

REPUBLIC OF TURKEY
BINGOL UNIVERSITY
INSTITUTE OF SCIENCE

**DETERMINATION OF *Saccharomyces cerevisiae* RESISTANT TO
PCM OBTAINED BY INVERSE METABOLIC STRATEGY AND
TRANS-EFFECT OF ITS METABOLITES ON PC3**

PhD THESIS

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BIOLOGY

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FOREWORD

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ABBREVIATIONS

GRAS	: Generally recognized as safe
PC3	: Human prostate cancer cell line
DMEM	: Dulbecco's Modified Eagle Medium
ROS	: Reactive Oxygen Species
TME	: The tumour microenvironment
SCFAs	: Short-Chain Fatty Acids
ORFs	: Open Reading Frames
H ₂ O ₂	: Hydrogen Peroxide
ESR	: Environmental Stress Response
YPD	: Yeast Peptone Dextrose
YMM	: Yeast Minimal Medium
EGM	: Endothelium Growing Medium
RPMI	: Roswell Park Memorial Institute medium
PBS	: Phosphate Buffer Saline
FBS	: Fatal Bovine Serum
DCFH-DA	: 2,7'-Dichlorofluorescein diacetate
EDTA	: Ethylenediaminetetraacetic acid
TCA	: Trichloro acetic acid
TBA	: 2-thiobarbituric acid
DAB	: Diaminobenzidine
EMS	: Ethyl methyl sulfonate
SDS	: Sodium dodecyl sulphate
HUVEC	: Human Vascular Endothelial Cells
PCM	: PC3 cultured medium
MCM	: MCF-7 cultured medium
HCM	: HUVEC cultured medium

WT	: Wild Type
MPN	: Most Probable Number
MYn	: Mutant Population obtained in YMM cultured
MPn	: Mutant Population obtained in PCM cultured
WT-DM	: DMEM cultured with WT
MP-DM	: DMEM cultured with MP2
WY-DM	: DMEM cultured with MY2
SFM	: Serum Free Medium
CYCS	: cytochrome c
CASP9	: caspase-9
CASP3	: caspase-3
QRT-PCR	: Quantative Real Time polymerase reaction
Ct	: comparative threshold
ECL	: Enhanced Chemiluminescence
TUNEL	: Transferase dUTP nick end labeling
MPTP	: Mitochondria membrane permeability transition pore
ESR	: Environmental Stress Response

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ABSTRACT

Cancer is a chaotic disease to treat, because of the cell variety and the multiplicity of intracellular pathways that are a major problem to find a simple and definitive solution. Furthermore, in order to investigate an accurate remedy to cancer, it is necessary to know the entire metabolism in detail, but the current level of knowledge and technology are not sufficient for that. Thus, it is required alternative approaches to struggle with cancer-like diseases. In this study; we aimed to use a natural strategy, which is based on to obtain the mutant strain of *S.cerevisiae* by an *in vivo* evolutionary engineering, which is able to easily grow in prostate cancer cells (PC3) environment. In addition, the counter effect of DMEMs (Dulbecco's Modified Eagle Medium), fermented with these individual mutants and WT (Wild Type) yeasts, in PC3 cells were assessed by several molecular assays such as cell growth, oxidative injury, cell migration and apoptosis related gene expressions.

Evolutionary engineering was applied to WT yeast population, which has the bad growth fitness in PCM (DMEM cultured with PC3 cells). The population treated with a chemical mutagen, EMS (Ethyl Methane Sulfonate), to randomly generate a variety of genetic phenotypes. The best individual mutants with PCM-resistant phenotype were selected with genetic stability assays applied. Additionally, the selected mutants and WT were separately cultivated in DMEM until logarithmic phase to gain a fermented medium. In order to clarify the effects of each DMEM cultured with mutant and WT yeasts (WT-DM, MP-DM, and MY-DM) in PC3 culture were examined by measuring oxidative damage, apoptotic index, cell migration and gene expression assays.

According to the findings of the present study, the growth fitness of mutant yeasts dramatically increased in PCM, which compared to WT. Therefore, the randomly EMS-mutagenized population probably consists of the desired colonies that can normally grow in PCM. This study further displayed that WT-DM, MP-DM, and MY-DM significantly decreased cell growth by inducing apoptosis in PC3 cell culture. However, MP-DM increased apoptotic index whereas it was downregulated the expression of apoptotic genes. Unlike WT-DM and MP-DM; MY-DM simultaneously activated many molecular pathways, for instance, elevated ROS production, suppressed cell migration and upregulation of apoptotic gene expressions, to promote apoptosis in PC3 cells. As a conclusion, in order to alter the situation that is the restricted growth of WT in PCM, the current study was successfully applied evolutionary engineering strategies to obtain the desired phenotypes (MY2 and MP2). Moreover, the results indicated that WT-DM and MP-DM, MY-DM include various effective metabolites to induce apoptosis in PC3 cells.

Keywords: Evolutionary engineering, cancer, PC3, *Saccharomyces cerevisiae*, yeast, fermented medium.

TERS METABOLİZMA YÖNTEMİ İLE PCM'E DİRENÇLİ *Saccharomyces cerevisiae* ELDE EDİLMESİ VE METABOLİTLERİNİN PC3 ÜZERİNDEKİ ETKİSİ

ÖZET

Kanser, kaotik bir rahatsızlıktır, çünkü hücre çeşitliliği ve hücre içindeki yolların çokluğu, basit ve kesin bir çözüm bulmayı zorlaştırır. Dahası, kansere karşı kesin bir çare bulmak için, metabolizmanın tamamını ayrıntılı olarak bilmek gerekiyor, ancak mevcut bilgi ve teknoloji seviyesi bunun için yeterli değil. Bu nedenle kanser benzeri hastalıklarla mücadele için alternatif yaklaşımlar gereklidir. Bu çalışmada amacımız doğal bir strateji olan evrimsel mühendislik yöntemi ile prostat kanser hücrelerinin kültürlendiği ortamda, kolayca büyüeyen *S. cerevisiae* mutant suşu elde etmektir. Buna ek olarak, elde edilen mutant ve WT (doğal suş) mayalar, DMEM ortamında kültürlenerek fermente besiyerileri elde edildi. Böylece bu fermente DMEM'lerin PC3 hücreleri üzerindeki karşı etkileri oksidatif hasar, hücre göçü, gen ifadesi ve hücre büyümesi gibi birkaç moleküler yöntemle değerlendirildi.

Evrimsel mühendislik yaklaşımı, PCM ortamında (PC3 hücreleriyle kültürlenmiş DMEM) iyi büyüemeyen WT maya popülasyonuna uygulanmıştır. Başlangıç popülasyonunda çeşitlilik oluşturmak için bir kimyasal mutajen olan EMS (Etil metansülfonat) rastgele mutasyon oluşturmak için kullanıldı. Genetik kararlık testleri kullanılarak, PCM'ye dirençli fenotip gösteren en iyi bireysel mutantlar belirlendi. Daha sonra, seçilen mutantlar ve WT, mayalanmış bir ortam elde etmek için logaritmik faza kadar ayrı ayrı DMEM'de büyütüldü. MY2, MP2 ve WT ile mayalanmış DMEM'lerin (WT-DM, MP-DM and MY-DM), PC3 hücreleri üzerindeki etkisini anlamak için oksidatif hasar, apoptotik indeks, hücre göçü ve gen ekspresyon testleri uygulandı.

Mevcut çalışmaya göre, evrimsel mühendislikle elde edilen mutant mayaların PCM içindeki büyüme becerisi, WT'ye kıyasla, bariz olarak arttı. Bu çalışma ayrıca, WT-DM, MP-DM ve MY-DM'nin PC3 hücre kültüründe apoptozu indükleyerek hücre büyümesini önemli ölçüde azalttığını göstermiştir. Bununla birlikte, MY-DM; WT-DM ve MP-DM'den farklı olarak, PC3 hücrelerinde apoptozu teşvik etmek için birçok moleküler yolu aynı anda aktive etti. Örneğin artan ROS üretimi, azalan hücre göçü ve apoptotik gen ifadelerinin artan regülasyonu. Diğer yandan ise, MP-DM apoptotik indeksi artırırken, apoptotik gen ekspresyonunu azaltmıştır. Sonuç olarak, mevcut çalışmada PCM'de kısıtlı büyüyen WT yerine, MY2 ve MP2 mutantları başarılı bir şekilde elde edilmiştir. Ayrıca, WT-DM ve MP-DM, MY-DM'nin PC3 hücrelerinde apoptozu uyaran çeşitli etkili metabolitleri içerdiğini de gösterdi.

Anahtar Kelimeler: Evrimsel mühendislik, kanser, PC3, *Saccharomyces cerevisiae*, maya, fermente besiyeri.

1. INTRODUCTION

Cancer is mostly defined as unchecked growth and division of normal cells in an advanced organism (Meng et al. 2012), so that causes a connection error between a maverick single cell and tissue. However, there is a sensitive balance between the internal and external environment of the cell to sustain communication with all tissues in a normal living system. There are many responsible factors in cells to disrupt this connection and induce cancerogenesis. For example, a cell metabolism involves countless mediators associated with internal and external pathways which open to many failures leading to cancers. Cumulatively repeated defect in any pathway can trigger cancer by removing the cell from the control mechanisms (Bertram 2001). Furthermore, the cancer types varied in advanced organisms because of the tissue-specific cell diversity, such as brain, blood, basal, renal, ductal cell carcinoma. In addition, a cancer type may give a different response to same treatment from person to person, because the cancer of each person may be promoted by a different defect in same cell type (Hanahan and Weinberg 2011). It is hard to find a common solution in such a disease, thus the each of them requires a specific treatment approach. Recently, accumulative knowledge about cancer has emerged that disease treatment approach should be unique to its development process (Dawson and Kouzarides 2012) because the conventional treatments and the existing medicines cannot enable a clear remedy for the complexity of the disease.

Hereafter, there is a need to research a simple and natural solution against cancer-like chaotic disorders. In order to find a right solution, nature may be a potential candidate with perfect systems against chaos. For example, all organisms have the ability to easily take a position in habitat according to micro and macro changes in the physical, social and psychological environment (Francescone et al. 2015). In short, they are robustness to survive in hard conditions, because there is a commensal, symbiotic or pathogenic relation between each of them. This property can be an example to investigate a treatment

for cancer-like serious disease causing extreme environmental conditions. Recent evidence shows the importance of interactions between host and mutualist organisms for the health of both of them (Cho and Blaser 2012).

Last ten years, researchers have revealed that the advanced organisms have an extra organ called "microbiota" spreading all over the body (Louis et al. 2014; Francescone and Hou 2015). The microbial integrity and diversity of this organ are important to protect their hosts against modern diseases such as cancer, diabetes and neurodegenerative (Ma et al. 2004; Bultman 2014). It is well-known that microbial production is crucial to balance homeostasis of the advanced organism (Vaishnava et al. 2008; Garrett et al. 2010). The most important points about the metabolite production of microbiota are, affecting each other and modifying environment according to their living conditions (Holmes et.al. 2012; Louis et al. 2014). These findings of microbial production may be used to improve a new approach for cancer and other diseases.

Organisms can be directed to regulate their habitat via their metabolite production. Especially, the adaptation of microorganisms to changing environmental parameters is easily followed by scientists because of their short life cycle. The most famous examples of this adaptation are antibiotic resistance lately worried the world (Laxminarayan et al. 2013) and rapid mutation of influenza type virus to escape vaccines (Zinder et al. 2013). The rapid adaptation capability of microorganisms may be used for desired metabolite production to modify extreme environment (Smanski et al. 2016; Zhang et al. 2016).

To change the environmental conditions, it is required a resistant phenotype which is able to adjust habitat according to itself. However, in order to obtain specific characteristics, the all molecular mechanisms in an organism must be known. Although the regulator effect of microbial fermented medium on the metabolism of various organisms have been assessed (Holmes et al. 2012; Fritz et al. 2013), the current technology and the data about cell metabolism are inadequate to obtain the desired phenotype through bioengineering, yet. That is required a new approach to attain a special phenotype.

The classic metabolic engineering (Bailey 1991) is mostly used to modify a phenotype by manipulating genes, enzymes, and pathways (Cakar 2009). However, the necessity of detailed information about the entire molecular mechanism is a major limitation of the classic way to create an aimed phenotype. In this case, the most important point is, determine the right methodology to access phenotype with the specific character which makes extreme environment habitable for itself. Inverse metabolic engineering was developed by Bailey and co-workers (1996) to select individuals with certain characteristics in population as a highly effective and successful method to be alternative to the classic metabolic engineering (Cakar 2012).

To achieve the desired phenotype with classic metabolic engineering method is needed to be master of all cell metabolism (Bailey et al. 1996). On the other hand, evolutionary engineering as an approach in inverse metabolic engineering provides advantages of studying with population rather than single individual because to find specific phenotypes, the random mutational changes should be created in each individual of the population (Sauer 2001; Cakar et al. 2012; Almario et al. 2013).

The evolutionary engineering was successfully applied to various microorganisms to obtain preferred individuals (Sen et al. 2011; Liu et al. 2015; Morales et al. 2017). Moreover, a number of studies have physiologically and productively improved *Saccharomyces cerevisiae* with evolutionary engineering strategy (Cakar et al. 2009; Lee and Oh 2015). *S. cerevisiae* is used as model organism in research for a long time. It is also generally recognized as safe (GRAS) to use in an industrial process such as bread, beer and wine production (Hong and Nielsen 2012). It has many advantages to be a model organism in studies such as short life cycle, rapid growing and representing advanced organism. Eventually, although the evolutionary engineering is a simple and outstanding method to generate numerous mutants in yeast population providing advantages to select desired one, it is not clarified yet which mechanisms are behind that.

The secret behind the quickness and easiness of biological events is still not understood. Recently, there are a lot of debates about the quantum phenomenon to might be responsible for the mystery in biological systems. Additionally, the current evidence

recommends that the quantum mechanics with unique features may be a secret part of the biological systems to provide various advantages to the organisms. These features are more than trivial quantum effects and may give the opportunity to the organisms to take advantages of quantum coherence on physiologically level (Lambert et al. 2012). If the quantum phenomenon is possible, it can also lead to evolutionary mutations (Bordonaro et al. 2014). Therefore, the quantum impact might be tried with the evolutionary engineering strategy used to obtain a phenotype with special ability to make cancer environment habitable.

