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DECOMPOSITION OF COTTON AND POLYESTER FABRICS IN SOIL AND RELATED FUNGI

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การศึกษาการย่อยสลายผ้าฝ้าย สามารถนำไปใช้ประโยชน์ในเชิงนิติวิทยาศาสตร์ได้ โดยการดูอัตราการย่อยสลายของผ้า ทำให้สามารถระบุช่วงเวลาของการเสียชีวิตได้ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาอัตราการย่อยสลายของผ้าฝ้ายและผ้าใยสังเคราะห์ในดินบริเวณป่าเขตร้อน และเพื่อเปรียบเทียบกับงานวิจัยที่เคยทำการศึกษาในดินเขตอบอุ่นบริเวณนอร์ทเวลส์ สหราชอาณาจักรด้วย การศึกษาอัตราการย่อยสลายทำโดยนำตัวอย่าง 3 ชนิด คือกางเกงในผ้าฝ้าย, ถุงผ้าฝ้าย 100% และถุงผ้าใยสังเคราะห์ แช่ในสารละลาย 5% ยูเรีย ที่ผสมกับ 2% กลูโคส เพื่อเลียนแบบของเหลวจากศพ จากนั้นนำตัวอย่างทั้ง 3 ชนิด ฝังลงในดินในช่วงฤดูร้อนและฤดูฝนของประเทศไทย ตรวจสอบอัตราการย่อยสลายของผ้าฝ้ายและผ้าใยสังเคราะห์ทุกๆ 2 สัปดาห์ เป็นระยะเวลา 10 สัปดาห์ด้วยตาเปล่าและกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (SEM) จากผลการศึกษาพบว่าเมื่อเวลาผ่านไปมีเชื้อราเจริญบนกางเกงในผ้าฝ้ายและถุงผ้าฝ้าย และอัตราการย่อยสลายของผ้ามีความแตกต่างกันในฤดูร้อนและฤดูฝน โดยในฤดูฝนใช้ระยะเวลาประมาณ 6 สัปดาห์ ส่วนฤดูร้อนใช้ระยะเวลาประมาณ 10 สัปดาห์ในการย่อยสลายผ้าฝ้ายทั้งหมด เมื่อนำผลการทดลองที่ได้ไปเปรียบเทียบกับผลการทดลองที่ทำในสหราชอาณาจักรพบว่าอัตราการย่อยสลายของผ้าฝ้ายในดินบริเวณป่าเขตร้อนของประเทศไทยสามารถเกิดได้เร็วกว่า 2-3 เท่า ทั้งนี้ขึ้นอยู่กับฤดูกาลด้วย นอกจากศึกษาอัตราการย่อยสลายแล้วงานวิจัยนี้ยังได้ทำการแยกราที่เจริญบนผ้าเพื่อนำมาทดสอบความสามารถในการในการสร้างเอนไซม์เซลลูเลส และมีการตรวจสอบติดตามกลุ่มประชากรราที่เจริญบนผ้าด้วยเทคนิค Denaturing Gradient Gel Electrophoresis (DGGE) อีกด้วย จากผลการทดสอบความสามารถในการสร้างเอนไซม์เซลลูเลสพบว่า *Trichoderma koningiopsis* เป็นราที่สามารถผลิตเซลลูเลสได้สูงสุด รองลงมาคือ ราในกลุ่ม *Penicillium* sp. อีก 2 ชนิด นอกจากนี้ผลจากการใช้เทคนิค DGGE ในการตรวจสอบกลุ่มประชากรรายังช่วยสนับสนุนว่า *Trichoderma koningiopsis* เป็นเชื้อราที่มีความสำคัญในการย่อยสลายผ้าฝ้ายอีกด้วย จากงานวิจัยนี้จะเห็นได้ว่าการศึกษาการย่อยสลายผ้าฝ้ายสามารถนำไปใช้ประโยชน์ในเชิงนิติวิทยาศาสตร์ได้ แต่ต้องมีการตรวจสอบคุณสมบัติของดิน สภาพแวดล้อมและระยะเวลาที่ทำการฝังด้วย เพื่อความน่าเชื่อถือในการนำไปใช้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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SASIKARN KOMKLEOW: DECOMPOSITION OF COTTON AND POLYESTER FABRICS IN SOIL AND RELATED FUNGI. ADVISOR: ASSOC. PROF. PRAKITSIN SIHANONTH, Ph.D., CO-ADVISOR: ASST. PROF. APHICHART KARNCHANATAT, Ph.D., 93 pp.

Previous studies have shown forensic mycology to be a potential tool from which post-burial interval can be determined. This work aimed to investigate decomposition of cotton and polyester materials in a tropical forest soil and to compare the obtained data with those obtained from a similar study in a temperate (North Wales, UK) soil. These experiments were carried out both during the dry season and rainy season in Thailand. The study utilized the soil burial method to determine the biodegradation rates of cotton briefs, 100% cotton bags and 100% polyester bags. Each textile sample was pre-soaked with a solution comprised of 5% urea and 2% glucose to simulate leakage of body fluid from a buried corpse. The weight loss, electron microscopy and visual examination revealed that cotton fabric samples (briefs and bags) are colonized by fungi and completely degraded within 6 weeks in the rainy season and 10 weeks in the dry season. When compared to the investigation in the UK, decomposition of cotton fabric in the Thai tropical soil took place at 2-3 times the rate of similar decomposition in a temperate soil in the UK according to season. In the enzyme assays for a selection of the fungi isolated from the cotton samples it was found that *Trichoderma koningiopsis* produced the most endogluconase, following by 2 *Penicillium* species. Moreover, the Denaturing Gradient Gel Electrophoresis (DGGE) confirmed that *Trichoderma koningiopsis* is the dominant fungus in both cotton samples. It is suggested that decomposition of clothing has potential in forensic investigations but reliability will require careful examination of the soil properties and the environmental conditions at the place and time of burial.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
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%	percentage
°C	degree Celsius
µg	microgram
µl	microlitre
µM	micromolar
µmol	micromole
bp	base pair
cm	centimeter
g	gram
kg	kilogram
kV	kilovolt
l	litre
M	molar
min	minute
ml	millilitre
mm	millimeter
ng	nanogram
nm	nanometer
psi	pound per square inch
rpm	revolutions per minute
s	second
U	unit activity
V	volt
w/v	weight by volume

CHAPTER 1
INTRODUCTION

Soil is a natural body of mineral and organic constituents differentiated into horizons of variable depth, which differs from the material below in morphology, physical make up, chemical properties and composition, and biological characteristics (1). Soil is fundamental for all life processes and its fertility governs the type and health of its associated vegetation. Apart from physical and chemical properties it is the soil population, mainly microorganisms, and nutrient sources of which organic materials are the most important which maintain a healthy nutrient status for plant growth. Although soil is populated by many organisms, including animals and microorganisms, but it is generally considered that it is the microorganisms which play the most important part in the release of minerals and carbon dioxide for plant growth. Thus it is the biological activities which ensure the level of nutrients in the soil is sufficient to maintain plant growth and continuation of plant populations (2). Most studies over the past thirty to forty years indicate that the soil microorganisms exist for most of their lives in an environment poor in nutrients or are oligotrophic (2). The fate of any introduced organic materials is therefore of fundamental importance in understanding the soil ecosystem and in most soils the organic material only accounts for 1-3% of the total weight.

Although live plants, animals and microorganisms provide nutrients for parasitic and symbiotic relationships it is dead organic matter which is the most widely distributed source of energy in the soil environment. Therefore its origin and subsequent decomposition is of fundamental importance to the soil. Dead organic matter in soil either originates from plants or animals although input from microorganisms themselves can sometimes be significant. Most of the dead plant material occurring in soil is in the form of leaves, twigs, fallen branches or shed bark. Cellulose is the most common component of the plant

material entering the soil and there have been many studies concerning its colonization, succession of microorganisms and its eventual decomposition (2). Cellulose is a polysaccharide composed of molecules of β-glucose linked by 1,4-β-glycosidic bonds. The number of glucose molecules in a chain ranges from 7 – 10 thousand. Chains may be arranged in a crystalline structure or an amorphous structure. These linear chains occasionally crosslink to from microfibrils, and may be used to produce textiles material. Native cellulose is highly crystalline with some amorphous sites; however, when processed the proportion of these amorphous sites increases (3). Increase in the proportion of these sites increases the susceptibility of cellulose to microbial attack.

The current investigations were therefore undertaken to examine decomposition of cotton and polyester materials in a tropical forest soil and to compare the data with those obtained from a similar study in a temperate (North Wales) soil (4). Particular attention was made to the fungi involved since under aerobic conditions in soil they are considered to be the major cellulose decomposers (5). In consideration of forensic aspects ladies briefs, either 100% cotton or 100% polyester, were examined following periods of burial in soil as being likely to be part of any forensic investigation of a deposed female corpse. The rate of breakdown of the clothing was evaluated for its potential in the forensic determination of the period of burial of a corpse and of clothing which may not be on a body.

CHAPTER 2

LITERATURE REVIEWS

2.1 The soil environment

Soil is a natural body of mineral and organic constituents differentiated into horizons of variable depth, which differs from the material below in morphology, physical make up, chemical properties and composition, and biological characteristics (1). The soil environment is a varying and complex mixture of both organic and inorganic compounds. It consists of three phases, all of which influence microbial activity; solid, liquid and gas, dispersed to form a heterogeneous matrix. The solid phase is comprised of organic matter and inorganic/mineral matter derived from the weathering of parent rock, resulting in varying particle size. It is the relative proportions of these different sized particles which determines the soil texture and subsequently the structure of the soil (6). Within this framework of particles exists the liquid and gas phases, also called the soil solution, and this is a major influence on microbial activity. The composition of the solution governs pathways of elements, clay particles are anions (negatively charged) and drawn to cations (positively charged) such as sodium ($Na^{+)}$, potassium ($K^{+)}$, calcium ($CA^{2+)}$, and water (7). Hydration of the ions releases energy creating heat. Different organic compositions of soil may affect the heat created (8).

Soil temperature also varies with depth and time; the sun's radiation only strikes the top layer of the soil which then must conduct the heat to the deeper soil layers. The delay in heat transfer time causes this heat to become lost resulting in the deeper soils having much lower temperatures than the top layers. Wilkins and Harris (9) found that the average monthly temperatures on the surface of a temperate forest soil varied from 2 ℃ to 19 ℃ whereas at only 7.5 cm depth the range was changed to 4 ℃ to 14 ℃. Soil temperature has an

obvious effect on the general metabolic activity of the microflora but rarely has a direct lethal effect. The extremes of temperature to which soil microbes are subjected depends on their position in the soil profile and the climatic conditions above the soil. Those growing on or near to the surface may be subjected to considerable changes in temperature during the course of a single day while microbes living in the lower parts of the soil may experience only small changes in temperature throughout the year. Tropical soils may have a high temperature throughout the year but in temperate regions the maximum temperatures are lower and the annual range is greater. Okafor (10) gave temperatures taken at 30 cm depth for a Nigerian soil and an English soil over one year. In the tropical soil the temperature was always between 28 °C and 30 °C but in the temperate soil it ranged from 2 °C to 15 °C. Interestingly he found that the organisms which colonized chitin buried in the soil in these two temperature ranges were quite different. At 10 °C fungi and bacteria were dominant but at 20 °C actinomycetes, nematodes and protozoa were important. Deeper soils also tend to have a higher carbon dioxide concentration than shallower soils as the exchange of gases between the soil and the atmosphere encourages oxygen to diffuse into the soil and carbon dioxide to move out (2, 11). Other gases present in the soil atmosphere and soil water include oxygen, methane and ammonia produced by microbial activity. Parr and Reuszer (12) found that reductions in oxygen levels in the soil atmosphere affected microbial populations, types and activity. The moisture content of a soil is directly related to its temperature as water has a much higher thermal conductivity than air. However, because the soil moisture is heated directly by the sun's energy it may not get the chance to heat up the soil particles before it evaporates. Therefore, a dry soil heats up and cools quicker than a saturated soil, except for clay soils which hold moisture better and consequently have reduced evaporation leading to increased temperature (2). Water is essential for the absorption of soluble nutrients by soil microorganisms into their cells (13).

Fungal metabolism cannot properly proceed when free water is not available and the activity of bacterial populations has also been found to fall (7). Seasonal changes can affect the aforementioned variables. Flooding, whilst increasing water saturation, can decrease oxygen levels in the soil atmosphere (12). In winter frozen soil water can reduce available moisture and cold temperatures can inhibit microbial activity. High temperatures and increased sunlight hours can increase soil temperature.

Soil pH regulates the solubility of plant nutrients as well as the bioavailability of potentially toxic heavy metals (14). It can also support or inhibit microbial extracellular enzymes. Soil processes such as biodeterioration and cycling of nutrients e.g. nitrification, create an acidic soil environment. The optimum pH for most microorganisms is pH 7.0; they are neutrophilic. However, fungi are generally more acid tolerant than bacteria and many grow optimally at pH 5.0. Although the bulk measurements of soil pH indicate that they are acidic, microsites within the soil exist where ion concentration may be significantly different (15) and where ammonification, the conversion of nitrogen to ammonia by microorganisms, may have raised the soil pH to a neutral level providing optimum pH for microbial growth. When amino acids, urea or chitin are added to an acid forest soil (pH 4.0), decomposition and subsequent release of ammonia can lead to an increase of soil pH to values of 6.0 - 7.0. Microscopic examination of such soil, flooded with a pH indicator, has shown that increases in pH may occur around particles of organic matter where ammonia is held. This included decomposing fungal mycelium (16).

2.2 The soil population

Soil is populated by many organisms, including animals and microorganisms, but it is generally considered that it is the microorganisms which play the most important part in the release of minerals and carbon dioxide for plant growth. It is the biological activities which ensure the level of nutrients in the soil is sufficient to maintain plant growth and continuation of plant populations (2). One of the most striking features of the soil microbial population is its diversity since fungi, bacteria, actinomycetes, algae and viruses, belonging to innumerable genera and species, can be found in almost any soil sample. The microorganisms found in soil are largely a reflection of the methods used to detect them (17-20). Firstly, the various groups of microorganisms are so nutritionally diverse that no one isolation medium is suitable to isolate all of them. Secondly there is the problem of counting colonies originating from numerous spores or hyphal fragments compared with those originating from single cells, mainly bacteria. Finally numbers of microorganisms do not necessarily indicate their relative importance since growth rate is most important in relation to their roles in various processes. Burges (21) and Garrett (22) recognized various groups of soil microorganisms based on their growth patterns. It is also the ability to survive in soil under adverse conditions which greatly effects the soil population and its long time existence. It has been estimated that in a normal agricultural soil a single gram may contain 5 metres of fungal mycelium, 10^8 bacterial cells and 10^6actinomycete spores together with numerous algae and viruses. On the basis that 1 metre of fungal hyphae weighs 9.4×10^{-5}g and that the average weight of a bacterial cell or actinomycete spore is 1.5×10^{-12}g (23) that is less than 0.06% of the total weight of the soil (2). In a sandy soil only 0.02% of the surfaces of soil particles were found to be colonized by bacteria (15). Thus in spite of the overall view that soil is heavily colonized by microorganisms in terms of

biomass the amount of microbial weight is extremely small. This is strongly believed to be a direct result of the low nutrient status of many soils (2).

2.3 Organic materials

Although live plants, animals and microorganisms provide nutrients for parasitic and symbiotic relationships it is dead organic matter which is the most widely distributed source of energy in the soil environment. Therefore its origin and subsequent decomposition is of fundamental importance to the soil. Dead organic matter in soil either originates from plants or animals although input from microorganisms themselves can sometimes be significant. It is however the input from plants which is the most significant. The total litter fall is dependent on the native vegetation and its climatic zone. Thus it has been stated that in a tropical soil total litter fall may reach 153,000 kg/hectare but in a temperate climate is much lower at about 25,500 kg/hectare(24, 25). It has been found that the total amount of fallen litter in an English pine forest was only 4,760 kg/hectare of which 4,080 kg was leaf litter and the remainder twig, cone and bark fragments (26). The fate of litter and its decomposition is greatly affected by a number of interrelated factors including climatic factors, edaphic factors, biotic and chemical factors. These all contribute to the rate of decomposition of all the litter and its incorporation into the soil (24). Thus a climate with lower temperatures and high rainfall causes slow rates of decomposition whereas soils with a high base content, high activity of soil microbes and animals exhibit much more rapid decomposition. Regardless of climate and soil conditions the chemical composition of leaves and plant material exerts a strong influence on their resistance to decomposition. There are various reports on the role of polyphenols in stabilization and therefore increased resistance to decomposition (27, 28).

Although it is the surface litter which is the main contributor to plant organic material in soil plant roots also make an important contribution. Based

on the root:shoot ratio of living plants it has been suggested that about half as much root material enters the soil compared with surface litter (2). There are also important contributions from a continuous supply of dead cells and root exudates from living roots (29). There are also seasonal changes occurring in the quantities of roots in soil (29). The rhizosphere effect provides one of the most active regions in soil based on microbial numbers and their metabolism (29, 30). The microbial population of the rhizosphere exhibits both qualitative and quantitative difference from root-free soil and although most groups of microorganisms show an increase in numbers it is bacteria which are the most stimulated (29).

