ลักษณะและการตรวจสอบ *Aureobasidium pullulans* ที่คัดแยกได้ในประเทศไทย

นางสาว มณฑารพ สุธาธรรม

CHARACTERISTICS AND IDENTIFICATION OF *Aureobasidium pullulans*   ISOLATED IN

THAILAND

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     Aureobasidium pullulans   เป็นยีสต์สีดำที่มีความสำคัญทางด้านอุตสาหกรรมเพราะ extracellular polysaccharide ซึ่งเป็นผลิตผลของมันถูกนำมาศึกษาด้วยจุดประสงค์เพื่อจะทำให้ผลผลิตของพูลลูแลนได้เพิ่มมากขึ้น   มีการพบสายพันธุ์ใหม่ที่แยกได้จากธรรมชาติในประเทศไทย การจัดจำแนกทำโดยวิธี การย้อมสี Diazonium blue B, ลักษณะทางสัณฐาน, กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด, กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน และ เทคนิคทางชีวโมเลกุล โดยวิธีInternal Transcribe Spacer (ITS) sequencing ของ rDNA ยีนส์ เทคนิคนี้ทำให้เกิดการพบสายวิวัฒนาการของ กลุ่ม Dothideales โดย *A. pullulans* จะอยู่รวมกันเป็นกลุ่ม (cluster) แต่พบว่าในสปีชีส์อื่นจะมีความแตกต่างกันมาก ซึ่งปรากฏการณ์นี้จะสามารถอธิบายได้หรือไม่ขึ้นกับการศึกษาต่อไปในภายหน้า สายพันธุ์ของเราเป็น *A. pullulans* สายพันธุ์ใหม่ จึงต้องได้รับการยืนยันว่าเป็น *A. pullulans* จริง ได้มีการวิเคราะห์โครงสร้างของพูลลูแลนโดย IR-spectrophotometer   ผลผลิตของพูลลูแลนถูกนำมาวัดโดยทำให้ตกตะกอนด้วยเอทธานอล ผลผลิตของพูลลูแลนจะได้สูงสุดเมื่อเลี้ยงในอาหารที่มีกลูโคสและแอมโมเนียมไนเตรตเป็นแหล่งคาร์บอนและแหล่งไนโตรเจน โดย *A. pullulans* CHULA-SU มีภาวะที่เหมาะสมต่อการผลิตที่ pH 6.5 ที่อุณหภูมิ 25 องศา *A. pullulans* CHULA-PR มีภาวะที่เหมาะสมต่อการผลิตที่ pH 7.5 ที่อุณหภูมิ 30 องศา และ *A. pullulans* CHULA-CU มีภาวะที่เหมาะสมต่อการผลิตที่ pH 6.5 ที่อุณหภูมิ 25 องศา ทั้งสามสายพันธุ์ ใช้ กลูโคสและแอมโมเนียมไนเตรต เป็นแหล่งคาร์บอนและแหล่งไนโตรเจนเช่นกัน

ภาควิชาพฤกษศาสตร์	ลายมือชื่อนิสิต..
สาขาวิชาพฤกษศาสตร์	ลายมือชื่ออาจารย์ที่ปรึกษา..............................
ปีการศึกษา 2544	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.........................
	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.........................

Aureobasidium pullulans, a black yeast with industrial significance because of its production of extracellular polysaccharides, was studied with the ultimate aim to enhance pullulan yield. New strains were isolated from nature in Thailand. Its identification was performed by Diazonium Blue B staining, morphology with the light microscopy, scanning and transmission electron microscopy, and sequencing of the Internal Transcribed Spacer domain of the rDNA gene. For the latter technique a large set of reference strains was founded, allowing the reconstruction of a phylogenetic tree of Dothideales. *A. pullulans* formed a well-delimited cluster, while the ITS was found to be heterogeneous within the species. A phenomenon could as yet not be explained and requires further study. Our strains were identical to the neotype culture of *A. pullulans*, and thus their correct identification was confirmed. Subsequently, the structure of pullulan was analyzed by IR-spectrophotometry. Pullulan production of our strains was measured by ethanol precipitation. Highest harvest was obtained when glucose and ammonium sulphate were used as C- and N-sources, respectively. Optimal pullulan production was achieved at a pH varying between 6.5-7.5, and at a temperature of 25-30°C. *A. pullulans* CHULA-SU showed optimal production of pullulan at pH 6.5, 25°C, with glucose and ammonium sulphate as a carbon and nitrogen sources, respectively. *A. pullulans* CHULA-PR produced most pullulan at pH 7.5, 30°C, using the same carbon and nitrogen sources. *A. pullulans* CHULA-CU produced pullulan optimally at pH 6.5, 30°C, using the same media.

Department	**Botany**	Student's Signature……………..………………….
Field of study	**Botany**	Advisor's Signature……….……………………….
Academic year	**2001**	Co-Advisor's Signature……….………………………
		Co-Advisor's Signature……….………………………

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ABBREVIATIONS

bp	=	base pair(s)
^{o}C	=	degree Celsius
CTAB	=	cetyl trimethyl ammonium bromide
CME	=	corn meal agar
DBB	=	diazonium blue B
DNA	=	deoxyribonucleotide triphosphate
Dnase	=	deoxyribonuclease
dNTP	=	deoxyribonucleotide triphosphate
DW	=	distilled water
EDTA	=	ethylene diamine tetra-acetic acid
EPS	=	Exopolysaccharide
g	=	gram
g/g	=	gram per gram
μg	=	microgram
hr	=	hour
ITS	=	Internal Transcribe Specer
l	=	liter
μl	=	microliter
M	=	molar
μM	=	micromolar
MEA	=	malt extract agar
mg	=	milligram
min	=	minute
ml	=	millimeter
ng	=	nanogram
nm	=	nanometer
PCR	=	polymerase chain reaction
PDA	=	potato dextrose agar
PDB	=	Potato dextrose broth

rpm	=	round per minute
sec	=	second
SDS	=	sodium dibecyl sulphate
TE	=	Tris/ EDTA (buffer)
Tris	=	Tris (hydroxymethyl) aminomethane

CHAPTER I

INTRODUCTION

Aureobasidium pullulans (De Bary) Arn. has been recognized as one of the very interesting black yeasts. It is polymorphic and capable of producing a carbohydrate polymer pullulan. Deshpande et al (1992) reported that *A. pullulans* is of widespread ecological occurrence. It is a phylloplane fungus. It grows on plant leave surface with low carbon and nitrogen content. It is found in polluted water because of the leaves fallen in the water. *A. pullulans* has been frequently isolated from moorland, peat bogs or podzol, forest soils including fresh water, estuarine and marine sediments and sea water. Yuen (1974) reported that *A. pullulans* is industrially important because of its capability to produce a polysaccharide "pullulan". Pullulan is now commercially exploited for its coating and wrapping potential and as a food ingredient. We have attempted to collect *A. pullulans* from several locations in Thailand in order to select the strain that is capable to produce pullulans production. In the molecular biology era, the proper identification of the black yeast strains may be done efficiently by using the PCR to elucidate the Internal Transcribe Space (ITS) sequence. De Hoog (1999) reported the taxonomy strains of *A. pullulans* collected from several locations in Europe by performing the ITS sequence, the phylogenetic tree of *A. pullulans* was constructed. The ITS sequence of the tropical strains of *A. pullulans* would be important to help finding the evolutionary relation between the tropical and temperate *A. pullulans* strains. These tropical *A. pullulans* are unique in characteristics. Moreover, *A. pullulans* is an important black yeast with many biological applications. Therefore the investigation of tropical *A. pullulans* is essential for the better understanding of the yeast.

Objective of the present study

To isolate *A. pullulans* from several locations in Thailand, identification by using the ITS sequence data for the proper identification of *A. pullulans* and the investigation of the exopolysaccharide (EPS) production.

CHAPTER II

LITERATURE REVIEW

Aureobasidium pullulans (De Bary) Arn. is a yeast-like fungus that was previously classified in the Fungi imperfecti or Deuteromycetes. The synonym is *Pullularia pullulans* (de Bary) Berkhout and *Dematium pullulans* de Bary (Hermanides-Nijhof, 1977). The fungus is commonly called a ' black yeast' because of its formation of budding cells in initial growth phases and melanin synthesis with obtained thick cell walls during prolonged cultivation (Takeo and de Hoog, 1991). In routine diagnostics it is frequently mistaken for a regular yeast. The diagnostic confusion is not surprising, since the taxonomy of black yeasts appears to be much more complicated than it was anticipated. With the application of molecular criteria, an impressive amount of species are now recognized among the strains used in earlier studies. The number of new taxa is expected to increase even more when detailed studies in biodiversity are performed.(de Hoog,1999). In addition, molecular phylogeny has enabled the establish best of more accurately the position of black yeasts within the fungal kingdom .

The affinity of *Aureobasidium* and relatives to Ascomycetes of the order Dothideales was surmised for a long time on using a combination of characters of conidiogenesis, expansion growth and assimilative abilities (de Hoog & Yurlova, 1994). On the basis of 18S ribosomal sequencing data confirms that it must be an anamorph of a member of the Dothideales, although a perfect stage (teleomorph) has not yet been found. Yurlova et al. (1999) found the ITS sequences of *A. pullulans* to be nearly identical to those of *Discosphaerina fulvida*.(F.R. Sanderson) Sivanesan, an ascomycete of which the anamorph, *Kabatiella lini* (Lafferty) Karakurin, is morphologically close to *A. pullulans*.

Taxonomic interrelationship of the genera *Aureobasidium, Kabatiella* and *Hormonema* has established through teleomorph connections known in a small number of species. *Kabatiella lini* (Lafferty) Karakulin is the anamorph of *Discosphaerina fulvida* (F.R. Sanderson) Sivanesan and *Hormonema dematioides* Lagerb. & Melin is the

anamorph of *Sydowia polyspora* (Bref. & v. Tavel) E. Müller. The teleomorph genera *Pringsheimia* and *Dothiora* in culture all are *Hormonema*-like (Yurlova et al., 1999).

On the basis of these data, *A. pullulans* is now classified according to the following schedule:

Division Ascomycota

    Class Euascomycetes

        Order Dothideales

            Family Dothideaceae

                Genus *Aureobasidium*

                    Species *Aureobasidium pullulans*

(de Hoog et al., 2000; Yurlova et al., 1999; Sterflinger et al., 1999).

The species has the following phenotypic characteristics: colonies grown on malt extract agar (MEA) expanding rapidly, appearing smooth, soon covered with a slimy exudate, cream-coloured or pink, later mostly becoming brown or black. Hyphae hyaline, 3-12 μm wide, locally converting into blackish-brown, thick-walled chlamydospores; marginal hyphae with regularly dichotomous branching. Conidiogenous cells undifferentiated, mostly intercalary in hyaline hyphae. Conidia produced synchronously in dense groups from small denticles, later formed percurrently and adhering in slimy heads. Conidia hyaline, ellipsoidal, very variable in shape and size (7.5-) 9.0-11.0 (-16.0) x (3.5) 4.0-5.5 (-7.0) μm, 1-celled, often with an indistinct hilum. Budding frequently observed. Endoconidia often present in intercalary cells. Temperature range for growth 2-35°C; optimum 25°C; maximum 35°C (de Hoog et al., 2000).

In old culture chlamydospore formation is common, giving the initially pale colonies, a blackish appearance. Other strains rapidly become dark due to the formation of thick-walled and dark hyphae, which often disintegrate into separate cells (Hermanides-Nijhof, 1977). In the past, two morphological varieties were therefore maintained: *A. pullulans* var. *pullulans*, with colonies remaining pink, light brown, or yellow, for at least three weeks, and *A. pullulans* var. *melanogenum* which soon becomes black or greenish-black due to dark hyphae which often falls apart into

separate cells (Hermanides-Nijhof, 1977). However, the varieties are genetically identical, the differences just due to different physiological conditions of isolates (Yurlova et al., 1996).

Nowadays, molecular techniques are applied to distinguish more fundamental entities. A new variety, *A. pullulans* var. *aubasidani* Yurlova, was described for strains producing aubasidan-like components. The new variety can be distinguished physiologically from *A. pullulans* var. *pullulans* by the absence of assimilation of mythyl-α-D-glucoside and lactose (Yurlova et al., 1996).

For the clinical case, it is found occasionally in the clinical laboratory as a contaminant in samples from skin and nail. Infections like keratitis are very rare, and then the portal of entry is always traumatic. Due to its growth on damp surfaces of medical devices, the species may be involved in peritonitis in patients on continuous ambulatory peritoneal dialysis (CAPD) (de Hoog et al., 2000). Nevertheless its BioSafety Level (BSL) classification, is BSL-1, which means the healthy host may be confronted daily with mass inoculant of such fungi without the development of any clinical symptoms, thus it is reasonable to allow handling in the laboratory without special precautions. Even laboratory accidents with such fungi are not likely to lead to human disease. In contrast, there is a potential risk with fungi that are resistant to hostile conditions and have the ability to behave as 'opportunists'. These are classified under BSL-2 (de Hoog 1996). Judging from vast amounts of older literature, BSL-2 fungi are not just emerging as a result of the recent immunocompromised hospital populations, but were already known in the last century as agents of traumatic mycoses. As they have inherent health risks, stricter safety regulations are necessary. This is particularly significant because agents of a local, chronic mycosis may disseminate when later immune defects occur (de Hoog, 1996). With *Aureobasidium*, such effects are extremely rare (de Hoog et al., 2000).

Aureobasidium pullulans has world wide distribution, particularly has been reported from the Northern temperate zone: Finland (Helander and Lehtimaki,1990), Canada, Alaska , Antarctica, British Isles, the Netherlands, Denmark, Germany, the United States. It has also been observed in tropical countries such as India, Jamaica

(Deshpande, Rale and Lynch, 1992) Brazil (Hagler, Rosa, Morais and Mendonca-Hagler, 1993) and Thailand (Takumasu, Tubaki and Manoch , 1997).

The fungus occurs very commonly in low-nutrient, somewhat osmotic environments as an epiphyte on the surface of leaves , needles and flowers (Dix and Webster, 1995). It is a saprophyte (Dix and Webster, 1995) and occasionally causes spoilage of foods (Pitt and Hocking, 1997). *A. pullulans* is one common fungus of the phylloplane and decaying leaves (Kuter, 1986 : Crawford, Carpenter and Harmon, 1990 : Pitt and Hocking , 1997), Moreover it is essentially epiphyte while the leaf is alive, it can become endophytic under certain circumstances. It is possible that some may even behave as weak parasites which the main route of entry is via stomata and attempts to penetrate via the epidermis. They are usually easily replaced by plant defence mechanisms. The most detailed studies of fungal successions on gymnosperm leaves have been carried out on *Pinus* species. (Pitt and Hacking , 1997) One of the fungal populations of conifer needles is   *A. pullulans* which may also be present in low numbers on conifer needles at a very early stage in the succession (Pitt and Hacking , 1997 : Takumasu et al., 1997)

Applied aspects

A. pullulans is industrially important because of its not only capable of producing a polysaccharide ' pullulan', but also itself can be employed for different applied purpose. For instant, the most widely study enzyme from *A. pullulans* is an industrially important enzyme (Federici, 1982) It is used for xylan-degradation (Deshpande, 1992 : Li et al, 1993. Christov et al., 1997). *A. pullulans* has been reported to be safe for use as a single-cell protein (SCP) for agro-industries in developing countries (Deshpande, Bhide, Gurjar and Rale, 1990 : Deshpande et al, 1992).

For medical application, In 1994, McCormack, Wildman and Jeffries reported that one of the yeast like fungi; *A. pullulans* can produce antibacterial compounds inhibitory to both *Pseudomonas fluorescens* and *Staphylococcus aureus* in an overlay biomass.The fungus has also been applied in environmental pollution control. Blackwell, Singleton and Tobin (1995) reported *A. pullulans* was among the fungi which can uptake metal cation by a mechanism for transporting of metal ions into microbial cells.

In 2000, Webb et al. presented that *A. pullulans* was the principle colonizing fungus, establishing itself on the PVC between 25 and 40 weeks of exposure.

Pullulan and its application

As we know that 'pullulan' : can be produced outside the cell by *Aureobasidium pullulans*. Pullulan is an extracellular, unbranched homopolysaccharide which composts of maltotriose and maltotetraose units with both α-(1,6) and α-(1,4) linkages. The regular alternation of α-1,4 and α-1,6 bonds resultsα in two distinctive properties, structural flexibility and enhanced solubility (Lee et al., 1999)

In 1998, Barry reported that the biosynthesis of pullulan could be considered to involve three stages, namely (1) the formation of UDP-glucose, which is the immediate precursor of pullulan, (2) a polymerization reaction to produce pullulan and (3) the transport of polymer across the cell membrane. The molecular weight of pullulan was found in the range of $5x10^4$ to $4x10^6$. However, it's can vary according to the substrates used and fermentation conditions (Lachke and Rale, 1995). Most pullulan molecular polymers are 50,000-3,000,000 D.P. (degree of polymerization).

The properties of pullulan are various. The product is used in medicine industry, food and cosmetics. Its has some similarity to styrene: it is transparent, shiny and elastic, digestible and poisonless. It has the properties of starch and cellulose in its ability to adhere to paper, wood, glass and metal. Since pullulan easily dissolves in water and is moisture retentive, its incorporation in foods prevents overdrying or retrogradation of food stuffs. Due to its natural water solubility, pullulan is compatible with water-soluble polymers, such as gelatin, polyvinylalcohol. It also improves the preservability of the product. Pullulan as a food or drink ingredient results in a low-calorie, tasteless, odourless and colourless additive.

Pullulan can be used to produce hard materials like thermoplastic polymer. By reactive esterification and ethoxylation, pullulan turns to impermeability membranes which can no longer be dissolved in water. The resulting films can be used as a barrier to prevent oxidation of foods and are therefore used for coating and as packaging material. Food products that are coated with pullulan can be kept longer than those protected with amylose. As is widely known, petrochemical films and plastic containers

cause environmental problems after disposal. Pullulan-based materials digest naturally and can be burnt without generating or releasing excess heat or toxic gasses. Pullulan coatings and packagings are effective in preventing formation of oxidized oil in high fat content nuts, such as peanuts, oily or greasy food products, such as dried sardines, dried meat products, fried confectionery and others (Yuen, 1974; Lachke & Rale, 1995).

Pullulan is produced in the form of flocculent material, dielectric material, syrup, cream, fiber which resemble nylon, and water-proof weaving products. Any possible fungal growth is delayed when pullulan is the predominant ingredient. In Japanese industry pullulan is produced from *Aureobasidium pullulans* by growth of the fungus in liquid medium. The product is cleansed to eliminate melanin pigment in the polymer and dried (Yuen, 1974; Deshpande,1992; Lachke & Rale, 1995)

Conditions for pullulan production
Carbon Source

Pullulan is synthesized under the condition in which an excess of a suitable carbon source is available. In general, *Aureobasidium pullulans* can use several substrates such as monosaccharide, disaccharide , but the favorable carbon source of pullulan production is glucose, fructose, xylose, maltose and sucrose (Catley, 1971: West and Reed-Hamer, 1991)

In 1994, Badr-Eldin et al. presented that *Aureobasidium pullulans* NRRL 6220 Synthesized polysaccharide most actively in media containing sucrose, fructose or maltose with $(NH_4)_2SO_4$ (0.6 g/l) or ammonium acetate giving highest yields of the polysaccharide. With the $(NH_4)_2SO_4$ at $\geq$ 1.2 g/l, production of polysaccharide decreased considerably. Polysaccharide production was highest at an initial pH of 6.5 while biomass formation was better at an initial pH below 5.5. Optimum phosphate concentration for polysaccharide production was 0.03M.

In 1995, Shabtai and Mukmenev studied the enhanced production of pigment-free pullulan by a morphogenetically arrested *Aureobasidium pullulans* (ATTC 42023) in a two-stage fermentation with shift from soy bean oil as a carbon source and glutamate as the nitrogen source. When the oil and glutamate source were nearly exhausted (below 5% of initial amounts), the cells were shifted to a production stage with sucrose

as the carbon source with continue nitrogen depletion. The production yield of pullulan on the sugar was about 0.6 g/g.

In 1998, Saha and Bothast shown that a color-varient strain of *Aureobasidium pullulans* (NRRL Y-12974) produced α-L-arabinofuranosidase (α-L-Afase) after growth in liquid culture on sugar beet arabinan, wheat arabinoxylan, L-arabinose, L-arabitol, xylose, xylitol, oat spelt xylan, corn fiber, or arabinogalactan. L-Arabinose was most effective for production of both whole-broth and extracellular α-L-Afase activity, followed by L-arabitol. Oat spelt xylan, sugar beet arabinan, xylose, and wheat arabinoxylan were intermediate in their ability to support α-L-Afase production. Lower amounts of enzyme activity were detected in corn fiber and arabinogalactan-grown cultures.

In 1999, Lee et al. cultured *Aureobasidium pullulans* under aerobic conditions with glucose, mannose, and glucose analog as energy sources. The exopolymer extracts produced under these conditions were composed of glucose and mannose. The molar ratio of glucose to mannose in the exopolymer extract and the molecular weight of the exopolymer extracts formed with glucose and mannose as a carbon sources was between 91 and 87%. The molecular weight decreased from 3.5×10^{6} to 2.12×10^{6} to 0.85×10^{6} to 0.77×10^{6} with culture time. As the culture time increased, the glucose content of the exopolymer extract formed with glucosamine decreased from 55 ± 3 to 29 ± 2 mol%, and the molecular weight increased from 2.73×10^{6} to 4.86×10^{6}. There was no evidence that glucosamine was directly incorporated into exopolymers. The molar ratio of glucose to mannose in exopolymer extracts ranged from $87 \pm 3 : 13 \pm 3$ to $28 \pm 2 : 72 \pm 2$ and were affected by the energy source added. On the basis of the results of an enzyme hydrolysis analysis of the exopolymer extracts and the composition changes observed, mannose (a repeating unit) was substituted for glucose, which gave rise to a new family of exopolymer analogs.

In 1999, Roukas investigated the production of pullulan from brewery wastes by *Aureobasidium pullulans* in shake culture. The maximum pullulan concentration (6.0 g/l) was obtained after 72 h of fermentation. The external addition of nutrients into the spent grain liquor improved significantly the production of pullulan. In the case, the highest values of pullulan concentration (11.0 $\pm$ 0.5 g/l), pullulan yield (48.2 $\pm$ 1.5%), and

sugar utilization (99.0 $\pm$ 0.5%) were obtained in the medium (pH 6.5-7.5) supplemented with K$_2$HPO$_4$ 0.5%, L-glutamic acid 1%, olive oil 2.5%, and Tween 80 0.5%.

Nitrogen Sources

Organics and inorganic nitrogenous substances can both be used as a nitrogen source for growth and pullulan production. Nitrogen sources include corn gluten, soybean, proteins, peptone, nitrates and ammonium salts (Lachke and Rale, 1995)

In 1991, West and Reed-Hamer examined on the effect of carbon and nitrogen source upon the amount of pullulan synthesized by *Aureobasidium pullulans* ATCC 42023.The carbon sources studied included sucrose, glucose, fructose, maltose and corn syrup while the nitrogen sources studied were ammonium nitrate, ammonium sulphate, ammonium tartrate, sodium nitrate and urea. After 5 days at 30°C, it was found that the level of pullulan found was maximal upon sucrose while the lowest level was noted on maltose. Cell dry weight was found to remain relatively constant independent of the carbon source used while the pH of the culture medium dropped significantly. It was demonstrated that the amount of pullulan synthesized was not proportional to the concentration of carbon source present. Pullulan synthesis seemed to be inhibited when the carbon source concentration was increased in batch culture. With respect to the nitrogen source screened, ammonium tartrate and asparagine stimulated maximal pullulan synthesis while urea allowed little pullulan synthesis.

