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THESIS

PHYLOGENETIC STUDY AND CHARACTERIZATION OF XYLANASE PRODUCED BY NEW SPECIES OF ACTINOMYCETES ISOLATED FROM THAI SOIL

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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A total of 13 isolates of actinomycete strains belonging to the family *Streptosporangiaceae*, isolated from Thai soil were tested for xylan degrading on xylan agar plate. It was found that 11 isolates belonged to the genera *Herbidospora*, *Microbispora*, *Microtetraspora* and *Nonomuraea*, showed the xylan degrading activity. Phylogenetic position, characteristic of the strains and their ability to degrade xylan had paid attention to strain DMKUA 205 and 245. Polyphasic taxonomy indicated that strain DMKUA 205 and 245 were proposed to be *H. sakaeratensis* sp. nov. and *M. siamensis* sp. nov., respectively. In addition, DNA-DNA relatedness values between *Streptosporangium claviforme* NBRC 15623^T and *H. cretacea* JCM 8553^T were higher than 70%, indicating that *S. claviforme* is related as *H. cretacea*. Therefore, the name *S. claviforme* should be treated as a synonym of *H. cretacea.* On the other hand, DNA-DNA relatedness values also showed that *M. amethystogenes* is a separate genomic species from *M. rosea* subsp. *rosea*. Therefore, use a combination of genotypic and phenotypic data *M. amethystogenes* was considered to merit species status. The thermotolerant strain DMKUA 245^T showed the highest xylanase activity when grown at 40°C. To improve the productivity of this strain, a three step-strategy was followed: screening of five factors (xylan, casein, $MgSO_4.7H_2O$, K_2HPO_4 and temperature), using a Plackett-Burman desing for the selection of the most critical variables, localization of the optima of the three most important quantitative factors, casein, $MgSO_4.7H_2O$ and temperature by response surface methodology with a Central composite design (CCD) and confirmation of the conditions determined with the quadratic model by comparison of the optimized conditions with the initial ones. Temperature was the main factor influencing the production of xylanase by the new thermotolerant *M. siamensis* DMKUA 245^T. The optimized medium consisted of (g/L): xylan, 10; casein, 0.16; $MgSO_4.7H_2O$, 0.05; K_2HPO_4, 0.1 and temperature of 45°C yielded 44 U/ml of xylanase activity in shaking flask experiments. Low casein concentration increased the xylanase activity and decreased proteolytic degradation of the xylanase. The maximum activity of 292 U/ml was achieved within 72 h cultivation with uncontrolled pH and an aeration rate of 0.5 vvm in the 3-L airlift fermenter, which increased by 49 folds compared to the un-optimized medium. The purified xylanase has specific activity of 219.4 U mg^{-1} proteins. SDS-PAGE demonstrated molecular weight of purified xylanase from the strain DMKUA 245 at about 65.8 kDa. The optimal pH and temperature for xylanase activity were 5.5 and 60ºC, respectively. The xylanase retained its activity over wide ranges of pH (4-11) and temperature up to 60ºC. The purified xylanase was stimulated by 1 mM Co^{2+}, K^+ and Mg^{2+}, whereas 1 mM Mn^{2+} and 5 mM EDTA inhibited enzyme activity. The purified xylanase was highly specific towards xylan, indicating that is a true xylanase. The K_m value for beechwood xylan was 3.3 mg mL^{-1}. According to hydrolysis production resulting in series of short-chain xylooligosaccharide, the purified enzyme was indicated to be an endo β-1,4-xylanase.

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Antika Boondaeng

May 2011

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LIST OF ABRREVIATION

Adjusted R^2	=	The adjusted coefficient of determination
DO or C_L	=	Dissolved oxygen concentration (% saturation and mg/l)
DP	=	Degree of polymerization
OUR	=	Oxygen uptake rate (s^{-1})
R	=	The Pearson correlation coefficient
R^2	=	The coefficient of determination
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PHYLOGENETIC STUDY AND CHARACTERIZATION OF XYLANASE PRODUCED BY NEW SPECIES OF ACTINOMYCETES ISOLATED FROM THAI SOIL

INTRODUCTION

Actinomycetes are Gram positive, aerobic, mycelial bacteria with high G+C ratio which form branching filaments that may persist as a stable mycelium or may break up into rod shaped or coccoid elements. The actinomycetes are universal occurrence in nature such as in soil, which have 10-50% of total microorganisms and found in fresh waters, in marine environment, in mud, in dust, in manures, in composts, on plant residues and on food products and their occurrence is greatly influenced by environmental conditions such as geography, agriculture, temperature, pH, organic matter, air distribution or humidity (Labeda and Shearer, 1990). They play an important role in decomposition of organic materials, such as cellulose and chitin and play a critical role in recycling organic matter and carbon cycle. Thus replenishing the supply of nutrients in the soil is as a vital part of the accumulated humus. They are the primary decomposers of tough plant materials by softening them up for their less enterprising relatives. They are the best known for their ability to produce bioactive compounds, such as antibiotics, vitamins and enzymes (McMarthy and Williams 1992; Sanglier *et al.,* 1996; Horan 1999; Lazzarini *et al*., 2000).

Among actinomycetes, approximately 70-90% of total actinomycetes are found in soil belonging to the streptomycetes and other are considered as non-streptomycetes (Hayakawa *et al.,* 1988). Twenty-nine genera of atinomycetes were isolated from Yunnan, China. In tropical zone, 86% and 14% of actinomycetes were found to be streptomycetes and non-streptomycetes, respectively (Xu *et al*., 1996). Amount of 67% non-streptomycetes and 33% streptomycetes were isolated from mangrove rhizospheres by Hatano (1997). The streptomycetes are being extensively used for commercial production of different bioactive compounds and some

important enzymes. As the search for producers of novel compounds continues, it becomes evident that many streptomycetes isolated from different environments produce the same compounds. Therefore, the chance of finding new biologically active molecules of actinomycetes is greatly reduced. However, non-streptomycetes, which are often very difficult to isolate and cultivate might represent a unique source of novel biologically active compounds (Baltz, 2006). Castiglione *et al.* (2007) reported planosporin producing *Planmonospora* sp., which is a novel antibiotic against gram-positive pathogens of medical importance, including multi-resistant clinical isolates. In 2011, an inulin fructotransferase was produced by *Nonomuraea* sp. isolated from Indonesian soil (Pudjiraharti *et al*., 2011). Suriyachadkun *et al.* (2003) identified 91 isolates of non-streptomycetes from Sakaerat Biosphere Reserve to be 14 genera, belonging to 5 families; *Nocardiaceae*, *Micromonosporaceae*, *Pseudonocardiaceae*, *Streptosporangiaceae* and *Thermomonosporaceae*. Some of these genera produced antibacterial and antifungal substances, and also produced enzymes to degrade organic polymer such as carboxymethylcellulose, lignin, chitin, starch and xylan.

Enzymes are the catalytic foundation of metabolism, and as such are the focus of intense worldwide research. They have played a central role in many manufacturing processes, such as in the production of wine, cheese, bread, modification of starch etc. Only in the past 2 decades, however, have microbial enzymes been used commercially in the pulp and paper industry. The use of xylanases in this industry has increased significantly with the discovery of Viikarri *et al*. (1986). Since then researchers have focussed their attention toward newer microbial isolates, the xylanases from which can be used in the pulp and paper industries.

Apart from its use in the pulp and paper industry, xylanases are also used in pre-treatment of forage crops and other lignocellulosic biomass, added to swine and poultry cereal-based diets to improve nutrient utilization, flour modification for bakery products, and saccharification of agricultural, industrial and municipal wastes (Sá-Pereira *et al*., 2002). Moreover, it is reported that xylanases have been widely used for clarifying fruit juices and wine (Hang and Woodams 1997), food processing in combination with cellulases (Biely, 1985), and improving the nutritional properties

of agricultural silage and grain feed (Kuhad *et al*., 1993). The attention on the applications of xylanase has led to discover many new enzymes with novel characteristics from various microorganisms (Wong *et al*., 1988).

In this study, 13 strains of actinomycetes belonging to Family *Streptosporangiaceae,* previously isolated from Sakaerat Biosphere Reserve (Suriyachadkun *et al.,* 2003) were determined for their ability on xylanase production. The highest xylanase producing strain was selected. Phylogenetic position of xylan degrading strains using 16s rRNA gene sequence was studied and a new strain were described. Statistical methods were applied to optimize the fermentation medium compositions for the improvement of xylanase production by the selected strain. The physical factors affecting the xylanase production was determined in an airlift fermenter. Subsequently, purification and characterization of the xylanase were investigated.

OBJECTIVES

1. To study phylogenetic position of β-xylanase producing actinomycete strains and describe new species.

2. To select the highest β-xylanase producing strain and investigate factors affecting β-xylanase production by the selected strain.

3. To develop fermentation processes of β- xylanase production by the selected strain using statistical method in shaking flasks and airlift fermenter.

4. To purify and characterize endo-β-xylanase from the selected strain.

LITERATURE REVIEW

1. Actinomycetes and their classification and identification

1.1 General Characteristics of Actinomycetes

Actinomycetes are fungi-like bacteria which comprise a group of branching filamentous microorganisms. They produce branching mycelium which may be of two kinds namely, substrate mycelium and aerial mycelium and form a various asexual spores such as conidia, chains of conidia including sporangia that contain spores. They are live mainly in soil and break down organic matter such as dead trees and leaves. They are well known as prolific producer of biologically active secondary metabolites of economic significance to the pharmaceutical, chemical and agricultural industries.

1.2 Morphology of actinomycetes

Among Gram-positive bacteria, actinomycetes exhibit the greatest morphological differentiation, which is based on a filamentous degree of organization. These filamentous elements are called hyphae. The same principle of growth is also known to apply to fungi. However, there is a fundamental difference: actinomycetes are typical prokaryotes while fungi form a separate kingdom within the eukaryotes (Vobis, 1997). The naked eye and the dissecting microscope give the information about colonial characters and mycelial characteristics; the optical microscope exhibits the morphological structures of hyphae and spores; the scanning electron microscope uses to discover additional external micromorphological fine detail; and finally, transmission electron microscopy uses to see the diversity of cellular and extracellular ultrastructures.

1.3 Formation of colonies

Colonies of actinomycetes are formed by mycelia, i.e. the mass of hyphae belonging to an organism. The growth of a new colony starts with an inoculum, which is transferred to sufficient culture medium. This may be a single spore, a sporangium, a fragment of a hypha or a small part of and old colony or stock culture. On solid agar media, the inoculum first develops a substrate mycelium, also known as a primary or vegetative mycelium. Vertically growing hyphae then penetrate the substrate and form a secondary mycelium, the aerial mycelium, which remains in permanent contact with the air (Figure 1). This provokes a change in the colony's physiological, ultrastructural and morphogenetical characters, e.g., spore production (Kalakoutskii and Agre, 1976). The difference between the hydrophilic nature of the substrate mycelium and the hydrophobicity of the aerial mycelium is very conspicuous. This is easy to distinguish by an impression preparation on a cover slip, viewed in a dry system with a light microscope: substrate hyphae are transparent and phase-dark, aerial hyphae are refractive and phase-bright.

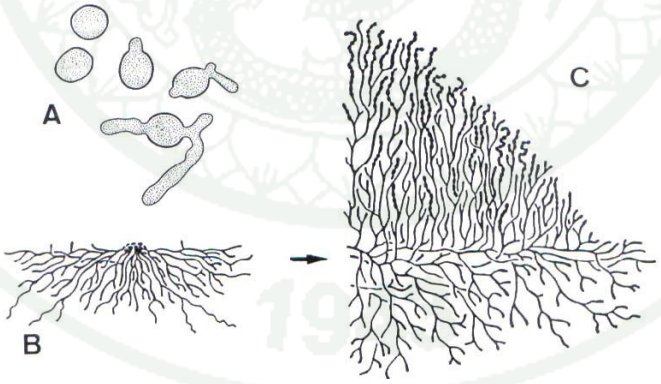


Figure 1 Development of mycelium in Streptomyces. (A) germination of spores, (B) formation of substrate (= primary) mycelium, (C) border of a grown up colony with sporulating aerial (=secondary) mycelium

Source: Vobis (1997)

1.4 Morphological characters of spores

Aside from the mycelial growth, spore formation is the most important morphological criterion that can be used to recognize an actinomycete. Conventionally, the formation of spores is restricted to the morphological group of sporoactinomycetes, where sporulation takes place in well defined parts of the mycelium. This is not the case in so-called nocardioform bacteria, where the hypahe eventually fragment into coccoid or rod-like elements that give rise to new mycelia (Prauser, 1981; Goodfellow and Cross, 1984). The spores produced individually or in short chains are in general thicker than the hyphae, while those which are developed in long chains, usually have the same diameter as the hyphae. Spores are about 1 or 2 μm thick and vary in terms of shape and surface characteristics (Figure 2).

1.5 Chemotaxonomy of actinomycetes

Phenotypic methods comprise all those that are not directed toward DNA or RNA; therefore, they also include the chemotaxonomic techniques. As the introduction of chemotaxonomy is generally considered one of the essential milestones in the development of modern bacterial classification, it is often treated as a separate unit in taxonomic reviews. The term "chemotaxonomy" refers to the application of analytical methods to collect information on various chemical constituents of the cell to classify bacteria. As for the other phenotypic and the genotypic techniques, some of the chemotaxonomic methods have been widely applied on vast numbers of bacteria whereas others were so specific that their application was restricted to particular taxa (Vandamme *et al*., 1996). Chemotaxonomy is concerned with the discontinuous distribution of specific chemicals, notably amino acids, lipids, proteins and sugars, and in this sense can be considered to provide good characters for classification and identification. It is, however, important that the observed variation in chemical composition is the result of genetic differences and not due to variation in cultivation conditions. Therefore, it is usually necessary to grow cultures under carefully standardized growth regimes before comparative chemotaxonomic work can be undertaken. Rigorously standardized cultivation conditions are particularly important

in studies involving quantitative analyses of chemical data (Goodfellow and O'Donnell, 1993). Several techniques are increasingly being used routinely in prokaryotic taxonomy.

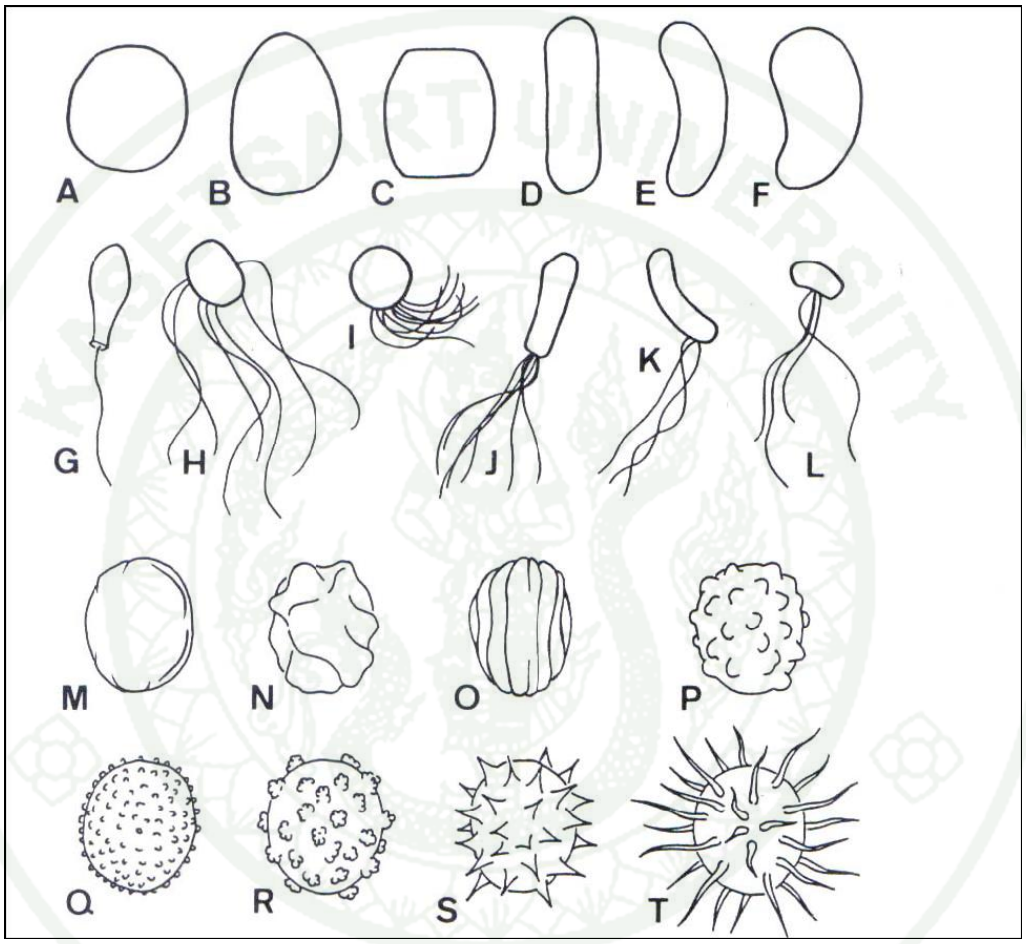


Figure 2 Morphological features of spores. General shape of spores: (A) globose, (B) ovoid, (C) doliform, (D) rod-shaped, (E) allantoid, (F) reniform. Type of flagellation: (G) monopolar monotrichous, (H) peritrichous, (I) polytrichous, (J) monopolar polytrichous (=lophotrichous), (K) Surface ornamentation: (M) smooth, (N) irregular rugose, (O) parallel rugose, (P) warty, (Q) tuberculate, (R) verrucose, (S) spiny, (T) hairy.

Source: Vobis (1997)

1.5.1 Cell wall composition.

The characteristic cell wall polymer of many prokaryotes, present in Gram-negative and Gram-positive bacteria and in the cyanobacteria, is peptidoglycan (Figure 3). The peptidoglycan type of Gram-negative bacteria is rather uniform and provides little information. Cell walls of Gram-positive bacteria, in contrast, contain various peptidoglycan types, which may be genus or species specific (Schleifer and Kandler, 1972). The variation in qualitative amino acid and/or sugar composition, especially the variation in the primary structure of the peptidoglycans of various Gram-positive bacteria has provided information of enormous taxonomic value. The cell wall composition of Gram-positive bacteria was one of the earliest useful chemotaxonomic characters. The amino acids present in the cell wall are now an accepted important part of the generic description and the sugars composition might help to distinguish between species. Cell wall types are summarized in Table 1.

1.5.2 Lipid composition

Among the prokaryote there are two distinct lipid categories. The eubacteria possess acyl lipids (ester-linked) while the archaeobacteria possess ether-linked lipids. Lipids occur in the cytoplasmic membranes of all eubacteria and in the cell wall complex of Gram-negative bacteria and certain Gram-positive bacteria. The fatty acid composition of the bacterial cell has proved useful in the classification of certain bacteria and in some cased the fatty acid pattern may be characteristic for a particular taxon. However, it should be noted that the fatty acid patterns obtained may be influenced by a number of factors: composition of growth medium, temperature of incubation, age of culture, and the techniques employed to analyze the sample.

Table 1 Cell wall type and sugar pattern of actinomycetes

Cell wall type			Whole Cell Sugar Pattern	
Type	**Major wall amino acid**	**Distinguising major constituents**	**Type**	**Diagnostic Sugar**
I	LL- DAP	-	-	-
II	meso-DAP	Glycine	D	Xylose, Arabinose
III	meso-DAP or OH-DAP	none	B C	Madurose None
IV	meso-DAP	Arabinose, Galactose	A	Arabinose, Galactose

Source: William (1989)

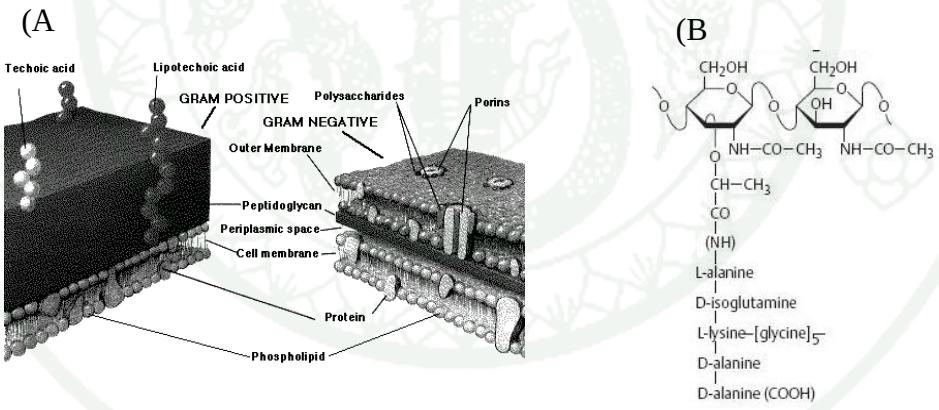


Figure 3 The structure of cell wall in bacteria (A) and peptidoglycan (B).

The most common polar lipid types are the phospholipids and the glycolipids. Phospholipids occur in many bacteria but certain actinomycetes and coryneform bacteria contain very characteristic phospholipids, the phosphatidylinositol mannoside. Glycolipids (glycosyl diacylglycerols) are widely distributed amongst Gram-positive bacteria can also be used as chemotaxonomic markers.

1.5.3 Isoprenoid quinones.

Isoprenoid quinones are a class of terpenoid lipids located in the cytoplasmic membranes of most prokaryotes and play important roles in electron transport, oxidative phosphorylation, and, possibly, active transport. Representatives of one, or more than one, of the three main types, ubiquinones, menaquinones, and demetylmenaquinones are present in the majority of prokaryotes so far examined. The majority of the aerobic and facultatively anaerobic gram-positive bacteria produce only menaquinones. The structure of isoprenoid quinones are shown in Figure 4. The large variability of the side chains can be used to characterize bacteria at different taxonomic levels. Gram-positive bacteria synthesize either menaquinones or demethylmenaquinones, or they lack isoprenoid quinones. Ubiquinones are not formed. The available data strongly suggest that these compounds will be of considerable value in the classification of micrococci, staphylococci, coryneform bacteria and certain actinomycetes.

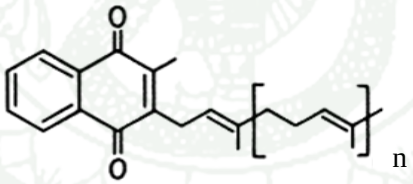


Figure 4 Poly-isoprenoid unsaturated side-chain of various lengths, with isoprene units varying from 4 to 13. These compounds are called menaquinones-n or MK-n. HΔ = hydrogenation on unsaturated side chain

1.6 Classification of Actinomycetes

The actinomycetes have DNAs rich in guanine plus cytosine (> 55 mol%) (Ruan, 1994). The cell wall composition consists of n-acetyl muramic acid and diaminopimelic acid, glutamic acid, glycine and alanine (Davis, 1973).

Shinji (1997) classified actinomycetes to 8 groups based on morphology and cell wall type composition according to the Atlas of Actinomycete. These groups are *Micrococcus, Microbacterium* and related genera, *Mycobacterium, Nocardia* and related genera, *Pseudonocardiaceae* and related genera, *Micromonosporaceae, Thermomonosporaceae, Streptosporangiaceae, Streptomycetaceae*, other genera.

A hierarchical classification structure for the taxa between the taxonomic levels of genus and class is proposed for the actinomycete line of descent as defined by analysis of small subunit (16S) rRNA and genes coding for this molecule (rDNA). The order *Actinomycetales* (Yokota, 1997) contains 90 genera classified into 8 groups (Figure 5). Stackebrandt *et al*. (1997) demonstrate the phylogenetic tree base on 16S rRNA sequences of totally 95 genera, belonging to 30 families and 10 suborders (Figure 6).

Nowadays, based on 16S rRNA gene sequence comparison, the phylogenetic relatedness of the order *Actinomycetales* in the class *Actinobacteria* contained 42 families and 13 suborders (Figure 7) (Zhi *et al*., 2009).

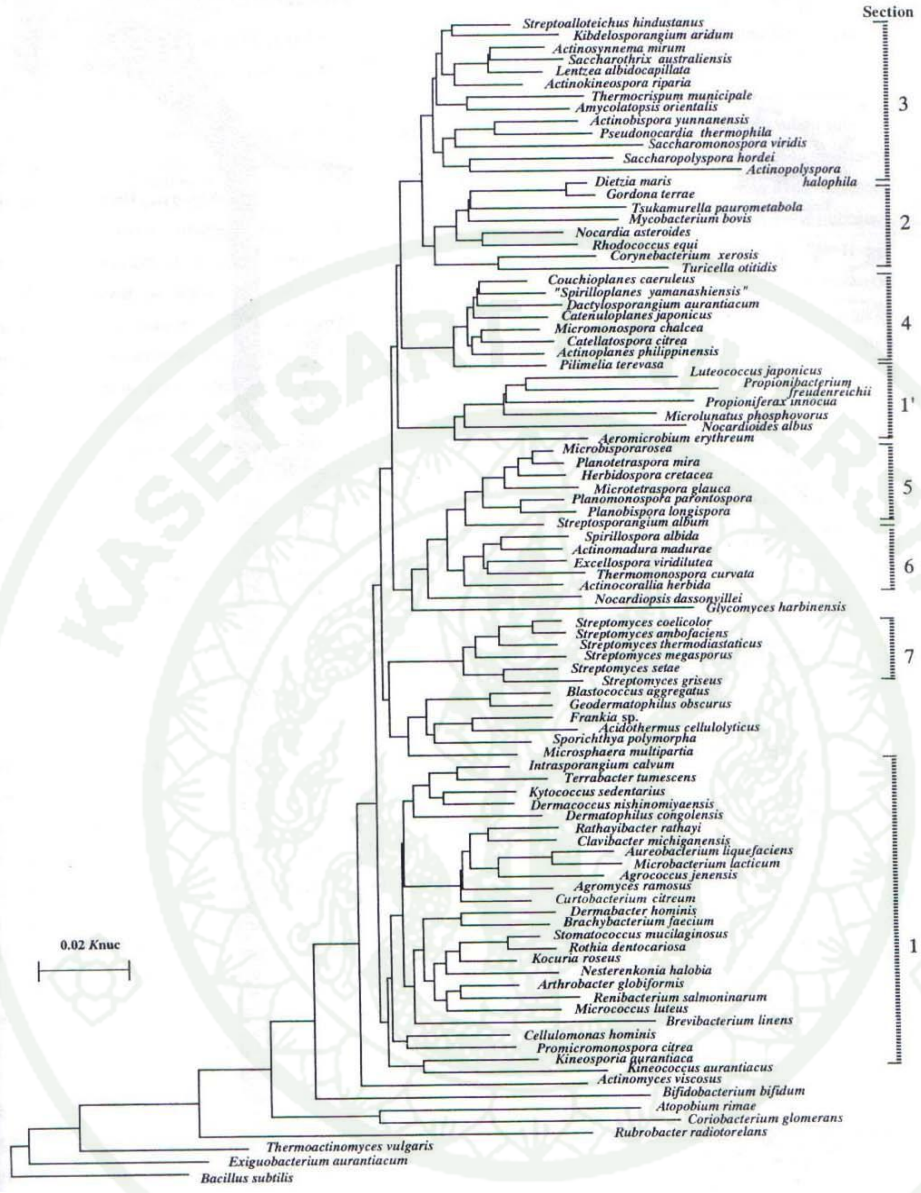


Figure 5 Phylogenetic relationship of 90 genera of actinomycetes and actinobacteria based on 16S rRNA gene sequences (The tree was constructed by neighbor-joining method) 1: *Micrococcus*, *Microbacterium* and related genera. 2: *Mycobacterium*, *Nocardia* and related genera. 3: Family *Pseudonocardiaceae* and related genera. 4: Family *Micromonosporaceae*. 5: Family *Streptosporangiaceae*. 6: Family *Thermomonosporaceae*. 7: Family *Streptomycetaceae*.

Source: Yokota (1997)

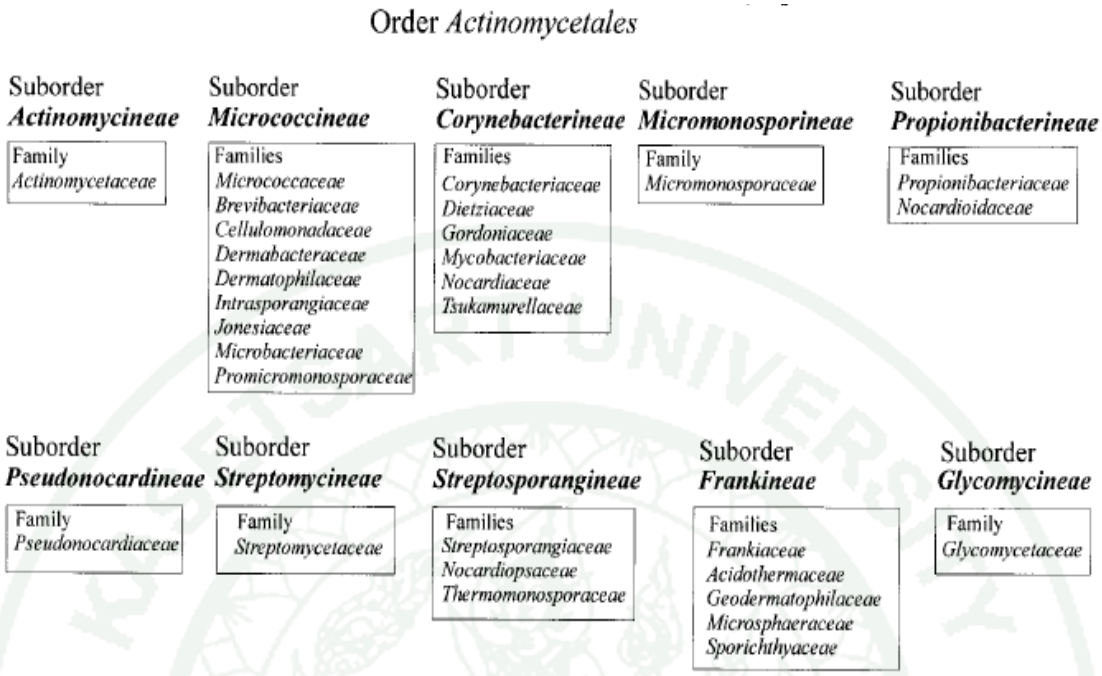


Figure 6 Proposed hierarchic classification system of the order *Actinomycetales* in the class *Actinobacteria* based on the phylogenetic analyses of the 16S rDNA/rRNA sequence data.

Source: Modified from Stackebrandt *et al*. (1997)

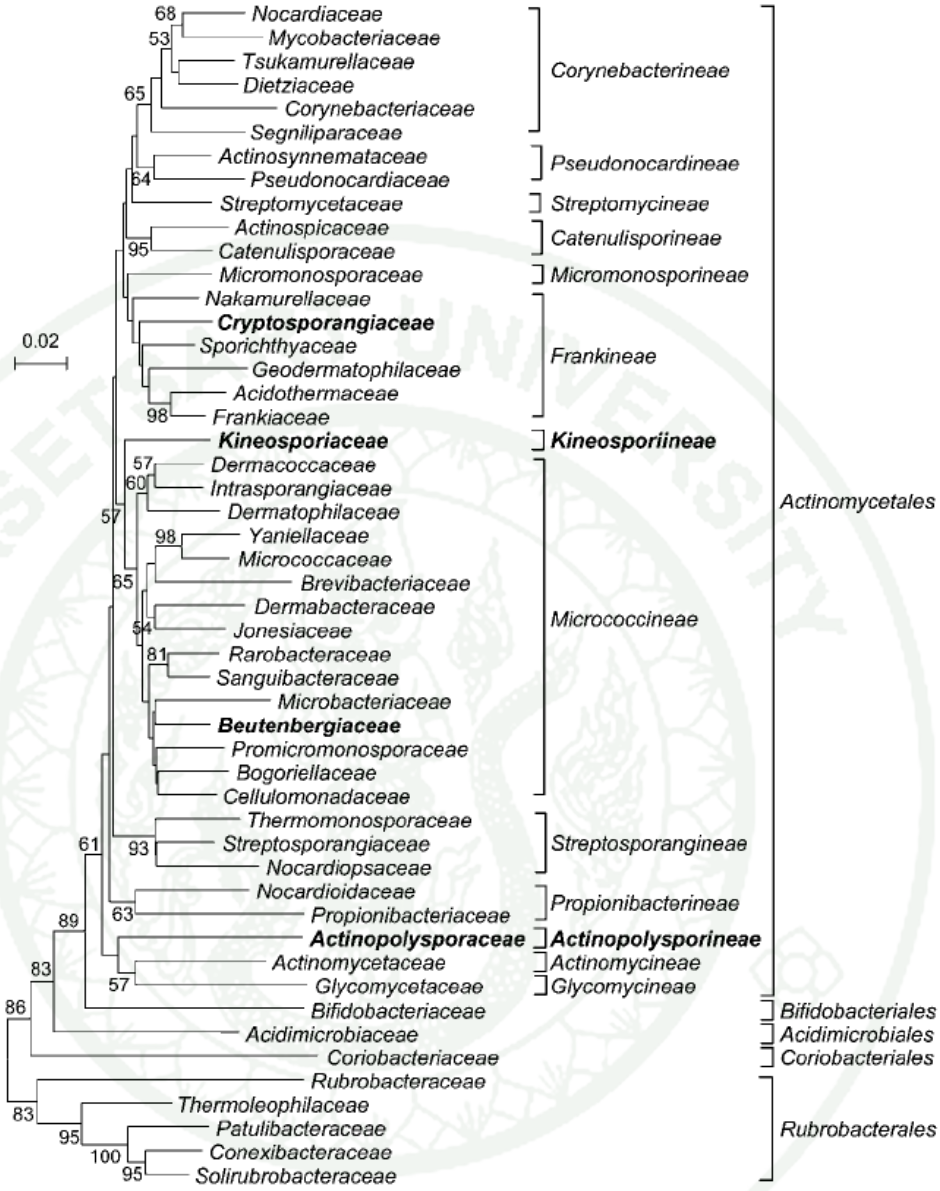


Figure 7 Intraclass relatedness of the class *Actinobacteria* showing the presence of five orders based on 16S rRNA gene sequence comparison. The phylogenetic relatedness of the families of the class *Actinobacteria* is outlined. Bootstrap values of 50% or more are indicated at branch points. Bar, 2 substitutions per 100 nucleotide positions.