In this study, there were two purposes associated with each other. The first one was based onto obtain resistant *S.cerevisiae* mutants that can grow in human prostate cancer cell line (PC3) cultured Dulbecco's Modified Eagle Medium (DMEM) by evolutionary engineering, an approach to inverse metabolism engineering. To obtain the desired mutants, the evolutionary engineering was applied in two different ways, normal and quantum impacts. The second aim was an assessment of the anticancer-activity of DMEMs fermented by the most resistant mutants which were obtained with the first aim. Therefore, PC3 cells were treated with DMEMs containing the metabolite production of best mutants to examine with the various molecular assays. The results were discussed to understand whether the survival instinct of microorganisms can be a potential treatment against cancer-like complex diseases.

There are six sections in this study. After the introduction, in section 2, it is mentioned about cancer development, cancer cell production and environment, its common treatment approaches and the important of microbial metabolites on homeostasis. Moreover, there is knowledge about *S.cerevisiae* as a model organism, its stress metabolism and evolutionary engineering, an approach to inverse metabolism engineering. In section 3, it is explained the materials and methods to obtain the desired phenotype and treated its metabolites on PC3. After that, the vital part, section 4 encloses the results of assaying metabolites of desired *S.cerevisiae* phenotype on PC3. The last part of the study discusses general findings and suggests some future aspects.

2. LITERATURE REVIEW

2.1. Cancer Progression and Environment, Prostate Cancer

Cancer is an anomaly that is the result of uncontrolled cells rejecting the normal cell cycle. Normal cells have perfectly controlled metabolic machinery with complex signalling networks between inner and outer environment to survive. The reproduction is an energy-dependent process, thus normal cells have to activate metabolic pathways to proliferate. As a result of this metabolic boost and with various outer signals, DNA damage and oxidative stress may be unavoidably promoted by reactive oxygen species (ROS) generated in energy metabolism. These kinds of alteration in cell metabolism may cause several damages in the sequence of oncogenes and tumor-suppressor genes that can remodel multiple intracellular signalling pathways to facilitating tumorigenesis (Vogelstein and Kinzler 2004).

A tumour can immediately modify its environment according to its own requirements. Cancer cell plasticity arises tumour heterogeneity as a consequence of phenotypic and functional effects of genetic change and environmental, in cell metabolism (Berridge et al. 2010). Therefore, tumour cells quickly adapt to unfavourable environmental conditions with alterations of metabolic pathways to generate sufficient energy (Chen et al. 2008). The tumour microenvironment (TME) has a significant impact on tumour pathogenesis. Over the last years, the importance of TME in cancer progression has also been determined to understand tumour behaviour. TME includes the accumulated metabolite production of cancer cells that cause less oxygen (hypoxia), high lactate and glucose and energy deprivation (Vogelstein and Kinzler 2004; Semenza 2010). As shown in Figure 1.1, tumour cells also secrete chemokines being responsible for paracrine interactions between cancer cells and the TME to modify tumour growth, homeostasis, and progression (DeBerardinis et al. 2008).

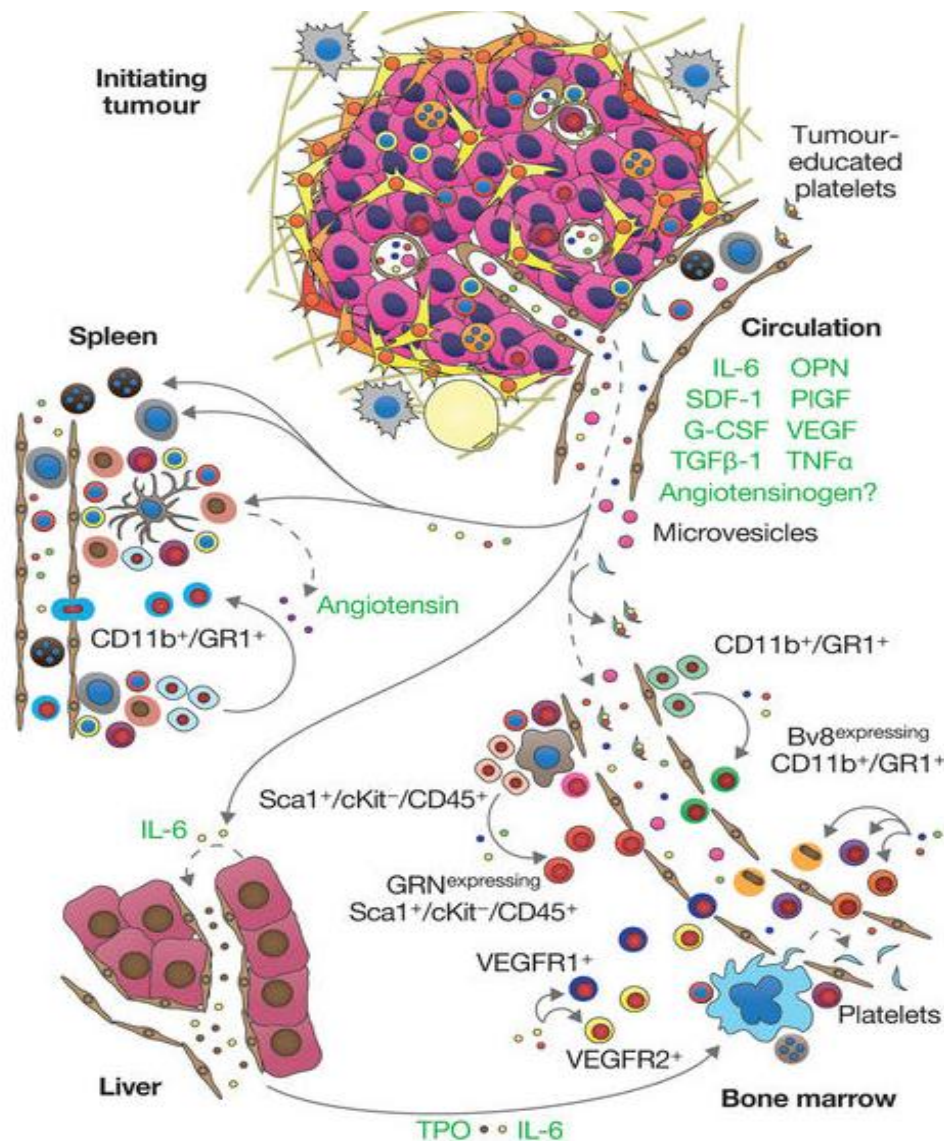


Figure 2.1. The scheme represents the molecule secretions of tumour cells in TME and the interactions of them between cancer cells and distant sites (McAllister and Weinberg 2014)

The world has troubles with the many cancer types being extremely heterogeneous characteristics according to tissue origin (Griguer et al. 2005). Each tissue has the specialized cells to perform its functions, thus any carcinogenesis in these cells will be unique to this tissue. Actually, it is possible to say that the number of cancer types is equal or more to tissue types. The most common ones are bladder, breast, colorectal, kidney, lung, pancreatic, prostate and lymphoma cancers.

Prostate cancer is also one of the most common types. The high number diagnosis of prostate cancer is related to multiple genetic and demographic factors such as age, family history and genetic susceptibility (Teerlink et al. 2016). Although there is a great effort to determine the profile of prostate cancer using modern molecular techniques, the biological processes promoting progression are still not clarified because of the difficulty in investigating small lesions. Therefore, it is still known as the first most common cancer in men (Siegel et al. 2015). The cancer studies published many data about the numerous factors in disease, which makes difficult to suggest an accurate solution for treatment. Recently, nature is attracted scientists to search for new approach against cancer. Furthermore, the importance of health balance between host body and microorganisms is well-understood in cancer-like diseases (Ohtani 2015; Roy and Trinchieri 2017).

2.2. Microbiota and Cancer

The relationship between microbiota and the human body begins at birth (Maynard et al. 2012) with the habitation and colonization of microbes, where the body directly in contact with air such as the gastrointestinal and urogenital tracts, mouth, skin and nostrils (Cho and Blaser 2012). However, the bacterial community of residence within each organ system differs from individual to individual, that is the consequence of diet, genetic, drug intakes such as antibiotic and other external environmental factors (Lozupone et al. 2012; Maynard et al. 2012). The quality and quantity of microbial composition in the human body are essential for human health.

The microbiota is named an extra organ of the human body due to its beneficial effects. It has several properties to maintain host homeostasis, for example producing toxins against pathogenic strains, altering the pH of the local environment and struggling with their competitors to metabolize key nutrients (Gamage et al. 2006; Fabich et al. 2008), and also protect epithelial integrity and mucosal layers by activating the host immune system (Garrett et al. 2010). The integrity of site-specific microbiota diversifies according to a location in the body, and this variety can be associated with human health.

There is a balance between microbial activity and host body, which is determined by both physical borders and the chemical integrity of metabolite production. For example, the epithelial barriers between microbiota resides should be maintained for the health of the organism. If these barriers, which provide a peaceful relationship between the microbial communities to protect the host from pathogens, is disrupted by alteration in the microbiota diversity (dysbiosis), that leads inflammatory pathologies and may induce to cancer initiation and progression (Rao et al. 2006; Jobin 2012; Goldszmid and Trinchieri 2012) (Fig 1.2).

The microbiota is associated with many diseases. At the first, the microbiota of gastrointestinal mucosa can modify local homeostasis, functions, and immunity (Vaishnava et al. 2008; Peterson and Artis 2014). There have been many described mechanisms for various bacterial products affecting mucosal physiology and pathology (Belkaid and Naik 2013; Peterson and Artis 2014). Furthermore, the recent data is correlated the intestinal microbiota with the pathogenesis of diseases in distant organs (Holmes et al. 2012) such as fatty liver (Henao-Mejia et al. 2012), arteriosclerosis (Koeth et al. 2013), and neural disease (Collins et al. 2012).

The intestinal microbiota produces various metabolites, the most-known are the three short-chain fatty acids (SCFAs), various gases and organic acids, which has a key role to suppress the inflammation and cancer in the intestine (Louis et al. 2014), by fermenting many of the undigested dietary components in the colon. According to recent evidence, these microbial metabolites can also induce inflammation and the DNA damage in the intestinal epithelial cells, leading certain types of cancer (Jobin 2012; Maynard et al. 2012; Puhr et al. 2016).

2.3. The Model Organism *S.cerevisiae*

The *S.cerevisiae* genome contains about 12 million base pairs in 16 chromosomes and the 23% of that sequences overlap with the human genome. There are nearly 6000 open reading frames (ORFs) in the yeast genome, that makes yeast has a larger expressed genome region (72%) rather than other higher eukaryotes. Therefore, *S.cerevisiae* is

chosen as a model organism to easily identify homologous genes on other eukaryotes and determine their functions (Dujon 1996; Goffeau 2000). Although using as a model microorganism, there is a competition between *S.cerevisiae* and *Escherichia coli*, yeast is mostly preferred because of its fast growth, fully understood the genetic system, easily obtaining mutants and open to various manipulations (Aa et al. 2006).

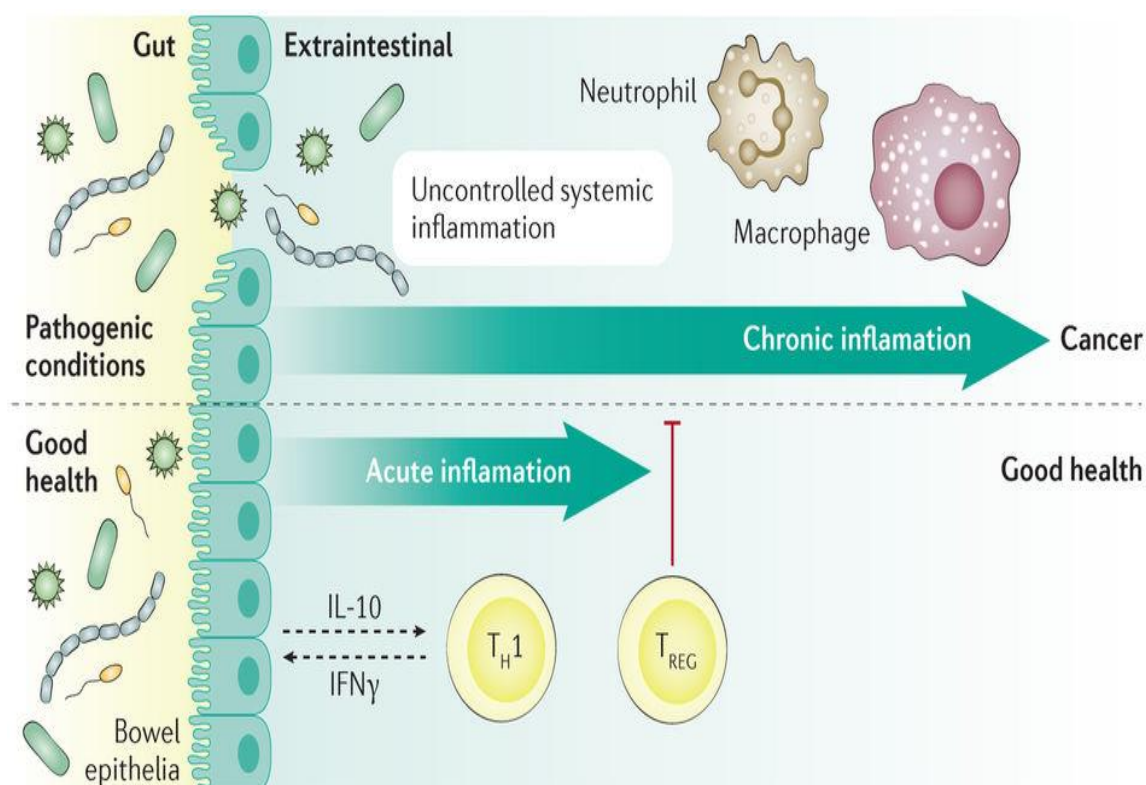


Figure 2.2. The relation between gut microbiota and extraintestinal environment (Prados 2016)

In research, it is an advantage of *S.cerevisiae* to be small like prokaryote, and also as a eukaryotic, it represents advanced organism to study complex diseases understand disease in detail (Hartwell 2002; Natter and Kohlwein 2013). *S.cerevisiae* is a monocellular model organism that is preferred in a number of research areas. It is also defined as baker's yeast or brewer's yeast. There are two important scientists in the history of yeast from 1935 to perform genetics studies, Ö.Winge from the Carlsberg Laboratory in Copenhagen and Carl C. Lindegren in Washington University (Hall and Linder 1993).