Although in general it is assumed that the contribution of dead organic material from animals and microbes is small in comparison with that from plants much of the decomposition products from plants become incorporated into animal and microbial cells. These in due course also decompose thus completing the mineralization process. In a temperate grassland soil it has been estimated that the main invertebrate groups contribute biomass of about 2,300 kg/hectare and Clark has calculated that there were 4,500 kg/hectare of live bacteria and a similar weight of fungi in an arable soil (31). It is also noteworthy that animals are usually motile and through migration in the soil can transfer nutrients from sites of higher nutrient status to those with fewer nutrients. Organic materials from plants and animals both above and below ground are constantly entering the soil ecosystem where they are decomposed by its microorganisms. Almost any naturally occurring chemical may be present in the soil at one time or another although some for only a short time. Activities of man also contribute with the addition of many new chemicals in the form of artificial fertilizers, herbicides, pesticides and industrial wastes. Many of these are xenobiotic and therefore their degradation is a slow sometimes very slow process. Organic matter which is not completely decomposed eventually contributes to the formation of amorphous humic

materials which are an important part of the soil environment. Humus is not a defined chemical component but is a complex mixture of many substances and some of its fractions e.g. humic acid is highly recalcitrant.

2.4 Succession

2.4.1 Succession on plant components

Succession is the ecological description of the sequence of organisms in a given time over a defined area. Within soil organic substances are colonized by successive waves of microorganisms with each wave altering the micro-habitat preparing it for the next wave. Thus, in microbial successions there is a step-by-step depletion of the original components. This is unlike the situation in plant successions where each stage in the succession results in a build up of species eventually reaching a climax (1). Sometimes within plant communities succession of soil microorganisms is related to higher plants in the ecosystem. It has been found that in a sand dune system roots of the dune grasses greatly influence the composition of the microbial population (32). The decay of the aerial parts of plants, in which fungi play a major role, is completed in the soil but begins on the senescing tissues before they are shed. Throughout the life of plants, fungal spores are being continuously deposited on their surfaces from the atmosphere. The number of spores in the atmosphere at any one time is influenced by seasonal weather patterns and follows a cycle which is mainly dictated by temperature and rainfall (33). In a temperate region fungal spores build up in the atmosphere in spring following renewed growth and activity of overwintering mycelia which then sporulate and reach their peak in late summer and early autumn. In the tropics it is the start of the rainy season which provides the big increase in spores in the atmosphere. The earliest invaders of living leaves are symbiotic endophytes and some of these are host specific. Many are parasites which cause disease but symptomless infection of leaves by fungi are very common (34). Many studies have shown that these endophytic

fungi are very widely occurring and that many different genera and species have been recorded. Studies on succession on leaf litter indicates that the early colonists of freshly fallen leaves tend to be uncommon species of fungi which may be weak parasites which have infected the leaves prior to leaf fall. It has been shown that *Lophodermium pinastri* and *Desmazierella acicula* are weak parasites of *Pinus sylvestris* and infect the needles whilst they are still on the tree (25); others may belong to the living leaf population (35). These early colonists are, however, rapidly replaced by common soil fungi such *Trichoderma viride*, as *Penicillium, Humicola, Fusarium, Mucor* and *Rhizopus* species.

The situation regarding plant roots is more difficult to follow since it is not possible to define an exact starting point. It is also more complicated because of the greater diversity of soil microorganisms on the living plant root, the rhizoplane. Most studies indicate that the successional process is similar to those on decomposing leaves with the pioneer colonists being replaced by a range of typical soil microorganisms as the host resistance declines and as the roots become moribund their internal tissues are invaded by some of the fungi also occurring on the living root surface (36). Similar findings were made by Dickinson and Pugh (37) for colonists of *Haliimone portulacoideds* plants and by Waid (38) for those colonizing rye grass roots with *Fusarium* and *Penicillium* species and *Trichoderma viride* replacing the earlier colonists. These are fungal species which possess greater saprophytic ability (39).

2.4.2 Succession on animal and microbial components

Succession on animal and microbial materials introduces a new range of different components to those occurring in plants. These include keratin found in skin, hair, feathers, nails and claws, chitin present in insect exoskeletons and fungal hyphae. Succession on hair buried in soil starts with the early colonization by common soil fungi but within 3-4 weeks true keratinophilic

fungi develop such as species of *Trichophyton* and *Microsporum* (40, 41). Species of these genera known collectively as dermatophytes are responsible for various skin infections usually referred to as ‘ring-worm’ (33). Bird feathers are also a common source of keratin with fungi such as *Arthroderma curreyi* and *Ctenomyces serratus* regular colonists (42). It is significant that sites where there is regular deposition of keratin through hair and feathers have a high isolation rate of kerationophilic fungi.

In the decomposition of chitin the fungal species *Mortierella marburgensis, Mortierella alpina* and *Trichoderma viride* are prominent chitin decomposing organisms. *Streptomyces, Psuedomonas* and *Bacillus* species can also be important (10, 43, 44). *Verticillium* and *Paecilomyces* species are also common chitinoclastic fungi in soil (33). The importance of other components present with the chitin has been shown to be significant regarding the decomposition of chitin. De-waxed and de-proteinized insect wings when buried in soil were completely decomposed whereas untreated, natural wings were still intact after 300 days in soil (45). *Mortierella alpina* and *Paecilomyces carneus* were found to be prominent in chitin decomposition under alkaline conditions and *Trichoderma viride* and *Mortierella marburgensis* in acid forest soils (44). Actinomycetes have been found to be major chitinoclastic organisms in agricultural soils, especially in the tropics (46), bacteria in water logged soils and fungi in temperate soils (10).

2.5 Colonization and decomposition of textiles

2.5.1 Natural cellulose materials

It is well known that most natural materials entering the soil system are colonized and broken down by the soil microbial population. Less complex materials are usually rapidly decomposed whereas complex ones such as cellulose and lignin are more resistant and their decomposition can therefore take place over a considerable period of time. In forensic investigations the decomposition of clothing and other accessories, on or off a buried body, may provide additional data of value to the investigating team. The interactions of the materials in different soils and under different environmental conditions provide a challenging situation which requires careful research.

It is however well documented that under the majority of soil conditions many textile materials do not survive long-term burial (47). It has also been reported that in an acid environment cellulosic materials, of which cotton is 90-99% comprised, are highly susceptible to hydrolysis of the 1,4-glucosidic link. This results in a shortening of the cellulose chains, a structural weakening of the fibre and finally total decomposition (48). The biodegradability of cellulose fabrics is therefore closely linked with the physical and chemical composition of the fabric as well as general environmental conditions. Cellulose is a linear polysaccharide comprised of β 1-4 linked glucopyranose residues and these linear chains occasionally crosslink to form microfibrils and may be subsequently used to produce cotton materials.

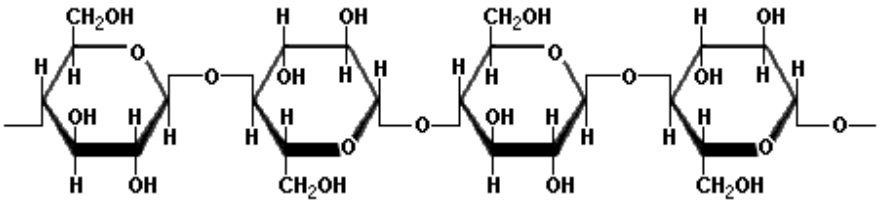

Figure 2.1 The structure of cellulose showing β1-4 linkages (http://www.scientificpsychic.com/fitness/carbohydrates2.html)

Raw cellulose is highly crystalline with some amorphous sites, however, when processed the proportion of these sites increases (5). A number of finishing products may be applied to cotton products such as crease-resistant chemicals and biocides. Easy-care crease-resistant chemicals such as imidazolidinone cross link with the macromolecules in the amorphous sites of the fibres and reduce moisture retention thus reducing susceptibility to biodeterioration (49). Many biocides are used to protect textiles against biodeterioration (50). Anti-mould treatments combat undesirable changes such as decolourization or discolouration by fungi such as *Aspergillus niger* whilst anti-bacterial treatments counter unpleasant odours caused by bacteria such as *Staphylococcus* species (51). Not all treatments are successful since it has been found that treatment of cotton with antimicrobial dye although inhibiting microbial growth the dissociation of ionic interactions in water resulted in low washing durability of the antimicrobial action (52). Although cotton is widely used in the manufacture of clothing synthetic fibres such as nylon and Meryl™ are commonly used in clothing products often in combination with cotton. Synthetic polymers are cheap to produce and usually have a longer life span than natural materials. They are also considered to result in more fashionable garments.

Cellulosic fibrous textiles are considered to be environmentally friendly because they are readily degraded in soil by microorganisms (48, 49). However, different cellulose fibres biodegrade uniquely, due to a difference in chemical composition and crystallinity; cotton has a relatively high crystallinity compared to other textiles such as rayon (48). Greater biodegradability has been observed in studies of polymers of lower crystallinity and lower molecular weight and amorphous cellulose is more readily attacked than the more crystalline cellulose (53). Processed cotton is more readily degraded than raw cellulose since the removal of cotton oils and other non-cellulosic matter during the bleaching process decreases the degree of polymerization (54).

Clearly the nature of the cotton and any blending with synthetic material will strongly influence the succession of microorganisms and the rate of decomposition.

Cellulolysis, or the hydrolysis of cellulose, is catalyzed by an enzyme complex called cellulase that consists of a number of extracellular β1-4 glucanases, some of which are endohydrolases randomly disrupting linkages throughout β1-4 glucan chains, producing glucose, cellobiose and higher molecular weight fractions, while others are exohydrolases or β1-4 cellobiohydrolases, which act only on the ends of β1-4 glucan chains releasing the disaccharide cellobiose. Glycohydrolases that release single glucose units from glucan chains are also part of the cellulase complex of some microorganisms. Beta 1-4 glucan endohydrolase and β1-4 glucan cellobiohydrolase may correspond to the C_x and C_1 enzymes of Reese, Siu (55). Both of these components are essential for the rapid hydrolysis of cellulose with a high crystalline content. Many fungi do not produce a C_1 component, so while their cellulases hydrolyse amorphous cellulose and manufactured carboxymethylcellulose they have little effect on the crystalline cellulose of cotton fibres.

Certain experimental evidence indicates that the C_1 component may not be a cellobiohydrolase and many have now expressed the view that C_1 is neither an exo-nor an endoglucanase and that both the endoglucanase and cellobiohydrolase components are part of the C_x complex. They suggest that the function of C_1 is to separate cellulose molecules on the surface of the fibrils allowing C_x access.

Beta 1-4 glucanases are thermostable glycoproteins which exist in multiple forms. Analysis of the β1-4 glucanase complex of *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*) showed that it is composed of five β1-4 endoglucanases of different molecular weights each working at its own optimum pH and *Trichoderma viride* produces at least four β1-4

cellobiohydrolases (56). Wood and McCrae (57) measured an increase in the rate of hydrolysis when several of the different forms of cellobiohydrolase from *Penicillium pinophilum* were incubated with cellulose in the presence of an endoglucanase. These workers suggested that the multiple forms of cellobiohydrolase are complementary because they are stereospecific in action. It is also possible that each cellobiohydrolase works best with its own endoglucanase and this could be why the latter also exist in multiple forms. An interesting observation that has ecological significance is that the β1-4 glucan cellobiohydrolases produced by one fungus may act with the β1-4 glucan endoglucanases produced by another. The decomposition of cellulose is finally completed by the transformation of trisaccharides and disaccharides to glucose by the action of β1-4 glucosidases within the hyphae.

All wood-rotting fungi undoubtedly degrade cellulose as do apparently many microfungi from soil and litter as measured by their ability to hydrolyse carboxymethylcellulose and pure cellulose in the laboratory (53). However, in nature cellulolytic activity depends upon a number of substratum-related factors, notably pH and mineral composition. This is reflected in differences in cellulolytic activity in different soils. It has been found that in extreme cases species that are very active in some soils show no activity in others. Where cellulose is embedded in a protective coat of lignin, as in xylem, cellulolysis tends to be limited to those fungi that can first modify or respire lignin. Thus the major decay of woody plant tissues is by specialized wood-rotting species.

The ability to hydrolyse cellulose is very variable. Some fungi have very low rates of utilization and others are unable to degrade cellulose at all. Examples of the latter can be found in all the divisions of the fungi, but it appears to be commonest among the Zygomycetes and Oomycetes, and so far most species which have been tested in the genera *Rhizopus*, *Mucor* and *Mortierella* and the majority of *Pythium* species have been shown to be inactive (53, 58, 59). Notable exceptions in the genus *Pythium* include

Pythium fluminum from fresh water (60, 61) and several common terrestrial species, *Pythium graminicola*, *Pythium irregulare* and *Pythium intermedium* (59). More have been found to hydrolyse cellulose when the fibres are in a swollen state (62). It must be said, however, that cellulolytic activity in *Pythium* species is usually rather weak. Few other genera in the Oomycetes have actually been tested but a few species in the Saprolegniales have also been shown to hydrolyse cellulose or carboxymethylcellulose (63). The ability to decompose cellulose (or other plant polymers) vigorously or not, as the case may be, has been used to classify fungi into several substrate-related ecological groups. However, low rates of hydrolysis of cellulose may not necessarily indicate poor adaptation to growth on cellulose for, as Garrett (64) has pointed out, it is not the rate of cellulolysis which is important but the rate in relation to the production of enough respirable carbohydrate sufficient for the need of a particular fungus. Successful growth on cellulose may not, therefore, necessarily be limited by the inherent rate at which the fungus hydrolyses cellulose. A measure of the potential for successful growth on cellulose is the cellulolysis adequacy index (CAI) of Garrett (64) which relates rates of cellulolysis to units of growth on media containing simple sugars. Deacon (59) suggested that this idea should be extended to provide a comparison of the degree of adaptation of fungi to growth on cellulose and could be used as a basis for the redefining of substrate groups.

A wide-ranging review of the ecology of microbial cellulose degradation has been produced by Ljungdahl and Eriksson (65). It is however important to understand that in a natural soil ecosystem it is the combined effects of soil microorganisms and soil animals which results in the complete breakdown of cellulose. Studies on plant litter placed in various litter bags has demonstrated that bags with a pore size which allows access to earthworms exhibited relatively rapid incorporation in to the soil whereas in bags of pore size which limited access the litter was slow to disappear.

2.5.2 Natural protein materials: wool and silk

Wool fibres are derived from sheep fleece and 99% of the fibre is composed of keratin with approximately 1% being composed of fats, sterols, and lipids (66). Wool fibres are decomposed by keratinophilic microorganisms under a range of conditions (67, 68) and the pattern of colonization and decomposition is similar to those shown in the decomposition of hair(33, 42). Even in a biologically active soil decomposition of buried wool is slower than cellulose based (69). Wool is often blended with synthetic fibres making decomposition more difficult.

Silk is usually used in luxury fabrics in Western countries because of its higher costs (70, 71) although it is widely used in Asia especially in China, India and Thailand. Silk is composed of a protein sericin and is highly hygroscopic and is resistant to rapid degradation. Also during processing it is often treated with metallic salts such as zinc and tin (72, 73) which increases its resistance to microbial degradation. Under aerobic conditions buried silk decomposes although slowly but under anaerobic conditions it may remain relatively unchanged for long periods of time even surviving for centuries (74). It is also apparent that there have been few long term burial experiments.

2.6 Synthetic materials

These include nylon, polyesters, acrylics, elastane. Nylon (alternatively named polyamide) was developed in the United States by DuPont in the late 1930s, with nylon yarn being used in experimental production of stockings in 1937. During World War II it was used for the manufacture of parachutes, glider tow ropes, nets, tents, and clothing especially ladies stockings (75). It is formed from a long-chain polyamide with recurring amide groups as an integral part of the polymer chain. Nylon is hydrophobic, is not attacked by insects, and is resistant to microbial attack (76). In fact nylon is very resistant to microbial degradation and has been shown to survive in soil for long periods.

Polyester textile fibres were developed during the 1940s. After World War II Imperial Chemical Industries (ICI) bought world rights to produce this fibre under the name of Terylene in the United Kingdom, whereas DuPont purchased the rights in the United States and produced the fibre under the name Dacron. The fibre consists of long-chain synthetic polymers composed of at least 85% by weight of an ester of dihydric alcohol and ter- phalic acid (P-HOOC-C_6H_4-COOH). It is used both on its own or often in mixed yarns with either wool or cotton. In recent years its low water absorbency means it is often used as the basis of technical fabrics such a fleece jackets, where it is often marketed under the trade name Polartec. It is not attacked by moth larvae and is resistant to microbial activity, although mildew may form on some sites and starches used in finishing. Although this will not weaken the fibre, this can cause discoloration (75). Acrylic yarn or acrylonitrile (vinyl cyanide, CH_2=CH.CN) began to be used in fibre making during the early 1950s. These fibres of long-chain synthetic polymers are composed of at least 85% by weight of acrylonitrile units and have been marketed under a number of trade names including acrilan, courtelle, and orlon (75). Acrylics have low water absorbency and since the 1960s have been used for easy-to-wash and easy-to-dry fabrics. They are highly resistant to chemical degradation, and the fibre is moth resistant on its own; however, in wool-acrylic mixtures moth larvae will attack through acrylic to get to the wool. Acrylics are naturally resistant to mildew and fungi. Elastane is often known under the trade names of Spandex or Lycra. Elastane is used to make highly elastic yarns (73, 76). Current use has been in highly flexible, close-fitting sports clothing. Despite the generic term Lycra for these garments, they are often mixed with other fibres to give better wear characteristics similar to elastain-nylon mixtures. Elastane fibres are also combined with cotton to provide garments such as underwear with some stretch. The fibre is formed from a long-chain synthetic polymer composed of at least 85% of segmented polyurethane. In 1958 the first spandex fibres were

produced by DuPont. In 1959 the name LycraTM was used in commercial production starting in 1960–61. Degradation of all of these synthetic materials is very slow but the textiles which are made with synthetic materials blended with natural materials are less resistant to microbial attack.

