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GENETIC CHARACTERIZATION OF LOCALLY ISOLATED YEAST STRAIN THROUGH MUTAGENESIS STUDY

TAN LI LUNG

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TAN LI LUNG

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Chairman : **Hirzun Mohd. Yusof, Ph.D.**

Faculty : **Food Science and Biotechnology**

Yeast biodiversity is considered to be almost untapped from a biotechnological viewpoint. Although *Saccharomyces cerevisiae* has been the most widely studied and exploited yeast species, other yeasts are fast emerging as microorganisms of important scientific and commercial value. In this research, five different yeast strains (isolated from several aquatic and food sources) were identified using API 20C AUX kit as belonging to the genus of *Rhodotorula*, *Pichia* and *Candida*. The isolate designated as YL3, was identified with high degree of confidence as a putative haploid strain of *Pichia ohmeri*. It has a rapid growth rate and was able to tolerate growth temperature of up to 42°C and salt osmolarity of 2.5 M NaCl. Subsequently, YL3 was chosen as the candidate strain for the investigation of its physiological and molecular characteristics. Mutants of YL3 were generated using UV mutagenesis to establish suitable genetic markers. The UV dose of 115 J/m^2 (that yielded 10% cell survival) was used for the large-scale irradiation. The screening of 3×10^3 UV-irradiated random mutants yielded

seven auxotrophic mutant with different amino acid biosynthesis defects; twenty temperature-sensitive (*ts*) mutants; and sixteen osmotic-sensitive (*os*) mutants. The stable auxotrophic mutants were two *adenine-deficient* (5Ax and 22Ax), one *methionine-deficient* (2Ax) and one *histidine-deficient* (25Ax). Among the *ts* mutation observed, three were absolute *ts* mutant (7D, 9D and 11A) which showed tight growth arrest at 37°C and 40°C. The *os* mutants showed varying growth sensitivity to NaCl concentrations of 100 mM to 2 M. The majority of the *ts* and *os* mutants showed abnormal cell morphology compared to the YL3 wild-type under the stressful conditions. Temperature and osmotic shift experiments revealed that two mutants, 9D (a *ts* mutant) and 3B (an *os* mutant), have showed profound decrease in cell viability at increased temperature and osmotic stress, respectively. Interestingly, 75% of the *os* mutants simultaneously showed *ts* phenotype, indicating a close relation between the two sets of mutations. Further characterization of 9D using 4',6-diamidino-2-phenylindole (DAPI) staining revealed that these cells formed chain-like morphology with each cell compartment contained multiple nuclei under temperature stress. It is evident that the normal cell division of 9D was strongly impaired.

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PENCIRIAN GENETIK STRAN YIS YANG TERPENCIL DARI SUMBER TEMPATAN MELALUI KAJIAN MUTAGENISIS

Oleh

TAN LI LUNG

Januari 2003

Pengerusi : **Hirzun Mohd. Yusof, Ph.D.**

Fakulti : **Sains Makanan dan Bioteknologi**

Kepelbagaian biologi yis adalah dianggap hampir tidak dipergunakan sebaik-baiknya dari sudut bioteknologi. Walaupun, *Saccharomyces cerevisiae* telah menjadi spesis yis yang paling dikaji and dieksploitasi, beberapa jenis yis lain telah mulai muncul sebagai mikroorganisma yang mempunyai nilai saintifik and komersial yang penting. Dalam kajian ini, lima yis yang berlainan stran (dipencilkan dari beberapa sumber akuatik and makanan) telah dikenalpasti dengan menggunakan kit API 20C AUX sebagai yis yang tergolong dalam genus *Rhodotorula*, *Pichia* dan *Candida*. Pencilan yang ditetapkan sebagai YL3 telah dikenalpasti sebagai satu stran haploid *Pichia ohmeri* putatif dengan ketepatan yang tinggi. Ia mempunyai kadar pertumbuhan yang cepat dan dapat menahan suhu pertumbuhan yang mencecah 42°C dan osmolariti unsur garam sebanyak 2.5 M NaCl. Seterusnya, stran YL3 telah dipilih sebagai calon untuk penyiasatan ciri-ciri fisiologi and molekularnya. Mutan-mutan YL3 telah dijanakan dengan menggunakan mutagenesis cahaya lembayung unggu untuk tujuan pengasasan penanda genetik yang