Sources: Zhi *et al*. (2009)

2. Family *Streptosporangiaceae* (Goodfellow, 2006)

The family *Streptosporangiaceae* corresponds to a tentative group "maduromycetes", which was characterized by *meso*-diaminopimelic acid (A_2pm) in cell walls and madurose in whole cells. However, the madurose-containing genus *Actinomadura* was not placed in the *Streptosporangiaceae* in the light of 16S rRNA cataloging and chemotaxonomic studies on cellular lipid compositions. The madurose-containing genera *Excellospora* and *Spirillospora* were also found to differ from members of the family in cellular lipid composition.

The diamino acid of peptidoglycan is *meso*-diaminopimelic acid (A_2pm). The diagnostic phospholipids are phosphatidylethanolamine (and/or its derivatives) and glucosamine containing phospholipid (type PIV). This phospholipid pattern is characteristic of this family. The menaquinones of all the constituents mainly consist of MK-9(H_2), MK-9(H_4) and MK-9 with exception of the genus *Herbidospora* which has MK-10(H_4) as a major component. Their tetrahydrogenated menaquinones are saturated at the third isoprenyl unit from naphthoquinone moiety as well as the second one from the ω-terminus of the isoprenyl side chain [MK-9(III, VIII-H_4) and MK-10(III, IX-H_4)], whereas tetrahydrogenation in other gram-positive bacteria including actinomycetes occurs at positions II and III. This mode of saturation is also characteristic of this family. The family indicates a complicated pattern of cellular fatty acids consisting of normal, monounsaturated, iso-branched, 10-methyl, and 2-hydroxy acids. Madurose, by which the members of this group are characterized, is not thought to be a reliable taxonomic marker for this family at present because its presence or absence varies at the strain level.

At present, the family *Streptosporangiaceae* is contained 11 genera, *Acrocarpospora, Herbidospora, Microbispora, Microtetraspora, Nonomuraea, Planobispora, Planomonospora, Planotetraspora, Sphaerisporangium, Streptosporangium* and *Thermopolyspora*. Phylogenetic studies using 16S rRNA gene sequences showed that these genera were coherent, and their chemotaxonomic properties also showed high similarity.

The morphological features of this family show a wide variety so that classification at the genus level is mainly based on the presence or absence of sporangia as well as on the number of spores produced on free sporophores or in sporangia.

2.1 *Acrocarpospora*

Non-fragmentary substrate mycelia are present. Spherical and clubshaped structures are borne on the tips of the aerial mycelium. These structures contain coiled spore chains. Spores are oval or short rod-like with a smooth surface and nonmotile.

2.2 *Herbidospora*

Chains contained 20-30 spores are formed in clusters at the tips of narrow sporophores directed built from the substrate mycelium. True aerial hyphae and zoospores are not observed. This genus can be clearly identified from the other members of the *Streptosporangiaceae* by chemotaxonomic properties such as menaquinone composition.

2.3 *Microbispora*

Aerial mycelium produced longitudinal pairs of spores that may be sessile or on short sporophores. Most members of the genus are mesophile, but some strains are thermophile with optimum growth at 50-60°C.

2.4 *Microtetraspora*

The organisms produced aerial mycelia bearing spores in chains. The significance of the distinction between the genera *Microtetraspora* and *Microbispora* should be evaluated in the light of phylogenetic studied.

2.5 *Nonomuraea*

The organisms formed branched substrate and aerial mycelia. Non-motile spores are produced. Chains of spores that may be hooked, spiral, straight, or enmeshed in pseudovesicles.

2.6 *Planobispora*

Aerial hyphae produced club-shaped sporangia borne in bundles on sporangiophores. Each sporangium contains a longitudinal pair of spores. Spores have motility.

2.7 *Planomonospora*

Aerial mycelium bears cylindrical sporangia in palm-leave pattern or parallel rows and each sporangium contain a single spore. Motile spores are released from the sporangium.

2.8 *Planotetraspora*

These microorganisms produce a filamentous growth which is differentiated into a vegetative mycelium and an aerial mycelium. Aerial hyphae produce long cylindrical sporangia at the ends of short sporangiophores. Each sporangium contains four spores in a single row. The sporangiospores are released from the sporangia when preparations are flooded with water and are motile by means of single polar flagella.

2.9 *Sphaerisporangium*

Non-fragmented substrate and aerial mycelia are produced. Aerial mycelium bears spherical globose sporangia containing non-motile spores. Spores are oval or spherical with a smooth, wrinkled and prominently ridged surface.

2.10 *Streptosporangium*

Globose to subglobose sporangia borne at the tip of sporangiophores were produced by aerial hyphae. Each sporangium contains a large number of spores arrange in an unbranched and coiled chain. Spores have non-motility.

2.11 *Thermopolyspora*.

The organisms formed short chains of spores on short sporophores. Aerial mycelia produce hooked or irregular spiral chains arranged in clusters on branched aerial. This genus is thermophile.

3. Methods and parameters used in prokaryotic species circumscription

This day, prokaryote taxonomists agree that a reliable classification can only be achieved by the exploration of the internal diversity of taxa by a wide range of techniques in what is generally known as the `polyphasic approach' (Vandamme *et al*., 1996). This approach implies that two sources of information must be investigated as extensively as possible: genomic information and phenotype. Genomic information is gained from all data that can be retrieved from nucleic acids, either directly trough sequencing or indirectly through parameters like DNA-DNA similarity or G+C mol%. Phenotype refers to the way in which the genotype is expressed, the visible or otherwise measurable physical and biochemical characteristics of an organism, a result of the interaction of genotype and environment.

3.1 Retrieving genomic information

The methods of genomic information retrieval are mostly directed toward DNA or RNA molecules. Nucleic acids are universally distributed and these are excellent tools to be used as standards for wide-ranging comparisons. The most complete genomic source of information is of course the entire bacterial genome. As large-scale sequencing of complete genomes is not feasible at present, several

alternative approaches have been taken. They include estimating the mean overall base composition of DNA, comparing genomic similarities by DNA-DNA pairing studies, generating unique sets of DNA fragments by digestion with restriction endonucleases (low-frequency restriction fragment analysis (LFRFA), pulsed field gel electrophoresis (PFGE), sequence comparisons of selected genes, DNA-rRNA hybridization and sequencing of rRNA (Rossello-Mora and Amann (2001).

3.1.1 DNA base composition (mol% G+C; G+C content; G+C %)

The primary structure of DNA results from the linear succession of the four nucleotide bases adenine (A), thymine (T), guanine (G) and cytosine (C), and this succession determines the genetic information of an organism's genome. Because of the double-stranded nature of DNA, where both strands are complementary with base pairing G-C and A-T, the ratios G/C and A/T usually remain constant at 1. However, the relative ratio [G+C]/[A+T] varies from genome to genome. The base ratio of a DNA molecule is generally described as the relative abundance of the pair G+C, and is commonly called G+C content. The DNA base ratio is calculated in percentage of G+C: [G+C]/[A+T+C+G]x100. This was the first nucleic acid technology applied to prokaryote systematics (Lee *et al*., 1956) and initially proved to be a useful and routine way of distinguishing between phenotypically similar and genomically different strains (Goodfellow and O'Donnell, 1993). It is usually one of the genomic characteristics recommended for the descriptions of species and genera. Among the prokaryotes, G+C contents vary between 20 and 80 mol% (Tamaoka, 1994). The greater the difference between two organisms, the less closely related they are. Theoretically, DNA molecules with differences of greater than 20-30 mol% can have virtually no sequences in common (Logan, 1994). Empirically, it has been shown that organisms that differ by more than 10 mol% do not belong to the same genus and that 5 mol% is the common range found within a species. While firm guidelines have yet to be set for the range of variation, values higher than 15 mol% can be taken as a strong indication for heterogeneity within a genus (Goodfellow *et al*., 1997). However, it should be noted that although differences in mol% are taxonomically useful for separating groups, similarities in base compositions do not

necessarily indicate close relationships because the determinations do not take the linear sequences of bases in the DNA molecules into account (the criterion can only be used negatively).

3.1.2 DNA-DNA hybridization (DNA-DNA pairing; DNA-DNA homology; DNA-DNA relatedness)

The determination of whole genome DNA-DNA similarity is today still the standard technique for species delineation (Stackebrandt and Goebel, (1994). A characteristic property of DNA and RNA is its ability for reassociation or hybridization. The complementary strands of DNA, once denatured, can, under appropriate experimental conditions, reassociate to reform native duplex structures. The specific pairings are between the base pairs A-T and G-C, and the overall pairing of the nucleic acid fragments is dependent upon similar linear arrangements of these bases along the DNA. Under standardized conditions, DNAs from different organisms reassociate depending on the similarity of their nucleotide sequences, thereby allowing quantification of the degree of relatedness, usually expressed as % similarity or homology. There are several methodologies to measure DNA-DNA relatedness, but all of them rely on the same principle (Figure 8). DNAs of two different organisms are mixed and denatured to give a solution of a mixture of single-stranded DNA molecules. Under controlled experimental conditions, DNA reassociation occurs and results in hybrid molecules: the higher the genetic similarity of the two organisms, the more nucleotide base sequences they have in common, and the more hybrid formation (hybridization) will occur. The comparison between the results obtained with the mixture of DNAs and pure reference DNA (homoduplex DNA) yields a degree of similarity (Wayne *et al*., 1987).

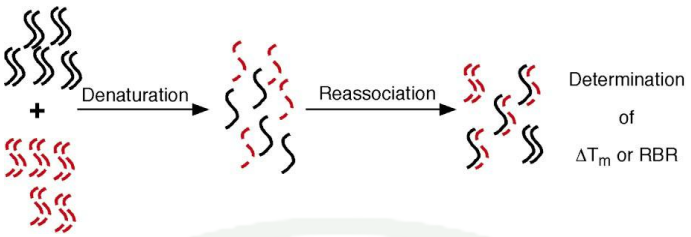


Figure 8 DNA-DNA reassociation assay.

Source: Rossello-Mora and Amann (2001)

3.1.3 rRNA anylysis

Techniques involving the analysis of rRNA or of the genes coding rRNA (rDNA) have revolutionized prokaryotic taxonomy. The conclusions drawn from these studies are based on the assumption that the rRNA genes are highly conserved because of the fundamental role of the ribosome in protein synthesis. rRNAs are molecules with universal, constant and highly constrained functions that were established at an early stage in evolution and that are not affected by changes in the organism's environment. As well as they are large molecules containing considerable genetic information, they have been chosen as the molecular basis for phylogenetic reconstructions at least in the prokaryotic world (Woese, 1992). Two additional assumptions are basic for the validity of this approach, namely that lateral gene transfer has not occurred between rRNA genes, and that the amount of evolution or dissimilarity between rRNA sequences of a given pair of organisms is representative of the variation shown by the corresponding genomes (Figure 9). If this holds true, the variations in the rRNA primary structures among the prokaryotes will reflect evolutionary distances among organisms.

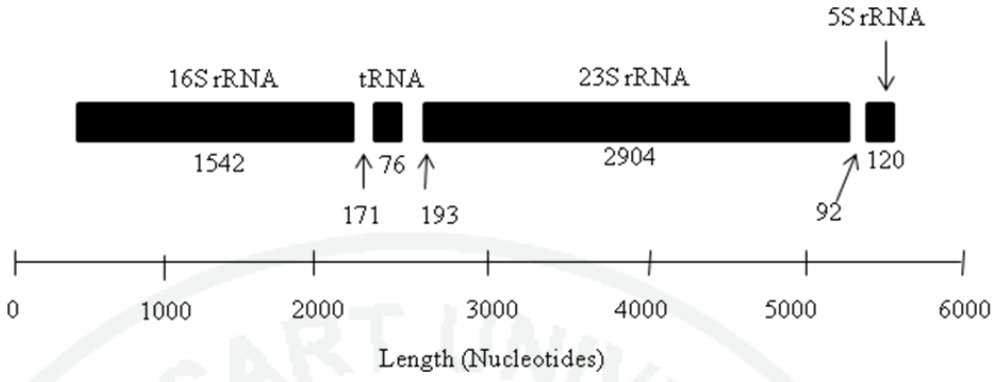


Figure 9 The length of rDNA coding region, including 16S rDNA.

Source: Christora *et al*. (1995)

3.1.4 DNA-based typing methods.

DNA-based typing methods generally refer to techniques which allow us to subdivide species into a number of distinct types. Classically, subtyping of species was performed by means of phenotypic analyses such as biochemical or tests, antibiotic susceptibility patterning, phage or bacteriocin typing, and many others. During the last few years, a battery of DNA-directed typing methods has been developed. Ideally, these techniques are universally applicable, they are reproducible and simple to perform, and they are highly discriminatory (Vandamme, 1996). One can differentiate between two basic techniques:

3.2 Phenotypic Methods

The phenotype is the observable expression of the genotype. Before molecular techniques were available to prokaryote taxonomists, the classification was exclusively based on morphology, physiology and growth conditions of the organisms. These investigations were directly linked to the use of pure cultures, and the laboratory capabilities to cultivate the organisms and analyze their properties. Thus, the classification schemes were biased towards aerobic heterotrophic microorganisms for

which an extensive retrieval of information was easy. One of the disadvantages of analyzing the phenotype is that the whole information potential of a prokaryotic genome is never expressed. Gene expression is directly related to the environmental conditions (e.g. growth conditions in the laboratory) (Smibert and Krieg, 1994).

4. Xylan degrading enzyme systems

4.1 Xylan

Xylans, the major portion of the hemicellulose of plant cell walls and grasses, are heteropolymers consisting principally of xylose and arabinose. Most xylans occur as heteropolysaccharides, containing different groups in the backbone chain and in the side chain (Biely, 1985). The common substituents found on the backbone of xylan are acetyl, arabinosyl, and glucuronosyl residues.

4.2 Xylanolytic enzymes

Owing to the heterogeneity of xylan, its hydrolysis requires the action of a complex enzyme system (Figure 10). Endo-1,4-β-D-xylanases randomly cleave the xylan backbone, β-D-xylosidases cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose while removal of the side groups is catalysed by α-L-arabinofuranosidases, α-D-glucuronidases, acetylxylan esterases, ferulic acid esterases and ρ-coumaric acid esterases. Indeed, complete xylanolytic enzyme systems, including all of these activities, have been found to be quite widespread among fungi, actinomycetes and bacteria. Heteroxylans contain different substituent groups in the backbone and side chain. Thus, the degradation of such a complex polysaccharide may involve synergistic action between the different components of the xylanolytic enzyme system (Biely, 1995).

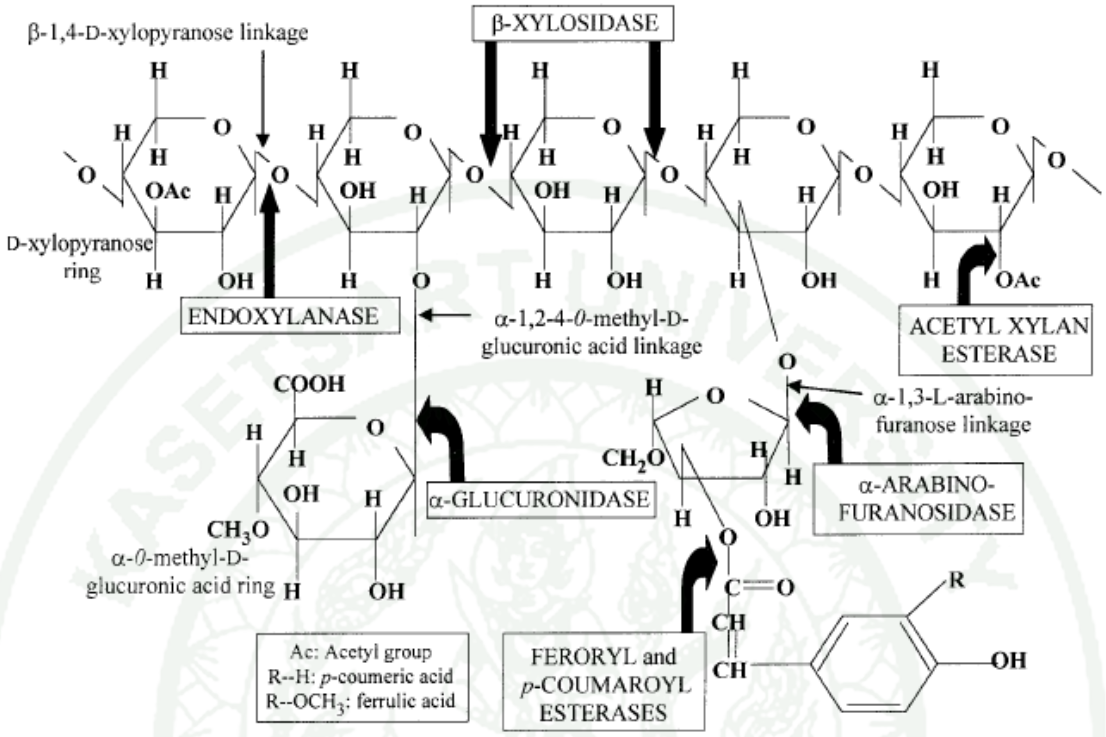


Figure 10 Structure of xylan showing different substituent groups with sites of attack by microbial xylanases.

Source: Beg *et al*. (2001)

4.2.1 Endoxylanase

As the structure and molecular mechanism of an enzyme are related to its primary structure, this classification system reflects both structural and mechanistic features. Enzymes within a particular family have a similar three-dimensional structure and similar molecular mechanism and it has also been suggested that they may have a similar specificity of action on small, soluble, synthetic substrates. During the early course of hydrolysis of xylan, the main products formed are xylooligosacchrides. Endo-acting xylanases have been differentiated according to the end product released from the hydrolysis of xylan (e.g., xylose, xyobiose and xylotriose, and/or arabinose). Xylanases can be divided into two major families of glycosyl hydrolases: Family 10 (F) and 11 (G) (Li *et al*., 2005; Kubata *et al*., 1994). The relation of enzyme within these families can be demonstrated by pairwise aligments of the protein sequences or by the basic local alignment search tool (BLAST) to discern sequence similarity. BLAST searches using recognized Family 10 (F10) or Family 11 (F11) xylanase protein sequences identify sets of enzymes that are mutually exclusive. Recently, xylanase is classified in glycosylhydrolase family 5, 7, 8, 10, 11 and 43 (Figure 11).

β-1,4-endoxylanase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.8) cleaves the internal glycosidic linkages of the heteroxylan backbone, resulting in a decreased DP of the substrate. The attack of the substrate is not random, and the bonds to be hydrolyzed depend on the nature of substrate (e.g., length and degree of branching of the substrate or the presence of substituents (Li *et al*., 2000).

4.2.2 β-Xylosidase

β-D-xylosidase (β-D-xyloside xylohydrolase; EC 3.2.1.37) are exoglycosidases that hydrolyzed short oligosaccharides and xylobiose from the nonreducing end to liberate xylose (Wong *et al*., 1988). True β-xylosidases are able to cleave artificial substrates like *p*-nitrophenyl-β-D-xyloside (Coughlan and Hazlewood, 1993). Among the xylooligomers, xylobiose is usually the best substrate.

The affinity of the enzyme toward xylooligosaccharides decreases with increasing degree of polymerization (Bajpai, 1997). Most of the β-xylosidase studied so far is completely inhibited by their hydrolysis product xylose. Many β-xylosidases have transxylosidation (transterase) activity, especially at high substrate concentrations, resulting in product of high molecular mass.

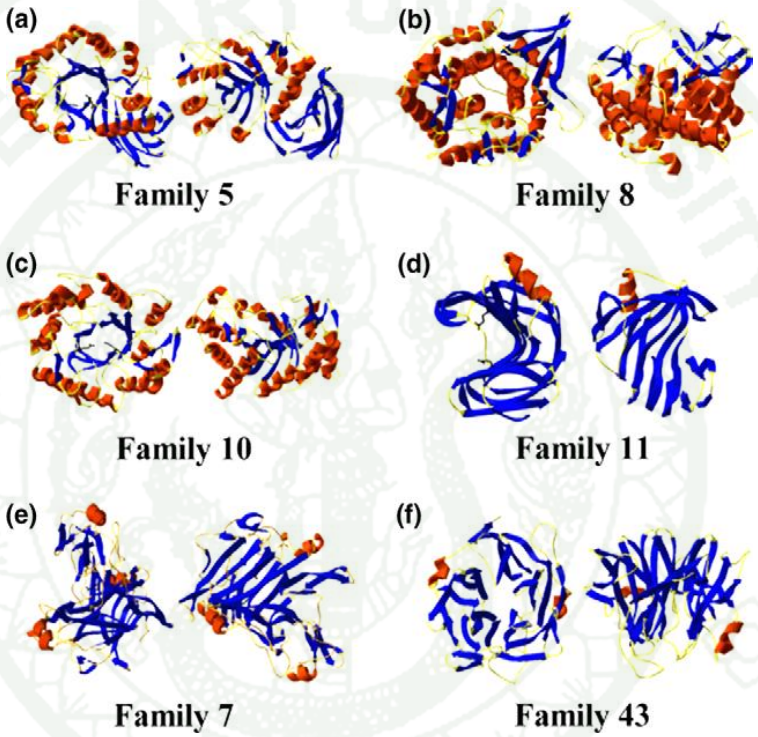


Figure 11 Representative structures of enzymes from various glycoside hydrolase families. (a) Structure of the family 5 enzyme, XynA, from *Erwinia chrysanthemi*. (b) Structure of the family 8 xylanase, pXyl, from *Pseudoalteromonas haloplanktis* TAH3a. (c) Structure of the *Streptomyces lividans* xylanase. (d) Structure of the *Trichoderma reesei* family 11 xylanase. (e) Structure of the *Trichoderma reesei* family 7 non-specific EGI. (f) Structure of the *Cellvibrio japonicas* family 43 a-L-arabinanase.

Source: Collins *et al*. (2005)

4.2.3 α-L-Arabinofuranosidase

In spite of the important role played by arabinosidases in the hydrolysis of xylan, only a few enzymes have been isolated and characterized. There are two types of arabinases, the exo-acting α-L-arabinofuranosidase (EC 3.2.1.55), which is active against *p*-nitrophenyl-α-L-arabinofuranosidase and on branched arabinans, and the endo-1,5- α-L-arabinase (EC 3.2.1.99), which is active only toward linear arabinans. These enzymes hydrolyze 1,5- α-L-arabinans, the production of arabinofunosidase in several actinomycetes seems to be induced, among others, by arabinan, xylan, and wheat bran.

α-L-Arabinofuranosidases capable of hydrolyzing both 1,3- and 1,5- α-L-arabinofuranosyl linkages in arabinoxylan have been reported in *Aspergillus niger* and *Bacillus subtilis*. This 1,4-β-D-arabinoxylan arabinofuranohydrolase is highly specific for arabinoxylans and is able to release only arabinose from arabinoxylan. During the arabinose release, the xylan backbone is not degraded and there is no production of xylooligosaccharides. The enzyme is not active toward α-L-1,3- or α-1,5-linked arabinose from arabinans, arabinogalactans, or *p*-nitrophenyl-α-L-arabinofuranoside.

4.2.4 α-Glucuronidases

α-D-Glucuronidases (3.2.1.-) hydrolyze the α-1,2 linkages between glucuronic acid and xylose residues in glucuronoxylan. The substrate specificities of α-glucuronidases differ according to the enzyme source. The enzymes from *Agaricus bisporus* and *Saccharomyces olivochromogenes* require a low-molecular-weight glucuronoxylan substrate. They release 4-O-methyglucuronic acid from 4-O-methylglucuronose substituted xylooligomers, but not from the polymer. This enzyme is active against low-molecular-weight glucuronoxylan fragments that result from the hydrolysis of xylan with endoxylanase. The α-glucuronidases is active only against substituted xylooligomers of DP greater than 2.

4.2.5 Acetylxylan esterase

Although xylan is highly acetylated in its native state, most of the xylans used to study xylanolytic enzyme systems were deacetylated xylans obtained from alkali extraction. However, acetylated xylooligosaccharides are the preferred substrate. Acetyl groups present on the xylan backbone inhibit the action of xylanase by steric hindrance. Acetylxylan esterase from Fluorescence succinogenes may play an important role in relieving this inhition by releasing acetic acid from xylan, thereby forming new unsubstituted sites on the polysaccharide backbone (Sunna and Antranikian, 1997). This, therefore, increases the susceptibility of the polymeric xylan to endoxylanases.

4.2.6 Ferulic and *p*-Coumaric Acid Esterases

Ferulic and *p*-coumaric acids are linked to xylan by ester bonds, ferulic acid esterase (EC 3.1.1.4) cleaves the ester linkages between arabinose side chains and ferulic acids in xylan. Similarly, *p*-coumaric acid and esterase (EC 3.1.1.4) cleaves the ester linkage between arabinose and *p*-coumaric acid. The enzyme releases ferulic acid from wheat bran only in the presence of xylanase and the best substrate for the production of the ferulic acid esterase by *Streptomyces olivochromogenes* is oat spelt xylan (Sunna and Antranikian, 1997).

The p-coumaroyl esterase is not able to release ester-linked acetyl groups from xylan. Furthermore, no reducing sugars are released from oat spelt xylan. The two feruloyl esterases release ferulic acid when incubated with ferulic acid containing tri-and tetrasaccharides.

The classifications of these enzymes into families are shown in Table 2.

Table 2 The hemicellulolytic enzymes, their classification into glycosyl hydrolase (GH) and carbohydrate esterase (CE) families.

Enzymes	**Substrates**	**EC**	**Family**
Endo-β-1,4-xylanase	β-1,4-xylan	3.2.1.8	GH 5, 8, 10, 11, 43
Exo-β-1,4-xylosidase	β-1,4-xylooligomers Xylobiose	3.2.1.37	GH 3, 39, 43, 52, 54
α-L-Arabinofuranosidase	α-Arabinofuranosyl (1→2) or (1→3) xylooligomers α-1,5-arabinan	3.2.1.55	GH 3, 43, 51, 54, 62
Endo-α-1,5-arabinanase	α-1,5-arabinan	3.2.1.99	GH 43
α-Glucuronidase	4-O-methyl-α-glucuronic acid (1→2) xylooligomers	3.2.1.139	GH 67
Endo-β-1,4-mannanase	β-1,4-mannan	3.2.1.78	GH 5, 26
Exo-β-1,4-mannosidase	β-1,4-mannooligomers mannobiose	3.2.1.25	GH 1, 2, 5
α-Galactosidase	α-galactopyranose (1→6) mannooligomers	3.2.1.22	GH 4, 27, 36, 57

Table 2 (Continued)

Enzymes	Substrates	EC	Family	
β-Glucosidase	β-glucopyranose (1→4) mannopyranose	3.2.1.21	GH	1, 3
Endo-galactanase	β-1,4-galactan	3.2.1.89	GH	53
Acetyl xylan esterase	2- or 3-O-acetyl xylan	3.1.1.72	CE	1, 2, 3, 4, 5, 6, 7
Acetyl mannan esterase	2- or 3-O-acetyl mannan	3.1.1.6		
Ferulic and *p*-cumaric acid esterases		3.1.1.73	CE	1

Source: Shallom and Shoham (2003)

4.3 Application of xylanase in biotechnology

Xylanolytic enzymes from microorganism have attracted a great deal of attention in the last decade, particularly because of their biotechnological potential in various industrial processes (Bajpai, 1999; Kuhad and Singh 1993; Niehaus *et al*. 1999; Wong and Saddler 1992), such as food, feed, and pulp and paper industries. Xylanases have shown an immense potential for increasing the production of several useful products in a most economical way. The main possibilities are the production of single cell proteins (SCPs), enzymes, liquid or gaseous fuels, solvents and sugar syrups, which can be used as such or as feed stock for other microbiological processes (Ball and McCarthy 1988; Kuhad and Singh 1993).

Currently, the most promising application of xylanases is in the prebleaching of kraft pulps (Bajpai 1999). Enzyme application improves pulp

fibrillation and water retention, reduction of beating times in virgin pulps, restoration of bonding and increased freeness in recycled fibers, and selective removal of xylans from dissolving pulps. Xylanases are also useful in yielding cellulose from dissolving pulps for rayon production and biobleaching of wood pulps (Bajpai *et al*. 1994; Srinivasan and Rele, 1999; Viikari *et al*. 1994).

Depression in weight gain and feed conversion efficiency in rye-fed broiler chicks has been associated with intestinal viscosity. Incorporation of xylanase into a rye-based diet of broiler chickens results in reduced intestinal viscosity, thus improving both the weight gain of chicks and their feed conversion efficiency (Bedford and Classen, 1992; Vanparidon *et al*., 1992).

The efficiency of xylanases in improving the quality of bread has been seen with an increase in specific bread volume. This is further enhanced when amylase is used in combination with xylanase (Maat *et al*. 1992). In 2008, xylanases have been found to improve the bread volume, crumb structure, reduce stickiness and staling and increased shelf life (Butt *et al*., 2008). There is an increasing trend in baking industry towards the application of xylanases in bread production.

Xylan is present in large amounts in wastes from agricultural and food industries. Hence, xylanases are used for conversion of xylan into xylose in waste water. The development of an efficient process of enzymatic hydrolysis offers new prospects for treating hemicellulosic wastes (Biely, 1985; Rani and Nand, 1996).

Corn cob is agricultural waste with large amounts in Thailand. Therefore, increasing value and utilization of corn cob xylan were studied. Xylanase and immobilized-β-xylosidase combination was found to be an effective method for conversion of corncob xylan into xylose. (Kitpreechavanich *et al*., 1994).

Xylanase treatment of plant cells can induce glycosylation and fatty acylation of phytosterols. Treatment of tobacco suspension cells with a purified

endoxylanase caused a 13-fold increase in the levels of acylated sterol glycosides and elicited the synthesis of phytoalexins (Moreau *et al*., 1994).

Xylanase are used concurrently with cellulase and pectinase for clarifying must and juices, and for liquefying fruits and vegetables (Biely, 1985), and in the pretreatment of forage crops to improve the digestibility of ruminant feeds and to facilitate composting (Gilbert and Hazlewood, 1993).

Alkyl glycosides are one of the most promising candidates for new surfactants. Commercially, they are produced from monomeric sugars such as D-glucose and a fatty alcohol. But the direct glycosylation using polysaccharide is more feasible for their industrial production, because hydrolysis of polysaccharide and subsequent steps can be omitted. Thus, use of xylanase in this process provides a challenging opportunity.

α-L-Arabinofuranosidase and β-D-glucopyranosidase have been employed in food processing for aromatizing musts, wines, and fruit juices (Spagna *et al*., 1998).

Some xylanases may be used to improve cell wall maceration for the production of plant protoplasts (Wong *et al*., 1988).

A recent application of a truncated bacterial xylanase gene from *Clostridium thermocellum* has been demonstrated in rhizosecretion in transgenic tobacco plants (Borisjuk *et al*., 1999).

Xylanase in synergism with several other enzymes, such as mannanase, ligninase, xylosidase, glucanase, glucosidase, etc., can be used for the generation of biological fuels, such as ethanol and xylitol, from lignocellulosic biomass (Dominguez, 1998; Kuhad and Singh, 1993; Olsson and Hahn-Hagerdal, 1996).

A potential application of the xylanolytic enzyme system in conjunction with the pectinolytic enzyme system is in the degumming of bast fibers such as flax, hemp, jute, and ramie (Puchart *et al*., 1999; Sharma, 1987). A xylanase-pectinase combination is also used in the debarking process, which is the first step in wood processing (Bajpai, 1999; Wong and Saddler, 1992). Replacement of slow natural retting by treatment with artificial mixtures of enzymes could become a new fiber liberation technology in the near future (Bajpai, 1999).

The strains reported for the commercial production of xylanases include *Trichoderma reesei* (Tenkanen *et al*., 1992), *Thermomyces lanuginosus* (Bajpai, 1999; Gubitz *et al*., 1997), *Aureobasidium pullulans* (Christov *et al*., 1999), *Bacillus subtilis* (Khanongnuch *et al*., 1999), and *Streptomyces lividans* (Ragauskas *et al*., 1994; Senior *et al*., 1992). Over the last decade, a number of microbial enzymes have been assessed for their potential applications in several industries. Several commercial products have been launched successfully worldwide in the past few years (Table 3).

5. Purification and characterization of xylanase

Several microorganisms including fungi and bacteria have been reported to be readily hydrolyzing xylans by synthesising 1,4-b-D endoxylanases (E.C. 3.2.18) and b-xylosidases (EC.3.2.1.37). In case of actinomycetes, few reports of purification and characterization of xylanase are available. Two endoxylanase with molecular weight of 26.3 and 16.8 kDa from *Microtetraspora flexuosa* SIIX were purified by ammonium sulfate fractionation, DEAE-Sepharose chromatography, gelfiltration on Sephacryl S 200 and fast protein liquid chromatography on Q-Sepharose. Optimal enzyme activities were obtained at 80°C and pH 6.0 (Berens *et al*., 1996).

Streptomyces sp. strain S38 secretes three xylanases (Xyl1, Xyl2, and Xyl3) that were purified by a Q-Sepharose HP column and Phenyl-Sepharose FF column. These enzymes enhanced the bleaching of kraft pulps (Georis *et al*., 2000).

Table 3 Commercial xylanases and their industrial suppliers.