2.7 Forensic aspects of buried clothing and accessories in soil

In forensic investigations a buried or dumped body may be naked, fully clothed or partially clothed. In many cases, however, the body is accompanied by a range of materials which include clothing and other textiles, metal objects, plastics and paper products. In some cases clothing and personal effects may provide assistance in the identification of a body. Decomposition of clothing and accessories such as belts, foot ware, bags and jewellery is very variable both as a result of the actual material and the soil environment in which they are buried. Metal fasteners, zips studs can all increase resistance to decomposition and the decomposition of clothing still present on a body is strongly influenced by modification of the immediate environment by fluids and decomposition products leaking from the corpse. Furthermore the presence of clothing on a body can greatly alter the pattern and time period of decomposition of the body by perhaps restricting access to the body by insects (77, 78). Clearly decomposition of textiles and accessories is a complex and interesting challenge for the forensic investigators. However, the microbial decomposition of clothing in soil is worthy of careful investigation and evaluation for its potential in forensic investigations. The decomposition of many textiles has been reviewed by Hawksworth and Wiltshire (79) have presented a detailed account of the role and potential of forensic mycology and list a number of areas where mycology might be of forensic assistance. They concluded that mycological evidence should be included as an integral part of the ecological assessment at crime scenes, especially outdoors. They also stressed that when time of death or deposition is uncertain, and fungal colonies

are evident on human remains, clothing, or associated items (indoors or out of doors) they can provide valuable data. This might be especially important if entomology is not appropriate. Other mycological aspects concerned presence of fungal spores in palynological preparations, possession of mushrooms on a suspect, in gut contents, or in food and drink associated with deaths or neurotropic behaviour. Finally large scale production of certain fungal species might be connected to various forms of extortion or terrorism.

CHAPTER 3
EXPERIMENTAL

3.1 Materials and Chemicals

2X Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific, USA)

3,5-Dinitrosalicylic acid (Sigma-Aldrich, USA)

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Qiagen, Germany)

Agarose gel (Thermo Fisher Scientific, USA)

Ammonium sulfate (Merck KGaA, Germany)

Ampicillin sodium salt (Sigma-Aldrich, USA)

Calcium chloride dihydrate (Merck KGaA, Germany)

Carboxymethylcellulose sodium salt (Sigma-Aldrich, USA)

Cetyl trimethyl ammonium bromide (Sigma-Aldrich, USA)

Cobalt (II) chloride hexahydrate (Merck KGaA, Germany)

Congo red (Ajax Finechem, New Zealand)

DCode electrophoresis reagent kit for DGGE (Bio-Rad, USA)

Ethanol (Merck KGaA, Germany)

Ethidium bromide (Thermo Fisher Scientific, USA)

Ferrous sulfate heptahydrate (Merck KGaA, Germany)

Glucose (Ajax Finechem, New Zealand)

Isopropyl alcohol (Sigma-Aldrich, USA)

Isopropyl-thio-2-D-galactopyranoside (Qiagen, Germany)

Loading dye (Thermo Fisher Scientific, USA)

Luria Bertani Broth, Miller (Himedia Laboratories, India)

Magnesium sulphate tetrahydrate (Merck KGaA, Germany)

Manganese (II) sulfate tetrahydrate (Merck KGaA, Germany)

Peptone (Becton, Dickinson and Company, USA)

Phenol:chloroform:isoamyl alcohol (Sigma-Aldrich, USA)

Potassium dihydrogen phosphate (Merck KGaA, Germany)

Rose bengal (Sigma-Aldrich, USA)

Sodium acetate (Sigma-Aldrich, USA)

Tris-borate-EDTA buffer (Sigma-Aldrich, USA)

Tween 80 (Merck KGaA, Germany)

Urea (Merck KGaA, Germany)

Zinc sulfate heptahydrate (Merck KGaA, Germany)

3.2 Equipments

Autoclave (Tomy ES-315, Japan)

Autopipette (Pipetman, Gilson, France)

Balance (Sartorius CP323S, Germany)

Gel electrophoresis device (Taitec pico-2, Japan)

Gel Doc™ XR+ Imaging System (Bio-Rad, USA)

High Speed Refrigerated Centrifuge (Kubota 6500, Japan)

Hot plate stirrer (HL instrument, Thailand)

Incubator (Shel Lab 1565, USA)

Laminar flow (Safety Lab, Thailand)

Life Express Thermal Cycler (Bioer, China)

Microcentrifuge (WiseSpin CF-10, Korea)

Minishaker (Ika MS1, Malaysia)

Orbital shaker (Biosan OS-10, Latvia)

pH meter (Mettler Toledo S20 SevenEasy™, USA)

The Dcode™ Universal Mutation Detection System (Bio-Rad, USA)

UV/Vis spectrophotometer (Thermo Fisher Scientific Genesys 20, USA)

Water bath (Genlab WB 122, England)

3.3 Location and vegetation

Khao Yaida is a forest in Rayong Province (Fig. 3.1) and is located in zone Latitude 12 Degree 38 Lipda and 12 Degree 47 North, Longitude 101

Degree 24 Lipda and 101 Degree 27 Lipda East. Khao Yaida is part of the Kachet-Phe-Klaeng National forest (Fig. 3.2). This forest is very important because it is the only water source in Rayong Province.

3.3.1 Soil type and parent rock material

The rocks that are the parent material of the soil are Schist, Gneiss and Mudstone. When these rocks were weathering soil formation occurred. The soil type of this forest consists of silty sand, clayey sand and sandy clay. These soil are poor in nutrients and almost 85 % of the soil is covered with the secondary dry-evergreen forest that developed following the successional climax.

3.3.2 Forest type and vegetation

The secondary dry-evergreen forest that developed has been protected from commercial exploitation and has three levels. The first level consists of trees of over 15.5 meters in height. The second level has trees between 8–15 . 5 meters and the third level contains trees of has less than 8 meters high. The important vegetation consists of Khanunpa (*Artocarpus lanceifolius* Roxb.), Tandum (*Diospyros transitoria* Bakh.), Lampankhao (*Duabanga grandiflora*), Banyan tree (*Ficus benjamina*) and Korlan (*Nephelium hypoleucum*). Records for total rainfall and temperature were obtained from The Thai Meteorological Department, Bang-Na. Full details are given in Appendix A.

Figure 3.1 Map of Thailand

Rayong arrowed

(http://www.visit-thailand.info/images/maps/political-map-of-thailand.gif)

Figure 3.2 Scenes of the forest area where the samples were buried (Khao Yaida, Rayong Province)

3.4 Methods

3.4.1 Soil properties testing

3.4.1.1 Soil pH

Soil pH was measured using a 1:2 mixture of soil : water (80). The solution was mixed by a mechanical shaker at 200 rpm for 1 hour and pH measured with a glass electrode.

3.4.2.2 Soil moisture

A clean and dry container was weighed (W_1) and a sample of soil of 100 g was placed in the container and weighed (W_2). The container was placed in the oven and dried at 80 °C to a constant weight. After drying, the container was removed from the oven and allowed to cool. The container with contents was weighed (W_3). The moisture content of the soil was calculated as a percentage of the dry soil weight.

$$MC\ (\%) = \frac{W_2 - W_3}{W_3 - W_1} \times 100\%$$

Where:

MC (%) = The percent moisture content of the soil

W_1 = Weight of container (g)

W_2 = Weight of moist soil + container (g)

W_3 = Weight of dried soil + container (g)

3.4.2 Prepare the fabric samples

In these experiments the textile material tested were ladies briefs, 100% cotton and 100% polyester. The ladies briefs were obtained either from Marks & Spencer or Tesco Lotus and white was chosen as it would be easier to observe any colour changes and also to ensure that no dye could interfere and alter the decomposition process. 100% cotton and 100% polyester obtained as rolls (Pahurat market, Bangkok) were used to make square bags (10x10 cm). All fabric samples were washed with tap water 3 times to remove any chemical dressing before burial in the soil. The briefs were wrapped around portions of synthetic sponge and portions of sponge were inserted in to the cotton and polyester bags (Fig. 3.3)

Figure 3.3 Briefs and textile bags ready for burial

3.4.3 Soil burial experiments

Investigation of the decomposition rate of clothing was undertaken following the burial of ladies briefs, 100% cotton and 100% polyester bags in a tropical soil at Khao Yaida, Rayong Province over a 10 week period. The test samples were buried at a depth of about 20 cm. These experiments were carried out both during the dry season and rainy season in Thailand. Each textile sample was pre-soaked with a solution comprised of 5% urea and 2% glucose to simulate leakage of body fluid from a buried corpse (4). Similar samples were soaked in water only. Three samples of briefs and material bags were retrieved from the forest every two weeks and transported to the laboratory for examination.

3.4.4 Determination of biodegradability

The extent of biodegradation was estimated from the weight loss of the fabric sample based on the following equation (81):

$$W_t(\%) = \frac{W_0 - W_t}{W_0} \times 100\%$$

Where:

W_t (%) = The percent weight loss after t days of incubation

W_0 = The original weight of the fabric sample before immersion (g)

W_t = The weight of the dry fabric sample after t days of incubation (g)

Dry weight of all samples was determined prior to burial and samples retrieved after determined periods of burial were gently washed in tap water to remove adhering soil, dried at 80°C for 48 hours and then weighed.

3.4.5 Observation of physical change

3.4.5.1 Light microscopy

All samples were retrieved from the forest at regular intervals and observed by eye and a stereo zoom light microscope (Olympus SZ-PT). As appropriate small portions of samples and associated fungi were mounted on a glass microscope slide and stained in lactophenol cotton blue. Microscopic examinations were carried out with bright field microscopy fitted with an Olympus DP71 camera and light microscopy with an Olympus BX51 research microscope using x10 and x40 dry objectives. Images were captured using an Olympus DP71 camera and analyzed by Infinity Analyze software provided with measurement functions and image enhancement options.

3.4.5.2 Scanning Electron Microscopy (SEM)

For examination by Scanning Electron Microscopy small sections of dried samples were mounted using Electrodag high conductivity paint (Acheson Colloids Company) on a 1cm aluminium stub. The specimens were sputter-coated with a film of gold approximately 500 A thick in an Emitech K550X coating unit. The coated specimens were then loaded into a FEI (Quanta 200) ESEM (Environmental Scanning Electron Microscopy, 2008) for examination over a range of magnifications.

Prior to coating the specimens were fixed in 2.5% Glutaraldehyde for 20 minutes to provide a rapid inter and intra-cellular penetration. The fixed specimens were dehydrated through a series of increasing concentrations of ethanol, ending in a 100% dehydrating liquid of the highest possible purity. Typically these are steps of 10, 20, 30, 50, 70, 90, 95 and absolute ethanol at 10 min for each, with 3 changes with absolute ethanol. Acetone was used as the intermediate fluid because it is miscible with carbon dioxide. Critical point drying (CPD) was used to prevent collapse in ESEM. This is achieved by replacing acetone with liquid CO_2 and then the liquid CO_2 is taken to a critical temperature and pressure (34. 5°C and 1200 psi (pounds per square inch)). At these parameters, the shearing forces and surface tension on the samples are minimal and the CO_2 is in equilibrium between liquid and gas. This stops the samples collapsing whilst they are dried. The samples were sputter-coated with gold using an Emitech K550X coating unit.

Another method for examination of living cells used a low-temperature environmental scanning electron microscopy (ESEM). Specimens were rapidly frozen in liquid nitrogen slush (at -180 ˚C) under vacuum and transferred to the Cryo-preparation chamber where the frozen samples were sublimated at -80 ˚C to remove surface ice crystals. Samples were then re-cooled to -130 ˚C and sputter coated with gold in the preparation chamber using a voltage of 1.2 kV Specimens were viewed at an accelerating voltage of 5 kV at -130 ˚C with a FEI Quanta 200 ESEM fitted with a Quorum Technology Cryo-preservation system.

Images for all methods were obtained using an image capture system (Oxford Instruments, INCA system, Oxford, UK) at Liverpool John Moores University.

3.4.6. Fungal isolation and identification

Fungi were isolated by dilution plate methods or single point inoculation from samples which were highly degraded. Fungal growth observed on the surface of decomposing samples was transferred to Potato Dextrose and Rose Bengal Agars containing the antibacterial antibiotic streptomycin sulfate (M & H. Manufacturing Co., Ltd. Samutprakarn, Thailand) at a final concentration of 50 µg per ml final concentration. The agars were sterilized by autoclaving at 15 psi at a temperature of 121℃ for 15 min. and the antibiotic then added once the agar had cooled to less than 50 ℃. Molten agars were then dispensed at a volume of around 15 ml in to 9 cm diameter sterile plastic Petri dishes (Grenier bio-one). Petri dishes were incubated on the bench at room temperature (25-30 °C) allowing cultures to experience periods of natural light. Once cultures showed signs of sporulation samples were mounted in lacto phenol cotton blue on clear glass microscope slides (Sail Brand China) with cover glasses 22 x 22 mm (Menzel-Glaser). Examinations were made by brightfield microscopy as before. Fungal identifications were obtained by comparison of morphological feature with those for published species using reference works (82-87).

3.4.7 Cellulase activity

3.4.7.1 Determination of cellulolytic enzymes on Carboxymethylcellulose Agar

Fungi isolated from fabrics were inoculated onto Carboxymethylcellulose Agar (CMC agar). After the incubation time (2-5 days), cellulolytic activities of isolated fungi were determined by using the congo red test with 2% w/v aqueous congo red to reveal the remaining CMC. The cellulose activity was estimated by measuring the size of the hydrolysis zone, and the leading edge of mycelial growth in the medium. The ratio: hydrolysis zone / colony diameter, provides additional information to compare strains (88).

3.4.7.2 Cellulolytic activity in liquid medium

Fungi shown to have good activity on CMC agar were selected for further testing in liquid media. The selected fungi were maintained on potato dextrose agar medium and actively growing fungal mycelia (5 agar plugs) from 7 day old cultures were transferred to 250 ml Erlenmeyer flasks containing 100 ml of Mandels medium (89) composed of; urea 0.3g/l, $(NH_4)_2SO_4$ 1.4 g/l, KH_2PO_4 2.0 g/l, $CaCl_2 \cdot 2H_2O$ 0.4 g/l, $MgSO_4 \cdot 4H_2O$ 0.3 g/l, peptone 1.0 g/l, $FeSO_4 \cdot 7H_2O$ 5.0 mg/l, $MnSO_4 \cdot 4H_2O$ 1.6 mg/l, $ZnSO_4 \cdot 7H_2O$ 1.4 mg/l, $CoCl_2 \cdot 6H_2O$ 2.0 mg/l, and Tween 80 2.0 ml/l, pH 5.5 to which 1% (w/v) of carboxymethylcellulose (Sigma-Aldrich) were added. The medium was sterilized by autoclaving at 121°C for 20 min. Inoculated flasks were incubated on a rotary shaker (New Brunswick Scientific) at 150 rpm at 25 °C for 21 days. Incubation was under natural light conditions. The culture fluids were centrifuged and the supernatant fluids were used as the crude enzyme preparations.

Endoglucanase activity was measured as described by Ghose (90) using the fungal supernatants and the reducing sugar produced was determined by the dinitrosalicylic acid method (91). One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1µmol of reducing sugar per min.

3.4.8 Molecular investigations

3.4.8.1 DNA extraction and PCR amplification

Fifteen fabric samples (five of each from ladies briefs, 100% cotton bags, and 100% polyester bags which were collected every two weeks), soil and selected fungal isolates were extracted for genomic DNA using CTAB method (details of the protocols used are given in Appendix E).

The extracted DNA samples were amplified by the primer set Fung-GC and NS1(Table 3.1) targeting the 18S rRNA gene region for DGGE analysis (Das et al., 2007).A total of 20 µl of PCR mixture contained 2X Phire Plant Direct PCR Master Mix, 0.5 µM of each of the primers and 10-100 ng DNA template. The amplification regime comprised a denaturing step (98°C/5 min), followed by 40 cycles of 98 °C/5 s, 50 °C/5 s, and 72 °C/20 s, and finally an extension step of 72 °C/ 1 min. Amplification products were separated by 1% agarose gel electrophoresis in 0.5X TBE buffer and visualized by ethidium bromide staining.