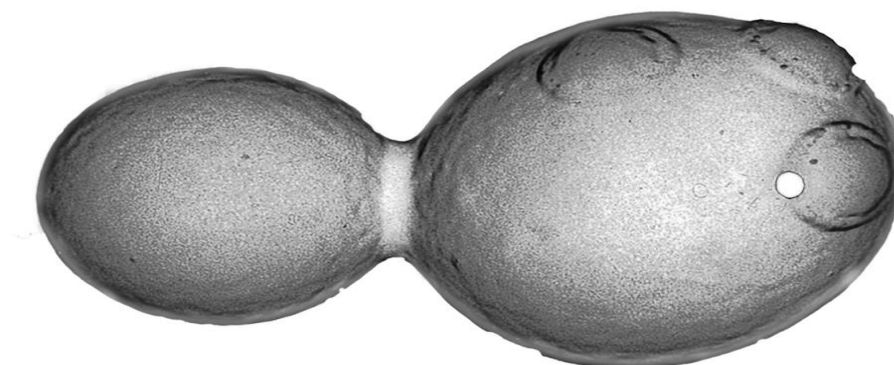


Figure 2.3. Scanning electrongraph image of *S.cerevisiae* cells grown (Coleman et al. 2008)

The first totally sequenced eukaryotic genome is *S. cerevisiae* S288c arrived other laboratories from C.C. Lindegren (Mortimer and Johnston 1986; Goffeau 2000). The mostly used *S.cerevisiae* strains in research are A364A, W303, FL100, CEN.PK, S1278b, SK1 and BY4716 (Schacherer et al. 2007).

Table 2.1. Size and composition of yeast cells (Sherman 2002)

Characteristics	Haploid Cell	Diploid Cell
Volume (μm^3)	70	120
Size (μm)	2-5	5-7
Composition 10^{-12}g		
Wet weight	60	80
Dry weight	15	20
DNA	0.017	0.034
RNA	1.2	1.9
Protein	6	8

S. cerevisiae can reproduce in two different ways, either vegetatively or sexually. It leaves from parent cell by budding during the vegetative reproduction. This process can repeat 20-30 times for each parent cell during its lifetime. Furthermore, the number of bud scars on the cell surface gives knowledge about the age of parent cell (Egilmez and Jazwinski 1989). There is a typical budding cell with the bud scars on the cell surface in Figure 2.3. The other reproduction way of *S.cerevisiae* is the sexual reproduction. It has the ability to grow as both haploid (n) and diploid (2n). As shown in Table 2.1, there are some differences between haploid and diploid cells of *S.cerevisiae* (Sherman 2002).

The sexual reproduction of *S.cerevisiae* is controlled by two mating types of haploid cells, *MATa* and *MAT α* which have a different allele of MAT locus on chromosome III (Elion 2000). There is communication between *MATa* and *MAT α* cells with secreted special pheromones, causing a “shmoo” form to respond to the opposite mating type (Fig. 2.4). The mixing of different mating types in equal amount provides an opportunity to obtain a diploid strain. In a subsequent process, the fusion of two shmoos forms a zygote with the diploid nucleus.

S.cerevisiae is generally grown in two kinds of media at 30°C; the complete medium like (2% ‘w/v’ glucose, 2% ‘w/v’ peptone, 1% ‘w/v’ yeast extract) and the synthetic medium (2% ‘w/v’ glucose, 0.67% ‘w/v’ yeast nitrogen base). According to ingredients of the medium, the doubling time of yeast at exponential growth phase can change between 90 and 140 min.

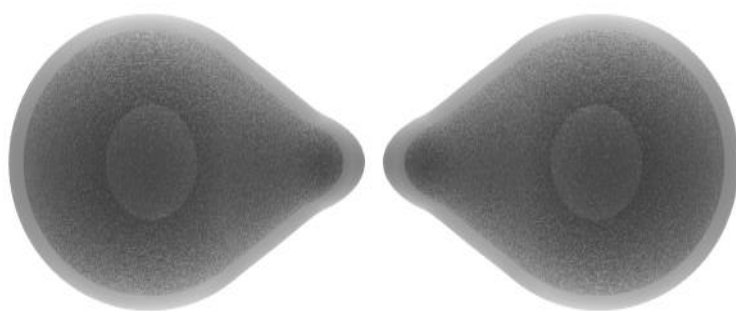


Figure 2.4. Shmoo formations in *S. cerevisiae* cells (Husnik, 2006)

2.4. The Industrially Importance of *S. cerevisiae*

Yeast is a well-known organism to produce beer, wine, and bread since Ancient Egypt. Although bacteria can also produce alcohol by breaking acids down to give a smoother taste in wine, *S.cerevisiae* differently perform that by converting glucose into ethanol in primary fermentation reaction. The other advantage of *S. cerevisiae* is lower alcohol inhibition to control alcohol level in industrial products. It nearly makes 18% alcohol production during an industrial fermentation (Madigan et al. 2008). During the bread production, *S. cerevisiae* also has an important role to give the bread a lighter and finer texture by producing alcohol and carbon dioxide in the fermentation process (Aidoo et al. 2006).

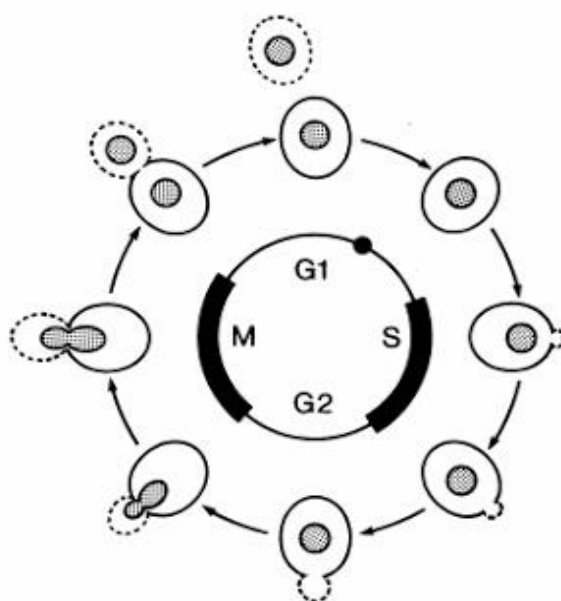


Figure 2.5. The cell cycle phases of *S.cerevisiae* (Herskowitz 1988)

There are many reasons drawing the attention of scientists to prefer *S. cerevisiae* as a model organism in research. One of them is sporulation mechanism to cope with stress conditions. The other important one is short life cycle that provides an advantage to produce new generations in less expensive and easier way (Fig. 1.5). When the GRAS feature of *S. cerevisiae* is added to these advantages, yeast could be a highly suitable system to use in experimental biology. (Wagner and Alper 2016)

2.5. Stress Responses of *S. cerevisiae*

There is always a stress response mechanism for all living organisms to survive in various stress conditions. Naturally, the most of the cells have the ability to perceive environmental challenges and response to them through the resistance mechanisms. Yeast has most suitable systems to study stress tolerance because of its robustness against hard environmental conditions.

The cellular stress mechanisms of yeast exposing to ethanol, freezing, heat, hydrogen peroxide (H₂O₂) and salt are highly understood (Gasch and Werner-Washburne 2002). During the life cycle, yeast may have to cope with various biological and physical stresses in instable temperature, osmolality, acidity, radiation, toxic chemicals and nutrient starvation. Therefore, it can rapidly evolve stress response mechanisms to minimize the effects of these harsh conditions unbalancing internal environment (González-Párraga et al. 2008)

Furthermore, yeast has cross-tolerance mechanism providing resistance more than one stress factors in the same time (Zheng et al. 2011). *S.cerevisiae* has cross-resistance mechanisms that can response stress conditions with nearly altering expression of 900 genes. These genes are assessed into the two groups; repressed and induced, as a part of the "Environmental Stress Response (ESR)". The repressed group in ESR, about 600 genes, are related to RNA metabolism, growth processes, and protein synthesis. In the other group, there are nearly 300 genes being responsible for energy metabolism, protein machinery, detoxification of ROS, cell wall modification and DNA damage repair. The regulation of ESR has not been clarified yet because there are more than one regulatory systems activating under different environmental conditions to control it (Nugroho et al. 2015).

2.6. Metabolic Engineering and Its Limitations

To reach a target, the chosen method is the most important point in a study. Scientists were lucky that the recombinant DNA technology has come to help to study in detail the most of the organism genomes and further innovations have revolutionized biology. Currently, new approaches provide crucial advantages to produce pharmaceutical proteins and hormones or obtain a mutant strain for extreme conditions (Chang and Keasling 2006). The basis of mentioned examples is “Metabolic Engineering” suggested by Bailey (1991) as a rational methodology (Bailey 1991). Metabolic engineering is used to obtain specific metabolites and mutant strains with desired properties by changing the cellular activity of genes, enzymes, and pathways (Petri and Schmidt-Dannert 2004).

Industrially, scientists apply to metabolic engineering for yeast research, especially *S.cerevisiae*, to improve expression of heterologous genes and eliminate the accumulation of unfavourable mutations (Nevoigt 2008). Actually, the classic metabolic engineering method is aimed to obtain desired phenotypes by manipulating cell component such as genes, enzymes, pathways (Cakar 2009). However, to know entire molecular metabolism of the cell is a limitation of metabolic engineering, so it is needed an alternative strategy to obtain the desired phenotype.

2.7. Evolutionary Engineering Strategy to Obtain Improved Microbial Phenotypes

The necessity of huge cell knowledge in metabolic engineering is a limitation to obtain the desired phenotype. Therefore, an alternative strategy has been compulsory to achieve the desired mutant strain. The inverse metabolic engineering method is an extremely effective and successful method for the selection of individual evolved as desired phenotype (Çakar et al. 2009). The evolutionary engineering is a specific approach in inverse metabolic engineering to improve these specific properties of phenotype (Patnaik 2008). As shown in Figure 2.6, the alternative strategy to metabolic engineering is inverse metabolic engineering that has three main stages: identification of the favoured strain, determination of the genetic basis of the desired phenotype and finally, experimental process to obtain the genetic ability in a model organism (Bailey et al. 1996).

Thanks to evolutionary engineering, the mutant strains with high efficiency and specific character have been obtained to use in various industrial productions (Almario et al. 2013; Küçükgoze et al. 2013). Especially, there are many well-characterized *S.cerevisiae* strains in molecular level obtained by evolutionary engineering (Koppram et al. 2012; Viktoria 2016; Fletcher et al. 2017).

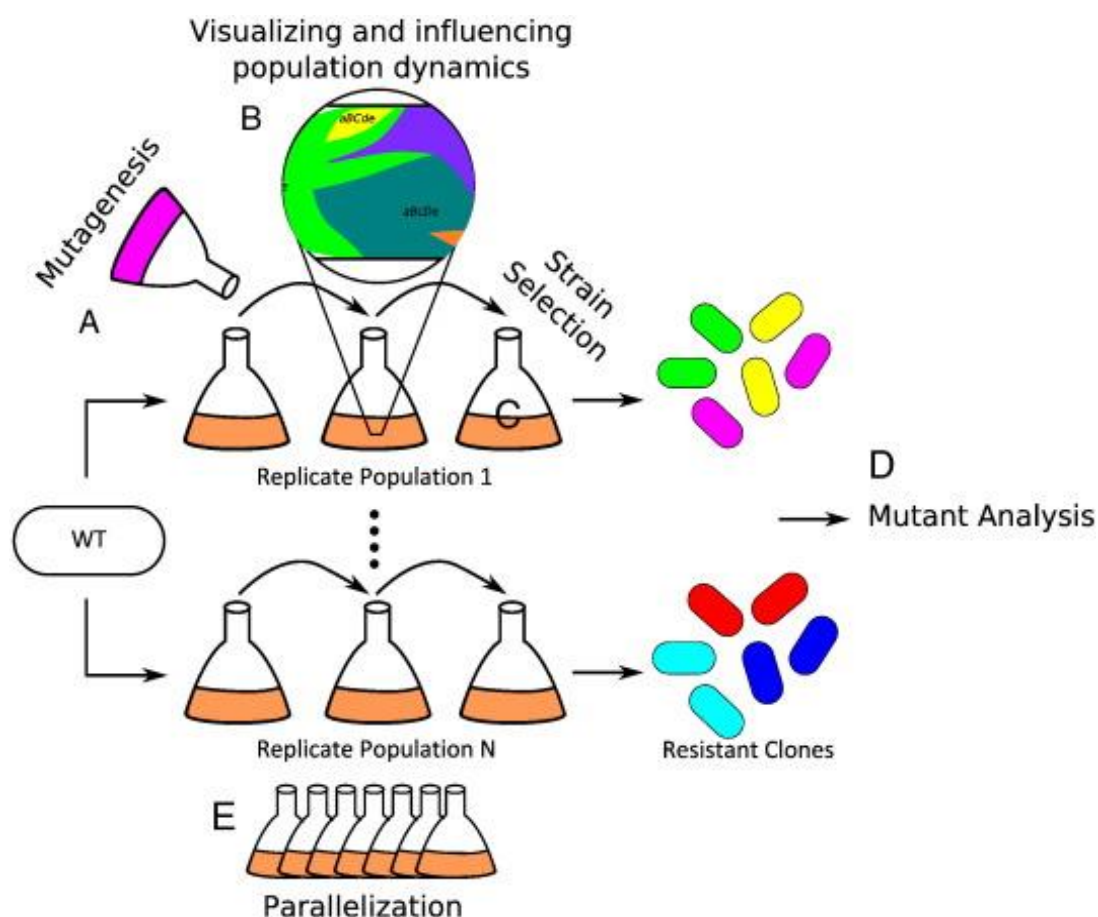


Figure 2.6. The experimental principals of evolution engineering process; A) use of chemical mutagens to create random mutagenic strains in population, B) the random mutations transform homogeneous population to heterogeneous with independent mutants, C) the desired phenotype strains are determined by selection media, D) high-throughput technologies are used to characterize mutants in molecular and physiological level and E) the favoured mechanisms are detected by increased parallelism (Winkler and Kao 2014)

3. MATERIALS AND METHODS

3.1. Material

3.1.1. Media

S.cerevisiae was grown in Yeast Peptone Dextrose (YPD) and Yeast Minimal Medium (YMM). On the other hand, Endothelium Growing Medium (EGM, Lonza), DMEM (Sigma-Aldrich) and Roswell Park Memorial Institute medium (RPMI, Sigma-Aldrich) were used in cell culture. Furthermore, the phosphate buffer saline (PBS), penicillin-streptomycin and endothelium growth factors; such as Insulin-like Growth Factor-1 (R3-IGF-1, Lonza), hydrocortisone (Lonza), gentamicinamphotericin-B (Lonza), ascorbic acid (Lonza), fetal bovine serum (FBS, Lonza), human Fibroblast Growth Factor-Beta (hFGF-, Lonza), vascular endothelium growth factor (VEGF, Lonza) were used for culturing process. The YMM and YPD were used for routine growth of the yeast cells and also synthetically defined in Table 3.1.

