DEVELOPMENT OF A HIGH-THROUGHPUT ASSAY FOR SCREENING PLANT EXTRACTS THAT CAN EXTEND THE CHRONOLOGICAL LIFE SPAN OF YEAST *SACCHAROMYCES CEREVISIAE*

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

CR	Calorie restriction
CLS	Chronological life span
CFU	Colony forming unit
^{o}C	Degree Celsius
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
g	Gram
x *g*	G-force
HT	High throughput
H_2O_2	Hydrogen peroxide
µL	Microliter
mg	Milligram
mL	Millilitre
mM	Millimolar
min	Minute
M	Molar
OD	Optical density
O_2	Oxygen
%	Percentage
PEs	Plant extracts
ROS	Reactive oxygen species
RLS	Replicative life span
n	Sample number
sec	Second
SEM	Standard error of mean
SC	Synthetic complete medium
v/v	Volume per volume
YPD	Yeast Peptone Dextrose medium

PEMBANGUNAN ASSAY BERDAYA TINGGI BAGI PEMERIKSAAN EKSTRAK TUMBUHAN YANG BOLEH MELANJUTKAN JANGKA HAYAT KRONOLOGI YIS *SACCHAROMYCES CEREVISIAE*

ABSTRAK

Sebatian bioaktif dihasilkan dalam bahagian tumbuhan berlainan dan sebatian tersebut boleh diekstrak sebagai ekstrak tumbuhan (PEs). Sesetengah sebatian bioaktif mempunyai sifat farmakologi seperti aktiviti antioksidan and perlanjutan jangka hayat pada organisma secara *in vitro* dan *in vivo*. Yis bertunas, *Saccharomyces cerevisiae* merupakan organisma model yang digunakan untuk mengkaji mekanisme penuaan kerana yis berkongsi laluan terpelihara dengan eukariot yang lebih tinggi. Jangka hayat kronologi (CLS) yis ditakrifkan sebagai keupayaan sel untuk kekal hidup dalam keadaan tidak bertunas. Dalam kajian ini, ekstak daun *Manihot esculenta*, *Wodyetia bifurcata* dan *Tabernaemontana divaricata* didapati boleh melanjutkan CLS yis. Ujian CLS berasaskan format 96-telaga telah dibangunkan untuk membolehkan penyaringan kuantitatif banyak PE pada masa yang sama untuk mengenal pasti ekstrak tumbuhan dengan aktiviti yang melanjutkan CLS yis. Sejumlah 223 PEs disaring, dan ekstrak *M. esculenta, W. bifurcata* dan *T. divaricata* telah diperhatikan boleh melanjutkan CLS yis secara konsisten dari hari ke-2 hingga ke-8. Pencirian lanjut ekstrak *M. esculenta* dan *W. bifurcata* menunjukkan bahawa mereka melanjutkan CLS yis sehingga 1.42- dan 1.38 kali ganda secara bergantung-kepada-dos. Kedua-dua ekstrak juga memberikan yis daya tahan terhadap tekanan oksidatif yang disebabkan oleh H_2O_2 tetapi bukan terhadap tekanan haba, menunjukkan bahawa PEs ini boleh melanjutkan CLS yis bergantung kepada, tetapi tidak terhad kepada, jalur tindak balas tekanan. Kesimpulannya, kajian ini menunjukkan bahawa tumbuhan tropika tertentu boleh

menghasilkan sebatian bioaktif yang boleh melanjutkan jangka hayat yis melalui tindakan anti-oksidatif.

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ABSTRACT

Bioactive compounds are produced in different parts of a plant, and the compounds can be extracted as plant extracts (PEs). Some bioactive compounds have pharmacological properties such as antioxidant and lifespan-extending activities on organisms *in vitro* and *in vivo*. The budding yeast *Saccharomyces cerevisiae* is a model organism for studying the mechanisms of ageing because yeast shares conserved pathways to higher eukaryotes. The chronological life span (CLS) of yeast is defined as the capacity of cells to remain viable in a non-dividing state. In this study, leaf extracts of of *Manihot esculenta*, *Wodyetia bifurcata* and *Tabernaemontana divaricata* were found to extend yeast CLS. A high-throughput 96-well based CLS assay was developed to enable quantitative screening of many PEs simultaneously to identify plant extracts with yeast CLS-extending activity. A total of 223 PEs were screened, and *M. esculenta*, *W. bifurcata* and *T. divaricata* PEs were observed to extend yeast CLS from Day 2 to 8 consistently. Further characterisation of *M. esculenta* and *W. bifurcata* extracts showed that they extended yeast CLS up to 1.42- and 1.38-fold respectively in a dose-dependent manner. Both extracts also conferred resistance to the yeast in H_2O_2-induced oxidative stress but not thermal stress, suggesting that these PEs may extend yeast CLS dependent on, but not limited to, the stress response pathway. Taken together, this study shows that certain tropical plants produce bioactive compounds that can potentially extend yeast lifespan through antioxidative action.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Ageing is an essential biological process that occurs throughout the life span of all living organisms and is controlled by both internal and extracellular factors (Vina *et al.*, 2007; Magalhaes, 2011). When time goes on, cellular and body systems start to decline with the accumulation of cellular damages and toxic substances in cells. In the human being, although physiological changes do not fully lead to pathology, it has been reported ageing is associated with many diseases such as reduce in sensory ability (Humes, 2015), cancers (Magalhaes, 2013), cardiovascular (North & Sinclair, 2012), neurodegenerative (Hou *et al.*, 2019) and metabolic diseases (Barzilai *et al.*, 2012). These ageing-related pathologies may appear in the younger group (≤ 55 years old), but they are more prone to be diagnosed in older adults.