Table 3.1 Primers used in the PCRs

Primer	Sequence (5´-3´)	Reference
Fung	ATTCCCCGTTACCCGTTG	MÖhlenhoff et al., 2001
Fung-GC	cgcccgccgcgccccgcgcccggcccgccgc ccccgccccATTCCCCGTTACCCG TTG	MÖhlenhoff et al., 2001
NS1	GTAGTCATATGCTTGTCTC	MÖhlenhoff et al., 2001

3.4.8.2 Denaturing Gradient Gel Electrophoresis (DGGE) analysis and sequence analysis of selected fragments

DGGE analysis was performed as described by Muyzer (93) using a BioRadDCode system. Amplified fragments obtained from every soil and fabric sample were loaded in an 8% polyacrylamide gel (ratio of acrylamide to bisacrylamide: 37:1) formed with a denaturing gradient of 25 to 55% (Appendix F). The DGGE was conducted at 80V, 60℃ for 18 hours. After electrophoresis, gels were stained for 30 minutes with ethidium bromide and visualized under UV light. Selected DNA fragments were excised from the gel and submerged for 2 days at 4 ℃ in 70 µl DNase/RNase-free distilled water. A PCR based on a 1 µl aliquot of the gel fragment extract as the template was performed under the same conditions as described above, with the same primer set without GC clamp (NS1-fung). Amplification products were purified using FavorPrep^{TM}GEL/PCR Purification Kit (Favorgen Biotech Corporation, Taiwan) and cloned using the TOPO® TA Cloning® Kit for sequencing. Cloning reactions were performed by the method of the manufacturer's instructions, with a minor modification (incubated the reaction for 1 hour at 22℃). After DNA ligation, the ligated plasmid vectors were transformed into the competent *E. coli* (TOP10) using chemical transformation (Heat shock at 42 °C for 30 seconds). Cloned sequences were determined at 1st BASE company (Malaysia). The complete sequences were taken and BLAST program at NCBI server to compare with GenBank database for identification.

CHAPTER 4
RESULTS

4.1 Soil properties testing

Soil physical properties such as temperature, moisture content and pH was measured during the dry season (March-May, 2013) and rainy season (September-November, 2013) were shown in Tables 4.1 and 4.2.

Table 4.1 Physical characteristics of soil in dry season (March-May, 2013)

Week	Temperature (°C)	Moisture content (%)	pH
0	27.5	2.3	6.4
2	29.0	1.6	7.0
4	30.0	1.8	6.9
6	29.0	7.7	6.0
8	27.0	2.0	6.7
10	27.0	5.7	7.0

Table 4.2 Physical characteristics of soil in rainy season (September-November, 2013)

Week	Temperature (°C)	Moisture content (%)	pH
0	25.5	19.2	6.9
2	27.2	16.9	7.0
4	25.1	16.0	6.6
6	26.2	16.2	7.4
8	25.6	17.5	7.1
10	25.1	15.8	7.0

Comparison of Tables 4.1 and 4.2 gives details of soil temperature, soil moisture and pH during the dry and rainy seasons and highlights a major difference in soil moisture content over these two experimental periods. During the dry season moisture content ranged from 1.6 to 7.7 % with an average soil moisture content of 3.5 % over the ten weeks. As expected the moisture content during the rainy season was significantly greater ranging from 15.8 to 19.2 % over the corresponding ten week period. The average moisture during this period at 16.9 % is 4.8 times that of the average dry season level. Interestingly there were only minor differences in the soil temperature between the two seasons with a temperature range of 27 ℃ to 30℃ (average 28.3°C) during the dry season and 25.1 °C to 27.2 ℃ (average 25.8 ℃) in the rainy season. Average air temperature during March and April (dry season) was 29.2 ℃ and 29.8 ℃ respectively and for October and November (rainy season) it was 26.9 °C and 27.4 ℃ respectively. It is also notable that pH in the dry season ranged from 6.0 to 7.0 compared with 6.6 to 7.4 for the rainy season with an average pH of 6.7 and 7.0 respectively.

These data suggests that it is soil moisture which is the most important physical factor in the decomposition of the samples as the soil pH and temperature are within a suitable range for most microorganisms to grow strongly.

4.2 Biodegradation rate of fabric samples

4.2.1 Dry season

The results obtained during the dry season clearly showed the major difference between cotton fabric (briefs and cotton square bags) and polyester samples (Table 4.3). The cotton samples showed varying degrees of degradation over the 10 weeks period but no visible or measurable changes could be determined for the polyester over the same period. The only visible change was slight discolouration of the surface of the polyester. The cotton square bag samples were showing signs of degradation by week 4. The weight loss of the cotton materials was 7.6-7.8%. A similar weight loss was recorded for the cotton briefs after 4 weeks. After 6 weeks clear changes had taken place with visible signs of decomposition for both cotton briefs and cotton square bags. The weight loss for the briefs was about 40% and for the cotton bags around 30%. The reason that there is a difference in weight loss between the briefs and the cotton bags may be a reflection on the thickness of the cotton being thinner in the briefs which may result in better access to moisture and the soil microorganisms during the dry period. By week 8 the weight loss of cotton samples was in the region of 70% with little difference in weight loss between the briefs and the bags. By week 10 the weight loss in the square bags was close to 100%. In the briefs the weight loss was around 80% which may be a result of better water retention by the cotton bags with their slightly thicker material. The treatment of the briefs with urea and glucose indicated little difference between briefs with urea and glucose compared to distilled water at 80% weight loss. The cotton bags however were different with weight loss of 96% for distilled water and 100% for urea and glucose treated samples. The greatest degradation occurred between weeks 4 and 8 as shown in Figure 4.1. These data are supported by visual appearance of the briefs (Fig. 4.2) and cotton bags (Fig. 4.3) as discussed later. The polyester samples showed no real change (Fig. 4.4).

Table 4.3 Weight loss and physical appearance of fabric buried in forest soil during the dry season

Fabric	Treatment	Week	Weight loss (%)	Fabric description	Degradation scale (1-5)
100% cotton (Brief)	Control (DW)	2	0.52 ± 1.10	Visually intact	1
		4	7.74 ± 2.32	Visually intact	1
		6	39.88 ± 0.04	Small to medium size holes and broken yarns	2
		8	70.67 ± 1.28	Medium size holes and broken yarns	3
		10	78.37 ± 2.16	Severe degradation	4
	Urea and Glucose	2	0.46 ± 0.06	Visually intact	1
		4	11.75 ± 1.56	Visually intact	1
		6	42.89 ± 3.97	Small to medium size holes and broken yarns	2
		8	71.16 ± 3.56	Medium size holes and broken yarns	3
		10	80.40 ± 2.56	Severe degradation	4

- Means of three replicates (%) ± S.D.
- Degradation scale (1-5)

1 = No visible degradation
2 = Small to medium size holes and broken yarns
3 = Medium size holes and broken yarns
4 = Severe degradation
5 = Complete degradation

Table 4.3 (continued) Weight loss and physical appearance of fabric buried in forest soil during the dry season

Fabric	Treatment	Week	Weight loss (%)	Fabric description	Degradation scale (1-5)
100% cotton (Bag)	Control (DW)	2	2.97 ± 3.47	Visually intact	1
		4	7.60 ± 0.03	Visually intact	1
		6	30.59 ± 0.12	Small to medium size holes and broken yarns	2
		8	67.87 ± 0.08	Medium size holes and broken yarns	3
		10	95.88 ± 4.26	Severe degradation	4
	Urea and Glucose	2	3.15 ± 2.42	Visually intact	1
		4	7.84 ± 7.89	Visually intact	1
		6	30.82 ± 6.95	Small to medium size holes and broken yarns	2
		8	69.54 ± 8.43	Medium size holes and broken yarns	3
		10	100.00 ± 0.00	Complete degradation	5

- Means of three replicates (%) ± S.D.
- Degradation scale (1-5)

1 = No visible degradation
2 = Small to medium size holes and broken yarns
3 = Medium size holes and broken yarns
4 = Severe degradation
5 = Complete degradation

Table 4.3 (continued) Weight loss and physical appearance of fabric buried in forest soil during the dry season

Fabric	Treatment	Week	Weight loss (%)	Fabric description	Degradation scale (1-5)
Polyester (Bag)	Control (DW)	2	0.00 ± 0.00	Visually intact	1
		4	0.00 ± 0.00	Visually intact	1
		6	0.00 ± 0.00	Visually intact	1
		8	0.00 ± 0.00	Visually intact	1
		10	0.00 ± 0.00	Visually intact	1
	Urea and Glucose	2	0.00 ± 0.00	Visually intact	1
		4	0.00 ± 0.00	Visually intact	1
		6	0.00 ± 0.00	Visually intact	1
		8	0.00 ± 0.00	Visually intact	1
		10	0.00 ± 0.00	Visually intact	1

- Means of three replicates (%) ± S.D.
- Degradation scale (1-5)

  1 = No visible degradation

  2 = Small to medium size holes and broken yarns

  3 = Medium size holes and broken yarns

  4 = Severe degradation

  5 = Complete degradation

4.2.2 Rainy season

Table 4.4 illustrated the major difference between dry and wet season. By week 6 all the briefs had been completely degraded with the 100% weight loss and clear visual appearance (Fig. 4.5). There distinct differences during the first 4 weeks and 85% degradation occurred. There was also significant degradation (24%) after only 2 weeks. The rate of decomposition for the cotton bags was also high with 24% weight loss after 2 weeks. Although weight loss was lower over week 4 and 6 by week 8 the cotton bags were completely degraded (Fig. 4.6). As for the dry season the polyester samples had not been degraded after 10 weeks.

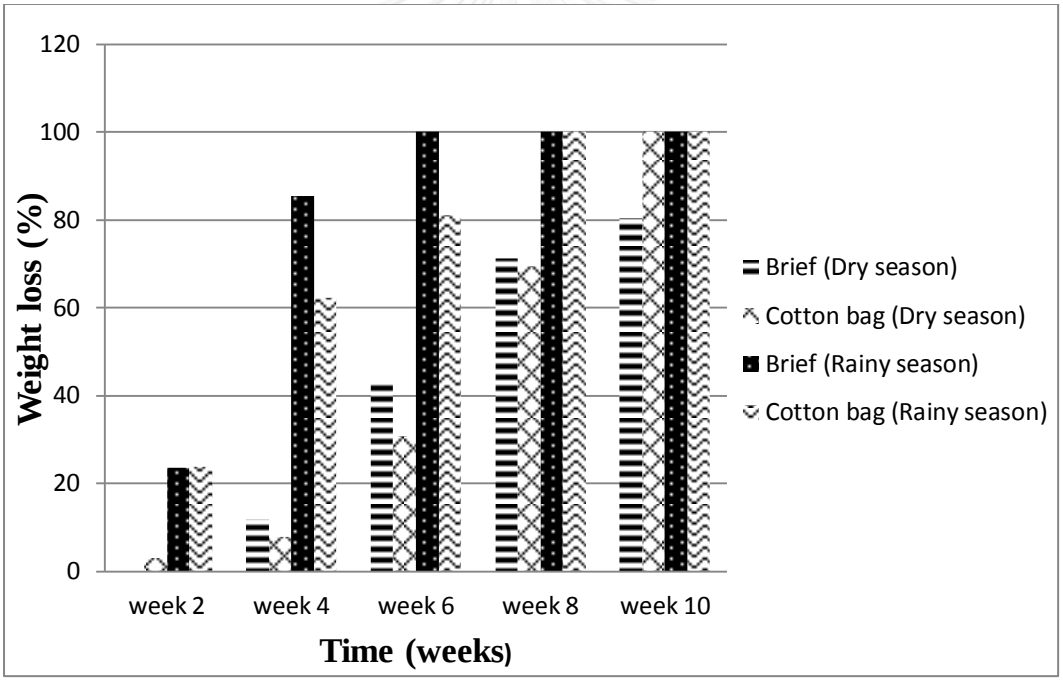

Figure 4.1 Comparison of weight loss of cotton samples week by week and between seasons

Table 4.4 Weight loss and physical appearance of fabric buried in forest soil during rainy season

Fabric	Treatment	Week	Weight loss (%)	Fabric description	Degradation scale (1-5)
100% cotton (Brief)	Urea and Glucose	2	23.59 ± 3.09	Visually intact	1
		4	85.37 ± 1.08	Severe degradation	4
		6	100.00 ± 0.00	Complete degradation	5
		8	100.00 ± 0.00	Complete degradation	5
		10	100.00 ± 0.00	Complete degradation	5
100% cotton (Bag)	Urea and Glucose	2	23.78 ± 11.36	Visually intact	1
		4	62.39 ± 2.99	Severe degradation	4
		6	81.03 ± 1.07	Severe degradation	4
		8	100.00 ± 0.00	Complete degradation	5
		10	100.00 ± 0.00	Complete degradation	5
Polyester (Bag)	Urea and Glucose	2	0.00 ± 0.00	Visually intact	1
		4	0.00 ± 0.00	Visually intact	1
		6	0.00 ± 0.00	Visually intact	1
		8	0.00 ± 0.00	Visually intact	1
		10	0.00 ± 0.00	Visually intact	1

- Means of three replicates (%) ± S.D.
- Degradation scale (1-5)

1 = No visible degradation
2 = Small to medium size holes and broken yarns
3 = Medium size holes and broken yarns
4 = Severe degradation
5 = Complete degradation

4.3 Observation of physical change

4.3.1. By eye

Figures 4.5, 4.6 and 4.7 of the cotton and polyester samples demonstrate a strong difference between cotton and polyester. As for the dry season there was no change in the polyester except for minor surface discolouration. Figure 4.5 illustrates the significant changes in the briefs starting with discolouration at week 2 and signs of degradation at week 4 with total degradation at week 6. A similar pattern can be seen for the cotton bags. Comparison of samples during the dry season confirms the slower rate of the decomposition of the cotton samples with the briefs being nearly completely degraded by week 10 with mainly the elastic components and polyester label remaining.

Figure 4.2 Decomposing briefs during dry season after 2 weeks (A), 4 weeks (B), 6 weeks (C), 10 weeks (D)

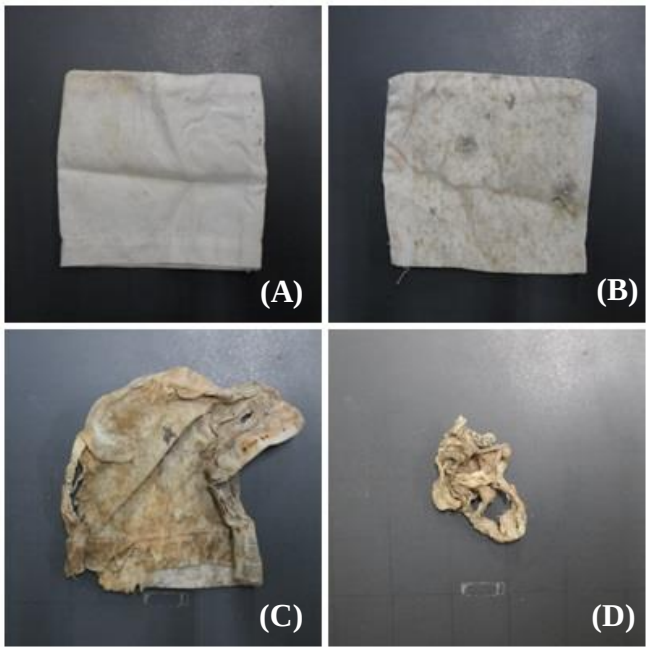

Figure 4.3 Decomposing cotton during dry season after 2 weeks (A), 4 weeks (B), 6 weeks (C), 8 weeks (D)

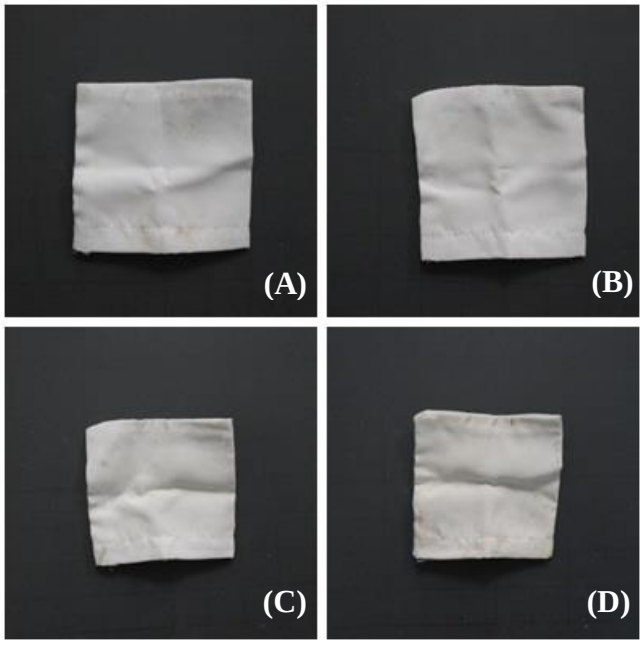

Figure 4.4 Decomposing polyester during dry season after 2 weeks (A), 4 weeks (B), 6 weeks (C), 10 weeks (D)