bersesuaian. Nilai dos lembayung unggu sebanyak 115 J/m^2 (yang menghasilkan kadar kehidupan sel sebanyak 10%) telah digunakan untuk radiasi secara skala besar. Penyaringan 3×10^3 mutan rawak yang diradiasikan oleh cahaya lembayung unggu telah menghasilkan tujuh mutan auksotropik yang mempunyai kecacatan biosintesis asid amino; dua puluh mutan yang sensitif terhadap suhu (*ts*); dan enam belas mutan yang sensitif terhadap keadaan osmotik (*os*). Mutan auksotropik yang stabil terdiri daripada dua mutan yang kekurangan-adenin (5Ax dan 22Ax), satu mutan yang kekurangan metionin (2Ax) dan juga satu mutan yang kekurangan-histidin (25Ax). Daripada kesemua ciri-ciri mutasi *ts* yang diperhatikan, tiga merupakan mutan *ts* mutlak (7D, 9D dan 11A) yang menunjukkan sekatan pertumbuhan ketat pada suhu 37°C dan 40°C. Mutan *os* telah menunjukkan sensitiviti pertumbuhan yang berbeza-beza terhadap kepekatan NaCl dalam linkungan 100 mM dan 2 M. Majoriti mutan *ts* dan *os* menunjukkan kecacatan morfologi sel di bawah keadaan tertekan berbanding dengan stran jenis liar YL3. Eksperimen peralihan suhu dan osmotik memperlihatkan bahawa dua mutan, 9D (mutan *ts*) dan 3B (mutan *os*), masing-masing menunjukkan penurunan jangka hayat sel yang amat jelas apabila tahap penekanan suhu dan osmotik ditingkatkan. Menariknya pula, 75% mutan *os* turut menunjukkan fenotip *ts*, menandakan hubungan yang erat wujud di antara dua set mutasi tersebut. Pencirian 9D dengan menggunakaan penandaan 4',6-diamidino-2-fenilindol (DAPI) selanjutnya telah memperlihatkan bahawa sel-sel ini membentuk morfologi menyerupai rantai, di mana setiap kompatmen sel mengandungi gandaan nukleus di bawah penekanan suhu. Ia membuktikan pembahagian sel normal oleh 9D telah tersekat.

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HIRZUN MOHD. YUSOF, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Chairman)

RAHA ABDUL RAHIM, Ph.D.
Associate Professor
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

ARBAKARIYA ARIFF, Ph.D.
Associate Professor
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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A	adenine
AFLP	amplified fragment length polymorphism
Amp	ampicillin
AOX	alcohol oxidase
API	Analytical Profile Index
ARS	autonomous replicating sequence
BAC	bacterial artificial chromosome
BME	β-mercaptoethanol
bp	base pair
BSA	bovine serum albumin
5-BU	5-bromouracil
C	cytosine
$CaCl_2$	calcium chloride
c.f.u.	colony-forming unit
CDW	cell dry weight
CEN	centromere
CsCl	cesium chloride
DAPI	4',6-diamidino-2-phenylindole
DES	diethylsulphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dsDNA	double stranded deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
EES	ethylethane sulphonate
EMS	ethylmethane sulphonate
ER	endoplasmic reticulum
ERC	extrachromosomal rDNA circles
EtBr	ethidium bromide
G	guanine
GRAS	generally regarded as safe

GTE	glucose-Tris-EDTA
HMW	high molecular weight
IPTG	isopropyl-D-thiogalactoside
IR	ionizing radiation
kb	kilobase pair
λDNA	lambda deoxyribonucleic acid
LB	Luria Bertani
M	molar (mol/L)
MCS	multiple cloning site
MMS	methylmethane sulphonate
mtDNA	mitochondrial deoxyribonucleic acid
MOPS	3-(*N*-morpholino) propanesulfonic acid
MOX	methanol oxidase
NCBI	National Centre for Biotechnology Information
NER	nucleotide excision repair
NTG	nitrosoguanidine
Ω	ohm
OD	optical density
ORF	open reading frame
ori	origin of replication
os	osmotic-sensitive
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
RAPD	randomly amplified polymorphic DNA
RbCl	rubidium chloride
rDNA	ribosomal deoxyribonucleic acid
RE	restriction endonuclease
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid

rpm	revolution per minute
SAB	Sabouraud glucose
SAP	shrimp alkaline phosphatase
SC	synthetic complete
SD	synthetic deficient
SDS	sodium dodecyl sulfate
T	thymine
TAE	Tris-acetate-ethylenediamine tetraacetate
TBE	Tris-borate-ethylenediamine tetraacetate
TE	Tris-ethylenediamine tetraacetate
TEL	telomere
ts	temperature-sensitive
U	uracil
UV	ultra-violet
WT	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YAC	yeast artificial chromosome
YBC	Yeast Biochemical Card
YCp	yeast centromeric plasmid
YEp	yeast episomal plasmid
YIp	yeast integrative plasmid
YM	yeast extract-malt extract
YNB	yeast nitrogen base
YPD	yeast extract-peptone-dextrose