Fundamental studies have been conducted to understand the molecular mechanisms of ageing as it plays a central role in cellular senescence and organismal ageing. According to GenAge database, there are more than two thousand genes modulate ageing of life span model organisms, and these genes are found to be evolutionarily conserved in eukaryotes mammals (Tacutu *et al.*, 2013).

Identification of novel natural compounds were carried out to improve an organism's health condition by delaying ageing process without interrupting the essential biological functions of cells (Lutchman *et al.*, 2016a). Phytochemical studies have shown that natural compounds derived from plants contain high pharmacological value towards living organisms and bioactive molecules have been successfully identified to extend the life span of model organisms (Baur *et al.*, 2006; Wilson *et al.*, 2006; Lutchman *et al.*, 2016b).

Model organisms have a relatively short generation time and life span ranged from weeks to months, and it is relatively simple to design experiments for ageing study. In this study, single eukaryotic yeast *Saccharomyces cerevisiae* is chosen as the model for ageing study because it shares pathways, which are conserved to a certain degree to higher eukaryotes.

In yeast ageing study, the life span of unicellular yeast cell can be categorised into replicative life span (RLS) and chronological life span (CLS). Replicative life span is defined as the total number of daughter cells produced by a single mother cell before it enters the stationary phase. Chronological life span measures the survival of cells in a non-dividing state.

Identification of small molecules and natural compounds that extended yeast life span have been reported previously, and this aids in the identification of anti- or pro-longevity biomarkers that further unwinds the complex network of ageing. Briefly, natural compounds in the form of plant extracts (PEs) are added into yeast culture medium, and the yeast life span is measured.

In short, this study involved the development of 96-well plate format CLS assay, high-throughput screening (HTS) of PEs using yeast CLS assay and the characterisation of PEs that extend yeast CLS.

1.2 Hypothesis and aim of this study

In previous studies, several natural compounds derived from plants are reported to have potential in extending the life span of model organisms by interacting with numerous biological processes and pathway related to ageing. However, limited studies were conducted on the discovery of tropical PEs that can extend life span, and the ageing biological activities not completely being identified. Hence, the main

objective of this study is to screen for potential plant extract(s) that contain(s) CLS-extending activity on yeast *S. cerevisiae* using high-throughput (HT) 96-well CLS assay.

This study is further divided into three specific aims: (a) to develop a yeast CLS assay, (b) to screen for tropical plant extracts (PEs) that can extend the yeast CLS using the developed assay, and lastly (c) to determine the dose-dependent manner and environmental stress response of yeast CLS-extending PEs. The approach used is outlined in Figure 1.1.

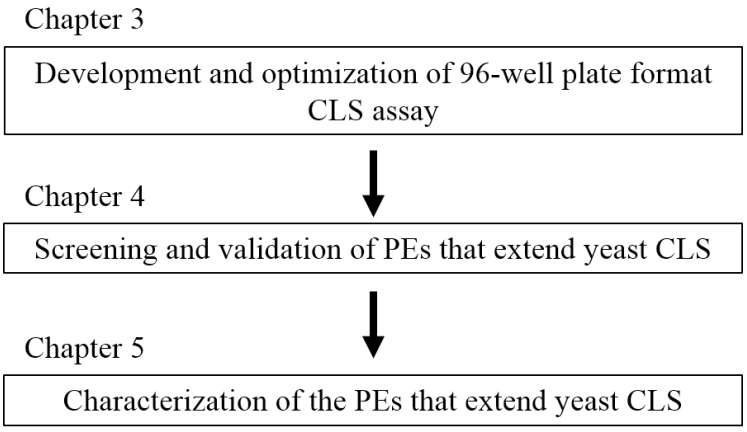


Figure 1.1 Outline and flow of the thesis

The experimental approach to screen and characterisation for PEs that extend yeast CLS. The main study was divided into three chapters with Chapter 3 on the development of the CLS assay, Chapter 4 on the screening of PEs on yeast CLS, and Chapter 5 on the characterisation studies.

CHAPTER 2

LITERATURE REVIEW

2.1 General concept of ageing in living organisms

Ageing is a natural phenomenon that occurs in all living organisms from the unicellular bacteria to multicellular organisms. It is a progressive accumulation of changes over time and these changes may increase the susceptibility of one to ageing-related diseases and death (Harman, 1981).

Ageing can be divided into whole organismal and single-cell ageing (Hayflick, 1985). Whole organism ageing is the study of the intercellular interaction of internal organs and body systems throughout their life span. For example, older people have reduced reflex receptor, slower nerve conduction velocity and brittle backbone. The physiological function of body cells starts to deteriorate, increases the vulnerability and mortality of cells and lower the capacity to respond to environmental challenges (Magalhaes, 2011). These phenotypes can be in visible form, or changes occur inside the body. Wrinkles on the skin, pigmentation spots on face and growing of white hairs are several examples of visible phenotypes affected by ageing (Passarino *et al.*, 2007). Elderly groups have a lower tolerance to extreme temperature and easily caught an infection from disease and request longer recovery time are due to reduced efficacy in altering the internal homeostasis of the body (Vina *et al.*, 2007).

Mortality of multicellular life is influenced by the death or dysfunction, or both of cells involving functions essential to the organisms (Harman, 1981). Neurodegenerative diseases are the consequences of aged cells being not able to carry out their function properly and eventually die off, while some elders also suffer from vision and hearing difficulties, experience bone fracture or osteoporosis and encounter weaker cardiovascular blood circulating system.