Supplier	Product trade name	Application
Alko Rajamaki, Finland	Ecopulp	Pulp bleaching
Sandoz, Charlotte, N.C. and Basle, Switzerland	Cartazyme	Pulp bleaching
Clarient, UK	Cartazyme HS 10, Cartazyme HT, Cartazyme SR 10, Cartazyme PS10, Cartazyme 9407 E, Cartazyme NS 10, Cartazyme MP	Pulp bleaching
Genercor, Finland; Ciba Giegy, Switzerland	Irgazyme 40–4X/Albazyme 40–4X, Irgazyme-10A, Albazyme-10A Multifect xylanase	Pulp bleaching
Voest Alpine, Austria	VAI Xylanase	Pulp bleaching
Novo Nordisk, Denmark	Pulpzyme HA, Pulpzyme HB, Pulpzyme HC, Biofeed Beta, Biofeed Plus Ceremix	Pulp bleaching Feed Brewing
Bicon India, Bangalore	Bleachzyme F	Pulp bleaching
Rohn Enzyme 0Y; Primalco, Finland	Ecopulp X-100, Ecopulp X-200, Ecopulp X-200/4, Ecopulp TX-100, Ecopulp TX-200, Ecopulp XM	Pulp bleaching
Meito, Nogaya Japan	Xylanase	Research
Rohm, Germany	Rholase 7118	Food
Solvay Interox, USA	Optipulp L-8000	Pulp bleaching
Thomas Swan, UK	Ecozyme	Pulp bleaching
Iogen, Canada	GS-35, HS70	Pulp bleaching
Sankyo, Japan	Sanzyme PX, Alpelase F Sanzyme X	Feed Food
Enzyme Development, USA	Enzeko xylanase	Baking, food, feed

Source: Beg *et al*. (2001).

Inaddition, Four xylanases, designated as FI-A, FI-B, FII-A, FII-B, were identified and purified from *Streptomyces actuosus* A-151. The optimum pH for FII-B was 4, and the others were near 5–6. The optimum temperatures for enzyme activities were 60 ◦C for FII-B, and 70 ◦C for the others. The molecular weights of FI-A, FI-B, FII-A, and FII-B xylanases were 30, 45, 26 and 20 kDa, respectively, by sodium dodecylsulfate–polyacrylamide gel electrophoresis and 30, 43, 25 and 21 kDa, respectively, by gel filtration (Wang *et al*., 2003).

Xylanase XYNB from *Streptomyces olivaceoviridis* A1 was purified by ammonium sulfate precipitation, anion exchange and gel filtration chromatography. The putative molecular weight is about 20.8 kDa. XYNB can be potentially utilized in fish feed industry (Wang *et al*., 2007).

Recently, Ninawe *et al*. (2008) reported the purification of extracellular xylanase from *Streptomyces cyaneus* SN32, with molecular weight of 20.5 kDa using DEAE–Sepharose column. The optimum pH and temperature are 6.0 and 60–65°C, respectively. The half-lives at 50 and 65°C are approximately 200 and 50 min, respectively. The purified enzyme was stable for more than 20 week at 4°C. Easy purification from the fermentation broth and its high stability will be highly useful for industrial application of this endoxylanase.

6. Airlift Bioreactor

Airlift bioreactors are known to be used for various gas-liquid reactions; they have recently gained interest also as fermentors. Due to the airlift bioreactor does not require mechanical agitation, does not have moving parts, and often little internal structure, reasonably high gas and nutrient transfer rates and relatively high yields at low input rates, its shear stress is considerably less than that in stirred-tank reactors helps reduce maintenance requirements and simplifies cleaning regimes (Siegel and Robinson, 1992; Park *et al*., 1998; Bai *et al*., 2001 and Kang *et al*., 2001). The energy required to mass transfer is minimal because the air injection at the base of the equipment contributes to both aeration and agitation. Its construction is relatively

simple and inexpensive, and contamination is minimal because there are no mobile mechanical parts for agitation, and cleaning and sterilization are more easily performed (Rossi *et al*., 2002).

Airlift bioreactors of various types have been constructed for use in a variety of biological experiments and in the biotechnological processes such as biochemical fermentation and biological wastewater treatment. The experimental results when organisms have been cultured in gas-lift columns at high viscosity have been relatively good. The combined cost associated with aeration and agitation could be reduced by about 40% by using a gas-lift column in place of a conventional stirred tank bioreactor (Kilonzo and Margaritis, 2004).

Oxygen concentrations in liquid cultures depend on the presence of oxygen in the gas phase above and in the air bubbles inside the medium, as well as in the dissolved oxygen in the medium. Air is sparged through a sparger located at the base of the bioreactor. Oxygen requirements may vary from one species to another, and concentration of oxygen in liquid cultures in bioreactors can be regulated by aeration, gas flow and air bubble size (Ziv, 2005). The small bubbles in the reactor lead to an increased surface area for oxygen transfer.

In spite of the fact that airlift bireactors are employed in several fermentation processes on an industrial scale, they are scarcely used as laboratory fermentors with a volume up. This is rather surprising, because small-scale airlift fermentors also have all the advantages of simplicity in construction: they are easy to make, reliable in use and low in cost.

In case of actinomycetes, airlift bioreactor was used for production of bioactive compound. Neomycin production by free and alginate immobilized *Streptomyces marinensis* cells was investigated in an airlift reactor (Srinivasulu *et al*., 2002).

Besides, the enzyme production was also investigated in an airlift fermentor. In 2009, PLA-degrading enzyme production in an airlift fermentor by a new thermophilic *Actinomadura* sp. T16-1 was reported (Sukkhum *et al*., 2009).

7. Experimental design

7.1 Plackett-Burman design

Plackett and Burman (1946) have developed saturated fractional factorial designs that allow the researcher to investigate accurately many factors simultaneously without having to investigate all the possible combinations of factors (Durig and Fassihi, 1993). This design allows determination of the effect of variables with a minimum number of experiments. The disadvantage of this design is that it does not yield estimates of the extent or type of interaction between variables (Motola and Agharkar, 1992). However, within the bounds of these limitations, the use of these screening procedures invariably results in a well designed, efficient experiment, the outcome of which can be supported with statistical significance. Thus, Plackett-Burman design has been recommended for preformulation compatibility studies.

Plackett–Burman design is a well established and widely used statistical technique for screening and selection of critical culture variables. This method has been successfully improved the production of xylanase by *Aspergillus niger* XY-1 (Xu *et al*., 2008), alpha amylase by *Bacillus amyloliquefaciens* (Gangadharan *et al*., 2007) and α-amylase by *Aspergillus oryzae* (Francis *et al*., 2003).

In case of actinomycetes, improving the enzyme production using Plackett-Burman design was investigated. In 2008, *a*-galactosidase production in submerged fermentation by *Streptomyces griseoloalbus* was studied using response surface methodology (Grace *et. al*, 2008).

8. Response Surface Methodology (RSM)

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. Originally, RSM was developed to model experimental responses (Box and Draper, 1987), and then migrated into the modelling of numerical experiments. The difference is in the type of error generated by the response. In physical experiments, inaccuracy can be due, for example, to measurement errors while, in computer experiments, numerical noise is a result of incomplete convergence of iterative processes, round-off errors or the discrete representation of continuous physical phenomena (Campen *et al*., 1990; Giunta *et al.*, 1996; Toropov *et al.,* 1996). In RSM, the errors are assumed to be random.

RSM is generally conducted in three phases, as emphasized in Myers and Montgomery (2002). Phase 0 involves the screening of explanatory variables to identify those which have a significant effect on the responses. Phase 1 is concerned with the location of optimum operating conditions by conducting a sequence of suitable experiments. Phase 2 involves the fitting of an appropriate empirical model, usually a second-order polynomial model, in order to examine the nature of the response surface in the vicinity of the optimum.

The response can be represented graphically, either in the three-dimensional space or as contour plots that help visualize the shape of the response surface. Contours are curves of constant response drawn in the *xi, xj* plane keeping all other variables fixed. Each contour corresponds to a particular height of the response surface. Example of three dimension plot and contour plot are shown in Figure 12.

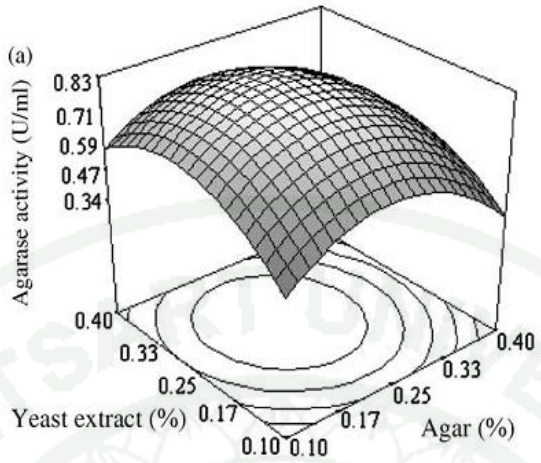


Figure 12 Response surface plots of agarase production by *Agarivorans albus* YWK-34. Interaction effect of agar and yeast extract on agarase production at fixed initial pH of 7.81.

Source: Fu *et al.* (2009)

8.1 Central composite design (CCD)

A Box-Wilson central composite design, commonly called "a central composite design" contains an imbedded factorial or fractional factorial design with center points that is augmented with a group of `star points' that allow estimation of curvature (Box and Wilson, 1951). This design is a very efficient design for fitting the second-order model. A CCD can be broken down into three parts, as shown in Figure 13.

- Two-level full or 2^{k}-fractional designs (the core).
- Axial points (outside the core).
- Center points.

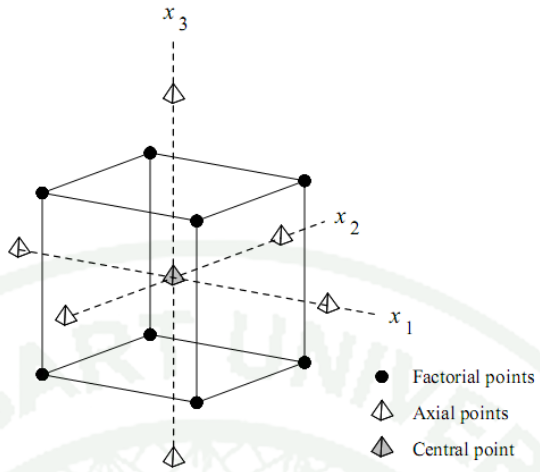


Figure 13 Central composite design for 3 design variables at 2 levels

If the distance from the center of the design space to a factorial point is ±1 unit for each factor, the distance from the center of the design space to a star point is ±α with |α| > 1. The precise value of α depends on certain properties desired for the design and on the number of factors involved (Park *et al*., 2008). Similarly, the number of center point runs the design is to contain also depends on certain properties required for the design. A central composite design always contains twice as many star points as there are factors in the design. The star points represent new extreme values (low and high) for each factor in the design. Table 4 summarizes the properties of the three varieties of central composite designs (Park and Kim, 1992). To maintain rotatability, the value of α depends on the number of experimental runs in the factorial portion of the central composite design:

$$\alpha = \left[\text{number of factorial runs}\right]^{1/4}$$

If the factorial is a full factorial, then

$$\alpha = \left[2^k\right]^{1/4}$$

Table 4 Determining α for rotatabiligy

Number of factors	Factorial portion	Scaled value for α relative to ±1
2	2^2	$2^{2/4} = 1.414$
3	2^3	$2^{3/4} = 1.682$
4	2^4	$2^{4/4} = 2.000$
5	2^{5-1}	$2^{4/4} = 2.000$
5	2^5	$2^{5/4} = 2.378$
6	2^{6-1}	$2^{5/4} = 2.378$
6	2^6	$2^{6/4} = 2.828$

The total number of experimental combinations is $2^k + 2k + n_0$, where k is the number of independent variables and n_0 is the number of repetitions of the experiments at the centre point. For statistical calculation, the experimental variables X_i have been coded as x_i according to the following transformation equation:

$$x_i = \frac{X_i - X_0}{\delta X}$$

where x_i is the dimensionless coded value of the variable X_i, X_0 is the value of X_i at the center point, and δX is the step change.

This methodology allows the modeling of a second order equation that describes the process. The models was analysed by multiple regression through the least squares method to fit the following equation (Myers, 1976):

$$Y = A_0 + \sum A_i X_i + \sum A_{ii} X_i + \sum A_{ij} X_i X_j$$

where Y is the predicted response variable; A_0, A_i, A_{ii}, A_{ij} are constant regression coefficients of the model, and X_i, X_j (i = 1, 3; j = 1, 3; $i \neq j$) represent the

independent variables in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2.

The RSM and CCD were increasingly used for optimization of various phases in some fermentation process such as physical parameters and factors, of many fermentation medium and process with various microorganisms. This method has been successfully applied to improve the production of xylanase by *Aspergillus niger* XY-1 (Xu *et al*., 2008), α -galactosidase by *Streptomyces griseoloalbus* (Sathyanesan *et al*., 2008) and α-amylase from *Bacillus amyloliquefaciens* (Gangadharan *et al*., 2008).

In case of using response surface method to enhance the enzyme production from actinomycetes strain, Techapun *et al* (2002) reported thermostable and alkaline-tolerant cellulase-free xylanase produced by *Streptomyces* sp. Ab106 using central composite design method. The optimum condition for xylanase production was 50°C and pH 7. The maximum yield of xylanase was about 15 IU without cellulase and manannase activities. Sathyanesan *et al*., (2008) reported statistical optimization of *a*-galactosidase production in submerged fermentation by *Streptomyces griseoloalbus* was investigated using response surface methodology. Using this statistical optimization method, the a-galactosidase production was increased from 17 to 50 U/mL.

MATERIALS AND METHODS

Meterials

1. ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, California, USA)
2. 3 L airlift bioreactor with 2 L working volume
3. Air pump (Heilea, China)
4. Autoclave (Sanyo, Belgium)
5. Balance (Satorious, Germany)
6. DO probe and controller (Meter Toledo, USA)
7. Electrophoresis (Hoef, USA)
8. Flow meter (Kofloc, Japan)
9. Glassware for analysis (Pyrex, USA)
10. High-Performance Liquid Chromatography (HPLC) (Shimadzu, Japan)
11. Light microscope
12. Liquid chromatography and Mass spectrometry (Shimadzu LCMS QP8000α)
13. Microplate reader (Labsystems Fluoroskan II Fluorescent Microplate Reader, WS-LABFII96)
14. MIDI Sherlock Microbial Identification System (Agilent Technologies 6890N Network GC System)
15. pH probe and controller (TOA, Japan)
16. Purification column size 5x30 cm and 10x45
17. pH meter (Eutech, Singapore)
18. TLC plates (Silica gel 60, 20x20cm; Merck)
19. Scanning electron microscope JSM6060 (JEOL, Japan)
20. Shaking incubator (Vision Scientific, Korea)
21. Spectrophotometer (UV-9200, Reyligh, China)
22. Ultrasonic processor model VCX 500 (Sonic and Materials, INC., Newtown, USA)

Methods

1. Screening of xylanase producing actinomycetes

Thirteen isolates of actinomycetes from Sakaerat Biosphere Reserve at Nakhonratchasima, previously identified as genera in Family *Streptosporangiaceae* based on morphology and chemotaxonomy using DAP and sugars in cell hydrolysate (Suriyachadkun, 2003) were used in this study.

The ability of xylanase production was screened on xylan medium containing (g/l) xylan, 10.0; peptone, 1.0; K_2HPO_4, 1.0; and $MgSO_4.7H_2O$, 0.2 and agar, 18 (pH 7.0). Xylanase activity on xylan agar plate was determined according to clear zone diameter. Each strain was point inoculated on the plate and incubated at 30°C for 7 days and measured clear zone diameter after flooding the plates with 0.1% aqueous Congo red for 15 min followed by repeated washing with 1 mol L^{-1} NaCl (Gessesse and Gashe, 1997). The average of duplicate clear zone was reported as xylan-degrading ability on the plate.

A loop of strain grown of yeast-malt extract agar slant was inoculated into xylan medium (50 ml) containing (g/L) xylan, 10.0; peptone, 1.0; K_2HPO_4, 1.0; and $MgSO_4.7H_2O$, 0.2 (pH 7.0), in a 250-ml flask and then cultured at 30°C on a rotary shaker for 7 days. After centrifugation at 10,000g for 10 min, the supernatant was used as crude enzyme.

Xylanase activity assay was measured with xylan as substrate. Enzyme solution (0.5ml) was added to 0.5 of 1% xylan in 100 mM acetate buffer, pH 5.5. After incubation at 60°C for 10 min, the reaction was terminated by adding 1 ml of 3, 5-dinitrosalicylic acid reagent (Miller, 1959). The mixture was heated in a boiling water bath for 5 min. Next, 5 ml of water was added and the A_{540} of the sample was measured. One unit of xylanase is defined as the amount of enzyme that release of 1 µmol of xylose in 1 min under the conditions described above.

The β-xylosidase and β-glucosidase activities were assayed using 0.9 mM *p*NP-β-D-xylopyranoside and *p*NP-β-D-glucopyranoside (Sigma Chemical Co.) as substrates, respectively, in 100 mM acetate buffer (pH 5.5) and enzyme to give a final volume of 1.1 ml. The reaction mixture was incubated for 30 min, and then 2.0 ml of 0.4 M sodium carbonate was added to terminate the reaction. The amount of *p*-nitrophenol released was measured by monitoring the optical density at 405 nm (Ratanakhanokchai *et al*., 1999.). The activity was expressed as µmol of *p*-nitrophenol released per minute per milliliter of enzyme solution.

Protease activity was determined as described by Hagihara *et al*. (1958). A mixture of 0.1 ml of the enzyme and 1 ml of 1 % (w/v) casein in 100 mM Phosphate buffer (pH 7.0) was incubated at 37°C for 10 min. The reaction was stopped by adding 3 ml of 10% trichloroacetic acid. The trichloroacetic acid-soluble fracion was measured at 275 nm photometric measurement. One unit (U) of the caseinolytic activity was difined as the enzyme activity releasing 1 µg of tyrosine equivalent per minute.

2. Morphological and biochemical characterization of the selected strains

2.1 Morphological characterization

The selected strain were grown on humic-vitamin (HV) agar plate at 30°C for 14 days and observed morphological structures using long working distance objective (400x and 1000x) of light microscope. Cultural characteristics were determined using 14-day cultures grown at 30 ^{O}C on various agar media according to the method of Shirling and Gottlieb (1966). The pigmentation and color of colony were examined. The selected strains were grown on yeast extract-malt extract (International *Streptomyces* Project (ISP-2)), oat meal agar (ISP-3), inorganic salt-starch agar (ISP-4), glycerol-asparagine agar (ISP-5) and tyrosine agar (ISP-7) as described in Appendix B1. The plates were checked at 7, 14 and 24 days. Color of colony and pigmentation determinations should be recorded only for mature cultures.

2.2 Light and electron microscope study of the selected strains

Morphological structures of the selected strains were observed using a scanning electron microscope (model JSM6060, JEOL, Ltd., Tokyo, Japan) and light microscope at high magnification.

The strains were grown on humic-vitamin (HV) agar plate at 30°C for 2 weeks. For scanning electron microscopy, the agar blocks containing the organism were fixed with the vapor of 2% osmium tetroxide at room temperature for 4 h. The specimen was dehydrated using a graded ethanol series, substituting isoamyl acetate for ethanol and then critical-point dried. After being sputter-coated with gold the specimen was observed under a scanning electron microscope. The yeast extract-malt extract medium was used for light microscope. An agar block small enough to fit under cover slip was cut out by a sterile block. Four sides of the agar block were inoculated with spores or mycelial fragment of the strain to be grown. A flamed cover slip was placed centrally upon the agar block. The plate was incubated at 30°C until growth and sporulation occurred. The cover slip was removed from the agar block. The strains were observed the morphology using scanning electron microscope.

2.3 Physiological and biochemical characterization

2.3.1 Ability to degrade substrates

The selected strains were examined for the ability to degrade cellulose, gelatin, casein and nitrate from nitrite and starch (inorganic salt-starch agar). Amount of 0.5 ml of inoculums which washed with normal saline was inoculated into each tube and incubated at 30°C for 7, 14 days. For observation cellulose degradation, filter paper will become soft due to degradation by the strain. In the case of gelatin liquefaction, after incubate the strain; the medium will be kept in refrigerator for 30 min. The positive result, gelatin become liquefies after kept in refrigerator compared with the control. Observation of casein degradation, the positive result is change of litmus paper color from blue to red (acid production in the medium). Reduction of

nitrate to nitrite is observed by using GR reagent (the positive is color change to red or pink) and Zn powder. Starch degradation, the result is observed clear zones on the plate.

2.3.2 NaCl tolerance and growth temperature

NaCl tolerance and growth temperature were assessed using yeast extract-malt extract medium. Washed inoculums were prepared. The uninoculated yeast extract-malt extract medium plates were dried by leaving them at room temperature for 4 hours after they were freshly poured or after removing them from refrigerator storage. Approximately 0.05 ml of washed inoculum was placed onto one edge of the agar surface. The drop was streaked straight across the dish. A second drop was placed onto the other edge. Only one culture per plate was used to avoid false positives due to cross feeding. Plates were observed after 10-16 days. The growth was compared with a control; growth on 0% NaCl in yeast extract-malt extract medium. The results were recorded as follows:

-Positive growth (++), when growth on tested NaCl concentration in basal medium was equal to or greater than growth on 0% NaCl in ISP-2 medium.

-Growth doubtful (+), when growth on tested NaCl concentration in yeast extract-malt extract medium was somewhat significantly less than on 0% NaCl in yeast extract-malt extract medium.

-Negative growth (-), when no growth on tested NaCl concentration in yeast extract-malt extract medium.

2.3.3 Utilization of carbon sources

Utilization of carbon sources were tested by using modified Pridham and Gottlieb medium (1948) (Appendix B1). Carbon sources for this test were no carbon source (negative control), D-glucose (positive control), L-arabinose,

cellulose, ducitol, D-fructose, D-galactose, I-inositol, maltose, D-mannitol, D-mannose, melezitose, D-raffinose, rhamnose, raffinose, D-sorbitol, D-xylose, and sucrose. They were sterilized without heat by the following methods: Filters sterilize 10% solution through bacteriological filter. Sterilized carbon sources were added to the basal mineral salts agar to give a final concentration of 1%. For example, 10 ml of 10% solution was added to 100 ml basal medium. After autoclaving, the basal agar medium was cooled to 60 °C and sterile carbon source was added aseptically to give a concentration of approximately 1%. The mixture was agitated and poured 25 ml of medium per dish into Petri dishes. Duplicate plates of each carbon compound were prepared for each culture to be tested. The medium was stored in refrigerator. A washed inoculum was prepared. The uninoculated plates were dried by leaving them at room temperature for 4 hours after they were freshly poured or after removing them from refrigerator storage. Approximately 0.05 ml of washed inoculum was placed onto one edge of the agar surface. The drop was streaked straight across the dish. A second drop was placed onto the other edge. Inoculate duplicate plates. Only one culture per plate was used to avoid false positives due to cross feeding. The plates were observed after 10-16 days. The growth from a given carbon source was always compared with the two controls; growth on basal medium alone, and growth on basal medium plus glucose. The results were recorded as follows:

-Strongly positive utilization (++), when growth on tested carbon in basal medium is equal to or greater than growth on basal medium plus glucose.

-Positive utilization (+), when growth on tested carbon is significantly better than on basal medium without carbon, but somewhat less than on glucose.

-Utilization doubtful (±), when growth on tested carbon is only slightly better than on the basal medium without carbon and significantly less than with glucose.

-Utilization negative (-), when growth is similar to or less than growth on basal medium without carbon.

Utilization of organic acids were also tested using defined medium of Gordon (1974) (Appendix B1). Organic acids for this test were benzoic acid, citric acid, fumaric acid, L-malic acid, mucic acid, succinic acid. The pH value of the medium was adjusted to 7.0 before the addition of 15 ml of 0.04% solution of bromocresol purple. They were autoclaved separately. After this agar medium was tubed and sterilized by autoclaving, 0.5 ml of a 10% solution of each organic acid was added aseptically to the tubes. The results were observed for acid color of the indicator after 7 and 28 days of incubation at 30°C.

3. Phylogenetic relationship based on 16S rDNA sequencing

The isolated strains were identified using 16S rRNA gene sequence and studied on phylogenetic relationship. Genomic DNA was extracted as described by Hopwood *et al*. (1985). These strains were grown in shaken TSB broth culture at 30°C for 3 days and harvested by centrifugation. Two gram (wet weight) of mycelium was suspended in 5 ml TE buffer (Appendix B2), lysozyme was then added. They were incubated at 30°C for 1 h by triturating every 15 min, 1.2 ml 0.5M EDTA was added; mixed gently and 0.13 ml protenase solution was added; mixed gently and incubated at 30°C, 5 min. Seven hundred µl of 10% SDS was added, tilted immediately then incubated at 37°C for up to 2 h. Five ml of phenol was added and shook thoroughly by hand for 10 min at room temperature. Five ml of phenol-chloroform was added and shook for 5 min at room temperature, centrifuged on a bench centrifuge (2000 rpm) for 5 min, transferred the upper (aqueous) phase with a shortened Pasteur pipette into a fresh screw cap bottle. Five ml of chloroform was added and shook for 5 min, centrifuged at 2000 rpm for 5 min, collected aqueous phase. Twelve ml absolute ethanol and 0.6 ml 3M sodium acetate were added, mixed gently and collected DNA pellet. Five ml of 70% ethanol was added, mixed and centrifuged for collecting DNA pellet. After that, it was dried by evaporator for 30 min, dissolved in 2 ml of TE buffer, stored at 4°C.

Further purification steps, including RNase treatment were carried out according to the method of Saito and Miura (1963). Added RNase to 10mg/ml and incubate 1 h at 37°C, repeated in steps adding phenol, then spooled DNA on a glass rod or sealed Pasteur pipette and transferred to a fresh screw cap. DNA was dissolved in 5 ml TE buffer overnight at 40°C. If DNA pellet was not completely dissolved, transferred to a 30°C orbital incubator. When DNA was completely dissolved, added 0.6 ml 3M sodium acetate and 12 ml absolute ethanol; mix. The DNA will precipitate immediately. Spooled DNA on a sealed Pasteur pipette and transferred to a fresh screw cap bottle. DNA was washed with 2 ml 70% ethanol. Removed the ethanol with a Pasteur pipette and finally dissolved the DNA in at least 1 ml sterile TE buffer, stored at 4°C.

A gene fragment specific for 16S rDNA gene-coding region was amplified by means of polymerase chain reaction (PCR). Two primers, 9F (5'-GAGTTT GATCCTGGCTCAG-3') (positions 9-27 on 16S rDNA) and 1541R (5'-AAGGAGG TGATCCAGCC-3') (positions 1541-1525 on 16S rDNA) were used. All primer positions were specified by the *Escherichia coli* numbering system (Brosius *et al*., 1978).

Amplification was carried out in 100 µl reaction mixture containing 100 ng of genomic DNA, 0.5 U of KOD plus DNA polymerase, 10 mM of dNTPs, 40 mM of each primer, 10 mM Tris-HCl and , 5 mM $MgCl_2$. and distilled water. The reaction was pre-denature at 94°C for 2 min, then repeated for 30 PCR cycles with denaturation at 94 °C for 15 sec, annealing at 50 °C for 30 sec and extension at 68 °C for 1.3 min. The amplified genomic DNA was purified by using PCR purification kit (spin type) according to the manufacturer's instruction. Visualization of the purified amplified genomic DNA was performed by electrophoresis using 1% agarose solution (Appendex B3) in 1X Tris-acetic acid EDTA (TAE) buffer and stained with ethidium bromide and observed under ultraviolet light.

The amplified and purified 16S rDNA genes were sequenced directly with an ABI PRISM Big Dye terminator V3.1 Cycle sequencing Ready Reaction Kit on an

ABI PRISM model 310 Genetic Analyzer (Applied Biosystems, Foster City, Califonia, USA).

The sequences of 16S rDNA were compared by BLAST Homology Search (http://www.ncbi.nlm.nih.gov/blast). The multiple alignments of the sequences obtained were made with program CLUSTAL X (version 1.81) (Thompson *et al*, 1997). Gaps and ambiguous bases were eliminated. The comparison of the aligned sequences was made for 1500 bases of 16S rDNA gene sequence in constructing phylogenetic trees by the neighbor-joining method (Saitou and Nei,1987) and MEGA 3 (Kumar *et al*, 2004). Distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). The confidence values of branches of phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

4. Identification of the selected strains

4.1 DNA analysis

4.1.1 Genomic DNA extraction

Genomic DNA was extracted from the selected strains as described in 3 (Hopwood *et al*., 1985).

4.1.2 DNA-DNA hybridization

DNA-DNA hybridization was used to determine genetic relatedness among representatives of the closely related species. Levels of DNA relatedness among the selected strains and related actinomycetes were determined with a method modified from Ezaki *et al*. (1989), using the β-galactosidase/4-methylumbelliferyl-β-galactoside system.

-Immobilization of DNA

DNAs of bacterial strains are prepared by the procedures as described above. A 100 µl portions of a heat-denatured, purified reference DNA solution (10 µg of DNA per ml) in phosphate-buffered saline (PBS) containing 0.1M $MgCl_2$ (Appendex B4) were incubated for 3 hours at 30°C in microdilution plates, discarded the solution and then dried at 55°C overnight. The solution was discarded, dried the plate and stored at room temperature.

-Labeling DNA with photobiotin

Amount of 1 mg of photobiotin (Appendex B4) was dissolved in 1 ml sterized milli Q water. A denatured DNA solution was mixed with an equal volume of photobiotin in eppendorf tube in the dark room. The uncapped eppendorf tube was stored on ice and irradiated with a sunlamp (300W) for 15 min. After irradiation, 0.1 M Tris-HCl buffer (pH 9.0) was added up to 200 µl. Then, 200 µl of 1-butanol was added and mixed by shaking. The upper layer was discarded after centrifugation at 15000 rpm for 5 sec (repeated 2 times). The solution was boiled for 5 min, cooled on ice and used immediately for hybridization experiments.

-Hybridization

For quantitative detection of biotylated DNA in microdilution wells, 200 µl portions of a pre-hybridization solution (Appendex B4) was added to microdilution wells with immobilized DNA, and then incubated at 37°C for 30 min. The pre-hybridization solution was removed from the wells and replaced with 100 µl portions of hybridization mixture containing 0.5 µg of heat-biotinylated DNA. The microplates were then covered and incubated at 55°C for 3 h. The solution was discarded and washed wells three times with 1X SSC buffer (Appendex B4).

-Binding of streptavidin-β-galactosidase complex (SABG) to DNA

A 100µl portion of a streptavidin-β-D-galactosidase solution (1 µg/) (Appendex B4) was added into wells, and incubated at 37°C for 30min. The solution was discarded and wells were washed three times with 300µl of 1X SSC buffer.

-Measurement of β-galactosidase activity (measurement of 4-MUF)

A 100µl portion of 4-methylumbelliferyl-beta-D-galactoside solution (0.01 µg/ml) (Appendex B3) was added into wells and incubated at 37°C for several periods of time. The fluorescence intensity was measured with a MicroFluor reader at a wavelength of 360 nm for excitation and 450 nm for emission.

-Calculation of % DNA similarity

% Similarity of DNA = 100X (X-N) / (P-N)

X: measured value of DNA to be tested
P: measured value of DNA as probe
N: measured value of DNA that has no relationship to DNA to be tested

4.1.3 Determination of DNA base composition

Genomic DNA was extracted from the selected strains as described above. The GC content of DNA was determined according to the method of Tamaoka and Komagata (1984) using HPLC. DNA was hydrolysed into nucleotides with nuclease P1 and bacterial alkaline phosphatase. DNA was dissolved in distilled water (1 mg/ml), then heated at 100 ^{o}C for 5 min and cooled rapidly in an ice bath. The

denatured DNA solution (10 µl) was mixed with 10 µl of nuclease P1 solution (0.1mg dissolved in 1 ml of 40 mM sodium acetate buffer containing 2 mM $ZnSO_4$, pH5.3), and incubated at 50 ^oC for 1 h. Then, 10 µl of bacterial alkaline phosphatase, 2.4 units/ml of 0.1 M Tris-HCl buffer, pH 8.1, was added to the sample, and incubated at 37 ^oC for 1 h. The hydrolysate was stored at -20 ^oC, and 5 µl of hydrolysate was applied to reversed-phase HPLC. The nucleosides were eluted by a mixture of 0.6 M $NH_4H_2PO_4$ (pH 4.0) and acetonitrile (20:1, v/v), at flow rate of 1 ml/min at room temperature. Each nucleoside was detected by UV absorbance at 270 nm.

Calculation of G+C content

Relative amounts of nucleosides were determined on peak areas, which represented integrated absorbance at 270 nm, and on coefficients of relative molar absorption using the following equation: relative amount of nucleoside in mol

$$= \frac{\text{peak area}}{\text{Coefficient of relative molar absorption}}$$

DNA base composition was calculated as follows:

$$\text{G+C mol\%} = \frac{Gr+Cr}{Ar+Gr+Cr+Tr}$$

(Nr = relative amount of each nucleoside in mol)

4.2 Chemotaxonomic of the selected strains

For biomass preparation, the selected strains were grown in shaken TSB broth culture at 30°C for 3 days, then harvested by centrifugation. Cells for the chemotaxonomic analysis were washed twice in distilled water and freeze-dried, for the molecular systematic works were washed in 0.1 NaCl-EDTA buffer and stored at -20°C until required.