In 1994, West and Reed-Hamer studied the effect of complex nitrogen sources, including tryptone, peptone, soytone, casmino acids and corn steep liquor, on pullulan production by *Aureobasidium pullulans* ATCC 42023 in a medium where sucrose or corn syrup served as the carbon source utilized. In general, all the complex nitrogen sources examined increased pullulan production relative to ammonium sulphate for either sucrose or corn syrup-grown cells. Cell weight of ATCC 42023 grown on either carbon source was lower when the culture medium contained a complex nitrogen source instead of ammonium sulfate. The pullulan content of the polysaccharide elaborated by sucrose-grown cells on day 5 was found to be highest on soytone, peptone or corn steep liqour. The pullulan content of the polysaccharide produced by corn-syrup-grown cells on day 5 was shown to be highest on peptone. The lowest

pullulan content of the polysaccharide synthesized by the fungus, using either carbon source, was found to occur after growth on casamino acids.

Temperature and pH

The influence of temperature and pH on the production of pullulan have been reported from the followings.

Temperature

In 1990, McNeil and Kristiansen examined the effect of temperature on polysaccharide formation and morphology of *Aureobasidium pullulans* in both batch and continuous culture in the range 20°C to 36°C. Apart from minor deviations at both the extremes of temperature, the response was similar in both modes of cultivation. It was found that the temperature affects the fermentation significantly, with 24°C being the optimum for product formation. The variation on polysaccharide concentration with temperature may be a result of changes in the growth rate or morphology of the producer organism.

In 1993, West and Reed-Hamer studied the effect of temperature on pullulan levels produced by *Aureobasidium pullulans* ATCC 42023 in relation to the carbon source present .Pullulan synthesis of fungal batch cultures, in which either sucrose or corn syrup was added as a carbon source, was followed for 5 days during which the incubation temperature was varied from 23 to 33°C . The optimal temperature for pullulan elaboration by *A. pullulans* was 26°C independent of the carbon source added. The polysaccharide concentration was lowest when the fungus was grown at 33°C on either carbon source. Dry weight of the fungal cells were also determined during 5 days at various temperature . The lowest cell dry weights were observed when the sucrose- or corn syrup-containing fungal cultures were maintained at 26°C. Fungal cell weights were usually highest at temperatures where pullulan production was relatively low. The pullulan content of the polysaccharide produced by the fungus on sucrose was highest on day 4 when it reached 54%. The pullulan content of the polysaccharide was 100% after the fungus had been grown on corn syrup for 4 days. Thus, both temperature and carbon source strongly influenced the elaboration of actual pullulan by *A. pullulans.*

pH

In 1984, Lacroxit, LeDuy and Choplin studied the effect of pH on the Batch fermentation of pullulan from sucrose medium by using *Aureobasidium pullulans* 2552 and 140B. In the batch fermentation, either in Erlenmeyer or in the fermentor, the pH of the culture medium was decreased rapidly from its initial pH value of 5.5 to the self-stabilized final value of 2.5 within 24 h. Experiments on the effect of initial pH on the fermentation revealed that at very low initial pH values, such as at pH 2, the polysaccharide production was insignificant. However the biomass concentration obtained was very high at this very low initial pH value. In the first stage of fermentation was conducted at the very acidic pH for the best production of biomass. When the biomass concentration reached its maximum value, the second stage of fermentation was initiated by adjusting the medium pH to a higher value for promoting the synthesis of the polysaccharide. Experiments conducted in Erlenmeyers and in the fermentor confirmed this concept.

In 1993, West and Reed-Hamer investigated the effect of pH on pullulan production relative to carbon source and yeast extract composition of growth medium by culture *Aureobasidium pullulans* ATCC 42023. The pH of a phosphate-buffered minimal medium, in which either sucrose or corn syrup was added as a carbon source, was varied from 2.0 to 7.5. In batch shake cultures, pullulan concentration was monitored over a period of 5 days at 30°C. The lowest pullulan concentration was detected at pH 2.0 for both carbon sources in a media which either contained or lacked yeast extract. The level of pullulan found for either carbon sources increased as the culture medium pH was elevated toward neutrality. The optimal initial pH of the medium differed with respect to the carbon source and to the present of yeast extract. The optimal pH for pullulan elaboration by the fungus after growth on medium containing yeast extract and sucrose was 6.5. The absence of yeast extract in the medium, the optimal initial pH decreased to 5.5. A broad pH optimum was found when ATCC 42023 was grown on a corn syrup-containing culture medium which was supplemented with yeast extract. Fungal pullulan elaboration was optimal between pH 5.0 and 7.0.In this

medium. In the absence of yeast extract, the optimal range for pullulan synthesis by *A. pullulans* decreased to between 3.5 and 5.0.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

A. Mycelia materials

The strains used in the study were collected from several locations in Thailand. A large number of reference strains from the culture collection of the Centraalbureau voor Schimmelcultures, the Netherlands, was used for property and characteristic sequence of comparison. (Appendix 1)

B. Primers use for PCR amplification and DNA sequencing

For set of primers used for amplify are
- primer ITS1 (5'-TCCgTAggTgAACCTgCgg-3')
- primer ITS4 (5'-TCCTCCgCTTATTgATATgC-3')
- primer V9G (5'-TTACGgTCCCTgCCCTTTgTA-3')
- primer LS266 (5'-gCATTCCCAAACAACTCgACTC-3')

C. Instruments

- Autoclave (Ta Chang, Taiwan)
- Automatic micropipette (Gilson)
- Centrifuge (Serval, USA)
- Centrifuge 14,000 rpm (Sigma 112, eppendorf 5417R)
- Critical-point-dried, (SAMDYI 780)
- Detector (LDC : 4100, USA)
- Electrophoresis-unit (ATTO Corporation, Japan)
- Electronic balance with 2 digits (U4600 P, Sartorious, Germany)

- Electronic balance with 4 digits (80A-200M, Presica, Switzerland)

- Haemacytometer (GenieII,Germany)

- Hot air oven (Memmert, Germany)

- Incubator shaker (Lab-Line,USA)

- Infrared spectroscopy (IR) (Perkin Elmer , 1760X, USA)

- Ion sputter (BALZERS UNION, SCD 040)

- Larminar air flow (ISSCO, TAB 123, Thailand)

- Light microscope (Olympus, Japan)

- Microcentrifuge (Eppendorf 5417R)

- PCR machine, adjustable to a rapid thermal ramp of 1°C/sec. (Hybaid., UK.)

- Scanning Electron Microscope (JEOL,JSM-5800LV)

- Transmission Electron Microscope (JEOL,JSM-200CX)

- Ultramicrotome (LKB)

- Vortex-Genie 2 (G-560 E Scientific Industries, INC., USA)

- Waterbaths (SU5, Grant Instruments (Cambridge) Ltd. Barrington, England)

D. **Other Supplies**

- Aluminium foil (3M, 0012-02-01 or Costar, 6524AL)

- Big Dye terminator cycle sequencing PR mix (Applied Biosystems, 4303153)

- Color print film, Gold 200, Eastman Kodak Company Rochester, USA.

- Column loader (MACL 09645)

- Electrophoresis-tray (1000 250ml.) and combs

- Erlenmeyer-flask (Pyrex, USA.)

- Eurogentic SmartLadder (Eurogentic MW-1700-02)

- GFX-columns, RNA and Gelband purification kit) (Amersham Pharmacia 27-9602-01)

- Latex glove

- Microcentrifuge tube 1.5 ml. (Sarstedt 72.690)

- 200 μl Microcentrifuge tube ultra thin (Biozym 179401)

- Microtiter plate 96-wells (Applied Biosystems, N801-0560)

- Micropestles (Eppendorf 0030 120.973)

- Multi screen HV plate (MAHV N45)

- Parafilm (Parafilm M laboratory film, Chicago, USA.)

- PCR vials ultra thin 200 μl (Biozym 179401)

- Pipettips 10, 20, 100 μl (Treff AG, CH-9113 Degersheim, Switzerland)

- Round bottom Eppondorf cups 2 ml.

- Sephadex G-50 superfine (Amersham Pharmacia, 17-0041-01)

- Tape

- Ultrapure water

- 96-well plate centrifuge (Sigma 3K15)

- Whatman paper no. 1 (Whatman International Limited, England)

E. **Chemicals**

- Agarose I (Amresco 0710)

- NH_4NO_3 (Fluk Cheimika)

- $(NH_4)_2SO_4$ (Calro Erba reagent)

- Bacto peptone (Difco, USA)

- Bromophenol blue (Biorad 161-0404)

- Celite 545 (Macherey, Nagel & Co.)

- Chloroform ($CHCl_3$) (1.02445, Merck, Germany)

- Corn Meal Agar (CME) (Difco, USA)

- CTAB (hexadecyltrimethylammoniumbromide, Sigma H-5882)

- DBB (Diazonium Blue B) (Sigma Chemical, USA)

- DNA-polymerase (Sphaero Q, TPO5c)

- DTT (Dithiotreitol) (Sigma D-0632)

- Ethanol 70% , 95% , 96% and 100%

- Ethidiumbromide (1,7-diamino-10-ethyl-9-phenylphenanthridiniumbromide, Serval 2238)

- Gelatine (Merck 4078)

- Glucose (Sigma Chemical, USA)

- Glycerol 87% (Merck 4093)

- HCl (Ferak 11448)

- Malt Extract Agar (MEA) (Difco, USA)

- $MgCl_2.6H_2O$ (Merck 5833)

- $MgSO_4.H_2O$ (Calro Erba reagent)

- Pancreatic RNAse 20u/mg (ribonuclear, Merck 24570)

- phenanthridiniumbromide, Serval 21238)

- KBr (Merck 4938)

- KCL (Merck 4936)

- KH_2PO_4 (Merck, Germany)

- Potato Dextrose Agar (PDA) (Difco, USA)

- Potato Dextrose Broth (PDB) (Difco, USA)

- Proteinase K (Merck 124568)

- Pullulan (Sigma Chemical, USA)

- iso-propanol (Sigma I-0398, Sigma Chemical, USA)

- NH_4-acetate (Fluka 09688)

- NaCl (Merck 6404)

- Na-EDTA (Titriplex III, Bio Rad 161-0729)

- Sodiumdobecylsulphate, SDS (Merck 13760)

- NaOH (Merck 11448)

- Sucrose (Calro Erba reagent)

- Sucrose (Merck 7653)

- TAE (BioRad 161-0743)

- Tris (hydroxymethyl)- aminomethane (Merck 8382)

- Triton X-100 (Merck 8603)

- Ultrapure dNTP set 100 mM (Pharmacia 27-2035-01)

  2'-deoxyadenosine-5'-triphosphate,2'-deoxythymidine-5'-triphosphate,

  2'-deoxycyticline-5'-triphosphate en 2'-deoxyguanosine-5'-triphosphate

METHODS

A. Isolation and Identification of *Aureobasidium pullulans*

1. Sample collection of *A. pullulans*

   Samples were collected from several locations in Thailand by using Corn Meal Agar (CMA) plate exposed at the time interval of 5, 10, 15, 20, 25 and 30 minutes. The sampling locations including Phurua National park (Loei province), Doi Suthep pine forest (Cheingmai province). Nam Now National park (Petchabun province), Tung Salang Laung National park (Phitsanulok province). Khao Yai National park (Nakornratchasima province) and a shady area at Chulalongkorn University , Asia Hotel (Ratchathewee district), House area (Bangsue district) in Bangkok.

2. Cultivation and Identification

   The exposed CMA plates were incubated at room temperature (30°C $\pm$ 2) for 7 days. The incidence of microorganism was observed. Any yeast-like colony was picked up and transferred for further studied on Potato Dextrose Agar (PDA) medium. The identification of the suspected yeast on the Malt Extract Agar (MEA) at 20°C and at room temperature.

   2.1 Diazonium Blue B (DBB) test modified by Barnett, et al. (2000).

   Each strain was cultured on MEA at room temperature at least 10 days. Then each culture was flooded with ice-cold DBB reagent. If the culture turned dark red within 2 min. at room temperature, the result was recorded as positive.

   2.2 Morphology Identification

   A. pullulans ATTC 42023 and *A. pullulans* NRRL 6992 were included for comparison. Identification of the strain was done following methods by de Hoog and Hermanides-NiJhof (1997), de Hoog and Guarro 2000). Confirmation of strains identifical was also done by sequencing the rDNA ITS region.

2.3 Scanning Electron Microscope (SEM) investigation

Morphology of selected strain was studied using Scanning Electron Microscope(SEM) following method modified from Gabriel (1982).

The mycelium was carefully cut off from agar plates where thickness was about 5 mm and then fixed , primary fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for overnight. Each specimen was washed three times in phosphate buffer, 10-15 min each. Specimens were optimally preserved by post fixation with 1% OsO_4 in 0.1 M phosphate buffer pH 7.2 for 2 hrs, and afterward washed three times in phosphate buffer, 10-15 min each. Specimens were dehydrated through a series of ethanol at concentration 30%, 50%, 70% 90% and 100%, 15 min each. Samples were critical-point-dried,(SAMDYI 780), attached to aluminium specimens stubs, and coated with gold in an Ion sputter (BALZERS UNION, SCD 040). Specimen were examined in the scanning electron microscope (JEOL ,JSM-5800LV) at 15 KV, and electron micrograph were taken.

2.4 Transmission Electron Microscope (TEM) investigation.

Morphology of selected strain was studied using TEM (Transmission Electron Microscope) following method modified from Napanitaya (1987).

The mycelium was fixed , primary fixation in 2.5% glutaraldehyde in 0.1 M Cacodehyde buffer, pH 7.2. Each specimen was washed three times in Cacodehyde buffer, 10-15 min each. Specimens were optimally preserved by post fixation with 1% OsO_4 in 0.1 M Cacodehyde buffer pH 7.2 for 1 hrs, and afterward washed in distilled water for 10-15 min. Specimens were dehydrated through a graded series of ethanol at concentration 30%, 50%, 70% 95%, 15 min each. and absolute alcohol three times , 15 min each. Specimens were filtrated in absolute ethanol : Spurr resin in ratio 3:1 for 2 hrs, 1;1 for 2 hrs and 1:3 for 1 hrs, and filtered in pure spurr resin three times, 2 hrs each. Specimens were embed polymerization at $70^{\circ}C$ for 8 hrs. Specimens were cut with ultramicrotome (LKB) about 60-90 nm thickness, replaced on Cu grid and were stained in uranyl acetate and lead citrate. Specimens were examined in the Transmission Electron Microscope (JEOL ,JSM-200CX) at 100 KV, and electron micrograph were taken.

3. Molecular   studies

Strains and culture conditions for ITS sequencing

Strains studied were listed in Appendix I. They were grown on MEA (Malt Extract Agar) for 1- 2 weeks at 24°C.

The techniques for molecular studies described by Gerrits van den Esde (2001). The steps were devided as follows.

DNA extraction

About 1 cm^2 of mycelium was transferred to a 2 ml microcentrifuge tube containing a 2:1 mixture of silica gel and celite 545 and 300 μl TES buffer. The material was ground with a micropestle for 1-2 min. Volume was adjusted to 500 μl by adding TES-buffer and vortex. 10μl of Proteinase K was added to the extract and incubated at 65°C for 10 min. Then 140 μl of 5 M NaCl was added to increase the salt concentration, followed by adding 65 μl of 10% CTAB and incubating again for 30 min at 65°C. One volume ($\sim$700 μl) of SEVEG was added and mixed carefully by inverting the tube several times, and placed on ice water for 30 min and centrifuged at 14,000 rpm for 10 min. After transferring the aqueous supernatant to a new microcentrifuge tube, 225 μl 5 M NH$_4$-acetate was added and mixed gently. Samples were incubated on ice water for 30 min, centrifuged for 10 min at 14,000 rpm and transferred the supernatant to a sterile 510 μl microcentrifuge tube to precipitate DNA, isopropanol was added to the supernatant mixed, and centrifuged for 5 min at 14,000 rpm. The pellets were rinsed twice with 500 μl ice-cold ethanol 70%. After air dried DNA pellet at room temperature. The sample was added to resuspended DNA 48.5 μl of TE-buffer, Removed of DNA was done by adding 1.5 μl RNAse solution, incubated 5-30 min at 37°C.

DNA purification was carried out using GFX PCR DNA and Gel band by placing a GFX-column in a collection tube and pipeting 500 μl Capture-buffer onto the column and add 50 μl amplicon. The sample was centrifuged for 30 sec at 11,500 rpm. Discard the flow-through by emptying the collection tube and place the column back onto the collection tube. One volume ($\sim$500 μl) of wash buffer was added to the GFX column and centrifuged at 11,500 rpm for 30 sec. Discard the collection tube and transfer the

column to a sterile 1.5 ml microcentrifuge tube 50 μl TE-buffer was added to elute DNA The sample was incubated at room temperature for 1 min, centrifuged for 1 min at 11,500 rpm to collect purified DNA. The sample was then the purified material.

PCR Amplification.

PCR amplification was performed in 50 μl volumes of a reaction mixture containing 1X PCR buffer, 0.2 mM dNTP, 50 pmol of each primer (ITS1 and ITS4), 1 U Taq DNA polymerase , 1.5 M MgCl$_2$ and adjust volume to 30 μl Ultrapure sterile water. The reactions were performed for 48 cycle at: denaturing step at 94°C, 45 sec; annealing step at 48 $^{\circ}$C, 30 sec; and elongation step at 72 $^{\circ}$C, 2 min and final elongation at 72°C for 6 min.

Preparing an agarose gel for PCR

The fragment lengths of the amplified DNA were determined on 1.2% agarose gel electrophoresis on basis of their molecular size. The DNA mixture consisted of 1X TAE-buffer, 1% ethidiumbromide, 2 μl loading buffer and 8 μl PCR product. The DNA fragment lengths were determined against the known SmartLadder of Eurogentic (Eurogentic MW-1700-02) electrophoresis was performed with 1X TAE-buffer as running buffer, at a constant voltage of 150 V for 1.5-2 hours. The gel was illuminated with UV light, and photo was taken using the ImageMaster VDS system.

DNA Sequencing.

With primers ITS1 and ITS4, amplification condition was done as follows: 25 cycled of 96°C, 10 sec 50°C,5 sec   60°C,4 min carried out with 15-50 ng of DNA for a 10 μl reaction mixture including 4 pmol primer and 4 μl BigDye PR Mix (Applied Biosystem, Nieuwerkerk and Ijssel, The Netherlands). The reaction was purified using sequencing reaction carried out in 10 μl containing 10-50 ng Amplicon, 4 pmol of primer, 4 μl of BigDye PR mix. The reaction was purified using sephadex G-50 superfine (Amersham Pharmacia) and following manufactured instruments. Capillary electrophoresis was done on an ABI Prism^{TM}310 Genetic Analyzer (Applied Biosystems).

Alignment and phylogenetic analysis.

The sequences were adjusted using SeqMan of Lasergene software (DNASTAR, Madison, Wisconsin, Inc.) and aligned using BioNumerics (Applied Maths, Kortrijk, Belgium). A distance tree was constructed with Neighbor-joining with correcting using the TREECON (version 1.3b) software package (van de Peer and De Wachter, 1994) Not all identical genotypes were included in the phylogenetic analysis; for additional data on strains, see Appendix II.

B. Exopolysaccharide production by *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU.

1. **Infrared spectroscopy (IR) investigation**

The EPS samples from *A. pullulans* 3 stains were used as a mull. Mull are prepared by throughly grinding 2-5 mg of a EPS in a smooth agate mortar. The suspended particles must be less than 2 μm to avoid excessive scattering of radiation. The mull is examined as a thin film between flat salt plates. The pellet (pressed-disk) (0.5-1.0 mg) is intimately mixed with approximately 100 ng of powered KBr. The mixture is pressed with special under a pressure of 10,000-15,000 psi into a transparent disk. Removal of the balls leaves a pellet in the nut that now serves as a cell.

IR spectra were obtained on a Perkin Elmer Model 160X Fourier Transform Infrared Spectrophotometer. Spectra of solid samples were recorded as KBr pellets (4000:1600 cm^{-1}).

2. **Growth study of *Aureobasidium pullulans***

Cultivated of *A. pullulans* in PDA (Potato Dextrose Agar) medium for 4 days. Then transferred to Erlenmeyer flasks 250 ml, containing 95 ml Potato Dextrose Broth medium (PDB). The initial cell suspension in the range 10^7 cell/ml by using Haemacytometer (see appendix IV) Pipet 5 ml cell suspension into 95 ml PDB. Each strain was cultivated at temperature 30°C, 150 rpm for 86 hrs.

3. **Study on Exopolysaccharide (EPS) production**

EPS was produced from the three isolates of *A. pullulans*. The yeast was maintained on a PDA slant at 4°C. The EPS production started with the preparation 5%

(v/v) inoculum (10^7cells per ml) in a Potato Dextrose Broth (PDB) medium. The optimal parameters for the pullulan production were determined including pH, temperature, carbon source, and nitrogen source.

3.1. Study on pH optimization

Cultivation of *A. pullulans* 3 strains was done in 9.5 ml. of a production medium (see Appendix V). Its initial pH was adjusted to a desired value 4.5, 5.5, 6.5 and 7.5 with HCl solution. The Erlenmeyer flasks contained 95 ml production medium with initial cell suspension. Each strain was cultivated under temperature 25°C, 150 rpm for 5 days.

Samples of each culture were removed and centrifuged. The supernatant containing EPS (Exopolysaccharide) was retained for 95% ethanol precipitation. The precipitated EPS was collected on Whatman No. 1 paper and dried. The filters were dried to constant weight at $60\,^{\circ}$C for 24 hrs and subsequently reweighed to determine the EPS content.

3.2 Study on temperature optimization

Cultivation of *A. pullulans* 3 strains was done at a favorable pH of each strain in the production medium as described in 2.1. The initial temperature was adjusted to a desired value $25\,^{\circ}$C, $30\,^{\circ}$C, and $35\,^{\circ}$C at 150 rpm for 5 days.

The EPS yield was detected as described in 3.1.

3.3 Study on carbon source optimization.

Cultivation of *A. pullulans* 3 strains was done at a favorable pH and temperature of each strain in the production medium as described in 3.1

Two different carbon sources, glucose at 5% (w/v) and sucrose at 5% (w/v).were used in the experiment. The EPS production was performed at 150 rpm for 5 days.

The EPS yield was detected as described in 3.1.

3.4 Study on nitrogen source optimization

Cultivation of *A. pullulans* 3 strains in favorable pH and temperature and carbon source of each strain in the production medium as described in 3.1

Two difference between nitrogen sources including $(NH_4)_2SO_4$ and Bacto peptone at 0.06%(w/v) was used in this experiment. The EPS production was performed at 150 rpm for 5 days.

The EPS yield was detected as described in 3.1.

Statistical analysis

Data on the EPS production were averaged from quadruplicating. All experiments were completely randomized designed. Statistical analyses included the Duncan's multiple range test (DMRT)

CHAPTER IV

RESULTS

A. Isolation and Identification of *Aureobasidium pullulans*.

1. Sample collection of *A. pullulans.*

      Among various locations in Thailand, sampling locations showing the presence of *A. pullulans* are indicated in Table 1.

Table 1: Sampling locations showing the presence of *A. pullulans*.