In a single-cell level, the fundamental mechanisms of ageing within a single cell are evaluated. Investigation of the genomics, transcriptomics and proteomics that involved in the process of life span progression assist in the understanding of the role of these biomarkers in the ageing at whole organismal level. For example, inhibition of the nutrient sensing signalling pathway in the cells upregulate the mitochondria respiration, and increase the stress tolerance towards oxidative stress, which has been proved to extend the life span of organisms (Bonawitz *et al.*, 2007; Pan *et al.*, 2011). Nowadays, several core cellular pathways have been discovered and well characterised to a certain degree to illustrate the network of the whole ageing system in a single cell. In short, these findings represent a major breaktrough in fully interpreting the complex ageing system and further delay the onset of ageing, diseases and cancers in human being.

2.2 Natural compounds provide beneficiary biological effect towards living organisms

Bioactive compounds are mainly the secondary metabolites produced in different parts of the plant that can be extracted as crude or refined plant extracts by using various extraction methods. These compounds elicit pharmacological or toxicological effects in humankind and animals (Bernhoft, 2010). They are produced and accumulated in plants alongside the primary growth, biosynthesis and reproduction. Bioactive compounds found in plants, which include phenolic, polyphenols, quinones, flavones, flavonoids, tannins, coumarin, terpenoids, alkaloids and sugar contain chemically active sites that interact with the target proteins in the organisms (Cowan, 1999). Although secondary metabolites are not involved in the primary biological processes, they are partially involved in intracellular protection and

extracellular defence mechanisms against predation from microorganisms, insects and pests (Cowan, 1999; Bernhoft, 2010). Other than the beneficiary effect, natural compounds that contain dosage-dependent toxicity effect towards cells and organisms can be identified.

Reactive oxygen species (ROS), which is mostly free radicals are produced from mitochondria respiration accumulate in cells across time that causes deleterious damage to cellular organelles (Harman, 1972; Bonawitz *et al.*, 2007; Iloki-Assanga *et al.*, 2015). Natural compounds such as flavonoids and carotenoids found in daily food contain antioxidants properties and can reduce the accumulation of ROS in organisms (Fabrizio *et al.*, 2003; Rahmat *et al.*, 2003). Evaluation of antioxidant capacity was carried out mostly *in vitro* via chemical-based assay instead of *in vivo* cellular assay (Lai & Lim, 2011; Ahumada-Santos *et al.*, 2013; López-Alarcón & Denicola, 2013; Bakar *et al.*, 2015).

Other than antioxidants properties, discovery of novel compounds that have various biological activities such as anti-microbial, anti-cancer, anti-inflammatory are conducted for novel drug innovation (Fernández *et al.*, 2001; Doughari, 2006; Choi & Ahn, 2008; Arung *et al.*, 2009; Awang *et al.*, 2010; Rao *et al.*, 2010; Okechukwu *et al.*, 2013; Gezahegn *et al.*, 2015; Shankar *et al.*, 2017). Although there are plenty of chemicals and drug prescription available for clinical treatment, some drugs are found to contain side effects on human, for example, skin rashes, inflammation, nausea and stomach upset. Medical history of many patients has reported that there is an increase of pathogenic strain with multidrug resistance ability that showed resistant to general antibiotics synthesised from microorganisms (Nikaido, 2009). Hence it is necessary to search for alternative natural compounds that exert anti-microbial activity.

2.2.1 Model organisms aid in the study of the lifespan-extending effect of natural compounds

Herbal plants have been used for medicinal purposes since the prehistoric period. Chinese herbs such as *Ginkgo biloba*, *Panax ginseng, Codonopsis pilosula,* and *Ziziphus jujuba* contain bioactive compounds that are commonly used as a health and anti-ageing supplement, benefits the long-term health of organisms (Liang & Jianhua, 1990; Wesnes *et al.*, 2000; Tahergorabi *et al.*, 2015). However, the effect of these plants was not due to a spontaneous reaction. There must be some interactions among the bioactive molecules and cells that tend to alter the internal cellular pathways (Kaeberlein, 2010; Wu *et al.*, 2014).

To determine the potential bioactive compounds and their mechanisms towards biological pathways, pharmacological studies and drug discovery are performed *in vivo* on model organisms (Pratchayasakul *et al.*, 2010; Steinberg *et al.*, 2010; Dutta & Patil, 2018). Hence, model organisms such as budding yeast *S.* cerevisiae, the fruit fly *Drosophila melanogaster*, non-parasitic roundworm *Caenorhabditis elegans*, mouse *Mus musculus* and zebrafish *Danio rerio* are introduced as the model in the laboratory for fundamental studies.

In the discovery of life span extending compounds, a number of studies have reported that at least 50 chemical and natural compounds extend life span of yeast flies, nematodes, mice, fishes and mammalian cells (Howitz *et al.*, 2003; Valenzano *et al.*, 2006; Anisimov *et al.*, 2008; Alvers *et al.*, 2009; Harrison *et al.*, 2009; Bjedov *et al.*, 2010; Choi *et al.*, 2010; Lee *et al.*, 2010; Steinberg *et al.*, 2010; Pietsch *et al.*, 2011; Leontieva & Blagosklonny, 2011; Wu *et al.*, 2014; Lutchman *et al.*, 2016a; Lutchman *et al.*, 2016b). Life span studies on ageing models and cell lines are robust, easily breed and manipulated under laboratory conditions, and they possess many biological

pathways which are conserved to a certain degree among each other and towards the human.

