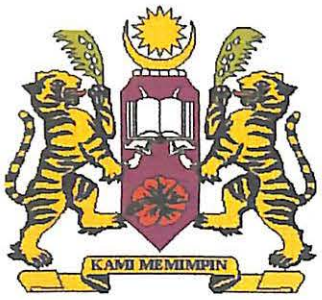

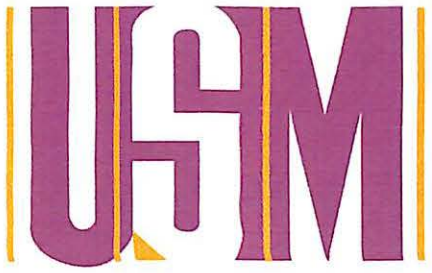

UNIVERSITI SAINS MALAYSIA

CONSTRUCTION OF GENOMIC LIBRARY FOR *Leucosporidium antarcticum*

By

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List of symbol and abbreviation

bp	Basepair
BSA	Bovine serum albumin
ddH_2O	Double distilled H_2O
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
EtBr	Ethidium Bromide
E. coli	*Escherichia coli*
kb	kilobasepair
L. antarcticum	*Leucosporidium antarcticum*
LB	Luria-Bertani
PBS	Phosphate buffered saline
RNase	Ribonuclease
PvP	polyvinyl pyrrolidano
SDS	Sodium dodecyl sulphate
TBE	Tris-Boric-EDTA
TE	Tris-EDTA
Tris	2-amine-2hydroxymethyl-1,3-propanediol
YPD	Yeast peptone dextrose
X-Gal	5-bromo-4-chloro-3-indolyl-D-galactosylpyranoside

Abstrak

L. antarcticum merupakan spesis yis yang dijumpai di Antartika. Dengan adaptasi spesifik yang membolehkannya hidup dalam suasana sejuk serta mencabar di Antartika, *L. antarcticum* mempunyai potensi untuk pelbagai aplikasi seperti protein anti-beku dan juga ciri-ciri lain yang berguna. Saiz genom untuk *L. antarcticum* adalah 14 Mb. Dalam penyelidikan ini, kami menggunakan vector plasmid untuk membina perpustakaan genetik *L. antarcticum* dalam strain *E. coli* Top Ten. *L. antarcticum* ditumbuhkan dalam media cecair YPD sebelum diasingkan. Tiga kaedah dibandingkan untuk mengekstrak DNA daripada *L. antarcticum* (kaedah pemecahan protein Proteinaise K, kaedah pemecahan protein Proteinaise K + Pronase and juga kaedah pemecahan nitrogen cecair). Kami mendapati kaedah pemecahan nitrogen cecair merupakan kaedah yang terbaik untuk digunakan bagi mengekstrak dan memecahkan DNA *L. antarcticum* pada saiz 1000 hingga 2000 bp. DNA ditulenkan bagi mendapat pecahan DNA pada saiz 1000 bp hingga 1500 bp. DNA yang ditulenkan dibaiki, didefosforilasikan dan dimasukkan ke dalam vektor pCR®4Blunt-TOPO® DNA berhujung tumpul. Plasmid rekombinan kemudian ditransformasi ke dalam *E. coli* menggunakan kaedah rejatan haba sebelum dikulturkan pada agar LB. Pengekstrakan plasmid dilakukan untuk melihat kehadiran serpihan DNA selitan. Kesimpulannya, kami berjaya menghasilkan lebih kurang 2976 klon *E. coli* iaitu merupakan 31.9 % daripada keseluruhan genom *L. antarcticum*.

