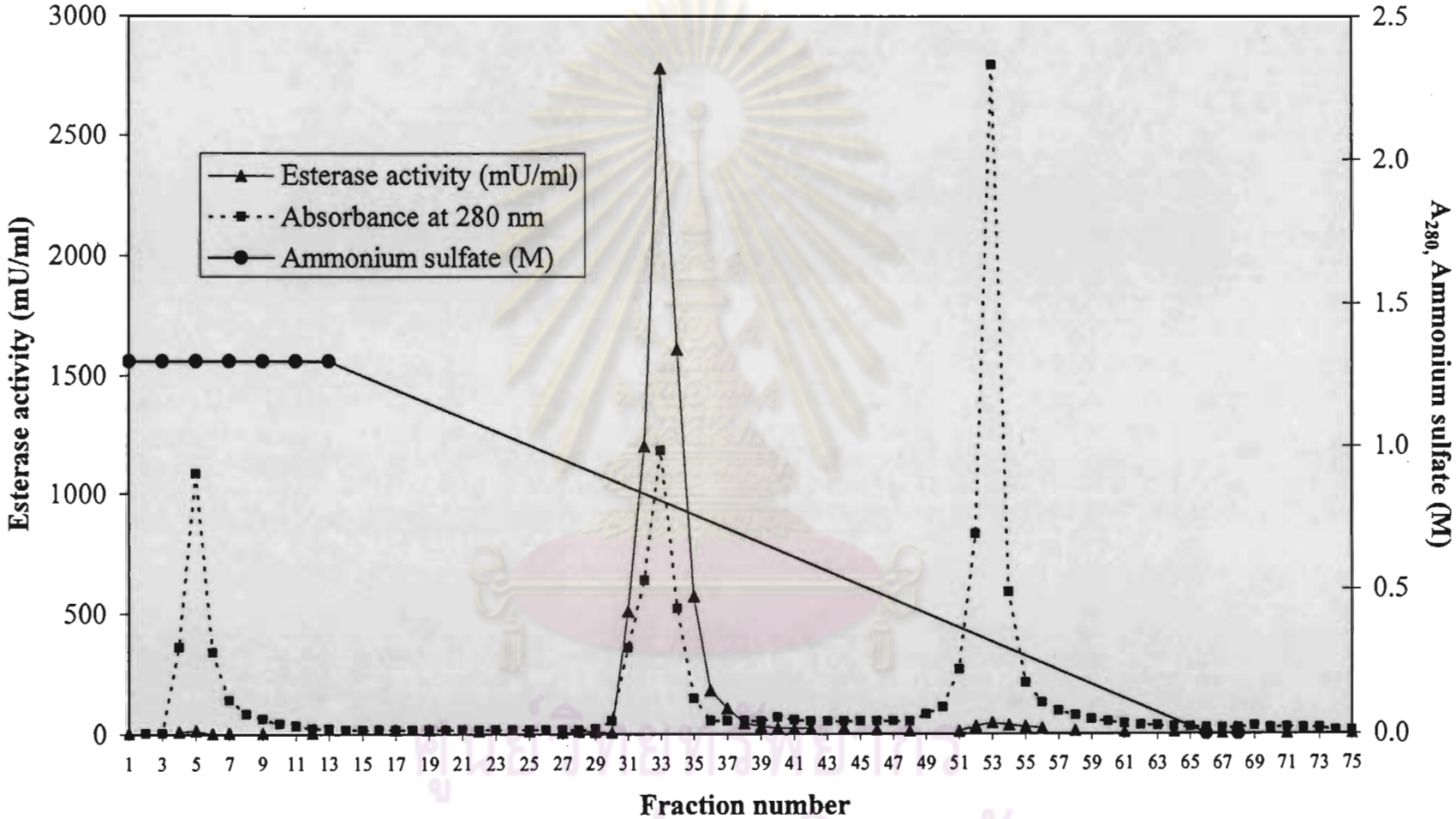


Figure 4.13 Purification of cutinolytic esterase from *F. solani* PBURU-B5 by Hitrap Phenyl HP Column

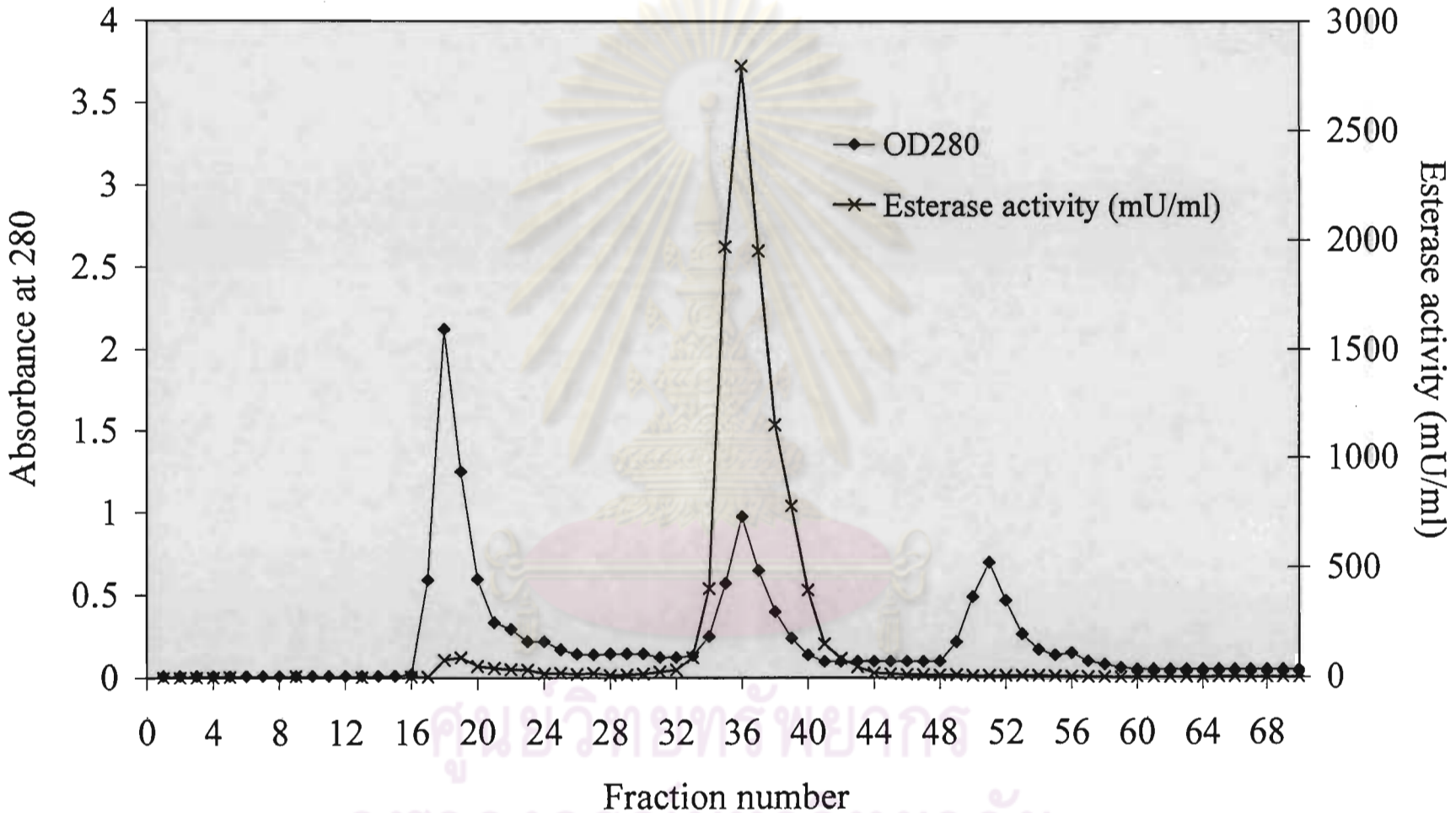


Figure 4.14 Purification of cutinolytic esterase from *F. solani* PBURU-B5 by HiPrep 16/60 Sephacryl S-200 High Resolution Column

4.5.6 Determination of enzyme purity and protein pattern on SDS-PAGE

The enzyme purity at each purification step was analyzed by SDS-PAGE (Section 3.5.6.4.1). In gel activity was compared with the silver staining. The enzyme from the HiPrep 16/60 Sephacryl S-200 High Resolution Column showed a single protein band 5 (Figure 4.15a) which corresponded with its activity by activity staining in lane 6 (Figure 4.15b).

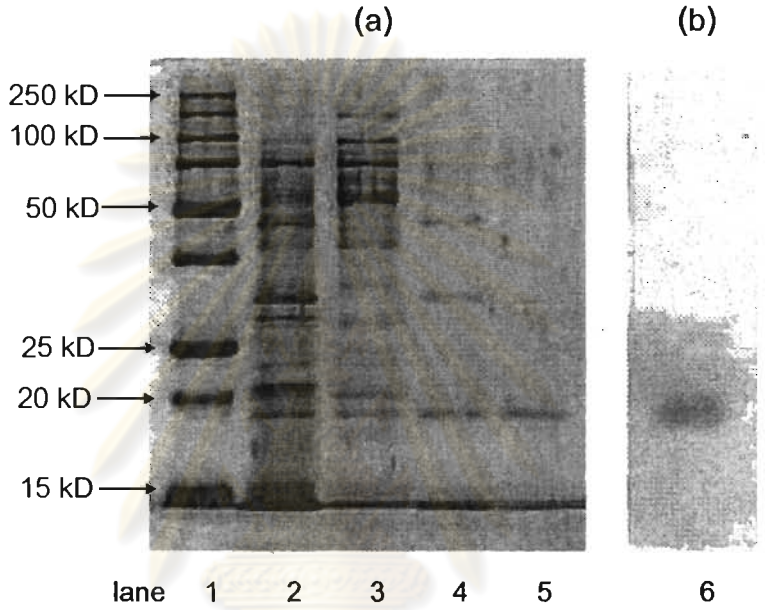


Figure 4.15 SDS-PAGE analysis of *F. solani* PBURU-B5 cutinolytic esterase at each purification - 12% acrylamide gel: (a) Silver staining (b) Activity staining.

(a) Silver staining

Lane 1 = Precision Plus Protein Standards (0.5 µl)

Lane 2 = 50-80% Ammonium sulfate precipitation (25 µg protein)

Lane 3 = Hitrap Q FF (25 µg protein)

Lane 4 = Hitrap Phenyl HP (10 µg protein)

Lane 5 = HiPrep 16/60 Sephacryl S-200 (10 µg protein)

(b) Activity staining

Lane 6 = HiPrep 16/60 Sephacryl S-200 (10 µg protein)

4.6 Characterization of purified cutinolytic esterase from PBURU-B5

4.6.1 Effect of pH on cutinolytic esterase activity

The optimum pH of the enzyme was determined as defined in section 3.5.7.1 (Figure 4.16). The 0.1 M of sodium acetate, potassium phosphate, Tris-HCl and borate were used as reaction buffers for pH 4.0-6.0, 6.0-8.0, 8.0-9.0 and 9.0-11.0, respectively. No buffer effect was observed at all pH ranges. Optimum pH was pH 9.0. The enzyme showed 60-85% of the activity at pH 6.0-8.0 while no activity was observed at pH 4.0. The activity less than 40% was found at pH 10.0-11.0.

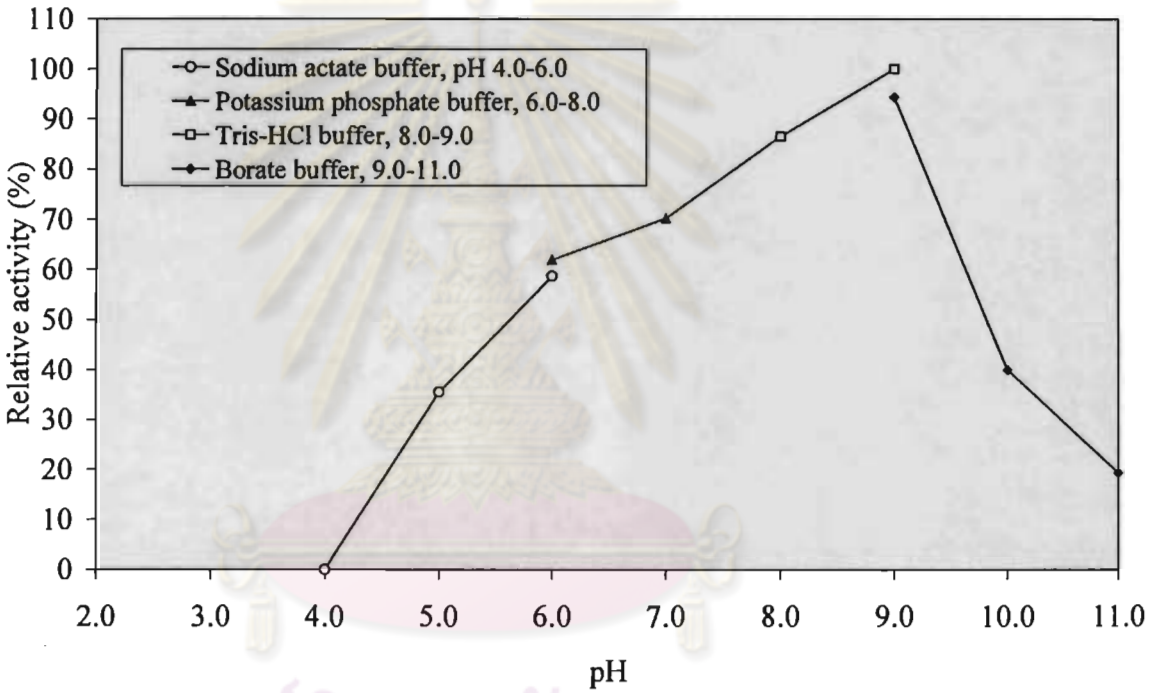


Figure 4.16 Effect of pH on cutinolytic esterase activity

4.6.2 Effect of temperature on cutinolytic esterase activity

The optimum temperature of the enzyme was determined by running the assay at various temperatures (Section 3.5.7.2). The enzyme showed the greatest activity at 45°C. At 50 and 55 °C, 84% and 57% of the activity remained after incubation for 30 min (Figure 4.17).

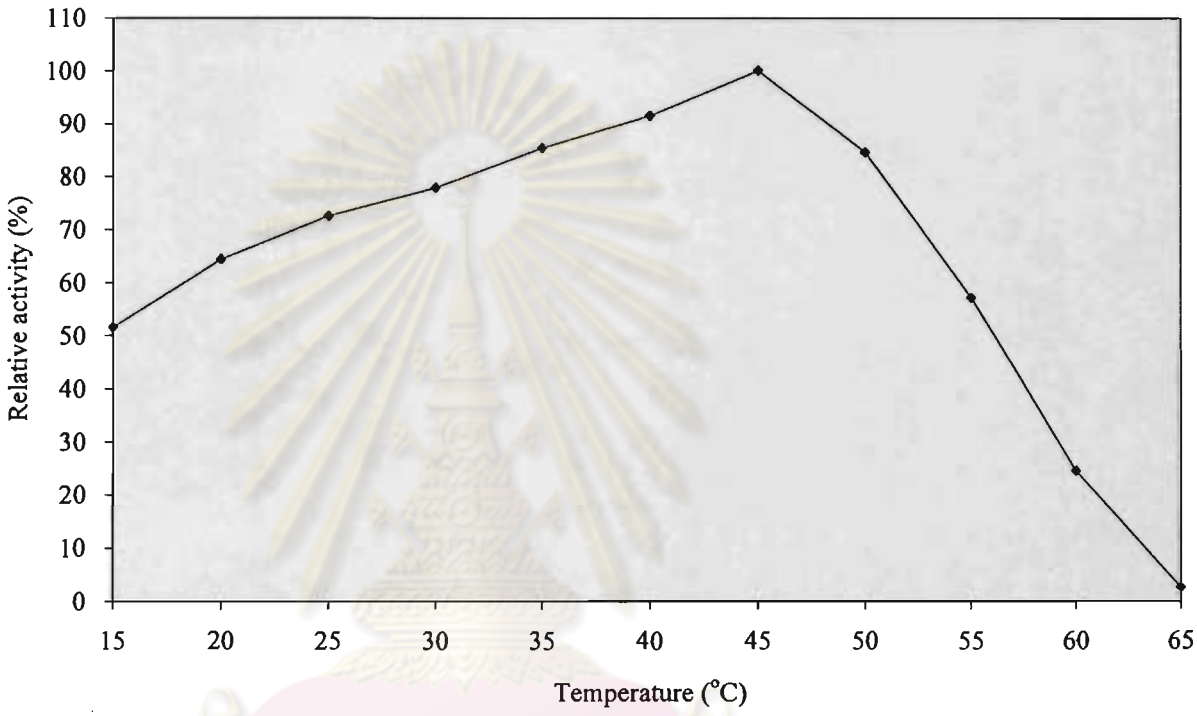


Figure 4.17 Effect of temperature on cutinolytic esterase activity

4.6.3 Effect of pH on cutinolytic esterase stability

The pH stability of the enzyme was measured by incubating the enzyme at pHs from 4.0-11.0 for 30 min (50 mM buffers) at 4°C and then residual activity was measured (Section 3.5.7.3). The enzyme was quiet stable to pH (6.0-10.0) at this cold temperature (Figure 4.18).