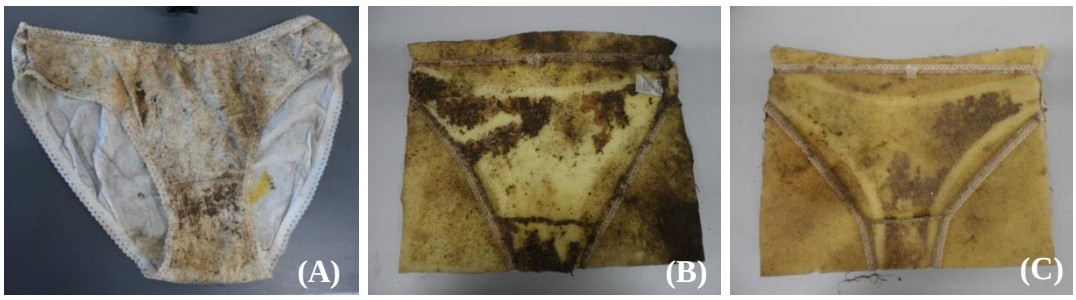

Figure 4.5 Decomposing briefs during rainy season after 2 weeks (A), 4 weeks (B), 6 weeks (C)

Note : Not observation in 10 weeks

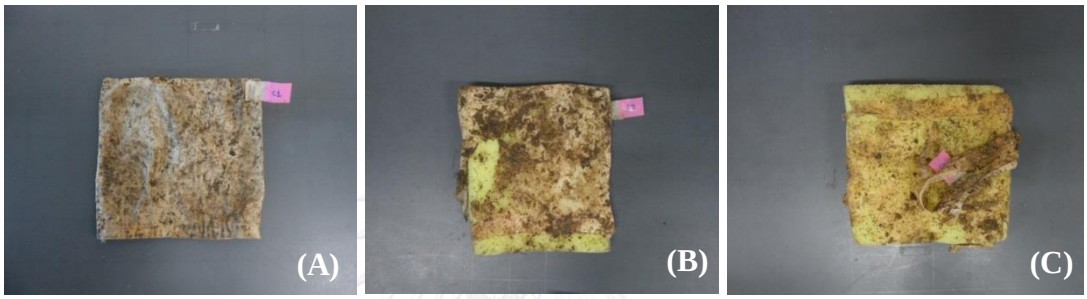

Figure 4.6 Decomposing cotton during rainy season after 2 weeks (A), 4 weeks (B), 6 weeks (C)

Note : Not observation in 10 weeks

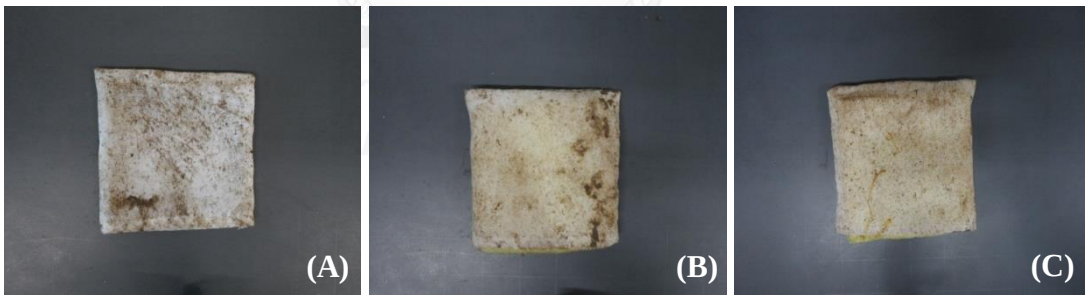

Figure 4.7 Decomposing polyester during rainy season after 2 weeks (A), 4 weeks (B), 6 weeks (C)

4.3.2. By Scanning Electron Microscopy (SEM)

Figure 4.8 confirms the presence of considerable fungal growth on the cotton briefs after 4 weeks burial during the dry season. Figure 4.9 at higher magnification indicates strong degradation of the cotton fibres with many of the fibres destroyed. A cotton bag buried for 4 weeks again during the dry season illustrates the presence of fungal hyphae but the cotton fibres are still mainly intact. After 6 weeks the fibre structure is still mainly intact although individual holes become apparent (Fig. 4.11). Following 8 weeks burial the cotton fibres can be seen to be at a more advanced state of degradation with the remains of actinomycete mycelium on the fabric surface (Fig. 4.12).

Figure 4.13 illustrates the presence of a Streptomycete spore chain on the cotton bag sample.

By comparison with the cotton samples (briefs and bags) the polyester samples remained intact with little evidence of microbial growth (Figs. 4.14 & 4.15).

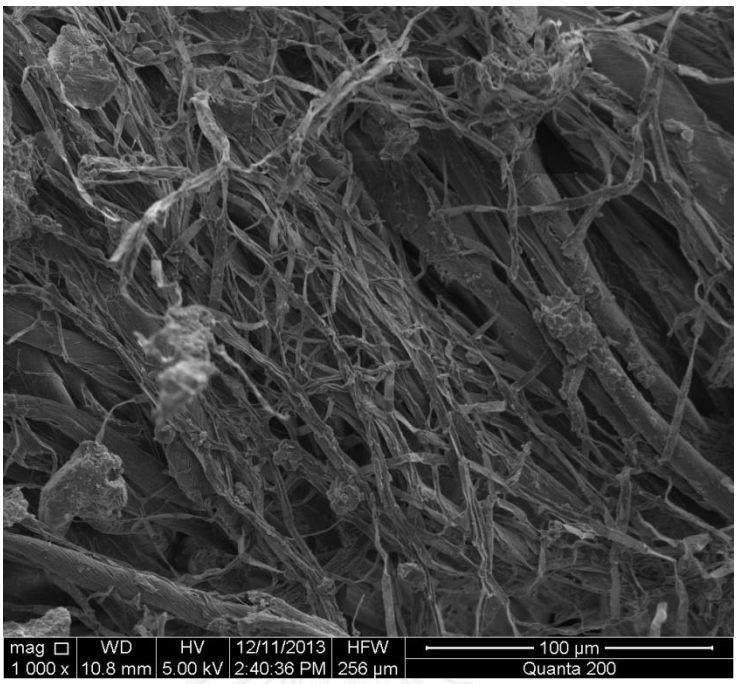

Figure 4.8 SEM of briefs after buried for 4 weeks

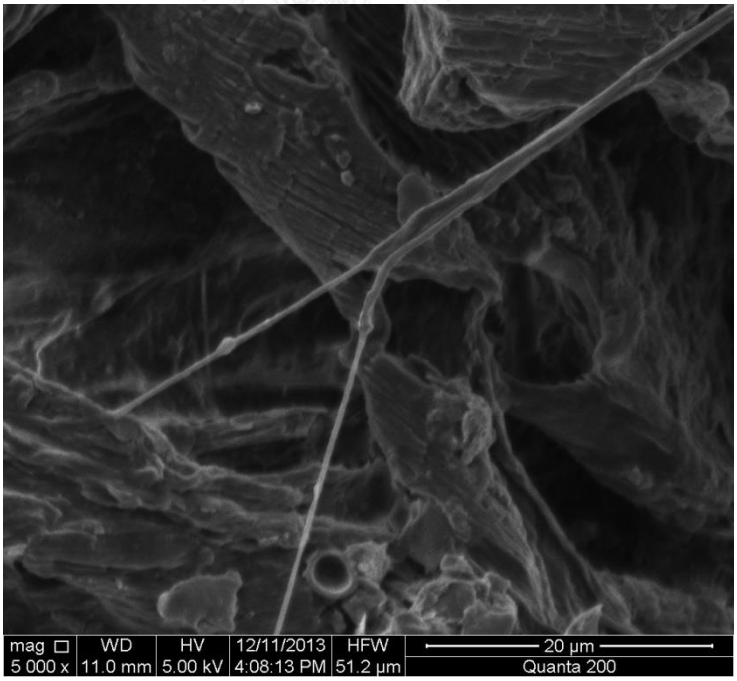

Figure 4.9 SEM of briefs after buried for 10 weeks

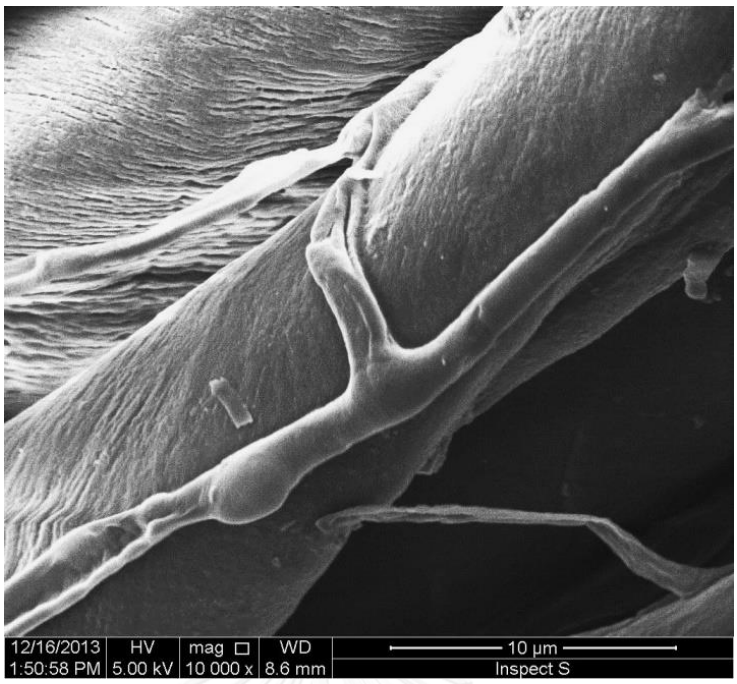

Figure 4.10 SEM of cotton (square bag) after buried for 4 weeks

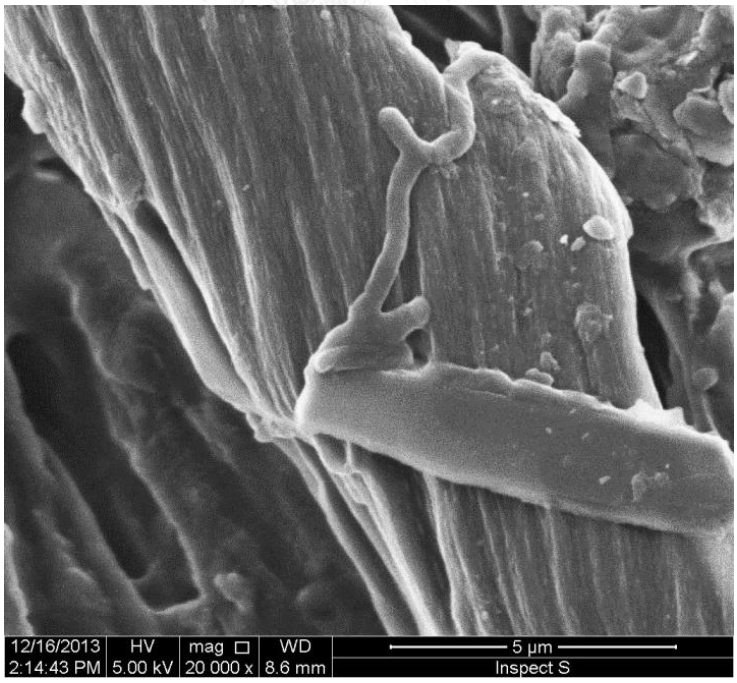

Figure 4.11 SEM of cotton (square bag) after buried for 6 weeks

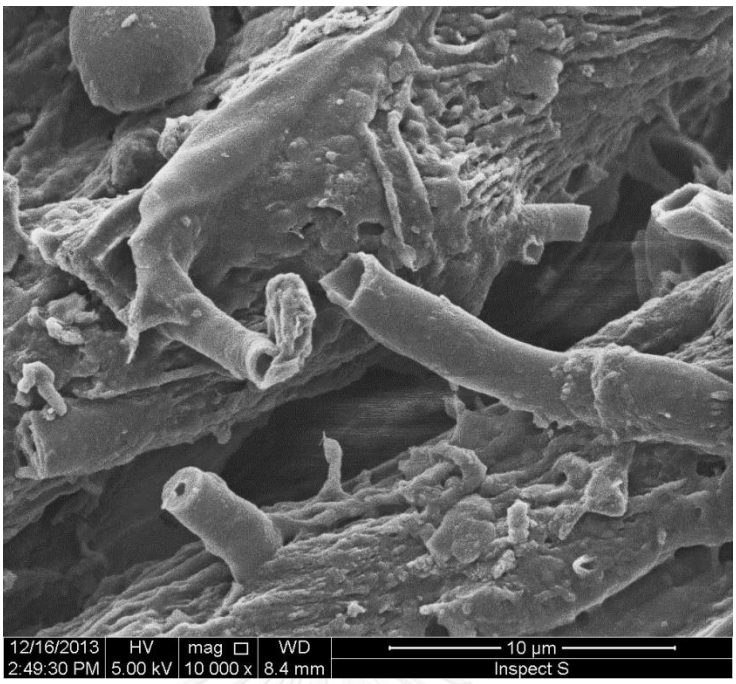

Figure 4.12 SEM of cotton (square bag) after buried for 8 weeks

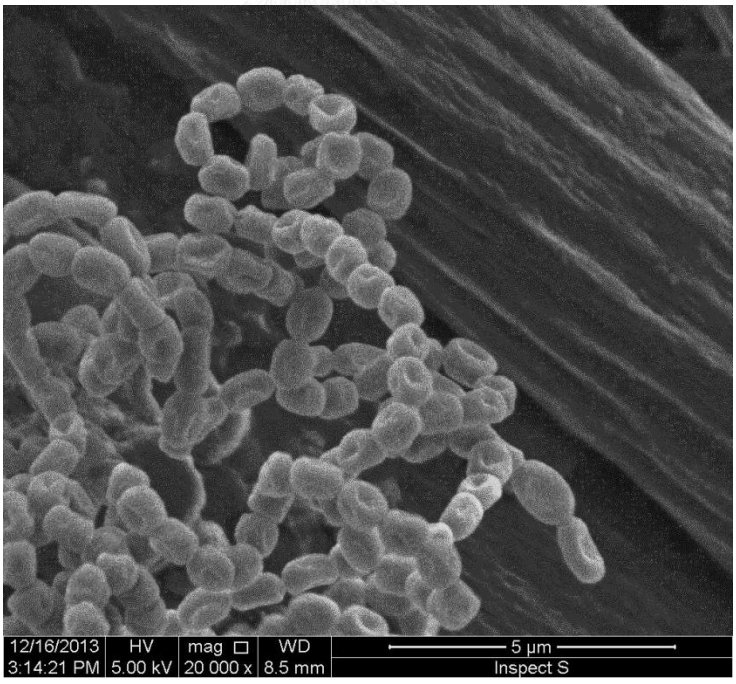

Figure 4.13 SEM of cotton (square bag) after buried for 10 weeks

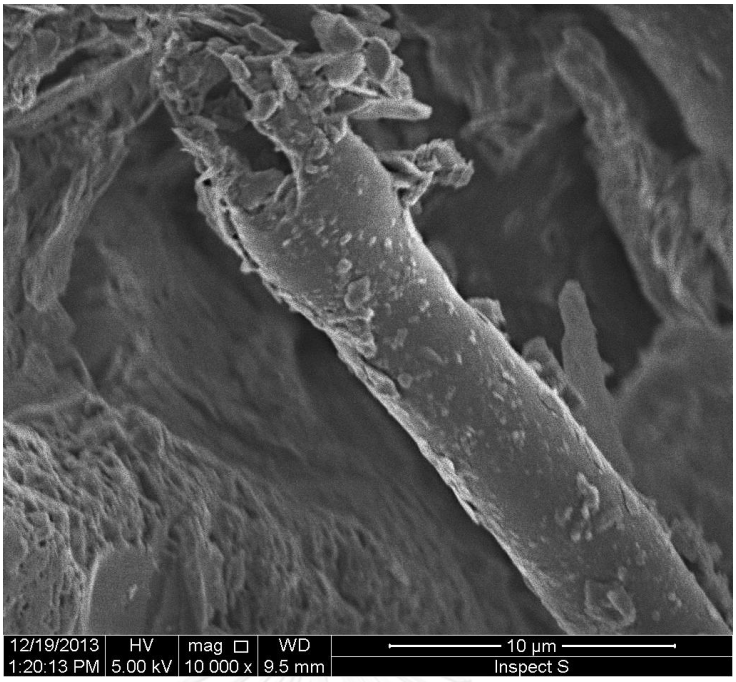

Figure 4.14 SEM of polyester (square bag) after buried for 8 weeks

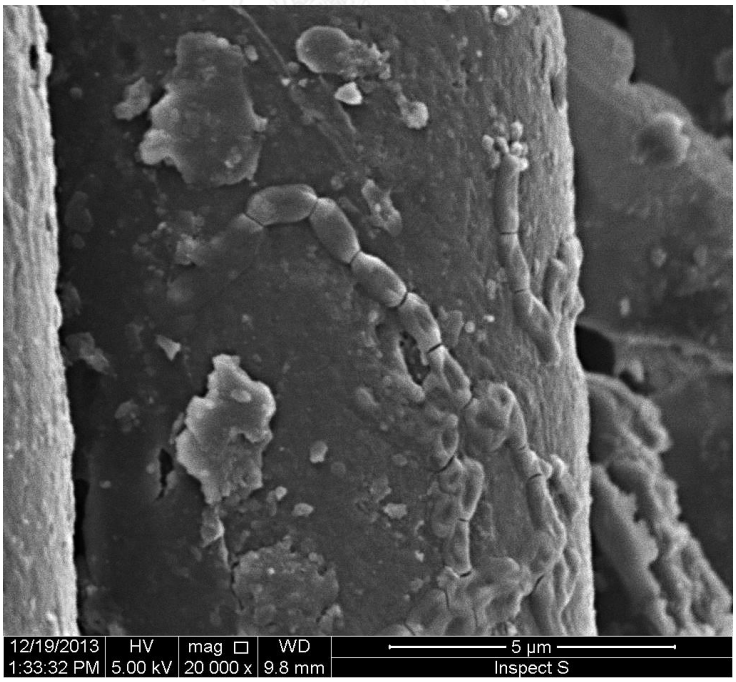

Figure 4.15 SEM of polyester (square bag) after buried for 10 weeks

4.4 Fungal isolation

Ninety five fungal isolates were obtained from buried fabric specimens by dilution plate methods or single point inoculation from samples which were highly degraded. A typical isolation plate is shown in Figure 4.16 below. A *Trichoderma* species with its characteristic green sporulation and a *Fusarium* species producing red pigmentation can be seen.