Ginkgo biloba extracts have been shown to improve the stress resistance and extend the median life span of wild type *C. elegans* by 8 %, while the purified components obtained from the extracts extend the life span of worms by 25 % (Wu *et al.*, 2002). Active components isolated from dried roots of *Salvia miltiorrhiza Bunge* including cryptotanshinone prolong the CLS of yeast through a nutrient-dependent mechanism similar to TOR signalling pathway (Wu *et al.*, 2014). By manipulating the nutrient component in ageing culture, bioactive compounds may regulate the signalling pathway that influences the ageing process (Wu *et al.*, 2013; Maruyama *et al.*, 2016). It also mediates the reactive oxidative species (ROS) production, one of the factors that affect CLS in yeast. *sod2*Δ mutants treated with cryptotanshinone has improved survival showed that plant extracts might protect the cells from oxidative stress (Wu *et al.*, 2014).

Another plant extract of *Rhodia rosea* (roseroot) increases the susceptibility of hydrogen peroxide H_2O_2 in yeast, but it extends the CLS of *S. cerevisiae* (Bayliak & Lushchak, 2011). These results showed that plant extracts could prolong the life span of organisms by maintaining the viability of cells during the stationary phase but not necessary support the protective mechanisms to certain intracellular substances. Thus, the investigation of compounds and their target pathways are vital for better understanding in the fundamental of ageing process.

2.2.2 The mode of action of natural compounds on model organisms

In general, not all the compounds in the secondary metabolites exert medicinal properties. Instead, only some or at least one compound isolated from the crude

metabolites is responsible for the biological activities, and these compounds are labelled as the “bioactive”. These compounds usually contain at least one active site at their chemical structure to interact with the extra- and intracellular component such as enzymes, transcription factors, neuroreceptors and DNA (Wink & Wyk, 2008; Osowski *et al.*, 2010; Wink, 2015).

Various type of chemical interactions between the active sites of bioactive compounds and the cellular components are proposed before the reactions are extended to some well-characterised biomarkers and pathways of ageing processes. These include ionic, covalent and non-covalent bonding between reactive atoms or molecules of both natural compound and cell organelles (Wink, 2015). For example, resveratrol was shown to inhibit enzymes and increase the survival of worms , fruit flies and rodents (Wood *et al.*, 2004; Baur *et al.*, 2006). Meanwhile, it also able to bind on mutagenic and carcinogenic aromatic compounds to prevent DNA intercalation that is triggered during cellular senescence. The latter is one of the unavoidable phenotypes in ageing (Lombard *et al.*, 2005; Osowski *et al.*, 2010; Lee *et al.*, 2013).

Characterisation of the action of bioactive compounds is also being studied at the molecular level. Some natural compounds extend yeast CLS dependently or independently on the ageing related signalling pathways (Alvers *et al.*, 2009; Kaeberlein, 2010b; Lutchman *et al.*, 2016a). Aromatic compounds such as flavonoids, carotenoids, terpenes, alkaloid act as antioxidant agent to protect cells from harmful reactive oxygen species that cause oxidative damages to the intracellular organelles (Cao *et al.*, 1997; Pietta, 2000; Stahl & Sies, 2003; Wu *et al.*, 2014; Lutchman *et al.*, 2016b; Young & Lowe, 2018). Nonetheless, the compound-protein interactions remain unknown and thus modelling of the structure of bioactive compounds and

proteins need to be conducted to predict the actual mode of action of these interventions in the extension of the life span of model organisms.

2.3 *S. cerevisiae* as a model organism for understanding the ageing mechanisms across evolutionary phyla

The study of ageing is difficult to be carried out directly on a human being due to the relatively long life span expectancy of 60 to 80 years old. Therefore, it is impractical for spending a long duration on a single study. Besides that, ageing is a complex biological process linking different signalling pathways in various body tissues . Thus, a few model organisms have been selected to create a clearer platform to understand the onset of ageing such as yeast, fruit flies and nematodes. *S. cerevisiae* is a notable tool for ageing study.

There are many advantages by selecting yeast for biological studies such as inexpensive growing culture, ease of manipulating the cell culture composition and rapid growth with short doubling time. Laboratory strain is usually non-pathogenic. Budding yeast is also susceptible in genetic engineering modification with various selection biomarkers, high efficiency in gene cloning through homologous recombination and contain many conserved genome regions compared to mammals (Sherman, 1991; Botstein *et al.*, 1997; Altmann *et al.*, 2007).

Budding yeast are selected to study the pathways and mechanisms of human diseases related to ageing alongside with *C. elegans,* fruit flies, mouse, and zebrafish (Rubinstein, 2003;Pandey & Nichols, 2011; Cox-Paulson *et al.*, 2012; Alexander *et al.*, 2014; Kõks *et al.*, 2016). The biomarkers of neurodegenerative diseases and cancer have been studied using yeast as a model by expressing the diseases related proteins

with the aid of genetic engineering tool (Duennwald *et al.*, 2006; Ehrnhoefer *et al.*, 2006; Guaragnella *et al.*, 2014).

One might question the appearance between yeast and human are significantly different, create an argument of selecting yeast as a model for ageing and disease research. Ageing-related pathways can be studied in yeast by identifying the related disease gene and introduced the human gene homolog (if any) into yeast genome and observing the cellular based mechanism (Karathia *et al.*, 2011; Denoth *et al.*, 2014; Pitt & Kaeberlein, 2015). Longevity pathways such as the target of rapamycin (TOR/Sch9), and Ras/adenylate cyclase/PKA in mammals are found to be conserved in model organisms ranging from yeast to mice provide a convincing platform for ageing research (Kaeberlein, 2010a; Longo & Fabrizio, 2011). It has been reported that mammalian proto-oncogene *ras* which has high abundancy in cancer cells has specific homologs in yeast gene (*RAS 1/RAS 2*) (DeFeo-Jones *et al.*, 1983; Bos, 1988). Yeast lacking both *Ras* 1 and *Ras* 2 genes encounters high mortality, and later Tatchell discovered that expressing the human *ras* gene in that mutant restored the viability of the cell (Tatchell, 1986). In short, these findings implicated to the understanding of ageing across phyla through conserved evolutionary genetic studies in yeast.