Abstract

L. antarcticum is a yeast species found in Antarctica. With special adaptation to survive in cold harsh environment of Antarctica, *L. antarcticum* have various potential applications like antifreeze proteins and useful features. The genome size of *L. antarcticum* is approximately 14 Mb. In this research, we tried to construct a gene library for *L. antarcticum* by using plasmid vector. *L. antarcticum* was cultivated in YPD broth before harvested. Three methods were evaluated for DNA extraction from *L. antarcticum* (Proteinaise K proteolytic lysis method, Proteinaise K + Pronase proteolytic lysis method and also liquid nitrogen lysis method). We found that liquid nitrogen lysis method was the best method to extract and fragmentize the *L. antarcticum* DNA at the required size of 1000 bp to 1500 bp. They were then purified to obtain DNA fragment of 1000 bp to 1500 bp. The purified DNA were repaired, dephosphorylated and inserted into pCR®4Blunt-TOPO® DNA vector. The recombinant plasmids were finally transformed into Top Ten *E. coli* strain using heat shock before plated on LB agar. Plasmid extractions were carried out on clones to screen for presence of DNA insert. In conclusion, this study managed to construct a gene library containing approximately 2976 *E. coli* clones which cover 31.9 % of the whole *L. antarcticum* genome.

Chapter One: Introduction

1.1 *Leucosporidium antarcticum (L. antarcticum)*

Leucosporidium antarcticum is a species of fungi found in the cold region of Antarctica in year 1966. (Fell et al, 1969)

Kingdom	Fungi
Phylum	Basidiomycota
Class	Pucciniomycotina
Order	Microbotryomycetes
Family	Leucosporidiales
Genus	*Leucosporidium*
Species	*antarcticum*

Table 1: *L. antarcticum* taxonomy (Fell et al, 1969).

Leucosporidium antarcticum is a cellular eukaryote organism of kingdom Fungi, phylum Basidiomycota, class Pucciniomycotina, order Microbotryomycetes, family Leucosporidiales, genus *Leucosporidium*; species *antarcticum.* (Fell et al, 1969)

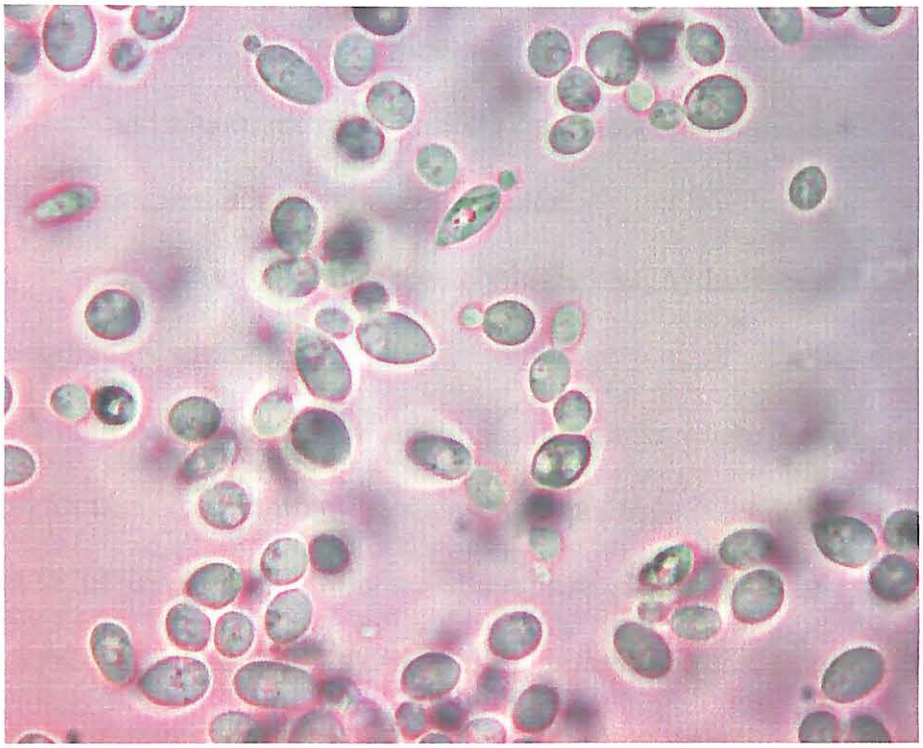

Figure 1: *Leucosporidium antarcticum* at 100 X magnification. (Light microscopy)

1.2 Characteristic of *L. antarcticum*

Leucosporidium antarcticum is an obligate psychrophilic, budding, non-sugar-fermenting, halotolerant marine yeast (Marzena *et al*, 2003). These fungi require cold environment to grow and survive. They reproduce by budding process similar to yeast.