4.2.1 Diaminopimelic acid (DAP, A_2pm)

The isomer of diaminopimelic acid in the cell hydrolysate was determined according to the method of Staneck and Roberts (1994). The selected strains were grown on ISP medium No.2 plate and incubated at 30°C. Amount of 30 µl of 6N HCl was added into the tube containing mycelium. The sealed tube was hydrolyzed by autoclave at 121°C for 15 min. one microliter of the hydrolyze was applied at the base line of the TLC plate (Cellulose, 20x20cm, Merck). Ascending TLC was performed with the solvent system methanol:distilled water:HCl:pyridine (80:17.5:2.5: 10, v/v) for approximately 3 h. After the chromatogram was air dried, spots were visualized by spraying with 0.2% ninhydrin in acetone and heating at 100 °C for 15 min. As a DAP standard, 1 µl of 0.01 M DL-DAP (Sigma Chemical Co.), which contained both *meso*- and L-DAP isomers, was used. The DAP spots were seen as gray-green fading to yellow, with the L isomer moving ahead of the *meso* isomer (Appendix Figure A1).

4.2.2 Analysis of whole-cell sugar

Fifty milligrams of freeze-dried biomass was hydrolyzed with 1ml of 1M H_2SO_4 at 100°C for 2 hours. After cooling, the hydrolysate was transferred to a 15-ml conical centrifuge tube, and saturated barium hydroxide ($Ba(OH)_2$) was added until pH was between 5.2 and 5.5 (determined with pH paper). The precipitate was removed by centrifugation. The supernatant fluid was evaporated in a 50-ml-beaker under a stream of air, and the residue was re-dissolved in 0.3 ml of distilled water (any insoluble material remaining at this step was removed by centrifugation); 1 µl of this hydrolysate was applied to the base line of the TLC sheet (Cellulose, 20x20cm, Merck) as well as 1 µl of each of two standard solutions. The first contains galactose, arabinose, and xylose, each at 1% concentration. The second solution contains rhamnose, mannose, glucose, and ribose, also each at 1%. Ascending TLC was performed with the solvent system n-butanol:distilled water:pyridine:toluene (10:6:6: 1, v/v) for approximately 4 hours. Spots were visualized by spraying the chromatogram with acid aniline phthalate (3.25 g of phthalic acid dissolved in 100 ml of water-saturated butanol

plus 2 ml of aniline) and heating at 100 °C for 4 min. Hexose spots were yellow after heating, and pentose spots were maroon. The carbohydrates migrated in the following sequence from the origin (slowest to fastest component): galactose, glucose, arabinose, mannose, xylose, ribose, and rhamnose. Madurose (3-o-methyl-D-galactose), if present in a hydrolysate, migrated the same distance as xylose but can be distinguished by its yellow color (Appendix figure A2).

4.2.3 Menaquinones

Menaquinones were extracted from 50-100 mg freeze-dried biomass by shaking in chloroform: methanol (2:1 v/v) overnight at cool temperature followed by evaporation then addition of acetone. Acetone was evaporated by N_2 and 100 µl ethanol was added. The purified menaquinone was analyzed by Liquid chromatography and Mass spectrometry.

4.2.4 Phospholipids

Phospholipids were extracted according to the method of Minnikin *et al.* (1979) and identified using two dimensional TLC. Phospholipids were extracted from 100 mg of freeze-dried biomass by shaking in Chloroform: methanol: 0.3%NaCl (2:4:16v/v) overnight at cool temperature. The solutions were transferred into separate centrifugation tubes and removed cell debris by centrifugation at 3000 rpm for 5 to 10 min. The upper layer was extracted from the biphasic mixtures and transferred them into a second set of centrifugation tubes. Amount of 1.75 ml of 0.3%NaCl and 1.75 ml of chloroform were pipette to both tubes. Before running a second centrifugation at 5000 rpm for 5 min, the suspension was mixed well on the vortex, and balanced tubes again afterwards. The lower chloroform phase in both tubes containing the lipids were transferred with Pasteur pipettes into separate 5 ml Pyrex bottles and brought them to dryness under a stream of nitrogen.

The dried lipid material was re-dissolved in 100 µl of ethanol and store at -20°C if not used on the same day. Amount of 5 µl of each of the merely

dissolved probes were spotted onto the lower left corner of the square TLC plates (Silica gel 60, 20X20 cm; Merck). The plates were developed in 2 dimensions using as mobile phase: in the first dimension a mixture of chloroform: methanol: water with a volumetric ratio of 65:25:4. Once the mobile phase reaches the upper limits of the plates, remove and dry them at room temperature for about 10 min; in the second dimension a mixture of chloroform: methanol: acetic acid: water with a volumetric ratio of 80:12:18:5. Once the mobile phase reaches the upper limits of the plates, remove and dry them at room temperature for about 10 min. TLC was detected and identified by the following staining procedures. Phosphorus-containing lipids were stained with ammonium molybdate stain. Amino-nitrogen-containing lipids were stained with ninhydrin stain. Carbohydrate-containing lipids were stained with periodate-Schiff stain. Mannose-containing lipids were stained with anisaldehyde detection. Acidic phospholipids were stained with rhodamine 6G stain. The absence of choline-containing compounds was demonstrated with Dragendorff reagent and with *cis*-aconinic acid anhydride.

4.2.5 Cellular fatty acid

Cellular fatty acid methyl esters were prepared and analysed according to the protocol of the MIDI Sherlock Microbial Identification System (Agilent Technologies 6890N Network GC System) (2002). Amount of 40 mg of bacterial cells were used. One milliliter of saponification solution (Appendex B5) was added to each tube containing cells. The tubes were securely sealed with teflon lined caps, vortexed briefly and heated in a boiling water bath for 5 minutes, at which time the tubes were vigorously vortexed for 5-10 seconds and returned to the water bath to complete the 30 minute heating. The cooled tubes were uncapped, 2 ml of methylation solution (Appendex B5) was added. The tubes were capped and briefly vortexed. After vortexing, the tubes were heated for 10 minutes at 80°C. Addition of 1.25 ml of extraction solution (Appendex B5) to the cooled tubes was followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes. The tubes were uncapped and the aqueous (lower) phase was pipetted out and discarded. About 3 ml of washing solution (Appendex B5) was added to the organic phase remaining in the tubes, the

tubes were recapped, and tumbled for 5 minutes. Following uncapping, about 2/3 of the organic phase was pipetted into a GC vial which was capped and ready for analysis.

A 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column has both the chromatographic performance and the column lifetime desired for routine analysis of bacterial extracts. The temperature program ramps from 170 °C to 270 °C at 5 °C per min. Following the analysis, a ballistic increase to 300 °C allows cleaning of the column during a hold of 2 min. The flame ionization detector allows for a large dynamic range and provides good sensitivity. Hydrogen is the carrier gas, nitrogen is the "makeup" gas, and air is used to support the flame.

5. Optimization of fermentation process for xylanase production

5.1 Effect of nitrogen source on xylanase production

The effect of nitrogen sources on the xylanase production by *M. siamensis* DMKUA 245^T was studied in the basal medium consisted of (g/L): xylan 10, peptone 1.0, K_2HPO_4 1.0 and $MgSO_4.7H_2O$ on a rotary shaker for 120 h at 40°C and 180 rpm. Six nitrogen sources (1.0 %, w/v): cassamino acid, casein, gelatin, malt extract, peptone and skim milk were used.

5.2 Plackett-Burman design

Plackett-Burman experimental design was used to determine the relative significance of 5 factors that influenced xylanase production by a new thermotolerant *Microbispora siamensis* DMKUA 245^T. The factors or independent variables considered for study included 5 factors (X_1 to X_5, representing xylan, casein, K_2HPO_4, $MgSO_4.7H_2O$ and temperature, respectively). All variables were numerical factors and were investigated at two widely spaced levels designated as –1 (low level) and +1 (high level). The factors under investigation as well as levels of each factor used in the experimental design were shown in Table 5, whereas Table 6 represents the design

matrix. Plackett–Burman experimental design is based on the first order polynomial model:

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

where Y is the response (enzyme activity), β_0 is the model intercept and β_i is the linear coefficient, and x_i is the level of the independent variable. This model is used to screen and evaluate the important factors that influence the response. In the present work, 5 assigned variables were screened in 8 experimental designs. All experiments were carried out in triplicate and the averages of the xylanase activity were taken as response (Table 6). From the regression analysis the variables, which were significant at 95% level ($P < 0.05$) were considered to have greater impact on xylanase production. The experimental design and statistical analysis of the data were done by using SPSS for window (version 11.5, 2002; USA).

Table 5 Experimental variables at different levels used for xylanase production by a new thermotolerant *M. siamensis* DMKUA 245^T using Plackett-Burman design.

Variable code	Variables	Levels	
		Low (-1)	High (+1)
X_1	Xylan (g /L)	10	30
X_2	Casein (g /L)	0.5	2.5
X_3	K_2HPO_4 (g /L)	0.1	1.9
X_4	$MgSO_4.7H_2O$ (g /L)	0.1	0.3
X_5	Temperature (°C)	35	45

Table 6 Plackett-Burman experimental design matrix with xylanase production by a new thermotolerant *M. siamensis* DMKUA 245^T

Run no.	X_1	X_2	X_3	X_4	X_5	dummy	dummy	dummy
1	1	1	1	-1	1	-1	-1	-1
2	1	1	-1	1	-1	-1	-1	1
3	1	-1	1	-1	-1	-1	1	1
4	-1	1	-1	-1	-1	1	1	1
5	1	-1	-1	-1	1	1	1	-1
6	-1	-1	-1	1	1	1	-1	1
7	-1	-1	1	1	1	-1	1	-1
8	-1	-1	-1	-1	-1	-1	-1	-1

5.3 Optimization of xylanase production by strain DMKUA 245^T

Response surface methodology was used to optimize the medium composition for enhancing xylanase production. In this study, the central composite design (CCD) with three factors (casein, $MgSO_4.7H_2O$ and temperature) and five levels, including three replicates at the center point, was used for fitting a second order response surface.

A CCD always contains twice as many star points as factors in the design. The star points represent new extreme values (low and high) for each factor in this design. To maintain rotatability, the value of α depends on the number of experimental runs in the factorial portion of the CCD.

In this study k = 3 factors (casein, $MgSO_4.7H_2O$ and temperature) could be written as:

$$\alpha = [2^k]^{1/4} = 1.68 \quad (2)$$

Table 7 and Table 8 showed the factors, their values and the experimental design. The variables were casein, $MgSO_4.7H_2O$ and temperature and the responses were the xylanase activity. A second-degree quadratic model was established as Eq. (3) by using the method of least squares as follows:

$$Y = a_0 + a_2X_2 + a_4X_4 + a_5X_5 + a_{24}X_2X_4 + a_{25}X_2X_5 + a_{45}X_4X_5 + a_{22}X^2_2 + a_{44}X^2_4 + a_{55}X^2_5 \quad (3)$$

where Y is the predicted response (xylanase yield); X_2, X_4 and X_5 the coded forms of the input variables (casein, $MgSO_4.7H_2O$ and temperature, respectively); a_0 a constant; a_2, a_4 and a_5 the linear coefficients; a_{24}, a_{25} and a_{45} a cross-product coefficient; a_{22}, a_{44} and a_{55} the quadratic coefficients. The relation between the coded forms of the input variable and the actual value of casein, $MgSO_4.7H_2O$ and temperature are described as Eq. (4):

$$x_i = \frac{X_i - X_0}{\delta X} \quad (4)$$

where x_i is the dimensionless coded value of the variable X_i, X_0 is the value of X_i at the center point, and δX is the step change (e.g., coded casein, +1 is equal to 0. 7). The center point in this study was obtained from Plackett-Burman design.

Table 7 Experimental range and levels of the independent variables used in central composite design.

Independent variables	**Level**				
	-1.68	**-1**	**0**	**1**	**1.68**
Casein (X_2)	0.16	0.3	0.5	0.7	0.84
$MgSO_4.7H_2O$ (X_4)	0.05	0.15	0.3	0.45	0.55
Temperature (X_5)	31.6	35	40	45	48.4

Table 8 Experimental design used in response surface methodology of 3 independent variables, (X_2) casein, (X_4) $MgSO_4.7H_2O$ and (X_5) temperature, with three center points.

Treatment Number	Code setting level			Actual level		
	X_2	X_4	X_5	X_2	X_4	X_5
1	-1	-1	-1	0.3	0.15	35
2	-1	-1	1	0.3	0.15	45
3	-1	1	-1	0.3	0.45	35
4	-1	1	1	0.3	0.45	45
5	1	-1	-1	0.7	0.15	35
6	1	-1	1	0.7	0.15	45
7	1	1	-1	0.7	0.45	35
8	1	1	1	0.7	0.45	45
9	-1.68	0	0	0.16	0.3	40
10	1.68	0	0	0.84	0.3	40
11	0	-1.68	0	0.5	0.05	40
12	0	1.68	0	0.5	0.55	40
13	0	0	-1.68	0.5	0.3	31.6
14	0	0	1.68	0.5	0.3	48.4
15	0	0	0	0.5	0.3	40
16	0	0	0	0.5	0.3	40
17	0	0	0	0.5	0.3	40

The data from the experimental design were subjected to a second-order multiple regression analysis using the least squares regression methodology to obtain the parameter estimators of the mathematical model. SPSS for windows and Statistica 5.0 software (Statsoft, USA) were used for regression analysis and graphical analysis of the data, respectively.

7. Batch fermentation in an Airlift fermenter

A schematic diagram of the experimental equipment is shown in Figure 14. Since this study was investigated on a scale 100 ml Erlenmeyer flask level, there is a need to scale-up the production of the enzyme. Therefore, the fermentation was carried out in 3 L airlift fermenter which was 185 mm in diameter and 632 mm high, with 2 L working volume. The fermenter, which surrounded by a water jacket for temperature control, was made from glass. The air sparger was a multi porous plate (10 mm in diameter) located at the bottom of the bioreactor. The DO probe, pH probe and antifoam sensor were positioned at the top of the bioreactor as shown in Figure 14.

The antifoam sensor was located 10 cm from the top of the upper broth surface. All the probes and sensor were interfaced with a control unit. The temperature in the fermenter was maintained at 45 ^{o}C. During fermentation, samples were collected at certain time intervals for further analysis.

7.1 The effect of pH

The effect of pH values on the enzyme production in an airlift fermenter was investigated at 45 ^{o}C and aeration rate of 0.5 vvm. Uncontrolled pH and controlled at pH 7.0 were studied. During fermentation, samples were collected from fermenter at certain time intervals (24 h) for xylanase activity assay.

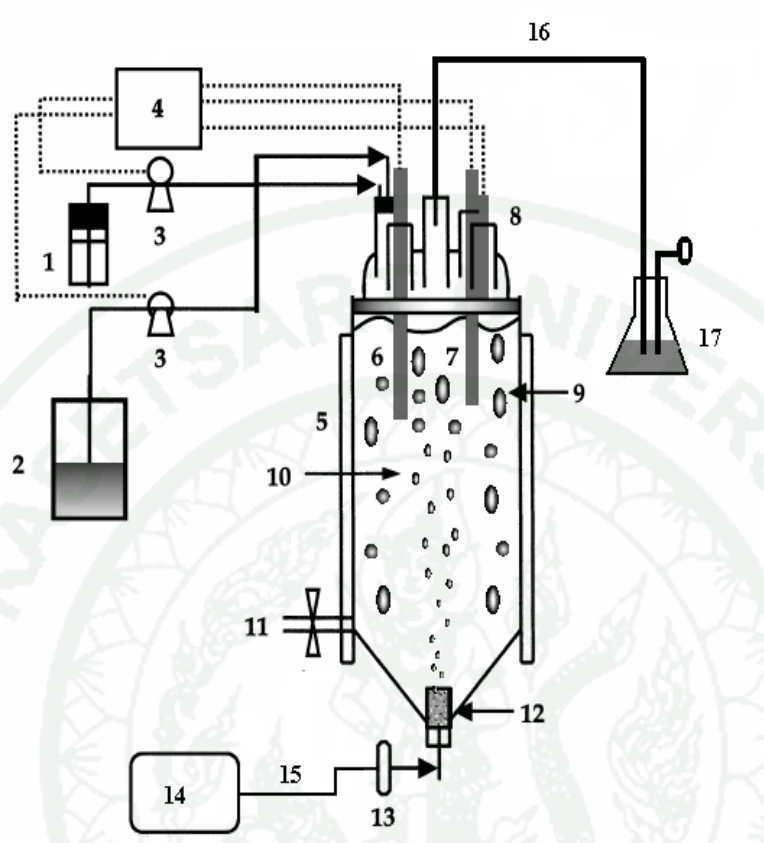


Figure 14 Schematic diagrams of 3 L airlift bioreactor used throughout this study. Experiment apparatus:

1. antifoam sensor	2. alkaline reservoir	3. pump
4. packed controller	5. water jacket	6. pH probe
7. DO probe	8. antifoam probe	9. dispersed bubble
10. sampling line	11. sparger	12. air filter
13. flow meter	14. air pump	15. air inlet line
16. air outlet line	17. 4% $CuSO_4.5H_2O$ solution	

Source: Modified from Miura *et al.* (2003)

7.2 The effect of aeration rate

To study of the effect of aeration rate, air flow meter was set at 0.25, 0.5 and 0.75 vvm. The condition for xylanase production in an airlift fermenter was uncontrolled pH and 45 ^{o}C. The samples were taken every 24 h for 120 h and used for xylanase activity assay.

Actinomycetes are also filamentous bacteria, usually formed pellet in the liquid medium. Growth estimation based on oxygen consumption assumes that the metabolism of these compounds is completely growth associated. The general principle is to interrupt the supply of oxygen and to measure the decrease of the dissolved oxygen concentration with time. To achieve the OUR of actinomycetes grown in fermenter during cultivation is shown in Appendix C1.

8. Purification of the xylanase produced by strain DMKUA 245^{T}

Xylanase was produced by strain DMKUA 245^{T} in the optimized medium. The culture supernatant was harvested by centrifugation at 10000 x *g* for 20 min. The supernatant was added in 100 ml DEAE-Sepharose at 4°C and left overnight. The upper layer was discarded. Then, the enzyme was eluted with 0.5 M NaCl and dialyzed with 50 mM Tris-HCl buffer (pH 8.0) overnight. The dialyzed enzyme was applied to a 100 ml DEAE-Sepharose column (10x45 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and eluted with a linear (0–0.5M) NaCl gradient. Active fractions were combined. Protein concentration was assayed using Lowry method (Appendix C2) with bovine serum albumin as a standard. Purified enzyme was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Appendix B6) to determined protein molecular weight.

9. Characterization of purified enzyme

9.1 Effect of pH and temperature on xylanase activity and stability

The purified enzyme was assayed at various pH values (3.0-12.0). The buffer system were: pH 3.0–5.5 acetate buffers, pH 5.5-7.0 citrate buffers, pH 7.0–8.0 phosphate buffers, pH 8.0–9.0 Tris buffers, and pH 9.0-12.0 glycine-NaOH buffers. The effect of pH on xylanase stability was studied by pre-incubating the enzyme at 4^{o}C for 24 h in buffers of pH 3.0-12.0. The enzyme assay was also determined at various temperatures (30-80^{o}C) (0.1 M acetate buffer, pH 5.5). Thermal stability of the enzyme was investigated by pre-incubation at various temperatures (30-80^{o}C) for 1 h. The samples were then assayed for residual xylanase activity as described above.

9.2 Activity on different substrates and kinetic parameters

The activity of purified enzyme was determined toward different substrates, beechwood xylan, wood xylan, insoluble xylan, corn cob xylan, *p*NP-β-D-xylopyranoside, *p*NP-β-D-glucopyranoside and carboxymethyl cellulose.

The activity toward beechwood xylan, wood xylan, insoluble xylan and corn cob was assayed in 100 mM acetate buffer, pH 5.5. After incubation at 60°C for 10 min, the reaction was terminated by adding 3 ml of 3,5-dinitrosalicylic acid reagent (Miller, 1959). The A_{540} of the sample was measured. One unit of xylanase is defined as the release of 1 µmol of reducing sugar/min under the conditions described above.

The activity of the purified enzyme on *p*NP-β-D-xylopyranoside and *p*NP-β-D-glucopyranoside was examined under the condition as described above.

The cellulase activity was measured under the same conditions as described above using carboxymethyl cellulose as a substrate.

For the kinetic experiments, different concentrations of each substrate were prepared in 100 mM acetate buffer (pH 5.5), and incubated with the purified xylanase at 60°C for 10 min. The K_m and V_{max} value were calculated from the kinetic data.

9.3 Thin layer chromatography (TLC)

Xylan hydrolysis products were determined by thin-layer chromatography on HPTLC plates cellulose (Merck; 10x10) with a mixture of n-butanol-acetic acid-water (2:1:1) as a solvent system (Kubata *et al*., 1994) The sugar spots were detected by heating the plates to 100C after spraying them with 4 g – diphenylamine, 4 ml aniline, 200 ml of acetone and 30 ml of 80% phosphoric acid (Ratanakhanokchai *et al*., 1999).

9.4 Effect of EDTA on enzyme activity

To remove the metal ion including in the purified enzyme molecule, the purified enzyme was pretreated with 5 mM EDTA at 4 ^{o}C overnight. The treated enzyme was dialysed in 0.05 M Tris-HCl buffer pH 8.0 to remove EDTA. The remained activity of EDTA treated pure enzyme was determined.

9.5 Effect of metal ions as chloride on enzyme activity

The influence of metal ions as chloride on EDTA treated pure enzyme was investigated by using dialysed enzyme from 8.3 and incubated with 1 mM each metal ion; Ca^{2+}, Co^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{1+} ,Zn^{2+} and 10 mM of SDS in the presence and absence of EDTA. Residual activity was measured using standard assay procedure.

RESULTS AND DISCUSSIONS

1. Screening of xylanase producing actinomycete strains and phylogenetic relationship based on 16S rDNA sequencing

A total of 13 isolates of actinomycete strains belonged to family *Streptosporangiaceae*, previously isolated from Sakaerat Biosphere Reserve at Nakhonratchasima (Suriyachadkun *et al*., 2003) were screened for xylanase production. The ability of xylanase production of each strain on xylan agar plate was shown in Table 9. All strains except DMKUA 222 and 225 formed a clear zone on the plate. The maximum of 3.5 cm in diameter of clear zone on xylan agar plate was exhibited by strain DMKUA 245 for 7 days. Therefore, DMKUA 245 was selected for optimization of fermentation process.

Phylogenetic study based on 16S rRNA gene sequences revealed the 11 strains of xylan degrading actinomycete as shown in Figure 15. The partial 16S rDNA sequences of the strains, consisting of 700-800 nucleotides, were compared with sequences from members of the family *Streptosporangiaceae*. Strain DMKUA 190, 195, 214, 215 and 216 were identified as *Microtetraspora* sp. Strain DMKUA 205 was identified as *Herbidospora* sp. Strain DMKUA 224, 245, 255and 261 belonged to *Microbispora* sp. and strain DMKUA 168 belonged to *Nonomuraea* sp. From the results of phylogenetic position, DMKUA 205 was identified in this research because of having only one species at that time, therefore it would have a posibility to earn new species. Previous study of DMKUA 245 was interesting in degradation of organic polymer such as xylan and chitin and showed the maximum of clear zone on xylan agar plate. Moreover, as phylogenetic tree results, it trended to be new species. Therefore DMKUA 245 was also focused in this research.

Table 9 Xylan degrading ability on xylan agar plates incubated at room temperature for 7 days.

Strain DMKUA	Diameter of colony (cm)	Diameter of clear zone (cm)
168	0.5	1
190	0.5	1
195	1	2
205	0.5	3
214	0.7	2
215	0.5	2.5
216	0.7	2.5
222	0	0
224	1	3
225	0	0
245	0.5	3.5
255	1	3
261	0.6	2.5

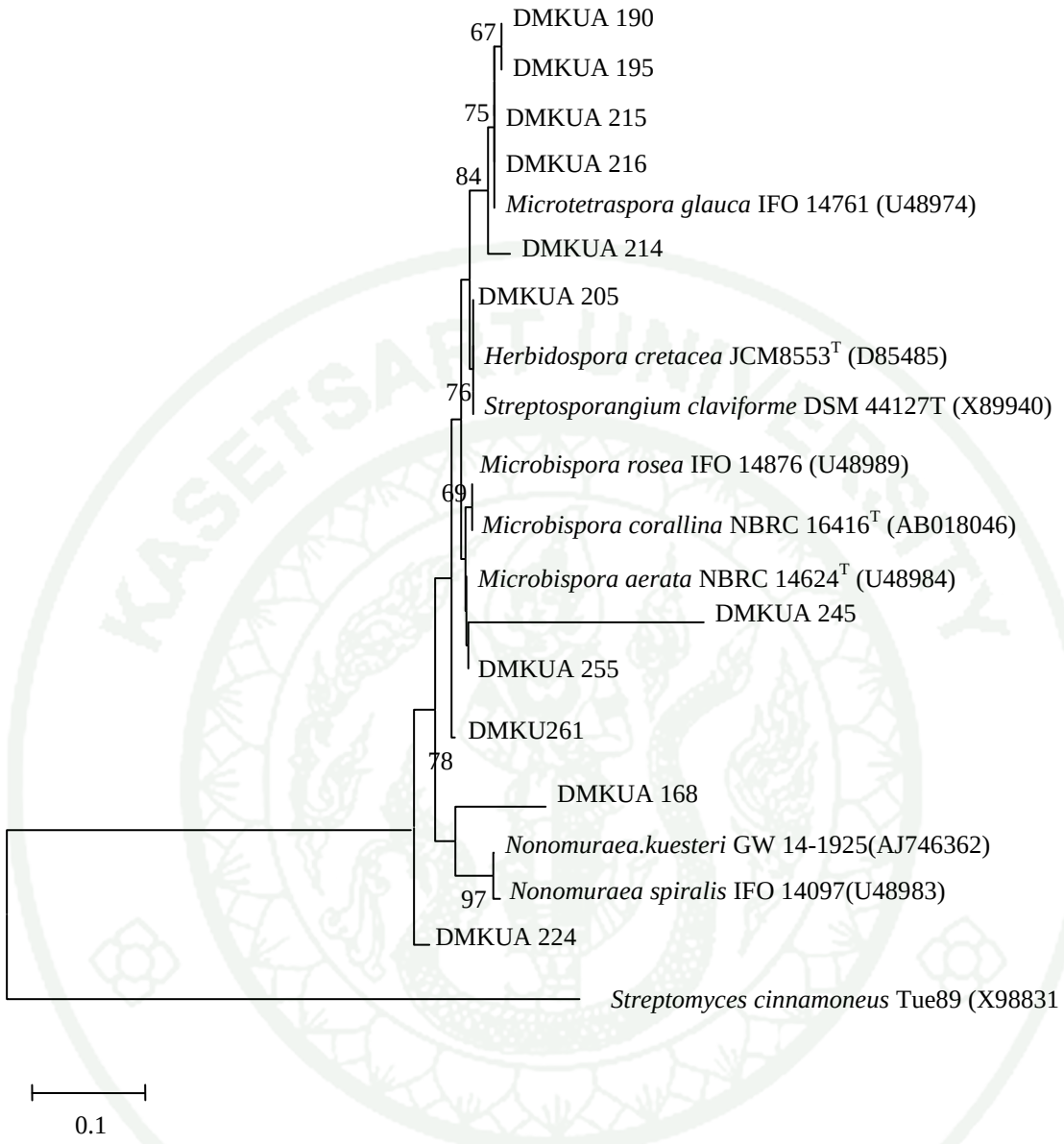


Figure 15 Neighbour-joining tree based on 700-800 neucleotides of 16S rRNA gene sequences showing the relationship of xylan-degrading actinomycetes. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates (only values >50% are indicated). Bar, 0.1 substitutions per nucleotide position.

2. Identification of strain DMKUA 205 and DMKUA 245

Actinomycete, strains DMKUA 205 and DMKUA 245 were identified using a polyphasic approach. In case of strain DMKUA 205, phylogenetic tree (Figure 16) based on 16s rRNA gene sequences, consisting of 1497 nucleotides, showed that strain DMKUA 205 was phylogenetically closely related to *Herbispora yilanensis* and *H. daliensis* species. (Figure 16). The 16S rRNA gene sequences of strain DMKUA 205 shared 99% similarity with *H. yilanensis*, and *H. daliensis* species. Furthermore, DNA–DNA relatedness values in reciprocal hybridizations (Table 10) were much lower than 70% between strain DMKUA 205^T and the following closely related organisms: *H. yilanensis* 0351M-12^T (35-54% %, reciprocally) and *H. daliensis* 0358M-1^T (58-65 %, reciprocally), indicating that strain DMKUA 205^T represents a separate genomic species. The G+C content of the DNA was 73 mol%.

The phylogenetic assignment of strain DMKUA 205 was supported by the results of chemotaxonomic studied (Table 11). The strain contains *meso*-diaminopimelic acid in cell wall amino acid and glucose, mannose, ribose, and madurose (3-O-methyl-D-galactose) in whole-cell hydrolysates (cell wall type IIIB of Lechevalier & Lechevalier, 1970). The phospholipid profile contained phosphatidylethanolamine, phosphatidylmethylethanolamine and glucosamine containing phospholipids. The major fatty acids were pentadecanoic acid (29% of total fatty acids), 14-methylpentadecanoic acid (17.4% of total fatty acids) and 10-methyl heptadecanoic acid (16.5% of total fatty acids). The predominant menaquinones were MK-10(H_4) and MK-10(H_6).

Morphological observation of 21-day-old culture grown on ISP-3 agar was shown in Figure 17. The phenotypic properties of strain DMKUA 205, *H. yilanensis* 0351M-12^T and *H. daliensis* 0358M-1^T were shown in Table 12. Strain DMKUA 205^T grew well on ISP-2 and ISP-3 (Appendix Figure A1). The vegetative mycelium was colorless to yellowish brown. The surface of the colony was white when sporulation occurred. No diffusible pigments were produced in any agar medium. While, *H. yilanensis* 0351M-12^T grew well on ISP-3 and ISP-5. The substrate mycelium were

pale yellow and colorless on ISP-3 and ISP-5, respectively. The aerial mycelium was white on ISP-3 and ISP-5. No diffusible pigments were produced in any agar medium. In case of *H. daliensis* 0358M-1^{T} grew well on ISP-3 and ISP-4. Substrate mycelium was strong yellow on ISP-3 and grayish yellow on ISP-4. The aerial mycelium was white and colorless on ISP-3 and ISP-4, respectively. Sporulation occurred on ISP-3. No soluble pigments were produced on any of the media tested.

The physiological and biochemical characteristics of strain DMKUA 205 and the phylogenetically closest species *H. yilanensis* 0351M-12^{T} and *H. daliensis* 0358M-1^{T} (Tseng *et al*., 2010) from Table 13 allowed differentiation among strain DMKUA 205, *H. yilanensis* 0351M-12T and *H. daliensis* 0358M-1^{T}. The temperature range for growth was 20-40°C. Strain DMKUA 205 grew on the medium containing 1.5% NaCl while strain 0351M-12^{T} and 0358M-1^{T} grew on 1% and 5% NaCl, respectively (Table 13).

It is evident from the genotypic and phenotypic data that strain DMKUA 205 is distinguished from previously described species of the genus *Herbidospora*. It is therefore proposed that this strain be classified as a new species of the genus *Herbidospora* and the name *Herbidospora sakaeratensis* sp. nov. is proposed for strain DMKUA 205^{T}.

DNA-DNA relatedness values in reciprocal hybridizations were higher than 70% (Wayne *et al*., 1987) between *Streptosporangium claviforme* NBRC 15623^{T} and *Herbidospora cretacea* JCM 8553^{T} (82.1 and 89.3%, reciprocally), indicating that *S. claviforme* is identified as *H. cretacea* (Table 10). Therefore, the name *S. claviforme* should be treated as a synonym of *H. cretacea.*

Description of *Herbidospora sakaeratensis* sp. nov.

Herbidospora sakaeratensis sp. nov. (sa.ka.e.ra.ten′sis. N.L. fem. adj. *sakaeratensis*, pertaining to Sakaerat Biosphere Reserve, the source of the soil from which the type strain was isolated).

Aerobic, Gram-positive, mesophile, non-motile actinomycetes that forms non-fragmented branched vegetative hyphae, but true aerial mycelium are not formed. Spores are short rod and have smooth surfaces. Temperature range for growth is 20-40°C. Growth occurs between pH 6.0-9.0. The maximun NaCl concentration for growth is 1.5%. Utilizes L-arabinose, D-fructose, D-galactose, D-glucose, D-mannitol, D-mannose, starch, sucrose and D-xylose as sole carbon sources, but not dulcitol, inositol, maltose, melezitose, D-raffinose, L-rhamnose, D-ribose and D-sorbitol. Utilizes fumaric acid, L-malic acid and succinic acid, but not benzoic acid and mucic acid. Cellulose degradation, gelatin liquefaction, nitrate reduction, milk peptonization and melanoid pigment formation are negative. Cell walls contain *meso*-diaminopimelic acid and *N*-acetylated muramic acid. Whole-cell hydrolysates contain madurose (chemotype IIIB). The phospholipid profile contains phosphatidylethanolamine, phosphatidylmethylethanolamine and glucosamine-containing phospholipids (phospholipids type PIV). The major cellular fatty acids were pentadecanoic acid, 14-methylpentadecanoic acid and 10-methyl heptadecanoic acid (fatty acid type 3c). Mycolic acids are absent. The predominant menaquinones are MK-10(H_4) and MK-10(H_6). The DNA G+C content of the type strain is 73 mol%. Habitat is soil. The type strain is strain DMKUA 205^T (=BCC 11662^T =NBRC 102641^T).