Table 3.1. The contents of YPD and YMM

Content	Percentage in YMM (w/v)	Percentage in YPD (w/v)
Glucose	2.00	2.00
Peptone	-	1.00
Yeast Extract	-	1.00
Yeast Nitrogen Base without amino acids and ammonium sulphate	0.67	-
Agar (for solid media)	2.00	2.00

3.1.2. Chemicals, Solutions/Buffers, Kits/Enzymes, Equipment

All the chemicals, solutions and buffers used in this study are respectively listed in Table 3.2 with their manufacturer and also in Table 3.3.

Table 3.2. Chemical components used in present study

Chemical	Company	Country
Sodium Chloride	Sigma-Aldrich	USA
Peptone	Sigma-Aldrich	USA
Yeast Extract	Sigma-Aldrich	USA
Yeast Nitrogen Base without amino acids and ammonium sulphate	Sigma-Aldrich	USA
Agar	Sigma-Aldrich	USA
Monohydrate Glucose	Sigma-Aldrich	USA
Tris Base	Sigma-Aldrich	USA
2,7'-Dichlorofluorescein diacetate (DCFH-DA)	Sigma-Aldrich	USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	USA
Hydrogen peroxide (H ₂ O ₂)	Sigma-Aldrich	USA
Tween	Sigma-Aldrich	USA
Trichloro acetic acid (TCA)	MERCK	Germany
2-thiobarbituric acid (TBA)	ACROS	USA
Diaminobenzidine (DAB)	Thermo-Fisher	USA
Malondialdehyde bis	Sigma-Aldrich	USA
Potassium Acetate	Sigma-Aldrich	USA
Glycerol	Sigma-Aldrich	USA
Ethanol	Sigma-Aldrich	USA
Agarose	Sigma-Aldrich	USA
Ethyl methyl sulfonate (EMS)	ACROS	USA
Nickel chloride (NiCl ₂)	Sigma-Aldrich	USA
DNA Markers	MERCK	Germany
Copper(II) Sulfate (CuSO ₄)	Sigma-Aldrich	USA

Table 3.3. The solutions and buffers that used in thesis study

Solutions/Buffers	Content
Potassium phosphate buffer	50 mM pH 7
Glycerol	30 % (v/v)
Sodium thiosulfate	10 % (v/v)
Ethanol	70 % (v/v) and 96 % (v/v)
Tris-HCl	50 mM pH 8
EDTA	10 mM
Sodium dodecyl sulphate (SDS)	1 %
NaOH	200 mM
TAE	50X
H ₂ O ₂	65 %

The commercially bought kits and enzymes are indicated in Table 3.4.

Table 3.4. Kits and enzymes used in this study

Kit/Enzyme	Company	Country
RNeasy Mini Kit	QIAGEN	Germany
High-Capacity cDNA Reverse Transcription Kit	APPLIED BIOSYSTEMS	USA
qPCR GreenMaster with UNG	JENA BIOSCIENCE	Germany
G-DEX IIc Genomic DNA Extraction Kit for Cell/Tissue	EUROPEAN BIOTECH	Belgium
ApopTag® Peroxidase In Situ Apoptosis Detection Kit	CHEMICON®	USA
QCM™ 24-Well Colorimetric Cell Migration Kit	CHEMICON®	USA
Total Protein Extraction Kit	ABCAM	USA
Glucose Colorimetric Assay Kit	CAYMAN	USA

As seen in Table 3.5, all equipment been part of the thesis study are presented. Moreover, there are also several websites and applications in Table 3.6 used to fix some materials and calculate results.

Table 3.5. The laboratory equipment used in present study

Equipment	Company	Country
Autoclave	HIRAYAMA	Japan
Deep Freezer	NUAIRE	England
Refrigerator	ARCELIK	Turkey
Electrophoresis Tank Systems	BIO-RAD	USA
Incubator	JENO TECH	Korea
5% CO ₂ Incubator (for cell culture)	ESCO	USA
Bio-safety Cabinet	HAIER	China
Light Microscope	OLYMPUS	USA
Spectrophotometer	SHIMADZU	Japan
Fluorescent Microscope	OLYMPUS	USA
Laminar Flow Hood	ESCO	USA
Micropipette Sets	EPPENDORF	USA
Magnetic Stirrer	IKA	Germany
Distilled Water Machine	HUMAN	China
Rotor-Gene Q	QIAGEN	Germany
Gel Imager	BIO-RAD	USA
Trans-Blotting System	BIO-RAD	USA
Thermal-Cycler	SENSQUEST	Germany
SpectraMax Plus 384 Microplate Reader	MOLECULAR DEVICES	Germany
UV-Spectrophotometer	SHIMADZU	Japan
iCELLigence Real Time Cell Analyzer	ACEA Bioscience	USA
X Ray Film Processor	CARESTREAM	USA
pH Meter	THERMO	USA
Ice Maker	HOSHIZAKI	Japan
Shaker	GERHARDT	Germany
Bench-Top Centrifuge	HERMLE	Germany
Spectrofluorometer	Perkin-Elmer LS-55	USA

Table 3.6. Software and websites used in this study

Software/Websites	Internet Link
Saccharomyces Genome Database	http://www.yeastgenome.org/
Human Genome Database	https://genome.ucsc.edu/
Primer3Plus	http://frodo.wi.mit.edu/primer3/
A Five Tube-MPN Tablo	www.jlindquist.com/generalmicro/102dil3a.html
GraphPad Prism 5	-
Image Lab	-

3.1.3. The Preparation Steps of Solutions Used in the Experimental Process

The solutions used for several assays as mentioned in Table 3.3 are presented at below with their preparation steps;

1. 50 mM Potassium phosphate buffer was prepared from the stock solutions of 0.1 M KH_2PO_4 and 0.25 M K_2HPO_4 with calculated amount of both the conjugate acid and base and add to 10 ml dH_2O . pK_a of $\text{KH}_2\text{PO}_4 = 7.20$

$$\text{pH} = \text{pK}_a + \log [\text{A}^-] / [\text{HA}]$$

$$7.0 = 7.20 + \log [\text{A}^-] / [\text{HA}]$$

$$0.20 = \log [\text{A}^-] / [\text{HA}]$$

$$1.584893192 = [\text{A}^-] / [\text{HA}]^*$$

$[\text{A}^-] / [\text{HA}] = 1.584893192$, we can say that $[\text{A}^-] / [\text{HA}] = 1.584893192 / 1$.

In this case $[\text{A}^-] = 1.584893192$; $[\text{HA}] = 1$.

3. 30% Glycerol solution was made in 10 ml dH_2O with taking 3,53 ml of 85% glycerol solution.
4. 10% Sodium thiosulfate was prepared in 50 ml dH_2O by taking 5 ml of pure solution.
5. 50 mM Tris-HCl was made with 0,6057 g Tris base to solve in 50 ml dH_2O . Then pH was arranged to 8 and the total volume was completed to 100 ml
6. 70% and 96% Ethanol was prepared from 99% absolute ethanol. 70 ml and 96 ml ethanol was separately measured and each of them completed to 100 ml with dH_2O
7. 10 mM EDTA was made with measuring 0,292 g EDTA and solved in 100 ml dH_2O
8. 200 mM NaOH solution was prepared by solving measured 0.8 g NaOH in 100 ml dH_2O
9. 50X TAE buffer was prepared in several steps; weight out 242 g of Tris base added in 750 ml deionized to completely solve. Add 100 ml of 0.5 M EDTA solution and 57.1 ml glacial acetic acid and adjust pH to 8.3.

3.2. Methods

3.2.1. Cell Culture

PC3 (ATCC[®] CRL1435[™]), human vascular endothelial cells [HUVECs (ATCC[®] CRL-1730[™])], were kindly supplied by Plant, Drug, and Scientific Research Centre (Eskişehir, Turkey) at passage 10. Both cells were checked for cross contamination and also tested for mycoplasma contamination by using EZ-PCR mycoplasma test kit (Biological Industries). PC3 cells were grown in DMEM and HUVECs were cultured in EGM, including growth supplements (Zumbansen et al. 2010) at 37°C in 5% CO₂ incubators for 24 hours. Furthermore, MCF-7 (human breast cancer cells) were friendly granted by Advanced Technologies Research Centre (Dumlupınar University, Kutahya) at passage 8 and cultured in RPMI 1640 complete media containing 10% fetal bovine serum and 1% penicillin-streptomycin solution. The supernatants of PC3 cells, MCF-7 cells and HUVECs were collected by centrifugation at 3000 rpm for 5 min. Both of them were double-filtered with 22 µm micro-filters and stored at +4°C until used.

3.2.2. Cultivation of Yeast Culture

In this study, CEN.PK 113-7D (MATa, MAL2-8c, SUC2) was used a reference strain of *S. cerevisiae* kindly provided by Dr. Laurent Benbadis (INSA-Toulouse, France). Yeast was cultivated in YMM and YPD. The culturing was performed at 30°C and 150 rpm, in 50-mL culture tubes containing liquid media. The growth rate was measured at the optical density (OD600) using a spectrophotometer. The stock cultures were prepared in 1 mL in YMM containing 30% (v/v) glycerol and stored at - 80°C.

3.2.3. Growth Fitness of *S. cerevisiae* in Cancer Cell Cultured Medium

The growth fitness of yeast was tested in PC3 cultured medium (PCM), MCF-7 cultured medium (MCM) and HUVEC cultured medium (HCM). At the first, each type of cell was cultured in their medium for 24 h. Then, each medium was collected and filtered in three times with 0.22 µm filter. After that, they stored in the fridge for later use. The pH value

of PCM, MCM and HCM were measured and adjusted to optimum growth conditions of *S. cerevisiae*. Moreover, the glucose concentration of PCM, MCM, and HCM was measured with glucose colorimetric assay kit to determine main stress factor on the growth fitness of *S. cerevisiae*.

S. cerevisiae was cultivated into 500 μ L of optimized PCM, MCM, HCM and YMM (control medium) for 24 h at 30°C and 150 rpm. The growth curve was analyzed with the iCELLigence analyzer.



Figure 3.1. The cell growth curves were analysed with iCELLigence Systems. The cells were inoculated in 500 μ L of medium in each well of E-plate L8, and then E-plate placed device set in 5% CO₂ incubator. The device connected to iPad tablet with wifi to periodically measure cell intensity in each well and send data to software to plot a growth curve

3.2.4. Determination of Desired Phenotype Strain in Yeast Population

The yeast population was randomly mutated with EMS to find the desired phenotype. The mutant colonies with specific properties to grow in PCM were randomly selected from solid PCM-agar. Then, the best mutant among selected colonies was determined with several cross-stress tests, which are used to examine the ESR of each mutant. The robustness of yeast was assessed according to the multi-stress resistance capacity.

3.2.4.1. Evolutionary Engineering Strategy to Obtain Yeast Mutants

To select PCM-resistant *S. cerevisiae* mutant strain, evolutionary engineering strategy was directly performed with slight modifications as described previously (Bailey 1991; Cakar 2009). The evolutionary engineering approach is based on creating random mutations in the genomic DNA of each individual of the population with a chemical mutagen. The method suggests that each individual mutant in population might be a unique phenotype. Thus, the some of them may be the desired one with a specific characteristic. The aimed mutant colonies can be selected in stress medium to characterize. In this study, EMS treatment was applied to increase the genetic diversity of the first population (Lawrence 2002). EMS is treated to two different *S.cerevisiae* cultures, one of them pre-cultured in PCM (to understand the effects of stress conditions on the direction of mutation) and the other one is in YPD. Initially, *S.cerevisiae* was pre-cultured in 10 mL YPD medium and PCM overnight at 30°C and 150 rpm. After that, about 5×10^7 cells/mL were taken and washed with 50 mM potassium phosphate (KH_2PO_4) buffer (pH 7) for each tube. 150 μL of EMS was added to culture tubes except for control. Tubes were vortexed and placed in an incubator at 30°C and 150 rpm. After 60 min, freshly prepared 5 mL sodium thiosulfate (10%, w/v) was added in order to inactivate EMS. The solution was mixed, centrifuged for 10 min at 5175 rpm. The pellets were then washed twice with YMM without glucose and resuspended in 10 mL YPD in a 50 mL culture tubes. After 24 h of incubation at 30°C, 150 rpm OD600 of the cultures was measured.

Table 3.7. Nomenclature of the mutants selected as single colony in selective media

Mutant Number	Name of the individual mutants	
	YPD-based	PCM-based
1 st mutant	MY1	MP1
2 nd mutant	MY2	MP2
3 rd mutant	MY3	MP3
4 th mutant	MY4	MP4

3.2.4.2. Direct Selection of Mutant Phenotypes on Plate

At the first, the growth fitness of EMS-mutagenized yeast population and wild type (WT) were determined with iCELLigence analyzer by cultivation into 500 μ L of PCM for 24 h. After that, EMS-treated cultures were directly inoculated to solid PCM contained plates with spread plate technique and kept at 30°C until the colonies were formed on the surface of the medium. Four single mutant colonies (MYn and MPn) were randomly selected for each petri dish and cultivated in YPD to prepare stock for further analysis (Table 3.6).

3.2.5. Growth Analysis of *S.cerevisiae* Mutants

The wild type and mutants routinely were pre-cultured in 50-mL Falcon-tube with 10 mL of YMM at 30°C and 150 rpm on a rotary shaker until late logarithmic phase. They were grown in 500 mL of PCM in E-plate at 30°C, with gentle shaking. The cell growth of each mutant was periodically measured with the iCELLigence analyzer to plot the growth curve.