Leucosporidium antarcticum thrive in salty cold environment of Antarctic Sea. The natural habitat of *L. antarcticum* has NaCl concentration of 2.5 to 3.5 % (Marzena et al, 2003). The highest concentration protein derived from this organism is at this concentration as NaCl play a role in enzyme biosynthesis (Marzena et al, 2003).

Physical examinations by microscope reveal that this organism has yeast like morphology with the width of 2.1 to 2.7 um and length of 2.9 to 4.0 um thus making it appeared as a bit oval in shape.

L. antarcticum are able to utilize various sugars present in the medium (maltose, sucrose) and also tributyrin as basic as carbon source (Marianna et al, 2003). It is noted that this organism is a non sugar fermenter.

This yeast secretes extracellular proteolytic enzyme, hydrolases and also lipase into its surrounding (Marianna et al, 2003).

Interestingly, it is able to grow in nitrogen free media where the only source of nitrogen is molecular nitrogen from the atmosphere, possibly this might be due to the yeast ability to utilize nitrogen from the atmosphere directly (Abdul et al, 2007). Another possible explanation for this characteristic is that this yeast lives in symbiosis with a prokaryote that can utilize and fix molecular nitrogen directly from atmosphere. The ability for organism to fix molecular nitrogen from atmosphere into biologically usable compound is a process restricted to some bacteria (Christoph et al, 2007).

The genome size of *L. antarcticum* is estimated to be around 10.5 to 14 Mb with nearly 76 % of gene analyzed is fungal in origin while the rest of it has similarity to genes from other group of organism (Abdul et al, 2007). Interestingly, 8 % of this yeast gene is similar to bacterial genes which are speculated to exist due to lateral gene transfer from bacteria to *L. antarcticum* or another possible explanation is that bacteria live in symbiosis with this yeast. Apart from this discovery, researcher also manage to uncover existence of several distinctive gene that codes for putative anti freeze protein, ice binding protein, lipase, protease etc (Abdul et al, 2007). Possibly these genes granted the yeast the needed advantage to survive in extreme cold region of Antarctica and possibly other undiscovered use yet.

These psychrophilic organisms were discovered in cold condition between altitude 60 S and 90 S which encompass the Antarctica region (Marzena et al, 2003).

Some biological process of this yeast while similar to organism existing in normal temperature region have special kinetic and features that enable efficient function and survival in cold energy deficient environment (Marzena et al, 2003). From other publications, it is noted that this yeast will not thrive in warm condition. It is noted that while the enzymes is adapted for maximum efficiency in cold environment, it is heat labile and would prevent the yeast from thriving in higher temperature. The *L. antarcticum* is a yeast that are specially adapted to survive in cold environment and thus are able to secure a habitat without intrusion from normal organism that could not survive the Antarctic freezing condition.

This antartic yeast has heat shock mechanism analogous to mesophilic yeast counterpart but with distinct protein stress protein profile favoring their survival in hostile Antartic condition (Michelle et al, 1998)

1.3 Scientific finding that enable *L. antarcticum* to survive in Antarctica

Antarctica lies in Antarctic circle that encompasses area of between latitude 66 S to latitude 90 S (Barry et al, 2004). Due to its position on earth, Antarctica received less overall sum of energy per unit surface compared to equator region. This resulted in extremely low temperature which is the normal condition all year around.

This is a very challenging place to live but many organisms managed not only to survive but adapted to thrive in this extreme condition. Challenges include rapid temperature changes which may cause repeated rapid freezing and thawing that will kill most unadapted organisms.