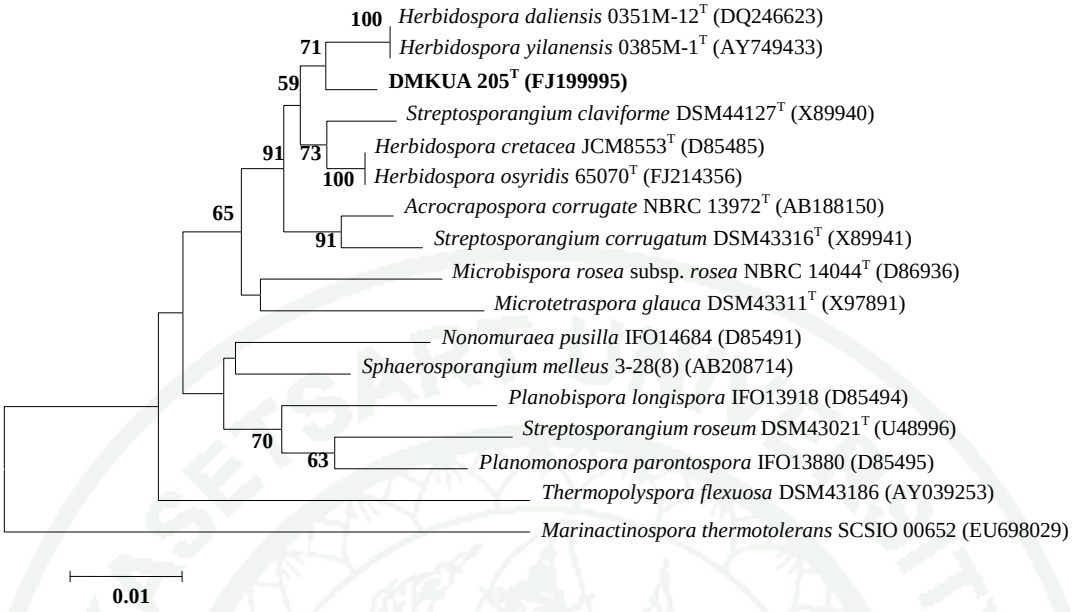


Figure 16 Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain DMKUA 205^T, *Herbidospora* species and representatives of the family *Streptosporangiaceae. Marinactinospora thermotolerans* SCSIO00652 (EU698029) is used as an outgroup. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates (only values >50% are indicated). Bar, 0.01 substitutions per nucleotide position

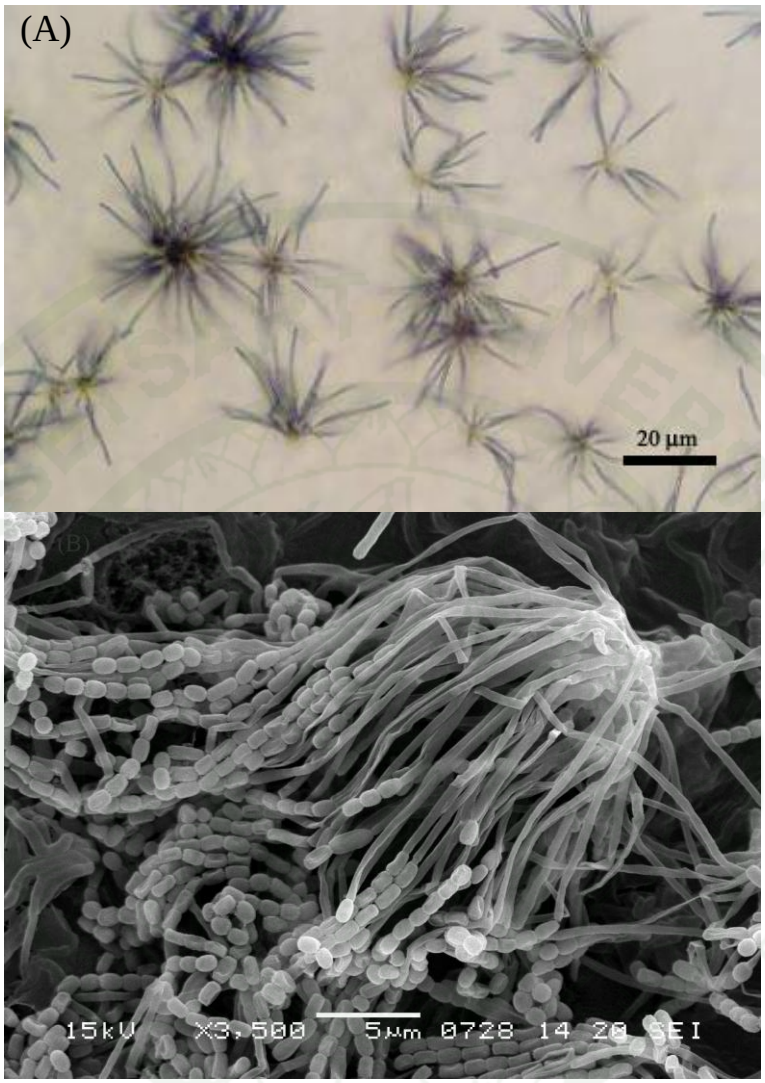


Figure 17 Photo micrograph (A) and scanning electron micrographs (B) of strain DMKUA 205^{T} grown on oatmeal agar (ISP-3) for 21 days at 30^{O}C. Magnification: X400(A) and X3,500(B).

Table 10 Levels of DNA relatedness among strain DMKUA 205^T, *H. yilanensis* 0351M-12^T, *H. daliensis* 0385M-1^T, *H. cretacea* JCM 8553^T and *Streptosporangium claviforme* NBRC 15623.

Strain	**G+C content (mol %)**	**% of DNA complementarity with labeled DNA from:**				
		DMKA 205^T	**0351M-12^T**	**0385M-1^T**	**NBRC 15623**	**JCM 8553^T**
DMKUA 205^T	73	100	35±13.4	58±4.9	43.7±2.6	46.5±0.7
0351M-12^T	70.6	54±13.4	100	53.6±0.9	ND	ND
0385M-1	70.7	65±4.9	54.9±0.9	100	ND	ND
NBRC 15623	ND	47.4±2.6	ND	ND	100	82.1±5
JCM 8553^T	70	45.5±0.7	ND	ND	89.3±5	100

The data are mean ± standard deviations from the mean.

ND, not determined.

Table 11 Chemotaxonomic characteristic of strain DMKUA 205

Characteristic	**Strain DMKUA 205**
Peptidoglycan	*meso*-DAP
Whole cell sugar	Type B (glucose, madurose and ribose)
Phospholipid	Phosphatidylethanolamine, phosphatidylmethylethanolamine, ninhydrin-positive glycophospholipids
Menaquinone	
MK-10(H_6)	2.1%
MK-10(H_4)	14.2%
Fatty acid*	
$C_{13:0}$	5.2%
Iso-$C_{14:0}$	3.5%
$C_{15:0}$	29.1%
2OH- $C_{15:0}$	3.7%
Iso-$C_{16:0}$	17.4%
Iso-2OH-$C_{16:0}$	4.3%
$C_{17:0}$	8.2%
Cis9-$C_{17:1}$	12.1%
10 methyl-$C_{17:0}$	16.5%

*Abbreviations for fatty acids: $C_{13:0}$, tridecanoic acid; Iso-$C_{14:0}$, 12-methyltridecanoic acid; $C_{15:0}$, pentadecanoic acid; 2OH- $C_{15:0}$, 2-hydroxy-pentadeacnoic acid; Iso-$C_{16:0}$, 14-methylpentadecanoic acid; Iso-2OH-$C_{16:}$0, 2- hydroxy-14-methylpentadecanoic acid; $C_{17:0}$, heptadecanoic acid; Cis9-$C_{17:1}$, cis-9-heptadecanoic acid; 10 methyl-$C_{17:0}$, 10-methyl heptadecanoic acid.

Table 12 Culture characteristics of strains DMKUA 205^T and *H. yilanensis* 0351M-12^T and *H. daliensis* 0358M-1^T

	DMKUA 205^T		**0351M-12^T**		**0385M-1^T**	
Agar medium	**Growth**	**Sporulation**	**Growth**	**Sporulation**	**Growth**	**Sporulation**
Yeast extract-malt extract (ISP- 2)	Good (Mustard gold)	None	None to poor (Deep yellow)	None	Poor (Deep yellow)	None
Oatmeal agar (ISP-3)	Good (Bamboo)	Good (White)	Good (Pale yellow)	Good (White)	Good (Strong yellow)	Good (White)
Inorganic salt-starch agar (ISP- 4)	None to poor (Colorless)	None	Poor (Yellowish white)	None	Good (Grayish yellow)	Poor (Colorless)
Glycerol-asparagine (ISP-5)	Poor (Colorless)	None	Good (Colorless)	Modurate	Moderate (Colorless)	None

Table 13 Differential characteristics of strains DMKUA 205^{T}, *H. yilanensis* 0351M-12^{T} and *H. daliensis* 0358M-1^{T}.

Carbon utilization			
L-Arabinose	+	-	+
Maltose	-	+	+
Mannose	+	+	-
Melezitose	-	+	+
myo-Inositol	-	+	-
Raffinose	-	+	+
Rhamnose	-	+	+
Xylose	+	-	-
Oraganic acid utilization			
Fumaric	+	+	-
DL-Lactic	-	+	-
L-Malic	+	-	-
Succinic	+	+	-
NaCl tolerance	1.5%	1%	5%

+, Positive; -, negative; w, weak reaction.

[a]All strains were positive for utilization of D-fructose, D-galactose, D-glucose, D-mannitol and sucrose and growth at 20-40°C. All strains were negative for cellulose degradation, utilization of benzoic acid, ducitol, mucic acid, D-ribose and D-sorbitol

In addition, strain DMKUA 245, phylogenetic tree based on 16s rRNA sequences, consisting of 1470 nucleotides, had the highest similarity values with sequences of members of genus *Microbispora* and the 100% bootstrap value clearly indicated the position of this isolate within a coherent cluster of the genus *Microbispora* (Figure 18). The 16S rRNA similarities between strain DMKUA 245 and the type strain of validly described *Microbispora* species ranged from 98.6% (*M. rosea* subsp. *rosea* and *M. rosea* subsp. *aerate*) to 97.8% (*M. corallina*). Furthermore, DNA–DNA relatedness values (Table 14) determined by reciprocal hybridizations were much lower than 70 % between strain DMKUA 245 and other members of the genus *Microbispora* (19 to 46%). Thus a fair conclusion was that strain DMKUA 245 represented a separate genomic species. The DNA G+C content of the type strain is 68 mol%.

The phylogenetic assignment of strain DMKUA 245 was supported by the results of chemotaxonomic studied (Table 15). Whole-cell hydrolysates of strain DMKUA 245 contained *meso*-diaminopimelic acid as the diagnostic diamino acid and madurose as the diagnostic sugar. Diphosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylinositol mannosides and ninhydrin-positive glycophospholipids were detected as polar lipids. This phospholipids pattern is similar to that of type PIV (Lechevalier *et al*., 1977). The major fatty acids were 14-methylpentadecanoic acid (67.8% of total fatty acids) and cis-9-14-methylpentadecanoic acid (7% of total fatty acids. The predominant menaquinones were MK-9(H_4), MK-9(H_2) and MK-9(H_0).

Morphological observation of 14-day-old culture grown on ISP-2 agar was shown in Figure 19. The phenotypic properties of strain DMKUA 245, *M. rosea* subsp. *Rosea, M. rosea* subsp. *Aerate, M. corallina* and *M. amethystogenes* were shown in Table 16. Strain DMKUA 245 grew well on ISP-2 and ISP-3 (Appendix Figure 1A). The color of substrate mycelium was colorless to yellow and the aerial mycelium was pale pink. Yellow and green soluble pigments were produced in ISP-2 and ISP-3 media, respectively. While, *M. coralline* NBRC 16416^T grew well on ISP-2

to ISP-5. The substrate mycelium of the strains on most media tested was pinkish to brownish red. When produced, the aerial mycelium was pink. Yellowish, soluble pigments were produced in ISP-3 and ISP-5. In case of *M. rosea* subsp. *rosea* NBRC 14044^T, *M. rosea* subsp. *aerat*a NBRC 14624^T and *M. amethystogenes* NBRC 101907^T grew well on ISP-2 to ISP-5 media. The substrate mycelium on most medium tested was beige to orange brown. The aerial mycelium was white on ISP-2 and ISP-3. No soluble pigments were produced in any agar medium.

The results of the physiological and biochemical characteristics of strain DMKUA 245 and the other strains used for comparison are summarized in Table 17. The results clearly demonstrated that strain DMKUA 245 could be differentiated from all other recognized species of the genus *Microbispora*. The temperature ranges for growth was 25-50°C.

It is evident from the genotypic and phenotypic data that strain DMKUA 245 can be distinguished from previously described species of the genus *Microbispora*. It is therefore proposed that strain DMKUA 245 represents a novel species of the genus *Microbispora*, for which the name *Microbispora siamensis* sp. nov. is proposed.

Miyadoh *et al.* (1990) proposed that *M. amethystogenes* is a later heterotypic synonym of *M. rosea* subsp. *rosea.* In this study, however, DNA–DNA relatedness values (from reciprocal hybridizations) between *M. amethystogenes* NBRC 101907^T and *M. rosea* subsp. *rosea* NBRC 14044^T were in the range of 49 to 53 % (Table 14). Since these values are lower than the 70% cut-off value, it is suggested that *Microbispora amethystogenes* is a separate genomic species from *M. rosea* subsp. *rosea* (Figure 18). In addition, *M. amethystogenes* NBRC 101907^T and *M. rosea* subsp. *rosea* NBRC 14044^T could be differentiated by characteristics such as gelatin liquefaction, milk peptonization, and the utilization of maltose, melezitose, D-sorbitol, fumaric acid and L-malic acid (Table 16). *M. amethystogenes* is therefore considered to merit separate species status on the basis of genotypic and phenotypic data (Nakajima *et al*., 1999).

Description of *Microbispora siamensis* sp. nov.

Microbispora siamensis (si.a.men′sis. N.L. fem. adj. siamensis pertaining to Siam, the old name of Thailand, the source of the soil from which the type strain was isolated).

An aerobic, Gram-positive, non-motile and thermotolerant actinomycete that forms non-fragmented branched vegetative hyphae. Substrate mycelia are colourless to yellow. During sporulation, the surface of the colony is pale pink. Yellow and green soluble pigments are produced in ISP-2 and ISP-3 media, respectively. Spores arranged in longitudinal pairs are formed on short sporophores that branch alternately from aerial hyphae. Spores are oval and have smooth surfaces. Substrates utilized as sole carbon sources include: L-arabinose, D-fructose, D-galactose, D-glucose, D-mannitol, D-mannose and sucrose, but not dulcitol, inositol, maltose, melezitose, raffinose, L-rhamnose or D-sorbitol. Citric acid, L-malic acid and succinic acid are used, but not benzoic acid, fumaric acid or mucic acid. Tests for cellulose degradation, gelatin liquefaction, nitrate reduction and milk peptonization are negative. The temperature range for growth is 25–50°C, but no growth occurs at 55°C. No growth occurs in the presence of 3% (w/v) NaCl. Strain DMKUA 245^T contains meso-diaminopimelic acid as the diagnostic diamino acid. Madurose is detected in whole-cell hydrolysates, indicating a cell wall chemotype IIIB (Lechevalier & Lechevalier, 1970). The phospholipid profile consists of diphosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylinositol mannosides and ninhydrin-positive glycophospholipids and indicates a phospholipid type IV chemotype. The major cellular fatty acids are 14-methylpentadecanoic acid and cis-9-14-methylpentadecanoic acid (fatty acid type 3c). The predominant menaquinones are MK-9(H_4), MK-9(H_2) and MK-9(H_0). The type strain, DMKUA 245^T (=BCC 14407^T=NBRC 104113^T), was isolated from soil. The DNA G+C content of the type strain is 68 mol%.

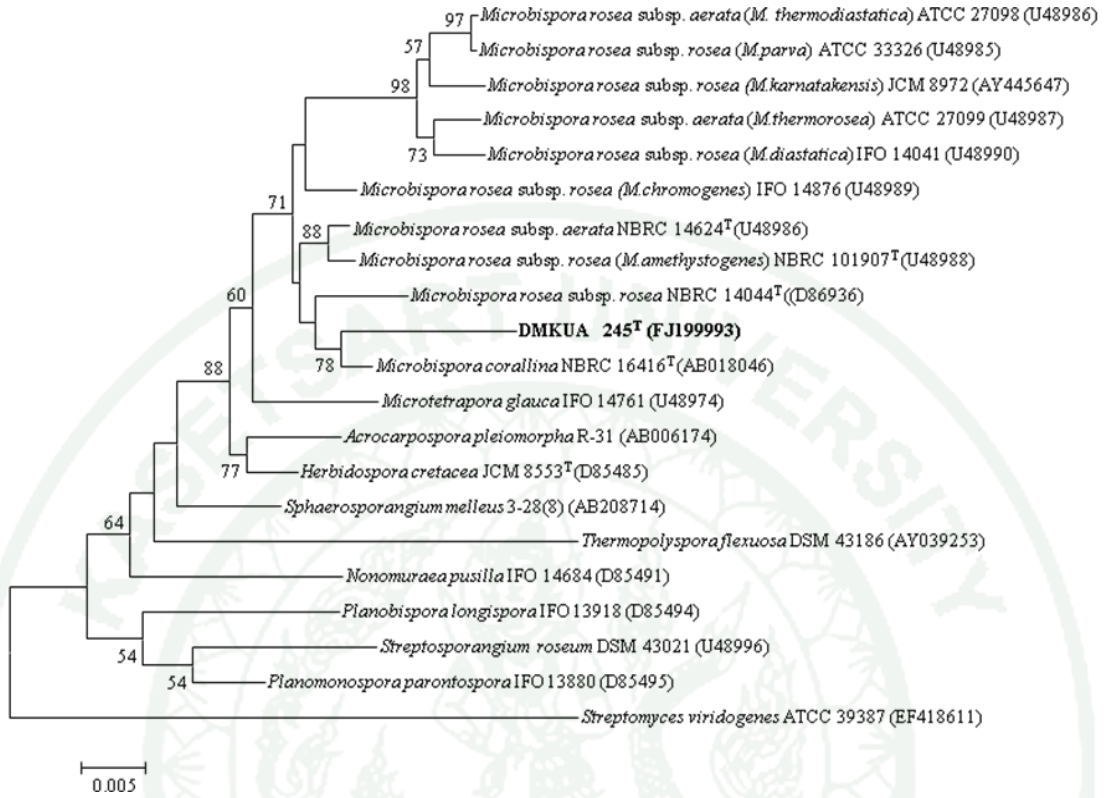


Figure 18 Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain DMKUA 245^{T}, *Microbispora* species and selected organisms belonging to the family *Streptosporangiaceae*. *Streptomyces viridogenes* ATCC 39387 (EF418611) is used as an outgroup. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates (only values >50% are indicated). Bar, 0.01 substitutions per nucleotide position.

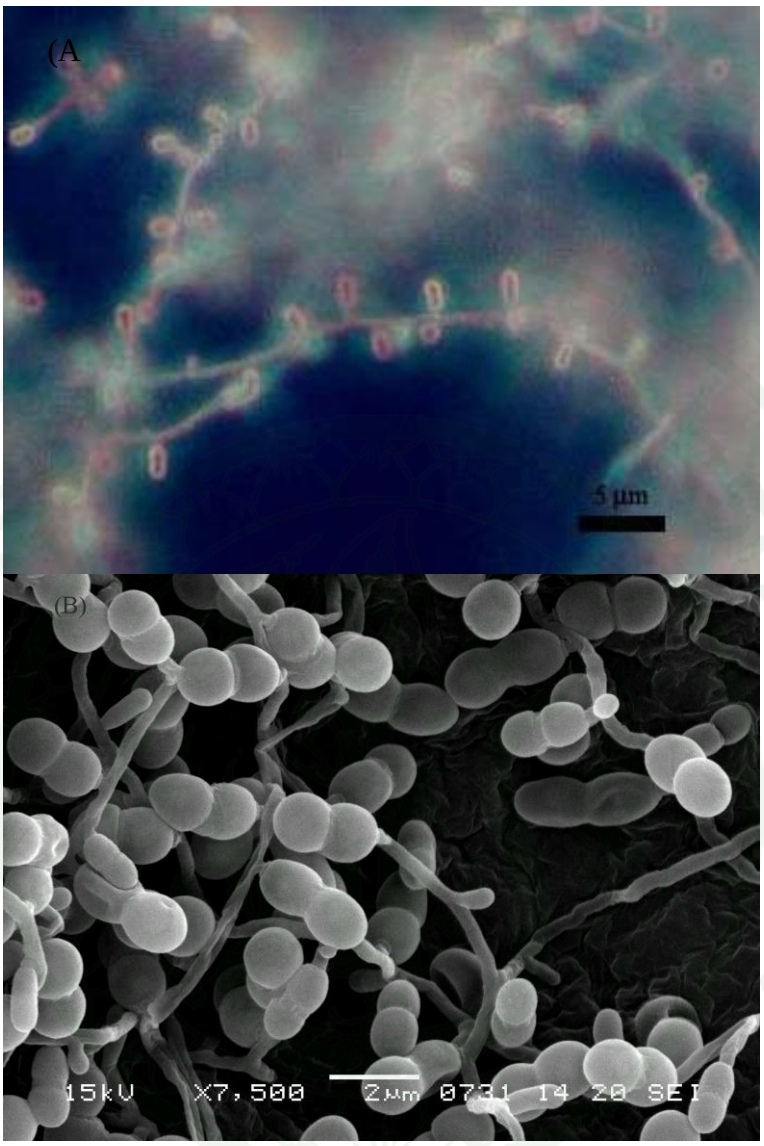


Figure 19 Photo micrograph (A) and scanning electron micrographs (B) of strain DMKUA 245^{T} grown on yeast extract-malt extract agar (ISP-2) for 14 days at 30^{O}C. Magnification: X400(A) and X7,500(B).

Table 14 DNA base composition and DNA-DNA relatedness between strain DMKUA 245^T and type strains of validly described *Microbispora* species.

Strain	G+C content (mol%)	Percentage DNA complementarity with labeled DNA from:				
		245^T	**16416^T**	**14044^T**	**14624^T**	**101907^T**
DMKUA 245^T	68	100	19	44	41	38
M. corallina NBRC 16416^T	72	27	100	34	31	31
M. rosea subsp. *rosea* NBRC 14044^T	71	44	45	100	71	53
M. rosea subsp. *aerata* NBRC 14624^T	67	46	35	62	100	47
M. amethystogenes NBRC 101907^T	67	36	34	49	51	100

Table 15 Chemotaxonomic characteristic of strain DMKUA 245^T

Characteristic	**Strain DMKUA 245^T**
Peptidoglycan	*meso*-DAP
Whole cell sugar	Type B (arabinose and madurose)
Phospholipid	diphosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylinositol mannosides and ninhydrin-positive glycophospholipids
Menaquinone	
MK-9(H_4)	28.3%
MK-9(H_2)	42.8%
MK-9(H_0)	29.0%
Fatty acid*	
Iso-$C_{14:0}$	2.8%
$C_{15:0}$	2.9%
Iso-$C_{15:0}$	5.3%
Iso-$C_{16:0}$	67.8%
Iso- $C_{16:1}$	7.1%
10 methyl-$C_{16:0}$	2.1%
10 methyl-$C_{17:0}$	4.5%
Unknown	7.4%

*Abbreviations for fatty acids: Iso-$C_{14:0}$, 12-methyltridecanoic acid; $C_{15:0}$, pentadecanoic acid; Iso-$C_{15:0}$, 13-methyltetradecanoic acid; Iso-$C_{16:0}$, 14-methylpentadecanoic acid; Iso-$C_{16:1}$, cis-9-14-methylpentadecanoic acid; 10 methyl-$C_{16:0}$, 10-methyl hexadecanoic acid, 10 methyl-$C_{17:0}$, 10-methyl heptadecanoic acid.

Table 16 Culture characteristics of strains DMKUA 245^T and the other four valid published species of the genus *Microbispora.*

	1[a]		2		3		4		5	
Agar medium	**Growth**	**Sporulation**	**Growth**	**Sporulation**	**Growth**	**Sporulation**	**Growth**	**Sporulation**	**Growth**	**Sporulation**
Yeast extract-malt extract (ISP- 2)	Good (Yellow)	Good (Pale pink)	Good (terra cotta)	Modurate (Shell pink)	Good (Orange brown)	Good (White)	Modurate (Brown)	Modurate (White)	Good (Orange brown	Good (White)
Oatmeal agar (ISP-3)	Good (White)	Good (Pale pink)	Good (Persim mon)	Poor (Pink tint)	Good (Orange brown)	Poor (White)	Modurate (Brown)	Modurate (Pale pink)	Good (Orange brown	Poor (White)
Inorganic salt-starch agar (ISP- 4)	Poor	None	Good (Coral)	Good (Cherry pink)	Good (Orange brown)	None	Poor	None	Good (Orange brown)	None
Glycerol-aspar agine (ISP-5)	Poor	None	Good (Resset)	Poor (Pink tint)	Good (Beige)	None	Poor	None	Good (Beige)	None

[a] 1, DMKUA 245^T; 2, *M. corallina* NBRC 16416^T; 3, *M. rosea* subsp. *rosea* NBRC 14044^T; 4, *M. rosea* subsp. *aerat*a NBRC 14624^T; 5, *M. amethystogenes* NBRC 101907^T.

Table 17 Physiological and biochemical characteristics of strains DMKUA 245^T and the other four valid published species of the genus *Microbispora.*

	Strain[b]				
Characteristics[a]	**1**	**2**	**3**	**4**	**5**
Gelatin liquefaction	-	-	+	+	+
Nitrate reduction	-	+	+	+	+
Milk peptonization	-	+	-	+	-
Carbon utilization:					
Dulcitol	-	-	w	-	-
Inositol	-	+	-	-	w
Maltose	-	+	+	-	-
Melezitose	-	+	-	-	-
D-Sorbitol	-	+	-	-	-
Organic acid utilization:					
Citric acid	+	-	-	+	-
Fumaric acid	-	+	-	-	-
L-Malic acid	+	+	-	-	-
Succinic acid	+	+	+	+	-
Growth at:					
25ºC	+	+	+	-	+
50ºC	+	-	-	+	-
55ºC	-	-	-	+	-
G+C content (mol%)	68	72	71	67	67

[a] +, Positive; -, negative; w, weak reaction.

[b] 1, DMKUA 245^T; 2, *M. corallina* NBRC 16416^T; 3, *M. rosea* subsp. *rosea* NBRC 14044^T; 4, *M. rosea* subsp. *aerat*a NBRC 14624^T; 5, *M. amethystogenes* NBRC 101907^T.

3. Optimization of fermentation process for xylanase production

3.1 Investigation of xylanase production at different temperature

The new thermotolerant *M. siamensis* DMKUA 245^T was selected based on the maximum diameter of clearzone on xylan agar plate. The strain was investigated for xylanase activity in xylan medium at different temperature for 120 h. As shown in Figure 20, it was found that the strain had the highest activity of 6 U/ml at 40°C.

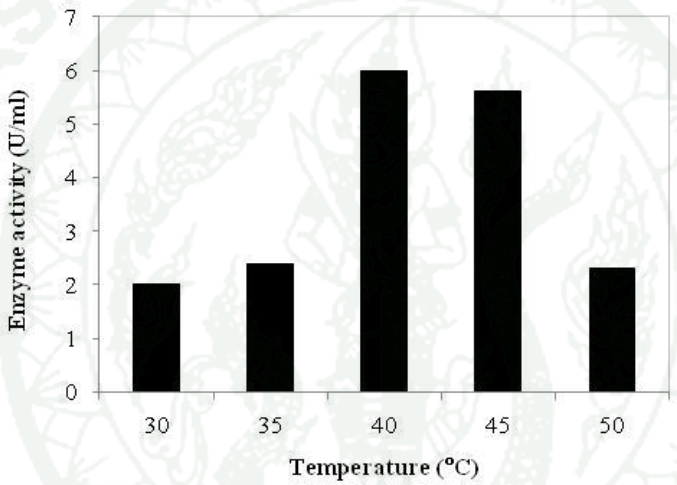


Figure 20 Xylanase activity of the new thermotolerant *M. siamensis* DMKUA 245^T at different temperature for 120 h.

3.2 Effect of nitrogen source on xylanase production

The effect of various nitrogen sources for xylanase production by the new thermotolerant *M. siamensis* DMKUA 245^T was investigated. A significantly increase of the enzyme production at a 95% confidence level was observe with maximum amount of xylanase using casein as nitrogen source (Figure 21). Dry weight obtained from the medium using skim milk had higher biomass but lesser xylanase activity compared to casein (Table 18). The xylanase production was not correlated with the dry weight formation.

Table 18 Xylanase activity and dry weight of the new thermotolerant *M. siamensis* DMKUA 245^T grown on different nitrogen sources.

Nitrogen sources	Enzyme activity (U/ml)	Dry weight (g/L)
Skim milk	11.5±1.1	0.276±0.004
Casamino acids	9.3±1.8	0.274±0.005
Gelatin	9.4±0.3	0.208±0.007
Casein	12.4±0.2	0.231±0.007
Malt extract	8.4±0.8	0.217±0.008
Peptone	11.2±1.2	0.256±0.005

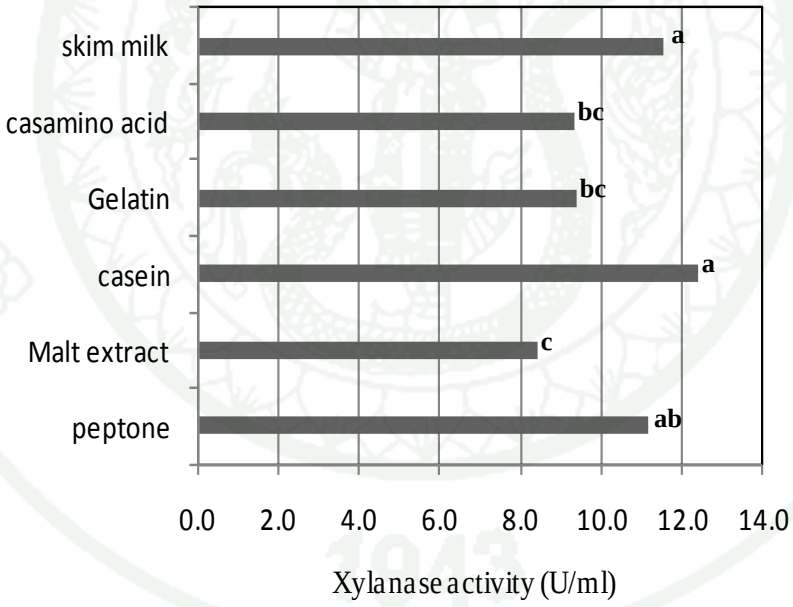


Figure 21 Effect of nitrogen sources on the xylanase production by *M. siamensis* DMKUA 245^T after 120 h cultivation. Different letters indicate significant differences *P*=0.05 level between treatment.

3.3 Plackett-Burman design

Based on effect of nitrogen sources, casein was the best nitrogen source for enzyme production by the new thermotolerant *M. siamensis* DMKUA 245^T, therefore it was used in next experiment. Plackett-Burman design was applied to obtain the optimum factors on enzyme production. The Plackett-Burman design matrix and response values are listed in Table 19. Among five variables, which were expected to play a critical role in enhancing xylanase production, three factors (casein, $MgSO_4.7H_2O$ and temperature) significantly affected enzyme production. The data of regression analysis for Plackett–Burman design are shown in Table 20. The model had a coefficient of determination (R^2) of 0.990, which can explain 99.0% variability of the data. Plackett-Burman design is a powerful technique for screening important variables and has successfully been used by many works (Xu *et al*., 2002; Kaur and Satyanarayana, 2005; Kumar and Satyanarayana, 2006). Model terms having values of '*p*' less than 0.1 are considered significant at 90 % of confidence level and hence, casein, $MgSO_4.7H_2O$ and temperature having the lowest values were found to be statistically significant in affecting xylanase production.

Table 19 Plackett-Burman experimental design matrix with xylanase production by the new thermotolerant *M. siamensis* DMKUA 245^T

Run no.	X_1	X_2	X_3	X_4	X_5	dummy	dummy	dummy	*Y*(U/ml)
1	1	1	1	-1	1	-1	-1	-1	25.5
2	1	1	-1	1	-1	-1	-1	1	13.6
3	1	-1	1	-1	-1	-1	1	1	7.2
4	-1	1	-1	-1	-1	1	1	1	12.0
5	1	-1	-1	-1	1	1	1	-1	24.4
6	-1	-1	-1	1	1	1	-1	1	29.0
7	-1	-1	1	1	1	-1	1	-1	29.6
8	-1	-1	-1	-1	-1	-1	-1	-1	7.7

Table 20 Results of regression analysis for the Plackett-Burman design.

Variable code	Variables	Coefficient estimate	Mean square	*F*-value	*P*-value
	Corrected model	24.615	112.827	64.023	0.001
X_1	Xylan (g /L)	1.241	3.474	1.971	0.233
X_2	Casein (g /L)	0.793	10.498	5.957	0.071[a]
X_3	K_2HPO_4 (g /L)	6.861	0.060	0.034	0.863
X_4	$MgSO_4.7H_2O$ (g /L)	6.673	25.875	14.682	0.019[a]
X_5	Temperature (°C)	0.00	445.881	253.010	0.000[a]

[a]Significant level=90%; R^2 Squared = .990 (Adjusted R Squared = .974).

4. Physical factors affecting xylanase production in an airlift fermenter

Since this research was investigated on a scale 50 ml Erlenmeyer flask level, there is a need to scale-up the production of the enzyme. Moreover, the physical parameter affecting xylanase production in the 3-L airlift fermenter was studied using the medium obtained from Plackett-Burman design. The medium contained (g/L): xylan 10, casein 0.5, K_2HPO_4 0.1, and $MgSO_4.7H_2O$ 0.3.