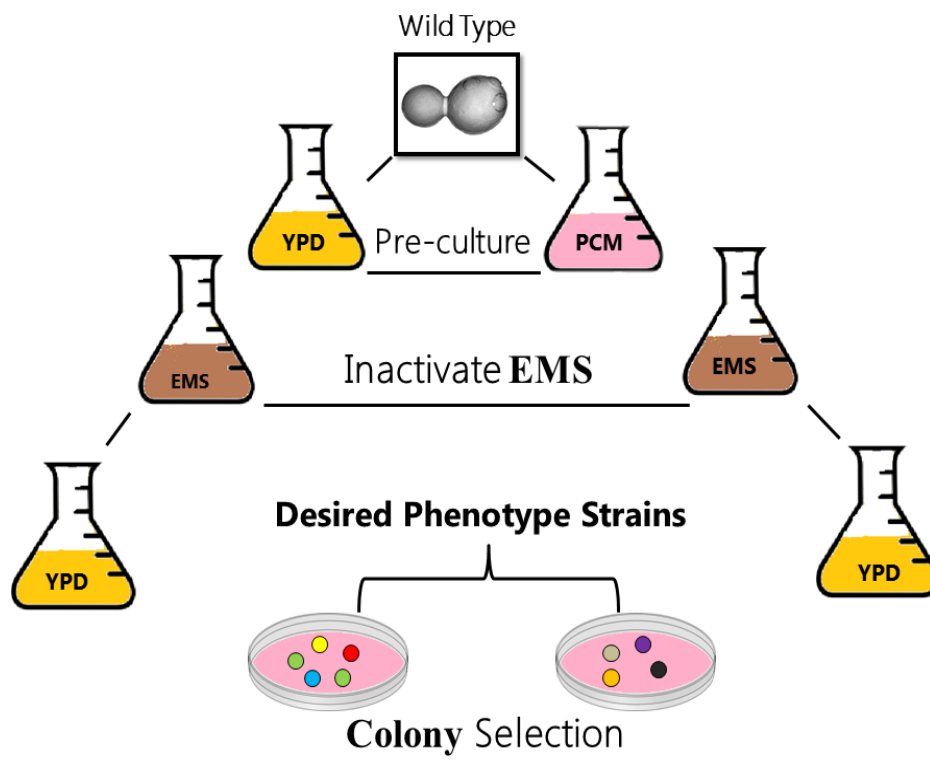


Figure 3.2. The inverse metabolic engineering approach to obtain favoured strains with random EMS mutagenesis. To select desired colonies, EMS-treated yeast population inoculated in solid selective media containing stress factors

3.2.6. Determination of the Best Mutant with Cross-Test Assays

3.2.6.1. Most Probable Number (MPN) Test

The cross-resistance of individual mutants to different stress types was assayed by a high-throughput, MPN test (Gamo and Shoji 1999). The assay is based on viable cell numbers in each-well containing 180 μL of PCM, MCM, and YMM with or without stressor. The yeast cells were serially diluted in 96-well plates from 10^{-1} to 10^{-8} five times in parallel for each sample. Cells were grown in PCM, MCM and YMM including 0.8 mM H_2O_2 for 72 h to estimate the survival number according to MPN tables. The survival rate of yeast in various stress condition (Cakar 2009) was calculated by dividing the viable cell number of treated sample to that of non-treated one.

3.2.6.2. Spot Assay

The sensitivity and resistance of yeast mutant strains to various compounds were tested with slightly change spot assay described previously (Memarian et al. 2007). Equal numbers of yeast cells were harvested in the logarithmic growth phase of YMM cultivated at 30°C and 150 rpm. The WT and mutant yeasts were pelleted by centrifugation at 10000 *g* for 5 min and resuspended in 100 µL water to dilute serially as 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} in micro-centrifuge tubes. Three µL from each dilution of samples was dropped onto solid control plates and plates containing stress factors (0.5 mM NiCl₂ and 8% (v/v) ethanol) in their media, and incubated at 30°C.

3.2.7. The Effect of DMEM Cultured with *S. cerevisiae* Mutant Strains to PC3

The best mutants (MP2 and MY2) were determined in consequence of cross-test assays. The further experiments were conducted with MY2, MP2 and WT strains. To understand the cross-metabolite effect of PCM-resistant strains to PC3, they were cultured in DMEM until in logarithmic growth phase.

3.2.7.1. The Cultivation of WT, MP2, and MY2 in DMEM

The pre-culture of WT, MP2 and MY2 yeast strains were cultivated in 50-mL Falcon-tube with 10 mL of YMM at 30°C and 150 rpm on a rotary shaker until late logarithmic phase. The equal number of cells was determined with OD600 measurement and inoculated in 500-mL Erlenmeyer with 100 mL of DMEM at 30°C and 150 rpm on a rotary shaker until logarithmic phase (approximately 8 h). DMEM cultured with WT (WT-DM), DMEM cultured with MP2 (MP-DM) and DMEM cultured with MY2 (MY-DM) were made ready by centrifugation three times at 14.000 rpm for 10 min and filtration with 0.22 µm filter.

3.2.8. Experimental Grouping

To understand the cross- metabolite effects of WT-DM, MP-DM, and MY-DM into the PC3 culture, the cell culture experiments were organized into four groups. In control group, PC3 was treated with just complete media (DMEM). In WT-DM group, cells were exposed to 3:1 WT-DM and DMEM. In MP-DM group, cells were treated with 3:1 WT-DM and DMEM. In MY-DM group, cells were also exposed to 3:1 MY-DM and DMEM. Initially, the cell groups were grown in 6-well plate with DMEM until reaching 70% confluence. After that, they were treated according to experimental grouping for 12 h.

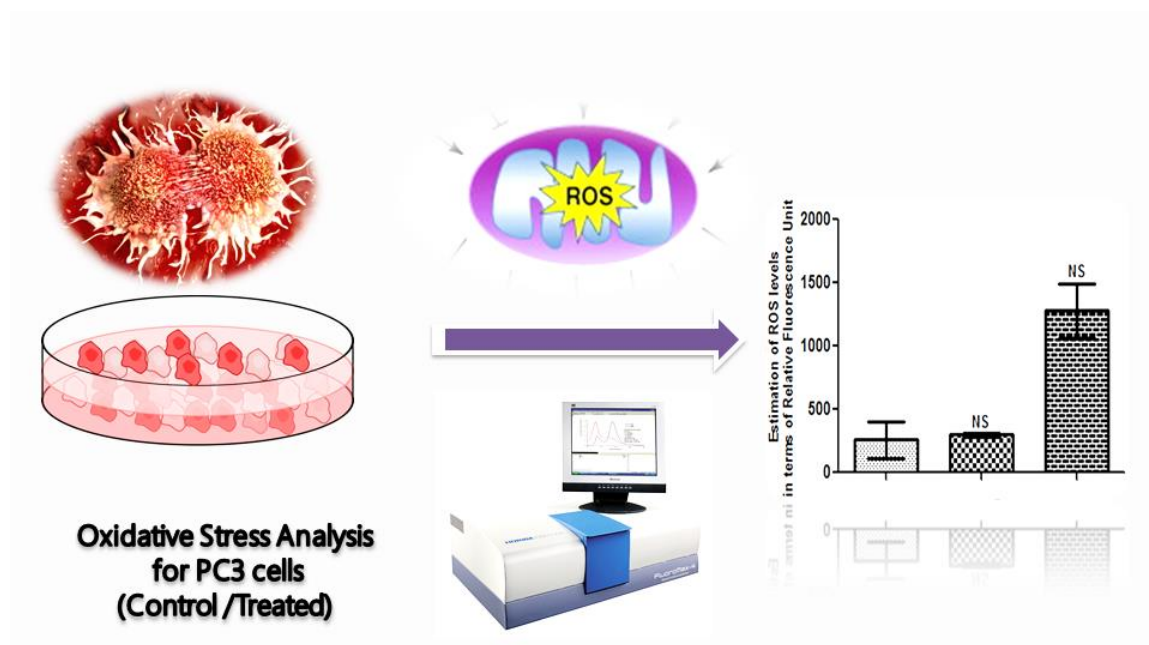


Figure 3.3. The oxidative damage in PC3 cells treated according to experimental grouping part (3.2.8) was manually detected with spectrofluorometre (Perkin-Elmer LS-55, USA) according to excitation (485 nm) and emission (525 nm) wavelengths.

3.2.8.1. Reactive Oxygen Species (ROS) Assay

The oxidative damage in PC3 with WT-DM, MP-DM, and MY-DM treatment was manually measured by using DCFH-DA. In brief, PC3 cells were cultured in 6-well plate with DMEM and then treated as stated in experimental grouping part (3.2.8). After that, 1×10^6 harvested cells of each group were exposed to 2 μ M DCFH-DA in PBS containing

ions (Ca^{+2} , Mg^{+2}) and glucose. Then, cells were held at 37°C for 45 min. Lastly, the fluorescence intensity of DCFH-DA was measured with a spectrofluorometer (Perkin-Elmer LS-55, USA) according to excitation (485 nm) and emission (525 nm) wavelengths (Oparka et al. 2016).

3.2.8.2. Cell Migration Assay

Cell migration was analysed according to QCM™ 24-Well Colorimetric Cell Migration Assay kit protocol. At the beginning, PC3 cells were cultured and re-seeded in the supplied insert with serum free medium (SFM) to starve them for 24 h. After that, the medium of supplied inserts was discarded. The inserts were replaced in wells containing medium as stated in experimental grouping part to incubate for 24h (3.2.8). The kit protocol was followed to measure cell migration for each well at 560 nm using SpectraMax Plus 384 Microplate Reader.

3.2.8.3. Tunnel Assay: *In Situ* Apoptosis Detection

The PC3 cell death was assessed with detection of apoptosis. Therefore, ApopTag In Situ Apoptosis detection peroxidase kit's protocol was followed to detect apoptosis in PC3 treated with media stated in experimental grouping part (3.2.8). Briefly, cells were cultured, fixed and stained according to protocol provided by manufacturer. The inverted light microscope was used to examine images of each sample. Furthermore, the apoptotic index of each sample was calculated by dividing counted apoptotic cell number to total cell number (Barut et al. 2005).

3.2.8.4. DNA Fragmentation Assay

The genomic DNA fragmentation in PC3 cells treated with media stated in experimental grouping part (3.2.8) was tested by agarose gel electrophoresis, which separates DNA fragments each other according to their size and shape. The concentration of agarose is important to determine pore size that DNA pass through. In this study, we firstly cultured PC3 cells and treated according to experimental grouping part. The harvested cells were

centrifuged at 2500 rpm for 3 min and washed in PBS to isolate DNA by using G-Dex extraction kit protocol. Genomic DNAs were run on 1% agarose gel and stained with Ethidium Bromide. The DNA fragmentation was imagined by Image Lab (GelDoc EZ).

3.2.8.5. RNA Isolation

Total protein of PC3 cells treated as mentioned experimental grouping part (3.2.8) was extracted according to protocol of RNeasy Mini Kit. The harvested cells were treated with lysis buffer according to their number (checked from kit table). The lysate was centrifuged at 14,000 rpm for 10 min and supernatant was filtered with g-elimination column to discard genomic DNA. After that, the RNA affinity column was used to purify total RNA from solution. The total RNA was collected from affinity column with elution buffer. The RNA integrity of samples was measured with nanodrop property of SpectraMax Plus 384 Microplate Reader. The quality of samples was also calculated by 260nm/280nm ratio.

3.2.8.6. cDNA Synthesis

The isolated total RNA is to use for cDNA synthesis with the reverse transcription of mRNAs. High-Capacity cDNA Reverse Transcription Kit manual sheet was followed step by step to perform cDNA synthesis reaction. Additionally, the initial concentration of total RNA added to reaction tube was also calculated from RNA quantity according to protocol provided by manufacturer. The cDNA samples were kept at – 20 °C for further experiments.

3.2.8.6. Gene Expression Analysis with cDNA

To understand mRNA levels of BAX, BCL-2, cytochrome *c* (CYCS), caspase-9 (CASP9), and caspase-3 (CASP3) in total RNA, gene expression analysis was performed with qPCR GreenMaster with UNG kit protocol. The initial amounts of cDNA were equally calculated to add each reaction tube. Quantative Real Time polymerase reaction (QRT-PCR) was started as stated in kit protocol with holding at 50°C for 2 min to

eliminate uracil residues from dU-containing DNA from previous cDNA reaction and then held at 95°C for 2 min to activate enzyme. The reaction was programmed on 35 cycles; each cycle including following conditions; denaturation at 95°C for 15 sec, annealing at 55°- 60°C for 30 sec and extension at 95°C for 30 sec. Annealing can be change due to the primers T_m . QRT-PCR was repeated three times for each samples mentioned at in experimental grouping part (3.2.8).