Low temperature affects organism by slowing down or inhibiting chemical reaction rates that catalyzes the biological process occurring in the organism (Salvino et al, 2002).

Several strategies that are usually employed by psychrophilic yeast include alteration of membrane lipid composition. By altering the degree of fatty acid unsaturation in the cell membrane, this reduces the damage caused by repeated freezing and thawing on the yeast cell (Serena et al, 2007). This alteration maintains membrane fluidity which allow the cell membrane to function normally even in freezing condition (Barry et al, 2004). This alteration also increases the efficiency of nutrient uptake at cold temperature in organism of cold origin while maintaining cell integrity (Barry et al, 2004).

Some of the Antarctic fungi synthesize cryoprotective carbohydrate (trehalose, mannitol, etc) that function to stabilize the membrane of yeast from cold damage (Serena et al, 2007). It is suggested that these cryoprotectant play a role in altering ions permeability and also altering water structure in the environment whether intercellular or extracellular allowing some control on water crystallization that lead to ice crystal formation in cold environment (Serena et al, 2007). Damaging ice crystal formation could be avoided in order to reduce cell injury.

Some Antarctic organisms synthesize antifreeze protein that act by preventing ice crystal formation thus preventing cell injury and enhance survival in frigid climate (Barry et al, 2004). These antifreeze proteins alter ice crystal formation by preventing ice crystal growth.

Another strategy used by *L. antarcticum* is the alteration of the enzyme and protein to be able to function efficiently in cold environment. A study on a protein of this yeast called serine proteinase derived from *L. antarcticum* shows that the protein

have low optimal temperature (25 °C), functioned at 0 °C, thermolabile, has high catalytic efficiency and low value of activation energy (Marzena et al, 2003). Psychrophilic organisms usually synthesize enzymes with higher specific activity at low and medium activity (Salvano et al, 2003). However, the synthesized enzymes are highly thermolabile and not efficient at tropical room temperature compared to tropical organism (Salvano et al, 2003).

1.4 The purpose of this work

Due to its ability to grow in extreme low temperature, this yeast generates research interest for various applications. Among these include catalytic protein encoded by this organism that work in low temperature efficiently unlike normal protein that require normal high optimum temperature to function at maximum efficiency.

Another possible application is in the field of cryobiology. Glycoprotein produced by *L. antarcticum* shows a partial benefit in improving rat liver preservation in conjunction with the presence of other preserving agent (Tilsˇer et al, 1996).

In order to begin studying the characteristic of *Leucosporidium antarcticum* (*L. antarcticum*) molecular origin, a gene library representing the whole *Leucosporidium antarcticum* (*L. antarcticum*) have to be constructed so that its entire DNA sequence could be mapped. This approach enable scientist to determine the number of functional protein coding gene (Ursula and Blomberg, 2006).

The first objective is to find the best cultivation condition that allows the yeast to populate.

The second objective is to find the best DNA extraction method to harvest the DNA from the yeast. Several methods are examined for this purpose. This includes using proteolytic lysis buffer (Proteinaise K) with sonication method; proteolytic lysis buffer (Proteinaise K and Pronase A) with sonication method; and the last method is liquid nitrogen cell lysis method.

The third objective is to construct a genomic library for *L. antarcticum*.

Chapter Two: Materials and methods

2.1 Test Organism

2.1.1 *L. antarcticum* strain

The *L. antarcticum* strain used in this experiment was derived from the sample taken back from Antarctica in year 2002 (A gift from Prof. Mohd Nazalan Mohd Najimudin).