4.1 Effect of pH on xylanase production

The effect on xylanase production was investigated at pH 7.0 and uncontrolled pH. Xylanase activity and oxygen uptake rate (OUR) which indicating the growth of strain in fermenter were performed. Figure 22 showed the data obtained from experiment. At uncontrolled pH (initial pH=7.0 and final pH=7.78), the maximal xylanase activity, 60 U/ml with OUR 1.14 S^{-1} was observed after 120 h cultivation. At pH 7.0, the maximum xylanase activity was dropped to 21 U/ml, while, OUR was decreased to 0.6 S^{-1}. The results indicated that the cultivation with uncontrolled pH had increased almost 3-folds compared with a controlled pH at 7.0 (21 U/ml).

Moreover, OUR indicated that at pH 7.0 increased the growth of strain (Figure 22B). This indicates that at pH 7.0 would yield a higher cell biomass but decreases the enzyme activity. According to lower yield of enzyme production at pH 7.0, this may due to certain pH for growth and it produced also high protease to digest effectively the target enzyme. Therefore, production of xylanase is pH-depent.

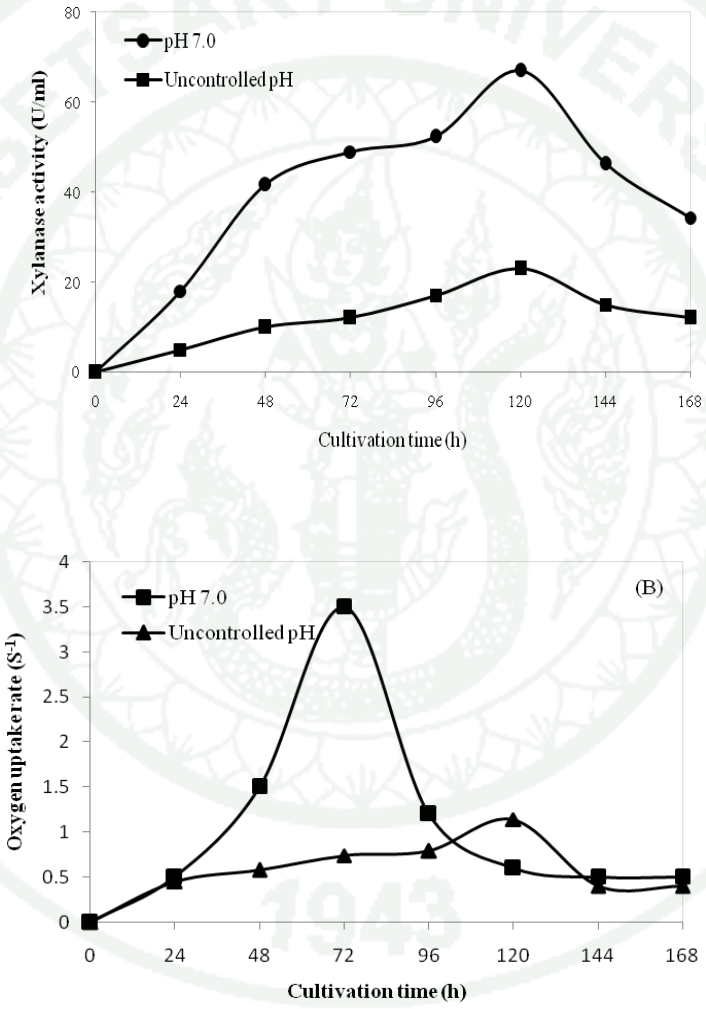


Figure 22 Effect of pH on xylanase production by the new thermotolerant *M. siamensis* DMKUA 245^{T} (A) and oxygen uptake rate (B) in an airlift fermenter at 45°C with aeration rate 0.5 vvm.

4.2 Effect on aeration rate on xylanase production

The enzyme was produced by the new thermotolerant *M. siamensis* DMKUA 245^{T} in a 3-L airlift fermenter and uncontrolled pH with aeration 0.25, 0.5 and 0.75 vvm, respectively. Figure 23 indicating that increasing in xylanase production was observed with the increasing in aeration rate up to 0.5 vvm (60 U/ml) and afterward a decline in the enzyme production at higher aeration was observed. The aeration rate at 0.75 and 0.25 vvm, reduced the enzyme activity was obtained as 50.5 and 27.3 U/ml, respectively. At uncontrolled pH, aeration rate at 0.5 vvm

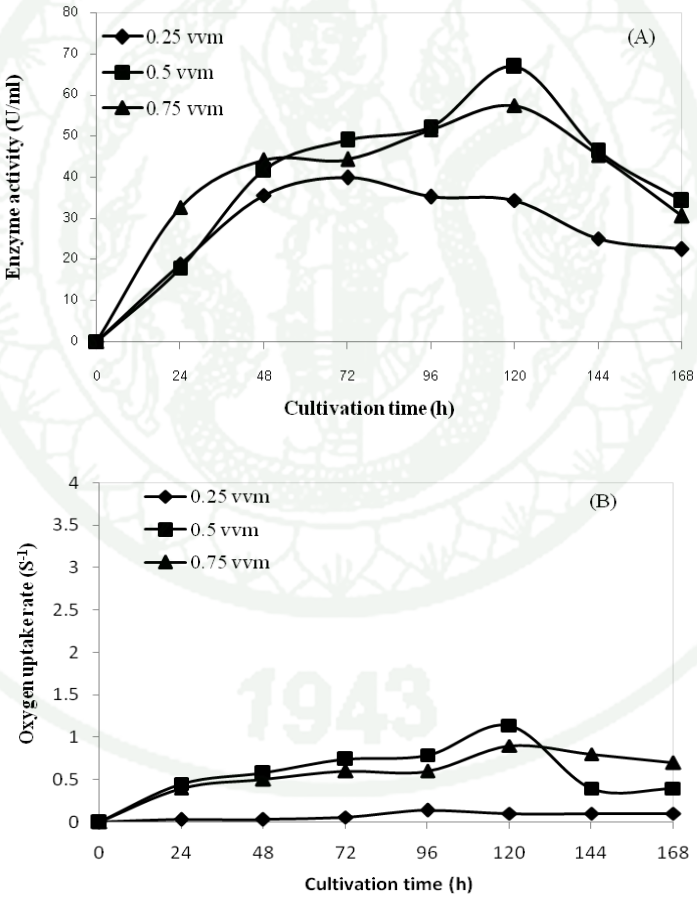


Figure 23 Effect of aeration rate on xylanase production by the new thermotolerant, *M. siamensis* DMKUA 245^{T} (A) and oxygen uptake rate (B) in an airlift fermenter at 45°C and uncontrolled pH.

showed the best results of enzyme activity which was also relative with growth (Figrue 23B). According to the effect of pH and aeration rate concluded that the maximum xylanase production by the thermotolerant *M. siamensis* DMKUA 245^T in an airlift fermenter was obtained under condition: uncontrolled pH and aeration rate 0.5 vvm.

5. Optimization of xylanase production

5.1 Regression model of response

CCD is a very useful tool for determining the optimal level of medium constituents and their interaction. Based on the results from Plackett-Burman design, casein, $MgSO_4.7H_2O$ and temperature were selected for the further evaluation of their effects on xylanase production by CCD. The experimental design matrix and results obtained for enzymes activities were shown in Table 21. Treatment runs 15-17 were the center points in the design, which were repeated three times for estimation of error. By applying multiple regression analysis on the experimental data, the following second order polynomial equation (Eq. 5) was used to explain the enzyme production.

$$Y = 13.42-0.32X_2+0.004691X_4+6.85X_5 +0.65X_2X_4-1.24X_2X_5 -0.064X_4X_5+2.12X_2^2+2.74X_4^2-1.92X_5^2 \quad (5)$$

where Y is the predicted response (xylanase production); X_2, X_4 and X_5 are coded values of casein, $MgSO_4.7H_2O$ and temperature, respectively.

The results of the second-order response surface model fitting in the form of ANOVA are given in Table 22. To test the fit of the model equation, the regression based determination coefficient R^2 was evaluated (Haider and Pakshirajan, 2007; Liu and Wang, 2007). Hu (1999) reported that the values of $R^2 \geq 0.75$, the model would explain the experiment. The nearer the values of R^2 to 1, the model would explain better for variability of experimental values to the predicted values

Table 21 Experimental design used in response surface methodology of independent variables, X_2, X_4 and X_5, with three center points, and the observed and predicted xylanase activity.

Run No.	Level			Actual level			Xylanase activity (U/ml)	
	X_2	X_4	X_5	X_2	X_4	X_5	Observed	Predicted
1	-1	-1	-1	0.3	0.15	35	6.7	9.2
2	-1	-1	1	0.3	0.15	45	29.3	25.5
3	-1	1	-1	0.3	0.45	35	5.0	8.0
4	-1	1	1	0.3	0.45	45	29.5	24.1
5	1	-1	-1	0.7	0.15	35	6.2	9.7
6	1	-1	1	0.7	0.15	45	25.9	21.1
7	1	1	-1	0.7	0.45	35	9.1	11.1
8	1	1	1	0.7	0.45	45	26.6	22.2
9	-1.68	0	0	0.16	0.3	40	18.6	19.8
10	1.68	0	0	0.84	0.3	40	17.6	18.9
11	0	-1.68	0	0.5	0.05	40	20.4	21.1
12	0	1.68	0	0.5	0.55	40	19.3	21.2
13	0	0	-1.68	0.5	0.3	31.6	4.0	0
14	0	0	1.68	0.5	0.3	48.4	19.4	19.5
15	0	0	0	0.5	0.3	40	13.5	13.4
16	0	0	0	0.5	0.3	40	13.7	13.4
17	0	0	0	0.5	0.3	40	13.5	13.4

(Sayyad *et al*., 2007; Liu and Wang, 2007). The model presented a determination coefficient (R^2 = 0.756) explaining 75.6% of the variability in the response (Table 22) and about 24.4% of total variation affected by other variables. The statistical significance of the regression model was checked by *F*-test, the results of ANOVA are shown in Table 22.

Model coefficients estimated by regression analysis for each variable is shown in Table 23. The significance of each coefficient was determined by *t*-values and *P*-values. The larger the magnitude of *t*-test value and smaller the *P*-value indicates the high significance of the corresponding coefficient (Karthikeyan et al., 1996; Tanyildizi et al., 2005). The results revealed that temperature (X_5) had a significant effect on xylanase production. However, no interactions between the two variables were found to contribute to the response at a significant level.

5.2 Model adequacy checking

It is necessary to examine the fitted model to ensure that it provides an adequate approximation to the true system and verifies that none of the least squares regression assumptions are violated. The residuals from the least squares fit play on important role in judging model adequacy. In order to checking the adequacy of model, graphic analysis of residuals was performed. If the model is adequate, the residuals should be structureless, they should contain no obvious. Figure 24 shows observed xylanase activity (the response) versus those from the empirical model Eq. (5). The dots represented the actual residuals which approximately along a straight line suggesting that normal distribution of residuals, therefore, the model was adequate. Figure 25 presents a plot of residuals versus the predicted response. The general impression is that the residuals scatter randomly on the display, suggesting that the variance of the original observation is constant for all values of *Y*. The plot was satisfactory, so it was concluded that the empirical model was adequate to describe the xylanase activity by response surface.

Table 22 Analysis of variance (ANOVA) for the model regression representing xylanase activity

Model	**Sum of Squares**	**df**	**Mean Square**	**F-value**	***p*-value**
Regression	881.259	9	97.918	2.410	.130[a]
Residual	284.369	7	40.624		
Total	1165.628	16			

R^2 = 0.756

Table 23 Regression coefficients and their significances for xylanase production from the results of CCD experimental design

Source	**Coefficient**	***t*-value**	***P*-value**
Intercept	13.42	3.656	0.1295
X_2	-0.32	-0.187	0.8565
X_4	0.004691	0.003	0.9979
X_5	6.85	3.967	0.0054
X_2X_4	0.65	0.288	0.7818
X_2X_5	-1.24	0.551	0.5989
X_4X_5	-0.064	0.028	0.9782
X_2^2	2.12	1.116	0.3009
X_4^2	2.74	1.441	0.1929
X_5^2	-1.92	-1.012	0.3452

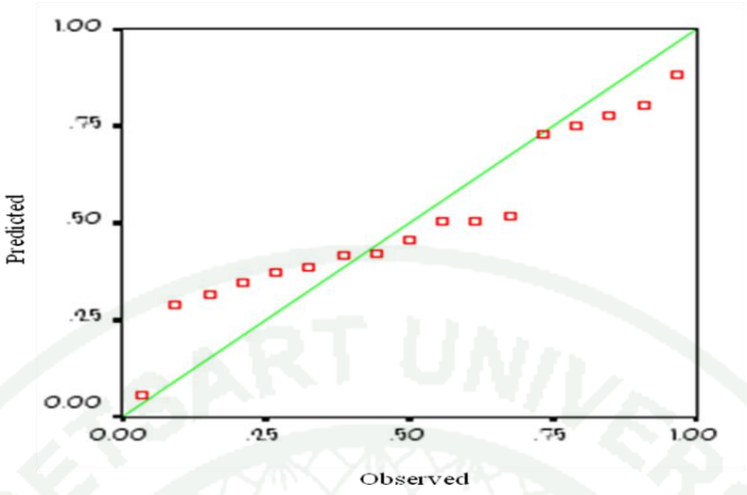


Figure 24 Observed xylanase activity vs. predicted xylanase activity under optimum medium compositions.

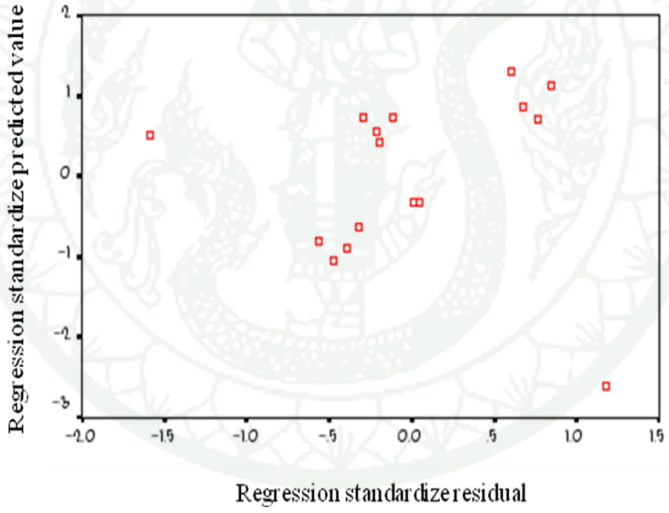


Figure 25 Plot of internally standardize residuals vs. predicted response.

5.3 Validation of the model

From the Table 23 indicated that temperature (X_5) had a significant effect on xylanase production, thus response plot and contour plot between temperature (X_5) and casein (X_2) and $MgSO_4.7H_2O$ (X_4) were drawn. Figure 26 showed the effect of temperature and casein concentration on xylanase activity.

Decreasing the concentration of casein and increasing temperature resulted in increasing of xylanase activity. Thus the α level (-1.68) of casein (X_2) and high level (+1) of temperature (X_5) were used as optimized value. The optimum value obtained from the Figure 26 corresponsed to the value from the regression equation (Eq. 5). Figure 27 showed that the highest xylanase yield was observed from the lowest concentration of $MgSO_4.7H_2O$ and the highest temperature which satisfied with the regression equation (Eq.5). To verify the optimization results, an experiment was performed under the predicted optimum conditions. The optimized condition of the test variables are as follows (g/L); casein 0.16, $MgSO_4.7H_2O$ 0.05 and temperature of 45°C.

The predicted xylanse activity for these conditions was 37 U/ml. The model was validated by repeating the experiment under the optimized conditions. The maximum experimental response for xylanase production was 44 U/ml after 120 h cultivation with productivity of 0.36 U/ml/h. The enzyme activity observed was 1.19 folds higher than the activity predicted by the optimized medium. The optimized condition of the test variables are as follows (g/L); casein 0.16, $MgSO_4.7H_2O$ 0.05 and temperature of 45°C.

According to the CCD result, the activity of xylanase produce by the strain of *M. siamensis* increased when the concentration of casein decreased, therefore to understand the mechanism of casein on the production of enzyme by this strain the effect of various casein concentration (0.16, 0.5 and 0.84 g/l) on growth, protease and xylanase productions was investigated. As shown in Figure 28, the highest xylanase activity was obtained from the medium with 0.16 g/l of casein after cultivation at 120 h. When amount of casein in the medium increased, the xylanase activity decreased (Figure 28A). In opposite, the highest protease activity (Figure 28B) and biomass concentration (Figure 28C) were obtained.

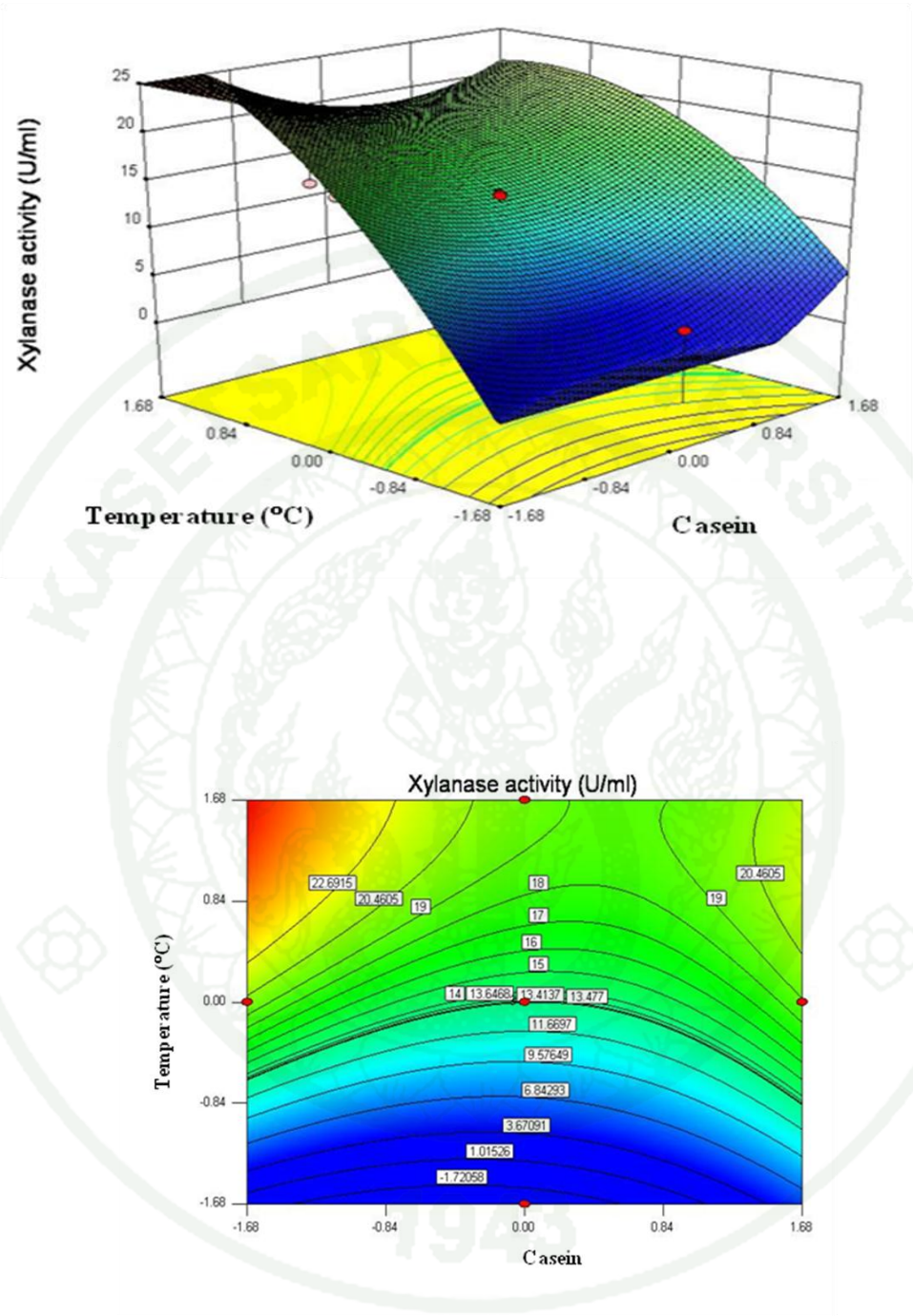


Figure 26 Response plot and contour plot of the combined effects of casein and temperature on the xylanase production by the new thermotolerant *M. siamensis* DMKUA 245^{T}.

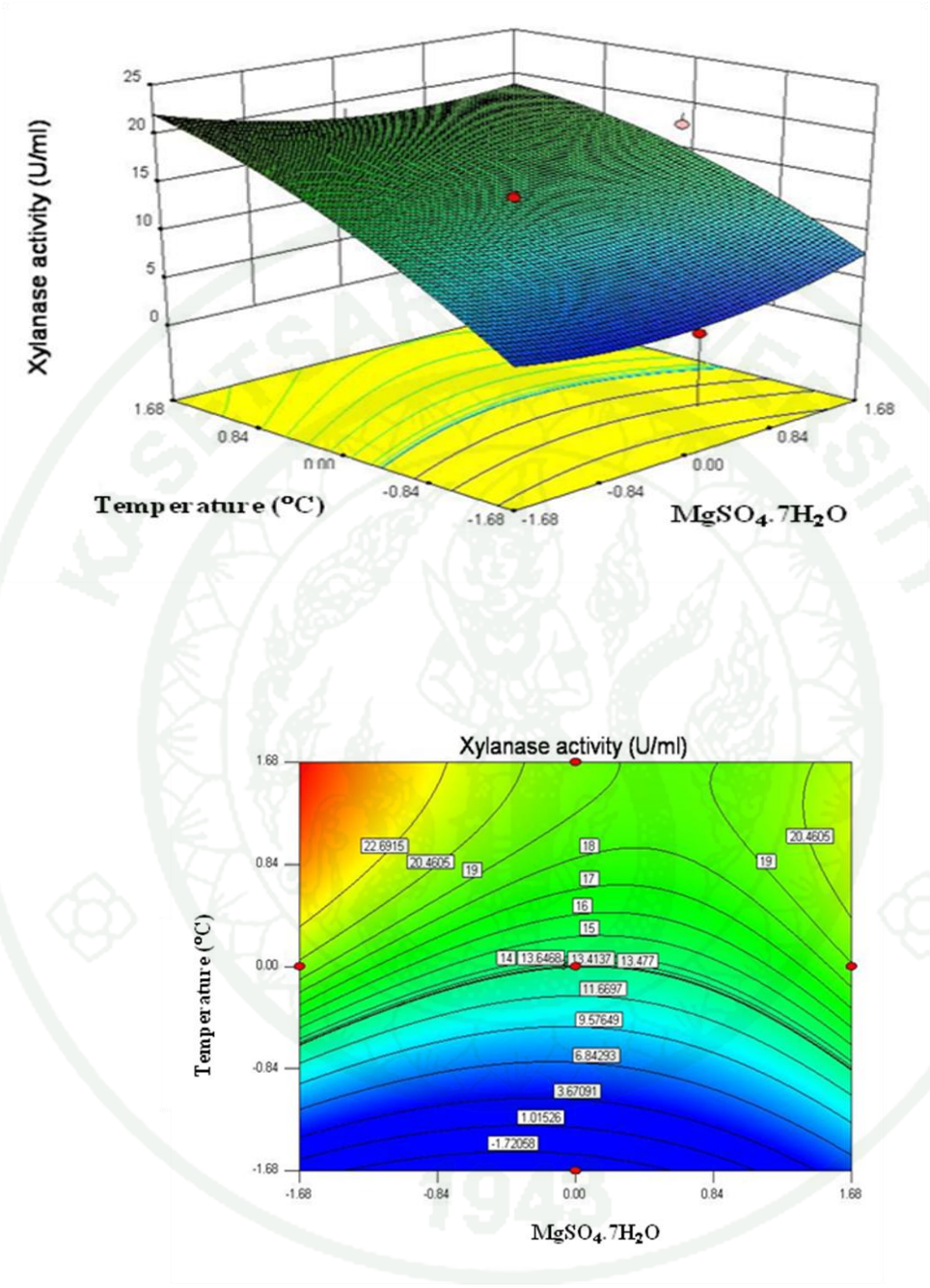


Figure 27 Response plot and contour plot of the combined effects of $MgSO_4.7H_2O$ and temperature on the xylanase production by the new thermotolerant *M. siamensis* DMKUA 245^T.

High casein concentration resulted in increased growth of *M. siamensis* with increased proteolytic enzyme activity which decreased the activity of the enzyme xylanase by the action of protease. Therefore, the less casein concentration though produced low biomass and proteolytic activity but increased the xylanase activity. The result was supported by the work of Thangamani *et al*. (2003) which reported that low level of xylanase produced by an alkali-tolerant *Aspergillus fumigatus* AR1 due to the action of high proteolytic degradation on xylanases during fermentation.

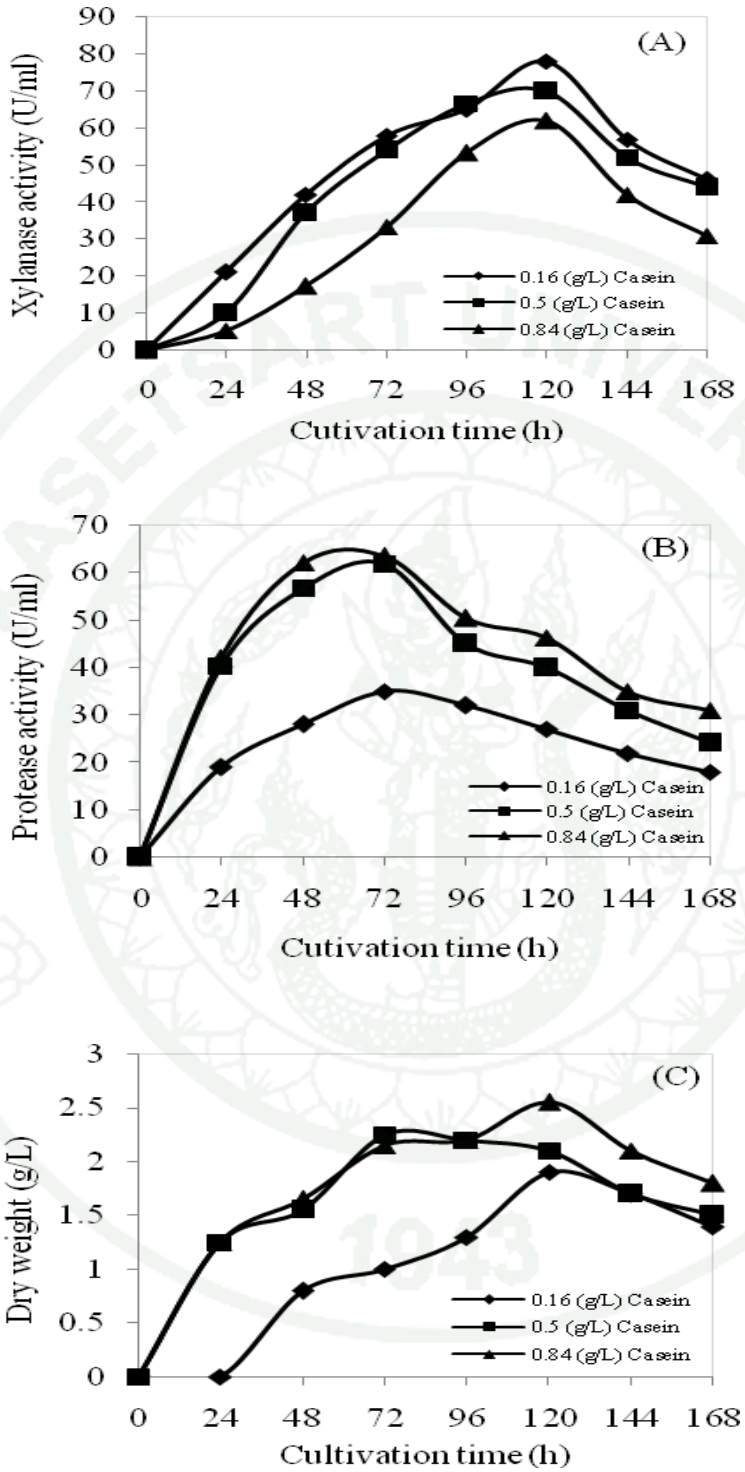


Figure 28 Effect of casein concentration on xylanase activity (A), protease activity (B) and dry weight (C) produced by the new thermotolerant *M. siamensis* DMKUA 245^{T}.

6. Batch fermentation in an airlift fermenter

The optimum conditions as determined by CCD experiments were carried out in the 3-L airlift fermenter at aeration rate of 0.5 vvm and uncontrolled pH obtained from Plackett-Burman experiment. The batch profile of xylanase production in the optimized medium was shown in Figure 28. The maximum xylanase production at 72 h cultivation was 292 U/ml with the productivity of 4.05 U/ml/h. From these studies, an increase of 6.6 and 11.3 folds in enzyme production and productivity, respectively, was observed in an airlift fermenter when compared to the shaking flask experiments.

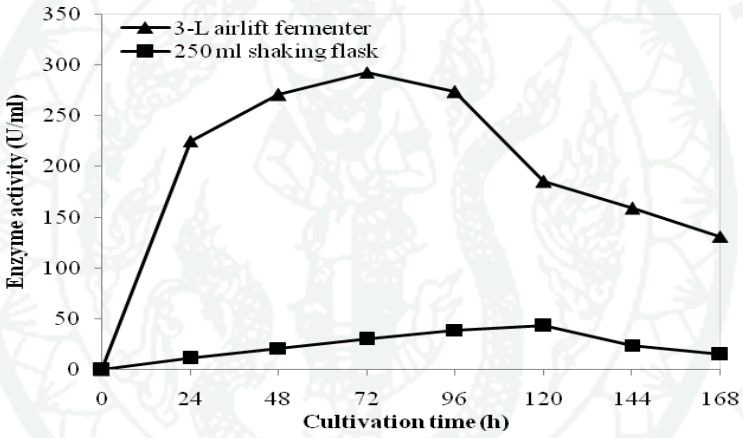


Figure 29 Time course of batch fermentation for xylanase production by the new thermotolerant *M. siamensis* DMKUA 245^T in the optimized medium.

Xylanase production by the new thermotolerant *M. siamensis* DMKUA 245^T was summarized in Table 24. Peptone was used as organic nitrogen source for un-optimized medium. The enzyme activity 6 U/ml was obtained. For the second step, casein, $MgSO_4.7H_2O$ and temperature were found to be a factor affecting the enzyme production and an activity of 29 U/ml was observed. The optimization of medium composition by using CCD in shake flasks was achieved with the concentration of

Table 24 Xylanase production from the new thermotolerant *M. siamensis* DMKUA 245^{T}.

Step	Method	Condition	Enzyme activity (U/ml)	
			Shaking flask	Airlift fermenter[a]
1	Un-optimized medium/condition	10 (g/L) xylan 1 (g/L) peptone 1 (g/L) K_2HPO_4 0.2 (g/L) $MgSO_4.7H_2O$	6	-
2	Screening of factors affecting the enzyme production using PB	0.5 (g/L) casein 0.3 (g/L) $MgSO_4.7H_2O$ temperature of 45 ˚C	29	60
3	Optimization of medium composition using CCD	0.16 (g/L) casein 0.05 (g/L) $MgSO_4.7H_2O$ temperature of 45 ˚C	44	292

[a] Aeration rate of 0.5 vvm and uncontrolled pH were added.

0.16 g/L casein, 0.05 (g/L) $MgSO_4.7H_2O$ and temperature of 45°C, with 44 U/ml of enzyme activity. At the step of the enzyme production in an airlift fermeter, the maximum enzyme activity, 60 and 292 U/ml, was obtained using Plackett-Burman experiment and CCD, respectively, at aeration rate of 0.5 vvm, uncontrolled pH and temperature 45 ˚C. In conclusion, xylanase production by *M. siamensis* DMKUA 245^T was significantly increased 49 folds.

7. Purification and characterization of xylanase

7.1 Purification of xylanase

Xylanase was produced by the new thermotolerant *M. siamensis* DMKUA 245^T in the optimized medium. Crude enzyme was purified using 100 ml DEAE-Seppharose. The profile of A280 and xylanase activity was shown in Figure 30. Table 25 summarizes the results of purification. The enzyme was purified to 88 folds with a recovery of 34% and a specific activity of 219.4 U mg^{-1} proteins. SDS-PAGE and zymogram analysis as shown in Figure 31 indicated that molecular weight of purified xylanase from the strain DMKUA 245^T was estimated to be 65.8 kDa. Zymogram revealed the presence of a zone of hydrolysis that corresponded with Commassie stained band of purified xylanase on SDS-PAGE, confirming the purified protein as xylanase (Figure 31).

7.2 Characterization of xylanase

7.2.1 Effect of pH and temperature on enzyme activity and stability

The purified xylanase was assayed at various pH and temperature as described in method. The optimal pH and temperature for the activity of the xylanase were 5.5 and 60°C, respectively (Figure 32).The xylanase was active in a pH range of 4–11 (Figure 33A). Moreover, the enzyme was also active in a temperature range of 30–60°C (Figure 33B), however, it was completely inactivated

when preincubated for half hour at over 70°C. These findings demonstrated the characteristics of tolerance and resistance of the new thermotolerant *M. siamensis* DMKUA 245^{T} xylanase against changes in pH and temperature which are desirable for biotechnological applications such as simultaneous saccharification and fermentation of xylan in agricultural wastes for lactic or ethanol production at acidic pH . Additionally, the enzyme is useful for the potential in feed industry becuase pH of animal's stomach is acidic, hence it can be used for animal feeding (Guo *et al*., 2010).