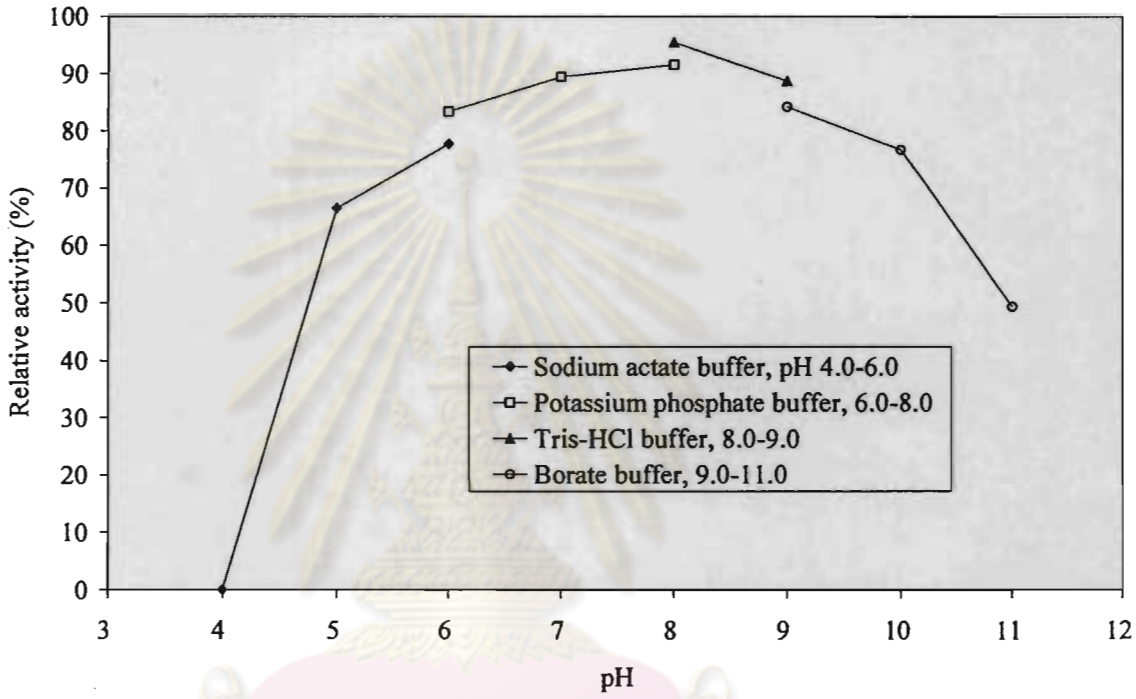


Figure 4.18 Effect of pH on cutinolytic esterase stability

4.6.4 Effect of temperature on cutinolytic esterase stability

The effect of temperature on stability of the esterase was determined by heating without substrate for 30 min at different temperature (section 3.5.7.4). Total enzyme activity remained when the enzyme was incubated at various temperatures from 10-65 °C for 30 min as shown in Figure 4.19. Heating from 50°C for 30 min let to gradual inactivation. More than 80% of activity remained when enzyme was incubated at 15°C to 45°C.

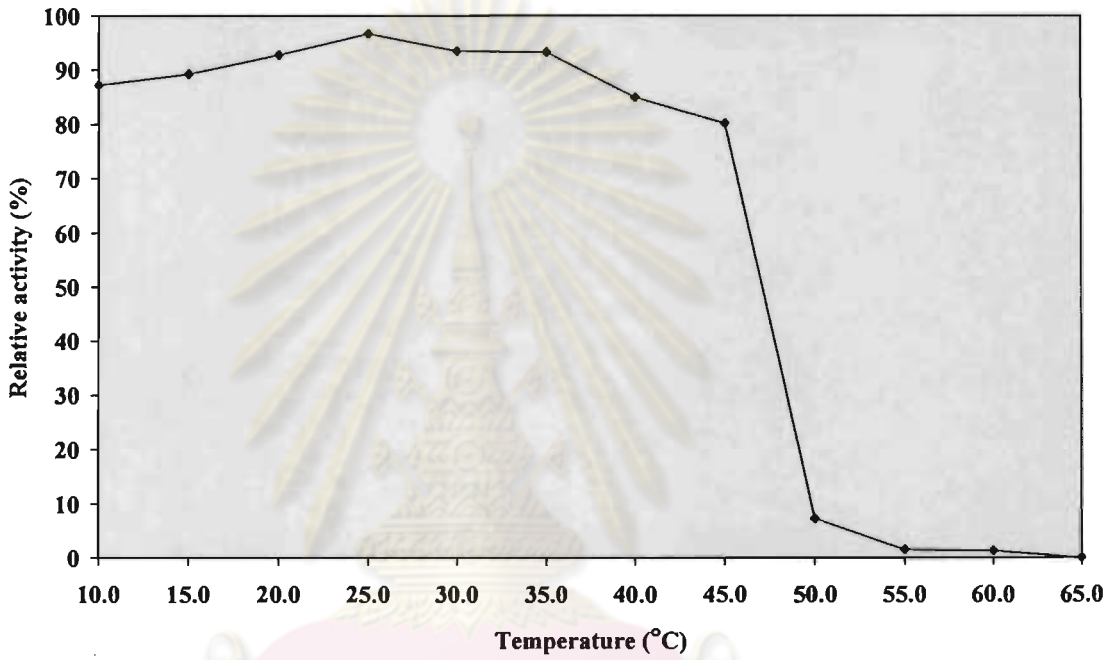


Figure 4.19 Effect of temperature on cutinolytic esterase stability

4.6.5 Effect of metal ions on cutinolytic esterase activity

The effect of metal ions on esterase activity was determined by adding the salts to the enzyme reaction mixture at various concentrations. The results are summarized in Table 4.7. No inhibition was observed with Ca^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} at 10 mM, though Cu^{2+} was inhibitory.

Table 4.7 Effect of metal ions on cutinolytic esterase activity

Metal ion	Relative activity (%)		
	Ion concentration (mM)		
	0.1	1.0	10
Control	100	100	100
$CaCl_2.2H_2O$	98.17	95.03	94.18
$CuSO_4.5H_2O$	97.03	95.43	50.84
$FeSO_4.7H_2O$	97.95	Nd[a]	Nd[a]
$HgCl_2$	99.50	98.64	91.16
$MgSO_4.7H_2O$	98.74	96.23	94.41
$MnSO_4.7H_2O$	98.78	97.73	90.93
$ZnSO_4.7H_2O$	99.94	95.61	94.92

[a]The activities of the enzyme were not determined due to $FeSO_4.7H_2O$ interference on the assay (a yellow color developed affecting the assay at 405 nm).

4.6.6 Effect of NaCl on cutinolytic esterase activity

The effect of NaCl towards esterase activity is shown in Figure 4.20. NaCl had a slight negative effect on the activity. More than 80% activity remained even when NaCl was applied at 0.7 M.

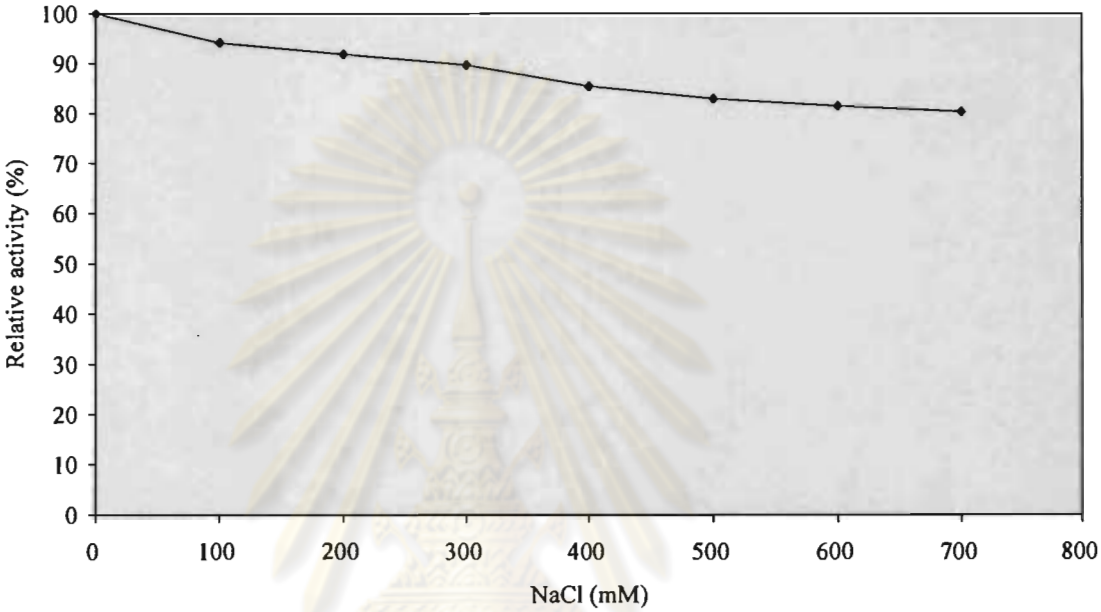


Figure 4.20 Effect of NaCl on cutinolytic esterase activity

4.6.7 Effect of ethylene diamine tetra-acetic acid (EDTA) on cutinolytic esterase activity

EDTA essentially had no effect on the activity of the esterase (Figure 4.21). There was over 80% residual activity even added 3 mM EDTA.

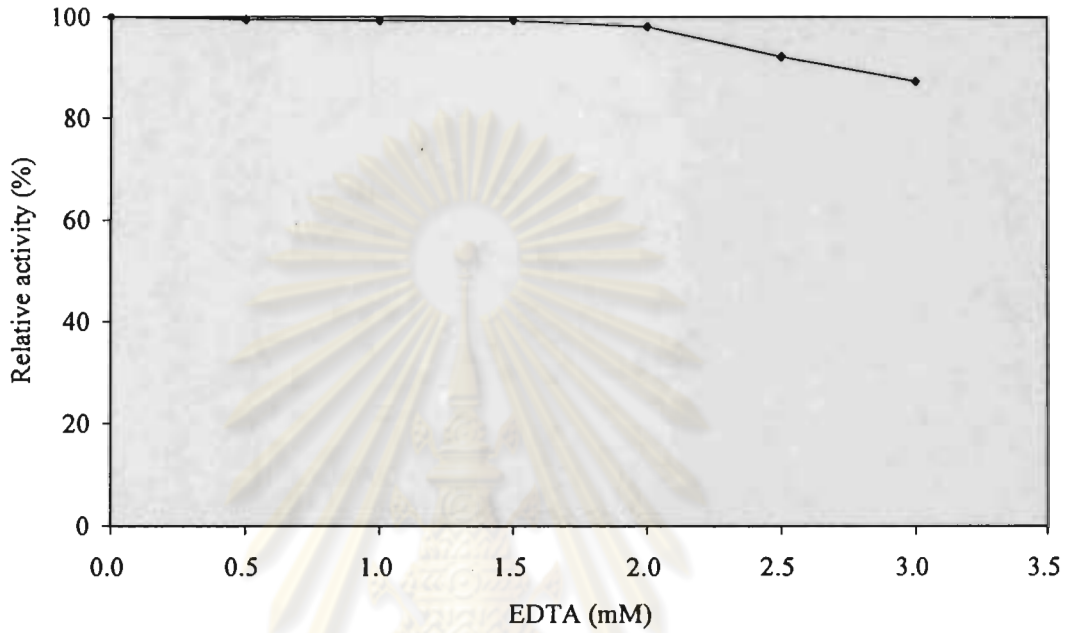


Figure 4.21 Effect of EDTA on cutinolytic esterase activity

4.6.8 Kinetic study of cutinolytic esterase

The kinetics of the esterase were evaluated by the Lineweaver-Burk analysis (Figure 4.22). concentrations of p-NPB from 0.1-1.0 mM were used. From x-intercept value (-1.899 = $-1/K_m$), Km was calculated to 0.53 mM while V_{max} was determined from y-intercept value (0.0963 = $1/V_{max}$). V_{max} value was 10.38 μmoles/min.

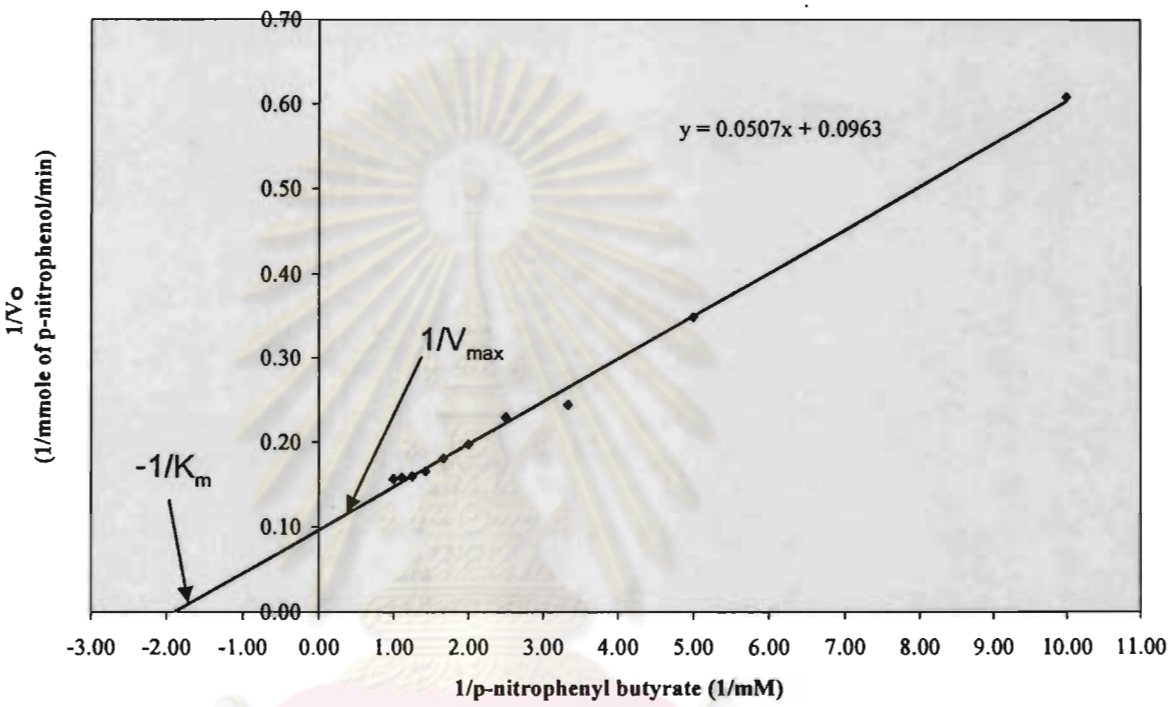


Figure 4.22 Lineweaver-Burk analysis of the cutinolytic esterase of *F. solani* PBURU-B5 with p-NPB as substrate

4.6.9 Molecular weight of cutinolytic esterase by gel filtration using a HiPrep 16/60 Sephacryl S-200 High Resolution Column

The native molecular weight of cutinolytic esterase was determined using calibrated a HiPrep 16/60 Sephacryl S-200 High Resolution Column (Section 3.5.7.9). The partition coefficient (K_{av}) values for each standard protein marker $[(V_e-V_0)/(V_t-V_0)]$ plotted against log molecular weight of each protein yielded a calibration curve (Figure 4.21). The K_{av} of the enzyme was used to determine its native molecular weight, and it's about 19 kDa (Figures 4.23 and 4.24).