2.1.2 *E. coli* strain

The *E. coli* strain used in this experiment is from Top Ten One Shot Chemically Competent *E. coli* genotype F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G from Invitrogen for general cloning and blue/white screening without IPTG. (Invitrogen) (Siah, 2007)

2.2 Media, buffer and solution preparation

2.2.1 Ampicillin stock preparation (50 mg/ml)

500 mg Ampicillin (Pharmmalaysia, Malaysia) were measured and dissolved into 10 ml sterile ddH_2O in a 15 ml falcon tube. The solution was filtered by using 0.2 um Milipore filter in a laminar air flow and aliquoted into 1 ml microcentrifuge tube. The microcentrifuge tubes were labeled and stored at -20 °C.

2.2.2 Kanamycin stock preparation (50 mg/ml)

500 mg Kanamycin (Amresco, USA) were measured and dissolved into 10 ml sterile ddH_2O in a 15 ml falcon tube. The solution was filtered by using 0.2 um Milipore filter in a laminar air flow and aliquoted into 1 ml microcentrifuge tubes. The microcentrifuge tubes were labeled and stored at -20 °C.

2.2.3 25 % Glucose stock solution preparation (50 ml)

12.5 g of glucose (R&M Chemicals, UK) were measured and dissolved into 50 ml of sterile ddH_2O in a 50 ml falcon tube. The solution was filtered by using 0.2 um Milipore filter in a laminar air flow into a new 50 ml sterile falcon tube and stored at 4 °C.

2.2.4 Tris HCl pH 8.0 preparation (100 ml)

15.76 g of Tris Cl (Promega, USA) were measured. ddH_2O was added until the volume reached 100 ml. The pH were adjusted to pH 8.0 using 5 M NaOH and stored at room temperature.

2.2.5 10 % (w/v) SDS solution preparation

10 g of SDS (Bio-Rad, USA) were dissolved in 100 ml ddH_2O. Solution was heated to 65 °C for 20 minute and stored at room temperature.

2.2.6 Proteinaise K preparation (10 mg/ml)

10 mg of Proteinaise K (Boeringer Manheim, Germany) were dissolved in 1 ml sterile ddH_2O. The prepared solution was aliquoted into 1.5 ml microcentrifuge tubes. These tubes were stored in –20 °C until use.

2.2.7 Pronase preparation (10 mg/ml)

1 ml ddH_2O were added to 10 mg Pronase (Boeringer Manheim, Germany) powder to make the final concentration of 10 mg/ml for the stock solution. The prepared solution was aliquoted into microcentrifuge tube and stored in –20 °C until use.

2.2.8 0.5 M EDTA pH 8.0 preparation (500 ml)

93.06 g EDTA (Promega, USA) were dissolved in 300 ml of ddH_2O. The pH was adjusted to pH 8.0 using 5 M NaOH. Then ddH_2O were added until the final solution volume is 500 ml. The prepared solution was autoclaved at 121 °C for 15 minutes and stored at room temperature.

2.2.9 3 M NaOAc / Sodium Acetate preparation (500 ml)

123 g of NaOAc (FlukaAG, Germany) were dissolved in 400 ml ddH_2O. Then ddH_2O were added until the final volume is 500 ml. The prepared solution was autoclaved at 121 °C for 15 minutes and stored at room temperature.

2.2.10 10 X Tris EDTA pH 8.0 buffer preparation (TE) (100 ml)

12.1 g Tris base (Amresco, USA) and 3.7 g EDTA (Promega, USA) powder were dissolved in ddH_2O until the final solution is 100 ml. The prepared solution was autoclaved at 121 °C for 15 minutes and stored at room temperature.

2.2.11 10 X PBS preparation (400 ml)

20 phosphate buffered saline tablets (GIBCO BRL, UK) were dissolved into 400 ml ddH_2O. The prepared solution was autoclaved at 121 °C for 15 minutes and stored at room temperature.