Table 25 Purification of xylanase from the culture supernatant of the new thermotolerant *M. siamensis* DMKUA 245^{T}.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U)	Purification factor (fold)	Recovery (%)
Crued enzyme	18000	7215	2.5	1	100
Batch-process of DEAE-Sepharose	7730	201.5	38.4	15	43
DEAE-Sepharose column	6098	27.8	219.4	88	34

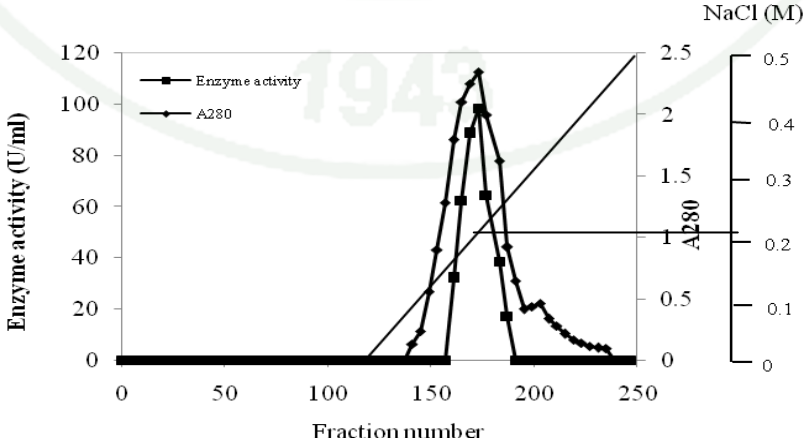
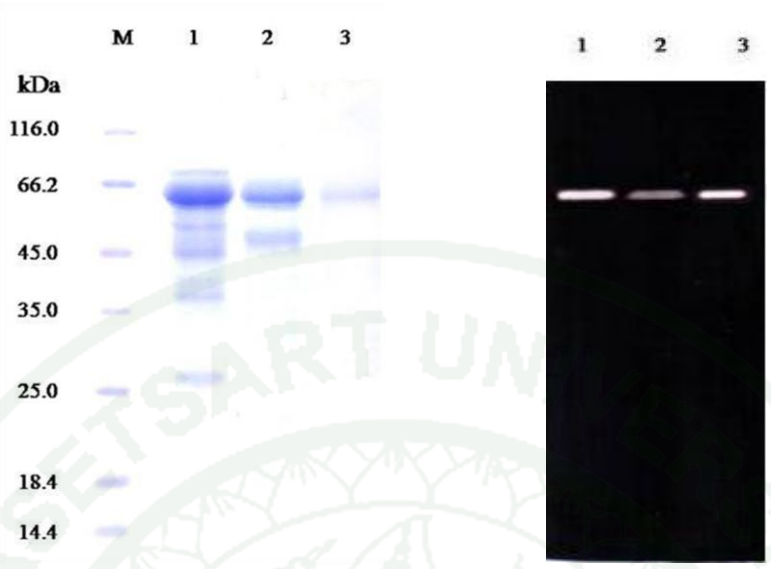


Figure 30 Profiles of A280 and xylanase activity in DEAE-Sepharose application.



(A) (B)

Figure 31 SDS-PAGE (A) and zymogram (B) of the purified xylanase from the new thermotolerant *M. siamensis* DMKUA 245^{T} Lane M: LMW protein marker; lane 1: crude enzyme; lane 2: batch-process of DEAE-Sepharose and lane 3: purified xylanase. The enzyme was electrophoresed in an SDS-PAGE (12%, w/v), and protein bands were stained with Coomassie brilliant blue R-205.

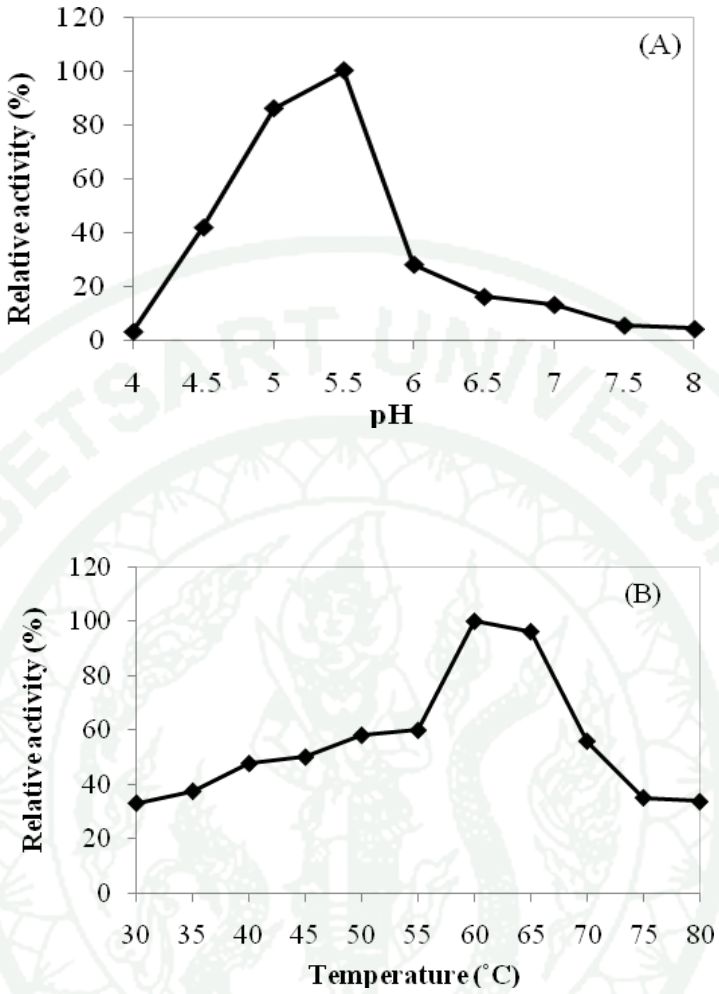


Figure 32 Optimum pH (A) and temperature (B) on the purified xylanase produced by the new thermotolerant *M. siamensis* DMKUA 245$^{T.}$

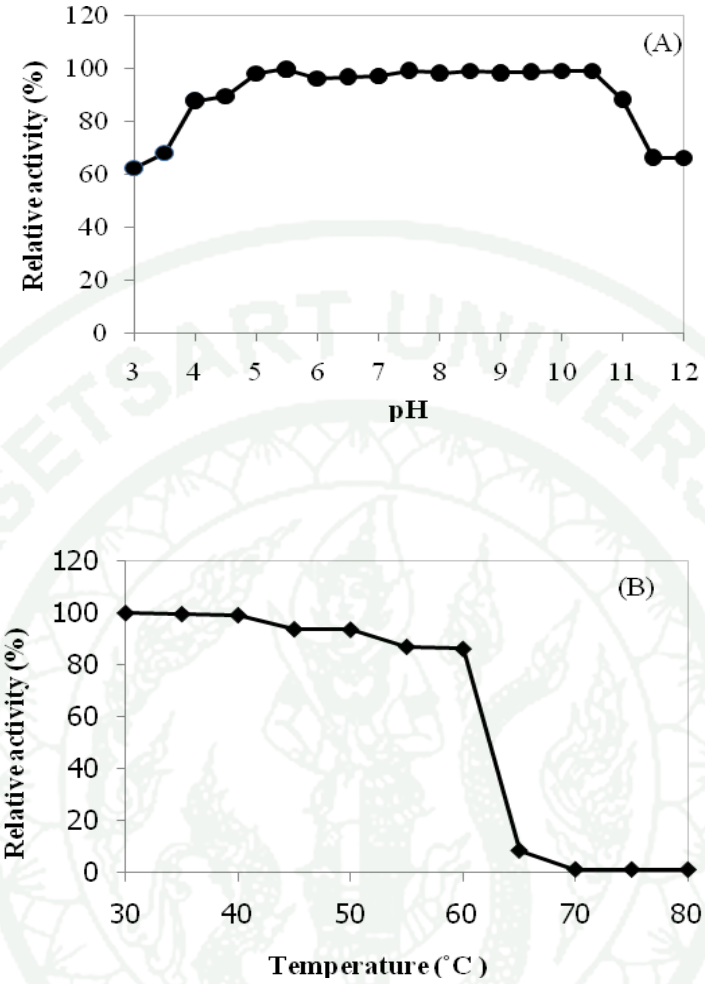


Figure 33 pH stability (A) and thermostability (B) of the purified xylanase produced by by the new thermotolerant *M. siamensis* DMKUA 245^{T}.

7.2.2 Activity on different substrate and kinetic parameters of the purified xylanase

The purified xylanase showed a high specificity toward the xylans tested (Table 26). The highest activity was observed for beechwood xylan. The specific activity of the xylanase towards insoluble xylan was higher than wood xylan and corn cob xylan. The xylanase did not act towards *p*NP-β-D-xylopyranoside, *p*NP-β-D-glucopyranoside, and carboxymethyl cellulose. These results indicated that the purified enzyme from *M. siamensis* DMKUA 245^{T} is a true enzyme.

Kinetic parameters with xylans as substrate were obtained at 60°C (Table 26). The K_m value was 3.3, 3.6, 4 and 4.4 mg mL^{-1} for beechwood xylan, insoluble xylan, wood xylan and corn cop xylan, respectively (Appendix Figure C2). Thus, the purified enzyme had the highest affinity for beechwood xylan. The kinetic properties of purified xylanase have been reported. The K_m from *Streptomyces rameus* L2001 was 5.3 mg mL^{-1} (Xiuting *et al*., 2010). The purified xylanase from *S. cyaneus* SN32 exhibited K_m value of 11.1 mg mL^{-1} (Ninawe *et al*, 2008). The K_m value determined *S. roseiscleroticus* NRRL B-11019 and *S.lividan* 66 towards oat spelt xylan were 7.9 and 3.71 mg mL^{-1}, respectively (Anthony *et al*., 1991; Dieter *et al*., 1990).

Table 26 Substrate specificity and kinetic parameters of the purified xylanase.

Substrate (1%, w/v)	**Specific activity (U/mg)**	**Relative activity (%)**	**V_{max} (mmol min^{-1} mg^{-1})**	**K_m (mg mL^{-1})**
beechwood xylan	219±1	100[a]	29.4	3.3
Insoluble xylan	206±15	94	16.7	3.6
wood xylan	181±11	83	18.2	4
corn cob xylan	109±2	50	14.3	4.4

[a]Activity for beechwood xylan was defined as 100%. The results presented are the average of three individual experiments.

7.2.3 Thin layer chromatography (TLC)

The hydrolysis products of xylans from different sources were shown in Figure 34. They were series of short-chain oligosaccharides and small amount of xylose, indicating that the purified enzyme was an endo type (endo-β-1,4-xylanase). From the hydrolysis products of xylobiose, the purified xylanase could not hydrolyze xylobiose. The pattern of hydrolysis product was similar to the action of xylanase produced by *Bacillus* sp. strain K-1 (Ratanakhanokchai *et al*., 1999) and by a thermotolerant *Streptomyces* T7 (Sulabha *et al*., 1989).

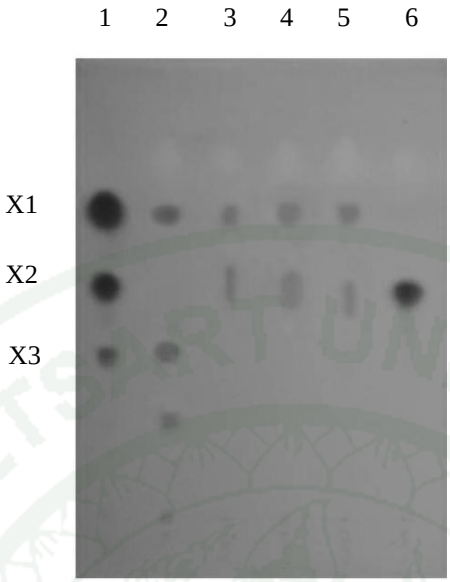


Figure 34 Thin layer chromatograph of sugar liberated from hydrolysate prodcuct of purified enzyme. Lane 1: standard of xylo-oligosacharides (X1=xylose; X2=xylobiose; X3=xylotriose); Lane 2-6: hydrolysate product of enzyme from: Lane 2: beechwood xylan; Lane 3: wood xylan; Lane 4: insoluble xylan; Lane 5: corn cob xylan and Lane 6: xylobiose.

7.2.4 Effect of metal ions as chloride and reagents on enzyme activity

The effect of metal ions as chloride, SDS and EDTA on xylanase activity of the EDTA treated pure enzyme was measured using dialyzed enzyme in the absence and presence of EDTA. It was found that xylanase activity was stimulated by Co^{2+}, K^{+} and Mg^{2+} to 143, 169 and 159%, respectively, but was inhibited by Mn^{2+} and EDTA down to 56 and 72%, respectively (Table 27). The activity was slightly affected by Ca^{2+}, Fe^{3+}, Na^{+}, Zn^{+} and SDS (Table 27). The results reported in this work were similar to other reports, such as enhancement on of activity by Co^{2+} and Mg^{2+} (Li *et al*., 2010) and inhibition by Mn^{2+} (Li *et al*., 2010; Nascimento *et al*., 2002). The xylanase activity from *Streptomyce rameus* L2001 was also found to be inhibited in the presence of EDTA (Li *et al*., 2010). These effects reveal which kind of ions should be precluded or included at appropriate concentrations in industrial processes. In the

presence of EDTA, the activity in all metal ions tested except Mn^{2+} was decreased. EDTA may chelate the metal ions and reduce their positive and negative effects on the enzyme activity.

Table 27 Effect of metal ions as chloride and reagent on the activity of EDTA-treated purified xylanase in the presence and absence of 5 mM EDTA. The data are mean ±standard deviations of the means.

	Relative activity of EDTA treated purified enzyme (%)	
Metal (1 mM)	**without EDTA**	**with EDTA (5 mM)**
Control	100±7	72±2
Ca^{2+}	97±2	38±1
Co^{2+}	182±5	69±1
Fe^{3+}	97±2	15±0
K^{+}	125±3	97±4
Mg^{2+}	159±2	51±2
Mn^{2+}	56±4	97±6
Na^{+}	108±8	49±1
Zn^{+}	82±7	16±3
SDS	93±2	90±8

According to the result of effect of metal ions on enzyme activity, Co^{2+}, K^{+} and Mg^{2+} stimulated the enzyme activity, therefore stability of EDTA-treated purified xylanase with these ions was investigated. The EDTA-treated purified xylanase was added with 1 mM Co^{2+}, K^{+} and Mg^{2+} at 4°C, overnight. After incubation at various temperatures (40-90°C), the residual activity was determined. Figure 35 indicated that after treatment the EDTA-treated purified xylanase with these ions, stability of the enzyme was increased compared to control, especially with Co^{2} still stable when the temperature was up to 90 °C. This result confirmed that Co^{2+}, K^{+} and Mg^{2+} enhanced the thermostability of the enzyme. This is the first report found that these ions enhanced the enzyme activity by thermostability.

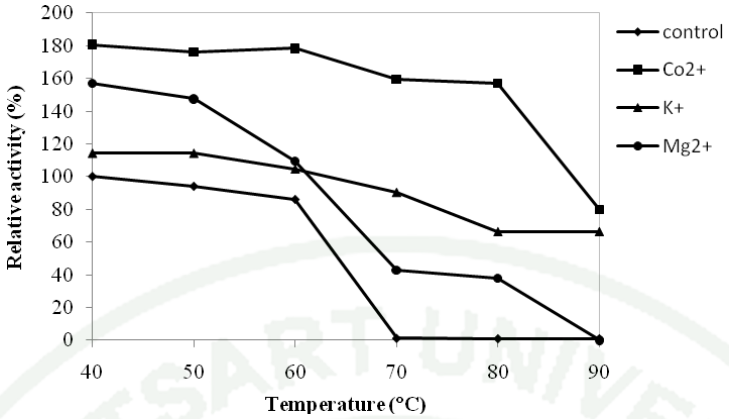


Figure 35 The effect of 1 mM Co^{2+}, K^{+} and Mg^{2+} on thermostability of the purified xylanase produced by the new thermotolerant *M. siamensis* DMKUA 245^{T}.

Since Co^{2+} was the best stimulatory ion on enzyme activity, therefore the effect of $CoCl_2.6H_2O$ addition at 1 mM (0.238 g/L) to the optimized medium obtained by CCD in shaking flask condition was investigated. In the presence of $CoCl_2.6H_2O$, the xylanase activity was higher than that of absence (Figure 36A). However, no difference on proteolytic activity was found in the medium with or without $CoCl_2.6H_2O$ (Figure 36B). This result confirmed $CoCl_2.6H_2O$ in enhancing thermostability of the enzyme.

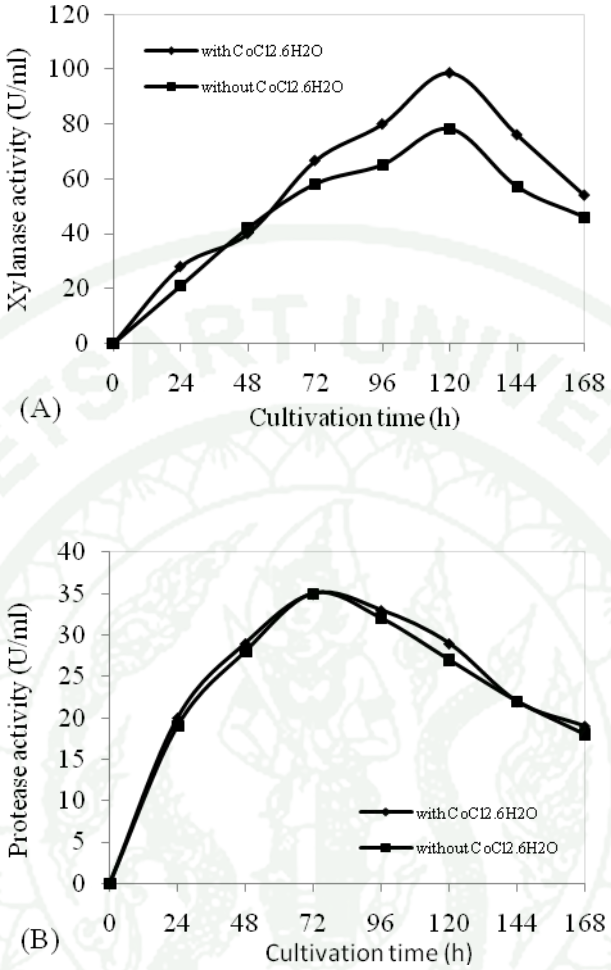


Figure 36 Effect of $CoCl_2.6H_2O$ on xylanase activity (A) and protease activity (B) produced by the new thermotolerant *M. siamensis* DMKUA 245^T.

CONCLUSION AND RECOMMENDATION

Conclusion

In this study, thirteen isolates of actinomycete belonged to family *Streptosporangiaceae* in 4 genera of *Herbidospora*, *Microbispora*, *Microtetraspora* and *Nonomuraea* were screened based on the ability to degrade xylan on xylan agar plate. Eleven isolates formed a clear zone on xylan agar plate. The maximum diameter of clear zone on xylan agar plate was exhibited by strain DMKUA 245. From the phylogenetic study, morphology of the strains and their ability to degrade xylan, strain DMKUA 205 and DMKUA 245 were selected for further identification and characterization. According to genotypic, phenotypic, phylogenetic properties and 16S rRNA gene sequences, strain DMKUA 205 and 245 were proposed to be *H. sakaeratensis* sp. nov. and *M. siamensis* sp. nov., respectively.

Strain DMKUA 205 formed colorless-yellowish brown on yeast extract-malt extract agar (ISP-2) and oatmeal agar (ISP-3). The surface of the colony was white when sporulation occurred. Spores are short rod and have smooth surfaces. Temperature range for growth is 20-40°C. Growth occurs between pH6.0-9.0. The maximun NaCl concentration for growth is 1.5%. Cell walls contain *meso*-diaminopimelic acid and *N*-acetylated muramic acid. Whole-cell hydrolysates contain madurose (chemotype IIIB). The phospholipid profile contains phosphatidylethanolamine, phosphatidylmethylethanolamine and glucosamine-containing phospholipids (phospholipids type PIV). The major cellular fatty acids are $C_{17:1}\omega9c$, 10-Methyl $C_{17:0}$, iso-$C_{16:0}$ and $C_{15:0}$ (fatty acid type 3c). Mycolic acids are absent. The predominant menaquinones are MK-10(H_4) and MK-10(H_6). The DNA G+C content of the type strain is 73 mol%. Strain DMKUA 205 shared 99% similarity with *H. yilanensis*, and *H. daliensis* species. DNA–DNA relatedness values in reciprocal hybridizations were much lower than 70% among strain DMKUA 205, *H. yilanensis* 0351M-12^T (35-54%, reciprocally) and *H. daliensis* 0358M-1^T (58-65 %, reciprocally). From the genotypic and phenotypic data that strain DMKUA 205 is

distinguished from previously described species of the genus *Herbidospora*. It is therefore proposed that this strain be classified as a new species of the genus *Herbidospora* and the name *Herbidospora sakaeratensis* sp. nov. is proposed for strain DMKUA 205^T.

Strain DMKUA 245 formed colorless-yellow substrate mycelium on yeast extract-malt extract agar (ISP-2) and oat meal agar (ISP-3). The aerial mycelium was pale pink. Yellow and green soluble pigments were produced in ISP-2 and 3, respectively. Spores are oval and have smooth surfaces. The temperature range for growth is 25–50°C, but no growth occurs at 55°C. No growth occurs in the presence of 3% (w/v) NaCl. Strain DMKUA 245^T contains meso-diaminopimelic acid as the diagnostic diamino acid. Madurose is detected in whole-cell hydrolysates, indicating a cell wall chemotype IIIB (Lechevalier & Lechevalier, 1970). The phospholipid profile consists of diphosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylinositol mannosides and ninhydrin-positive glycophospholipids and indicates a phospholipid type IV chemotype. The major cellular fatty acids are iso-$C_{16:0}$, iso-$C_{16:1}$ and iso-$C_{15:0}$. The predominant menaquinones are MK-9(H_4), MK-9(H_2) and MK-9. The DNA G+C content of the type strain is 68 mol%. The similarity values of sequences among strain DMKUA 245 and the closely related species, *Microbispora corallina*, *M. rosea* subsp. *rosea*, *M. rosea* subsp. *aerate* and *M. amethystogenes* were 98.4%, 97.4%, 97.0% and 96.9%, respectively. DNA–DNA relatedness values determined by reciprocal hybridizations were much lower than 70 % between strain DMKUA 245^T and other members of the genus *Microbispora* (19 to 46%). Thus a fair conclusion was that strain DMKUA 245^T represented a separate genomic species. . It is therefore proposed that this strain be classified as a new species of the genus *Microbispora* and the name *Microbispora siamensis* sp. nov. is proposed for strain DMKUA 245^T.

Inaddition, DNA-DNA relatedness values in reciprocal hybridizations were higher than 70% between *Streptosporangium claviforme* NBRC 15623^T and *Herbidospora cretacea* JCM 8553^T (82.1 and 89.3%, reciprocally), indicating that *S. claviforme* is identified as *H. cretacea*. Therefore, the name *S. claviforme* should be

treated as a synonym of *H. cretacea.* Morever, DNA–DNA relatedness values between *Microbispora amethystogenes* NBRC 101907^T and *Microbispora rosea* subsp. *rosea* NBRC 14044^T in reciprocal hybridizations experiments were in the range of 49 to 53%, which is lower than 70%, indicating that *Microbispora amethystogenes* is a separate genomic species from *Microbispora rosea* subsp. *rosea*.

The new thermotolerant *M. siamensis* DMKUA 245^T showed the maximum diameter of clear zone on xylanase agar plate. Therefore the strain DMKUA 245^T was selected for xylanase production. The optimal condition for the ezyme production by this strain was investigated. The basal medium contained (g/L): xylan, 10; peptone, 1; $MgSO_4.7H_2O$, 0.2 and K2HPO4, 1. Casein was the best nitrogen source on enzyme production. The result of Plackett-Burman showed that among five variables (xylan, casein, $MgSO_4.7H_2O$, K2HPO4 and temperature), casein, $MgSO_4.7H_2O$ and temperature significantly affected enzyme production at 90% of confidence level. The medium consisted of (g/L): xylan, 10; casein, 0.5; K_2HPO_4, 0.1 and $MgSO_4.7H_2O$, 0.3 gave the maximal activity of 29 U/ml. The CCD experiment estimated the optimum values of the factors obtained from Plackett- Burman desing for maximal xylanase production. Under following condition (g/L): casein, 0.16; $MgSO_4.7H_2O$, 0.05; temperature of 45°, the model predicted xylanase activity of 37 U/ml. Verification of the optimization showed that xylanase production of 44 U/ml was observed in shaking flask experiments. High casein concentration resulted in high biomass and high proteolytic enzyme production, whereas the xylanase production was decreased. This phenomenon is because of the action of protease on xylanases degradation during fermentation. The maximal activity of 60 and 292 U/ml was achieved using the medium obatained from Plackett-Burman design and CCD experiment, respectively, in the 3-L airlift fermenter with uncontrolled pH and an aeration rate of 0.5 vvm.

The enzyme produced by strain DMKUA 245^T was subjected to the purification and characterization study. The molecular weight of purified xylanase from the strain DMKUA 245^T was estimated to be 65.8 kDa. The optimal pH for the activity of the purified xylanase was about 5.5, and it was active in a pH range of 4–11. Moreover, the enzyme had an optimal temperature of 60°C and was also active

in a temperature range of 30–60°C. The purified xylanase was stimulated by and 1 mM Co^{2+}, K^{+} and Mg^{2+}, but was inhibited by 1 mM Mn^{2+} and 5 mM EDTA. The specific activity of the purified xylanase towards beechwood xylans was the highest and the K_m value was 3.3 mg mL^{-1}. From these results indicate that the purified enzyme had the highest affinity for beechwood xylan**.** The hydrolysis products were a series of short-chain xylooligosaccharide, indicating that the purified enzyme was an endo β-1,4-xylanase. From the characters of the purified xylanase demonstrated the characteristics of tolerance and resistance of the new thermotolerant *M. siamensis* DMKUA 245^{T} xylanase against changes in pH and temperature which are desirable for biotechnological applications such as simultaneous saccharification and fermentation of xylan in agricultural wastes for lactic or ethanol production at acidic pH or use as feed enzyme.

Recommendation

1. Because the xylanase enzyme produced by this novel strain of *M. siamensis* is endotype and performs well in acidic condition, therefore, the study on application of this xylanase as animal feed or for the production of prebiotic xylooligosacharides from the degradation of lignocellulosic agricultural wastes should be investigated.

2. Sequencing of xylanase gene from the new thermotolerant *M. siamensis* DMKUA 245^{T} should be further examine to search for new type of xylanase enzyme.

3. Induction of xylanase by carbon sources and inorganic nitrogen sources should be investigated.

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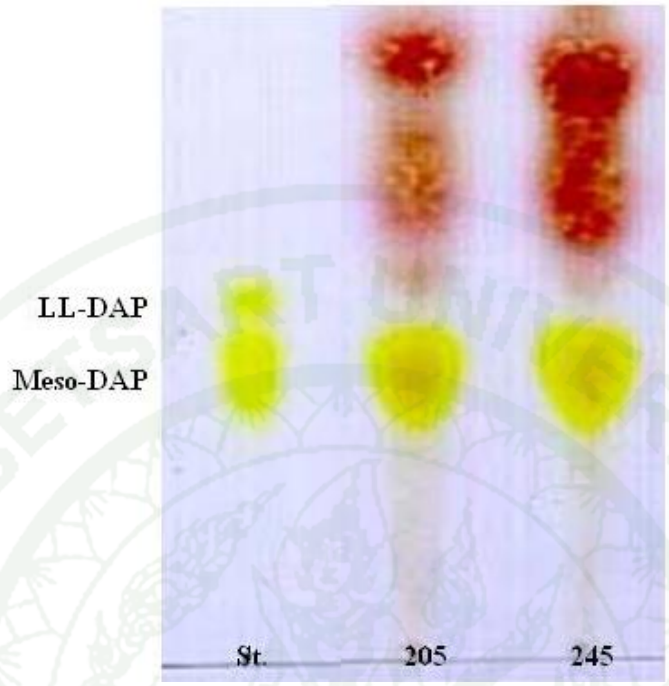
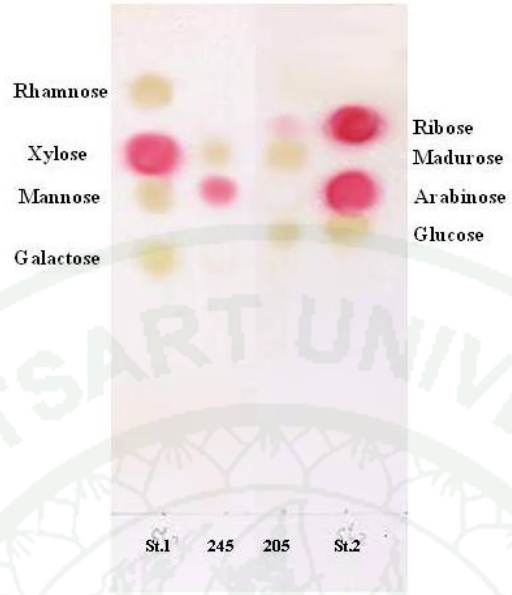
APPENDICES

Appendix A

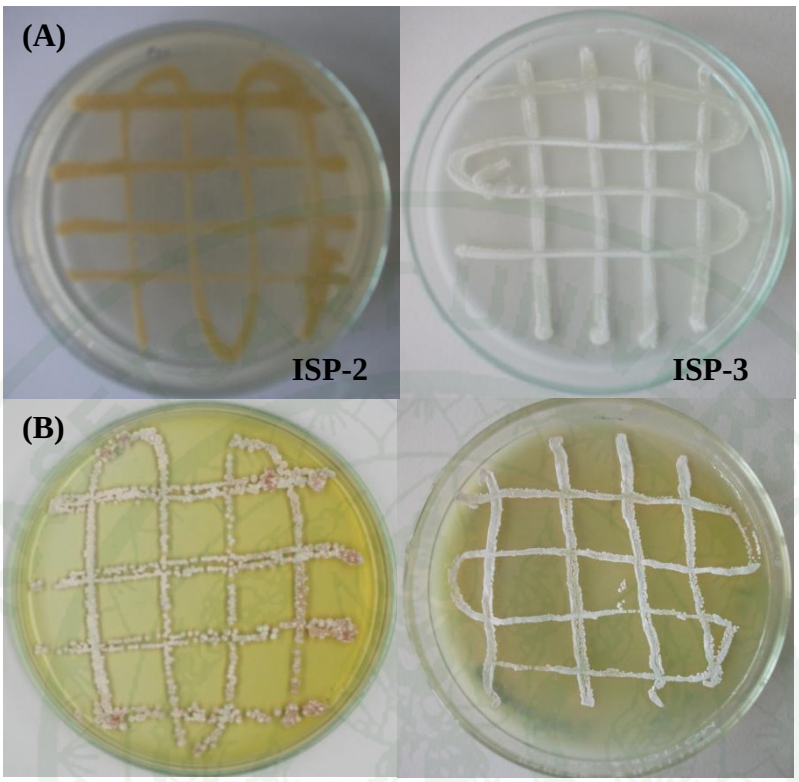
Experimental results



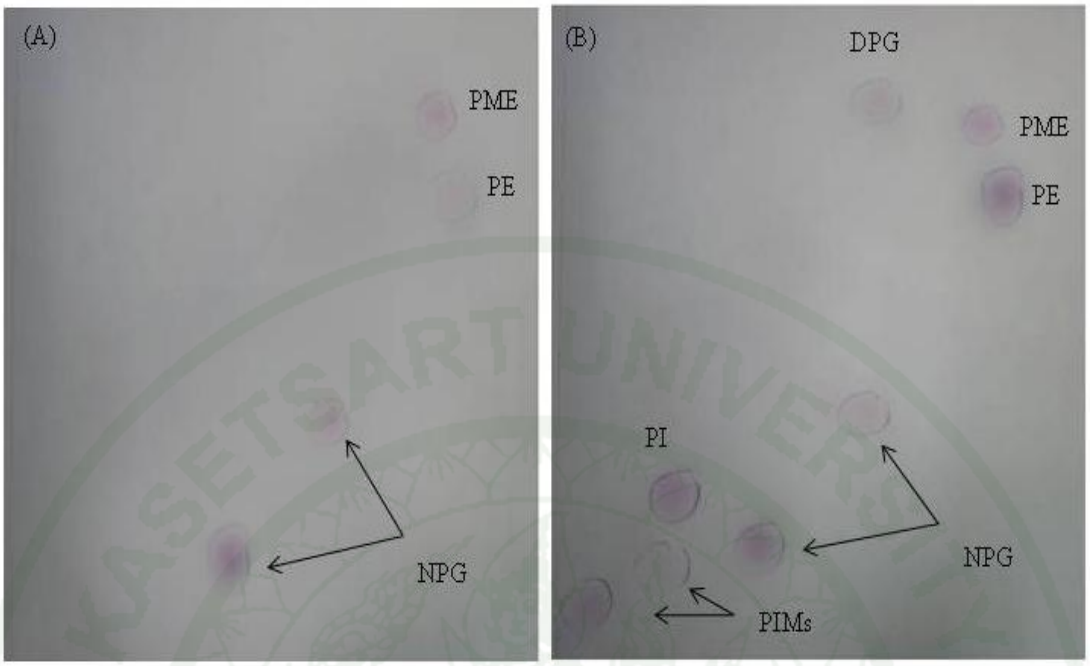
Appendix Figure A1 TLC chromatograms of diaminopimelic acid (A_2pm) of strain DMKUA 205^T and 245^T, St. is the standard solution of A_2pm (LL-A_2pm and *meso*-A_2pm).



Appendix Figure A2 TLC chromatograms of sugars of strain DMKUA 205^{T} and 245^{T}, St. 1 and 2 are standard solution of sugar mixture.