Locations	Sampling Date	Sources	Result
1. Tab building Chulalongkorn University (Bangkok)	12th Nov. 1999.	air	+
2. Botany building Chulalongkorn University (Bangkok)	1st Oct. 2000.	*Ixora* sp.	+
3. Asia Hotel (Bangkok)	7th Oct. 2000.	public fountain	+
4. House area (Bangkok)	13th Oct. 2000.	Soil	+
5. Doi Suthep pine forest (Cheingmai province)	19-24th Mar. 1999. 5-7th Aug. 1999	Air	+
6. Khao Yai National park (Nakornratchasima province)	15-17th May.1998.	Air	-
7. Nam Now National park (Petchabun province)	11-14th Aug. 1999.	Air	-
8. Phurua National Park (Loei province)	11-14th Aug. 1999.	Air	+
9. Tung Salang Laung National Park (Phitsanulok province)	11-14th Aug. 1999.	Air	-

Remark : + = detected of *A. pullulans*

     - = not detected of *A. pullulans*

2. Cultivation and Identification

2.1 Diazonium Blue B (DBB) test

Table 2: Diazonium Blue B (DBB) test.

No.	Name	Other reference	Positive test
1.	*A. pullulans* CHULA-SU	-	-
2.	*A. pullulans* CHULA-PR	-	+
3.	*A. pullulans* CHULA-CU	-	-
4.	*Cryptococcus albidus*	CBS 142	+
5.	*Pichia anomala*	CBS 110	-

Remark:

+ = positive test

- = negative test

Cryptococcus albidus (CBS 142) was used as a positive control

Pichia anomala (CBS 110) was used as a negative control

2.2 Morphology Identification

Morphology identification of *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU, including *A. pullulans* ATTC 42023 and *A. pullulans* NRRL 6992 were used comparison.

Colony characteristic

For all 3 strains, colonies on CMA, MEA, and PDA grew rapidly, slightly at the center, attaining up to 8 cm. diam in 1-2 weeks. Aerial mycelium absent covered with a slimy exudate, cream-coloured later becoming black (for *A. pullulans* CHULA-SU and *A. pullulans* CHULA-PR) and deep red in *A. pullulans* in CHULA-CU. (Figure 1,2 and 3)

Microscopy characteristics

Description based on cultures grown on MEA, PDA and CMA at 25°C.

A. pullulans has some morphological features, like yeast cells and hyphae which produce synchronous conidia when they are young. Hyaline hypha wide average 2.60 μm. for all 3 strains. (Figure 1, 2 and 3)

The conidia then fall off and depending on the nutritional condition they will germinate with yeast cells or with hyphae. Conidia hyaline, ellipsoidal shape for all 3 strains.

A. pullulans CHULA-SU, conidia vary in size, (3.9)-(10.4)X(6.5)-(23.4) μm. *A. pullulans* CHULA-PR, conidia size (2.6)-(5.2)X(5.2)-(10.4) μm. For *A. pullulans* CHULA-CU conidia is rather regular shape than the others 2.6x2.6 μm.

Also dark thick walled chlamydospores are produced, but this is the case in the whole order of Dothideales and thus is not only characteristic of *A. pullulans*. Sometimes also endoconidia are produced as we observed in *A. pullulans* CHULA –SU (Figure 1(b)). It is common in *A. pullulans*.
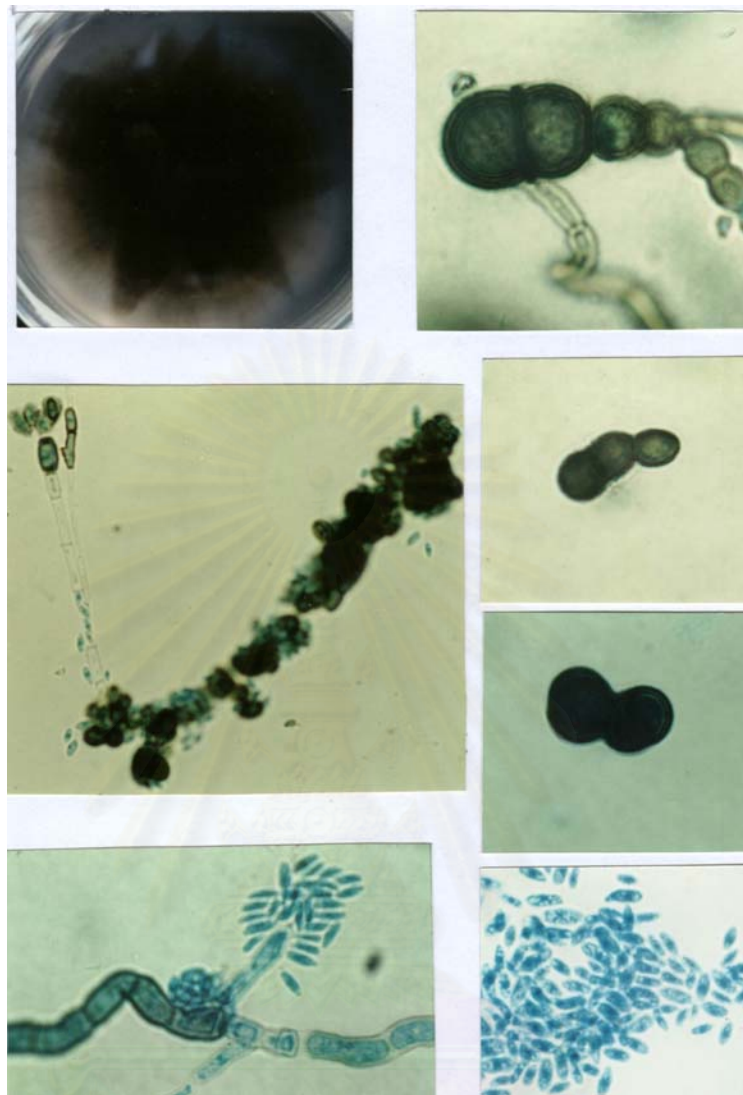
Figure 1: a) *A. pullulans* CHULA-SU: **b)** Hypha which present endoconidia (bar=25μm):
c) synchronous conidia (bar=10μm): **d-f)** dark chlamydospore (bar=10μm): **g)**
conidia(bar=10μm)
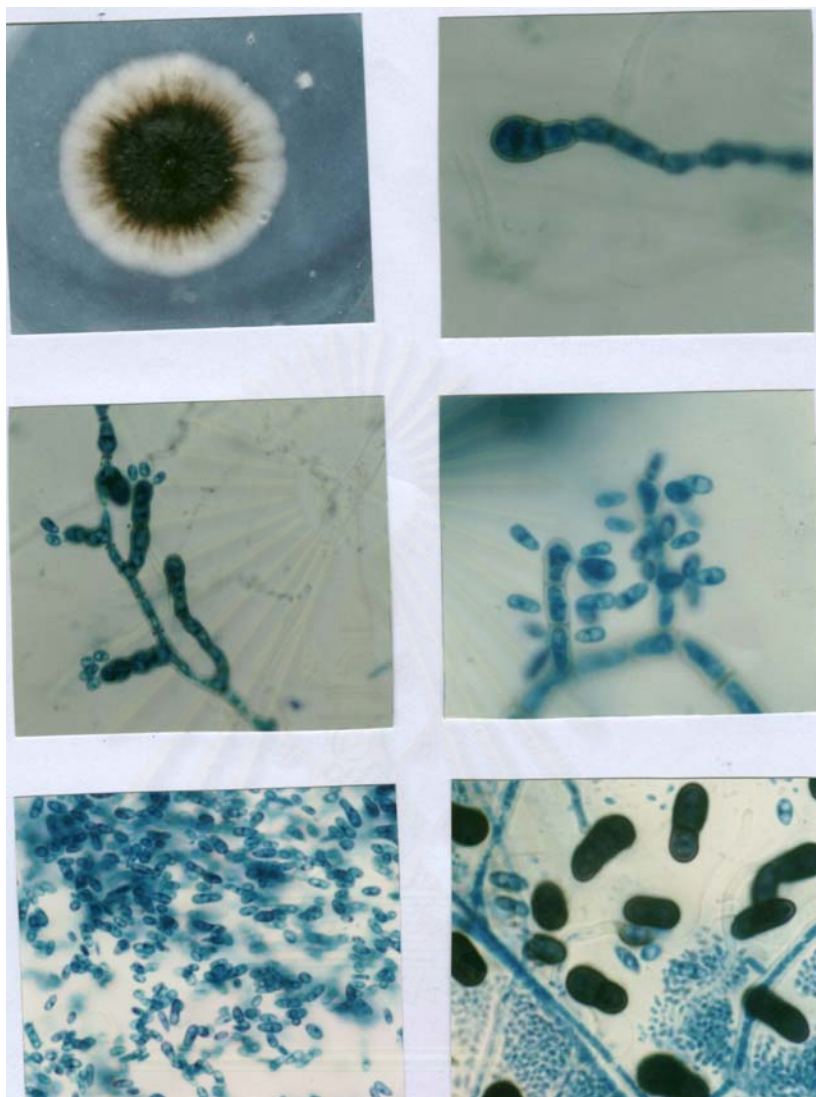
Figure 2: a) *A. pullulans* CHULA-PR: **b)** dark hypha with the chlamydospore at the hyphal tip (bar=10μm): **c-d)** conidial apparatus (c: bar=25μm; d: bar=10μm): **e)** conidia(bar=10μm): **f)** dark arthroconidia (bar=25μm)
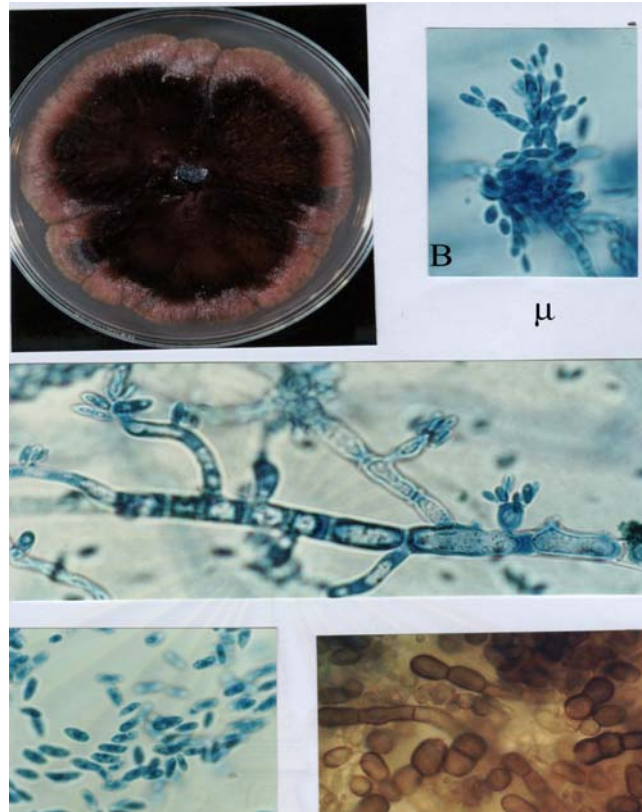
Figure 3: **a)** *A. pullulans* CHULA-CU: **b-c)** conidial apparatus and produce the conidia synchronously (bar=10μm): **d)** conidia (bar=10μm): **e)** intercalary and discrete chlamydospores (bar=10μm)

2.3 SEM (Scanning Electron Microscopy) investigation

Hyphae and yeast cells are visible . In Figure 4-15. A hyphal end is seen with several conidia arrached and all of the same size, suggesting synchronous conidiation Conidial scars can be seen for all 3 strains (Figure 4-15)
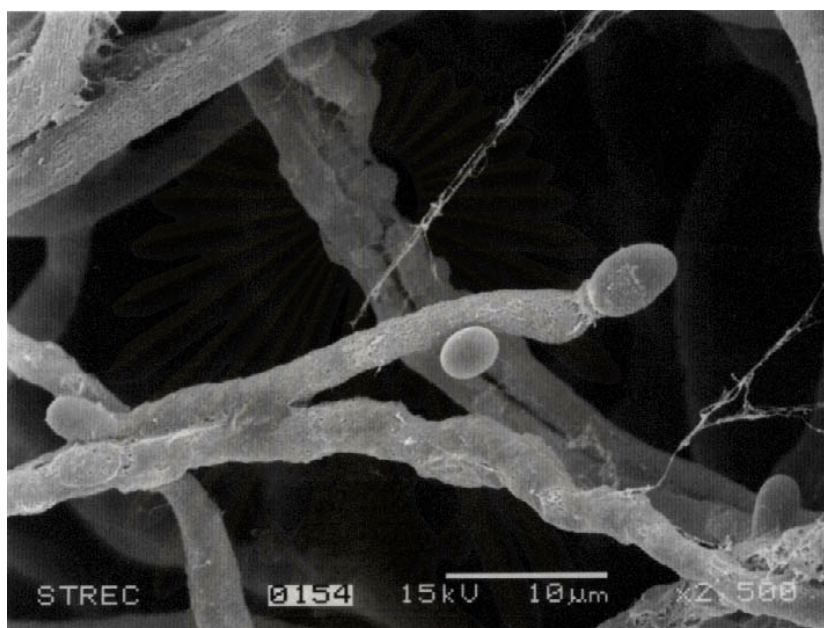
Figure 4: *A. pullulans* CHULA-SU: young hyphae with conidia at the end of hyphal tip. (x 2,500)
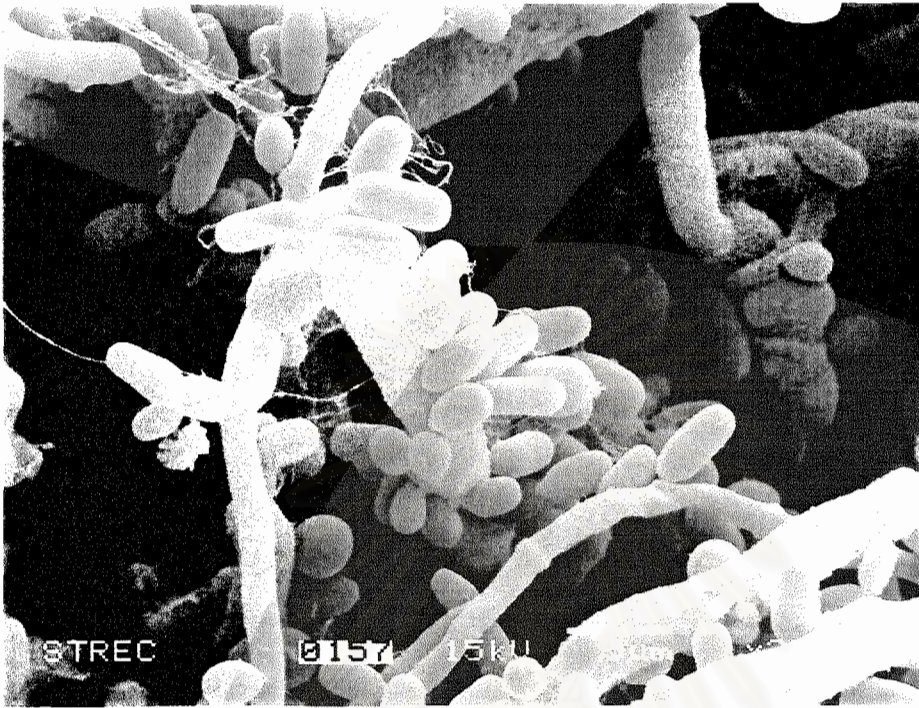
Figure 5: *A. pullulans* CHULA-SU: hyphal element with synchronous conidia. (x 2,500)
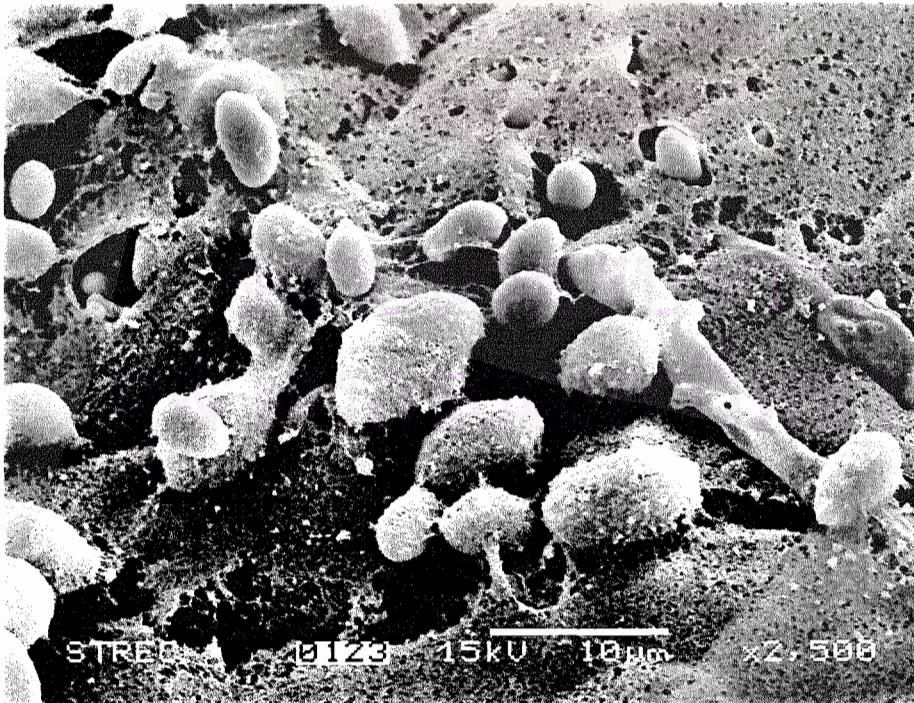
Figure 6: *A. pullulans* CHULA-SU: conidia. (x 2,500)
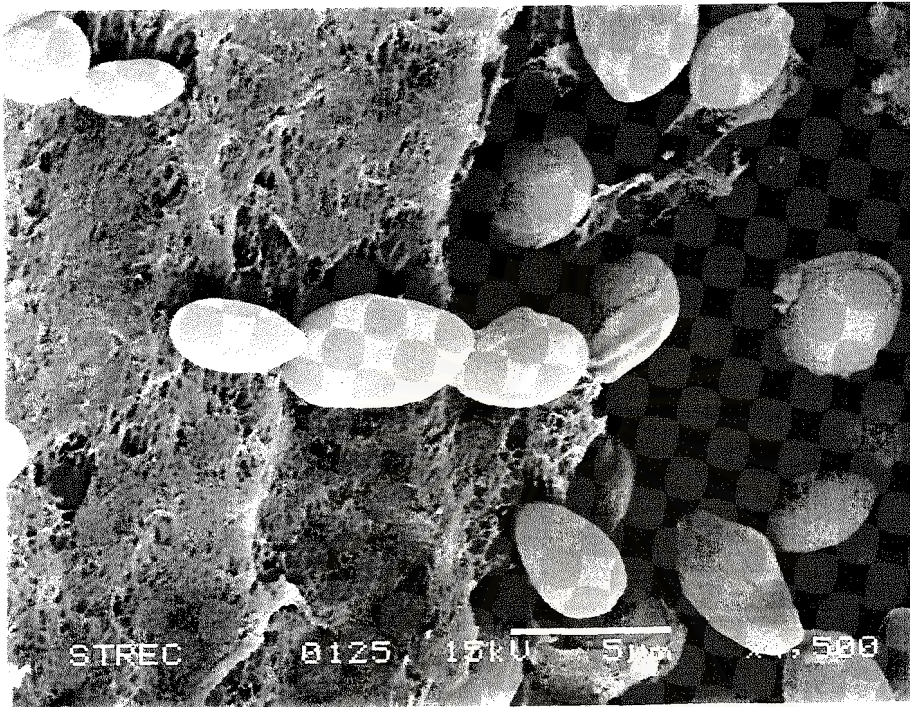
Figure 7: *A. pullulans* CHULA-SU: a branch with a clump of conidia adhering. (x 4,500)
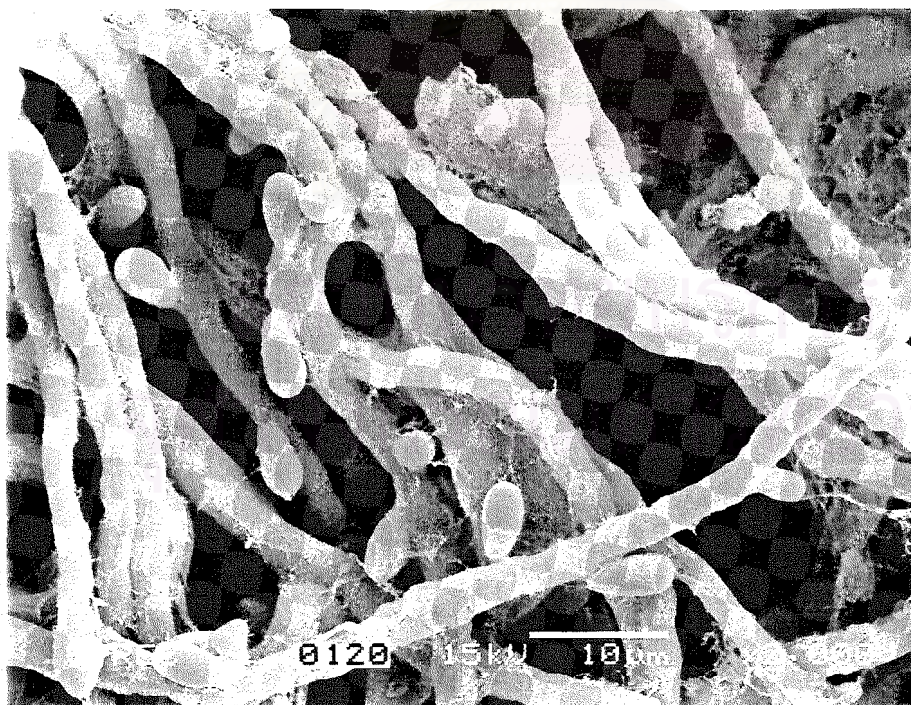
Figure 8: *A. pullulans* CHULA-PR: hyphae with conidia. (x 2,000)
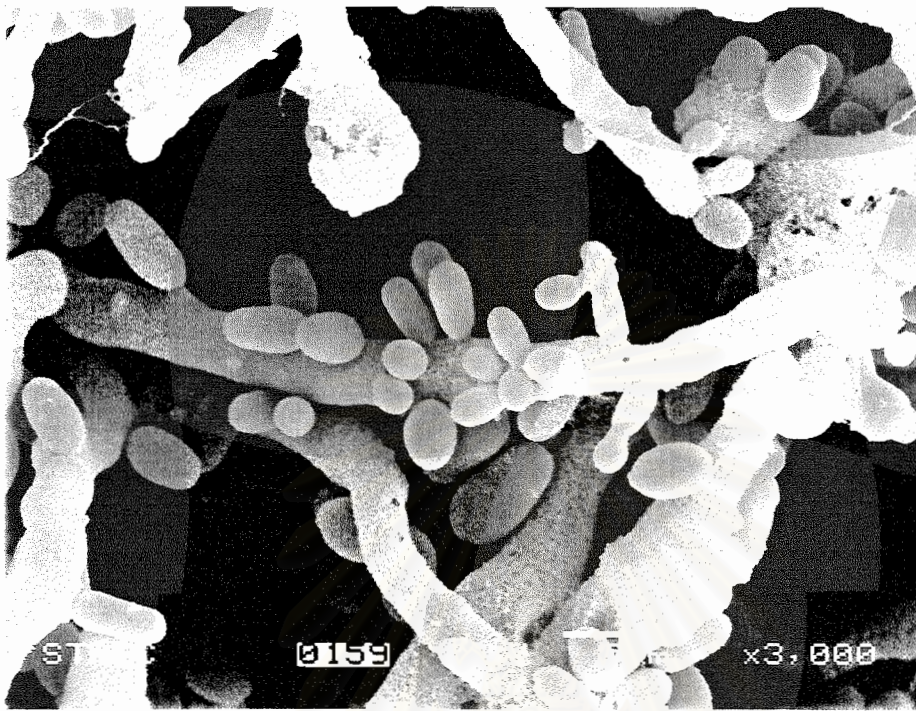
Figure 9: *A. pullulans* CHULA-PR: hyphae with synchronous conidia. (x 3,000)