2.3.1 Replicative life span (RLS) of yeast

S. cerevisiae appears to be the simplest model to study ageing-related mechanisms and signalling pathways. Many protocols have been established to ease the identification of molecular mechanisms and factors that modify the life span of yeast (Murakami *et al.*, 2008; Murakami & Kaeberlein, 2009; Goldberg *et al.*, 2010; Jung *et al.*, 2015).

RLS is the quantitative measurement of the number of daughter cells produced by each mother cell before senescence (MacLean *et al.*, 2001; Kaeberlein *et al.*, 2007). The first paper about ageing in yeast was published at 1959 by Mortimer and Johnston, and they concluded that cells have a finite mitotic replicative capacity which does not divide forever (Mortimer & Johnston, 1959). Yeast cells divide asymmetrically by budding from the mother cells. The replicative capacity of an individual mother cell decreases when more daughter cells bud off from the same individual. RLS studies can be carried out by either using a conventional micro-dissection method (Steffen *et al.*, 2009) or HT microfluidic devices such as flow cytometry (Chen *et al.*, 2017). Both methods have a similar objective: to distinguish between mother and daughter cells for further ageing experiments. Bud scars generated on the membrane of mother cells during the budding process act as a biomarker for RLS measurement as well (Allen *et al.*, 2006).

2.3.2 Chronological life span (CLS) in yeast

Chronological life span (CLS) of yeast measures the ability of non-dividing cells to maintain viability over time, the mean and maximal surviving life span (MacLean *et al.*, 2001; Fabrizio & Longo, 2008). CLS of yeast can be quantified by direct measurement such as viability staining method or by monitoring the outgrowth of cells. The outgrowth of yeast can be determined as the ability of cells to re-enter mitotic cycle when fresh, rich media are introduced into the cell, on either solid agar form or liquid medium (Kaeberlein, 2010a).

In the stationary phase, yeast halt from dividing mainly due to the depletion of carbon source in the media. After that, yeast will switch from fermentation to respiration metabolism state. *S. cerevisiae* first utilises glucose or other fermentable

sugar such as fructose and galactose for cell division and metabolic activities. When glucose is depleted, yeast catabolizes ethanol accumulated during the log growth phase and produce acetic acid, encountered a diauxic shift followed by post-diauxic state (Fabrizio & Longo, 2003). Accumulation of acetic acid decreases the pH of the medium and caused lethal damage to the yeast cells.

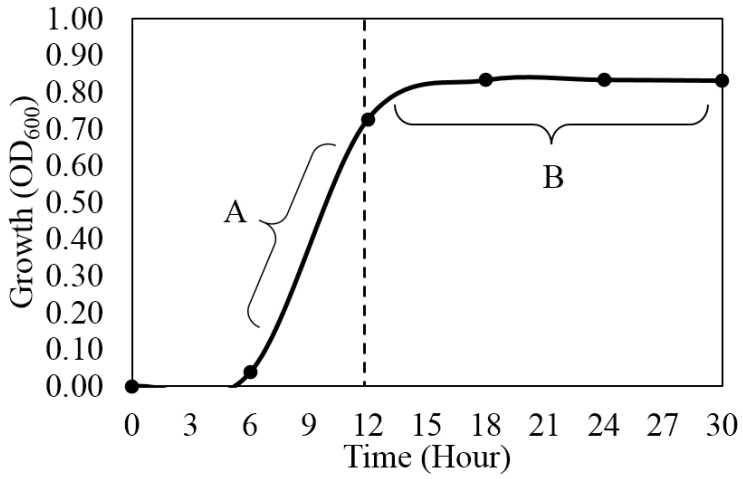


Figure 2.1 The growth curve of yeast and their two major models of ageing
(A) Replicative life span (RLS) studies are carried out when the growth of yeast is at the log phase. **(B)** Chronological life span (CLS) studies are carried out when the yeast cells reached the stationary phase.

In a standard CLS experiment, yeast cells are cultured in designed synthetic defined medium without substituting with fresh medium throughout the experiment. to ensure the cell cycle is arrested due to nutrient depletion, mainly the carbon and nitrogen source. The viability of cells is measured by using the conventional method: colony forming cell count (CFU), the counting of colonies formed on fresh, rich media plate with the aid of serial dilution. This method is seldom used nowadays due to time-consumption and high labour cost. Instead, many HT protocols firstly started by Murakami *et al.* enable researchers to determine the viability of aged cells in a

microplate format by monitoring the OD value of the aged cells throughout the experiment (Murakami *et al.*, 2008; Murakami & Kaeberlein, 2009; Goldberg *et al.*, 2010; Jung *et al.*, 2015).

The quantitative analysis generates numerical data regarding the survival and mortality of the ageing yeast cell. This method has been validated by comparing the results obtained from CFU methods and OD measurement protocol (Murakami *et al.*, 2008).

2.4 Assessment of multiple chronological life span assays in yeast

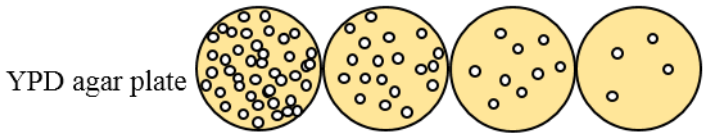
Assessment of live yeast cells is classified into cell viability and vitality. Viability studies the proportion of live cells in a whole cell population. Cell vitality examines the various physiological state of cells such as metabolic and enzyme activities, mitochondria membrane potential and cellular ATP content (Kwolek-Mirek & Zadrag-Tecza, 2014). For yeast life span study, cells are assumed alive when colonies were formed on the agar surface and considered dead if no colonies are observed. But in certain life span studies, some chemical and physiological stress (pH, osmotic and oxidative) does not lead to direct cell death (Loveless *et al.*, 1954). Instead, these factors affect the physiological state of cells by altering the intracellular metabolic pathway switch, which inhibits cell proliferation and hence failed to reproduce on solid agar and in a liquid medium.