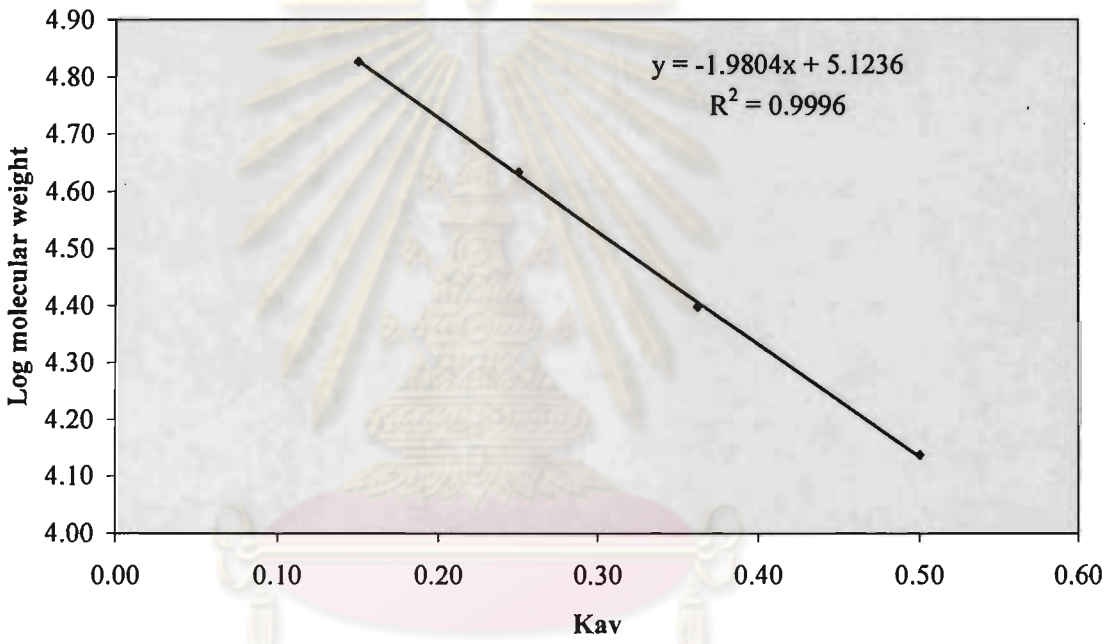


Figure 4.23 Assessment of the native molecular weight of PBURU-B5 cutinolytic esterase by gel filtration chromatography using a HiPrep 16/60 Sephacryl S-200 High Resolution Column

Ribonuclease A	(MW 13,700)
Chymotrypsinogen	(MW 25,000)
Ovalbumin	(MW 43,000)
Albumin	(MW 67,000)

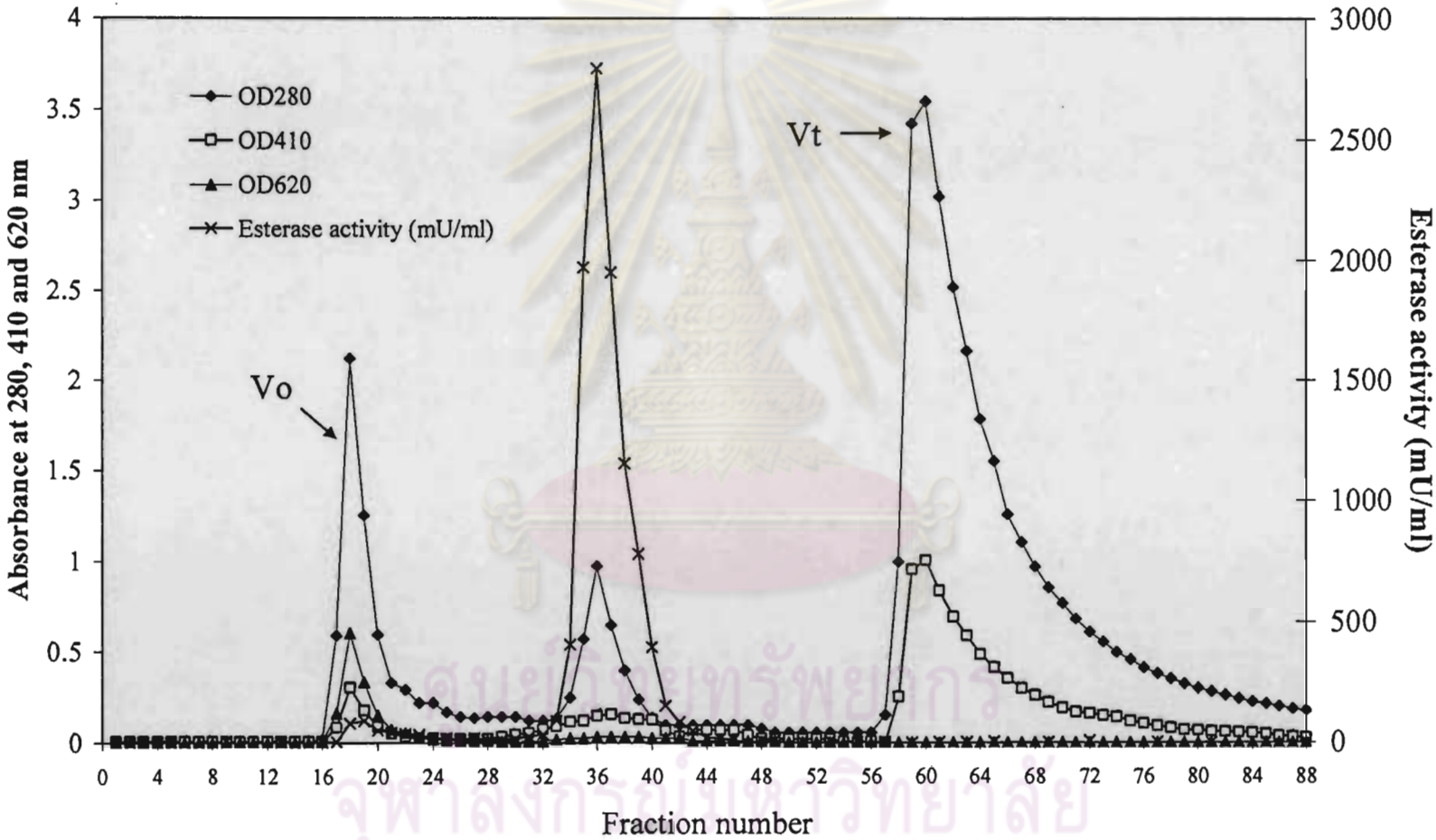


Figure 4.24 Molecular weight determination of cutinolytic esterase from PBURU-B5 by HiPrep 16/60 Sephacryl S-200 High Resolution Column

4.7 Applications of the enzyme on PET surface modification

The effect of cutinolytic esterase towards PET cloth was observed using the partially purified enzyme (50-80% ammonium sulfate cut). Concentrated crude enzyme (987 ml - 553 mg total protein and with 2,578 activity units) (specific activity 4.7 U/mg), was used for all PET modification experiments.

4.7.1 Assay for enzymatic hydrolysis of PET fibers based on ultraviolet light (UV) absorbance assay

The release of terephthalic acid (TPA) from PET was estimated by incubating pieces of PET yarn in 100 ml 50 mM Tris-HCl buffer (pH 9.0) at 45°C with a range of enzyme concentrations for up to 168 h (Section 3.5.8.2) (Figure 4.25). The activity of the enzyme towards PET was concentration dependent. This response was not completely proportional to enzyme concentration probably a reflection of the insoluble nature of PET, the enzyme simply attacking the surface area of the PET, resulted in releasing of TPA and the amount of TPA increased when the higher concentration of enzyme employed as shown in Figure 4.25.

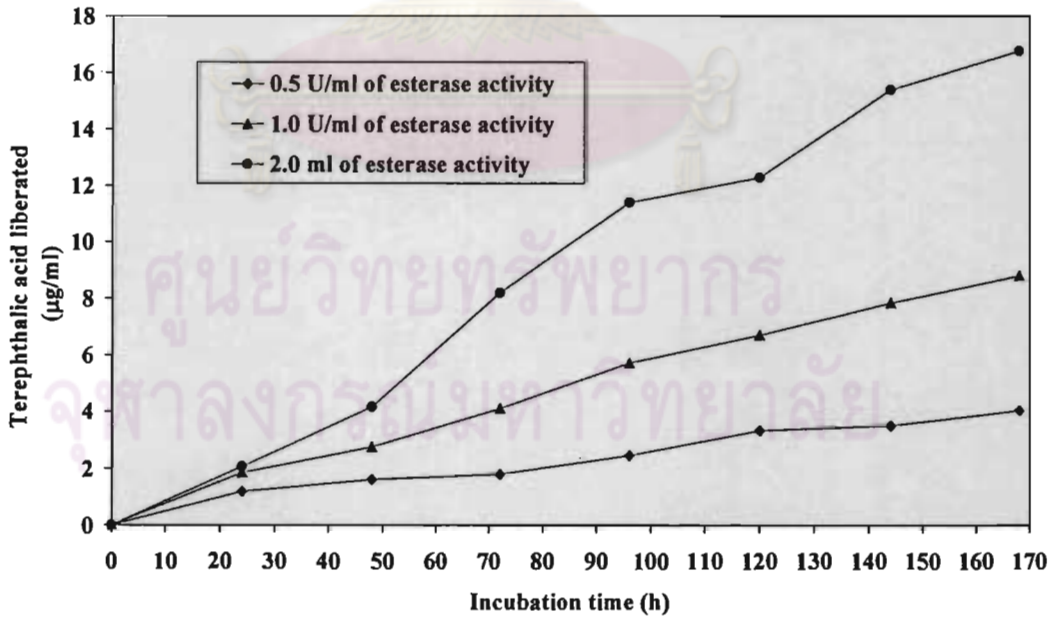


Figure 4.25 *F. solani* PBURU-B5 esterase activity towards PET fibers assessed by terephthalic acid release based on ultraviolet light (UV) absorbance assay.

4.7.2 Enzymatic hydrolysis of PET fibers based on fluorescence analysis

The action of the enzyme towards PET was assessed by incubating PET fiber with enzyme and the enzyme action monitored by fluorescence (Figure 4.26). The TPA released fairly closely followed enzyme concentration.

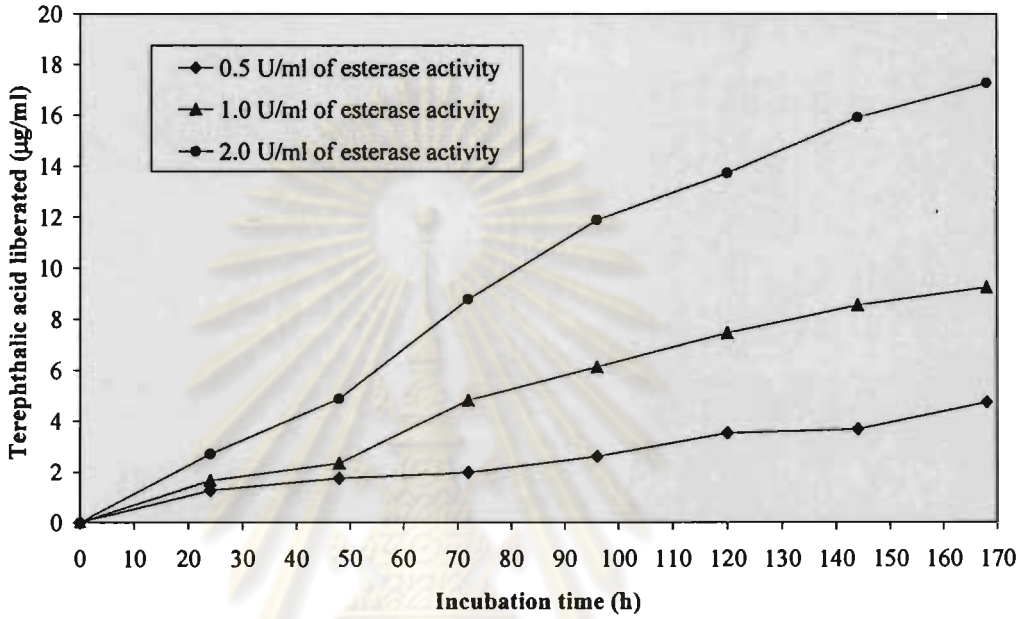


Figure 4.26 *F. solani* PBURU-B5 esterase activity towards PET fibers assessed by terephthalic acid release based on fluorescence method

4.7.3 Measurement of wettability of the enzyme and alkaline treated PET fabrics

The wetting time of untreated PET fabric is greater than that of enzyme and also alkali treated PET fabrics (Figure 4.27). The wetting time of buffer and inactivated enzyme did not change compared to the untreated control. However, there was reduction of wetting time of enzyme and also alkali treated fabrics. Although the PET fabrics treated with 3.0 M NaOH resulted in greatest wettability (Figure 4.27), those treated with all concentrations of enzyme also gave significant level of reduction in wetting time. The enzyme or alkali attacked ester bond of fabric thus bulk of carboxyl and hydroxyl groups were occurred and the hydrophilicity of fabric was enhanced.

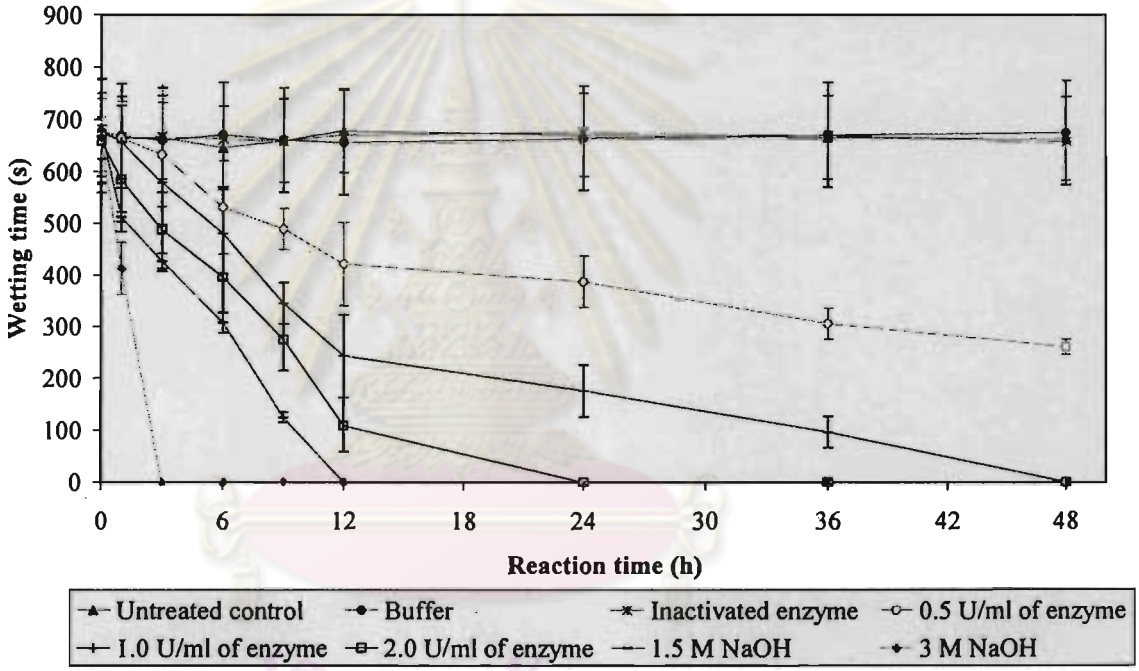


Figure 4.27 The wettability of untreated, enzyme treated, and alkali treated PET fabrics measured in horizontal area

4.7.4 Water absorption of the enzyme and alkaline treated PET fabrics

Water absorption of treated PET fabrics with enzyme or alkaline showed sharply increased compared to untreated control, buffer treated and inactivated enzyme treated PET fabrics (Figure 4.28). The rising heights of water of all enzyme treated fabrics were dramatically increased by the amount of enzyme treatment (time of incubation and enzyme concentration). Water absorption of enzyme treated PET fabrics increased the hydrophilicity of PET fabrics in similar manner to the alkali treated fabrics.

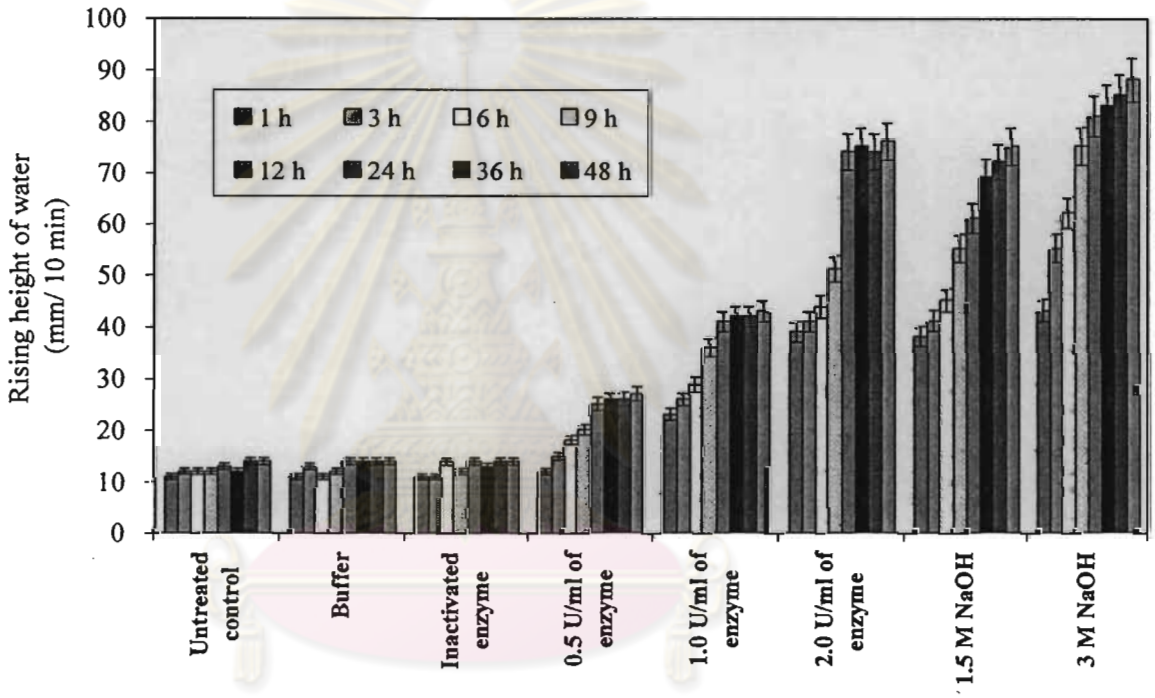


Figure 4.28 Water absorption of untreated and enzyme and alkaline treated PET fabrics

4.7.5 Moisture content of enzyme and alkaline treated PET fabrics

The uptake of water by PET makes it more a meanable, and gives a softer feel of the fabric to the wear. Moisture content of fabric following enzyme and also alkali treatment was followed using infrared treatment to remove absorbed water coupled with monitoring weight change (Infrared moisture balance - Section 3.5.8.7) as described in Figure 4.29. The moisture uptake increased during the first 24 hours and then remained static for all treated samples. The increasing percentage of moisture content of enzyme treated fabric was less than 1% due to the action of enzyme to fabric only occurred on the fabric surface. Control cloth did not show water uptake.