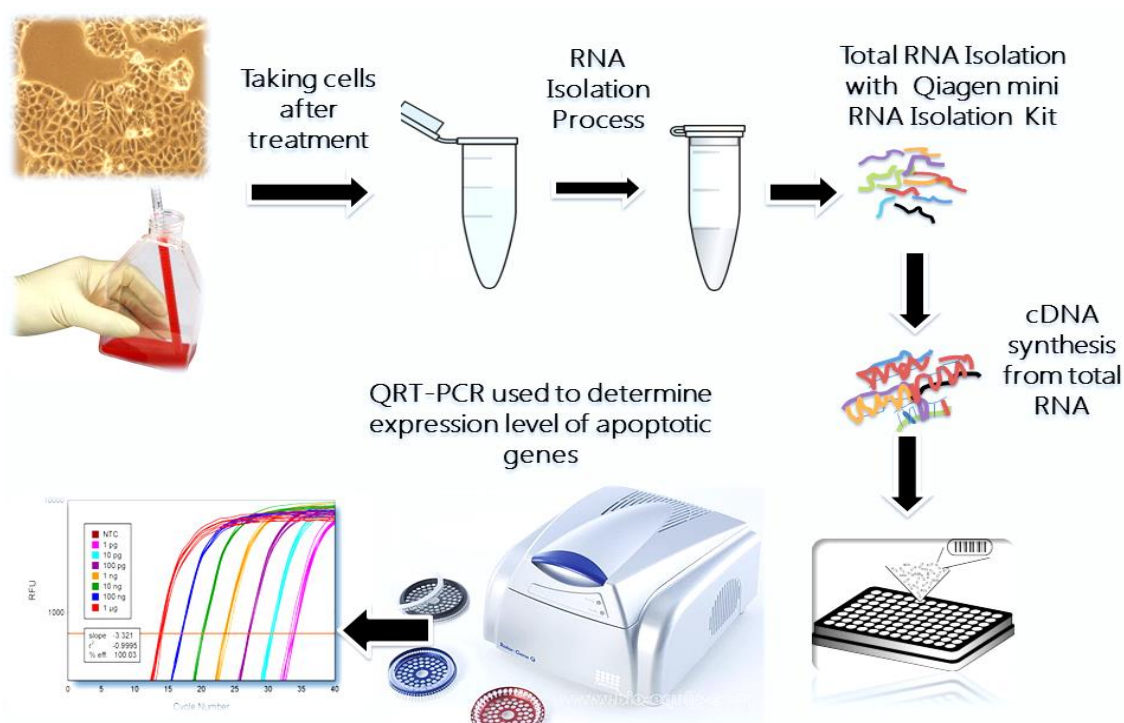


Figure 3.4. The mRNA levels of Bax, Bcl-2, cytochrome *c*, caspase-9, and caspase-3 and β -actin in PC3 cells were treated with media as stated in experimental grouping part (3.2.8). To analyse gene expression with QRT-PCR, at first total RNA was isolated, and then the mRNAs in total RNA were reversely transcribed to cDNA with specific kits

Table 3 8. The pair of primer of genes were assessed for apoptosis with QRT-PCR

Gene Name	Primer Sequences at 5'-3' Direction
BAX (F)	TGGAGCTGCAGAGGATGATTG
BAX (R)	CGGGGATTGATCAGACACGTAA
BCL-2 (F)	TTTAATTGTATTAGTTATGGCCT

Table 3.8. (Devam): The pair of primer of genes were assessed for apoptosis with QRT-PCR

BCL-2 (R)	CAATAAACAATTCTGTTGACG
CYCS(F)	AACAAAGGCATCATCTGGGGAG
CYCS (R)	CACAGGTGAATCTTGCTTGGT
CAS9 (F)	ATTGTGAACATCTTCAATGG
CAS9 (R)	AGTAGGACACAAAGATGTCA
CAS3 (F)	TAGTTGCAATTGAATTAAATTAGGA
CAS3 (R)	TAGAATACACAGTCTTAAGTGG

The fold changes of gene expressions were analysed with comparative threshold (Ct) method, comparing treated samples with the control sample. ACTB and GAPDH were used as a housekeeping gene to normalize the expressions of other genes. The fold changes of gene expression were calculated according to Ct values used to define the mRNA levels in PC3 cells by the following formula;

$$\Delta Ct_{\text{calibrator}} = Ct(\text{apoptotic genes}) - Ct(\beta\text{-Actin})$$

$$\Delta Ct_{\text{Sample}} = Ct(\text{apoptotic genes}) - Ct(\beta\text{-Actin})$$

$$\Delta\Delta Ct = \Delta Ct_{\text{Sample}} - \Delta Ct_{\text{Calibrator}} \text{ (Yuan et al, 2006).}$$

3.2.8.7. Protein Extraction

Total protein was isolated to determine protein levels of Bax and procaspase-3. The PC3 cells were cultured and treated as mentioned in experimental grouping part (3.2.8), and then cells were harvested and washed up in the cold PBS. The ice-cold lysis buffer (Table 3.8) was added all samples due to cell number to obtain total cellular protein lysates. Then, the samples with lysis buffer were incubated on ice for 1h. After that, they were centrifuged at 14,000 rpm for 10 min at 4°C and, the supernatants were carefully collected in micro-centrifuge. Total protein concentration was measured with Bradford Assay (Kruger 1994). The Laemmli sample buffer 5X (Table 3.8) was added to all protein samples and heated at 95°C for 5 min. Finally, they were stored at -20 °C for next steps.

Table 3.9. The ingredient of lysis buffer and sample buffer used to isolate total protein

Lysis Buffer Content	Concentration	Sample Buffer (5X)	Concentration
Tris-HCl	50 mM, pH 7.4	Tris-HCl	60 mM, pH 6.8
NaCl	150 mM	SDS	2%
Triton-X 100	1%	Glycerol	10%
SDS	0.1%	Bromophenol blue	0.01%
EDTA	1 mM	β -mercaptoethanol	5%
Phenylmethylsulfonyl fluoride (PMSF)	1 mM	Tris-HCl	60 mM, pH 6.8
Dithiothreitol (DTT)	1 mM		
Deamino Benzamidine	1 mM		

3.2.8.8. Western Blotting Analysis

At first, 12 % stacking and separation polyacrylamide gel (Table 3.9) were prepared to run total protein samples. The proteins were run in electrophoresis tank systems being full with running buffer (Table 3.10) at 20 mA during stacking gel condition, then at 40 mA for separation gel. After that, the completed gels were placed between sandwich equipment with 0,2 μ m nitrocellulose membrane to transfer total protein to the membrane by adding transfer buffer (Table 3.10) in tank systems and running at 200 V for 2h.

Table 3.10. The content of Separation (12%) and Stacking (12%) gels to run total protein

	12% Separation Gel	12% Stacking Gel
Solution	Volume (5 ml)	Volume (2 ml)
ddH ₂ O	1,7 ml	1,4 ml
30% polyacrylamide	2.0 ml	330 μ l
Tris-HCl 1,5 M pH: 8,8	1.3 ml	250 μ l
10% SDS	50 μ l	20 μ l
10% APS(Ammonium Persulfate)	50 μ l	20 μ l
TEMED	5 μ l	2 μ l

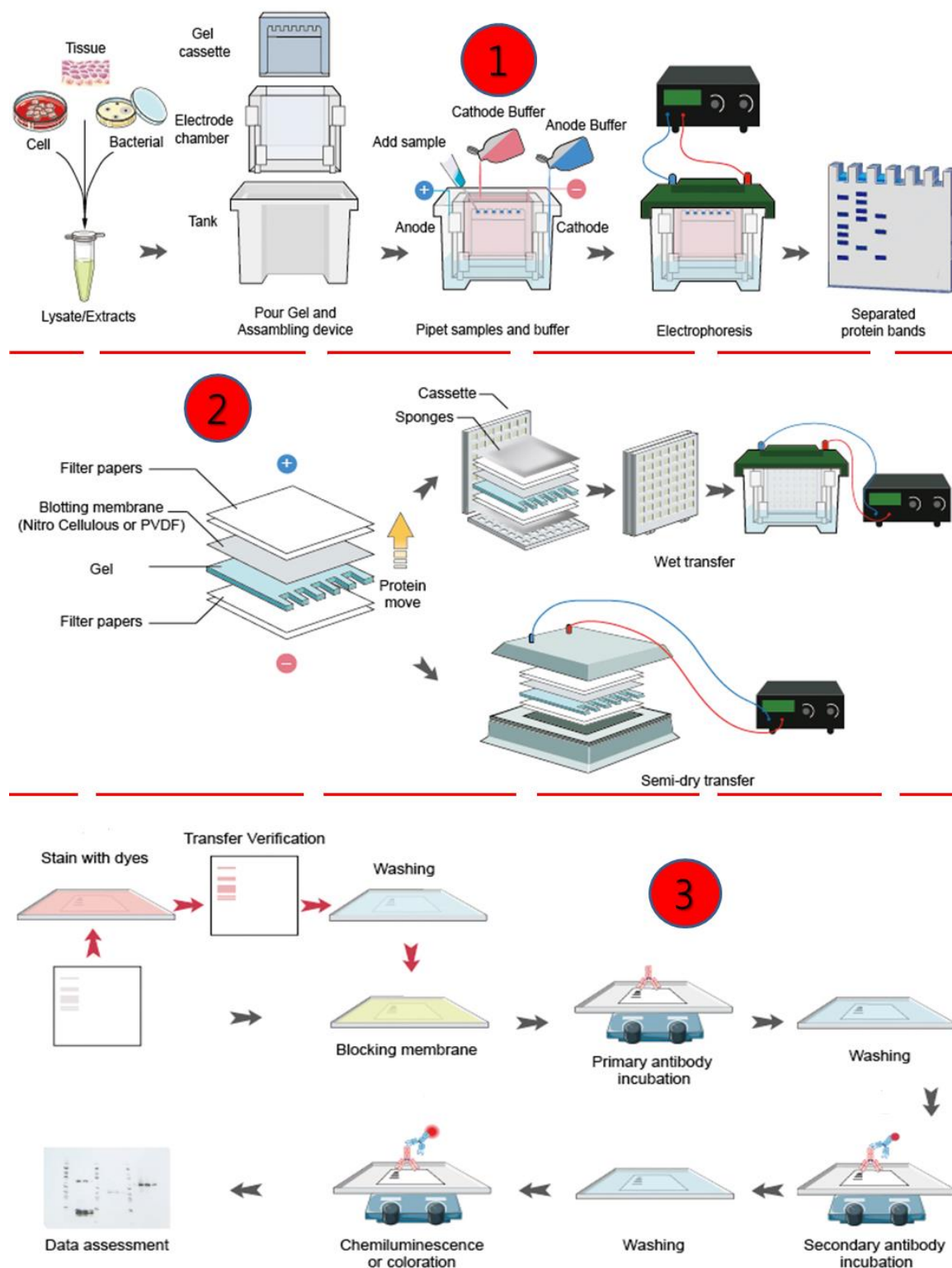


Figure 3.5. The protein levels of Bax, procaspase-3, and GAPDH in PC3 cells treated with media as stated in experimental grouping part (3.2.8) were determined by western blotting assay. 1) Total protein was run in stacking and separation gels. 2) The separated proteins were transferred to a membrane. 3) Target protein was blotted with specific antibodies to image

Following the transfer process, the membranes were gently shaken in 5% (w/v) Bovine Serum Albumin (BSA) in TBS-Tween 20 (TBS-T; 0.05%) for 1h to block binding sites. In next step, membranes were kept in the 1:500 dilution of primary monoclonal antibodies for GAPDH (sc-47724, Santa Cruz Biotechnology); Bax (sc-20067, Santa Cruz Biotechnology) and Procaspase 3 (sc-271759, Santa Cruz Biotechnology) at room temperature with gentle shaking for 2h. Briefly, the samples were washed with TBS-T for 5 min in five times, then secondary antibodies (1:1,000 dilution, Goat Anti-Mouse IgG H&L(HRP), sc- 2005, Santa Cruz Biotechnology) was added to membrane box to incubate for 90 min at room temperature with gentle shaking. The membranes were cleanly washed with TBS-T for 5 min in five times, before adding enhanced chemiluminescence (ECL) solutions. Lastly, the chemiluminescence of membranes was imaged to film by X Ray Processor. The protein levels of GAPDH, Bax and procaspase 3 were calculated by GelDoc EZ and analysed Image Lab. 5.2.1 software. Furthermore, the fold changes in protein levels were normalized to the intensity of referenced protein (GAPDH) according to with the following article (Degasperi et al. 2014).

Table 3.11. The ingredients of running and transfer buffers used for Western Blotting

Runnig Buffer Content (1X)	Amount
Trisbase	30.3 gr
Glycine	14.4 gr
SDS	10 gr
ddH ₂ O	equal to 1 L
Transfer Buffer Content (1X)	
Trisbase	6.8 gr
Glycine	28.8 gr
Methanol	200 ml
ddH ₂ O	1.6 L

4. RESULTS

4.1. The Cultivation of *S.cerevisiae* in Cell Cultured Media

The yeast growth fitness was assessed in YMM, DMEM, HCM, PCM, and MCM to clarify the medium restriction on *S.cerevisiae*. Figure 4.1 shows that the cell proliferation in PCM and MCM dramatically 6-4 fold decreased in comparison to YMM and DMEM ($p < 0.05$). Furthermore, there has been a nearly 1.5 fold decrease between in HCM and control media (YMM and DMEM). The cancer cells cultured media (especially PCM and MCM) significantly restricted yeast growth

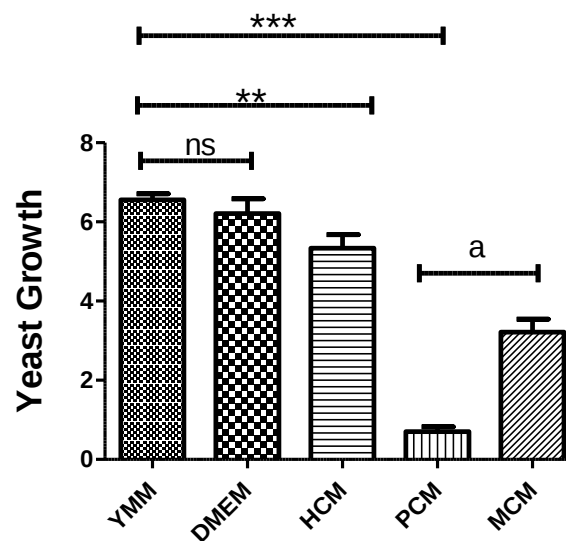


Figure 4.1. The yeast growth in YMM, DMEM, HCM, PCM, and MCM. The growth rate of *S.cerevisiae* was independently measured three times with iCELLigence Real Time Cell Analyzer. The data were expressed by mean $\pm$ SEM ($n \geq 3$), *** $p < 0.001$ PCM, MCM vs DMEM, YMM, a $p < 0.001$ MCM vs PCM

4.1.1. Elimination of the Other Stress Factors

To determine the reasons for PCM restriction on yeast growth, the glucose consumption of PC3 in DMEM (Liu et al., 2010) was measured as 3 gr/L with Glucose Colorimetric Assay Kit and also its pH was arranged to the physiological level. The yeast growth fitness was tested in the various media containing different glucose concentrations. (Table 4.1)

Table 4.1. The various media prepared with different glucose concentrations

Medium	Glucose Concentration
YMM	20 g/L
YMM-L	3 g/L
DMEM-H	4,5 g/L
DMEM-L	1 g/L

Figure 4.2 displays that there are no a significant yeast growth differences between media in Table 4.1. The glucose level and pH of PCM is not a real stress factor on *S.cerevisiae* to declining growth rate.