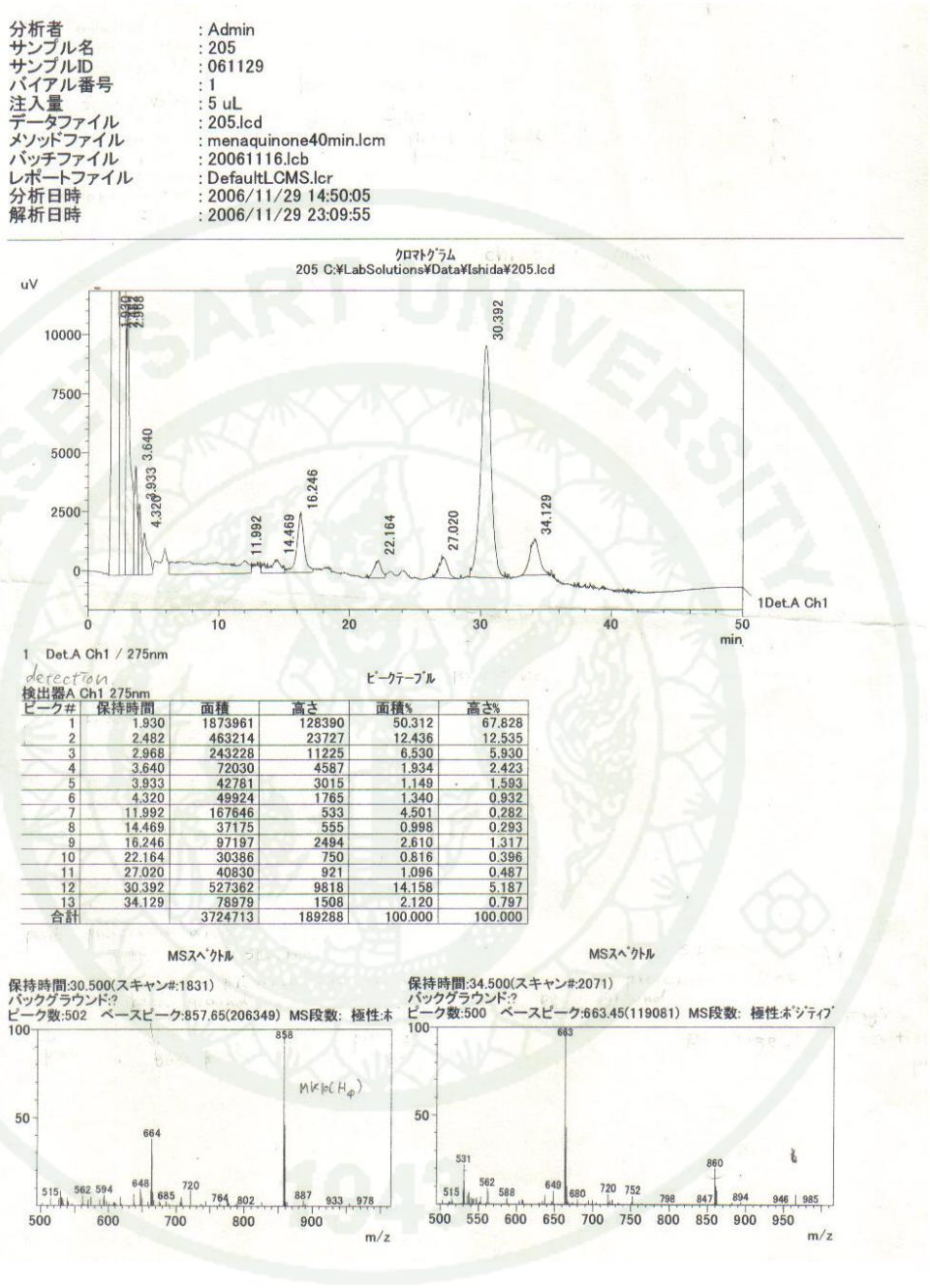


Appendix Figure A3 Morphological characters of strain DMKUA 205^{T} (A) and DMKUA 245^{T} (B) grown on ISP-2 and ISP-3 for 14 days at 30°C.



Appendix Figure A4 Polar lipids appearing on a two-dimension thin- layer chromatogram of strain DMKUA 205^T (A) and DMKUA 245^T (B); PE, phosphatidylethanolamine; PME, phophatidyl-*N*-methylethylethanolamine; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositolmannosides; NPG, ninhydrin-positive phosphoglycolipids.

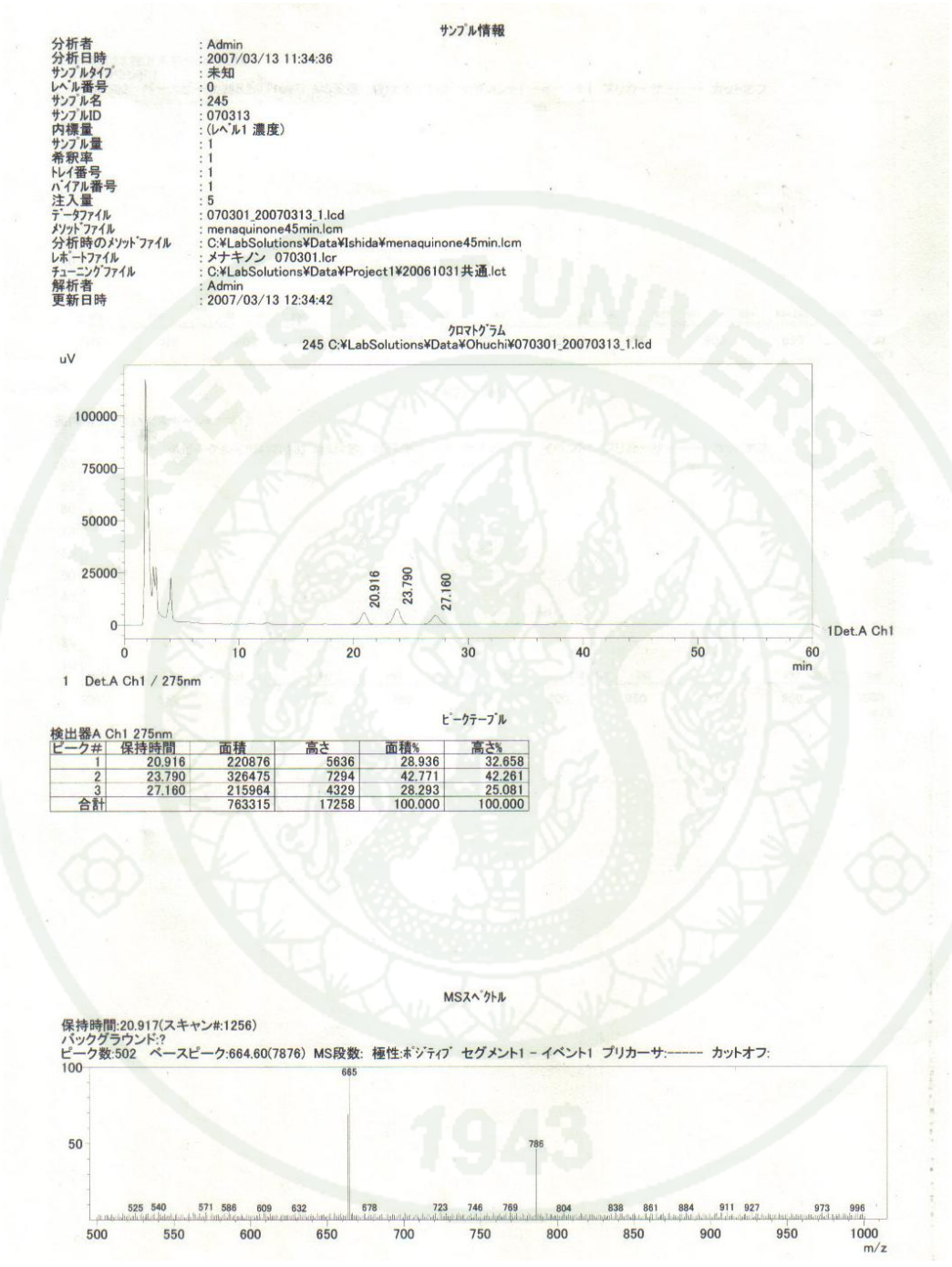
(A)



ピーク#	保持時間	面積	高さ	面積%	高さ%
1	1.930	1873961	128390	50.312	67.828
2	2.482	463214	23727	12.436	12.535
3	2.968	243228	11225	6.530	5.930
4	3.640	72030	4587	1.934	2.423
5	3.933	42781	3015	1.149	1.593
6	4.320	49924	1765	1.340	0.932
7	11.992	167646	533	4.501	0.282
8	14.469	37175	555	0.998	0.293
9	16.246	97197	2494	2.610	1.317
10	22.164	30386	750	0.816	0.396
11	27.020	40830	921	1.096	0.487
12	30.392	527362	9818	14.158	5.187
13	34.129	78979	1508	2.120	0.797
合計		3724713	189288	100.000	100.000

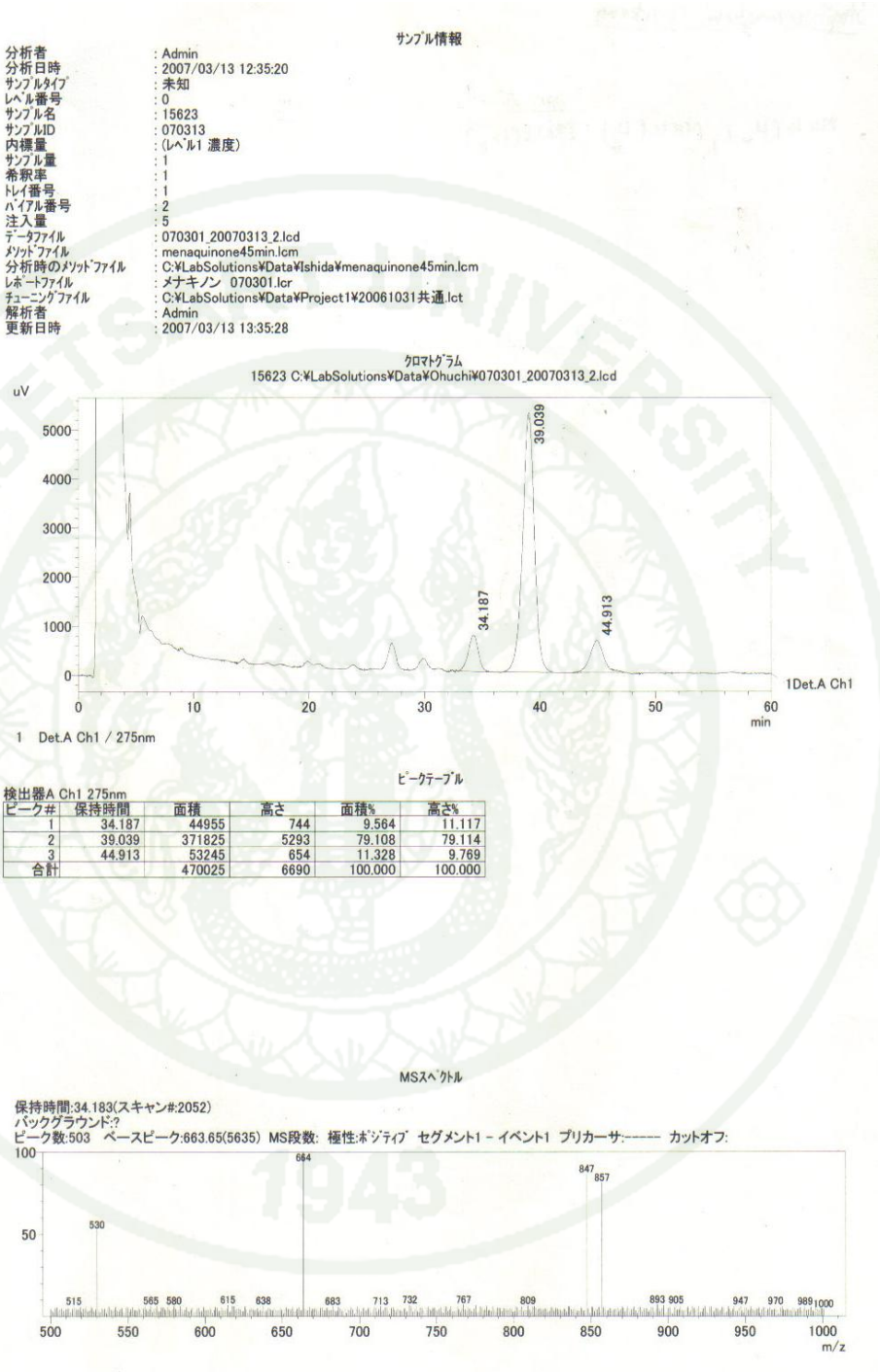
Appendix Figure A5 LC/MS profile of menaquinone type of strain DMKUA 205^T (A), DMKUA 245^T (B) and *Streptosporangium claviforme* NBRC 15623.

(B)



Appendix Figure A5 (Continued)

(C)



Appendix Figure A5 (Continued)

Appendix Table A1 The MIDI profile of fatty acid composition of strain DMKUA 205^{T}.

RT	Response	Ar/Ht	RFact	ECL	PeakName	Percent	Comment 1	Comment 2
1.561	4.506E+8	0.025	…..	7.031	SOLVENT PEAK	…..	<min rt	
1.683	440	0.034	…..	7.277		…..	<min rt	
1.828	1038	0.023	…..	7.568		…..	<min rt	
5.780	1745	0.029	1.005	13.000	13:0	5.15	ECL deviates 0.000	Reference 0.001
6.623	1219	0.030	0.989	13.618	14:0 ISO	3.54	ECL deviates 0.000	Reference 0.001
8.681	10306	0.035	0.962	15.000	15:0	29.14	ECL deviates 0.000	Reference 0.001
9.724	6211	0.036	0.954	15.626	16:0 ISO	17.42	ECL deviates 0.000	Reference 0.001
10.723	1334	0.041	0.949	16.217	15:0 2OH	3.72	ECL deviates 0.000	
11.716	4361	0.040	0.945	16.791	17:1 CIS9	12.10	ECL deviates -0.001	
11.834	1532	0.042	0.944	16.860	16:0 ISO 2OH	4.25	ECL deviates -0.002	
12.077	2969	0.041	0.943	17.000	17:0	8.23	ECL deviates 0.000	Reference 0.001
12.796	5948	0.041	0.941	17.408	17:0 10Methyl	16.45	ECL deviates -0.002	

Appendix Table A2 The MIDI profile of fatty acid composition of strain DMKUA 245^{T}.

RT	Response	Ar/Ht	RFact	ECL	PeakName	Percent	Comment 1	Comment 2
2.505	1356	0.028	…..		SOLVENT PEAK	…..	<min rt	
2.563	286	0.032	…..			…..		
2.623	153	0.018	…..			…..		
2.721	300	0.026	…..			…..		
6.623	1910	0.031	0.987	13.618	14:0 ISO	2.83	ECL deviates 0.000	Reference 0.000
8.102	3601	0.034	0.977	14.622	15:0 ISO	5.29	ECL deviates 0.001	Reference 0.002
8.682	1994	0.034	0.975	14.999	15:0	2.92	ECL deviates -0.001	Reference 0.000
9.449	4848	0.041	0.972	15.460	16:1 ISO	7.08	ECL deviates -0.001	
9.725	46439	0.035	0.972	15.626	16:0 ISO	67.82	ECL deviates 0.000	Reference 0.001
10.432	5070	0.035	0.970	16.049	Unkonow 16.048	7.39	ECL deviates 0.001	
11.096	1456	0.040	0.969	16.433	16:0 10Methyl	2.12	ECL deviates 0.000	
1..796	3123	0.041	0.967	17.409	17:0 10Methyl	4.54	ECL deviates -0.001	

Appendix A1 16S rDNA sequences of strains

Strain DMKUA 205^{T}

GCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAG
CGGAAAGGCCCTTCGGGGTACTCGAGCGGCGAACGGGTGAGTAACACGT
GAGTAACCTGCCCCTGACTCTGGGATAAGCCTGGGAAACTGGGTCTAATA
CCGGATATGACATCCTTCGGCATCGTCGGGTGTGGAAAGTTTTTTCGGTCA
GGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTAGTGGCCTACCAA
GGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGC
AATGGGCGGAAGCCTGACGCAGCGACGCCGCGTGGGGGATGACGGCCTT
CGGGTTGTAAACCTCTTTCAGCAGGGACGAAGTTGACGTGTACCTGTAGA
AGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGTGGCTTGTC
GCGTCTGCCGTGAAAGCCCGCGGCTTAACTGCGGGTCTGCGGTGGATACG
GGCAGGCTAGAGGCTGGTAGGGGCAAGCGGAATTCCTGGTGTAGCGGTG
AAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGCTTGCTGG
GCCAGTTCTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCTAGGTGTGGGGTGCT
TCCACGTGTCCCGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAG
TACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGG
TTTGACATCACCCGGAAACGGCCAGAGATGGTCGCCTCTTCGGACTGGGT
GACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTGCCAGCAACCTCTTCGG
AGGGTTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG
GGGATGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAACATGCT
ACAATGGCCGGTACAGAGGGTTGCGATACCGTGAGGTGGAGCGAATCCCT
AAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGT
CGGAGTCGCTAGTAATCGCAGATCAGCAATGCTGCGGTGAATACGTTCCC

GGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGCAACACCCGAA
GCCCGTGGCCCAACCACTTGTGGGGGGAGCGGTCGAAGGTGGGGCTGGC
GATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGG
AATCACCTCCT

Strain DMKUA 245^{T}

GTTTAAAGTAGTGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGG
CGTGCTTAACACATGCAAGTCGAGCGGAAAGGCCCTTCGGGGTACTCGAG
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TTGTTCCATGTTGCCAGCACGCCCTTTGGGGTGGTGGGGACTCATGGGAG
ACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATG
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GGGGAGCGGTCGAAGGTGGGGCTGGCGATTGGGACGAAGTCGTAACAAG

Appendix B

Reagents

Appendix B1 media composition and preparation

- Trace salts solution (Use as directed in ISP-3, 4, 5 and 7)

$FeSO_4.7H_2O$	0.1	g
$MnCl_2.4H_2O$	0.1	g
$ZnSO_4.7H_2O$	0.1	g
Distilled water	100	ml

- ISP medium no.1 Tryptone-yeast extract broth

Tryptone	5	g
Yeast extract	3	g
Distilled water	1	L
pH 7.0 to 7.2 before autoclaving		

Dispense 5 ml of broth into test tubes with a diameter of 20 mm or more. Use these test tubes for initialing growth from lyophilized pellet. One tube will be needed for each culture studied. Dispense 50 ml of the broth into each 250 ml Erlenmeyer flask. These flasks will be used for preparation of washed inoculum. One flask will be needed for each culture study.

- ISP medium no.2 Yeast extract-malt extract agar

Yeast extract	4	g
Malt extract	10	g
Dextrose	4	g
Distilled water	1	L
Agar	20	g
Adjust to pH 7.3		

Liquefy agar by steaming at 100ºC for 15-20 minutes. Dispense appropriate amount for a slanting into at least 6 tubes for each culture. Sterilize by autoclaving; cool tubes as slants. Use the agar for preparation of stock cultures. Also sterilize medium 2 in flasks for pouring the sterilized medium into Petri dishes.

- ISP medium no.3 Oatmeal agar

Oatmeal	20	g
Agar	18	g

Cook or steam 20 g oatmeal in 1000 ml distilled water for 20 minutes. Filtrate, add distilled water to restore volume of filtrate to 1000 ml, add trace salts solution 1 ml. Adjust to pH 7.2 with NaOH. Add 18 g agar; liquefy by steaming at 100°C for 15-20 min. Sterilize in flasks for pouring into Petri dishes. Swirl medium before pouring to assure even distribution of the oatmeal.

- ISP medium no.4 Inorganic salt-starch agar

Solution I: soluble starch 10.0 g.

Make a paste of the starch with a small amount of cold distilled water and bring to a volume of 500 ml

Solution II:

K_2HPO_4	1.0	g
$MgSO_4.7H_2O$	1.0	g
NaCl	1.0	g
$(NH_4)_2SO_4$	2.0	g
$CaCO_3$	2.0	g
DW	500	ml
Trace salts solution	1.0	ml
Adjust to pH 7.0-7.4		

Mix starch suspension and salts solution. Add agar 20.0 g. Liquefy agar by steaming at 100 °C for 15-20 min. Sterilize in flasks for pouring into petri dishes.

- ISP medium no.5 Glycerol-asparagine agar

L-asparagine	1.0	g
Glycerol	10.0	g
K_2HPO_4	1.0	g
Distilled water	1.0	L
Trace salts solution	1.0	ml
Adjust to pH 7.0-7.4		

Agar	20.0	g

Liquefy agar by steaming at 100°C for 15-20min. Sterilize in flasks for pouring into Petri dishes.

- ISP medium no.7 Tyrosine agar

Glycerol	15.0	g
L-tyrosine	0.5	g
L-asparagine	1.0	g
K_2HPO_4	0.5	g
$MgSO_4.7H_2O$	0.5	g
NaCl	0.5	g
FeSO4.7H2O	0.01	g
Trace salt solution	1.0	ml
Distilled water	1.0	L
Adjust to pH 7.2-7.4		
Agar	20.0	g

Liquefy agar by steaming at 100 °C for 15-20 min. Sterilize in flasks for pouring into petri dishes.

- Pridham and Gottlieb trace salts: 100 ml

$CuSO_4.5H_2O$	0.64	g
$FeSO_4.7H_2O$	0.11	g
$MnCl_2.4H_2O$	0.79	g
$ZnSO_4.7H_2O$	0.15	g

Only 1 ml of this solution was used per liter of final medium. Stored at 3-5°C until required for use. Bring to room temperature before use. Prepare fresh solution each month.

- Cellulose medium: 100 ml

Gilter paper (Whatman No.1) 1 x 13.5cm		
$NaNO_3$	0.2	g
K_2HPO_4	0.1	g

KCl	0.05	g
$MgSO_4.7H_2O$	0.05	g
$FeSO_4.7H_2O$	0.001	g
pH 7.0-7.2		

- Glucose-peptone-gelatin medium: 100 ml

Glucose	2	g
Peptone	0.5	g
Gelatin	20	g
pH 7.0		

- Potassium nitrate broth:

Beef extract	0.3	g
Peptone	0.5	g
KNO_3	0.1	g
pH 7.0		

- Defined medium of Gordon

NaCl	1.0	g
$MgSO_4.7H_2O$	0.2	g
(NH4)2HPO4	1.0	g
K_2HPO_4	0.5	g
0.04% phenol red	20	ml
Distilled water	1.0	L

The medium was prepare by adding 2 g of each organic acids and the pH value of the malate agar was adjusted to 7.2 with 1 N NaOH; the pH value of of the other agars was adjusted to 6.8 before autoclaving. Utilization of the organic acids was established by the alkaline color of the phenol red after 28 days.

Appendix B2 Composition of reagents of DNA extraction was as follows:

1. TE buffer :	10.8 g Tris-HCl
	0.83 g EDTA-2Na
	pH was adjusted to 8.0 with HCl
	Add distilled water to 1,000 ml
2. TAE buffer (10X)	12.1 g Tris-base
	2.9 ml 100% acetic acid
	5 ml 0.5 M EDTA (pH 8.0)
	Add distilled water to 250 ml
3. SET buffer:	75 mM NaCl,
	25 mM EDTA pH8
	20 mM Tris-HCl pH 7.5
4. Lysozyme	50 mg/ml in water
5. Proteinase K:	20 mg/ml in water
6. 10% SDS	
7. 5M NaCl	
8. Chloroform	
9. isopropanol	
10. 70% ethanol	

Appendix B3 Composition of reagents of agarose gel electrophoresis was as follows:

1. Loading dye	1.25 g Bromophenol blue
	1.25 g Xylene cyanol FF
	15 g Glycerol
	Add distilled water to 50 ml
2. 1% agarose gel	0.8 g Agarose
	80 ml TBE buffer/TAE buffer
	10 mg/ml Ethidium bromide 4 µl
3. TBE buffer (10x)	27 g Tris-base

13.75 g H_3BO_3 (Boric acid)

2.32 g EDTA-2Na

Add distilled water to 250 ml

Appendix B4 The reagents for DNA-DNA hybridization

Stock solution

1. 0.2M $MgCl_2$		100	ml
2. 20xSSC		100	ml
	NaCl	175.3	g
	Trisodium citrate	77.4	g
	D.W	1000	ml
3. 20xPBS		100	ml
	Na_2HPO_2	2	g
	KH_2PO_4	4	g
	NaCl	160	g
	KCl	4	g
	D.W	1000	ml
4. 0.1M tris-HCl, 1 mMEDTA (pH 9.0)		50	ml

5. Distilled water. autoclave

Prepare before use

Chemical	**hybridization mixture**	**Pre-hybridization mixture**
Dextran sulfate	0.125 g	-
D.W	1.4 ml	2.9 ml
20xSSC	500 µl	1 ml
Formamide	2.5 ml	5 ml
Denatured salmon DNA (boil 5 min)	50 µl	100 µl
50xDenhardt (not vortex)	500 µl	1 ml

Streptoavidin-enzyme solution

Bovine serum albumin	0.02 g
1xPBS	(2 ml 2xPBS, 2 ml DW)
Streptoavidin-β-galactosidase conjugate	4µl (1U)
Substrate solution	
4-methylumbeliferyl-β-D-galactopyranoside	0.0010 g
DMSO	20 µl (1 mg/20 µl)
Add 1x PBS (2 ml 2xPBS, 2 ml D.W)	4 ml

Appendix B5 The reagents for fatty acid determination

Reagent 1, Saponification—45 g sodium hydroxide, 150 ml methanol, and 150 ml distilled water. Dispensing through use of an autopipet assures reproducibility and allows for large numbers of assays in a day.

Reagent 2, Methylation—325 ml certified 6.0N hydrochloric acid and 275 ml methyl alcohol. This drops the pH of the solution below 1.5 and causes methylation (for the increased volatility in a partially polar column) of the fatty acid. The fatty acid methyl ester is poorly soluble in the aqueous phase at this point.

Reagent 3, Extraction—200 ml hexane and 200 ml methyl tert-butyl ether. This will extract the fatty acid methyl esters into the organic phase for use with the gas chromatograph.

Reagent 4, Sample Cleanup—10.8 g sodium hydroxidedissolved in 900 ml distilled water. This procedure reduces contamination of the injection port liner, the column, and the detector. More than 10,000 analyses can be performed on a column prior to needing any maintenance.

Appendix B6 Composition of reagents of SDS-PAGE was as follows:

The enzyme was precipitated by adding equal vol. of colded 10% (w/v) Trichloroacetic acid (TCA). The solution was centrifuged at 10,000 rpm for 5 min. The precipitated enzyme was dissolved in sample buffer.

1. Acrylamide-bis Stock, 100 ml:	30% acrylamide
	0.8% N,N'-methylene bis acrylamide
2. 2x SDS-Running Buffer, 100 ml:	0.75 M Tris-HCl, pH 8.8
	0.2% SDS
3. 2x SDS-Stacking Buffer, 100 ml:	0.25 M Tris-HCl, pH 6.8
	0.2% SDS
4. 5x SDS-Electrode Buffer, 100 ml:	0.125 M Tris-HCl, pH 8.3
	0.96M glycine
	0.5% SDS
5. TEMED full strength	
6. 2x SDS-SAB	0.125 M Tris-HCl, pH 6.8
	4% SDS
	20% glycerol
	0.002% Bromphenol blue
	10% mercaptoethanol
	(adjust before use)

Compositions of coomassie brilliant blue stain and destain solution were as follows:

1. Stain:	450 ml water
	500 ml methanol
	75 ml acetic acid
	5 g Coomassie brilliant blue
2. Destain I:	1.0 L water
	1.0 L methanol

	200 L acetic acid
3. Destain II:	150 L methanol
	225 L acetic acid bring to 1.0 L with water

Appendix C

Analysis

Appendix C1 Determination of the oxygen uptake rate (OUR) using dynamic gassing method (Tagushi and Humphrey, 1966)

Since biomass sampling in fungal fermentation in bioreactor is difficult because fungal cell and fermentation medium not homogenized (Taguchi and Humphrey, 1966). Actinomycetes are also filamentous bacteria, usually formed pellet in the liquid medium. Growth estimation based on oxygen consumption assumes that the metabolism of these compounds is completely growth associated. The general principle is to interrupt the supply of oxygen and to measure the decrease of the dissolved oxygen concentration with time. If there is no further transfer from the gas to liquid phase, the decrease in oxygen concentration is only due to consumption by the cells. Consequently the linear decrease over the time interval is proportional to the slope of the curve.

To achieve the OUR of actinomycetes grown in fermenter during cultivation, the air supplied was stopped. Due to the respiration of the culture, the dissolved oxygen concentration linearly declined which can be monitored by a DO probe. The decreasing in oxygen concentration was recorded. The plot of dissolved oxygen concentration versus time shows a linear tendency whose negative slope is the value of OUR (s^{-1}), as shown in Appendix Figure C1 (line AB). The aeration supply should be interrupted for a few second, to ensure that the microorganisms are not going to be damaged due to lack of oxygen. After that air was pumped into the culture and the dissolved concentration increase as a function of time as shown in line BC of Figure 19. The for example graph of OUR determination in this study is shown in Appendix Figure C1.

Appendix C2 protein concentration assay

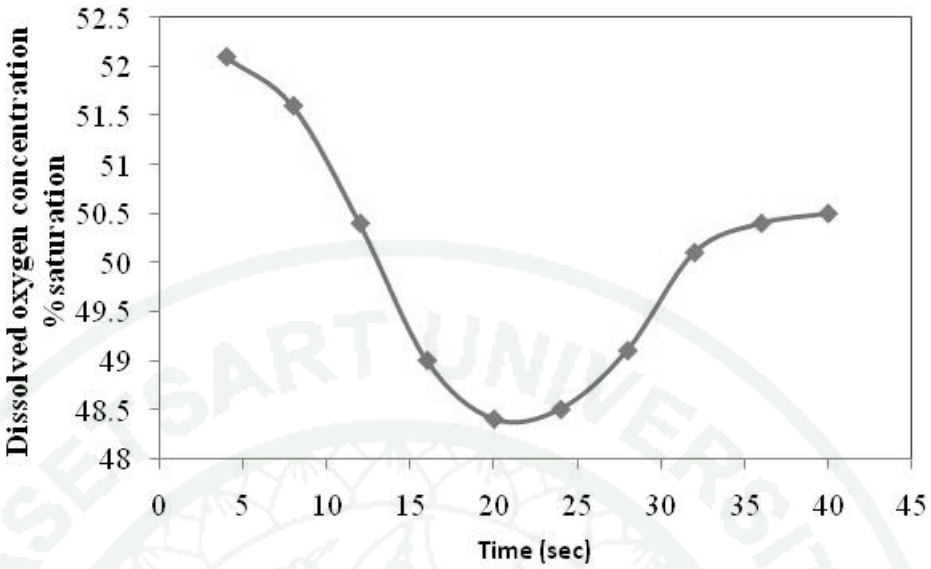
1. Complex-forming reagent: Prepare immediately before use by mixing the following stock solutions in the proportion 100:1:1 (by volume), respectively:

- Solution A: 2% (w/v) Na_2CO_3 in distilled water.
- Solution B: 1% (w/v) $CuSO_4.5H_2O$ in distilled water.
- Solution C: 2% (w/v) sodium potassium tartrate in destilled water.

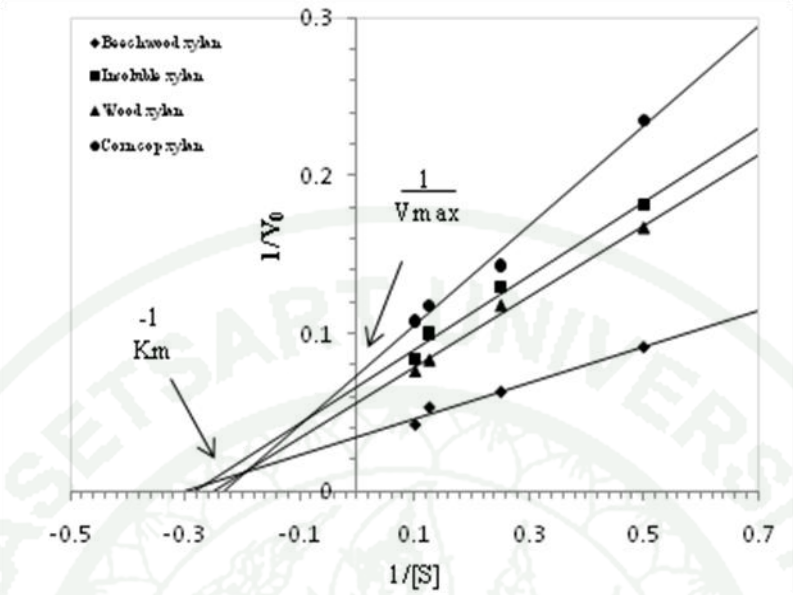
2. Folin reagent (commercially available): Use at 1N concentration.
3. Standard: Use a stock solution of standard protein (e.g., bovine serum albumin fraction V) containing 2 mg/ml protein in distilled water, stored frozen at -20°C. Prepare standards by diluting the stock solution with distilled water as follows:

Stock solution (µl)	0	5	25	50	250	500
Water (µl)	500	495	475	450	250	0
Protein conc. (µg/ml)	0	20	100	200	1000	2000

One milliter freshly mixed complex-forming reagent was added to 0.1 ml of sample or standard. Let the solution stand at room temperature for 10 min. Add 0.1 ml of Folin reagent, using a vortex mixer, and let the mixture stand at room temperature for 30-40 min. Read the absorbance at 750 nm if the protein concentration was below 500 µg/ml. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations.



Appendix Figure C1 Oxygen uptake rate (OUR) of *A.keratinilytica* in 3 L airlift bioreactor during fermentation.



Appendix Figure C2 K_m for purified xylanase from *M. siamensis* DMKUA 245^T using each xylan as substrate.

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