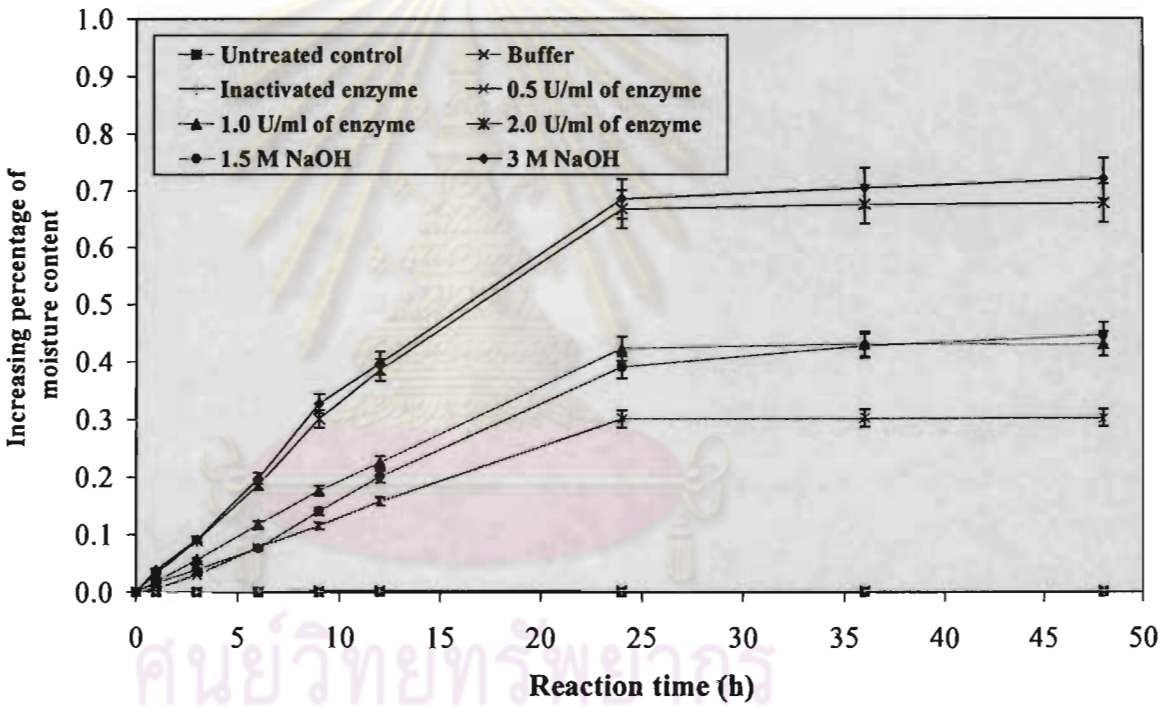


Figure 4.29 Increasing percentage of enzyme and alkaline treated PET fabrics

4.7.6 Susceptibility of enzyme and alkali treated PET fabrics to dye

4.7.6.1 Disperse dye (Dianix Red CC)

The "dyeability" of PET fabrics after enzyme and alkaline treatments were evaluated based on disperse dye treatment (Section 3.5.8.8.1). The results are shown in Figure 4.30. The color strength (expressed as K/S value at maximum absorption wavelength – 500 nm), indicated that the color strength of enzyme treated PET fabric was significantly increased and this was proportional to enzyme concentration or time of treatment (Figure 4.30). Clearly enzyme and also alkali treatment enhanced the susceptibility of the cloth to dye.

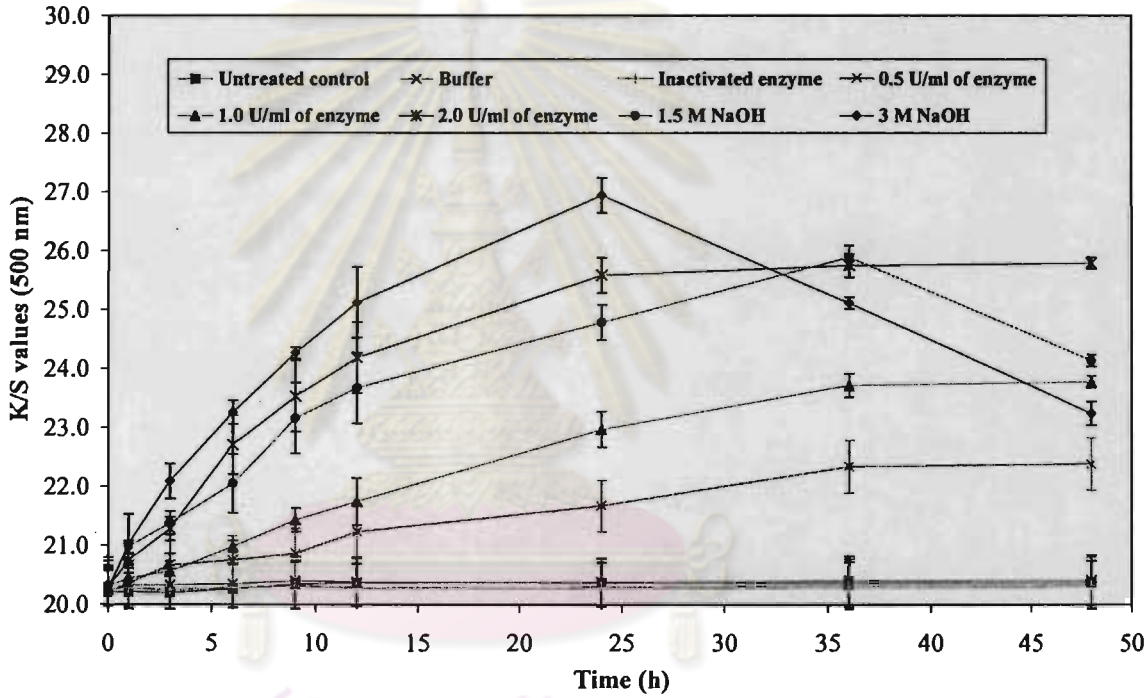


Figure 4.30 Color measurement of untreated and enzyme or alkaline treated PET fabrics dyed with dispersed dye (Dianix Red CC, supplied by Dystar Thai Ltd.) (Section 3.5.8.1)

4.7.6.2 Dyeing with the basic dye (Astrazon Red FBL 200% 03)

The uptake of the dye after enzyme or alkali treated PET fabrics showed that all treatment gave in enhancement (see the K/S values – Figure 4.31). The 3 M NaOH-treated fabrics yielded the most intensely dye fabric, followed by treatment of 2 U/ml of enzyme, 1.5 N NaOH, 1.0 U/ml and 0.5 U/ml of enzyme. No color changes were observed in the control samples. The treatment appears of basic practical value.

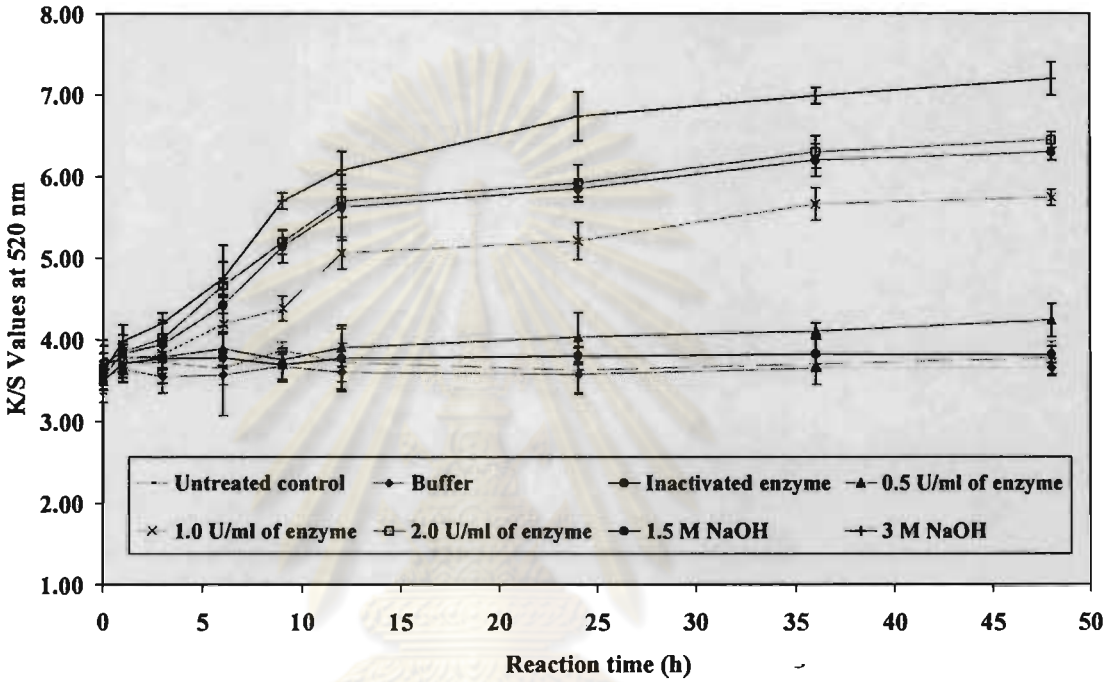


Figure 4.31 Dye susceptibility of enzyme and also alkali treated PET fabrics. (Astrazon Red FBL 200% 03, supplied by Dystar Thai Ltd. – section 4.3.8.8.2.)

4.7.7 Tensile strength of enzyme and alkali treated PET fabrics

The tensile strength of enzyme and alkali treated PET fabric were subjected to test to analyze any changes in this parameter of tensile strength (Figure 4.32). The tensile strength of 3 M and 1.5 N NaOH treated PET fabric was dramatically decreased (Figure 4.32). In comparison, there was a little change in enzyme treated samples or control (Figure 4.32). The molecule of enzyme was large; it couldn't penetrate inside the fabric. The reaction of enzyme towards fabric was only presented at the surface of fabric. On the other hands, alkali could penetrate into fabric and attacked a bulk of ester bond, resulted weak strength of fabric. As regards a reduction in tensile strength the enzyme treatment was superior to that of use the alkali.

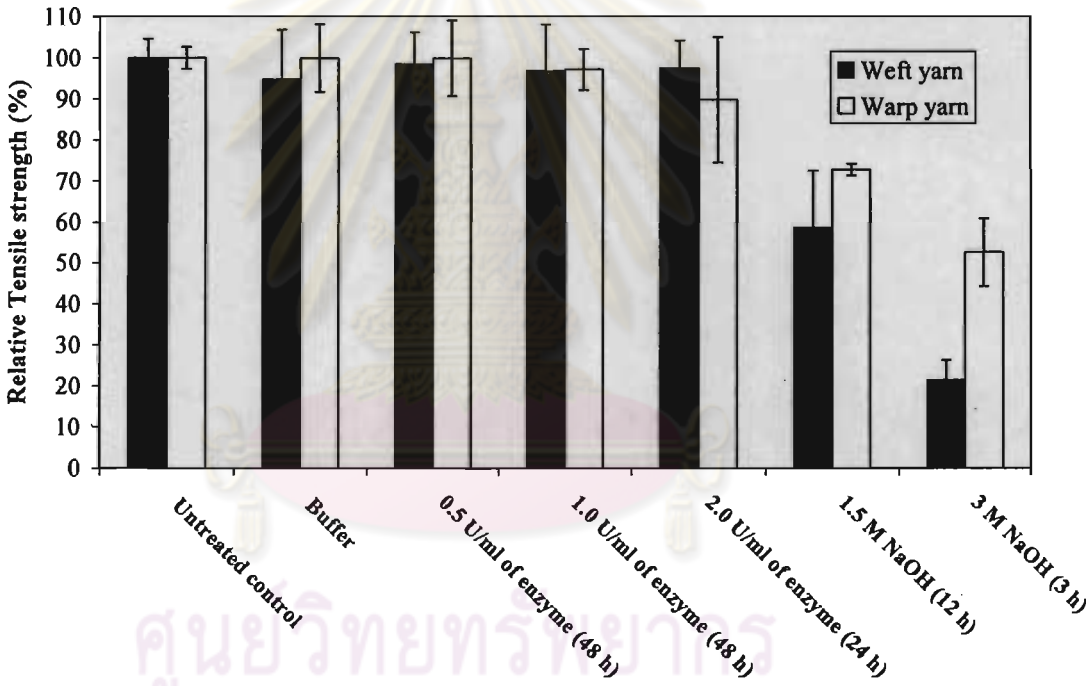


Figure 4.32 Tensile strength of untreated and enzyme or alkaline treated PET fabrics

4.7.8 Surface chemical analysis of enzyme and alkaline treated PET fabric

ATR-FTIR analysis which monitors changes in did not reveal any major differences between an enzyme, alkali and control samples (Figure 4.33). This analysis is not useful for further enzyme action (Figure 4.33).

4.7.9 Surface morphological analysis of enzyme and alkaline treated PET fabrics

SEM images of morphological changes of treated PET fabrics using enzyme or alkali are shown in Figure 4.34. As illustrated in Figure 4.34 (a, b and c, d) the original surface of untreated PET fabric was smooth as was buffer treated PET fabric. The enzyme treated fiber showed breaking (Figure 4.34 ef, gh, and ij). Alkali treatment also resulted in surface pitting on PET fibers (Figure 4.34 kl and mn). Scanning electron microscope is useful in illustrated surface attack of enzyme and also alkali treated fiber, but does not indicate the distinction of the weight loss between the two fabric treatment methods. Probably cross-sectional transmission electromicroscopy could illustrate the weight loss in the alkaline treated samples.

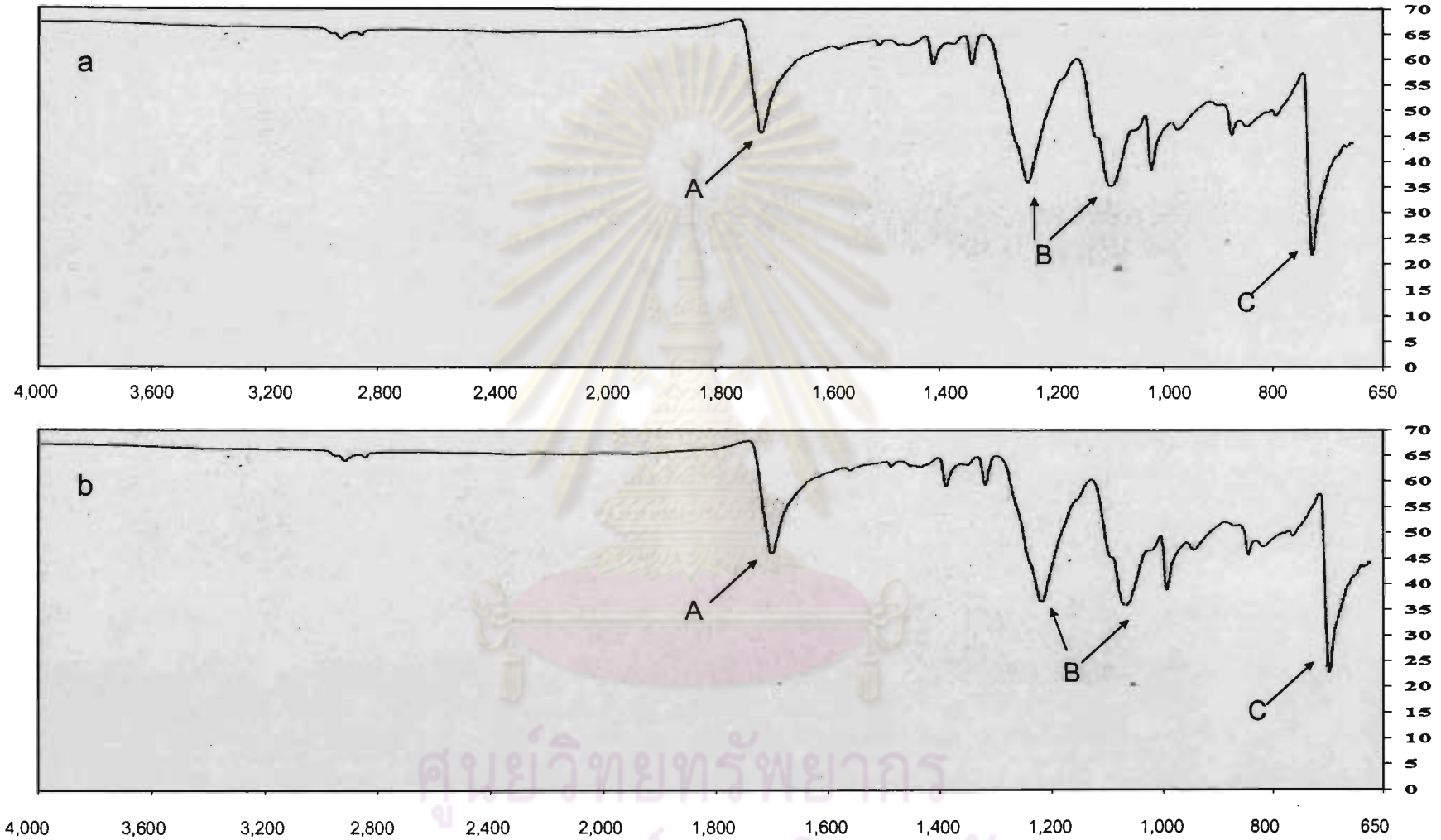


Figure 4.33 ATR-FTIR spectra of untreated PET fabric (a) and buffer treated PET fabric (b)