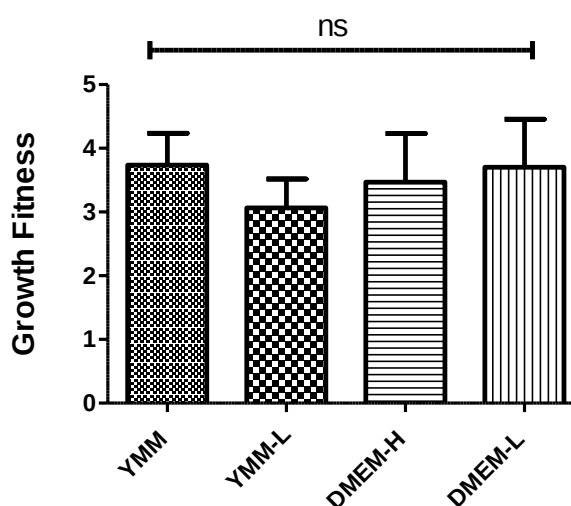


Figure 4.2. The yeast growth fitness in YMM, YMM-L, DMEM-H, and DMEM-L. The growth rate of *S.cerevisiae* was measured in various media containing different glucose concentration to determine glucose stress level. The data were expressed by mean $\pm$ SEM ($n \geq 3$), ns $p < 0.05$

4.2. An Inverse Metabolism Engineering Approach: Evolutionary Engineering

4.2.1. Ethyl Methyl Sulfonate (EMS) Treatment of the WT Strain

The EMS mutagenesis was applied two groups of WT (Fig. 3.2) to obtain the desired phenotype in the initial population of *S.cerevisiae*. After EMS treatment, yeast was incubated with YPD for overnight to check population healthy with measuring at OD600. EMS-treated population (MPn and MYn) and WT were inoculated to PCM at 30°C for 24h. The yeast growth curves were assayed with iCELLigence Real Time Cell Analyzer. The MPn and MYn have better growth fitness in PCM compared to WT (Fig. 4.3A). There is a dramatically increase in cell proliferation both MPn and MYn. As a result, the mutant population probably consists of the desired phenotype.

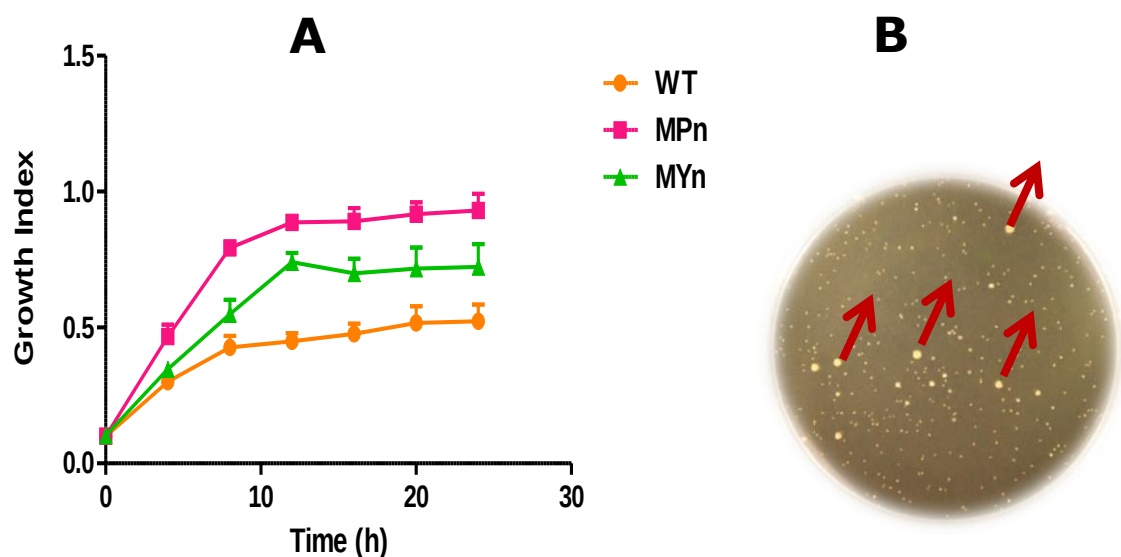


Figure 4.3. The growth curve of WT, MPn, MYn **A**) They were measured in PCM by iCELLigence Real Time Cell Analyzer. There is a significant growth difference between both MPn and MYn with WT. ($p < 0.05$). **B**) The colonies were randomly selected from PCM agar plate.

4.2.2. The Colony Selection with Direct Method and Determining the Best One with Cross-resistance Tests

To obtain the desired phenotype, the four individual mutant colonies were randomly selected from each of PCM solid plates prepared with spread plating of MYn and MPn.

(Table 3.6) The equal number of each selected mutant (Table 3.6) taken from pre-cultured YMM was seeded in PCM to determine the growth fitness of them. Figure 4.4 demonstrates that MP2 and MY2 have doubled growth rate compared to WT and other mutant strains. As seen in Figure 4.4, all selected mutants can normally grow without any defect in PCM, thus it is unnecessary turning back to colony selection. If the selected mutants cannot grow in liquid PCM, the colony selection must be repeated again.

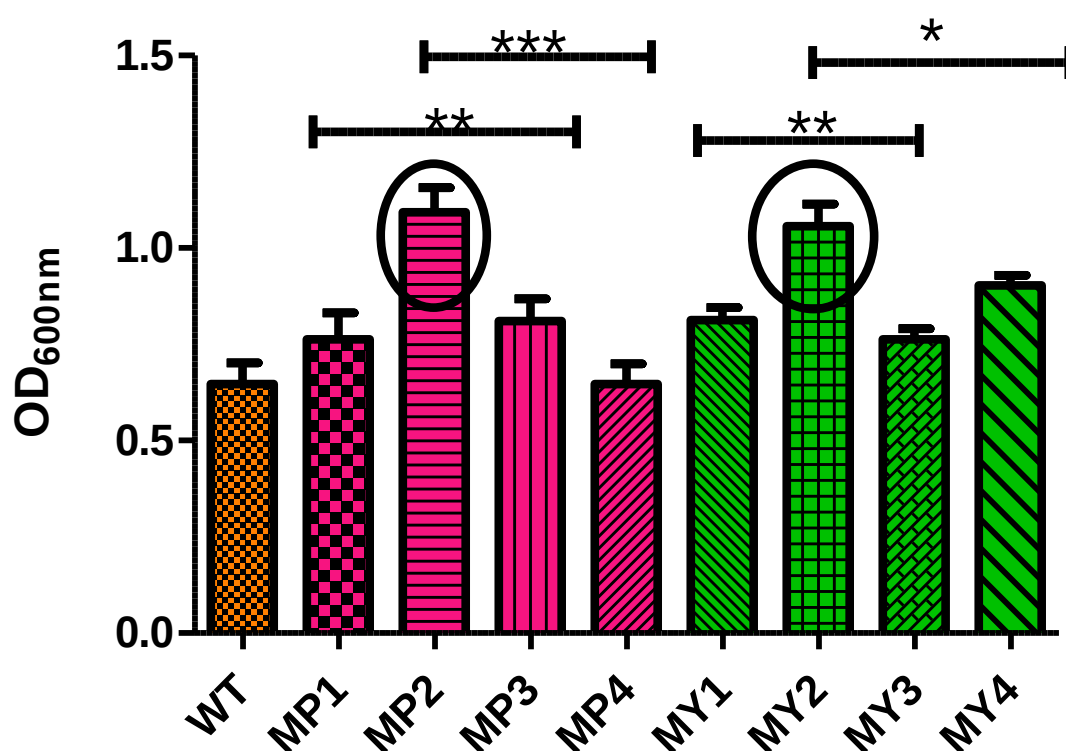


Figure 4.4. To determine the best mutant in selected colonies, the election tests were performed. The grown fitness of the selected mutants in PCM was analysed with OD600 measurement. The data were expressed by mean $\pm$ SEM ($n \geq 3$), * $p < 0.05$

Furthermore, the cross-resistance tests applied to the selected mutants in various stress conditions to determine the best one (Fig. 4.5). All samples were pre-cultured in YMM at overnight for 24 h and then centrifuged to spot onto the YMM agar plates containing 0.5 mM NiCl_2 and 8 % (v/v) ethanol according to serial dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}). It is clear in Figure 4.4 and 4.5 that the best mutants in selected colonies are MP2 and MY2 according to the results of MPN and Spot Assay. MP2 and MY2 were more stable in PCM

and also shown cross-resistance in YMM agar containing 0.5 mM NiCl₂ and 8 % ethanol. The further experiments have been designed with WT, MP₂, and MY₂ to determine their counter-effect in PC3 cells.

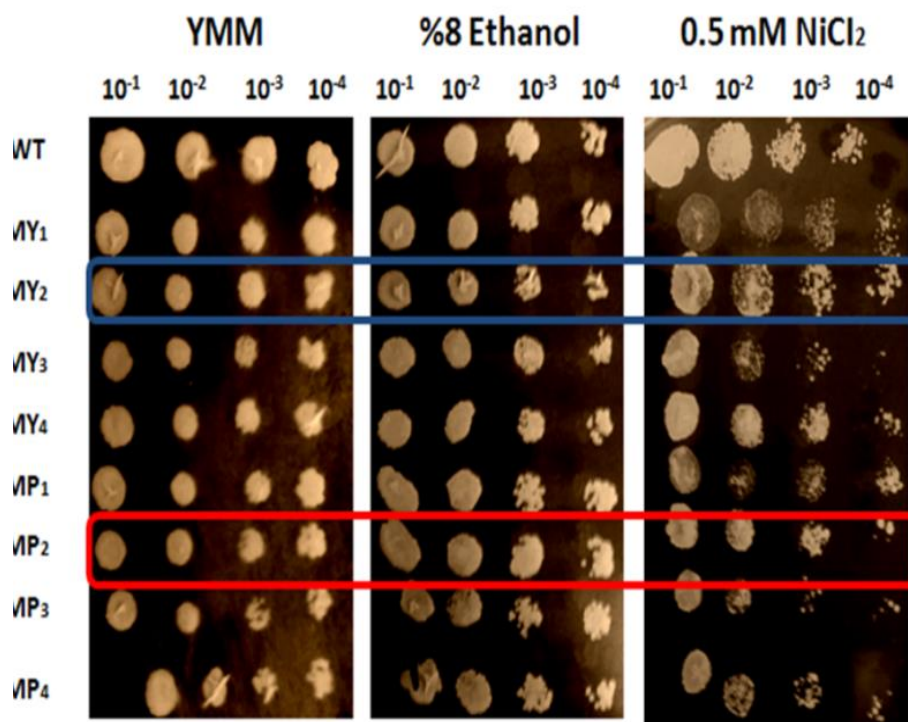


Figure 4.5. The direct selection strategy and determine cross-resistance of individual mutants, the spot test results of WT, and individual mutants on YMM plates containing different stress factors to determine cross resistances (Red and blue frame box indicate MY₂ and MP₂, the most resistant mutants)

4.3. Cultivation of *S. cerevisiae* Strains in DMEM

4.3.1. Culturing of WT, MY₂, and MP₂ in DMEM

To understand the counter-effects of WT, MY₂ and MP₂ metabolites in PC3, they were inoculated in DMEM at 30°C, 150 rpm until the middle of the logarithmic growth phase. The DMEM cultured with yeasts (WT-DM, MP-DM, and MY-DM) were triple-filtered with 0.22 µm filter to avoid any contaminants and stored up at 4°C for use in further experiments.

The growth analysis of PC3 was tested in each WT-DM, MP-DM, and MY-DM. Figure 4.6 indicates that all types of fermented DMEM (WT-DM, MP-DM, and MY-DM) significantly decreased PC3 proliferation as compared to control. Furthermore, the MP-DM and MY-DM were more effective to decrease cell growth rather than WT-DM. There is a significant counter-effect of fermented DMEM with yeast strains in PC3 cells.

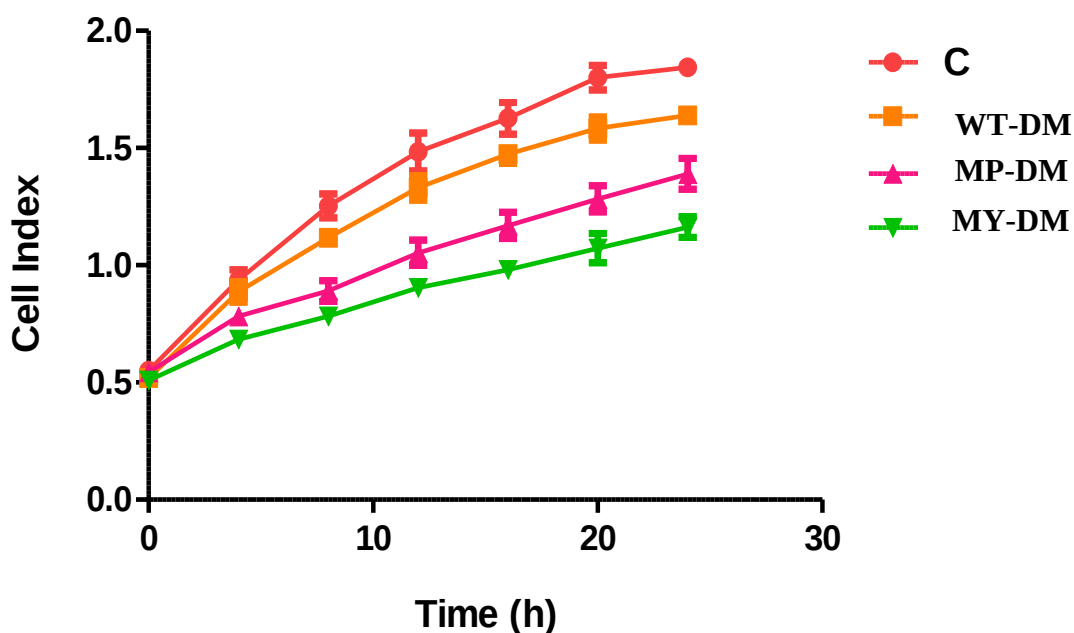


Figure 4.6. The growth fitness of PC3 was assayed in fermented DMEM and also control medium (DMEM) by iCELLigence Real Time Cell Analyzer. The significance of data was statistically analyzed by linear regression and correlation ($p < 0.05$)

4.4. ROS and Migration Analysis of PC3 in WT-DM, MP-DM, and MY-DM

Intracellular oxidative damage in PC3 with fermented DMEM was manually measured. The ROS level in PC3 was boosted with WT-DM and MY-DM while there is no significant change in MP-DM (Fig. 4.7A). Although there is an increase both in WT-DM and MY-DM, the MY-DM caused 2-fold more oxidative injury in PC3 rather than WT-DM. In short, MY-DM incomparably raised intracellular ROS level of PC3.