The most commonly used method is the colony-forming unit (CFU) and spot assay. This method has been widely applied in yeast CLS studies (MacLean *et al.*, 2001; Fabrizio *et al.*, 2003; Bonawitz *et al.*, 2007; Smith Jr *et al.*, 2007; Wang *et al.*, 2008). A small volume (≤ 10 μL) of aged cells is serially diluted then plated or spotted onto a rich agar plate and incubated at least 48 hours until colonies are formed. Cell

viability was determined by counting colonies that appear on the agar plate (≤ 300 colonies on each plate), and the number of CFU per mL is calculated (Figure 2.2 A). Plating and spotting methods are relatively easy for fewer samples, but it is not practical for the screening of a large number of samples, taking into consideration of the number of dilutions, biological replicates and then the total number of agar plates needed. On the other hand, spot assay is applied for phenotype studies on the growth of cells. Cell viability of aged cells decrease over time can be observed on the reduction of colonies spotted onto the agar plate with different dilution (Figure 2.2 B).

HT quantitative analysis for yeast CLS studies was developed to replace the laborious CFU method (Murakami *et al.*, 2008; Wu *et al.*, 2013; Wu *et al.*, 2014). The aged cell population is transferred to fresh liquid medium, and the outgrowth of cells is monitored by taking OD readings every 10~30 minutes up to 24~48 hours. This CLS assay is performed on microplate which can screen at least 96 samples in one experimental run.

A CFU plate assay



Day	2	4	6	8
Colonies	255	140	86	20
Survival (%)	100	54.9	33.7	7.8

B Spot plate assay

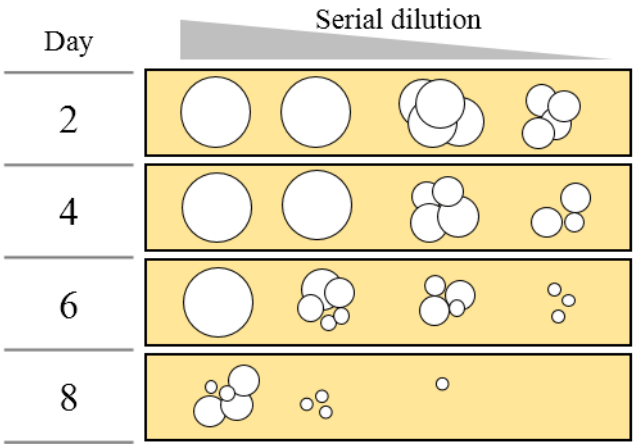


Figure 2.2 Semi-quantitative analysis for estimation of yeast survival

(A) CFU plating method and **(B)** spot plate assay for determination of yeast CLS. A small volume of ageing cell suspension is serially diluted and plated or spotted onto YPD agar plate in several biological replicates. Plates are incubated for 48 hours until colonies are formed. Number of colonies formed on the first day of CLS (Day 2 in the diagram) is referred to 100 % survival. The number of colonies formed on the plate decrease indicating ageing of cells and the viability is reduced across time. *Number of colonies on the CFU plate assay was not according to scale*.

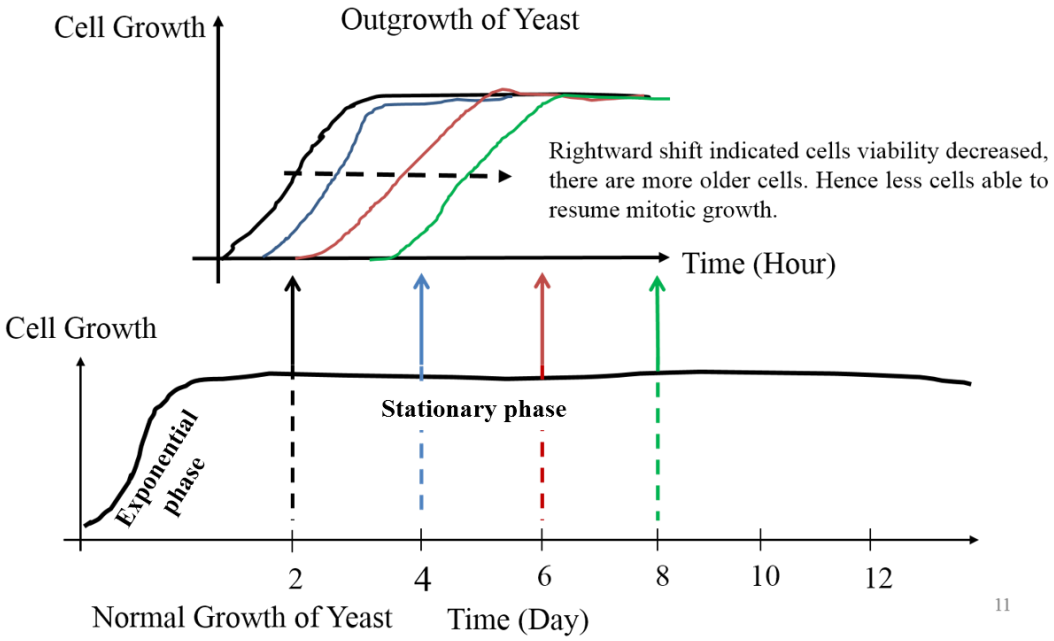


Figure 2.3 HT quantitative analysis of yeast CLS and survival

In the normal growth of a unicellular organism, cell population enter the exponential phase and divide until it reaches the stationary phase. CLS assay is initiated when cells are non-dividing. After two days incubation, few µL of cell aliquot is transferred into a microplate containing 100-150 µL of sterile rich liquid medium. The outgrowth of cells is monitored by collecting OD_{600} measurement. Outgrowth measurements are repeated on several days as required and the outgrowth curves are plotted. A rightward diauxic shift is observed indicating a reduction in the viability of the cell population in the ageing culture.