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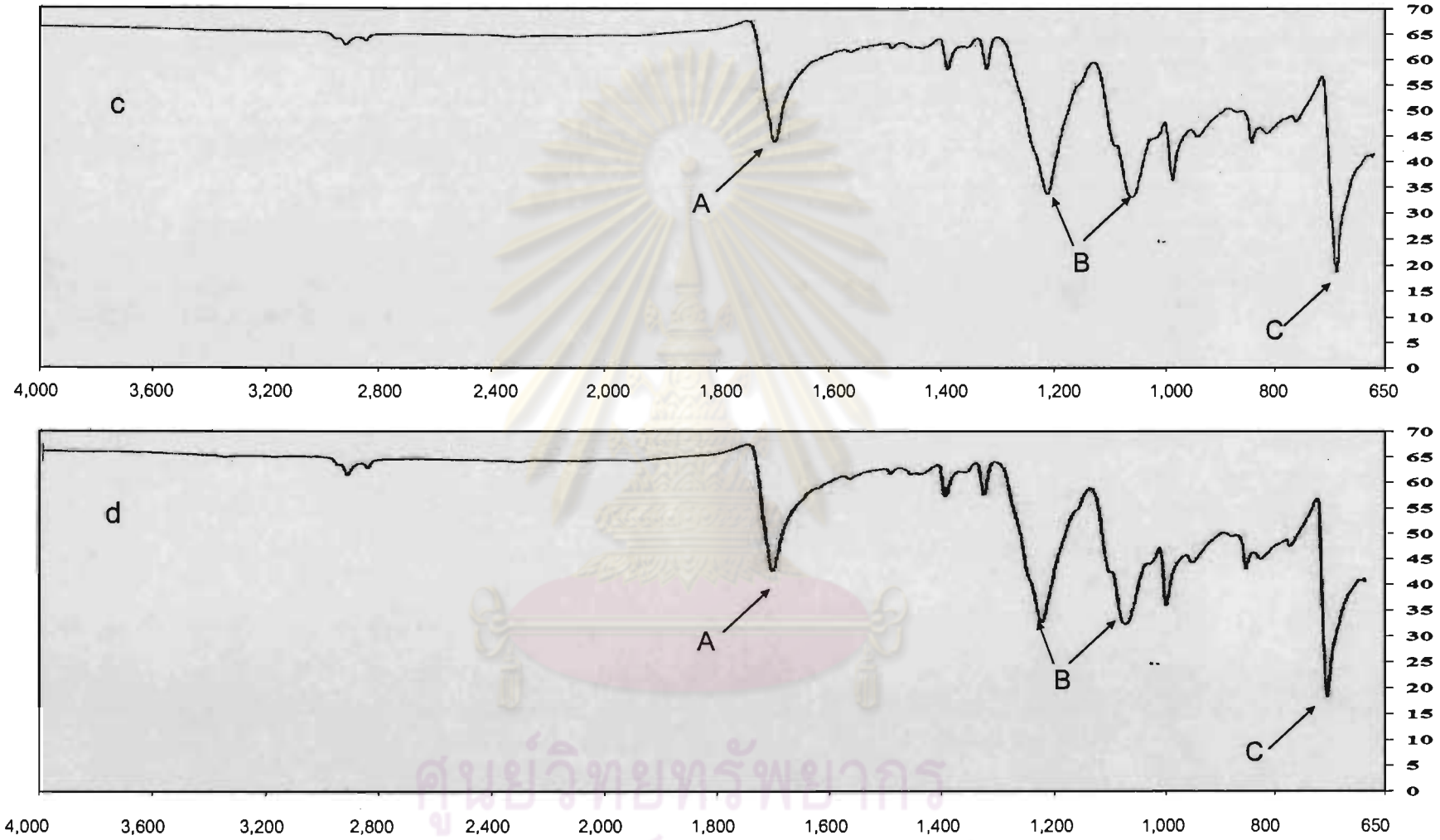

Figure 4.33 (cont.) ATR-FTIR spectra of fabric treated with 0.5 U/ml enzyme (48 h) (c) and 1.0 U/ml enzyme (48 h) (d)

107

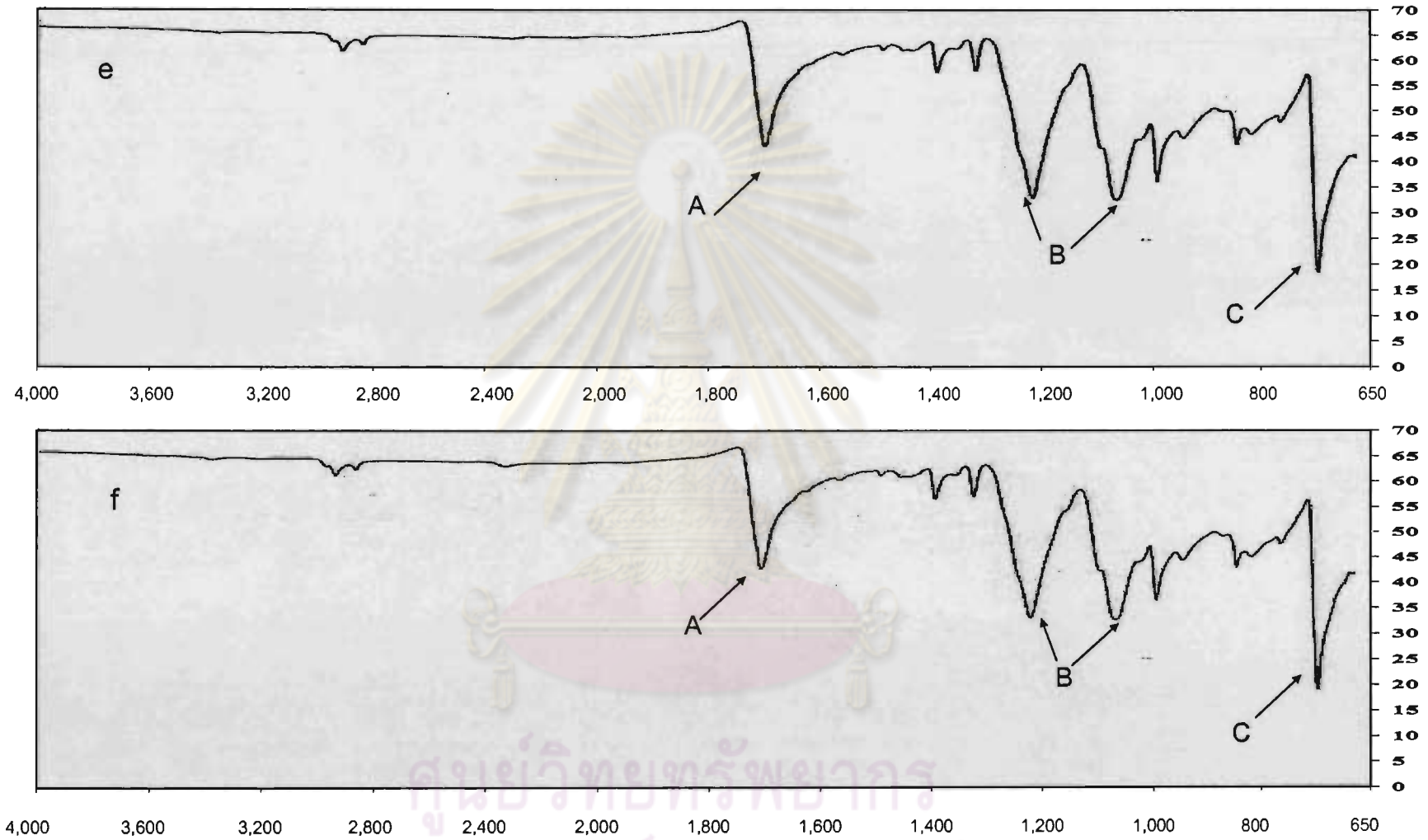

Figure 4.33 (cont.) ATR-FTIR spectra of 2 U/ml enzyme (24 h) (e) and 1.5 M NaOH (12 h) (f)

108

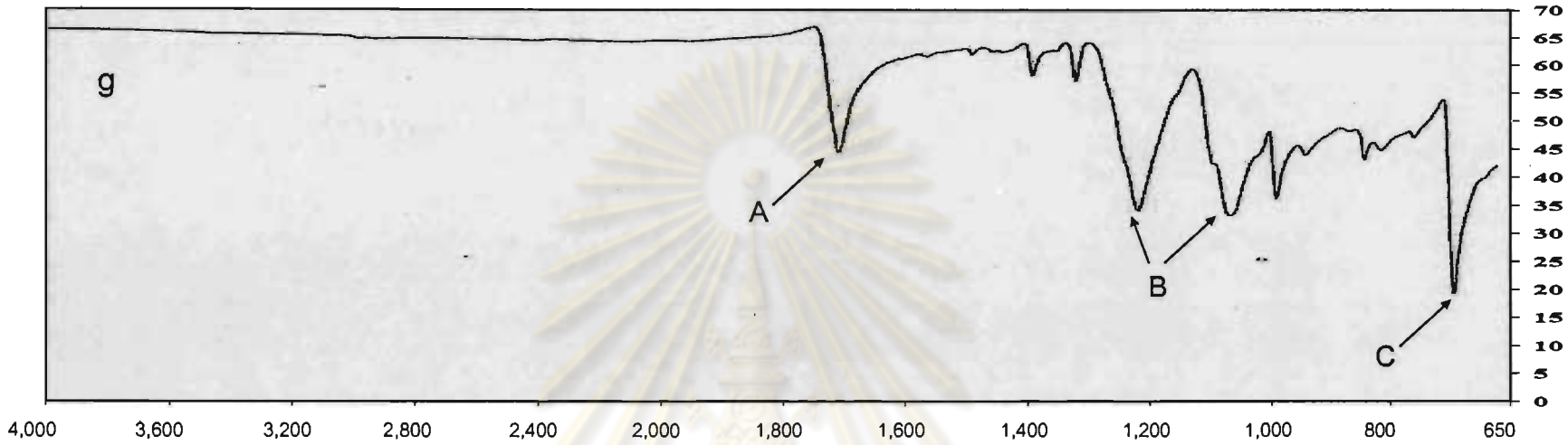

Figure 4.33 (cont.) ATR-FTIR spectra of 3.0 M NaOH (3 h) (g)

Interpretation:

A: C=O stretching of the ester carbonyl group

B: The ester C-O-C asymmetric and symmetric stretching vibrations

C: Out of plane bending of the two carbonyl substitutes on the aromatic ring

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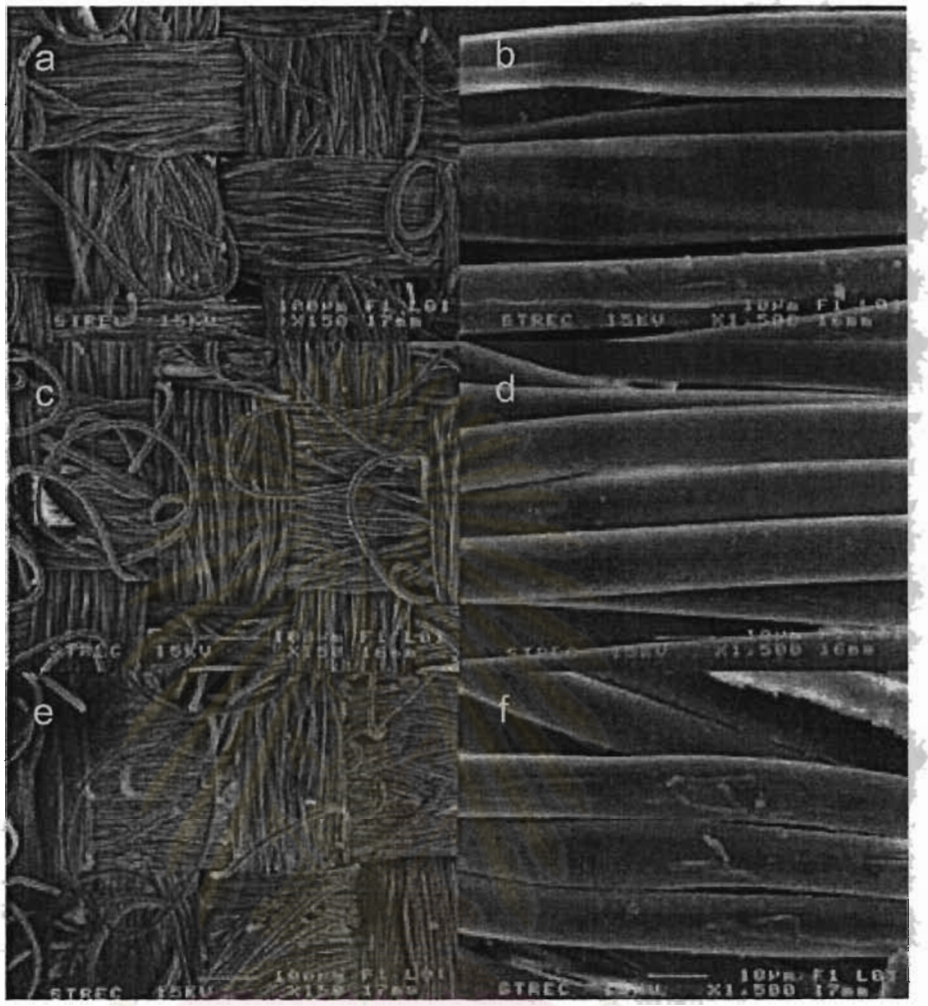


Figure 4.34 SEM photographs at x150 (left) and x1,500 (right) of untreated control (a and b), buffer (c and d) and 0.5 U/ml enzyme (48 h) (e and f).

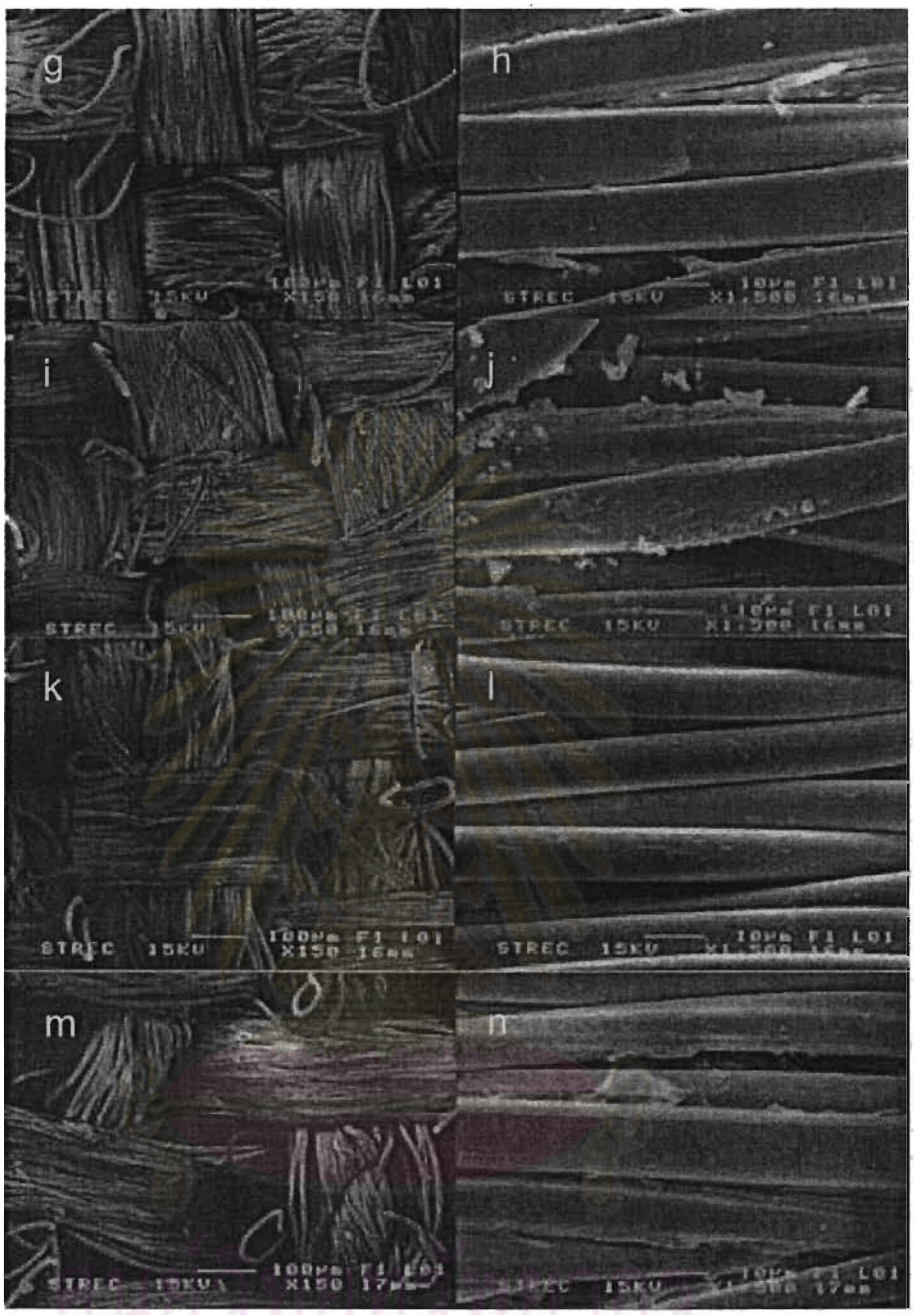


Figure 4.34 (cont.) SEM photographs at x150 (left) and x1,500 (right) of1.0 U/ml enzyme (48 h) (g and h), 2 U/ml enzyme (24 h) (i and j), 1.5 M NaOH (12 h) (k and l) and 3.0 M NaOH (3 h.) (m and n).