Figure 10: *A. pullulans* CHULA-PR: mature hyphae with chlamydospore at the end of hyphal tip. (x 2,500)
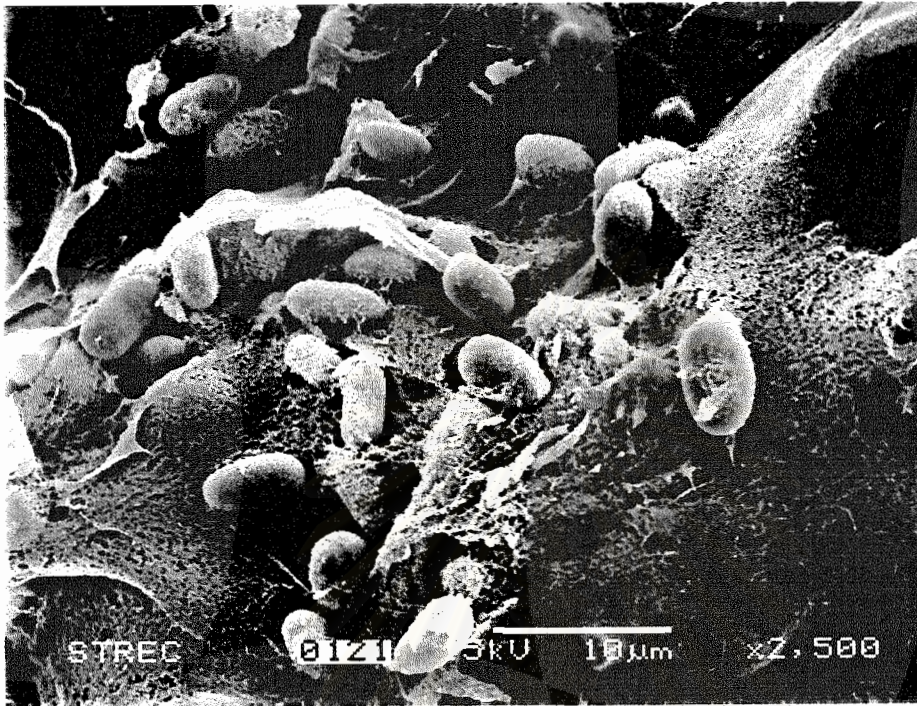
Figure 11: *A. pullulans* CHULA-PR: conidia. (x 2,500)
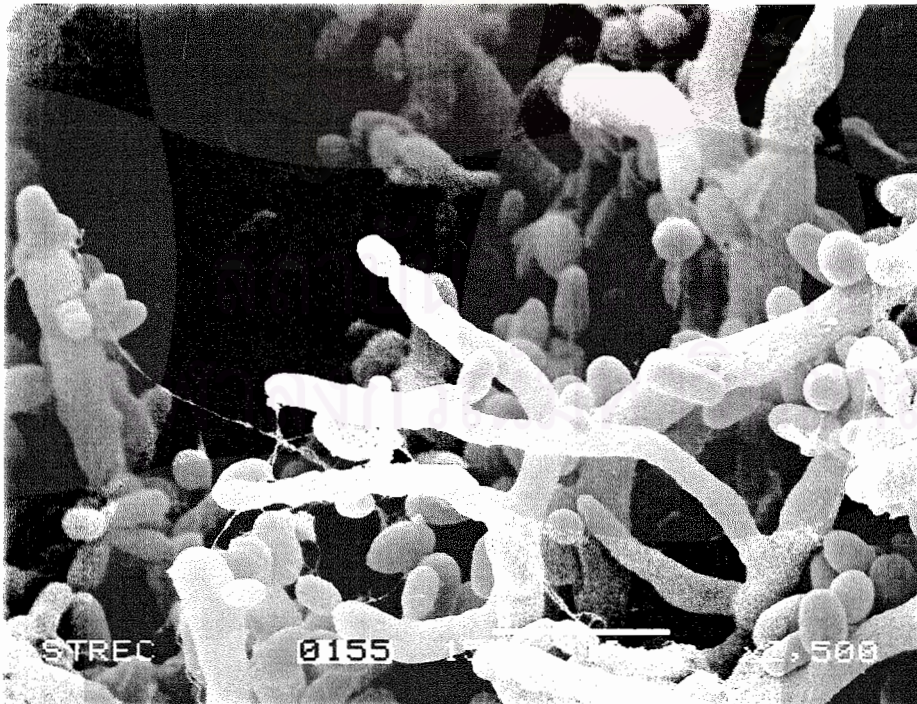
Figure 12: *A. pullulans* CHULA-CU: young lateral hyphae with conidia. (x 2,500)
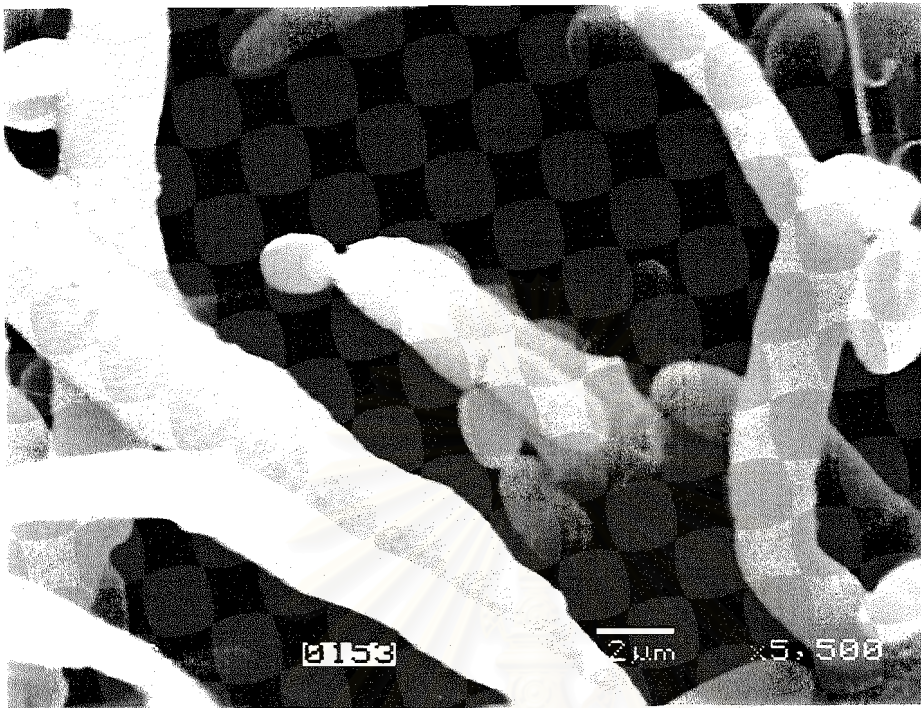
Figure 13: *A. pullulans* CHULA-CU: hyphae with several conidia of nearly the same size and one scar. (x 5,500)
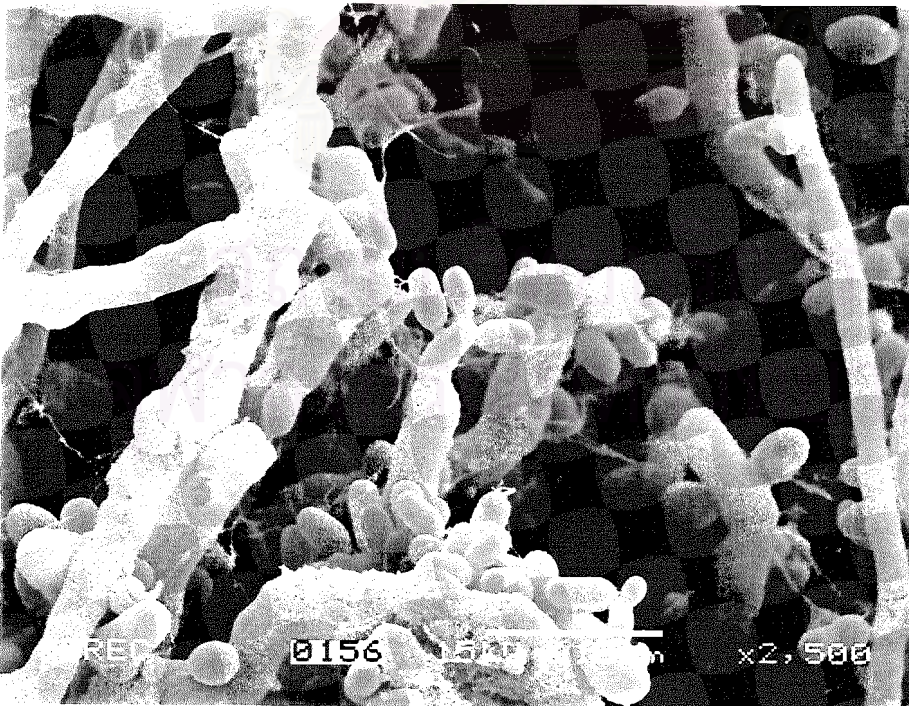
Figure 14: *A. pullulans* CHULA-CU: mature hyphae with adhering conidia. (x 2,500)
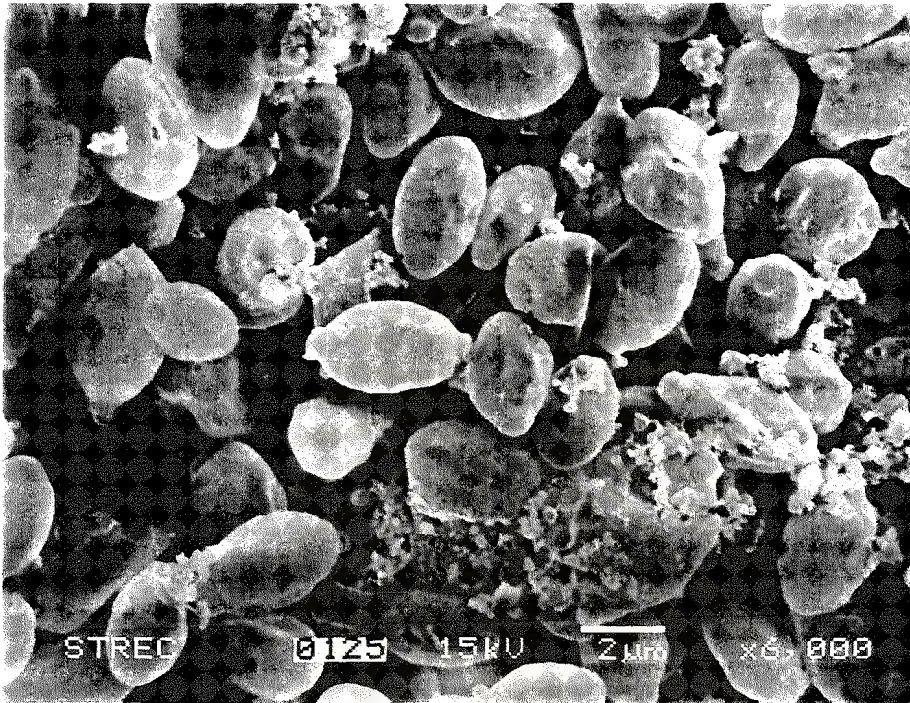
Figure 15: *A. pullulans* CHULA-CU: yeast like cells protruding scar are characteristic.
(x 6,000)

2.4 Transmission Electron Microscope (TEM) investigation

For all pictures show electron-transparent, not multilayered wall, so it is show that these are an ascomycete. (Figure 16-19)

Figure 16: *A. pullulans* CHULA-SU: conidium, scar can be visible.(x 1,650)
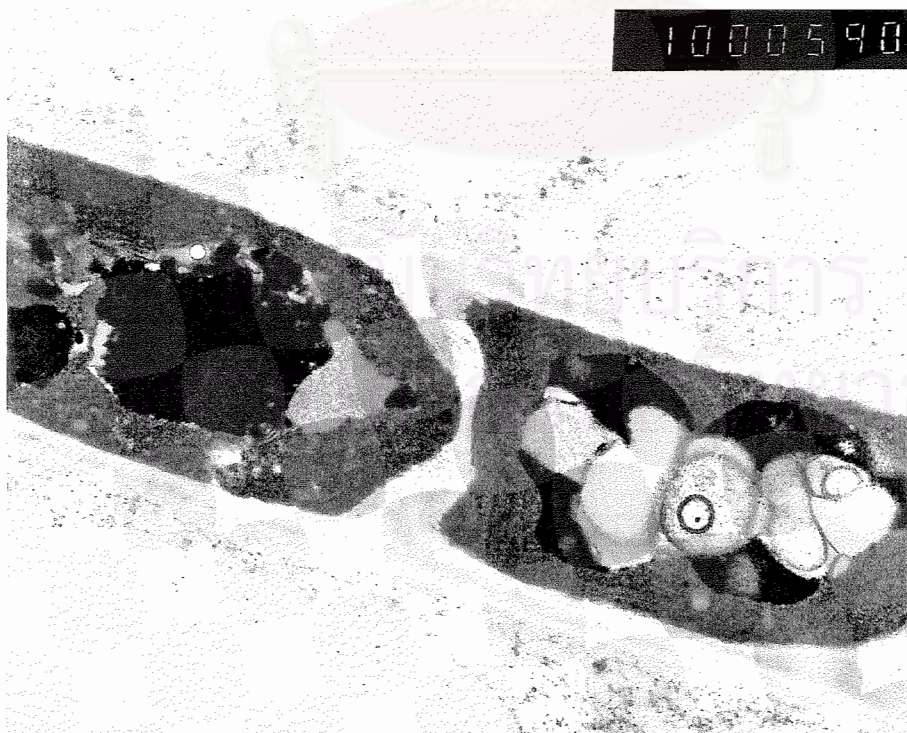
Figure 17: *A. pullulans* CHULA-PR: septum without pore. (x 1,650)
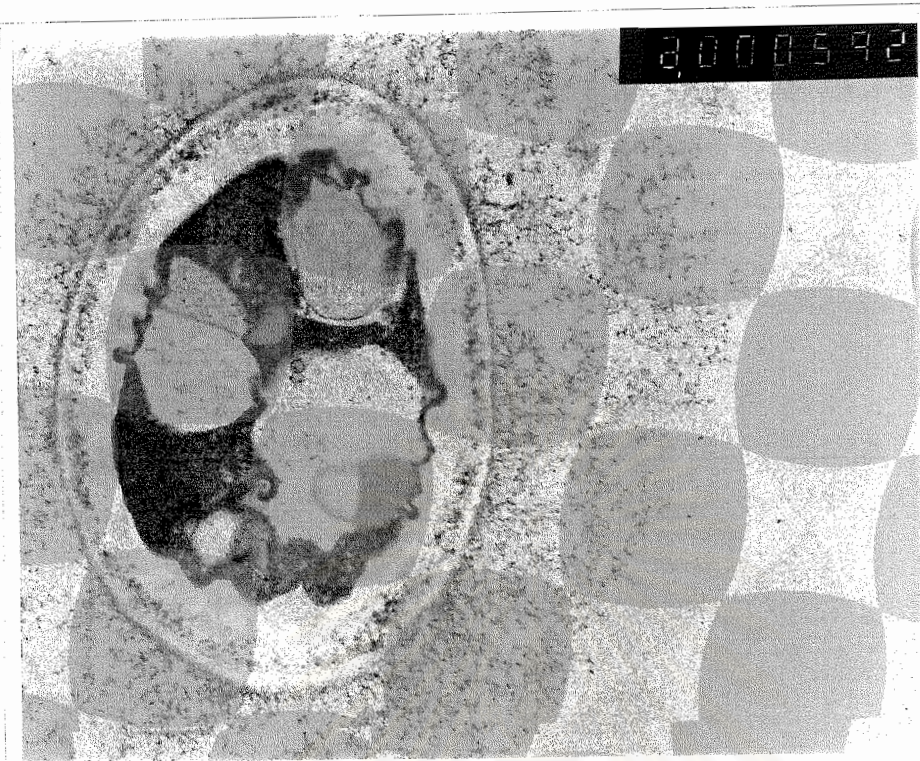
Figure 18: *A. pullulans* CHULA-PR: conidium with melanosome, lots of extracellular materials. (x 1,650)
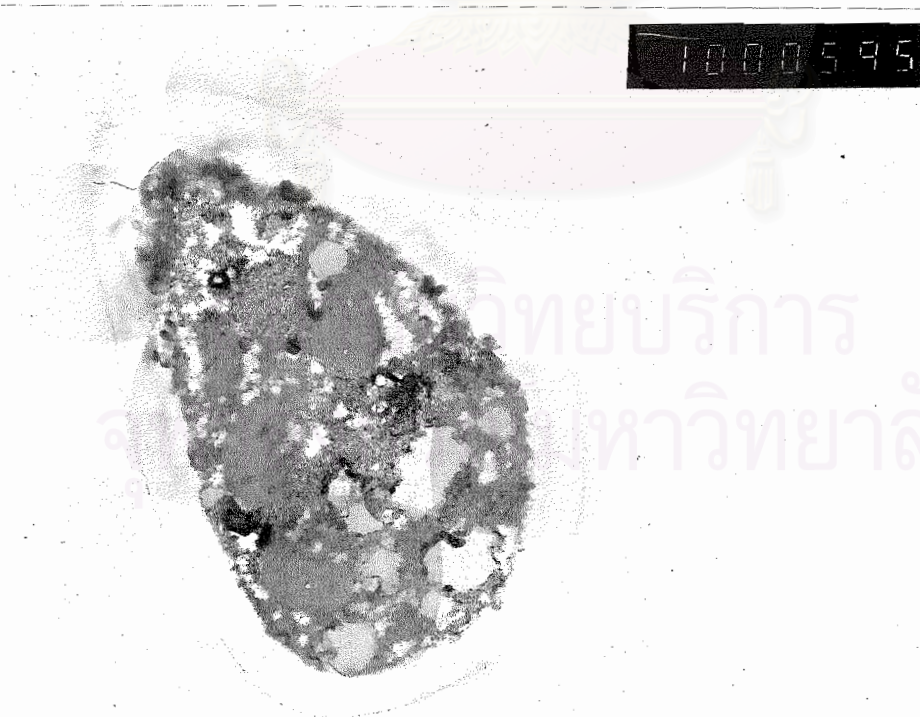
Figure 19: *A. pullulans* CHULA-CU: conidium with thick wall, scar at the left. (x 1,650)

3. Molecular studies

ITS Sequencing

Figure 20: (a-d) Gels showing PCR products of ITS region . Approximatly 1,000 bp. In 79 species studied. Using number 1-79for each species refer to Appendix I.

a)

1-   19

b)
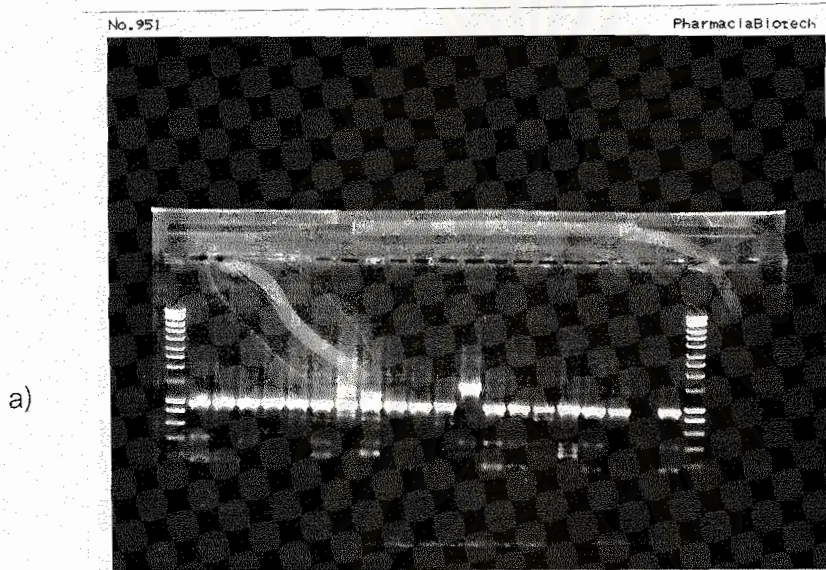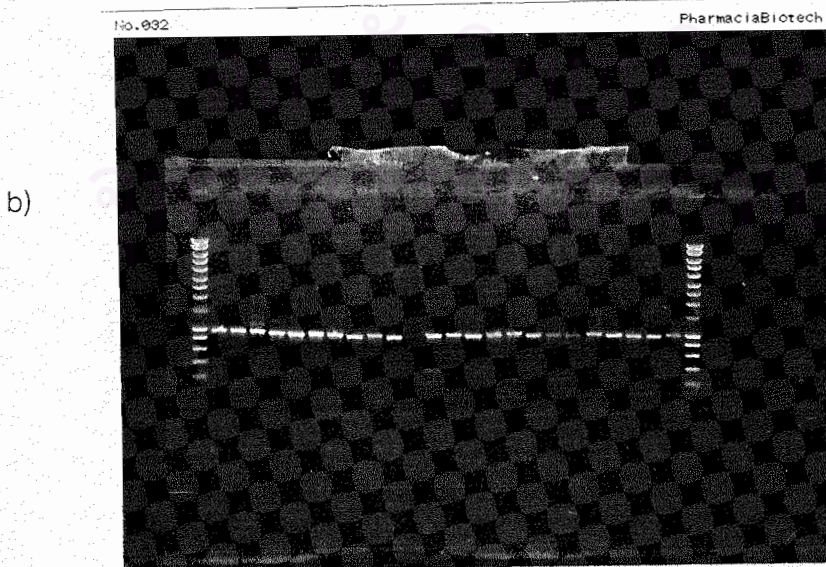
20 -   42
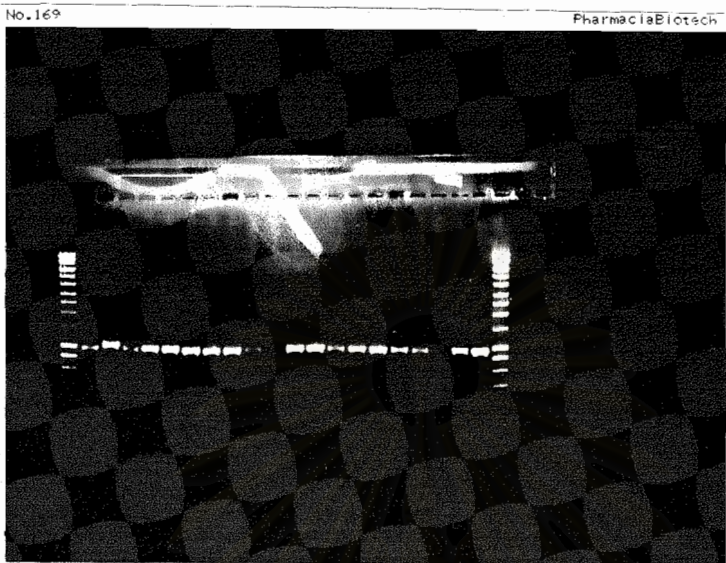
c)