Some CLS studies were performed using microscopy and biological stain-based method (Mirisola *et al.*, 2014). Tryphan blue and propidium iodide stained only dead cell with compromised cell membrane that allows the dye to enter into cell (López-Amorós *et al.*, 1995; Fang & Trewyn, 2012). Methylene blue is diffused into both into lived and dead cells but lived cells are able to pump out the dyes and remain colourless while dead cells remained stained (Bapat *et al.*, 2006). FUN-1 stained metabolically active cells by transporting the dye across the vacuole and formed a red compact intravacuolar structure (CIVS) (Millard *et al.*, 1997; Essary & Marshall,

2009). Rhodamine 123 is used as a probe to monitor the mitochondria membrane potential which is one of the biomarker for ageing study (Ludovico *et al.*, 2001; Baracca *et al.*, 2003). The advantage of using staining and fluorescence illuminance method is that the results can be obtained within a couple of minutes or hours compare to days where CFU and outgrowth methods are used. But these methods also create a limitation on the number of samples processed. Preparation of stained sample can be labour intensive and for microscopic operating, scoring of hundreds of cells is time consuming if there are many samples to be viewed, which leads to operator fatigue and increase the probability of human error.

In short, the definition of survival of yeast cells is highly depended on the experimental design of each research, whether to check for ability to re-mitosis or determine the physiological state of the cells regardless the capability to reproduce due to metabolic and pathway alteration.

2.5 Conserved pathways that modulate yeast CLS

The target of rapamycin (TOR) signalling pathway is a complex bio-regulator that monitor the inter- and extracellular condition of yeast in response to growth factor signals, cell metabolism, nutrient availability and cellular stress (Evans *et al.*, 2011; Leonov *et al.*, 2017). Previous studies have found that TOR pathway is highly conserved across model organisms ranging from yeast to mice, which is involved in many cellular functions such as cell growth, proliferation differentiation and survival. Hence, scientists prompt to explore this pathway and determine the effects of longevity genes through a diverse model organism.

Yeast TOR proteins are serine-threonine kinases that are divided into two parts: rapamycin-sensitive manner TORC1 and rapamycin-insensitive manner TORC2

(Loewith *et al.*, 2002). TOR proteins in *S. cerevisiae* shares approximately 45% similarity to the mammalian TOR (mTOR) (Raught *et al.*, 2001). Powers *et al.* conducted a yeast deletion collection to conduct an unbiased CLS assay by screening approximately 4,800 of single-gene deletion yeast strains and found out that deletion of one of the key-component *tor1* gene extends the CLS of yeast (Powers *et al.*, 2006). In this study, depletion of amino acids or treatment with rapamycin drug inhibits the downstream of the TOR pathway and autophagy is activated. Treatment of rapamycin on TOR pathway is specific and worked on a dose-dependent manner whereby a relatively low amount of the drug can slow down, but does not inhibit the cell division of yeast (Powers *et al.*, 2006).

Calorie restriction (CR) is another most-studied aspect for ageing sciences, and many studies have shown that yeast cells cultured in medium with glucose concentration less than 2 % could extend the chronological life span of budding yeast (Smith Jr *et al.*, 2007; Murakami *et al.*, 2008). The degree of CR is referred to the glucose concentration, from mild restriction with 0.5 % to extreme restriction of 0.05 % glucose concentration in the culture medium. However, the extension of yeast CLS does not depend solely on CR. CR extends yeast CLS through a complex link to many cellular mechanisms involved in cell cycle regulation. In depletion of carbon source, four main nutrient-sensing protein complexes start to modulate the downstream process of the cell which are TOR-Sch9 signalling pathways, protein kinase A (PKA), sucrose non-fermenting protein (Snf1) and phosphate metabolism protein 85 (Pho85) (Leonov *et al.*, 2017).

Autophagy is required for rapamycin to extend yeast CLS. Autophagy defected yeast strains with deleted *atg* gene cannot extend CLS validates the fact that inhibition of TOR pathway by rapamycin extends yeast CLS through upregulation of autophagy

in the cells (Alvers *et al.*, 2009). Later in the findings, CR was reported to induce autophagy and with the presence of leucine and promote mitochondria respiratory proficiency to extend yeast CLS (Aris *et al.*, 2013).

Some of the mutants with deletion of *ras2* gene has an unusual extended life span, and these mutants are firstly discovered by studying the effect of superoxide dismutase on yeast by Longo. Deletion of *ras2* and *cyr1* genes increase the heat and oxidative stress tolerance and extend the yeast CLS (Fabrizio & Longo, 2003).