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Screening for PET-hydrolyzing enzymes

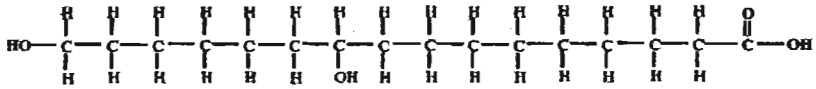
A manifold variety of plant surfaces and soil from a range of environmental niches yielded 22 fungi out of 115 isolates (Table 4.1) that were capable of degrading polycaprolactone (PCL), as indicated by a zone of clearing on MM-PCL agar plates (Figure 4.1). Such zones could be easily observed after 3-5 days of incubation. This diversity is not remarkable in that cutin is abundant and is widespread occurring on the surfaces of all aerial parts? of plants (stems, petioles, leaves, flower parts, fruits, and some seed coats, but also on internal parts such as juice sacks of citrus (Kolattukudy, 2001a). The total amounts are considerable. In higher plant leaves, cutin ranges from 20 to 600 $\mu g/cm^2$ of the surface area, while in some fruits with a well developed cuticle, the cutin content may reach 1.5 mg/cm^2 (Kollatukudy, 2001a). Thus with the ubiquity of cutin, it was not surprising that cutin degraders were generally isolated from different aerial parts of plants (fruit peel, leaf and bark) and also soil near by roots, and all of which produced cutinolytic esterase (Table 4.2). In analogous interpretation in that cutin is widely prevalent, it was not remarkable to find PET degrading fungi from low (0.5 m, Samutsakorn Province) and high (1,100 m, Chiangmai Province) altitudes, at ranges from 20.2 $^{\circ}C$ (Chaingmai) to 32.0 $^{\circ}C$ (Bangkok), and from seven provinces. The sampling collections included the rainy season (June, July, August and September) and the dry season (March). Noting that this is a limited survey, what was apparently remarkable was the dominance of *Fusarium* cultures, 16 of 22 isolates (Table 4.1), perhaps reflecting the common phyllosphere status of Fusaria and the selective isolation technique. A few of the more generally occurring *Aspergillus* and *Penicillium* species were isolated from soil and found to attack polycaprolactone (Table 4.1).

There was no obvious correlation between esterase activities produced by the fungi and sizes of clearing zones (Figure 4.1 and Table 4.2). However, the size of the clearing zone includes the amount of enzyme, its release, and also the rate of growth of the fungus. Such a correlation between enzyme yield and clearing zone size is further

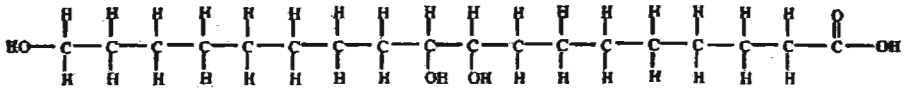
obscured in that some enzyme apparently remains mycelial bound (Table 4.5). Further enzyme was released following Triton X-100 treatment.

PCL has been shown to be degraded by enzymes secreted by a number of bacteria (Benedict, Cameron and Huang, 1983) and fungi (Benedict,1983; Cameron and Costa, 1987). Many phytopathogens secrete cutinase, a serine esterase that breaks ester bonds in cutin. Nishida and Tokiwa showed that some fungal pathogens degrade PCL and suggested an involvement of cutinase (Nishida and Tokiwa, 1994). Genetic, regulatory and enzymatic evidence showed that the PCL depolymerase from *Fusarium* that was required for utilization of PCL for growth is a cutinase (Murphy *et al.*,1996). Cutinase activity is induced and released in the supernatant of *F. solani* f. sp. *pisi* and other fungal phytopathogens grown in medium containing cutin as a carbon source (Köller and Parker,1989; Lin and Kolattukudy,1980). Murphy *et al.* (1996) showed that no depolymerase activity in supernatants of the cutinase-negative mutant of *Fusarium*, even when cutin or PCL was present in the growth medium. Stahl and Schäfer (1992) reported that the mutant grew very poorly in medium containing cutin as a sole source of carbon and lacked cutinolytic activity. Cutinase synthesis is induced naturally by a variety of C_{16} and C_{18} omega hydroxy fatty acids that are released from cutin by cutinase (Lin and Kolattukudy, 1978). One of these, 16-hydroxyhexadecanoic acid, induces PCL depolymerase in wild-type *Fusarium* strains. The products of PCL hydrolysis produced by several fungal PCL depolymerases are a mixture of monomers, dimers, and trimers (Murphy *et al.*,1996). PCL dimers and trimers are structurally similar to natural inducers of cutinase (Figure 5.1).

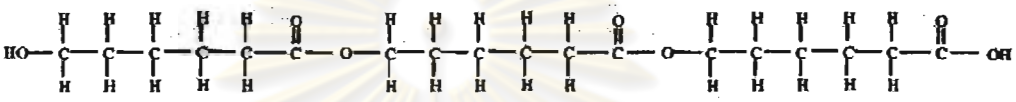
The use of polycaprolactone (PCL) as a selective isolation substrate merits comment. Cutinases secreted by many phytopathogenic fungi including *Fusarium solani* f. sp. *pisi* can have low substrate specificity. Certain cutinases hydrolyze cutin and are also capable of degrading PET (Lin and Kolattukudy, 1978; Vertommen *et al.*, 2005). Cutinase is induced by cutin or suberin (Murphy *et a*l., 1996), and can be repressed by glucose (Lin and Kolattukudy, 1987). On these general bases, PCL hydrolysis was used as an initial screen to find fungal isolates producing enzyme that may be active on aromatic synthetic polyesters such as PET.



10,16-dihydroxy C-16 acid



9,10,18-trihydroxy C-18 acid



Polycaprolactone trimer

Figure 5.1 Comparison of the chemical structures of two cutin monomers that are inducers of cutinase with the PCL trimer (Murphy *et al.*,1996).

All isolates readily grew in a mineral medium with potato suberin as the sole carbon source and produced esterases with maximum activities ranging from 11 (PBURU-B14) to 1,126 mU/ml (PBURU-B19) (Table 4.2). Suberin contains aliphatic domains composed of esterified hydroxy and epoxy fatty acids and the corresponding dicarboxylic acids similar to those found in cutin (Kolattukudy, 1980). Thus it would appear that microbial cutinase should be able to hydrolyze suberin and perhaps releases the suberin monomer as an inducer of cutinase. Degradation of suberin probably involves not only hydrolysis of the aliphatic components, but also degradation of the aromatic domains of the polymer (Fernando *et al.*, 1984). In the case of suberin-degrading enzymes, consideration should be include enzymes which degrade either or both of the aliphatic and aromatic components. Recently, hydrolytic enzymes acting on cutin and suberin have shown to have a potential application in modification of polyester synthetic fiber (Nimchua *et al.*, 2007; Silva *et al.*, 2005). The hydrolysis of polyethylene terephthalate (PET) by a fungal cutinase from *F. solani* f. sp. *pisi* and a bacterial hydrolase from *Thermobifida fusca* has been demonstrated (Müller et al., 2005;

Vertommen *et al.*, 2005). Such broad evidence showing that cutinase degraded suberin could hydrolyze the ester bond of PET because of their similarity of structure. In my study, it is noteworthy that only one fungus (PBURU-B5) was able to grow to any reasonable extent on PET fibers yielding activity (61 mU/ml) after 21 days growth (Table 4.2). Thus, this isolate was selected for further study.

5.2 Identification of the fungal isolate PBURU-B5

Examination by bright field light microscopy of the morphology of PBURU-B5 grown on Banana leaf agar (BLA) for 5 days showed typical Fusaria characteristics (Barnett and Hunter, 1999; Seifert, 1993). It produced both macro- and microconidia (Figure 4.2). Macroconidia are hyaline and canoe-shaped in side view. Microconidia were ellipsoidal, produced from long monophialides in aerial mycelium. Chlamydospore usually occurred singly or in pairs. The colony grew to 5 cm diameter in 4 days and was whitish (Figure 4.2). On morphological grounds it was *Fusarium solani*.

The sequence of the ITS region of rDNA, portion of the ERG gene and portion of the TEF gene of PBURU-B5 yielded sequences highly similar to *F. solani* sequences in Genbank, including the following: NRRL 22354 (DQ236829; AF178338; AF178402) and FRC#s1592 (DQ237259; DQ094746; DQ247446). This molecular biological analysis gave the corresponding identification with the morphological characteristics. On this basis isolate PBURU-B5 is identified as *Fusarium solani*. Deposit in a Culture Collection is under consideration.

5.3 Optimization of culture conditions for cutinolytic esterase production from PBURU-B5 and its growth

The optimal initial pH and incubation temperature for cutinolytic esterase production from PBURU-B5 were pH 11.0 (Figure 4.9) and 25 °C (Figure 4.10), respectively. The obtained pH was corresponded to the other cutinases. *T. fusca* cutinase exhibited a very basic pH optimum at 11.0 for cutinase production. Most of the fungal cutinases also have a basic pH optimum (pH 9.0) (Kolattukudy, 1985), although fungal cutinases with pH optimum below 7.0 have been reported (Koller and Parker, 1989; Trail and Koller,1993). The bacterial cutinase produced by *P. mendocina* ATCC

55613 has a pH optimum at 10.5 when tested against apple cutin (Sebastian and Kolattukudy, 1988).

Effect of incubation temperature affects cutinase production. *Fusarium oxysporum* produced the most esterase activity at 30 °C (Macedo and Fraga, 2007). Enzyme production of *Pseudomonas aeruginosa* was most rapid at 28 and 37°C for strains K799 and DAR41352, with highest levels achieved at 28°C for K799 and 22°C for DAR41352 (Fett *et al.*, 1992). The most cutinase production from thermophilic actinomycete *Thermomonospora fusca* was obtained from incubating the culture at 50 °C (Fett *et al.*, 1999).

pH 11.0 is most basic, and could cause the ester bond of suberin in production medium to be hydrolyzed (Zeronian and Collins, 1989). The fatty acid monomer was easily released from the suberin substrate by alkaline degradation compared to the other pHs lower than 11.0. The fatty acid obtained from hydrolysis reaction activated the cutinase producing gene (*cut1* gene). The continuous activation of these fatty acid monomers led to the secreting of a bulk of cutinase to the culture. The esterase was first detected on day 2, followed by a rapid increase until day 4 and was stable at this level to day 10. The pH dropped from 11.0 to 8.75 within 4 days, while glucosamine content indicated rapid growth until day 4, followed by a slow down (Figure 4.11).

5.4 Purification of cutinolytic esterase from PBURU-B5

A variety of techniques that permit rapid and effective protein purification are available. The principle properties of enzymes can be exploited in separation including size, charge, solubility and the possession of specific binding sites. Most purification protocols require more than one step to achieve the desired level of product purity. The use of sequential techniques causes some loss of product. Consequently, the key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required (Janson and Ryden, 1998)

The first step in the purification of a protein is the preparation of an extract containing the protein in a suitable form and extraction procedures should be selected according to the source of the protein. In this work, cutinolytic esterase, an extracellular

enzyme, was obtained from culture supernatant of *F. solani* PBURU-B5. The apparent yields released to the extracellular fluid appear only half of total esterase activity (Table 4.5) in part reflecting that the enzyme can bind to the cell and/or the residual suberin in the medium. Such bound enzyme was readily recovered by treatment with buffer containing Triton X-100 (Fernando *et al.*, 1984). The same results were obtained from other isolates. Cutinolytic esterase from *Melanocarpus albomyces* was also found to be bound to fungal mycelium about 50% of the total activity and 30% could be released by one extraction with 0.1% Triton-X100 (Kontkanen, Tenkanen and Reinikainen, 2006). The enzyme from *F. solani* f. sp. *pisi* bound more tightly to the mycelia and suberin and more than 90% of the esterase activity could be recovered by washing the residual suberin and mycelium using 0.4% Triton-X100 (Fernando *et al.*, 1984). The yield from PBURU-B5 of 0.27 U/ml is reasonable compared to other microbes. For instance out of 232 gram-positive and gram-negative bacterial isolates, only *Pseudomonas aeruginosa* (10 isolates) produced more than 0.5 U/ml esterase (Fett *et al.*, 1992). A *Botrytis cinerea* strain yielded 1.2 U/ml cutinase (Gindro and Pezet, 1999) and that from *Monilinia fructicola* showed 8.92 U/ml of esterase activity (Wang *et al.*, 2000). Other fungi give lower yields, for example *Venturia inaequalis* gave only 0.12 U/ml (Köller and Parker, 1989). Thus the esterase yields from culture extract of PBURU-B5 (0.27 U/ml) was average compared to the other isolates, the range being from 0.011 to 1.13 U/ml (Table 4.2).

Solubility differences in salt are frequently exploited to separate proteins in the early stages of purification protocols (Bollag and Edelstein, 1991). Ammonium sulfate is the salt of choice and was effective (Figure 4.6). In the 50-80% ammonium sulfate precipitation step, over 70% of other proteins were removed with about 90% enzyme recovering. (73% of total activity with 3.15-fold increase of specific activity) (Table 4.5).

Column chromatography is often useful for efficient protein purification. Ion exchange chromatography gives high resolution combined with large sample loading (Janson and Ryden, 1998). QAE Sepharose an anion exchanger has been used in the purification of several cutinases (Fernando *et al.*, 1984; Purdy and Kolattukuty, 1975b). Its popularity stems from the possibility of high resolving power, versatility, reproducibility and ease of performance. This column greatly contributed to the column

purification procedures, with less loss of esterase activity, while about 80% of other proteins were eliminated (Table 4.5), (Figure 4.12) also indicates that the net charge of the enzyme in working buffer, 50 mM Tris-HCl buffer, pH 8.5, was negative because the binding of the enzyme illustrates an isoelectric point (pI) less than pH 8.5. The pI of *F. solani* f. sp. *pisi* cutinase is 7.8 (Petersen *et al.*, 2001), while from *Monilinia fructicola* (Wint.) Honey its pI value is about 8.2 (Wang *et al.*, 2000), and a 40.8 kDa cutinase from *Botrytis cinerea* Pers.: Fr. is 4.2 (Gindro and Pezet, 1999).

Amongst the purification method for cutinolytic esterase, hydrophobic interaction chromatography (HIC) has been effective (Gindro and Pezet, 1999; Kontkanen *et al.*, 2006). HIC takes advantage of the hydrophobicity of proteins promoting their separation on the basis of hydrophobic nteraction between immobilized hydrophobic ligands on the chromatographic medium and on the non-polar regions on the surface of the proteins (Queiroz *et al.*, 2001). Hitrap Phenyl HP or Phenyl Sepharose has phenyl groups on the surface. The sample ionic strength is adjusted with salt to increase hydrophobicity of protein (Queiroz *et al.*, 2001). Thus, care must be taken regarding the concentration of the salt. It should be lower than the concentration used in precipitation of the enzyme. The salt concentration of 1.3 M was used for adsorption in this work (Figure 4.13). The elution of proteins is achieved by decreasing the salt concentration in order to increase their hydrophilicity. Ammonium sulfate was chosen as its effectively promotes hydrophobic interactions and is widely used (Feeserle *et al.*, 1995; Glavin *et al.*, 1994; Queiroz *et al.*, 2001). In my Hitrap Phenyl HP column, a decreasing gradient of ammonium sulfate from 1.3 M to 0 M. Other proteins were also eliminated (Table 4.5). The elution by 20mM sodium phosphate buffer pH 7.5 without ammonium sulfate suggests that this enzyme is a relatively strong hydrophobic protein.

Some purification guidebooks suggested that HIC is ideal for used immediately after salt precipitation where the ionic strength of the sample will enhance hydrophobic interaction and also for avoiding the desalting step (Janson and Ryden, 1998; Queiroz *et al.*, 2001). However, preliminary experiments of direct application of the enzyme (ammonium sulfate precipitation step) to the Hitrap Phenyl HP column resulted in removal of many proteins but were extremely slow. The purification sequence using ammonium sulfate precipitation followed by Hitrap Phenyl HP column and then Hitrap Q

FF column (QAE Sepharose) did not give acceptable yield of enzyme activity (5%) (Figure 4.5). Better yield can be obtained through further experimentation.

Gel filtration chromatography is useful for purification of cutinases (Gindro and Pezet, 1999; Kontkanen *et al.*, 2006; Sebastian and Kolattukudy, 1988). In gel filtration, proteins are fractionated mainly on the basis of their size. Protein molecules small enough to enter the beads have a longer path as they travel through the column matrix than do larger molecules. Large protein molecules excluded from the matrix thus emerge in the "flow-through" faction (Janson and Ryden, 1998). I used HiPrep 16/60 Sephacryl S-200 High Resolution Column. Sephacryl HR is a cross-linked copolymer of allyl dextran with N, N-methylenebisacrylamide. Gel filtration was effective (Table 4.5).