43 –   59
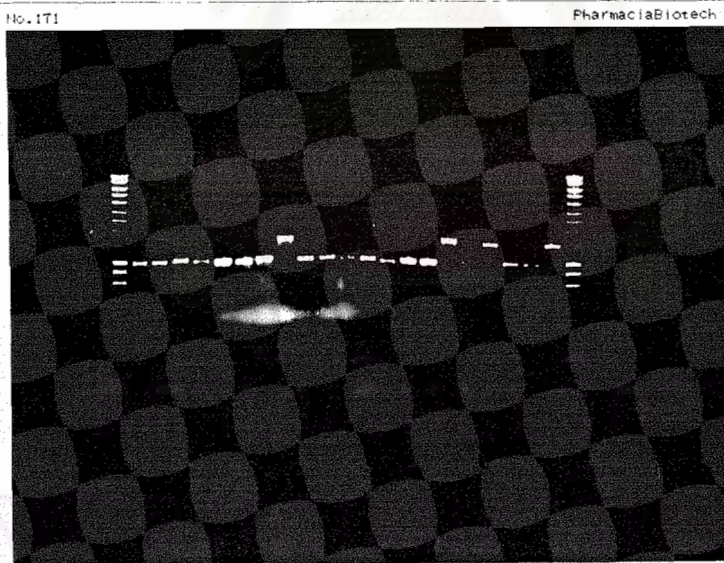
d)

60 –   79

Identification *A. pullulans* by using sequencing of the rDNA ITS domains

suggested that these 5 isolates were similar to the neotype strain of *A. pullulans*. Blastn

searches in Genbank also revealed the highest similarity with *A. pullulans* as shown in

Figure 21
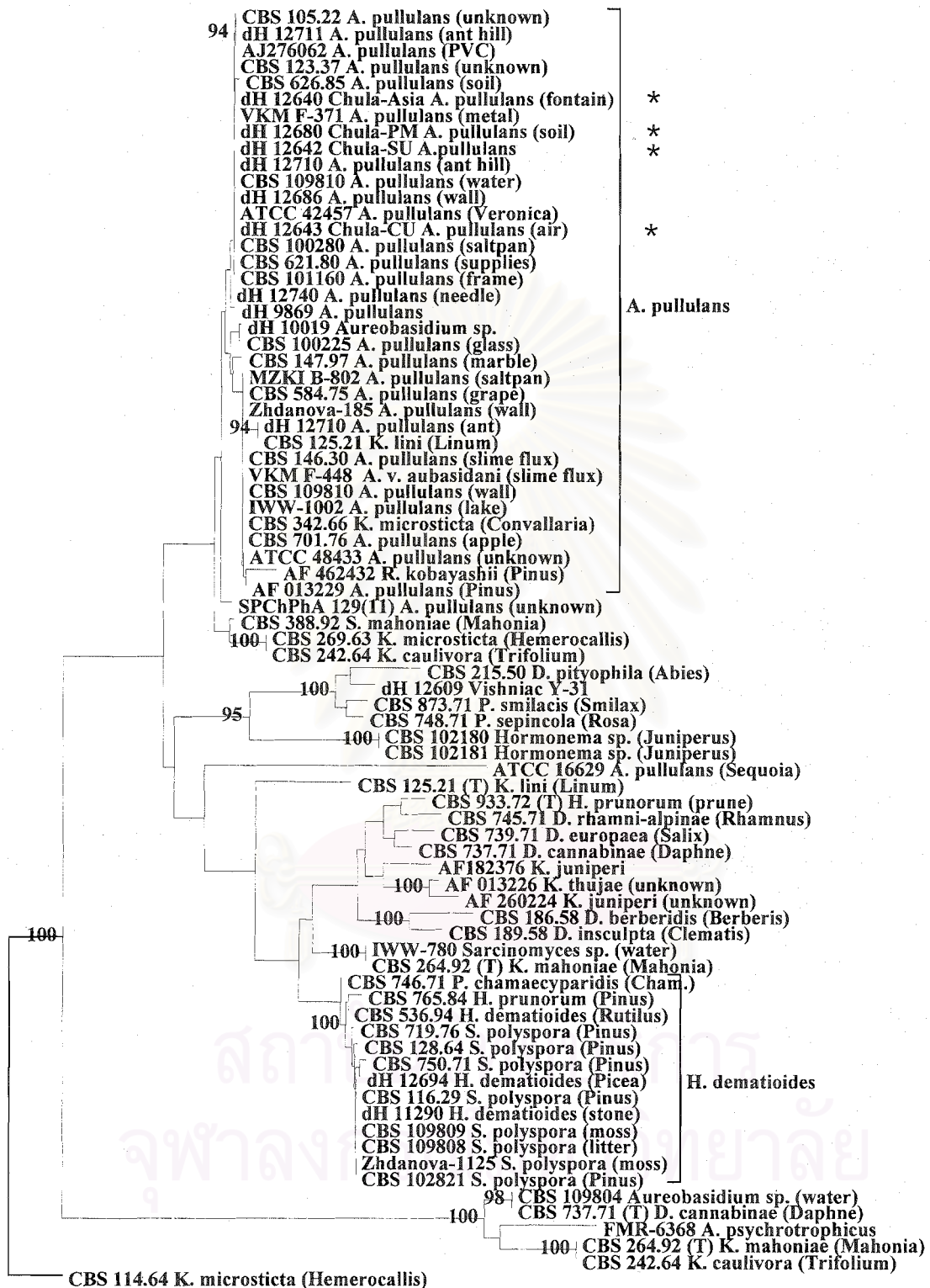
Figure 21: Neighbor-joining tree constructed with the TREECON package on the basis of ITS1 and ITS4 sequences.Bootstrap values > 90 are shown with the branches. *Kabatiell microsticta* CBS 114.64 was used as out group.

B. Exopolysaccharide production by *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR
    and *A. pullulans* CHULA-CU

1. Infrared spectroscopy (IR) investigation

The result of the experiment of the exopolysaccharide produced by *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU.

The analysis of the structure of the exopolysaccharide produced by *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU by IR-spectrophotometer compared with the pullulan standard (Sigma, USA) results in the table 3 and Figure 22,23,24 and 25

Table 3: Comparison of IR-spectrum of *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU.

vibration	Pullulan std. (cm^{-1})	*A. pullulans* CHULA-SU (cm^{-1})	*A. pullulans* CHULA-PR (cm^{-1})	*A. pullulans* CHULA-CU (cm^{-1})
OH streching	3442.54	3429.10	3422.39	3422.39
C-H streching	2918.66	2945.52	2925.37	2918.66
C-O ⎰ O streching	1635.82	1649.25	1629.10	1635.82
C-OH streching	1414.18	1427.61	1414.18	1427.61
C-O streching	1024.63	1038.06	1031.34	1038.06

The value of the transmittance of the pullulan standard and exopolysaccharide which each strain produced has the similar structure (Figure 22,23,24 and 25) It might confirmed that exopolysaccharide from *A. pullulans*, 3 strains is pullulan.
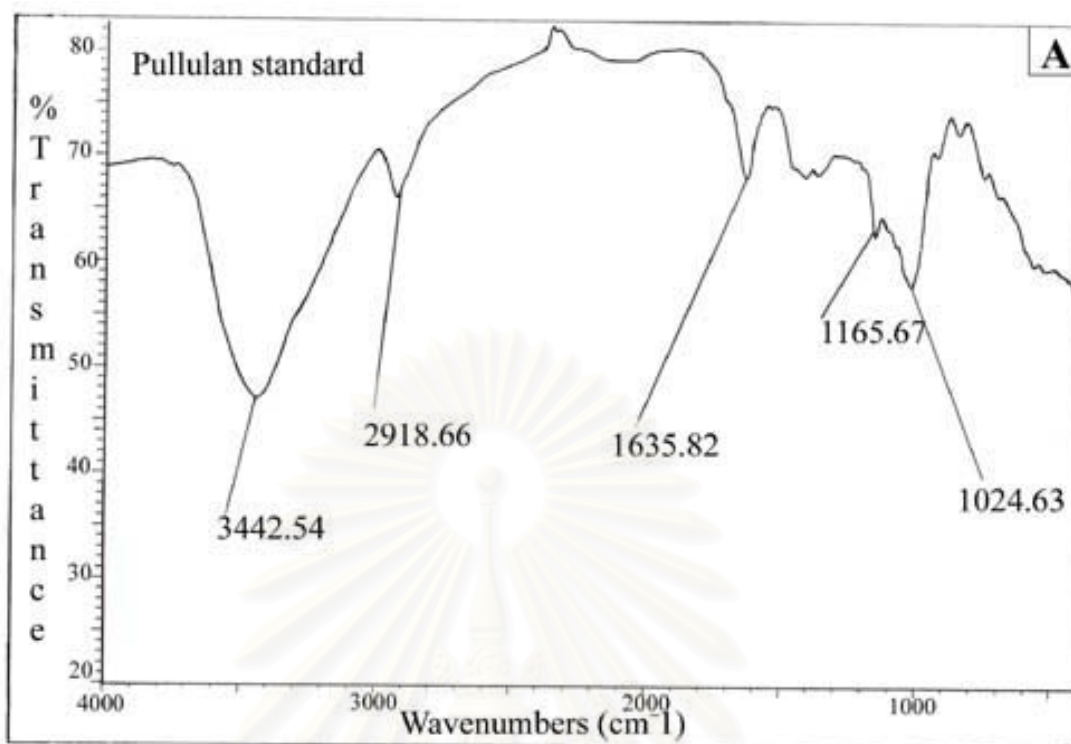
Figure 22: IR-spectrum of pullulan standard
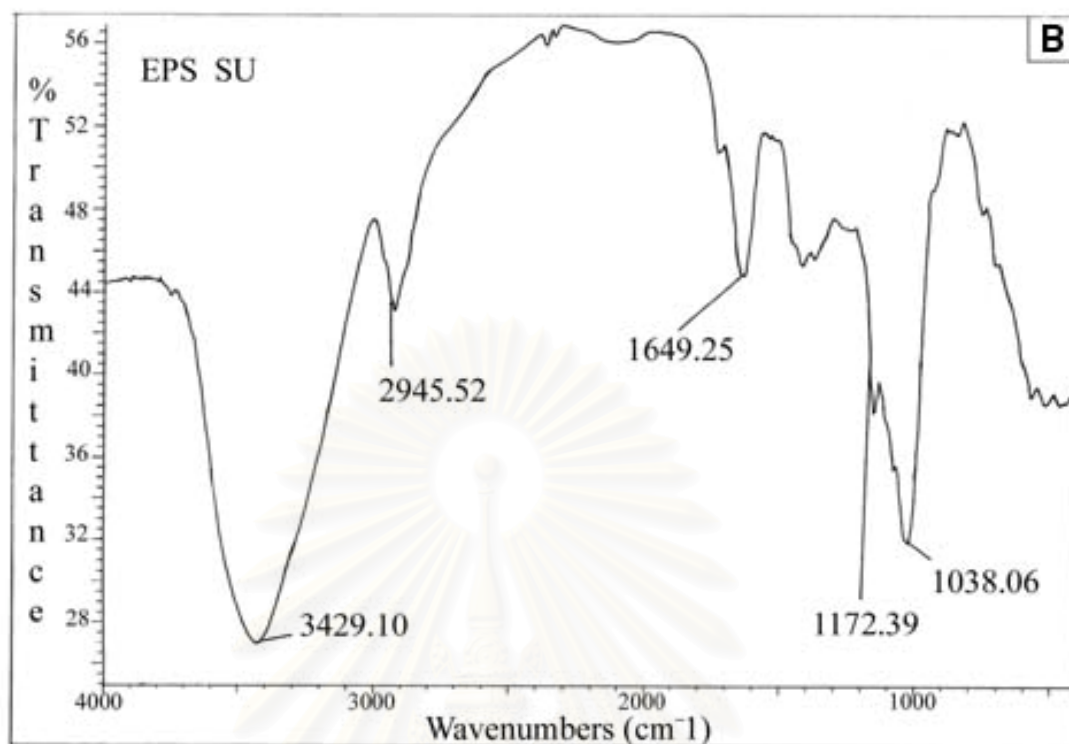
Figure 23: IR-spectrum of exopolysaccharide precipitate from A. pullulans CHULA-SU
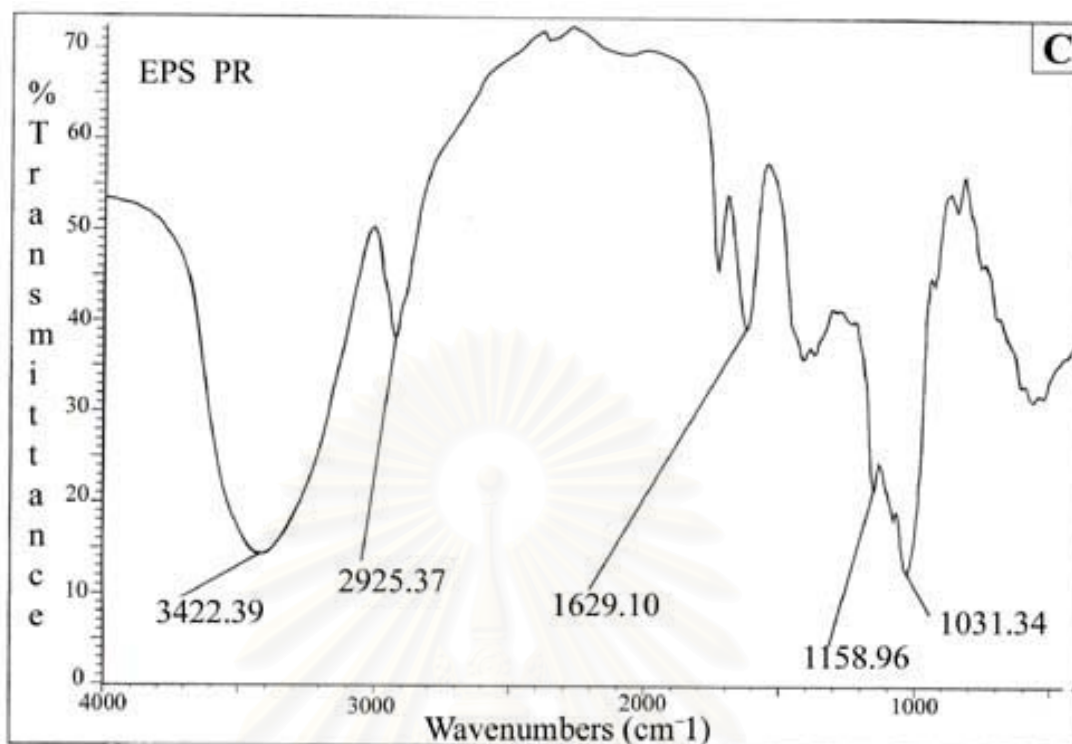
Figure 24: IR-spectrum of exopolysaccharide precipitate from A. pullulans CHULA-PR
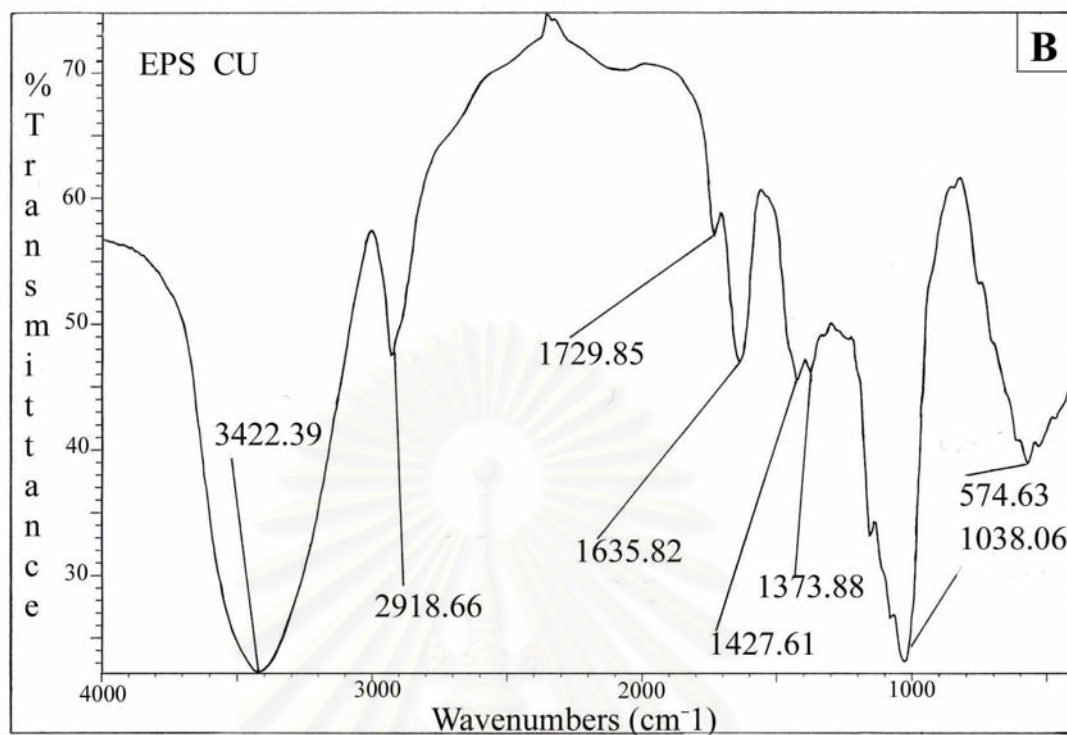
Figure 25: IR-spectrum of exopolysaccharide precipitate from A. pullulans CHULA-CU

2. Growth profile of *Aureobasidium pullulans* isolated in Thailand.

Cultivation of *A. pullulans* all 3 strains was done in PDB medium for 84 hours. *A. pullulans* CHULA-SU took 6 hours to grow in the log phase. Then the cells increased up to the highest amount within 30 hours. The growth of cells was in the form of stationary phase and continued to the death phase in 72 hrs.

A. pullulans CHULA-PR took 6 hours to grow in the log phase. Then the cells increased up to the highest amount within 30 hours. The growth of cells is in the form of stationary phase and continue to the death phase in 72 hrs.

A. pullulans CHULA-CU took 6 hours to grow in the log phase. Then the cells increased up to the highest amount within 30 hours. The growth of cells is in the form of stationary phase and continue to the death phase in 72 hrs.

The data of *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU as shown in Figure 26
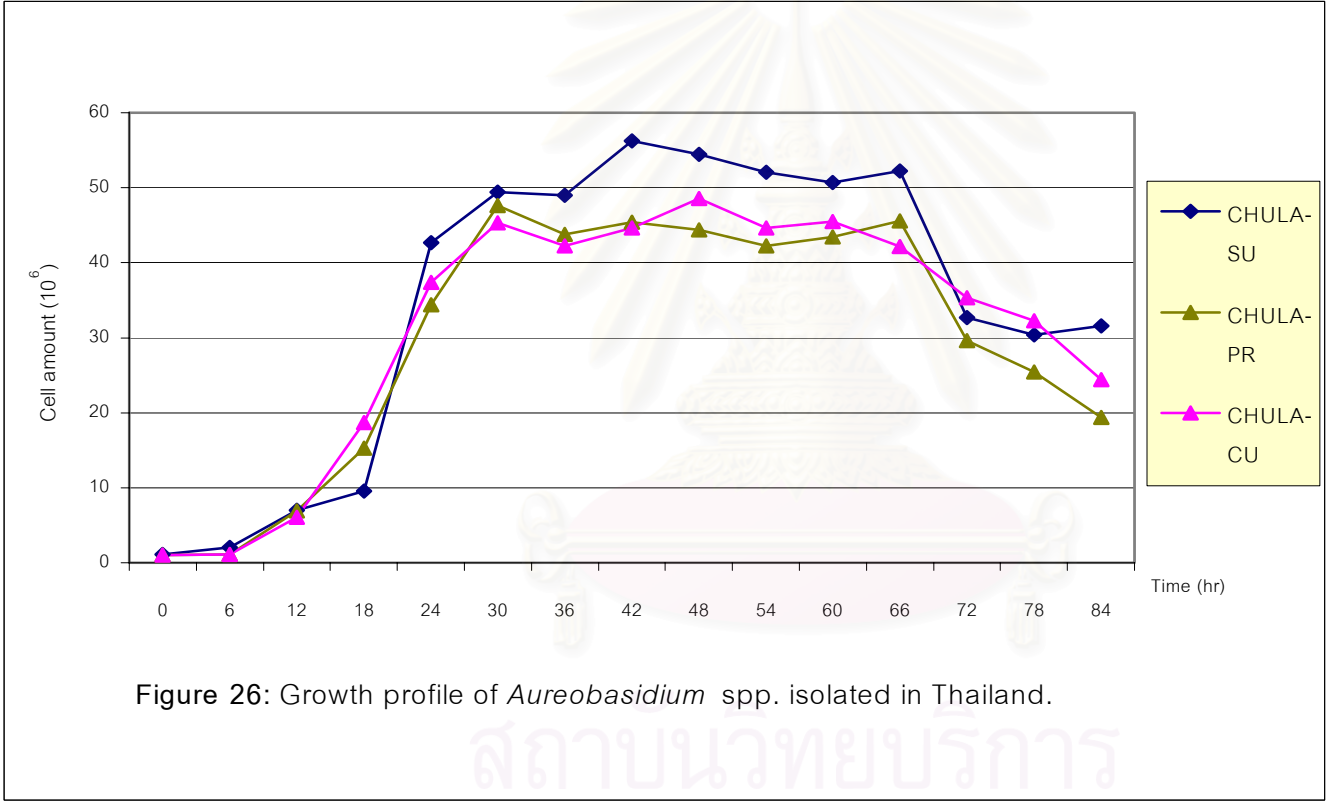
Figure 26: Growth profile of *Aureobasidium* spp. isolated in Thailand.

3. Study on Exopolysaccharide (EPS) production.

3.1 Study on pH optimization

A. pullulans strains CHULA-SU, CHULA-PR and CHULA-CU were grown in liquid production medium with initial pH values adjusted to 4.5, 5.5, 6.5, 7.5. Strains were cultivated at 25°C, shaken at 150 rpm for 5 days

With *A. pullulans* CHULA-CU, an average yield of pullulan of 1.699 g/g C-source was obtained at pH 7.5, which was significantly higher ($p<0.05$) than that of *A. pullulans* CHULA-PR which produced pullulan yield of 0.0522 g/g C-source at pH 6.5 and *A. pullulans* CHULA-SU of which the highest pullulan yield was 0.2382 g/g C-source at pH 6.5.