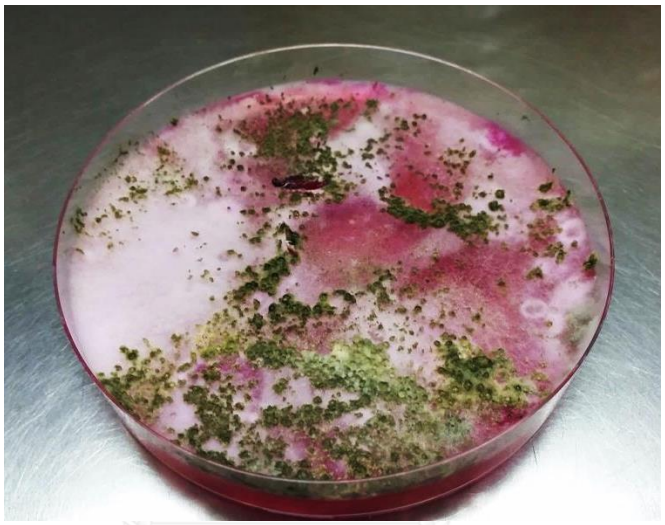

Figure 4.16 Typical of fungi grown on Rose Bengal Agar was prepared by dilution plating method of buried fabric specimens.

4.5 Celluase activity

4.5.1 Determination of cellulolytic enzymes on Carboxymethylcellulose Agar

To select the cellulase-producing fungi, ninety five fungal isolates were cultured on Carboxymethylcellulose Agar. Determination of cellulose degradation based on carboxymethylcellulose assays following the methodology of Pointing (88) is presented in Table 4.5. The most active isolates according to the ratio of the hydrolysis zone diameter to fungal colony diameter were isolates 11, 91 and 21 with ratios of 3.00, 2.67 and 2.50. A typical CMC hydrolysis plate is given in Figure 4.17.

Table 4.5 Screening of cellulase producing fungi on Carboxymethylcellulose Agar

Fungal isolate no.	Diameter of colony (cm)	Diameter of clear zone (cm)	Ratio of hydrolysis zone diameter to colony diameter
Isolate 2	3.1	3.7	1.19
Isolate 7	1.4	3.4	2.43
Isolate 8	1.2	1.4	1.17
Isolate 11	1.8	5.4	3.00
Isolate 12	1.6	3.6	2.25
Isolate 21	1.6	4.0	2.50
Isolate 32	2.8	3.6	1.29
Isolate 40	0.8	1.9	2.38
Isolate 75	1.5	3.7	2.47
Isolate 83	0.8	1.0	1.25
Isolate 87	1.5	2.8	1.87
Isolate 91	1.5	4.0	2.67

Figure 4.17 CMC hydrolysis zones around fungal colonies

4.5.2 Cellulolytic activity in liquid medium

The above three isolates were further investigated in liquid culture of 1% CMC in Mandels medium. The results are presented in Figure 4.18. This shows that the isolate number 11 (subsequently identified as *Trichoderma koningiopsis*) exhibited the highest endogluconase activity after 3 days. The two *Penicillium* isolates also had the greatest activity after 3 days. These activities were measured at 7.74 U/ml (isolate 11), 6.33 U/ml (isolate 91) and 4.78 U/ml (isolate 21). Activity declined after 3 days to 4.01 U/ml, 3.65 U/ml and 2.75 U/ml respectively. Isolates 11 and 91 then peaked again after 9 days at 6.38 U/ml and 6.02 U/ml. After 21 days isolate 91 had an activity level of 6.66 U/ml and isolate 11 6.35 U/ml. Isolate 21 had lower activity at 4.66 U/ml. The highest rate of endogluconase activity in all 3 isolates occurred after only 3 days incubation and is therefore rapid when compared with other organisms e.g *Daldinia eschscholtzii* (Ehrenb. : Fr.) Rehm where maximum activity of 8.64 U/ml was achieved after 10 days (94). It is worth noting that commercial production of endogluconases usually involves fungal species belonging to

Trichoderma and *Aspergillus* (95). Interestingly *Trichoderma koningiopsis* and *Penicillium* species were found to be the predominant cellulolytic fungi in tropical and subtropical forests in China with *Trichoderma koningiopsis* producing the highest level of β-glucosidase (96).

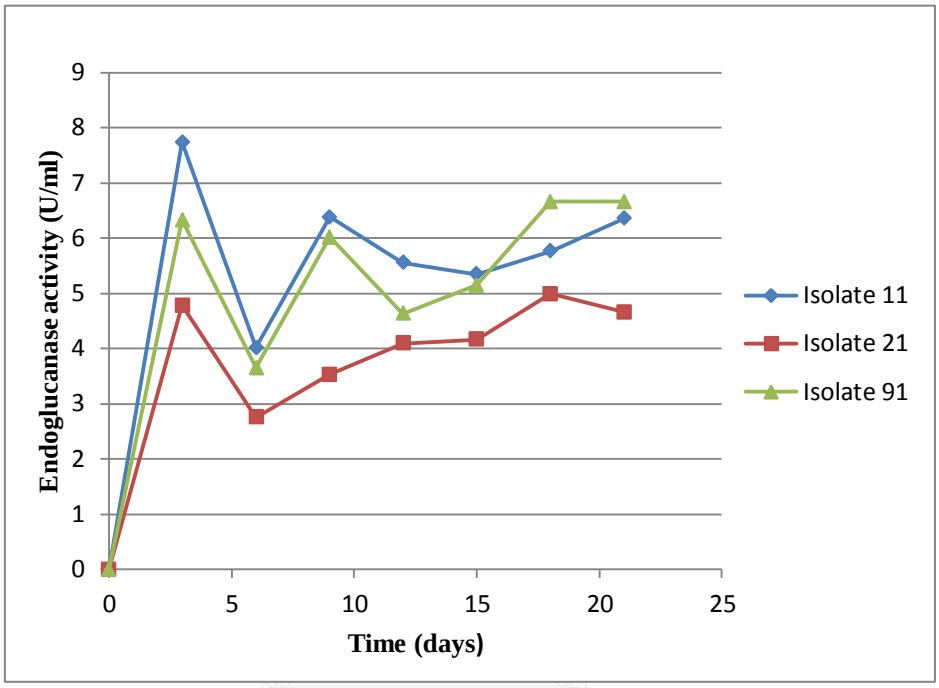

Figure 4.18 Comparisons time course of endoglucanase production by isolate 11, isolate 21 and isolate 91

4.6 Fungal identification

Identification was based on light microscopy of characteristic morphological features, mainly sporing structures, and when possible isolates were assigned to known genera. DNA sequences were also used and these enabled confidant identification of a number of the fungi isolated.

Isolate 11

Growth of fungal isolate11 and spores under the microscope are shown in Figure 4.19. This is identified as a *Trichoderma* species on the basis of its cultural characteristics and conidiophore morphology.

To confirm species level of fungal isolate 11, molecular investigation was also used. The 18S rRNA gene region of fungal isolate 11 was partially amplified, sequenced and submitted to GenBank database. The obtained sequence was compared with those in the National Center for Biotechnology Information Nucleotide Sequence Database (NCBI) by using the Basic Local Alignment Search Tool (BLAST) algorithm. The sequence of fungal isolate 11 approximated size 350 bp exhibited the highest level of homology (99% identity) with *Trichoderma koningiopsis* (accession number JQ278020.1)

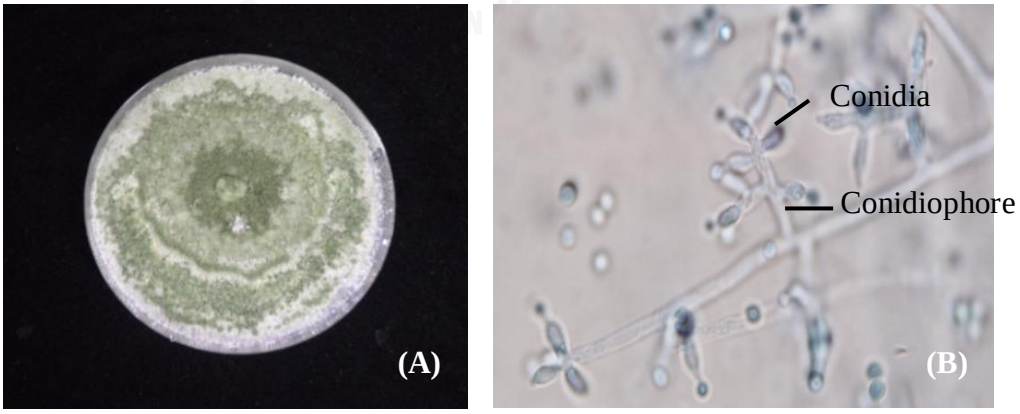

Figure 4.19 Growth of *Trichoderma koningiopsis* (isolate 11) on Potato Dextrose Agar after 7 days of incubation (A), *Trichoderma koningiopsis* conidiophores and conidia under the microscope (B)

Isolate 21

Growth of fungal isolate 21 and spores under the microscope are shown in Figure 4.20. This is a species of *Penicillium* on the basis of its typical conidiophore morphology.

To confirm species level of fungal isolate 21, molecular investigation was also used. The sequence of fungal isolate 21 approximated size 350 bp exhibited the highest level of homology (100% identity) with *Penicillium commune* (accession number KF018446.1)

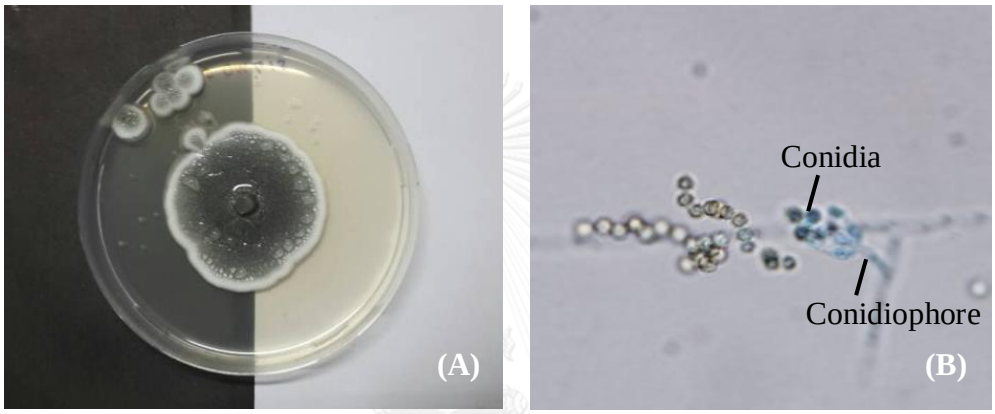

Figure 4.20 Growth of *Penicillium commune* (isolate 21) on Potato Dextrose Agar after 7 days of incubation (A), *Penicillium commune* conidiophores and conidia under the microscope (B)

Isolate 91

This is also a *Penicillium* species but is not the same species as isolate 21 (Fig. 4.21).

To confirm species level of fungal isolate 91, molecular investigation was also used. The sequence of fungal isolate 91 approximated size 350 bp exhibited the highest level of homology (99% identity) with *Penicillium decumbens* (accession number KF857287.1)

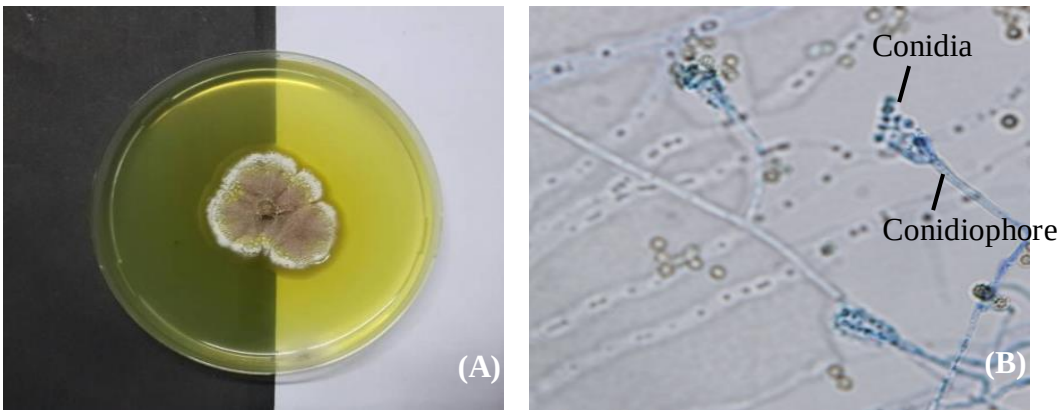

Figure 4.21 Growth of *Penicillium decumbens* (isolate 91) on Potato Dextrose Agar after 7 days of incubation (A), *Penicillium decumbens* conidiophores and conidia under the microscope (B)

4.7 Molecular investigation using DGGE

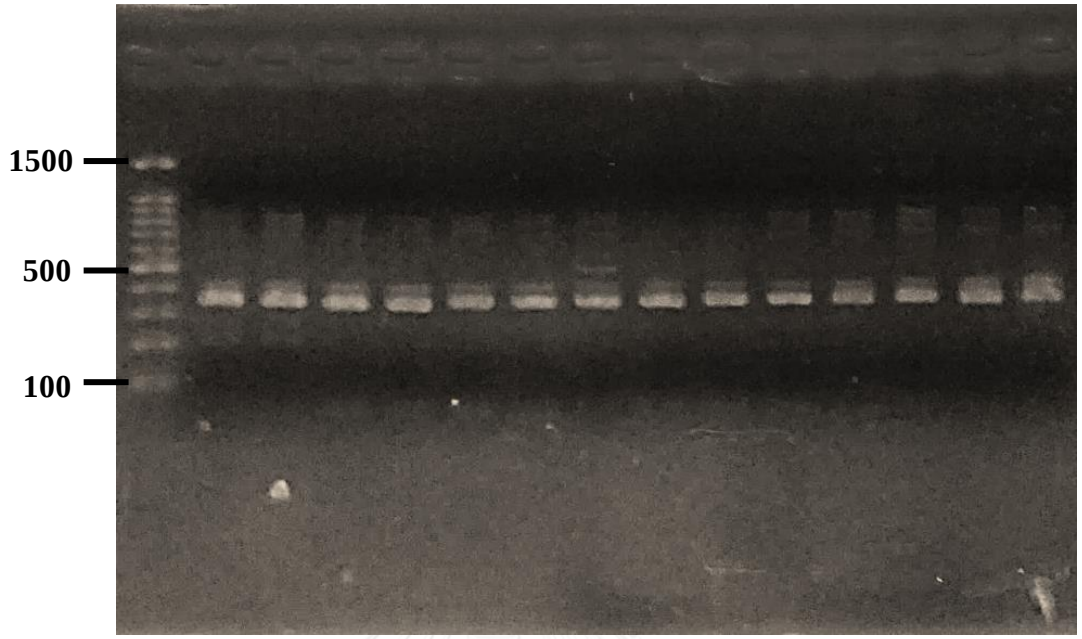

Figure 4.22 Agarose gel electrophoresis of PCR products (350 bp)

DNA was extracted from every soil and fabric sample and amplified by the primer set Fung-GC and NS1 targeting the 18S rRNA gene region. PCR products were approximately 350 bp in length (Fig. 4.22). Amplicons of the same length but different nucleotide compositions are separated in a denaturing gradient gel of polyacrylamide, based on their differential denaturation profile (97, 98). After the DGGE was performed the 16 selected DNA fragments were taken for sequencing. The BLAST results are given in Tables 4.6 and 4.7. The tables confirmed that many fungi were found with *Trichoderma koningiopsis*, *Penicillium decumbens*, *Fusarium solani*, *Trichoderma viride* and *Iodowynnea auriformis* being known cellulase producers.

The DGGE profiles from gel 1 (Fig. 4.23) show the DNA bands extracted from soil, briefs and cotton bags. It was found that *Trichoderma koningiopsis* and *Trichoderma viride* are dominant microorganism in both cotton samples. The bands A-1, B-1, C-2, D-2, E-2, F-1, H-1, I-1, J-1, K-2 and L-2 had the same migration distances as *Trichoderma koningiopsis* (isolate 11). It can be suggested that these bands are probably of *Trichoderma koningiopsis* as well. Another row of DGGE profile that was found every week were detected as *Trichoderma viride*. Moreover, isolate 21 (*Penicillium commune*) was found mainly in all the cotton bag samples.