In conclusion, effective purification of *F. solani* PBURU-B5 was obtained by used of Triton X-100 extraction, ammonium sulfate precipitation, followed by QAE sepharose, Phenyl Sepharose and Sephacryl S-200 columns to yield an enzyme of high purity (Table 4.5).

There has not been a lot of cutinases purify so comparatively Purdy and Kolattukudy (1975) purified cutinase from *F. solani* f. sp. pisi to homogeneity by ammonium sulfate precipitation, Sephdex G-100, QAE Sephadex A-25, and SE-Sephadex. Efficient purification of cutinase from *Pseudomonas putida* was accomplished through acetone precipitation followed by use of DEAE cellulose, QAE Sephadex A-25, Sepharose 6B and Sephadex G-100 column. Here purification gave a 207-fold enrichment of the enzyme activity but, the overall yield of the purified enzyme was only 3% (Sebastian and Kolattukudy, 1988). Wang *et al.* (2000) successfully purified cutinase from *Monilinia fructicola* (Wint.) Honey, passing the crude enzyme to QAE Sepharose, S Sepharose and Phenyl Sepharose columns yielded a single cutinase band on SDS-PAGE. By the same purification methods cutinase was purified from *Colletotrichum kahawae* and *C. gloeosporioides*, the process involved ammonium sulfate fractionation, and two column-chromatographic steps, namely DEAE sephadex and SP Sephadex (Chen *et al.*, 2007). A symmetrical peak consisting solely of the 21 kDa cutinase protein was eluented from the final column and demonstrated a single cutinase band on SDS-PAGE.

5.5 Characterization of purified cutinolytic esterase from PBURU-B5

Optimum pH and temperature

Cutinase from *F. solani* PBURU-B5 showed the optimum pH of 9.0, corresponding with certain other cutinases (Sebastian and Kolattukudy, 1988). The cutinase from *F. solani* f. sp. pisi and *F. roseum* has the optimum pH at 10.0 (Soliday and Kolattukudy, 1976; Trail and Köller, 1992). In contrast enzyme activity can be at lower pH. Cutinase from *V. inaequalis* has an optimum at pH at 6.0 (Köller and Parker, 1989). The *Fusarium* cutin esterase thus fits between the ranges of 6.0 to 10.0 of other microbial cutinases. Tris-HCl buffer (pKa 7.8 – Figure 4.16, 4.18) was the appropriate buffer for the PBURU-B5 enzyme.

Cutinolytic esterase from *F. solani* PBURU-B5 was stable in a wide pH range of 6.0-10.0 (Figure 4.18) upon incubation at 4°C for 30 minutes. This was similar to alkalophilic *F. solani* f. sp. pisi cutinase (Trail and Köller, 1992) though different from that of *P. putida* (Sebastian and Kolattukudy, 1988) and *F. roseum* (Soliday and Kolattukudy, 1976) which were only stable in the pH ranges of 8.5-10.5 and 8.0-11.0, respectively. Clearly, the stability of the cutionolytic esterase in relation to pH is species specific.

The optimum temperature of cutinase from *F. solani* PBURU-B5 was 45 °C. For, other cutinases the range of optimum temperature was from 40-50 °C (Carvalho *et al.*, 1998). Specifically the optimum temperature for cutinases from *V. inaequalis* (Köller and Parker, 1989) and *F. solani* f. sp. pisi cutinase (Trail and Köller, 1992) were 40 °C and 50 °C, respectively. The distinctive optimum temperature of 80 °C was observed with *Streptomyces scabies* cutinase which is high for a soil borne potato pathogen (McQueen and Schottel, 1987). That of thermophilic *Thermomonospora fusca* is 80 °C (Müller *et al.*, 2005).

Size of enzyme

The molecular weight of the cutinolytic esterase PBURU-B5 was estimated to be 19 kDa by SDS-PAGE. Gel filtration gave the same molecular weight suggesting the protein to be a monomer. In similar vein, most cutinases are monomeric, ranging in molecular weight between 18-60 kDa (Soliday and Kolattukudy, 1976; McQueen and

Schottel, 1987; Sebastian and Kolattukudy, 1988; Köller and Parker, 1989; Trail and Köller, 1992; Gindro and Pezet, 1999; Wang *et al.*, 2000). However, the majority of the cutinases are all molecular weight range (18-20 kDa).

5.6 Effect of chemicals and Kinetics

When cutinolytic esterase from *F. solani* PBURU-B5 was tested for the effects of metal ions, EDTA and NaCl on enzyme activity, only Cu^{2+} caused inhibition (Table 4.7). The esterase activity was reduced 50% when the concentration of Cu^{2+} was presented (Table 4.7). The chemicals are sometime used as protectants and that it was significant that EDTA had no effect on the activity of this enzyme (Figure 4.21). As EDTA is an inhibitor of metallo-enzymes, thus the PBURU-B5 cutinolytic esterase is not a metallo-enzyme (Figure 4.21). Also NaCl showed no effect on activity of PBURU-B5 esterase (Figure 4.20) and advantage as several purification steps including ion exchange chromatography were performed at high concentration.

To determine the kinetic parameters of cutin esterase, p-NPB was used as substrate. Substrate binding affinity (K_m) was 0.53 (Figure 4.22). Table 5.1 summarizes kinetic parameter of the other purified cutinases. The K_m of our enzyme was similar to that of *F. solani* f. sp. *pisi* (Trail and Köller, 1992). It was lower than that of *S. scabies* (McQueen and Schottel, 1987) and *M. fructicola* (Wang *et al.*, 2000).

Table 5.1 Comparsion of K_m of purified cutinase

Microbial strains	K_m (mM)	References
Streptomyces scabies	125	McQueen and Schottel, 1987
Fusarium solani f. sp. *pisi*	0.47	Trail and Köller, 1992
Monilinia fructicola	1.23	Wang *et al.*, 2000
Pseudomonas putida	0.27	Sebastian and Kolattukudy, 1988

5.7 Applications of the enzyme on PET surface modification

The enzyme preparation from *F. solani* PBURU-B5 cultivated in media containing potato suberin was active towards PET fiber as indicated in UV (Figure 4.25) and fluorescence (Figure 4.26) assays. Terephthalate and its esters released from PET fiber have characteristic strong absorbance peaks around 240 nm. The UV assay with increases the absorbance at this wavelength was found to correlate with the increase enzyme concentration (Figure 4.25), indicating the release of terephthalate and its esters.

I obtained similar results in the fluorescence assay. Enzyme action cleaves ester bonds (hydroxyl and carboxylic acids at the fiber surface) and releases terephthalic acid and ethylene glycol into solution. The terephthalic acid yields hydroxy-terephthalic after reaction with peroxide under boiling conditions. As the hydroxy-terephthalic acid has a sharp emission peak at 425 nm using an excitation at 315 nm, it can be determined by fluorescence. This method has been adapted from quantification of radical formation by ultrasound using a similar procedure. In alkaline solution, terephthalic acid produces terephthalate anions which react with hydroxy radicals ($HO^{\bullet}$) from hydrogen peroxide to produce highly fluorescent hydroxy-terephtalate ions (HTA) (Mason *et al.*, 1994). The formation of terephthalic acid was detected after cutinase treatment of PET fibers, confirming the enzymatic hydrolysis of PET fibers occurs with the enzyme (Figure 4.26). Terephthalic acid formed by enzymatic hydrolysis increased in relation to incubation time, and also concentration of the enzyme. The intensity of the sharp peak at 425 nm increased even with long enzyme exposure (168 hours) (Figure 4.26).

Kellis *et al.* (2001) and Yoon *et al.*, (2002) used absorbance 240 nm to show the effective action of the enzyme towards PET yarn. A cutinase from *P. mendocina* was shown to display a high activity in this same assay (Poulose *et al.*, 2001). The enzyme preparations from the actinomycetes *Thermomonospora fusca* KW3b and also isolates M5, M9 have also released aromatic compound from PET. Monitored attack of the PET yarn by monitoring the increase of absorpbance from PET yarn is generally useful (Alisch *et al.*, 2004).

PET fabric characteristics include hydrophobicity and low reactivity with most chemical agents as described previously (Alisch *et al.*, 2006). The low hydrophobicity makes the fibers less suitable to be in contact with the human skin while the low reactivity causes the fiber to be unsuitable as a carrier for chemical finishing agents. The conventional industrially approach to render polyester hydrophilic is an alkali treatment, thus hydrolyzing the polyester bonds (Zeronian and Collins, 1989). Although hydrophilicity is achieved, the favorable bulk properties of polyester, particularly the strength, are also decreased. Furthermore, the high amount of sodium hydroxide (30%) and the high operating temperatures (130 ^{o}C) necessary are a large disadvantage. The process is not environmentally benign. The use of hydrolytic enzymes for the modification of the fabric becomes an alternative since the activity of the enzymes is limited to the fabric surfaces, and the textile treatment can be performed under mild and compliant conditions. In my study, I compared the effect of alkali and enzymatic treatments to PET fabrics including the hydrophilicity, dyeing property, surface chemical structure and surface morphology after each treatment was applied to the fabrics.

The wettability, moisture content and water absorption of enzyme treated PET fabrics and alkali treatment were increased compared those of untreated control, buffer treated and inactivated enzyme treated fabrics respectively (Figure 4.27, 4.28 and 4.29). The enzyme or alkali was degraded ester bond of PET fabric resulted in presence of many carboxyl and hydroxyl groups. These chemical groups increased the hydrophilicity of fabrics. The wettability of the enzyme treated PET fabrics was nearly equivalent to that of the alkali treatment. However, it took longer than the chemical treatment. The untreated control did not absorb measurable amounts of water (Figure 4.27).

The relative percentage moisture and water absorption increases were in proportion to the amount of enzyme applied. The enzyme treatment clearly decreases the hydrophobicity of the PET fabrics (Figures 4.28, 4.29). The alkali treated fabrics similarly showed increase of hydrophilicity since the higher moisture content and water absorption were obtained (Figures 4.28, 4.29). The increased water and moisture absorption by treated PET fabrics are analogous to those obtained with microbial

enzymes from *Aspergillus* sp. strain St 5 (Fischer *et al.*, 2004) and *Thermomonospora fusca (*Alisch *et al.*, 2006).

The main underline reason in enhancing the hydrophilicity of fabrics is an increase the amount of carboxyl and hydroxyl groups caused by hydrolysis of ester bond by enzyme of alkali treatment. Hydroxyl groups are more polar, thus greater wettability, and moisture and water absorption result.

The dyeability of PET fabrics after enzyme or alkaline treatments was evaluated in this study (Figures 4.30, 4.31). After treatment with enzyme or alkali, PET fabrics could be dyed with either disperse or basic dye. Fixation of disperse dyes to fibers depends on their particle size and uniformity and on the nature of the dye dispersing agent. Although disperse dyes were initially developed in the 1920's for dyeing cellulose acetate and cellulose triacetate fibers, these dyes are now primarily used for dyeing of synthetic polyester and polyester fiber blends (Cooke, 1983). The disperse dyes are held in the fiber, the larger the dye molecule, the better the alignment along the PET fiber chain and the stronger the dye/ fiber interaction. The enzyme treated PET fabric gave greater color intensity (K/S value) over incubation time while increasing of color intensity was detected from the beginning of incubation time, followed by rapidly dropped.

Basic dyes are water-soluble and are cationic. These dyes are applied primarily to acrylics and occasionally to polyester and polyamide fibers. Although basic dyes gave brilliant colors on wool, silk and cellulosic fibers, they show poor fastness. Thus, they fell into disuse for these fabrics (Vigo, Bruno and Goynes, 1991). My enzyme hydrolyzes ester bonds on the surface of PET fabrics to yield carboxylic acid groups. The generation of carboxylic acid groups was also illustrated by an increased dyeability with basic dye at the PET surface after enzyme treatment (Figure 4.31). The same result was obtained from alkali treated PET fabric. The untreated control, buffer and inactivated enzyme treated PET fabrics showed no significant changes in color intensity (K/S value). Thus, the increasing of K/S value in PET treated fabric was caused by enzymatic treatment. The results from both dye methods strongly supported the concept leading to the loss of the dyeability with disperse dye.

Surface modification of PET fabrics treated with 3.0 and 1.5 M NaOH showed dramatically decreased in tensile strength compared with samples treated with enzyme, buffer and untreated controls (Figure 4.32). This reaction leads to a more hydrophilic fiber. It reduces the quality of the fibers such as tensile property and causes a loss of fiber material in the process. PET is hydrolyzed under strong alkaline conditions yielding a massive weight-loss and a highly hydrophilic fiber. The alkali hydrolysis is an "all-or-nothing" mechanism providing more than surface changes (Shukla et al., 1997). Thus, though the alkali method could be considerably improved. In contrast, since enzymes are large molecules, diffusion inside the fibers will not happen, and only superficial formation of hydroxyl and carboxylic groups occurred. The enzyme method could be the approach to the future.

The ATR-FTIR studied of untreated and enzyme or alkaline treated PET fabrics were performed to assess any structural changes, the introduction of new functional groups, or the alteration of existing groups on the surface of fabrics. The result of ATR-FTIR yielded the similar results for all treatments including enzyme and alkali and also untreated control (Figure 4.33). The carboxylic acid groups caused by both enzyme and alkaline hydrolysis had the same intensity of the peak at 1,710-1713 cm^{-1} corresponding to C=O stretching of the ester carbonyl group including ester bond. Thus, in the control sample there was a peak at that range of wavelength and a bulk of ester bonds remained on the fabric. The total of the remaining ester bonds and the carboxylic groups that resulted from hydrolysis have yielded similar amount. But no changes were detected from this study, even on the surface of 3 M NaOH treated PET fabrics. ATR-FTIR did not appropriate useful in the particular treatment.

The original surface of untreated PET fabric was smooth as well as buffer treated PET fabric. When different concentrations of enzyme applied, the breaking of fiber was observed as seen in Figure 4.34 e, f, g, h, i and j. Alkaline treatment was found to be attack the polyester and caused surface pitting on PET fibers. This is the main cause of weight loss after treatment of polyester fabric with NaOH. The changes in the surface morphology of the fabrics observed after the enzyme or alkaline treatment are explained by the degradation of polymer by enzyme or alkaline treatment.

Aromatic polyesters have previously been considered as very resistant to degradation by hydrolytic enzymes (Müller, Kleeberg and Deckwer, 2001). However, the current results add to the accumulating evidence that esterases and cutinases from various fungi and bacteria can hydrolyze ester bonds in PET (Müller *et al.*, 2005; Nimchua *et al.*, 2007; Yoon *et al.*, 2002). The screening using polycaprolactone was useful in selecting a variety of fungi producing esterases. However, in that only one strain produced an enzyme with major activity towards PET fiber, a caution is raised in using this substrate in an absolute manner. That does not detract from the isolation of a truly positive strain of *F. solani*. The overall results support the concept of the enzymatic modification of aromatic synthetic polyesters and potential use in textile finishing processes.

Summary statement

This study illustrated the selective isolation of a cutinolytic esterase. In the selection this type of esterase was predominantly occurred in twenty two of one hundred and fifteen fungal samples. Remarkably, sixteen of twenty two occurred in *Fusarium* spp. The best esterase that also attacked PET yarn was analyzed, a purification protocol developed and the enzyme characterized when applied to PET fabric increased wettability and greater susceptibility dye resulted yet without loss of tensile strength. When applied to the shiny crisp PET fabric, a softening of surface texture was gain with result enhance wearing properties. The fabric must soft to touch.

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APPENDICES

Appendix A

Culture media

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	500	mg
Agar	15	g

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

3. Suberin powder

Suberin was isolated from potatoes as described by Walton and Kolattukudy (1972). Peels from potatoes (*Solanum tuberosum* Linn.) were boiled in oxalate buffer (4 g of oxalic acid and 16 g of ammonium oxalate per liter) for 2 to 4 h. Suberin layer was collected by filtration through cheesecloth, washed several times with distilled water, dried, and ground in a mill. Powdered suberin was extracted with chloroform-methanol (2:1, V/V) overnight with mild stirring. A second extraction was done with chloroform in a Soxhlet apparatus for 24 h. The suberin was dried at 80 °C and then washed several times with distilled water. Pectin and cellulose were removed by treatment with *Rhizopus* sp. pectinase (1

g/liter) and *Aspergillus niger* cellulase (5 g/liter; Sigma Chemical Co., St. Louis, Mo.) in pH 4.0 acetate buffer (50 mM) at 22 °C for 48 h. Suberin was recovered by filtration, washed with distilled water, and dried at 60 °C.

4. **Banana leaf agar**

Dry banana leaves (1-2 cm squares)	4	pieces
Corn meal	76	g
Agar	15	g

Added distilled water to 1000 ml and then autoclaved. Autoclaved banana leaves were then placed on the surface of corn meal agar.

5. **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.

6. **Potato dextrose broth (PDB)**

Glucose	20	g
Infusion from potato	1,000	ml

7. **Production medium**

Suberin	2	g/l
Peptone	5	g/l

Dissolved in LMM 1,000 ml

Appendix B

Reagents

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

1. **Tris-HCl buffer (50 mM, pH 7.5)**

TrisBase	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.