CHAPTER 1

INTRODUCTION

In the last 40 years, advances in molecular biology and genetic engineering have made possible not only the genetic selection, but also the genetic modification of microorganisms. As the study of microbes moves into the era of functional genomics, there is an increasing need for molecular analysis of a wide diversity of microorganisms, particularly those which employed yeast systems. Yeasts are becoming increasingly important in the "biotechnological revolution" by virtue of both their features and their very long and safe use in human nutrition and industry.

Yeast is suited for fundamental studies and heterologous protein production as it has a well-defined genetic system, established genetic manipulation procedures, and ease of growth on simple and cheap energy source. In the last couple of decades, yeast expression systems has become the system of choice for the production of eukaryotic proteins of pharmaceutical and industrial importance (therapeutic proteins, novel vaccines and drugs). Although its genetic complexity greatly exceeds that of prokaryotic counterpart, yeast expression systems hold the advantage of having the ability to perform post-translational modifications, thus producing protein products that are biologically active. The yeast system proved to be the most economically feasible and less demanding in terms of time and effort. These discoveries have led to the availability of new yeast strains fit to fulfill requests of industrial production and fermentation. The yeast expression systems have also contributed to protein function determination, gene and cell therapy strategies, and can serve as biocatalysts.

To date, more than 700 species of yeasts have been described (Boekhout and Kurtzman, 1996). Most of the genetic and biochemical studies have, however, been carried out with strains of *Saccharomyces cerevisiae*. Although a considerable amount of knowledge has been accumulated on the fundamental processes and biotechnological applications of *S. cerevisiae*, it has been found to be a non-optimal host for the large-scale heterologous protein production because of its low secretory capacity, which results in lower yield. Its inability to perform certain complex post-translational modification such as certain types of phosphorylation, amidation and undesirable overglycosylation, can result in potentially immunogenic products. Presently, *Pichia pastoris* (a methylotroph) is considered to be the best yeast expression system for commercial purposes, achieving yield of 5-40% of total cell protein. Other yeast species that has received similar attention include *Schizosaccharomyces pombe, Kluyveromyces lactis, Hansenula polymorpha, Candida boidinii, Candida utilis, Pichia methanolica, Pichia stipitis, Schwanniomyces occidentalis* and *Yarrowia lipolytica.*

The large variety of other yeast genera and species may offer various advantages for experimental study as well as for product formation in biotechnology. The genetic investigation of these "unconventional" yeasts are poorly developed and information about corresponding data is limited. It is estimated that there are 62,000 and 669,000 of undescribed genera and species, respectively for ascomycetous yeasts (Hawksworth and Mouchacca, 1994). The richness of flora and fauna in Malaysia gives high probability of obtaining potentially valuable microorganism of industrial application, particularly methylotrophic yeast strains, comparable to the established systems mentioned above.

The generation of vast genomic information from the Human Genome Project and the calls for functional genomics and proteomics can be facilitated by the development of such a novel expression system. It may contribute to the fundamental understanding of, not only the yeasts, but also eukaryotic genes at the molecular level. This brings to the main aspect of this study whereby several locally isolated yeasts and mutants generated from one of the selected yeast strain were identified and characterized. The yeasts may be of good potential to be subjected to genetic manipulation for its commercial value. As the yeasts are of novel characteristics, this study has undertaken the forward genetics approach, which utilizes the standard classical method of mutagenesis (using UV as the mutagen), with the subsequent cloning of the resulting mutant genotype by complementation with a yeast genomic library. The subtracted genomic library of a selected wild type yeast isolate (YL3), to be constructed during the course of this project, would serve as a important tool for the purposes mentioned above.

It is hoped that this study would contribute to the fundamental understanding of a locally isolated unconventional yeast, that could ultimately led to the development of expression systems comparable to the established strains such as *Pichia pastoris*. It is the aim of this project to investigate and discuss the main results of the characterization of the candidate yeast; and to clarify some aspects of the mutants generated from this strain with the objectives stated below:

1. To identify and characterize local yeast isolates and mutants generated via UV mutagenesis.
2. To develop and characterize suitable mutant yeast strains.