The DGGE profiles from gel 2 (Fig. 4.24) show DNA bands extracted from polyester. It has the same dominant microorganisms as gel 1, *Trichoderma koningiopsis* and *Trichoderma viride*. The band of *Trichoderma koningiopsis* showed high intensity every week while *Trichoderma viride* showed high intensity only in weeks 4, 6 and 8. In addition, some uncultured fungi were recorded.

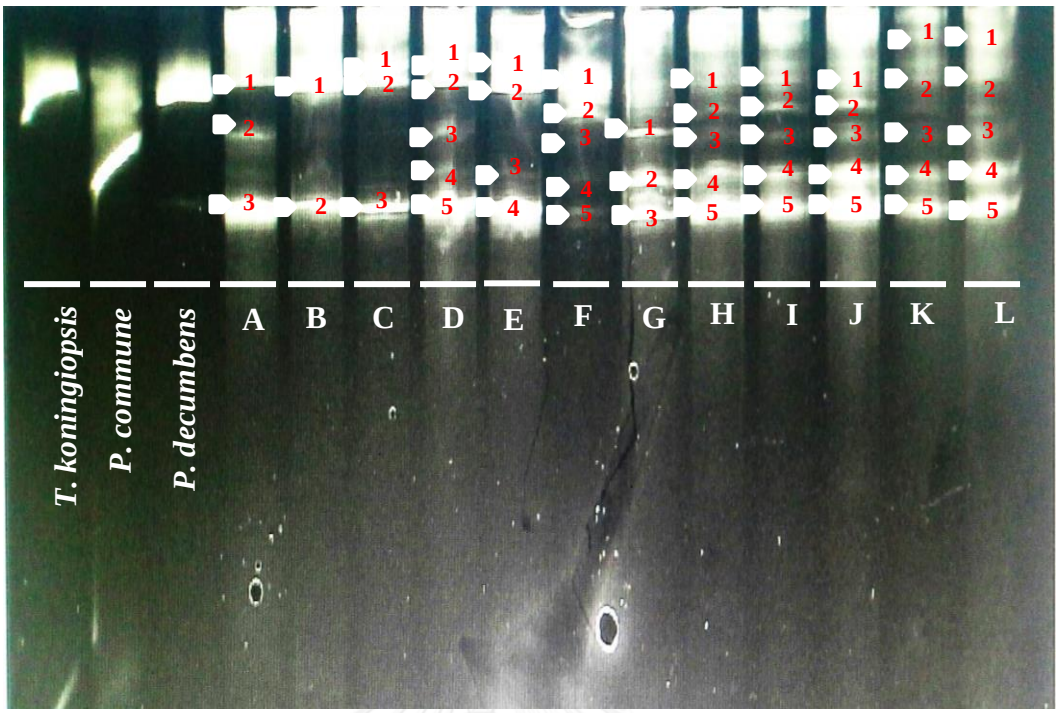

Figure 4.23 (gel 1) DGGE profiles of fungal partial 18S rRNA gene amplicons amplified from the soil and fabric samples (briefs and cotton bags). The lane labels are shown below.

Lane A = Soil week 0
Lane B = Soil week 10
Lane C = Brief week 2
Lane D = Brief week 4
Lane E = Brief week 6
Lane F = Brief week 8
Lane G = Brief week 10
Lane H = Cotton (square bag) week 2
Lane I = Cotton (square bag) week 4
Lane J = Cotton (square bag) week 6
Lane K = Cotton (square bag) week 8
Lane L = Cotton (square bag) week 10

Table 4.6 Most closely related sequences to those derived from selected 18S rRNA gene seperated by DGGE (gel 1)

Band	Most closely related fungal sequence	Accession no.	Identity (%)
K-1, L-1*	*Fusarium oxysporum* strain F-14	KJ126877.1	98%
C-1*, D-1, E-1	*Myceliophthora hinnulea* strain ATCC 52474	KF356953.1	98%
Isolate 11*, A-1, B-1, C-2,D-2, E-2, F-1, H-1, I-1*, J-1, K-2, L-2	*Trichoderma koningiopsis* strain T-440	JQ278020.1	99%
Isolate 91*, J-2	*Penicillium decumbens* strain ZQ001	KF857287.1	99%
H-2, I-2*	*Melanocarpus albomyces* strain ATCC 16460	JQ067911.1	98%
A-2*, D-3, G-1	*Fusarium* sp. MBS1	FJ613599.1	99%
Isolate 21*, F-3, H-3, I-3, J-3, K-3, L-3	*Penicillium commune* strain 4.5	KF018446.1	100%
D-4*, E-3, F-4, G-2, H-4, I-4, J-4, K-4, L-4	*Fusarium solani* strain 421502	EF397944.1	99%
A-3, B-2, C-3, D-5, E-4, F-5, G-3, H-5*, I-5, J-5, K-5,L-5	*Trichoderma viride* strain HS-F9	FJ598872.1	99%

* = selected 18S rRNA gene amplicons

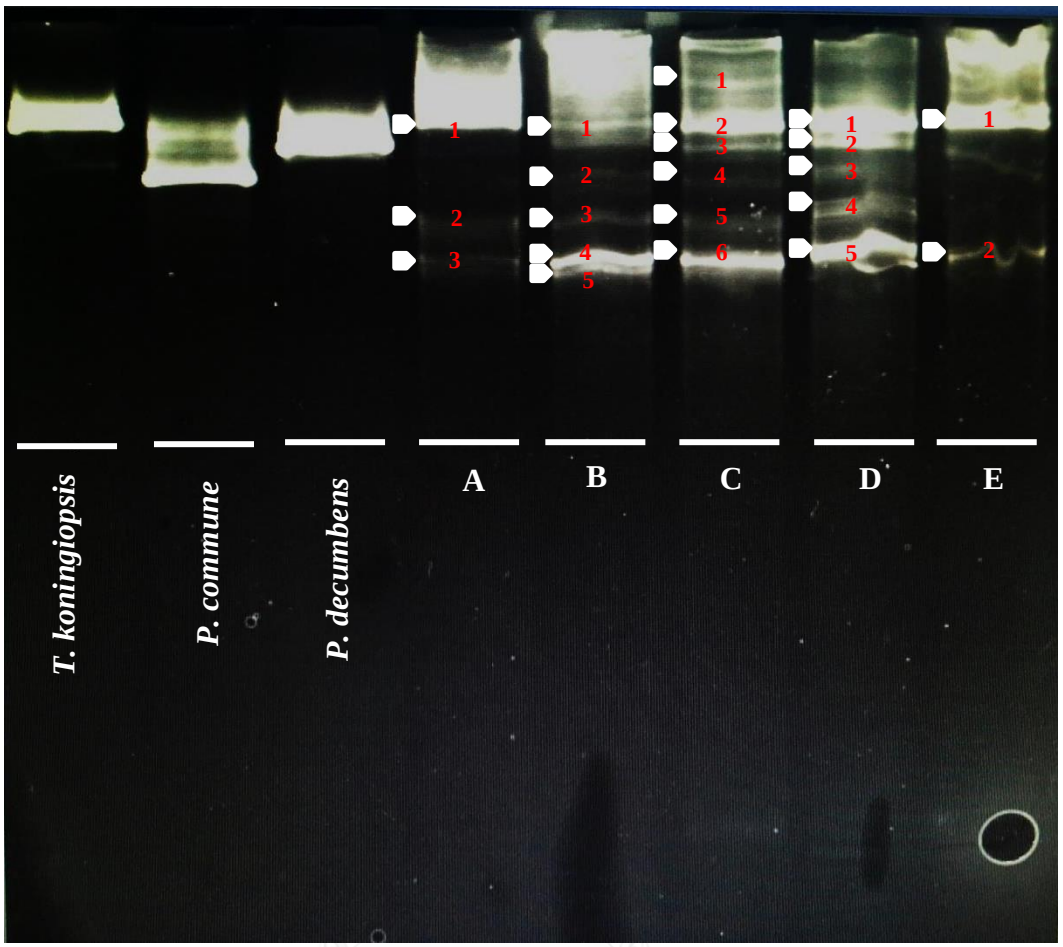

Figure 4.24 (gel 2) DGGE profiles of fungal partial 18S rRNA gene amplicons amplified from the polyester fabric samples. The lane labels are shown below.

Lane A = Polyester square bag week 2

Lane B = Polyester square bag week 4

Lane C = Polyester square bag week 6

Lane D = Polyester square bag week 8

Lane E = Polyester square bag week 10

Table 4.7 Most closely related sequences to those derived from selected 18S rRNA gene seperated by DGGE (gel 2).

Band	Most closely related fungal sequence	Accession no.	Identity (%)
Isolate 11*, A-1, B-1, C-2, D-1, E-1	*Trichoderma koningiopsis* strain T-440	JQ278020.1	99%
D-2*	*Gliocladium* sp. AAT-TS-4	EU581866.1	98%
C-3*	*Iodowynnea auriformis*	DQ646530.1	99%
Isolate 91*	*Penicillium decumbens* strain ZQ001	KF857287.1	99%
Isolate 21*, C-4, D-3	*Penicillium commune* strain 4.5	KF018446.1	100%
B2*	Uncultured fungus clone Pa_1(33)	HM534578.1	87%
A-2*, B-3, C-5	Uncultured eukaryote clone 2 isolation-source soil oxic incubation T0h	KF357064.1	99%
A-3*, B-4, C-6, D-5, E-2	*Trichoderma viride* strain HS-F9	FJ598872.1	99%
B5*	*Fusarium oxysporum*	GU212653.1	98%

* = selected 18S rRNA gene amplicons

CHAPTER 5
DISCUSSION AND CONCLUSIONS

5.1 Discussion

The investigation into the microbial degradation of a natural textile (cotton) and a man-made textile (polyester) was undertaken with 2 main themes involved. Firstly the degradation of the two different textiles was compared and consistent with published data (5) cotton could be completely degraded over either 6 weeks or 10 weeks depending on the season of burial. As was expected decomposition was fastest in samples buried during the rainy season. Two different cotton samples were tested and in general, although there were differences between their degradation in some weeks, by the end of the experimentation of 10 weeks in the dry season or 6 weeks in the rainy season the cotton was completely degraded. The degradation was followed week by week by visual observation by eye, light microscopy, scanning electron microscopy and by following weight loss. All the results obtained demonstrated the more rapid degradation during the rainy season and with little difference in soil temperature between the two seasons moisture content of the soil is concluded to be the most important factor. Although visual observation proved to be useful in following changes weight loss over a timed period is a more reproducible method. Tensile strength testing is generally not feasible since it was found that even with the slower rate of degradation in the UK soil the briefs were very susceptible to tearing after only 6 weeks burial (4). In common with other investigations polyester clothing remained virtually unchanged (4).

A second aspect concerned differences in decomposition between burial in a tropical soil compared to burial in a temperate soil in the UK. The investigation in the UK was undertaken to determine the potential value of decomposition of clothing buried in soil as a useful source of data for forensic

evidence. This investigation involved the burial of ladies briefs which had been immersed in a solution of 5% urea and 2% glucose prior to burial to simulate the leakage of body fluids from a buried corpse (4). The briefs were buried at a depth of 15-20 cm in Cilcain Hall Woods, Mold, Flintshire, North Wales, UK, a deciduous woodland soil of pH between 6.7 -7.3. Each of the briefs were wrapped around a sponge, which had also been pre-treated with urea and glucose, ensuring that the briefs were in good contact with the soil and also prevented folding of the garments. In order to make the current investigation comparable with the UK investigation ladies briefs of the same manufacture were used and pre-treated in similar fashion. Surprisingly the pre-treatment and burial with sponges soaked in urea and glucose showed little differences in breakdown over the period of burial in the Thai soil. This differed from the results obtained in the UK where the presence of urea and glucose stimulated cellulose decomposition with *Copriniopsis echinospora* (Buller) Redhead, Vilgalys & Moncalvo being identified as the major decomposer (4). As it is also an ammonia-loving fungus (99) and therefore the leakage of urea into the soil is likely to stimulate *Copriniopsis echinospora* (99). There are differences in the soil types involved and therefore microorganisms available but the lower temperatures in the UK during the burial period are considered to be a major factor in the slower rate of decomposition. In Thailand the greatest rate of degradation was found to be 6 weeks in the rainy season and 10 weeks in the dry season. In the UK it was 20 weeks for almost complete decomposition although good fungal colonization of the briefs was evident from week 6 (4).

The Thai investigation involved more detailed and different approaches to the UK investigation with greater emphasis on comparison of degradation by time period using weight loss, direct observation of the samples including scanning electron microscopy, enzymes assays for a selection of the fungi isolated from the cotton samples and DNA studies. Molecular identification is invaluable when working with fungi which are difficult to identify to species

level using traditional taxonomic methodology. DGGE was also particularly useful in following changes in populations with time and in the current investigation provided a different insight into the succession of fungi on the cotton samples investigated. BLAST searches on DNA sequences from fungi isolated enabled the identification of a number of the fungal isolates including well known cellulose decomposing *Trichoderma viride*, *Trichoderma koningiopsis* and *Fusarium oxysporum*. The enzyme assays found *Trichoderma koningiopsis* to produce the most endogluconase followed by 2 *Penicillium* species which is in close agreement with a recent Chinese study by Zhang, Liu (96). Although not isolated a *Streptomycete* species was observed on Thai samples when they were examined by SEM. *Streptomyces* and other actinomycetes are known to be able to degrade cellulose (101).

It has been shown that decomposition of cellulose as cotton fabrics occurs at a greater rate in the Thai soil as opposed to that of the temperate UK soil. However another important aspect of the current investigation was to assess the potential forensic value of decomposition of clothing as if it was on a buried corpse. Although there have been many studies on changes occurring with time of burial these have concentrated on temperate and occasionally subtropical soils (69). Surprisingly there have been very few studies undertaken with tropical soils. One exception is from Coral Atolls in the tropical Pacific (102) and therefore this investigation was intended to provide further information on this aspect but in a tropical forest soil. The Thai investigation confirmed that ladies briefs made from cotton can decompose completely with time with only the elastic waist band and polyester label remaining. Likewise in common with other reports on microbial degradation of man-made materials the polyester briefs remained virtually unchanged following prolonged burial. To what extent the decomposition of the cotton briefs, and other clothing, can be of value as forensic evidence is still to be proven but a major review on mycology in forensic studies highlights a number of aspects worthy of

consideration (79). The studies in the UK and Thailand demonstrate that decomposition of cotton clothing occurs rapidly in soils with a tropical Thai soil resulting in total decomposition by 6 weeks during the rainy season. In the UK decomposition was slower but even so major destruction of the cotton briefs was evident after 16 weeks with virtually complete degradation at 20 weeks.

5.2 Conclusions

It is concluded that decomposition of cotton fabric in the Thai tropical soil takes place at 2-3 times the rate of similar decomposition in a temperate soil in the UK according to season.

The environmental factors which are indicated as most important are soil moisture levels in Thailand but temperature in the UK.

The effect of urea and glucose were greater in the UK soil with the ammonia-loving *Copriniopsis echinspora* being an important cellulose decomposer. In the Thai soil *Trichoderma* species, *Fusarium oxysporum* and *Penicillium* species were prominent and were identified by traditional taxonomic methods and their identity confirmed by 18S rDNA sequences aligned with GenBank databases using BLAST searches. Studies on cellulose decomposers from tropical and subtropical soils in China showed *Trichoderma* and *Penicillium* species to be predominant and as found in the current study *Trichoderma koningiopsis* exhibited the greatest endogluconase activity (96). Interestingly other well known cellulose decomposing fungi such as *Chaetomium globosum* Kunze.: Fr, (5) were not recovered in either the UK or Thai investigations,

It is suggested that decomposition of clothing has potential in forensic investigations but reliability will require careful examination of the soil properties and the environmental conditions at the place and time of burial. Ladies briefs were chosen since many burials involve bodies buried clothed or

partially clothed however the trend in wearing polyester underclothing in place of cotton makes decomposition to have little forensic application. However investigations similar to this may be of value in determining whether the body has been moved from one site to another.

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APPENDICES

APPENDIX A

Rainfall and Temperature Profiles

1. Amount of rainfall (mm)

Station: 478201-Rayong, Rayong province

Table A.1 The amount of rainfall in Rayong province

Month/Year	Total
Jan-13	116.4
Feb-13	5
Mar-13	36.3
Apr-13	96.9
May-13	130
Jun-13	338.1
Jul-13	212.7
Aug-13	166.4
Sep-13	265.8
Oct-13	295.7
Nov-13	69.1
Dec-13	11.4

2. Average air temperature (°C)

Station: 478201-Rayong, Rayong province

Table A.2 The average air temperature in Rayong province

Month/Year	Average air temperature
Jan-13	26.6
Feb-13	28.1
Mar-13	29.2
Apr-13	29.8
May-13	30.3
Jun-13	29.0
Jul-13	28.5
Aug-13	28.5
Sep-13	28.1
Oct-13	26.9
Nov-13	27.4
Dec-13	24.1

APPENDIX B
Media

1. Potato Dextrose Agar (PDA)

Potato	200	g
Dextrose	20	g
Agar	15	g
Distilled water	1,000	ml

To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1,000 ml distilled water for 30 minutes. Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form). Mix in other ingredients and boil to dissolve. Sterilization in an autoclave for 15 min at 121°C. Then aseptically transfer to Petri dishes.