2.2.12 SOC media preparation (50 ml)

1 g tryptone (Amresco, USA), 0.25 g yeast extract (Amresco, USA), 0.025 g NaCl (Merck, Germany) were dissolved in 40 ml ddH_2O. 500 ul of 250 mM KCl were then added to the solution. pH of the solution was adjusted to 7.0 using 5 M NaOH. ddH_2O were added until final volume is 50 ml. The prepared solution was autoclaved at 121 °C for 15 minutes and cooled down to 55 °C. 500 ul of sterile 1 M $MgCl_2$ and 72 ul 25 % glucose were added. The prepared solution was stored at 4 °C.

2.2.13 5 M NaCl preparation

29.2 g of NaCl (Merck, Germany) were dissolved with ddH_2O until the end volume is 100 ml. The prepared solution was autoclaved at 121 °C for 15 minutes and stored at room temperature.

2.2.14 RNAse preparation (10 mg/ml)

10 mg RNase (Fermentas, USA) were dissolved in 1 ml diluent (10 mM Tris, 15 mM NaCl). The prepared solution was heated to 100 °C for 15 minutes before allowed to slowly cool down to room temperature. The solution was aliquoted into microcentrifuge tube and stored in – 20 °C. (Sambrook et al, 1989)

2.2.15 5 M HCl preparation

416 ml of 12 M HCl (BDH Chemicals, UK) were added to 583 ml of ddH_2O. The prepared solution was mixed and stored at room temperature.

2.2.16 5 M NaOH preparation

20 g of NaOH (BDH Chemicals, UK) were dissolved in 100 ml ddH_2O and stored at room temperature.

2.2.17 80 % (w/v) glycerol preparation

80 ml 100 % glycerol (BDH Chemicals, UK) were added to 20 ml ddH_2O and mixed. This solution was autoclaved at 121 °C for 15 minutes and stored at room temperature.

2.2.18 EtBr / Ethidium Bromide preparation (10 mg/ml)

2 g of EtBr (Sigma, USA) were dissolved in 200 ml ddH_2O. The prepared solution was stored in sealed plastic container at room temperature. (Sambrook et al, 1989)

* Precaution for laboratory safety:

Ethidium bromide is a powerful mutagen and is considered moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated. (Sambrook et al, 1989)

To decontaminate ethidium bromide, following procedure was employed. Water was added until the concentration of ethidium bromide was less than 0.5 mg/ml. 1 volume of 0.5 M $KMnO_4$ was added and mixed carefully. Then 1 volume of 2.5_N HCl was added. The solution was mixed carefully and left to stand at room temperature for several hours. Then 1 volume of 2.5_N NaOH was added and mixed. The solution was discarded. (Sambrook et al, 1989)

2.2.19 250 mM $MgCl_2$ preparation (100 ml)

5.083 g $MgCl_2$ (Amresco, USA) were dissolved in 70 ml ddH_2O. Then ddH_2O were added until the final volume is 100 ml. This solution was autoclaved at 121 °C for 15 minutes and stored at room temperature.

2.2.20 1 M $CaCl_2$ preparation (100 ml)

14.7 g $CaCl_2$ (Amresco, USA) were dissolved in 70 ml ddH_2O. Then ddH_2O were added until the final volume is 100 ml. This solution was autoclaved at 121 °C for 15 minutes and stored at room temperature.

2.2.21 10X TBE stock solution preparation (1000 ml)

108 g of Tris base (Amresco, USA) , 55 g of Boric acid (Promega, USA) and 40 ml 0.5 M EDTA pH 8.0 were measured and mixed into 200 ml sterile ddH2O in a 1 L bottle. After the solid have dissolved completely, ddH_2O were added until the volume is reached 1000 ml. The prepared solution was stored at room temperature.

2.2.22 DNAse preparation

10 mg DNAse (Sigma, USA) were dissolved in 10 ml diluent (0.1 M iodoacetic acid, 0.15 M sodium acetate pH 5.2). The prepared solution was heated to 55 °C for 45 minutes and cooled down to 0 °C. 1 M $CaCl_2$ were added until its concentration is 5 mM. The solution was then aliquoted into microcentrifuge tube and stored in – 20 °C. (Sambrook et al, 1989)