2. **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
ddH_2O to	100	ml

Dissolved by gentle heating

3. **TE buffer, pH 8.0**

1 M Tris-HCl, pH 8.0	1,000	µl
0.5 M EDTA (di-sodium), pH 8.0	200	µl
ddH_2O to	100	ml

B. Glucosamine assay

1. **Phenolphthalein solution, 0.5%**

Phenolphthalein	0.5	g
Ethyl alcohol	60	ml
Distilled water to	100	ml

2. **Potassium hydrogen sulfate, 1%**

Potassium hydrogen sulphate	1	g
Distilled water to	100	ml

3. **Acetylacetone reagent**

Acetylacetone	1	ml
0.5 N sodium carbonate solution	50	ml

4. **Erhlich's reagent**

p-dimethylamino benzaldehyde	2.67	g
Ethanol	50	ml
Concentrated hydrochloric acid	50	ml

C. **Protein purification**

1. **Bradford's reagent**

Coomassie blue brilliant blue G-250	100	mg
95% Ethanol	50	ml
85% Phospharic acid	100	ml
Distilled water to	1,000	ml

2. **50 mM Tris-HCl, pH 8.5**

TrisBase	6.1	g
Distilled water	800	ml

Set pH to 8.5 with conc. HCl and adjusted the volume to 1,000 ml by distilled water.

3. **50 mM Tris-HCl, pH 8.5 containing 1 M NaCl**

TrisBase	6.1	g
NaCl	58	g
Distilled water	800	ml

Set pH to 8.5 with conc. HCl and adjusted the volume to 1,000 ml by distilled water

4. **50 mM Tris-HCl, pH 8.5 containing 0.15 M NaCl**

TrisBase	6.1	g
NaCl	8.8	g
Distilled water	800	ml

Set pH to 8.5 with conc. HCl and adjusted the volume to 1,000 ml by distilled water

5. **20 mM sodium phosphate buffer (pH 7.5)**

$NaH_2PO_4.H_20$	0.8	g
Na_2HPO_4	1.9	g
Distilled water	800	ml

Adjusted pH to 7.5 ml by NaOH or HCl and then set volume to 1,000 ml by distilled water

6. **20 mM sodium phosphate buffer (pH 7.5) containing 1.3 M $(NH_4)_2SO_4$**

$NaH_2PO_4.H_20$	0.8	g
Na_2HPO_4	1.9	g
$(NH_4)_2SO_4$	172	g
Distilled water	800	ml

Adjusted pH to 7.5 ml by NaOH or HCl and then set volume to 1,000 ml by distilled water

D. SDS-PAGE

1. **Stock reagents**

30% Acrylamide mix, 100 ml

Acrylamide	29.2	g
N,N'-methylene-bis-acrylamide	0.8	g

Adjusted volume to 100 ml with distilled water.

2. 1.5 M Tris-HCl pH 8.8, 100 ml

TrisBase 18.17 g

Adjusted pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water

3. 1.0 M Tris-HCl pH 6.8, 100 ml

TrisBase 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water

4. 0.5 M Tris-HCl pH 6.8, 100 ml

Tris base 6.06 g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water

5. 5X Electrophoresis buffer, 1 litre

TrisBase 15.1 g

Glycine 71.3 g

SDS 2.5 g

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

6. 10% Ammonium persulfate, 1 ml

Ammonium persulphate 0.1 g

Dissolve in distilled water to 1 ml.

7. 10% SDS, 50 ml

SDS 5 g

Dissolve in distilled water to 50 ml.

8. 5X sample buffer

1 M Tris-HCl pH 6.8	0.6	ml
50% Glycerol	5.0	ml
10% SDS	2.0	ml
2-Mercaptoethanol	0.5	ml
1% Bromophenol blue	1.0	ml
Distilled water	0.9	ml

One part of sample buffer was added to four parts of sample. The mixture was heated 5 min. in boiling water before loading to the gel.

9. 12% Separating gel (for 10 ml)

Distilled water	3.3	ml
30% Acrylamide mix	4.0	ml
1.5 M Tris-HCl, pH 8.8	2.5	ml
10% SDS	0.1	ml
10% Ammonium persulfate	0.1	ml
TEMED	0.004	ml

10. 5% Stacking gel (for 3 ml)

Distilled water	2.1	ml
30% Acrylamide mix	0.5	ml
0.5 M Tris-HCl, pH 6.8	0.38	ml
10% SDS	0.03	ml
10% Ammonium persulfate	0.03	ml
TEMED	0.004	ml

E. Silver staining

1. Fixing solution

50% MeOH	500	ml
12% Acetic acid	120	ml
37% H_2CO	0.1	ml
Distilled water to	200	mL

2. Thiosulfate solutions, 0.02%

$Na_2S_2O_3$	50	mg
ddH_2O	250	ml

3. $AgNO_3$ solution, 0.2%

$AgNO_3$	0.4	g
ddH_2O	200	ml

4. Na_2CO_3, 6%

Na_2CO_3	30	g
ddH_2O	500	ml

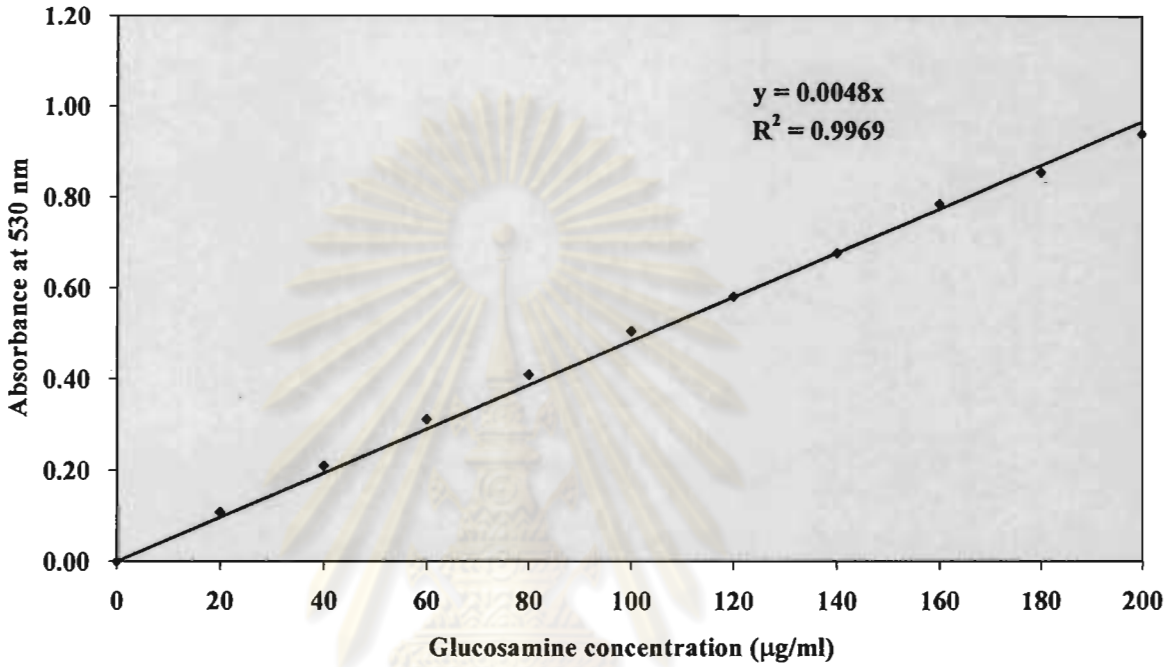
5. Developing solution

6% Na_2CO_3	196	ml
37% formaldehyde	0.1	ml
0.02% $Na_2S_2O_3$	4	ml

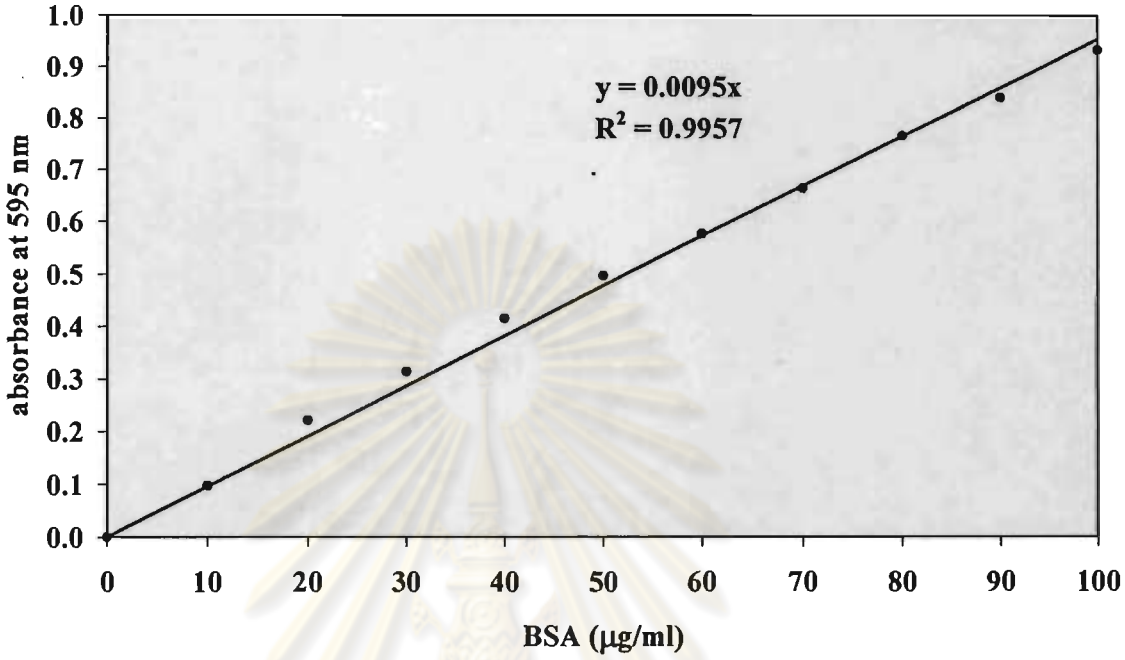
Appendix C

Standard curve

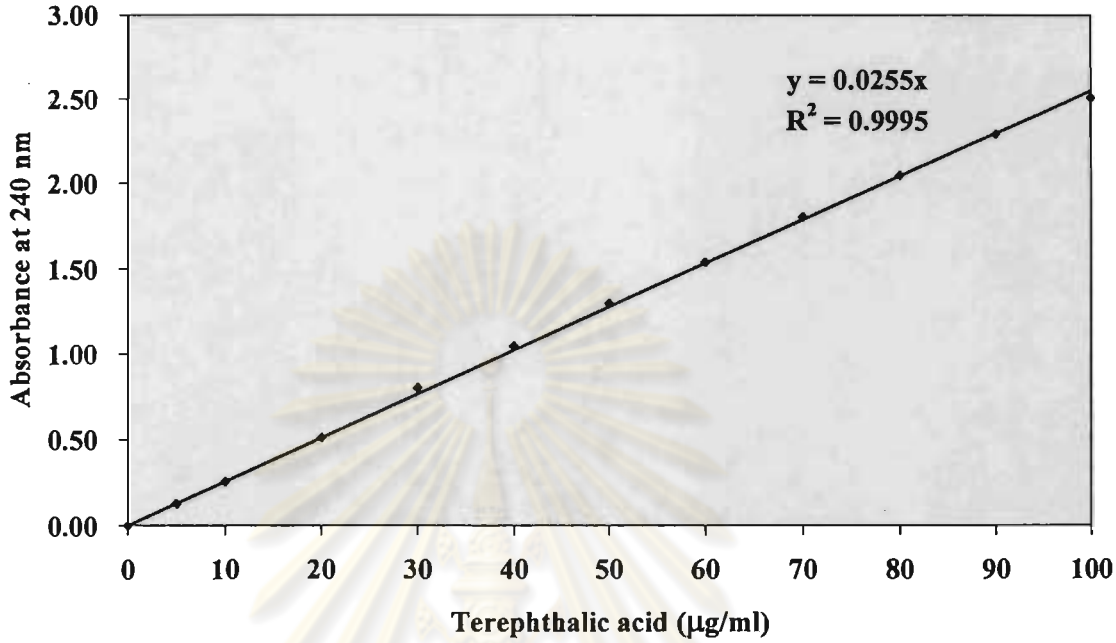
A. Stardard curve for glucosamine determination



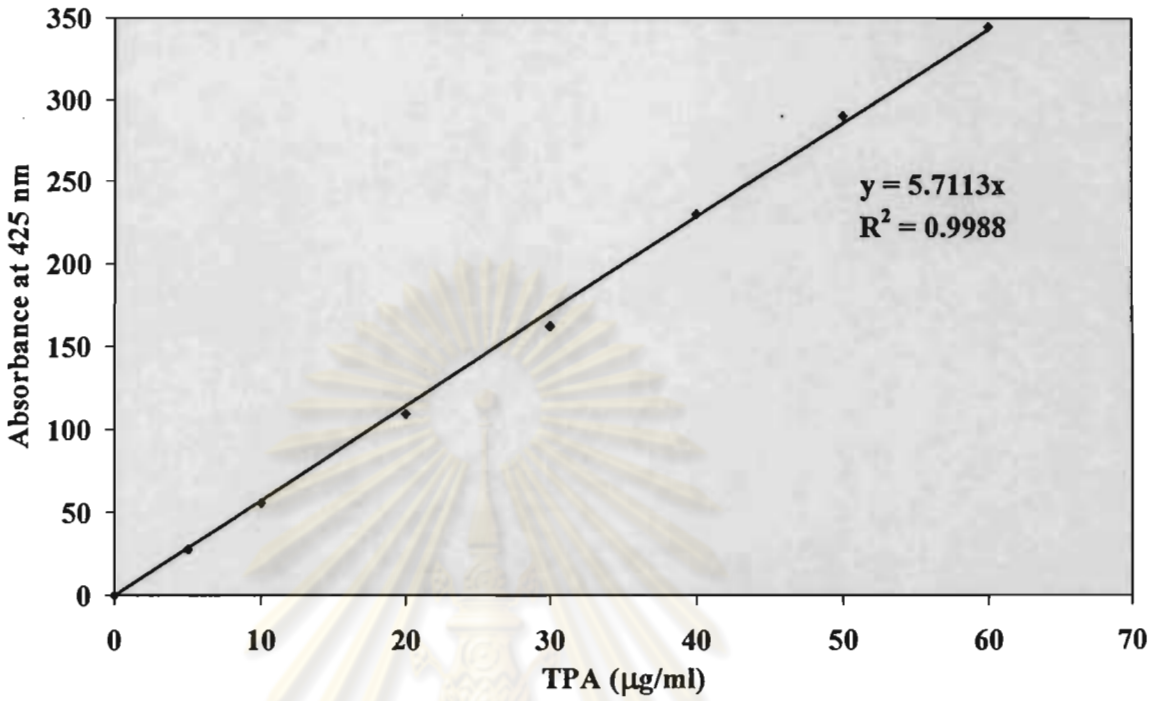
B. Stardard curve for protein determination by Bradford's assay



C. Stardard curve for terephthalic acid (TPA) determination by UV assay



D. Stardard curve for terephthalic acid (TPA) determination by fluorescence assay



Appendix D

1. Esterase activity calculation

A solution of 10 mM p-nitrophenyl butyrate (p-NPB) dissolved in absolute ethanol was used as substrate. The reaction mixture consisted of 50mM Tris-HCl buffer (pH 7.0), enzyme solution and p-NPB solution. One-cm quartz cuvette was used for the measurements on a spectrophotometer at 25°C. The wavelength was 405 nm and a time scan over 60 seconds where $\Delta A/\Delta t$ was calculated. Three measurements were made on each protein and it was crucial that $\Delta A/\Delta t$ was almost equal. The reference only consisted of buffer and p-NPB. The measurement was made after the addition of p-NPB to start it up. The enzyme volume added depends on the ratio between the reference (the spontaneous reaction) and the enzyme measurement. The enzyme activities were calculated using the Lambert-Beer law.

$A = \varepsilon \times C \times d$

Remark: A = the being measured absorbance at 405 nm

ε = Extinction coefficient at pH 7.0 of p-Nitrophenol ($7{,}461\ mM^{-1}cm^{-1}$)

C = the concentration of reaction (mM)

d = the diameter of cuvette (1 cm)

An enzyme unit (1 U) was defined as the amount of enzyme required to release 1 μmol of p-nitrophenol per minute at pH 7.0 and 25 °C.

2. **Calculation of ammonium sulfate solids to reach % satuaration to add to an enzyme solution**

Amount of ammonium sulfate (solid) to be added to an enzyme preparation was calculated from the following equation.

For 1 liter of enzyme solution:

$$g = \frac{533\ (S_2 - S_1)}{100 - 0.3S_2}$$

Remark: g = the amount of ammonium sulfate to add in grams

S_1= the original saturation percentage of salt

S_2= the desired saturation percentage of salt

VITA

Miss Thidarat Nimchua was born on June 21th, 1980 in Lampang province, Thailand. She graduated from Lampang Kanlayani School in March 1999 and received the Bachelor of Science degree with a major in Genetics from Chulalongkorn University in April 2003. After that, she continued to study for the Degree of Philosophy of Science in Biological Sciences Program, at the Faculty of Science, Chulalongkorn University in June 2003.