2. Rose Bengal Agar

Dextrose	10	g
Peptone	5	g
KH_2PO_4	1	g
$MgSO_4 \cdot 7H_2O$	0.5	g
Agar	15	g
Rose Bengal	33	mg
Distilled water	1,000	ml

Dissolve the above compositions in 1,000 ml of distilled water. Sterilization in an autoclave at 121ºC for 15 min. Cool to 45-50ºC and add 1 ml of streptomycin to the medium (Dissolve 0.3 g streptomycin with 1,000 ml sterilized distilled water). Then aseptically transfer to Petri dishes.

3. Cellulolysis basal medium (CBM)

$C_4H_{I2}N_20_6$	5	g
Yeast Extract	0.1	g
KH_2P0_4	1	g
$CaC1_2 \cdot 2H_20$	0.001	g
$MgS0_4 \cdot 7H_20$	0.5	g
Distilled water	1,000	ml

Basal medium may be conveniently stored as a 10 x sterilized stock. It is also possible to simplify the procedure by using less defined basal growth medium such as peptone plus yeast extract, or malt extract. Such basal media used at a concentration of 0.1-0.2 % w/v are sufficient to support cellulose degrading fungi.

4. Carboxymethylcellulose agar (CMC agar)

Carboxymethylcellulose sodium salt	20	g
Agar	16	g
Cellulolysis basal medium (CBM)	1,000	ml

Dissolve Carboxymethylcellulose sodium salt with CBM 900 ml thoroughly. After that the CBM was added to reach 1,000 ml. Sterilization in an autoclave at 121ºC for 15 min. Then aseptically transfer to Petri dishes.

APPENDIX C

Dye Staining of Carboxymethylcellulose Agar

Dye staining of carboxymethylcellulose agar (CMC agar) (88)

Procedure

1. Prepare CBM medium supplemented with 2% w/v low viscosity CMC and 1.6% w/v agar and autoclave.
2. Aseptically transfer to Petri dishes.
3. Inoculate with test fungus.
4. Incubate at 25 ℃ in darkness. When the colony diameter is approximately 30 mm (2-5 days), stain agar plates as follows:
5. Flood the plates with 2% w/v aqueous congo red (C.I. 22120) and leave for 15 min.
6. Pour off stain and wash the agar surface with distilled water.
7. Flood the plates with 1 M NaCl to destain for 15 min.
8. Pour off destain. CMC degradation around the colonies will appear as a yellow-opaque area against a red colour for undegraded CMC**.**

APPENDIX D
Dinitrosalicylic Acid Method

Reducing sugar determination by Dinitrosalicylic Acid Method (91)

1. Reagent and preparation

3, 5-dinitrosalicylic acid	10	g
Na_2SO_3	0.5	g
Na-K tartrate	200	g
NaOH	10	g
Phenol	2	g
Distilled water	1000	ml

NaOH 10 g are added into 500 ml of distilled water and mixed in order to add the 200 g Na-K tartrate. When the solution dissolved, 3, 5-dinitrosalicylic acid 10 g is then added and continuously stired. After that, the 0.5 g of Na_2SO_3 and 2.0 g of phenol are dissolved, respectively. Finally, the volume is adjusted to 1,000 ml by distilled water and kept away from light.

2. Procedure

1. The diluted sample 1.0 ml is mixed with 1.0 ml of DNS solution. The mixture is boiled for 5 min.

2. The sample is cooled down by immersing the sample tube into cold water immediately. 10 ml of distilled water is added. The mixture was mixed well, and measured at absorbance 550 nm.

3. Absorbance 550 nm is converted to glucose concentration with standard curve.

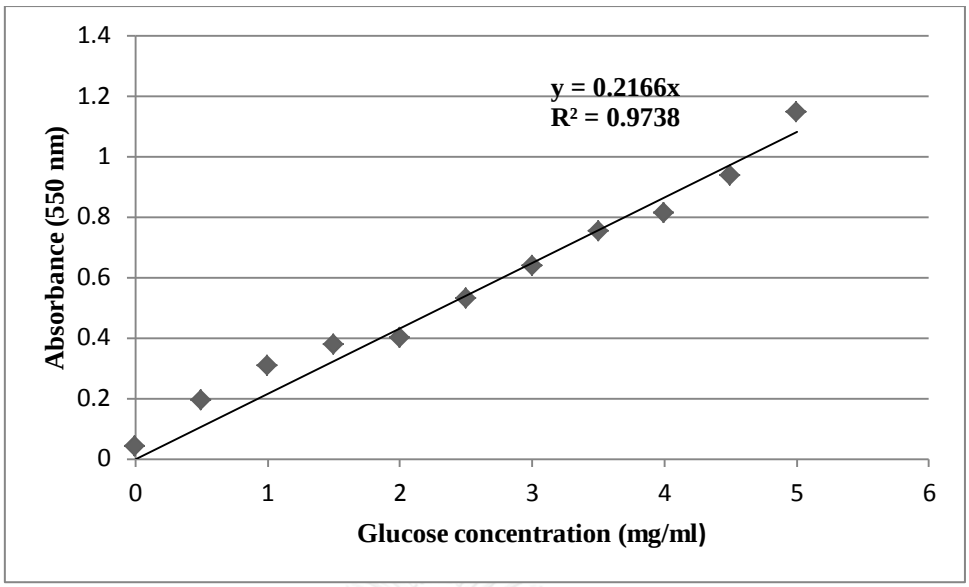

Figure D.1 Standard curve for reducing sugar determination by Dinitrosalicylic Acid Method

APPENDIX E
DNA Extraction by CTAB Method

Procedure

1. Grind fungal mycelium to a fine paste in approximately 400 µl of CTAB buffer and Rnase A 4 µl.

2. Incubate the CTAB/fungal extract mixture for 1 hour at 65 °C in a water bath.

3. After incubation, spin the CTAB/fungal extract mixture at 13,000 rpm for 10 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.

4. To each tube add 400 µl of Chloroform and mix the solution by inversion. After mixing, spin the tubes at 13,000 rpm for 10 min.

5. Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.

6. To each tube add 1/10 of total mixture volume of 3 M Ammonium Acetate followed by 1x of total mixture volume of ice cold isopropanol.

7. Invert the tubes slowly several times to precipitate the DNA.

8. DNA precipitate can be isolated by spinning the tube at 13,000 rpm for 15 min. Remove the supernatant and wash the DNA pellet by adding two changes of ice cold 70% ethanol.

9. After the wash, spin the DNA into a pellet by centrifuging at 13,000 rpm for 10 min. Remove all the supernatant and allow the DNA pellet to dry (approximately 1 hour).

10. Resuspend the DNA in sterile DNase free water (approximately 40-100 µl H_2O).

11. Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

APPENDIX F

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

Table F.1 Composition of gel stock solution for DGGE (8% gel)

Composition	Denaturing gradient	
	25% denaturing solution	55% denaturing solution
40% acrylamide/bis 37.5:1	20 ml	20 ml
50x TAE	2 ml	2 ml
Formamide (deionized)	10 ml	22 ml
Urea	10.5 g	23.1 g
Ultrapure water	68 ml	56 ml

APPENDIX G

Nucleotide Sequences

Trichoderma koningiopsis **(Isolate 11)**

TGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTA
TAAGCAATTATACCGCGAAACTGCGAATGGCTCATTATATAAGTTAT
CGTTTATTTGATAATACTTTACTACTTGGATAACCGTGGTAATTCTA
GAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGTTGTATTTAT
TAGATTAAAAACCAATGCCCCTCGGGGCTCTCTGGTGAATCATAAT
AACTAGTCGAATCGACAGGCCTTGTGCCGGCGATGGCTCATTCAAA
TTTCTTCCCTATCAACTTNCGA

Penicillium commune **(Isolate 21)**

NNTTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA
AGTATAAGCAACTTGTACTGTGAAACTGCGAATGGCTCATTAAATC
AGTTATCGTTTATTTGATAGTACCTTACTACATGGATACCTGTGGTA
ATTCTAGAGCTAATACATGCTAAAAACCCCGACTTCAGGAAGGGGT
GTATTTATTAGATAAAAAACCAACGCCCTTCGGGGCTCCTTGGTGAA
TCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCA
TTCAAATTTCTGCCCTATCAACTT

Penicillium decumbens **(Isolate 91)**

CCNTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA
AGTATAAGCACTTTATACTGTGAAACTGCGAATGGCTCATTAAATCA
GTTATCGTTTATTTGATAGTACCTTACTACATGGATACCTGTGGTAA
TTCTAGAGCTAATACATGCTAAAAACCCCGACTTCAGGAAGGGGTG
TATTTATTAGATAAAAAACCAACGCCCTCCGGGGCTCCTTGGTGAAT
CATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCAT
TCAAATTTCTGCCCTATCAACTTCGA

Fusarium oxysporum **(Band L-1, Gel 1)**

NTTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAA
GTATCAGCAATTATACCGCGAAACTGCGAATGGCTCATTATATAAG
TTATCGTTTATTTGATAATACTTTACTACTTGGATAACCGTGGTAATT
CTAGAGCTAATACATGCTAAAGATCCCGACTTCGGAAGGGATGTAT
TTATTAGATTAAAAACCAATGCCCTCCGGGGCTCACTGGTGATTCAT
GATAACTCCTCGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCA
AATTCTTCCCTATCAACTTCGA

Myceliophthora hinnulea **(Band C-1, Gel 1)**

NNCCTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCGTGTCT
AGGTATAAGCAATTATACAGCGAAACTGCGAATGGCTCATTAAATC
AGTTATCGTTTATTTGATATTACCTTACTACATGGATAACCGTGGTA
ATTCTAGAGCTAATACATGCAAAAAATCCCGACTTCGGAAGGGATG
TATTTATTGGATTAAAAACCAATGCCCTTCGGGGCTCTCTGGTGATT
CATAATAACTTCTCGAATCGCACGGCCTTGCGCCGGCGATGGTTCAT
TCAAATtTCTGCCCTATCAACTT

Trichoderma koningiopsis **(Band I-1, Gel 1)**

TTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAG
TATAAGCAATTATACCGCGAAACTGCGAATGGCTCATTATATAAGTT
ATCGTTTATTTGATAATACTTTACTACTTGGATAACCGTGGTAATTCT
AGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGTTGTATTT
ATTAGATTAAAAACCAATGCCCCTCGGGGCTCTCTGGTGAATCATA
ATAACTAGTCGAATCGACAGGCCTTGTGCCGGCGATGGCTCATTCA
AATTCTTCCCTATCAACTTCGATGTT

***Melanocarpus albomyces* (Band I-2, Gel 1)**

NTTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAA
GTATAAACACGTTATACTGTGAAACTGCGAATGGCTCATTAAATCA
GTTATCGTTTATTTGATAGTACCTTACTACATGGATAACCGTGGTAA
TTCTAGAGCTAATACATGCTGAAAATCCCGACTTCGGAAGGGATGT
ATTTATTAGATTAAAAACCAATGCCCTTCGGGGCTCTCTGGTGATTC
ATAATAACTTCTCGAATCGCACGGCCTTGCGCCGGCGATGGTTCATT
CAAATTTCTGCCCTATCAACTTCGACGG

***Fusarium* sp. (Band A-2, Gel 1)**

NTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGT
ATAAGCAATTATACAGCGAAACTGCGAATGGCTCATTAAATCAGTT
ATCGTTTATTTGATATTACCTTACTACATGGATAACCGTGGTAATTC
TAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATT
TATTAGATTAAAAACCAATGCCCTCCGGGGCTCACTGGTGATTCATG
ATAACTCCTCGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAA
ATTTCTTCCC

***Fusarium solani* (Band D-4, Gel 1)**

NNTTGTAGTCATaATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA
AGTATAAGCAATTATACAGCGAAACTGCGAATGGCTCATTATATAA
GTTATCGTTTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAA
TTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGT
ATTTATTAGATTAAAAACCAATGCCCTCCGGGGCTCACTGGTGATTC
ATGATAACTCCTCGAATCGCATGGCCTTGCGCCGGCGATGGTTCATT
CAAATTTCTTCCTATCAACTTTCGAGTTGG

***Trichoderma viride* (Band H-5, Gel 1)**

NNTTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA
AGTATAAGCAATTATACAGCGAAACTGCAAATGGCTCATCATATAA
GTTATCGTTTATTTGATAGTACCTTACTACATAGATAACCGTGGTAA
TTCTAGAGCTAATACATGCTAAAAATCCCAACTTCGGAAGGGATGT
ATTTATTAGATTAAAAACCAATGCCCCTCGGGGCTCTCTGGTGAATC
ATAATAACTAGTCGAATCGACAGGCCTTGTGCCGGCGATGGCTCAT
TCAAATTCTTCCC

***Gliocladium* sp. (Band D-2, Gel 2)**

TTTGTAGTCATAATCTTGTCTCAAAGATTAAGCCATGCATGTCTAAG
TATAAGCAATTATACGGCGAAACTGCGAATGGCTCATTATATAAGT
TATCGTTTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAATT
CTAGAGCTAATACATGCTTAAAATCCCGACTTCGGAAGGGATGTAT
TTATTAGATTAAAAACCAATGCCCTTCGGGGCTCTCTTGGTGATTCA
TGATAACTTCTCGAATCGCACGGCCCCGCGCCGGCGATGGTTCATTC
AAATTCTTCCCTATCAACTTC

***Iodowynnea auriformis* (Band C-3, Gel 2)**

NTTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAA
GTATAAGCAATCTATACAGTGAAACTGCGAATGGCTCATTAAATCA
GTTATCGTTTATTTGATGATACCTTACTACTTGGATAACCGTGGTAA
TTCTAGAGCTAATACATGCACAAAAATCCCGACCCCTGGAAGGGAT
GTATTTATTAGATAAAAAACCAATGCCTTCGGGCTCTTTGGTGATTC
ATAATAACTTAACGAATCGCATAGCCTTGTGCTGGCGATGGTTCATT
CAAATTTCTGCCCTATCAACTT

Uncultured fungus clone Pa_1 (33) (Band B-2, Gel 2)

NTTTGTATTCATTAGCTGTTTTCAAAGATTAACCCATCAATGTTTAA
GTATAACCAATTATACNGGGAAATTAGGAATGGTTCTTTATATAAGT
TTTGGTTTATTTGATAGTCCCTTATTACAGGAATAACGGTGGTAATT
TTAGAGTTAATCCATGTTAAAATTCCGAATTTGGGAAGGAAGGTATT
TATTGGATTAAAAACAAATCCCTTCTGGGCTCCTTGGTGATTCATGA
TAACTTCTCGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAA
TTCTTTCCCTATCAACTTTCGAGTTG

Uncultured eukaryote clone 2 isolation-source soil oxic incubation T0h (Band A-2, Gel 2)

NNTTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA
AGTATAAGCAATTATACCGCGAAACTGCGAATGGCTCATTATATAA
GTTATCGTTTATTTGACAATACTTTACTACTTGGATAACCGTGGTAA
TTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGTTGT
ATTTATTAGATTAAAAACCAATGCCCCTCGGGACTCTCTGGTGAATC
ATAATAACTAGTCGAATCGACAGGCCTTGTGCCGGCGATGGCTCAT
TCAAATTCTTCCCTATCAACTT

Trichoderma viride **(Band A-3, Gel 2)**

NNTTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA
AGTATAAGCAATTATACCGCGAAACTGCGAATGGCTCATTATATAA
GTTATCGTTTATTTGATAATACTTTACTACTTGGATAACCGTGGTAA
TTCTAGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGT
ATTTATTAGATTAAAAACCAATGCCCNTCGGGGCTCTCTGGTGAATC
ATAATAACTAGTCGAATCGCCAGGCCTTGTGCCGGCGATGGCTCATT
CAAATTCTCCC

***Fusarium oxysporum* (Band B-5, Gel 2)**

NNNTTGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTA
AGTATAAGCAATTATACAGCGAAACTGCGAATGGCTCATTATATAA
GTTATCGTTTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAA
TTCTAGAGCTAATACATGCTTAAAATCCCAACTTCGGAAGGGATGT
ATTTATTAGATTAAAAACCAATGCCCTCCGGGGCTCACTGGTGATTC
ATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATT
CAAATTTCTGCCCTNTCA

VITA

Miss Sasikarn Komkleow was born on February 24, 1989 in Rayong province, Thailand. She graduated with a Bachelor Degree of Science from Faculty of Science, Department of Microbiology, Chulalongkorn University in 2010. She had been studies for a Master Degree of Science in Microbiology, the Faculty of Science, Chulalongkorn University since 2014. She got a Scholarship from the Graduate school, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibala Aduladeja. Her thesis was presented in the 25th Annual Meeting of the Thai Society for Biotechnology and International Conference, The Emerald Hotel on October 16-19, 2013.