The data as shown in Figure 27,28 and 29
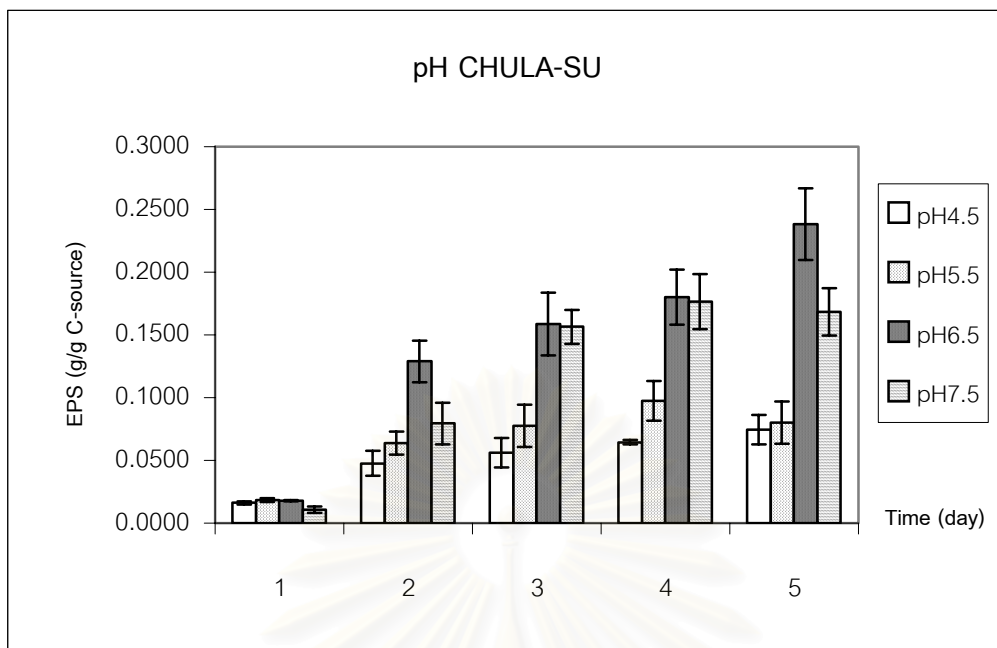
Figure27: Effect of pH upon the production of pullulan from *A. pullulans* CHULA-SU.
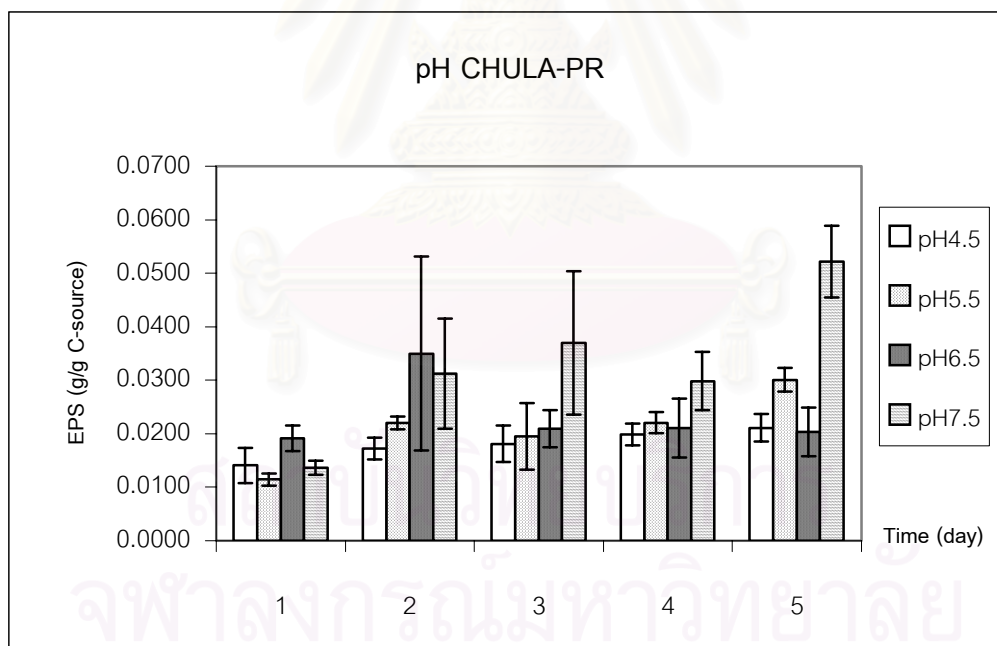
Figure 28: Effect of pH upon the production of pullulan from *A. pullulans* CHULA-PR.
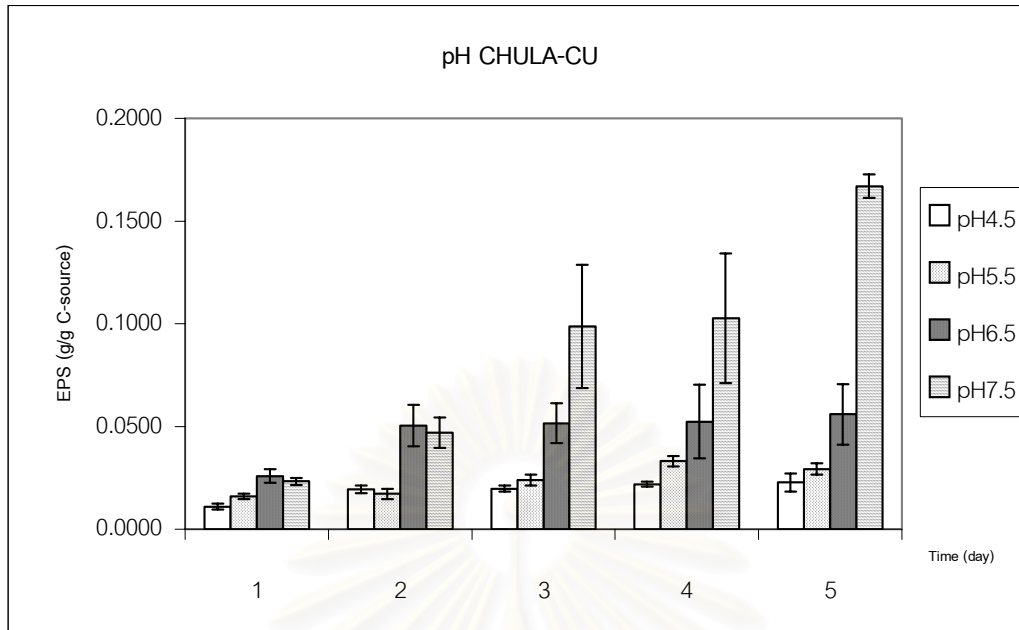
Figure 29: Effect of pH upon the production of pullulan from *A. pullulans* CHULA-CU.

3.2 Study on temperature optimization.

Cultivation of *A. pullulans* CHULA-SU, CHULA-PR and CHULA-CU depended on incubation temperature with initial favorable pH of each strain, was done in production medium. The range of temperature studied was 25°C, 30°C and 35°C.

It was found that the temperature affected the pullulan highest yield significantly ($p<0.05$) at 30°C of *A. pullulans* CHULA-PR. The highest weight of pullulan should be 0.2245 g/g C-source.

A. pullulans CHULA-CU at the temperature 25°C-30°C gave the highest weight of pullulan yield. The production of pullulan was 0.1852-0.1859 g/g C-source.

A. pullulans CHULA-SU gave the highest weight of pullulan at the temperature 25°C. The production was 0.1580 g/g C-source.

All 3 strains gave the highest yield on the fifth day of pullulan production.

The data as showed in Figure 30, 31and 32.
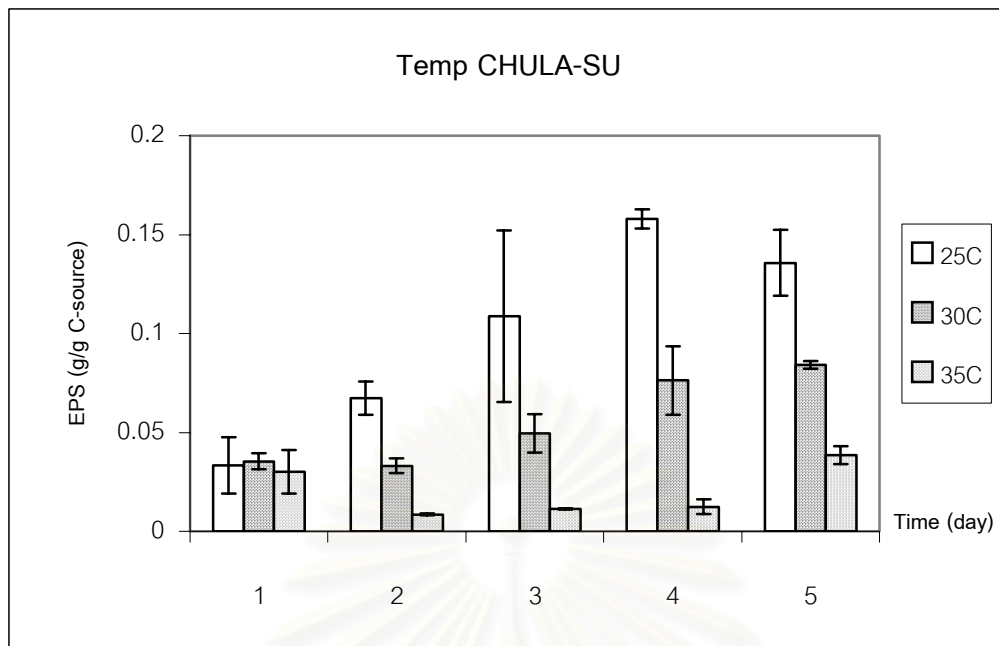
Figure 30: Effect of temperature upon the production of pullulan from *A. pullulans* CHULA-SU.
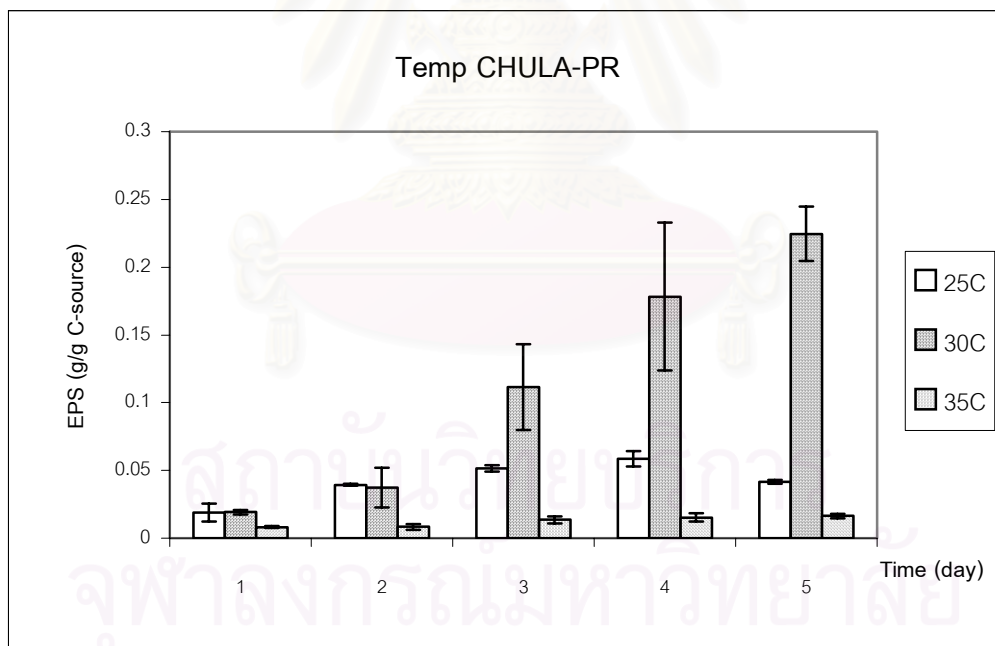
Figure 31: Effect of temperature upon the production of pullulan from *A. pullulans* CHULA-PR.
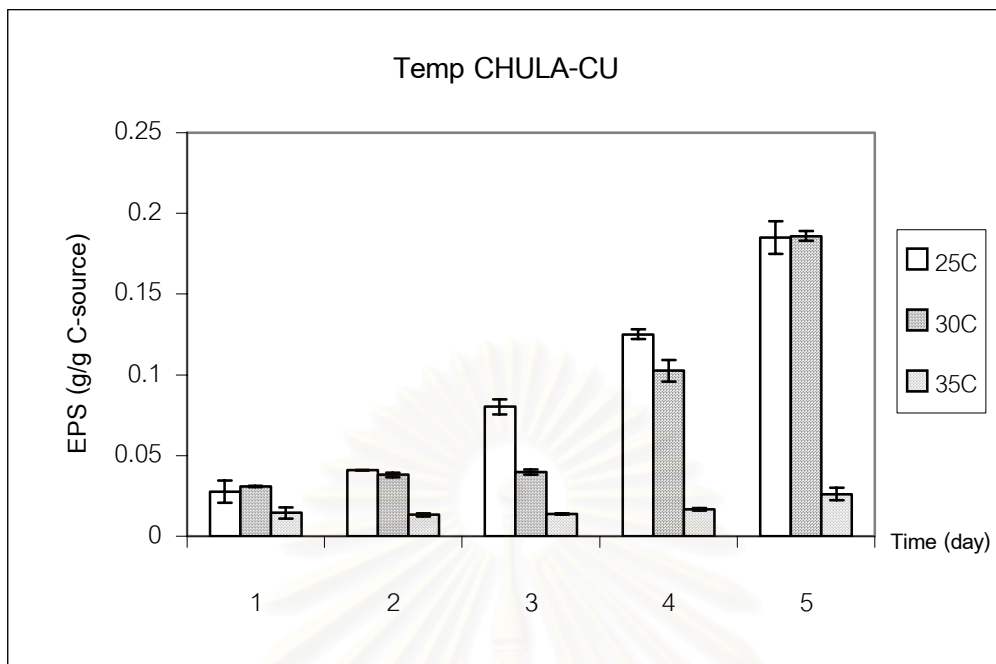
Figure 32: Effect of temperature upon the production of pullulan from *A. pullulans* CHULA-CU.

3.3 Study on Carbon source optimization

The production of pullulan by *A. pullulans* CHULA-SU, CHULA-PR and CHULA-CU was examined by two different carbon sources; sucrose and glucose. Our goal is to observe the media which obtains the pullulan in maximal yield. It is shown that glucose is the preferred C-source for all three strains.

A. pullulans CHULA-PR produced the highest significance; 0.2245 g/g C-source on the fifth day of the production. While *A. pullulans* CHULA-CU produced only 0.1852 g/g C-source on the fifth day of the production. *A. pullulans* CHULA-SU produced pullulan 0.1580 g/g C-source on the fourth of the production.

The data as shown in Figure 33.
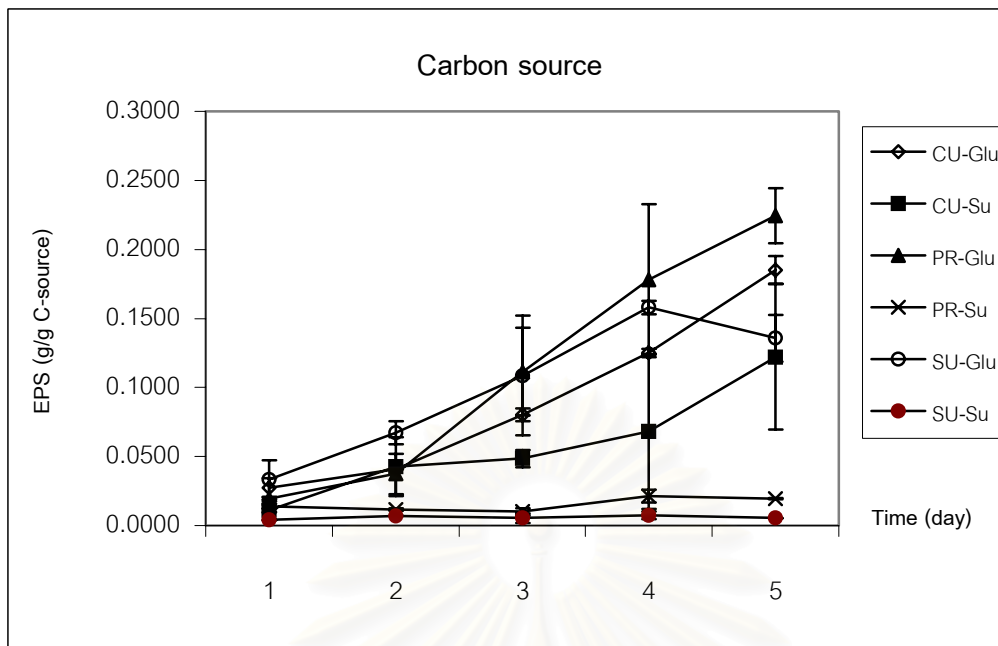
Figure 33: Effect of carbon source upon the production of pullulan from *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU produced from two types of carbon sources.

3.4 Study on Nitrogen source

The production of pullulan by *A. pullulans* CHULA-SU, CHULA-PR and CHULA-CU was examined by two different nitrogen sources; $(NH_4)_2SO_4$ and peptone. Our goal is to observe the media which obtains the pullulan in maximal yield.

This result shows that *A. pullulans* all 3 strains preferred to use $(NH_4)_2SO_4$ a nitrogen source. *A. pullulans* CHULA-PR produced the highest significant pullulans production on the fifth day of the pullulan production. The weight of pullulan 0.2245 g/g C-source was more than *A. pullulans* CHULA-CU, which produced pullulan 0.1852 g/g C-source on the fifth day of the production. *A. pullulans* CHULA-SU produced highly on the fourth day of the production. That was 0.1580 g/g C-source.

The data as shown in Figure 34.

CHAPTER V

DISCUSSION

A. Isolation and Identification of *Aureobasidium pullulans*

1. Isolation of *Aureobasidium pullulans*

The presence of *A. pullulans* in Thailand was found on various locations. Sampling sites and isolation details are indicated in table 1. The six samples taken, three were negatives, including: Khao Yai National park in Nakornratchasima province, Nam Now National park, Petchabun province, and Tung Salang Laung National park, Phitsanulok province. This indicates that *A. pullulans* is an ubiquitous fungus which is common everywhere, also in Thailand (de Hoog et al , 2000).

2. Cultivation and Identification

2.1 Diazonium Blue B (DBB) test

For the Diazonium Blue B (DBB) test *Cryptococcus albidus* (CBS 142) (Basidiomycetous yeast) and *Pichia anomala* (CBS 110) (Ascomycetous yeast) were used as a positive and negative control. We can observe easily by eyes when the culture turns dark red within 2 minutes after being flooded with ice-cold DBB reagent at room temperature (Barnett et al, 2000).

DBB and related diazonium dyes are known to form coloured complexes under a number of conditions. Diazo-substituted phenols may be oxidized not only by chemical substances such as naphthols or catechols but also by the action of certain enzymes, such as peroxidase. It is therefore conceivable that the DBB reaction may be a reflection of chemically different phenomena in different yeasts (Lachance 1982).

In this experiment the DBB test gave positive result in *A. pullulans* CHULA-PR, but negative result in *A. pullulans* CHULA-SU and *A. pullulans* CHULA-CU. and Summerbell (1985) tested *A. pullulans* and found the negative result same as we did. However, Summerbell (1985) reported that there are two major impediments to staining filamentous fungi with DBB. Firstly, the staining reaction is not as intense with positively-staining mycelial colonies as it is with yeast colonies. This is because of the relatively

diffuse nature of mycelial growth. Secondly, the aerial mycelial of most filamentous fungi is sufficiently hydrophobic to repel the stain's aqueous solvent.

De Hoog and Yurlova (1994) said that DBB gave mostly a weak reaction strains and were difficult to interpret in black yeast, despite their ascomycetous character which indeed should give the negative result. It was concluded that DBB gave reliable results only with the true yeasts.

2.2 Morphology Identification

Identification of species in *Aureobasidium, Kabatiella* and *Hormonema* is important view of their potential roles in biodeterioration and plant pathology. Classically *Aureobasidium* and *Kabatiella* on the one hand and *Hormonema* on the other are distinguished by synchronous vs. percurrent modes of conidiogenesis (Hermanides-Nijhof, 1977). However, de Hoog and Yurlova (1994) noted that each of the adjacent , synchronously formed scars in *A. pullulans* remain productive, showing percurrent conidiation as in *Hormonema*. The taxa differ only by the number of conidiogenous loci per hyphal cell : 1-2 in *Hormonema,* up to 14 in *Aureobasidium* and even more in *Kabatiella*. None of the physiological difference between the two most common species, *A. pullulans* and *H. dematioides*, allowed reliable distinction of the species.

2.3 Scanning Electron Microscope (SEM) investigation.

Among *Aureobasidium* group, *A. pullulans* is distinct from other *Aureobasidium* species because of the size and shape of the conidia and its probably saprophytic nature. In old cultures, chlamydospores may be formed, giving the colonies a blackish appearance. Some strains rapidly become dark due to the formation of thick-walled, dark hyphae, which often disintegrate into separate cells (Hermanides-Nijhof, 1977). The only significant morphological feature for the recognition of *A. pullulans* microscopically is therefore the synchronous conidium production on young hyphal cells. In SEM picture, conidial scars are known to remain productive (Yurlova and de Hoog, 1994). So that in a later stage of development the difference with *Hormonema* is not visible anymore.

With SEM the conidia were confirmed to be arranged in groups which were all of the same size, and therefore they were likely to have been produced all at the same time; their growth and maturation are synchronous. This is typical for *A. pullulans* and

not known in *H. dematioides*. Some scars could be revealed, which still show no sign of repetitive conidium production.

2.4 Transmission Electron Microscope (TEM) investigation

With TEM, the cell was shown to be electron-transparent without a multilayered substructure. Thus the species must be an ascomycete. In some of the cells, melanosomes were visible, indicating the points that the fungus turned black. Thus the species could not be a yeast (Hemiascomycetes), which was also confirmed by the absence of micropores. In stead, in Figure 17 a septum is observed which is considerably thinner in the middle. That must be the area close to the septal pore, where the septum of Euascomycetes is much thinner.

In Figure 18, extracellular material is visible. This is likely to be polysaccharide. The presence of EPS in our black yeast fits with the above conclusion that the fungus is *A. pullulans*; the material then must be pullulan.

3. Molecular studies

Aureobasidium pullulans strains constitute a well-delimited main group I the tree (Figure 21) which includes isolation from temperature as well as from tropical climates Morphologically the species is similar to *Hormonema dematioides*, particularly in older parts of colonies where percurrent conidiogenesis prevails (Yurlova and de Hoog, 1996). Yurlova and de Hoog (1996) found only two diagnostic typically is found in environments which are low in nitrogen. It is very common as a phylloplane fungus on leaves, flowers and fruits, and frequently found on moist surface without showing invasion, such a damp walls, metallic equipment, PVC tubes glass and painted window frames. (Webb,2000). On the other hand, *Kabatiella* has been introduced for invading leaves of host plant, the fungus being present with an acervular structure ((Hermanides-Nijhof, 1977). Nevertheless infraspecific diversity is significant. A main cluster comprises the type strains of both the varieties *pullulans* (CBS 584.75) and *aubasidani* (CBS 100524), as well as *Kabatiella lini*, the anamorph of *Discosphaerina fulvida.* Strains originate from slime flux of trees and from sugary surfaces, damps walls and once from polluted water. *Kabatiella lini* and *K. microsticta*, with identical ITS sequences, were isolated from leaf infections of herbaceous plants. The second main group is

consistently different from the cluster around the type strains mentioned above. Strains are derived from low-nutrient damp surfaces and soil.

B. Exopolysaccharide production by *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU.

1. Infrared electron transparent

The experiment of the exopolysaccharide produced by *A. pullulans*, 3 strains, is pullulan.

The analysis of pullulan, shown on each graph is that percentage value of transmittance is different. It may depend on the different amount of the material. If we look at value of the vibration in each graph, it is very similar. So we may conclude that it is the real pullulan.

The experiment shows that the structure of the pullulan produced from *A. pullulan*, 3 strains may be different from the pullulan standard. It is like the opinion of Ueda (1963) and Catley & Whelm (1971), shown on their reports that the structure might be different in each strain.