Leonov *et al.* have reviewed some cellular proteins and signalling pathways required for various phytochemical compounds to extend the life span of evolutionarily related organisms from yeast to mammals (Leonov *et al.*, 2015). Discovery of natural compounds that can extend yeast CLS aid in understanding the ageing mechanisms. Extracts of *S. miltiorrhiza Bunge*, *C. racemose*, *Valeriana officinalis L.*, *Passiflora incarnate L.*, *G. biloba*, *Apium graveolens L.* and *S. alba* have been reported to extend yeast CLS through several cellular processes and signalling pathways by examining different mutant strains that are responsible to the ageing biomarkers (Wu *et al.*, 2014; Lutchman *et al.*, 2016a; Lutchman *et al.*, 2016b).

2.6 Environmental stress tolerance of yeast

The common environmental stress face by *S. cerevisiae* are oxidative and thermal stress. Hence, yeast has several defensive mechanisms to overcome stress, and the expression of these mechanisms are elevated during the stationary phase whereby the metabolic rate is high.

Reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and oxygen free radicals are produced in yeast cells during mitochondria respiration (Perrone *et al.*, 2008; Farrugia & Balzan, 2012). For facultative anaerobes, production

of cellular ROS is unavoidable due to mitochondria respiration and oxygen consumption, hence the detoxification of ROS is essential to protect cells from oxidative damages (Pan, 2011). Hence, antioxidant defence mechanisms are induced to reduce the concentration of ROS that leads to oxidative stress (Moradas-Ferreira & Costa, 2000).

Thermal stress is also a common environmental stress encountered by *S. cerevisiae*. Thermal stress responses are mediated by the heat shock proteins HSPs when heat shock is introduced to the cells (Boy-Marcotte *et al.*, 1999; Verghese *et al.*, 2012). Failure of response to thermal stress is lethal because it can cause physiological changes to cells such as inhibition of cell growth and proliferation, misfolding and aggregation of proteins, alteration of the permeability of cell walls and membrane (Rowley *et al.*, 1993; Verghese *et al.*, 2012).

In *S. cerevisiae*, inhibition of TOR-Sch9 and PKA pathways extend yeast CLS by enhancing the stress response transcription factors Msn2/Msn4 and serine/threonine-protein kinase Rim15 (Boy-Marcotte *et al.*, 1999; Powers *et al.*, 2006; Deprez *et al.*, 2018). Overexpression of mitochondria superoxide dismutase gene *SOD1* (), exposure of yeast cells to addition of adenine and nucleobases, calorie restriction and elevated mitochondria ROS during the exponential growing phase increases the stress tolerance and extend yeast CLS (Fabrizio et al., 2003; Pan *et al.*, 2011; Ocampo *et al.*, 2012; Spencer *et al.*, 2014). This suggested that yeast cells easily adapted to stress by upregulating the stress tolerance defence systems as at the early growing phase.

In summary, yeast extends CLS by several factors but not limited to downregulation of nutrient and growth sensing pathway connecting the upstream and downstream of these core hubs, calorie restriction, alteration in the nutrient

composition and upregulation of autophagy process and overexpress of stress response genes.

CHAPTER 3

DEVELOPMENT OF CHRONOLOGICAL LIFE SPAN ASSAY

3.1 Introduction

High-throughput (HT) methods allow researchers to perform a large-scale genome-wide and chemical screens for identification novel biomarkers of ageing and diseases, virulence factors of pathogenic microorganisms, small compounds for extension of life span, with further approaches in genomics, proteomics and transcriptomics studies.

In yeast CLS studies, HT methods were established as well for large-scale screening of compounds or genes involved in the extension of yeast life span. Instead of counting colonies formed in the CFU method, HT CLS assay monitors the outgrowth of cells by taking OD measurement in fixed age-point and time-interval using an automated microplate reader. From there, the doubling time, growth rate, time to reach the early exponential phase at each age-point, lastly the survival percentage and integral can be calculated based on OD readings.

HT CLS assay has been applied by Powers *et al.* to determine the CLS of approximately 4,800 single-gene deletion strains of yeast, to identify the possible biomarkers of ageing mechanisms in yeast (Powers *et al.*, 2006). The CLS assay was further improved and modified by several research groups to generate an accurate and a reliable quantitative analytical data for ageing studies using yeast as a model (Murakami *et al.*, 2008).

Development of 96-well plate format CLS assay is important in this study to suit the local laboratory conditions and equipment. This development will be applied in the HT screening of more than two hundred plant extracts to investigate the potential in extending the CLS of yeast.

Absorption spectrometry is widely used in research to quantify biological components such as the concentration of DNA, protein, enzyme and cells (Nilapwar *et al.*, 2011). However, optical density is an arbitrary reading that can be affected by cell density, cell size, type of medium (either SC or YPD medium in this study) and the condition of different model of the spectrophotometer. Same strain with similar cell concentration will also produce different OD values depending on the values was measured using a cuvette or microplate. There, the relative variability of OD measurements using the Bio Microplate Reader HiTS versus serial dilution and plating for CFU was examined as well to determine the relationship between OD values and cell colonies count.

In this study, yeast CLS and survival was estimated based on the OD values collected during the outgrowth measurement due to the high-throughput requirement to screen up to 96 samples simultaneously in a relatively short period.

3.2 Experimental approach

S. cerevisiae yeast strains 1783 and MLC30M were cultured in the volume of 1 mL (Bijou bottle) and 180 µL (96-well microplate) to evaluate their outgrowth and the diauxic shift of outgrowth curves. Both methods were carried out simultaneously to compare the CLS results and consistency between two different ageing method. A standard curve for yeast measured with OD at the wavelength of 600 nm versus serial dilution and plating for CFU was performed to examine the correlation between OD measurement using Bio-Microplate Reader HiTS and number of cells in the unit of CFU/mL. In short, two *S. cerevisiae* laboratory strains were evaluated for the development of CLS assay using the local laboratory conditions to observe the CLS extension and to select the suitable strain for further CLS studies.