CHAPTER 2

LITERATURE REVIEW

2.1 General Characteristics of Yeast

Yeast is a general term for unicellular fungi, comprising phylums of Ascomycotina, Basidiomycotina and Deuteromycotina. Yeasts are grouped into 60 genera with over 700 species that have been described (Kreger-van Rij, 1987; Boekhout and Kurtzman, 1996). Yeasts are chemoorganotrophic organisms characterized by a wide dispersion of natural habitats (Phaff, 1990). The most well known and commercially significant yeasts are the related species and strains of *Saccharomyces cerevisiae*.

2.1.1 Cell and Reproduction

Yeast cells exhibit great diversity with respect to size, shape and colour. Different yeast cells can be shaped as ellipsoidal, ovoid, cylindrical, apiculate, spherical, curved, flask-shaped, triangular and elongated (Campbell, 1988). Even individual cells from a pure culture can display morphological and colourimetric heterogeneity, which are induced by alterations in physical and chemical conditions. The surface of different yeast colonies exists in several forms such as smooth, raised in the centre with a central pit, plicate, rugose, crispulate, verruculose or with concentric striations (Kocková-Kratochvílová, 1990). Powdery textures, however, are often characterized by profusion of conidia on the surface of yeast colony.

Yeasts multiply as single cells that divide by budding (the budding yeasts), by direct division through binary fission (the fission yeasts), or they may grow as simple irregular filaments (mycelium), as either haploid or diploid cells. They differ from most fungi, which grow as thread-like hyphae. Some yeasts form pseudohyphae that are chains of elongated cells formed by budding (Gimeno *et al.*, 1992). These structures differ from true hyphae by being constricted at the septa, forming branches that begin with a septation. Pseudohyphal often grow vertically into the agar below the colony, a characteristic originally thought to be typical of *C. albicans*, but found to be evident in other yeasts as well (Kron, 1997; Gancedo, 2001).

Yeast cells exist in two sexual mating types, which are hereditary phenotypes denoted by two different alleles (Sprague, 1995). The yeast sexual states are not enclosed in a fruiting body as compared to most other fungi. In sexual reproduction, most yeasts form asci, which normally contain four haploid ascospores. These ascospores may multiply through vegetative division (budding or fission) or undergo sexual conjugation with ascospores of the opposite mating type (Hammond, 1996).

2.1.2 Physiology and Biodiversity

Yeasts grow typically in moist environments where there are plentiful supplies of simple and soluble nutrients. Sources of carbon for yeast metabolism are diverse, that include basic saccharides, aliphatic alcohols, polyols, hydrocarbons, organic acids, fatty acids and polymeric compounds (Rose, 1987). Yeasts can also respire aerobically by utilizing sugars and other organic substrates. With few exceptions, they are unable to degrade biopolymers, such as starch and cellulose that are used by many hyphal fungi.

Yeasts exhibit a wide range of temperature limits for growth. The vast majority of yeasts are grouped in the category of mesophilic, where their optimum growth temperatures ranges from 10°C to 48°C (Watson, 1987).

Yeasts exhibit great specialization for habitat, which are of potential value in biotechnology (Phaff, 1986). They are common on plant leaves, flowers, fruit surfaces, roots, tree exudates and necrotic tissues of cacti (Phaff and Starmer, 1987). Yeasts associated with plant source include *Saccharomycodes ludwigii, Candida ernobii, Trichosporon penicillatum*, *Hansenula uringei* and *Pichia minuta*. Yeasts are also found in soil and aquatic sources, where they contribute to the decomposition of plants and algae. Yeasts occur in soils of different texture, chemical composition, humidity and pH values. Yeast populations are the highest in fresh water, but significantly decrease in marine waters (Phaff, 1990). The most common yeasts in aquatic sources are the red yeasts comprising almost 50% of the microbial population. These include yeasts from the genus of *Rhodotorula, Rhodosporidium* and *Sporobolomyces*.

2.1.3 Association with Humans and Animals

Yeasts can be found on the skin surfaces of insects (Phaff and Starmer, 1987), crustaceans (Hagler and Ahearn, 1987) and in the intestinal tracts of warm-blooded animals. They may live symbiotically or as parasites and pathogens of humans and animals. Most yeasts associated with humans and animals are grouped in the phylum Deueteromycotina comprising of five genera: *Candida*, *Cryptococous*, *Malassesia*, *Rhodotorula* and *Trichosporon* (Hazen, 1995); with *Candida* being the most widely occurring yeasts associated with humans (Phaff, 1990; Odds, 1994; Nguyen *et al.*,