2. Growth profile of *A. pullulans* isolated in Thailand

The growth velocity of *A. pullulans* was determined in order to have optimal starter cultures for the experiment and to find the appropriate time of monitoring biomass production in relation to pullulan production. The growth curve of *A. pullulans* CHULA-SU, CHULA-PR and CHULA-CU shows log phase, lag phase and stationary phase (Figure 26), the stationary phase being reached in 30 to 66 hrs. This means that optimal production of pullulan takes place during stationary phase. Starter cultures should preferably be incubated no longer than 66 hrs, i.e., be used before stationary phase begins.

3.Study on Exopolysaccharide (EPS) production

3.1 Study on pH optimization

A. pullulans CHULA-SU, CHULA-PR and CHULA-CU needed appropriate optimization of the pH of the growth medium to reach optimal pullulan production.

A. pullulans CHULA-SU produced the highest amount of pullulan compared with A. pullulans CHULA-CU and A. pullulans CHULA-PR. The pullulan yield in this strain was 0.2382 g/g C-source on the fifth day of production at pH 6.5. Also strain CHULA-CU reached highest pullulan production on the fifth day, the pullulan yield being equal to 0.1669 g/g C-source. The same was found for A. pullulans CHULA-PR with a pullulan yield of 0.0522 g/g C-source. This result is in accordance with data published by Lacroix et al. (1994), West & Reed-Hamer (1994), and Lachke & Rale (1995). These authors explained the effect of pH optimization for pullulan production by A. pullulans by the fact that the optimum pH of synthetic media for pullulan production is near neutrality; further growth of A. pullulans after day 5 leads to acidification of the medium. A. pullulans grows well at an acidic level of pH 2.0, but it does not synthesize pullulan under this condition.

We may conclude that feeding to produce pullulan must begin with adjusting an acidic pH in order to raise the number of cells in the medium, then shift the pH to near-neutrality in order to induce A. pullulans to synthesize pullulan.

3.2 Study on temperature optimization

This part of the work monitors pullulan production by A. pullulans CHULA-SU, CHULA-PR and CHULA-CU under different incubation temperatures using an initial pH favorable for each strain and using glucose as carbon source. The range of temperatures studied included 25°C, 30°C, and 35°C.

It was found that the temperature affected the pullulan yield most significantly at 30°C on the fifth day of production in strain CHULA-PR. The highest harvest of pullulan obtained was 0.2245 g/g C-source.

A. pullulans CHULA-CU produced the largest amount of pullulan on the fifth day of production when incubated at temperatures of 25°C - 30°C. The production of pullulan was 0.1852-0.1859 g/g C-source.

A. pullulans CHULA-SU after 5 days gave the highest amount of pullulan at a temperature of 25°C. The production was 0.1580 g/g C-source.

The results of these experiments were similar to those of McNeil & Kristiansan (1990) and West & Reed-Hamer (1993) in that the appropriate temperature to produce the pullulan by A. pullulans is between 25°C – 30°C. The experiment shows that A.

pullulans CHULA-PR and *A. pullulans* CHULA-CU give good pullulan yield in the higher temperature range (30°C), which is the average day temperature in Thailand. So it is recommended to improve pullulan production in Thailand by using these two slightly more thermophilic strains.

3.3 Study on Carbon source optimization

Production of pullulan by *A. pullulans* CHULA-SU, CHULA-PR and CHULA-CU was examined by using two different carbon sources: sucrose and glucose. Our goal is to find the medium which promotes pullulan in maximal yield. It is shown that glucose is the preferred C-source for all three strains.

A. pullulans CHULA-PR produced the highest amount of 0.2245 g/g C-source on the fifth day of the production by using glucose as a carbon source, while strain CHULA-CU produced only 0.1852 g/g C-source on day 5. *A. pullulans* CHULA-SU produced 0.1580 g/g C-source pullulan on the fourth day of production.

Carbon source is the most important factor for pullulan production. It has been reported that glucose and fructose are the most efficient small-molecule natural inducers of pullulan synthesis. Maltose and sucrose also serve well, although highest pullulan yields have been obtained from starch-grown cultures. Other carbon sources; such as lactose, xylose, and xylan may be utilized for growth, but do not induce polysaccharide synthesis (Silman et al., 1990).

Our three strains of *A. pullulans* utilized glucose better than sucrose. Silman et al. (1990) compared the production of polysaccharide from strains of *A. pullulans* for these compounds. Their result showed that only one of their strains, NRRL Y-12974, gave a relatively viscosity using glucose which was equivalent to that produced from sucrose. Our findings that the *A. pullulans* strains from Thailand can use glucose better than sucrose may be explained by the fact that glucose, being a small molecule, is taken up immediately, but if the incubation time is extended with a day, sucrose may prove to give higher pullulan yields.

In 1993, Saha et al. suggested that glucose was a good carbon source for the generating amylase activity. This report shows that *A. pullulans* produced a complex amylolytic enzyme system consisting of an α-amylase, two forms of glucoamylase, and an α-glucosidase necessary for extensive starch degradation. On contrast, Li et al.

(1993) and Wymelenberg et al. (1999) observed that xylanase activity by *A. pullulans* was strongly repressed when glucose was used as C-source. This was explained by the fact that xylan and D-xylose, the end products of xylan degradation, both are able to induce transcription only when glucose is absent or reaches certain low levels in the culture. The establishment of the exact regulatory mechanism for xylanase gene expression in this fungus needs further investigation (Li & Ljungdahl, 1994). If the hypothesis is correct, it means that *A. pullulans* CHULA-SU, CHULA-PR and CHULA-CU can not only give high pullulan production which can used in commercial pullulan production, but also *A. pullulans* can be used for enzyme production. For each of these functions, appropriate conditions have to be adjusted in each strain.

3.4 Study on Nitrogen source

The variation of the initial nitrogen source was expected to influence the pullulan production. As we know that either organic or inorganic nitrogenous substances can be used as a nitrogen source for pullulan production (Lachke and Rale, 1995). It is up to each microorganism to be favorable and suitable for one of these nitrogen sources.

This result shows that *A. pullulans* all 3 strains are preferable to use $(NH_4)_2SO_4$ a nitrogen source. *A. pullulans* CHULA-PR produced the highest significant pullulans production on the fifth day of the pullulan production. The weight of pullulan 0.2245 g/g C-source was more than *A. pullulans* CHULA-CU, which produced pullulan 0.1852 g/g C-source on the fifth day of the production. *A. pullulans* CHULA-SU produced highly on the fourth day of the production. That is 0.1580 g/g C-source.

This conclusion is similar to the experiment of Catley (1971a) studied the role of pH and Nitrogen limitaion in the elaboration of the extracellular polysaccharide pullulan by *Pullularia pullulans*. That is increasing $(NH_4)_2SO_4$ in the culture with the glucose as a carbon source will increase the pullulan production.

CHAPTER VI

CONCLUSION

A. Isolation and Identification of *Aureobasidium pullulans*

1. Isolation of *Aureobasidium pullulans*

   A. pullulans is an ubiquitous fungus.

2. Cultivation and Identification

   2.1 Diazinium Blue B (DBB) test

   DBB test gave positive result in *A. pullulans* CHULA-PR, but negative result in *A. pullulans* CHULA-SU and *A. pullulans* CHULA-CU.

   2.2 Morphology Identification

   A. pullulans CHULA-SU , *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU are *A. pullulans* for a description of the standard morphology of *A. pullulans* , see Page 3. The fungus is an Euascomycete with melanized cell walls and synchronous conidium production; this combination of characters fits with *Aureobasidium pullulans*.

   2.3 Scanning Electron Microscope (SEM) investigation.

   With SEM the conidia were confirmed to be arranged in groups which were all of the same size, and therefore they were likely to have been produced all at the same time; their growth and maturation are synchronous. This is typical for *A. pullulans* and not known in *H. dematioides*. Some scars could be revealed, which still show no sign of repetitive conidium production.

   2.4 Transmission Electron Microscope (TEM) investigation

   For all pictures show electron-transparent, not multilayered wall, so it is show that these are an ascomycete.

3. Molecular studies

   The strains from Thailand are confirmed to be *A. pullulans* because of ITS identity with the type strain. The infraspecific variability found in other strains of *A. pullulans* can as yet not be explained.

B. Exopolysaccharide production by *A. pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU

1 Infrared spectroscopy (IR) investigation

The experiment of the exopolysaccharide produced by *A. pullulans*, 3 strains, is pullulan.

The analysis of pullulan, shown on each graph is that percentage value of transmittance is different. It may depend on the different amount of the material. If we look at the value of the vibration in each graph, it is very similar. So we may conclude that it is the real pullulan.

2. Growth profile of *Aureobasidium pullulans* isolated in Thailand.

Cultivation of *A. pullulans* all 3 strains in PDB medium for 84 hours, *A. pullulans* CHULA-CU, CHULA-PR and CHULA-SU took 6 hours to grow in the log phase. Then the cells will increase up to the highest amount in 30 hours. The growth of cells is in the form of stationary phase and continue to death phase in 72nd of observed time.

3. Study on Exopolysaccharide (EPS) production.

3.1 Study on pH optimization

A. pullulans strains CHULA-CU, CHULA-PR and CHULA-SU were grown in liquid production medium with initial pH values adjusted to 4.5, 5.5, 6.5, 7.5, Strains were cultivated at 25°C, shaken at 150 rpm for 5 days

3.2 Study on temperature optimization.

It was found that the temperature affected the pullulan yield highest significantly ($p<0.05$) at 30°C of *A. pullulans* CHULA-PR. The highest weight of pullulan should be 0.2245 g/g C-source.

A. pullulans CHULA-CU, at the temperature 25°C - 30°C gave the highest weight of pullulan yield. The production of pullulan was 0.1852-0.1859 g/g C-source.

A. pullulans CHULA-SU gave the highest weight of pullulan at the temperature 25°C. The production was 0.1580 g/g C-source.

All 3 strains gave the highest yield in the fifth day of pullulan production.

3.3 Study on Carbon source optimization

It is shown that glucose is the preferred C-source for all three strains.

A. pullulans CHULA-PR produced the highest significance; 0.2245 g/g C-source on the fifth day of the production. While *A. pullulans* CHULA-CU produced only 0.1852 g/g C-source on the fifth of the production. *A. pullulans* CHULA-SU produced pullulan 0.1580 g/g C-source on the fourth of the production.

3.4 Study on Nitrogen source

This result shows that *A. pullulans* all 3 strains were preferable to use $(NH_4)_2SO_4$ a nitrogen source. *A. pullulans* CHULA-PR produced the highest significant pullulans production on the fifth day of the pullulan production. The weight of pullulan 0.2245 g/g C-source was more than *A. pullulans* CHULA-CU, which produced pullulan 0.1852 g/g C-source on the fifth day of the production. *A. pullulans* CHULA-SU produced highly on the fourth day of the production. That is 0.1580 g/g C-source.

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APPENDICES

APPENDIX I

The mycelia used in the study were collected at several locations in Thailand.

For strains which used for molecular part was support by Prof. Dr. Sybren de Hoog , Centraalbureau voor Schimmelcultures, the Netherlands.

No.	Number	Name
1.	CBS 116.29	*Sydowia polyspora*
2.	CBS 114.64	*Kabatiella microsticta*
3.	CBS 536.94	*Hormonema macrosporum*
4.	dH 264.92	*Hormonema* sp.
5.	CBS 264.92	*Kabatina mahoniae*
6.	CBS 388.92	*Selenophoma mohoniae*
7.	CBS 767.71	*Kabatiella aeae*
8.	CBS 765.84	*Hormonema prunorum*
9.	CBS 242.64	*Kabatiella caulivora*
10.	CBS 105.22	*Aureobasidium pullulans*
11.	CBS 873.71	*Pringsheimia smilacis*
12.	CBS 748.71	*Pringsheimia sepincola*
13.	CBS 750.71	*Sydowia polyspora*
14.	CBS 128.64	*Sydowia polyspora*
15.	CBS 342.66	*Kabatiella microsticta*
16.	CBS 269.63	*Kabatiella microsticta*
17.	dH 11290	*Hormonema dematioides*
18.	dH 10019	*Aureobasidium* sp.
19.	CBS 215.50	*Sydowia polyspora*
20.	CBS 719.76	*Sydowia polyspora*
21.	dH 12680	*Aureobasidium pullulans* (CHULA-PM)
22.	dH 12640	*Aureobasidium pullulans* (CHULA-ASIA)

No.	Number	Name
23.	dH 12641	*Aureobasidium pullulans* (CHULA-IXORA)
24.	dH 12643	*Aureobasidium pullulans* (CHULA-CU)
25.	dH 12687	*Trimmatostroma abietis*
26.	dH 12686	*Aureobasidium pullulans*
27.	dH 12629	*Trimmatostroma* sp.
28.	dH 12694	*Hormonema* sp.
29.	dH 12623	*Aureobasidium pullulans*
30.	dH 12625	*Aureobasidium pullulans*
31.	dH 12626	*Aureobasidium pullulans*
32.	dH 12627	*Aureobasidium pullulans*
33.	dH 12628	*Aureobasidium pullulans*
34.	dH 12629	*Aureobasidium pullulans*
35.	dH 12631	*Aureobasidium pullulans*
36.	dH 12632	*Aureobasidium pullulans*
37.	dH 9967	*Aureobasidium pullulans*
38.	CBS 101160	*Aureobasidium pullulans*
39.	CBS 626.85	*Aureobasidium pullulans* var. *pullulans*
40.	dH 12633	*Aureobasidium pullulans*
41.	dH 12634	*Aureobasidium pullulans*
42.	dH 12635	*Aureobasidium pullulans*
43.	dH 12636	*Aureobasidium pullulans*
44.	dH 12637	*Aureobasidium pullulans*
45.	dH 12639	*Aureobasidium pullulans* (CHULA-PR)
46.	dH 10041	*Aureobasidium pullulans*
47.	CBS 621.80	*Aureobasidium pullulans* var. *pullulans*
48.	dH 9968	*Aureobasidium pullulans*
49.	CBS 123.37	*Aureobasidium pullulans* var. *pullulans*
50.	CBS 298.56	*Aureobasidium pullulans* var. *pullulans*
51.	CBS 100280	*Aureobasidium pullulans* var. *pullulans*

No.	Number	Name
53.	CBS 100593	*Aureobasidium pullulans*
54.	dH 12609	NO Name
55.	dH 12044	*Aureobasidium* (IWW 499)
56.	CBS 745.71	*Dothiora rhamni-olpinae*
57.	CBS 707.95	*Hormonema schizolunatum*
58.	dH 12642	*Trimmatostroma* sp.
59.	dH 12710	*Aureobasidium pullulans*
60.	dH 12711	*Aureobasidium pullulans*
61.	CBS 279.86	*Spilodochium* sp
62.	CBS 100225	*Aureobasidium pullulans*
63.	CBS 701.76	*Aureobasidium pullulans* var. pullulans
64.	CBS 146.30	*Aureobasidium pullulans* var. pullulans
65.	CBS 584.75	*Aureobasidium pullulans* var. pullulans
66.	CBS 147.97	*Aureobasidium pullulans* var. pullulans
67.	CBS 747.71	*Pringsheimia euphoebiae*
68.	CBS 933.72	*Hormonema prunorum*
69.	CBS 125.21	*Kabatiella lini*
70.	dH 12642	*Aureobasidium pullulans* (CHULA-SU)
71.	dH 12694	*Hormonema* sp.
72.	dH 12697	*Hormonema* sp.
73.	CBS 737.71	*Dothiora cannabinae*
74.	dH 12739	Hormonema like
75.	dH 9962	*Pringsheimia* sp.
76.	CBS 749.71	*Pringsheimia sepincola*
77.	dH 12738	Zygomycete like
78.	dH 746.71	*Pringsheimia chamaecyparidis*
79.	CBS 303.84	*Trimmatostroma* sp.

APPENDIX II

List of strains studied (Phylogenetic tree)

No.	Name	Number	Original substrate	Geography
1	*Antarctiomyces psychrotrophicus*	FMR 6368 (T)		Antarctica
2	*Aureobasidium pullulans v. aubasidani*	CBS 100524 = VKM-F = 448 (T)	Slime flux, *Betula* sp.	Russia
3	*Aureobasidium pullulans v. pullulans*	CBS 123.37	Unknown source	Unknown
4	*Aureobasidium pullulans v. pullulans*	CBS 100225	Moist glass	Netherlands
5	*Aureobasidium pullulans v. pullulans*	CBS 621.80	Army supplies	USA
6	*Aureobasidium pullulans v. pullulans*	CBS 100280	Hypersaline water	Slovenia
7	*Aureobasidium pullulans v. pullulans*	CBS 101160	Window frame	Sweden
8	*Aureobasidium pullulans v. pullulans*	CBS 105.22	Unknown	Unknown
9	*Aureobasidium pullulans v. pullulans*	CBS 109800 = dH 11797	Peritoneal dialysis	Greece
10	*Aureobasidium pullulans v. pullulans*	CBS 109810 = dH 12237 = Zhdanova 184	Wall surface	Ukraine
11	*Aureobasidium pullulans v. pullulans*	CBS 146.30	Slime flux, *Quercus*	Germany
12	*Aureobasidium pullulans v. pullulans*	CBS 147.97	Marble	Namibia

List of strains studied (continue)

No.	Name	Number	Original substrate	Geography
13	*Aureobasidium pullulans v. pullulans*	CBS 584.75	Fruit, *Vitis vinifera*	France
14	*Aureobasidium pullulans v. pullulans*	CBS 626.85	Sandy soil	Spain
15	*Aureobasidium pullulans v. pullulans*	CBS 701.76	Fruit, *Malus* sp.	Unknown
16	*Aureobasidium pullulans v. pullulans*	ATCC 48433 = AF 121285	Unknown source	Unknown
17	*Aureobasidium pullulans v. pullulans*	dH 12168 = IWW-A 1002	Lake water	Antarctica
18	*Aureobasidium pullulans v. pullulans*	dH 12711	Ant nest	Brazil
19	*Aureobasidium pullulans v. pullulans*	dH 12710	Ant nest	Brazil
20	*Aureobasidium pullulans v. pullulans*	dH 12235 = Zhdanova 185	Radioactive wall surface	Ukraine
21	*Aureobasidium pullulans v. pullulans*	MZKI B-802	Hypersaline water	Slovenia
22	*Aureobasidium pullulans v. pullulans*	SPChPhJ129(11)	unknown	Unknown
23	*Aureobasidium pullulans v. pullulans*	VKM-F 371	Metallic equipment	Russia
24	*Aureobasidium pullulans v. pullulans*	dH 12740	Surface living needle, *Pinus*	Netherlands
25	*Aureobasidium pullulans v. pullulans*	dH 9869 = det 60/93 H3		asked 15-3-02
26	*Aureobasidium pullulans v. pullulans*	CHULA-Asia = dH 12640	Public fountain	Thailand
27	*Aureobasidium pullulans v. pullulans*	CHULA-SU = dH 12642	Air under *Pinus* sp.	Thailand
28	*Aureobasidium pullulans v. pullulans*	CHULA-CU = dH 12643	Air	Thailand

List of strains studied (continue)

No.	Name	Number	Original substrate	Geography
29	*Aureobasidium pullulans v. pullulans*	CHULA-PR = dH 12648	Air	Thailand
30	*Aureobasidium pullulans v. pullulans*	CHULA-Ixora = dH 12641	*Ixora* sp.	Thailand
31	*Aureobasidium pullulans v. pullulans*	AF013229	*Pinus sylvestris*	USA
32	*Aureobasidium pullulans v. pullulans*	ATCC 42457 = AF 121284	Flower, *Veronica spicata*	Czech Republic
33	*Aureobasidium pullulans v. pullulans*	AJ 276062	PVC	UK
34	*Aureobasidium pullulans v. pullulans*	dH 12686 = det 396 396/01 A1	Painted outdoor wall	Sweden
35	*Aureobasidium* sp.	ATCC 16629 = AF 121283	Bark, *Sequoia sempervirens*	USA
36	*Aureobasidium* sp.	dH 10019 = det 421/93 HRA-Z		asked 15-3-02
37	*Aureobasidium* sp.	dH 12044 = IWW 499	Drinking water	Germany
38	*Aureobasidium* sp.	dH 12609 = Vishniac Y-31		asked 18-3-02
39	*Dothichiza pityophila*	CBS 215.50	Dead bark, *Abies concolor*	Norway
40	*Dothidea berberidis*	CBS 186.58		Switzeland
41	*Dothidea insculpta*	CBS 189.58 = AF027764	*Clematis vitalba*	France
42	*Dothiora cannabinae*	CBS 737.71 (T)	Twig, *Daphne cannabina*	Himalaya

List of strains studied (continue)

No.	Name	Number	Original substrate	Geography
43	*Dothiora europaea*	CBS 739.71 (T)	Twig, *Salix helvetica*	Switzerland
44	*Dothiora rhamni-alpinae*	CBS 745.71 (T)	Twig, *Rhamnus alpina*	Italy
45	*Hormonema dematioides*	CBS 116.29 (T)	*Pinus*	unknown
46	*Hormonema dematioides*	CBS 536.94	*Rutilis rutilus*, gills	Russia
47	*Hormonema dematioides*	dH 11290 = Sterfl	Stone	asked 15-3-02
48	*Hormonema dematioides*	dH 12694 = det 397/2001 100	Wood, *Picea*	Sweden
49	*Hormonema prunorum*	CBS 933.72 (T)	Fruit, *Prunus domestica*	UK
50	*Hormonema prunorum*	CBS 765.84	Log, *Pinus radiata*	Chile
51	*Hormonema* sp.	CBS 102180 = dH 11410 = Hodges 6063	Branch, Cupressocyparis leylandii	USA
52	*Hormonema* sp.	CBS 102181 = dH 11411 = Hodges 5450	Branch, Cupressocyparis leylandii	USA
53	*Kabatiella caulivora*	CBS 242.64	Leaf, *Trifolium* sp.	USA
54	*Kabatiella lini*	CBS 125.21 (T) = AJ 244252	*Linum* sp.	UK
55	*Kabatiella microsticta*	CBS 114.64	Leaf, *Hemerocallis* sp.	Netherlands

List of strains studied (continue)

No.	Name	Number	Original substrate	Geography
56	*Kabatiella microsticta*	CBS 342.66	Leaf, *Convallaria*	Germany
57	*Kabatiella microsticta*	CBS 269.63	Leaf, *Hemerocallis* sp.	Netherlands
58	*Kabatiella zeae*	CBS 767.71	Leaf spot, *Zea mays*	Germany
59	*Kabatiella* cf. *zeae*	CBS 102365 = dH 11470	Foodstuffs	Netherlands
60	*Kabatina juniperi*	AF 260224		USA
61	*Kabatina juniperi*	AF182376		Syst Appl Micr 23:333-343, 2000
62	*Kabatina mahoniae*	CBS 264.92 (T)	*Mahonia repens*	USA
63	*Kabatina thujae*	CBS 238.66 (T) = AF 013226	Shoot, Thuja occidentalis	Germany
64	*Pringsheimia euphorbiae*	CBS 747.71 (T)	Stem, Euphorbiaceae sp.	Greece
65	*Pringsheimia chamaecyparidis*	CBS 746.71 (T)	Twig, Chamaecyparis lawsoniana	France
66	*Pringsheimia sepincola*	CBS 748.71	Twig, *Rosa canina*	Switzerland

List of strains studied (continue)

No.	Name	Number	Original substrate	Geography
67	*Pringsheimia smilacis*	CBS 873.71 (AUT)	Twig, *Smilax aspera*	Italy
68	*Rhizosphaera kobayashii*	ATCC 46389 = AF 462432	*Pinus pumila*	Japan
69	*Sarcinomyces* sp.	dH 12067 = IWW 780	Drinking water	Germany
70	*Selenophoma mahoniae*	CBS 388.92 (T)	Leaf, *Mahonia repens*	USA
71	*Sydowia polyspora*	CBS 102821 = Sterfl D8 = dH 11940	*Pinus sylvestris*	Germany
72	*Sydowia polyspora*	CBS 109808 = dH 12232 = Zhdanova 1161	Forest litter	Ukraine
73	*Sydowia polyspora*	CBS 109809 = dH 12234 = Zhdanova 1162	Moss in radioactive bog	Ukraine
74	*Sydowia polyspora*	CBS 128.64	Wood, *Pinus sylvestris*	Netherlands
75	*Sydowia polyspora*	CBS 719.76	Needle, *Pinus sylvestris*	Netherlands
76	*Sydowia polyspora*	CBS 750.71	Twig, *Pinus strobus*	Canada
77	*Sydowia polyspora*	dH 12233 = Zhdanova 1125	Moss in radioactive bog	Ukraine

Abbreviations used:

ATTC = American type Culture Collection , Rockville, U.S.A. ;

de Hoog = G.S. de Hoog, personal collection;

CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands;

CHULA = Chulalongkorn University Collection, Bangkok, Thailand;

MZKI = Microorganism Culture Collection, National Institute of Chemistry, Ljubljana, Slovenia;

SPChPhA = Chemical-Pharmaceutical Institute, St. Petersburg, Russia;

VKM = All-Russian Collection of Microorganisms, Puschino, Russia;

AUT = authentic strain

NT = ex-neotype culture

T = ex-type culture

FMR = Facultad de Medicina, Reus, Tarragona, Spain.

IWW = Rheinisch Westfhlisches Institut fёr Wasserforschung, Mёlheim an derRuhr, Germany

NRRL = Agricultural Research Service Culture Collection, Northern UtilizationResearch Branch, US Dept of Agriculture, Peoria, Illinois, USA

APPENDIX III

I-1. Pullulan yields (g/g carbon source) from the cultivation of *Aureobasidium pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU in the production medium with initial pH 4.5, 5.5, 6.5 or 7.5 at 25 ^{o}C., 150rpm for 5 days.

Strains	pH	Time (day)				
		1	2	3	4	5
SU	4.5	0.0162 $^{m-o}$	0.0475 $^{i-l}$	0.056 $^{h-j}$	0.0645 $^{g-i}$	0.0746 ^{gh}
	5.5	0.0184 $^{m-o}$	0.0640 $^{g-i}$	0.0775 ^{fg}	0.0973 ^{ef}	0.0800 ^{fg}
	6.5	0.0177 $^{m-o}$	0.1289 ^{d}	0.1587 ^{bc}	0.1800 ^{b}	0.2382 ^{a}
	7.5	0.0110 ^{o}	0.0794 ^{fg}	0.1564 ^{c}	0.1768 ^{bc}	0.1684 ^{bc}
PR	4.5	0.0141 $^{m-o}$	0.0172 $^{m-o}$	0.0181 $^{m-o}$	0.0199 $^{m-o}$	0.0211 $^{m-o}$
	5.5	0.0114 ^{no}	0.0221 $^{m-o}$	0.0195 $^{m-o}$	0.0220 $^{m-o}$	0.0301 $^{k-o}$
	6.5	0.0192 $^{m-o}$	0.0350 $^{j-n}$	0.0209 $^{m-o}$	0.0211 $^{m-o}$	0.0203 $^{m-o}$
	7.5	0.0137 $^{m-o}$	0.0312 $^{k-o}$	0.0370 $^{j-m}$	0.0299 $^{k-o}$	0.0522 $^{i-k}$
CU	4.5	0.0109 ^{o}	0.0194 $^{m-o}$	0.0197 $^{m-o}$	0.0219 $^{m-o}$	0.0227 $^{m-o}$
	5.5	0.0158 $^{m-o}$	0.0172 $^{m-o}$	0.0238 $^{m-o}$	0.0330 $^{k-o}$	0.0293 $^{k-o}$
	6.5	0.0258 $^{l-o}$	0.0503 $^{i-k}$	0.0515 $^{i-k}$	0.0524 $^{i-k}$	0.0559 $^{h-j}$
	7.5	0.0232 $^{m-o}$	0.0469 $^{i-l}$	0.0986 ^{ef}	0.1027 ^{e}	0.1669 ^{bc}

[1]Superscript letters indicate that a value followed by the same letter did not differ significantly

(P<0.05) in Duncan's multiple range test from other values with the same letter

Analysis of variance (pH optimization)

SOV	DF	SS	MS	F
TRETMENTS	59	0.4951	0.0084	58.1963 **
ERROR	120	0.1073	0.0001	
TOTAL	179	0.5124		

(cv.)= 22.00%

*,** = SIGNIFICANT AT 95%, 99% LEVEL

ns = NON SIGNIFICANT AT 95% LEVEL

I-2. Pullulan yields (g/g carbon source) from the cultivation of *Aureobasidium pullulans* SU, *A. pullulans* PR and *A. pullulans* CU in the production medium at 25, 30 or 35 $^{\circ}$C, initial pH of each favorite strain as shown in pH optimization,shaken 150 rpm for 5 days

Strains	Temp. ($^{\circ}$C)	Time (day)				
		1	2	3	4	5
SU	25	0.0333 $^{j-q}$	0.0673 $^{j-i}$	0.1086 ^{e}	0.1580 ^{c}	0.1357 ^{d}
	30	0.0354 $^{j-p}$	0.0331 $^{j-q}$	0.0495 $^{i-k}$	0.0763 ^{gh}	0.0841 ^{fg}
	35	0.0302 $^{k-q}$	0.0085 ^{q}	0.0114 ^{pq}	0.0124 $^{o-q}$	0.0386 $^{j-o}$
PR	25	0.0190 $^{l-q}$	0.0393 $^{j-o}$	0.0514 $^{i-k}$	0.0586 $^{h-j}$	0.0416 $^{j-l}$
	30	0.0193 $^{l-q}$	0.0375 $^{j-p}$	0.1115 ^{e}	0.1782 ^{bc}	0.2245 ^{a}
	35	0.0082 ^{q}	0.0083 ^{q}	0.0135 $^{n-q}$	0.0153 $^{l-q}$	0.0163 $^{l-q}$
CU	25	0.0275 $^{k-q}$	0.0409 $^{j-m}$	0.0802 $^{f-h}$	0.1251 ^{de}	0.1852 ^{b}
	30	0.0307 $^{k-q}$	0.0380 $^{j-p}$	0.0399 $^{j-n}$	0.1025 ^{ef}	0.1859 ^{b}
	35	0.0145 $^{m-q}$	0.0132 $^{n-q}$	0.0139 $^{n-q}$	0.0167 $^{l-q}$	0.0261 $^{k-q}$

[1]Superscript letters indicate that a value followed by the same letter did not differ significantly

(P<0.05) in Duncan's multiple range test from other values with the same letter

Analysis of variance (Temperature optimization)

SOV	DF	SS	MS	F
TRETMENTS	44	0.4191	0.0095	53.4844 **
ERROR	90	0.0160	0.0002	
TOTAL	134	0.4351		

(cv.)= 22.00%

*,** = SIGNIFICANT AT 95%, 99% LEVEL

ns = NON SIGNIFICANT AT 95% LEVEL

I-3. Pullulan yields (g/g carbon source) from the cultivation of *Aureobasidium pullulans* CHULA-SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU in the production medium having glucose or sucrose as the sole carbon source with initial pH and temperature of each favorite strain, shaken 150 rpm for 5 dyas.

Strains	C-source	Time (day)				
	(g/g C-source)					
		1	2	3	4	5
SU	Glucose	0.0333 $^{g-j}$	0.0673 $^{f-h}$	0.1086 ^{de}	0.1580 ^{bc}	0.1357 ^{cd}
	Sucrose	0.0040 ^{j}	0.0071 ^{ij}	0.0055 ^{j}	0.0073 ^{ij}	0.0053 ^{j}
PR	Glucose	0.0193 ^{ij}	0.0375 $^{g-j}$	0.1115 ^{de}	0.1782 ^{b}	0.2245 ^{a}
	Sucrose	0.0139 ^{ij}	0.0116 ^{ij}	0.0100 ^{ij}	0.0212 ^{ij}	0.0196 ^{ij}
CU	Glucose	0.0275 $^{h-j}$	0.0409 $^{f-j}$	0.0802 ^{ef}	0.1251 ^{cd}	0.1852 ^{b}
	Sucrose	0.0111 ^{ij}	0.0427 $^{f-j}$	0.0485 $^{f-i}$	0.0681 ^{fg}	0.1221 ^{cd}

[1]Superscript letters indicate that a value followed by the same letter did not differ significantly

(P<0.05) in Duncan's multiple range test from other values with the same letter

Analysis of variance (C-source optimization)

SOV	DF	SS	MS	F
TRETMENTS	29	7.9179	0.2730	26.4755 **
ERROR	60	0.6188	0.0103	
TOTAL	89	8.5367		

(cv.)= 33.22%

*,** = SIGNIFICANT AT 95%, 99% LEVEL

ns = NON SIGNIFICANT AT 95% LEVEL

I-4. Pullulan yields (g/g carbon source) from the cultivation of *Aureobasidium pullulans* CHULA- SU, *A. pullulans* CHULA-PR and *A. pullulans* CHULA-CU in the production medium having $(NH_4)_2SO_4$ or peptone as a nitrogen source with initial pH, temperature and carbon source of each favorite strain, shaken 150 rpm for 5 days.

Strains	N-source (g/g N-source)	Time (day)				
		1	2	3	4	5
SU	$(NH_4)_2SO_4$	0.0333 [i-m]	0.0673 [hi]	0.1086 [fg]	0.1580 [be]	0.1357 [cf]
	Peptone	0.0081 [m]	0.0285 [j-m]	0.0360 [i-m]	0.0304 [j-m]	0.0248 [k-m]
PR	$(NH_4)_2SO_4$	0.0193 [m]	0.0375 [i-m]	0.1115 [fg]	0.1782 [b]	0.2245 [a]
	Peptone	0.0217 [lm]	0.0185 [m]	0.0564 [h-l]	0.1594 [bd]	0.1677 [bc]
CU	$(NH_4)_2SO_4$	0.0275 [km]	0.0409 [i-m]	0.0802 [gh]	0.1251 [ef]	0.1852 [b]
	Peptone	0.0157 [m]	0.0412 [i-m]	0.0595 [h-k]	0.0645 [h-j]	0.1314 [d-f]

[1]Superscript letters indicate that a value followed by the same letter did not differ significantly

(P<0.05) in Duncan's multiple range test from other values with the same letter

Analysis of variance (nitrogen optimization)

SOV	DF	SS	MS	F
TRETMENTS	29	7.4602	0.2572	17.2216 **
ERROR	60	0.8963	0.0149	
TOTAL	89	8.3565		

(cv.)= 31.41%

*,** = SIGNIFICANT AT 95%, 99% LEVEL

ns = NON SIGNIFICANT AT 95% LEVEL

APPENDIX IV

Using a hemocytometer to determine total cell counts

1. With the cover-slip in place, use a Pasteur pipette or other suitable device and transfer a small amount of cell suspension to both chambers of the hemocytometer by carefully touching the edge of the cover-slip with the pipette tip and allowing each chamber to fill by capillary action. Do not overfill or under the chambers.

2. Starting with 1 chamber of the hemocytometer, count all the cells in the 1 mm center square and four 1 mm corner squares. (see Diagram I).

   Note: Count cells on top and left touching middle line of the perimeter of each square. Do not count cells touching the middle line at bottom and right sides. (see Diagram II).

3. Repeat this procedure for chamber 2.

   Note: If greater than 10% of the cells appear clustered, repeat entire procedure making sure the cells are dispersed by vigorous pipetting in the original cell suspension. If less than 200 or greater than 500 cells (20-50 cells per square) are observed in the 10 squares, repeat the procedure adjusting to an appropriate dilution factor.

4. Cell Counts-Each square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations:

   CELLS PER ml = the average count per square x dilution factor x 10^4 (count 10 squares)

   Ex: If the average count per square is 45 cells x 10^4 = 4.5×10^5 cells/ml.

   TOTAL CELLS = cells per ml x the original volume of fluid from which cell sample was removed.

   Ex: 4.5×10^5 (cells per ml) x 10 ml (original volume) = 4.5×10^8 total cells.

5. Withdraw a second sample and repeat counting procedure to ensure accuracy.
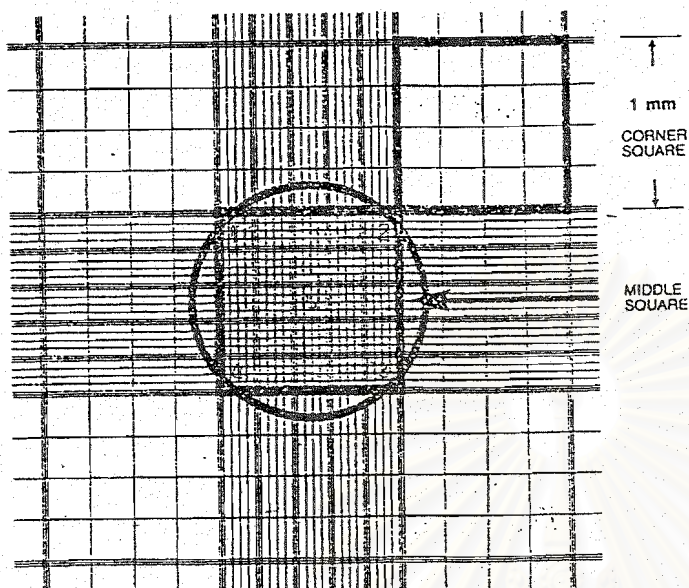
DIAGRAM I

STANDARD HEMOCYTOMETERCHAMBER

DIAGRAM II

CENTERSQUARE

(ENLARGEMENT)
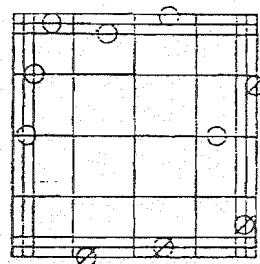
Count cells on top and left
Touching middle line(O)
Do not count cells touching
Middle line at bottom and
right.

The circle indicate the approximate area covered at 100x microscope magnification (10x ocular and 10x objective). Include cell on top and left touching middle line (O). Do not count cells touching middle line at bottom and right (∅). Count 4 corner sqares and middle sqare in both chambers (one chamber represented here).

APPENDIX V

Corn Meal Agar (CMA)

Ground corn	60 g
Glucose	10 g
Agar	15 g
Distilled water	1 l

Malt Extract Agar (MEA)

Malt extract	25 g
Agar	15 g
Distilled water	1 l

Final pH 5.5

Potato Dextrose Agar (PDA)

Potatos (boil & filtered)	200 g
Glucose	20 g
Agar	15 g
Distilled water	1 l

Potato Broth Agar (PDB)

Potatos(boil & filtered)	200 g
Glucose	20 g
Distilled water	1 l

Production medium

Glucose	50 g
$(NH_4)_2SO_4$	0.6 g
K_2HPO_4	5.0 g
$MgSO_4.7H_2O$	0.4 g
NaCl	1.0 g
Yeast extract	0.4 g.
Distilled water	1 l

APPENDIX VI

1. Chemical preparation for Extraction of ribosomal DNA (Gerrits van den Ende,2001)

-   10% CTAB

Dissolve 10 g CTAB in 100 ml ultrapure water. Keep at room temperature. Do not autoclave.

-   Ethanol 96% (-20°C)

Remark: Ethanol 96% should not be non-denatured ethanol.

-   Ethanol 70% (-20°C)

Mix 700 ml. Ethanol 96% with ultrapure water to a volume of 960 ml.

-   5 M NaCl

Dissolve 29 g NaCl in 100 ml. Ultrapure water.

-   5 M NH_4-acetate

Dissolve 38.54 g CH_3COONH_4 in 100 ml. Ultrapure water.

-   RNAse solution

Dissolve 10 mg Pancreatic RNAse in 1 ml. 0.01 M Na-acetate, pH 5.2. Heat the solution till 100°C during 15 min. Cool slowly to room temperature. Adjust pH by adding 100 μl 1 M Tris, pH 7.4. Make aliquot of 100 μl and store at –20°C.

-   Proteinase K 10 mg/ml

Dissolve 50 mg. Proteinase K in 5 ml ultrapure water. Make aliquots of 500 μl and store at -20°C.

-   SEVAG

Mix 240 ml. Chloroform with 10 ml. Isoamylalcohol.

- 0.01 M sodium acetate ; pH 5.2

Fill a Falcon tube with 45 ml ultrapure water. Discard 150 μl and add 150 μl sodium acetate 3M

- 3 M sodium acetate ; pH 5.2

Dissolve 20.4 g CH3COONa.3H2O in 50 ml Ultrapure water. Adjust pH to 5.2 . Make aliquots of 1 ml. In eppendorfcups. Store at -20 °C.

- TE-buffer

Add 0.12 g Tris and 0.04 g Na-EDTA to 80 ml. Ultrapurewater. Adjust pH 8.0 with 1 N HCL. Heat the solution if EDTA does not dissolve very well. Adjust volume to 100 ml with ultrapure water. Aliquot the buffer in 50 ml tubes. Autoclave the solution during 15 min. at 121 °C. Store at room temperature.

- TES-buffer

Add 1.2 g Tris, 0.38 g Na-EDTA and 2 g sodiumdodecylsulphate (SDS) to 80 ml. Ultrapurewater. Adjust pH 8.0 with 1 N HCL. Heat the solution if EDTA does not dissolve very well. Adjust volume to 100 ml with ultrapure water. Aliquot the buffer in 50 ml tubes. Filter sterilize the solution if need be. Do not Autoclave Store at room temperature.

- 1 M Tris ; pH 7.4

Dissolve 121.1 g Tris in a total volume of 800 ml Ultrapure water. Adjust pH to 7.4 with 5 N HCl and later with 1N HCl. Add ultrapure water to a volume of 1000 ml.

2. Chemical preparation for Purifying rRNA with GFX-column (Gerrits van den Ende, 2001)

- TE-buffer, pH 8

Add 0.12 g Tris and 0.04 g Na-EDTA to 80 ml. Ultrapurewater . adjust pH 8.0 with 1 N HCL. Heat the solution if EDTA does not dissolve very well. Adjust volume to 100 ml with

ultrapure water. Aliquot the buffer in 50 ml tubes. Autoclave the solution during 15 min. at 121°C. Store at room temperature.

- 10 mM Tris-buffer; pH 8

Add 0.12 g Tris to 80 ml. Ultrapurewater.Adjust pH 8.0 with 1 N HCL. Adjust volume to 100 ml with ultrapure water. Aliquot the buffer in 50 ml tubes. Autoclave the solution during 15 min. at 121°C. Store at room temperature.

3. Chemical preparation for Sequencing using capillary electrophoresis on the ABI Prism 3700 (Gerrits van den Ende, 2001)

- Sequencing buffer 5x

Dissolve 4.84 g Tris and 0.2 g MgCl2.H20 in 80 ml. Ultrapure water. Adjust pH to 9.0 with 1 N HCl. Add ultrapure water to 100 ml.

4. Chemical preparation for Excuting a PCR with ribosomal DNA (Gerrits van den Ende, 2001)

- PCR-buffer 10X

Add 9.32 g potassium chloride, 3.03 g Tris and 1.53 g magnesiumchloride hexahydrate to 220 ml ultrapure water. Adjust pH to 8.3 by adding $\pm$ 10 ml 1N HCl. Add 0.25 g gelatin and 2.5 ml Triton X-100, heat the solution for 30min. at 56°C. Adjust the total volume to 250 ml with ultrapure water. Make aliquots of 50 ml. The buffer may be autoclaved.

- 0.05M Tris pH 8.3

Add 6.05 g Tris to 500ml ultrapure water. Adjust pH to 8.3 by adding $\pm$ 20 ml 1N HCl. Add ultrapure water to a volume of 100 ml.

- 0.5 M EDTA pH 8.0

Add 186.1 EDTA to 800 ml ultrapure water and stir on a magnetic stirrer. Add while stiring ± 20 g sodium hydroxyde. The pH will be approx 8.0. Adjust total volume to 1000 ml with ultrapure water.

- 1M DDT

Dissolve 1.54 g DTT in 10 ml ultrapure water. Aliquot in portions of 1 ml and store at –20°C.

- dNTP-mix 5 mM

Pipet 50 μl dATP, 50 μl dTTP, 50 μl dCTP, 50 μl dGTP and 800 μl ultrapure water sterile water into a sterile eppendorfcup. Dilute this solution to 1 mM before use.

- DNA-polymerase dilutionbuffer

Mix 0.2 ml 1 M Tris, pH 8.0, 0.2 ml 0.5 M EDTA pH 8.0, 0.1 ml 1 M DTT, 49.5 ml ultrapure water and 50 ml glycerol 87% thoroughly. Store solution at 4°C.

- DNA-polymerase user solution 1 unit/μl

Mix 20 μl DNA-polymerase 5 unit/μl with 80 μl DNA-polymerase dilutionbuffer and vortex. Store solution at –20°C.

- Loading buffer

Dissolve 100 mg bromophenolbleu in a few drops of ethanol. Add 10 ml ultrapure water. Dissolve 10 g sucrose separately in 40 ml ultrapure water. Mix both solutions and store at 4°C.

- TE-buffer

Add 0.12 g Tris and 0.04 g Na-EDTA to 80 ml ultrapure water. Adjust pH at 8.0 with 1 N HCl. Heat the solution if EDTA does not dissolve very well. Adjust volume to 100 ml. Aiquot the solution in 50 ml tubes. Autoclave the solution for 15 min. at 121°C. Store at room temperature.

- Low Range marker-solution

Pipet 20 μl low range marker, 20 μl loading buffer and 60 μl TE-buffer pH 8.0 and mix. Use 6-8 μl marker-solution perlane.

- 1 Kb marker-solution

Pipet 30 μl Promega 1Kb-marker, 3 μl loading dye 6x and 27 μl TE-buffer pH 8.0 and mix. Use 3-5 μl marker-solution per lane.

- SmartLadder

The SmartLadder of Eurogentec is user ready. Per lane 5 μl is used. With this marker the size and the concentration of the amplicon can be estimated.

5. Chemical preparation for diazonium blue B reagent. (Barnett et al, 2000.)

- Dissolving diazonium blue B salt (Brentamine Blue B of ICI plc of Fast Blue Salt B of Hoechst AG) in cold 0.1 M-trisiHCl buffer, pH 7.0, at 1 mg/ml

BIOGRAPHY

Miss Montarop Sudhadham was born on March 28, 1976 in Bangkok, Thailand. She finished the primary education from Watwetawantanmawat School in 1988, and the secondary education from Benjamarachalai School in 1994. She enrolled in Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang in 1995, and got a grant from the International Association of Agriculture Students Exchange Program (IAAS) for 3-month training session in Horticulture in Norway in 1997.After got a Bachelor of Science in 1998, she began working on a Master's degree in Botany at the Department of botany, Faculty of Science, Chulalongkorn University in 1998. She was award a scholarships from Biodiversity Research and Training Grant (BRT) in 1999, and attended the 5th Graduate Congress in Singapore in 2000. In 2001, Miss Montarop Sudhadham spent three months at the Centraalbureau voor Schimmelcultures, the Netherlands, working on the molecular portion of her thesis under the supervision of Prof. Dr. G. Sybren de Hoog.