Moreover, the migration capacity of PC3 in fermented DMEM was also determined. Cells were starved in FBS free medium for 24 h. After that, they were placed in an insert dividing with semi-permeable membrane from WT-DM, MP-DM, and MY-DM. Finally, the migrated cells were counted in the bottom of each membrane to measure migration capacity. Figure 4.7B shows that PC3 cells 2-3 fold less migrated to fermented media in comparison to control medium. The least migration is with MY-DM while WT-DM is 2-fold and MP-DM is 3-fold higher than it.

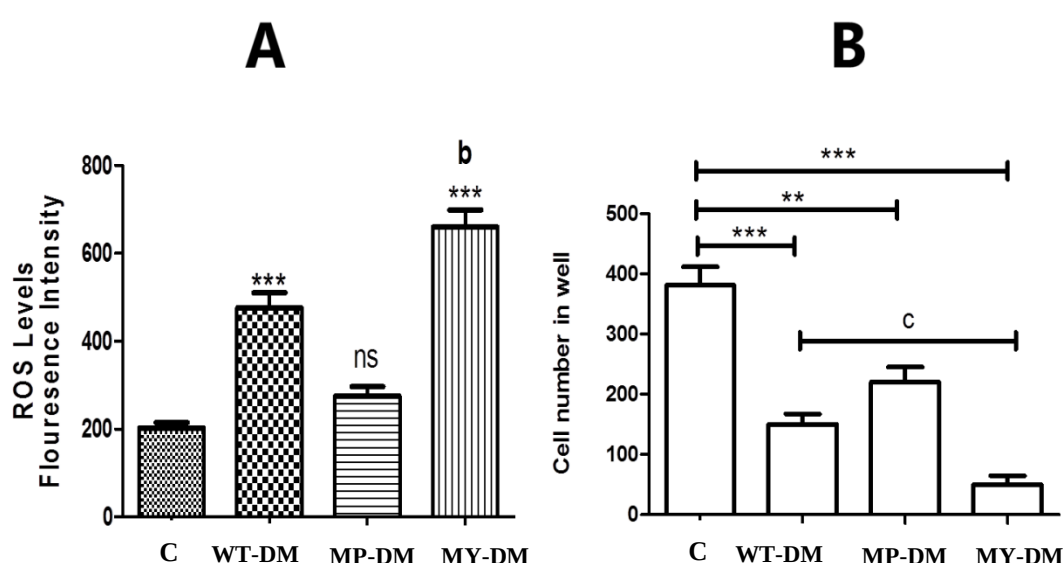


Figure 4.7. The counter effects of WT-DM, MY-DM and MP-DM in PC3 culture, **A)** the oxidative injury and **B)** cell migration number of PC3 were analysed in each WT-DM, MP-DM, and MY-DM and also control medium (DMEM). The data were shown as mean SEM (n 3). *** p < 0.001 C vs WT-DM and MY-DM, b p < 0.01 WT vs MY-DM. (C=Control)

4.5. Apoptotic Index of PC3 with WT-DM, MP-DM, and MY-DM

Apoptosis is a programmed cell death which is mostly bypassed by cancer cells. The cell viability experiment demonstrated the decreased cell growth (Fig. 4.6), therefore terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was used to detect one type of cell death, apoptosis. ApopTag in Situ Apoptosis Detection Peroxidase Kit detects DNA fragmentation that is the result of apoptotic signalling cascades. The apoptosis was detected according to Lawry (2012)

Apoptotic cells shrink and lost their structure without splintering. The apoptotic cells were imaged in PC3 cultured with WT-DM, MP-DM, and MY-DM (Fig. 4.8A). The apoptotic index was also calculated as seen in Figure 4.8B. The numbers of apoptotic cell in fermented DMEMs are significantly difference compared to control medium. Moreover, MY-DM has more apoptotic cells rather than WT-DM and MP-DM. Fermented DMEMs increased apoptosis in PC3 culture compare to normal DMEM.

4.6. Quantitative Real Time–PCR Analysis

The apoptosis related genes expressions (BAX, BCL-2, CASP3, CASP9 and CYCS) were analysed with qPCR Green Master with UNG kit protocol. Briefly, to understand gene patterns in the cell; total RNA is isolated; the mRNAs are used to synthesize cDNA with reverse transcription. Lastly, the cDNA pool is used to assay gene expression with specific primers in QRT-PCR. The fold changes of gene expression were calculated according to Ct values used to define the mRNA levels in PC3 cells in Table 1 (Livak and Schmittgen 2001). The gene arrives threshold according to their copy number in cDNA pool because the gene with high number copies firstly reaches threshold line. On the other hand, the gen with few number copies lastly appears on the target line.

Table 4.2. The comparative threshold (Ct) of gene expressions measured with QRT-PCR

	GAPDH	BAX	BCL-2	CASP3	CASP9	CYCS
C	18,9±1,3	27,4±1,3	15,9±1,0	17,1±0,5	20,5±1,7	14,2±0,2
WT-DM	11,3±0,4	24,1±1,1	16,9±2,0	15,9±1,3	18,8±3,4	14,1±0,8
MP-DM	8,6±0,6	27,7±0,8	10,0±1,3	16,9±0,3	19,0±1,6	14,9±0,2
MY-DM	21,7±0,6	26,6±1,2	9,5±2,4	15,9±1,2	20,2±1,8	14,4±0,3

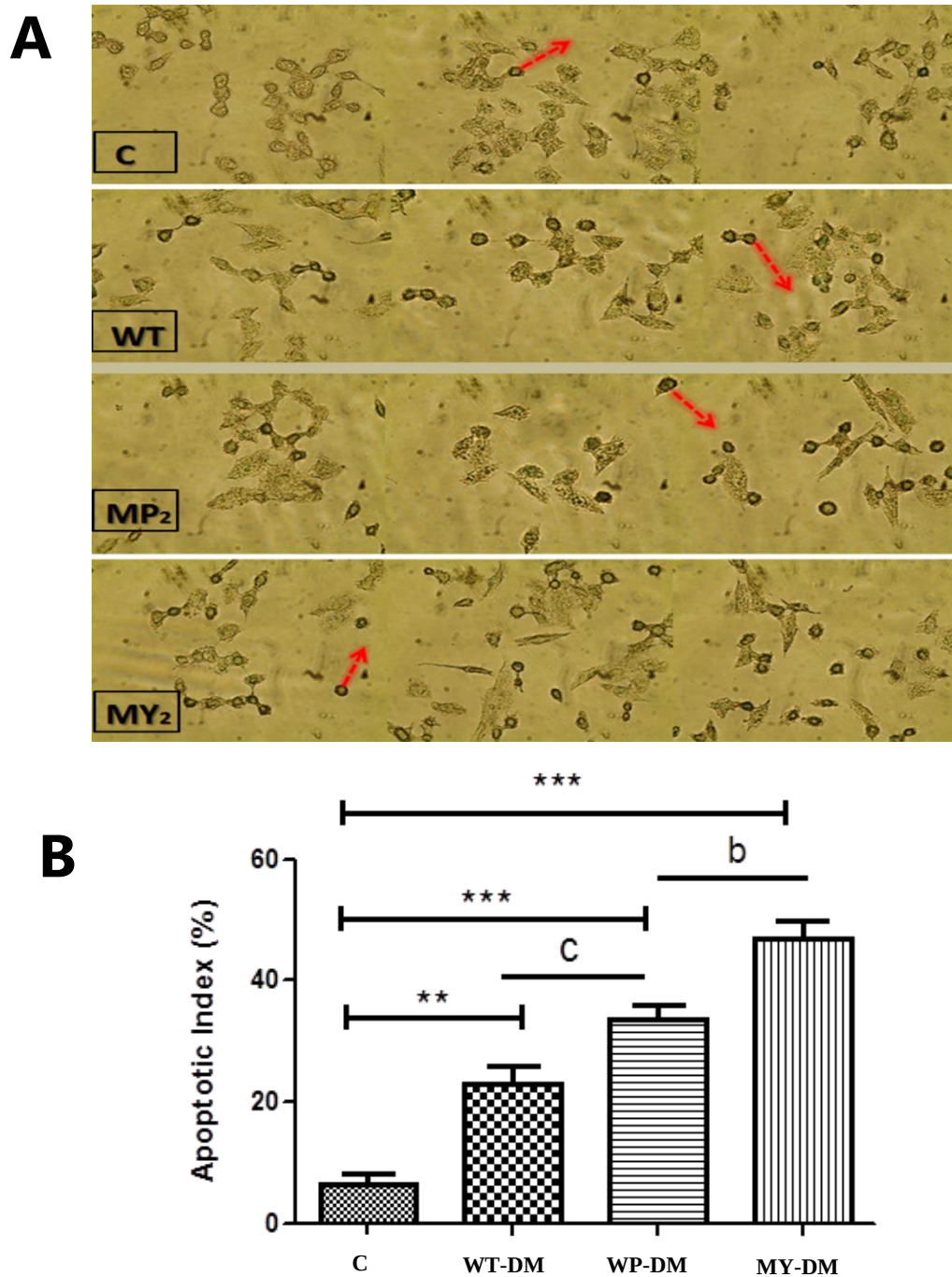


Figure 4.8. The apoptotic effect of WT-DM, MP-DM, and MY-DM in PC3 culture. Each fermented DMEM induced apoptosis in PC3 as evidenced by TUNEL staining. **A)** PC3 cell culture imaged under the inverted microscope and the alterations in nuclear morphology were evaluated by TUNEL staining (shown by red arrows). **B)** Apoptotic index was calculated by dividing apoptotic cells to total cell number. Data were presented by mean $\pm$ SEM, (n = 3). *p < 0.05 WT-DM vs C *** p < 0.001, b p < 0.05 MY-DM vs MP-DM

Figure 4.9 presents that, the apoptosis-related genes (CASP3, CASP9, and CYCS) are 10-fold up-regulated with MY-DM compared to WT-DM and MP-DM nearly 50-fold downregulated. Moreover, the balance between the expression levels of the BAX and BCL-2 is important for cell survival and death (Reed 1997). Therefore, the BAX/BCL-2 ratio was also calculated as an apoptotic marker and observed 20-50 fold increase in MY-DM and WT-DM when there is a decrease more than 50-fold changes with MP-DM. According to all results normalized to control, the apoptotic markers were significantly increased in PC3 cells treated with MY-DM. However, there is also a dramatic apoptotic downregulation in PC3 cells treated with WT-DM and MP-DM.

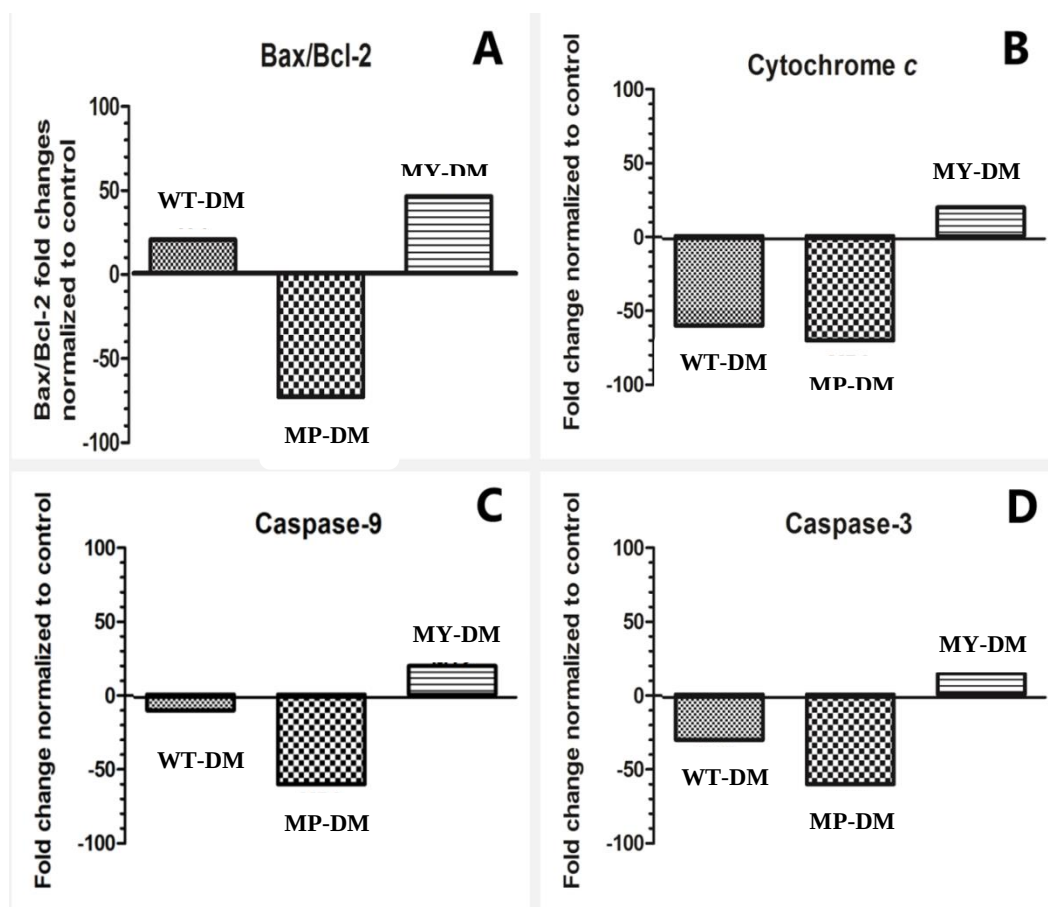


Figure 4.9. The effect of DMEM cultured with yeast strains on the level of apoptosis related gene expressions in PC3 cells. Cells were treated with WT-DM, MP-DM, and MY-DM. (A) The gene expression ratio of pro-apoptotic BAX and anti-apoptotic BCL-2, (B) CYCS, (C) CASP9 and (D) CASP3 were measured by using QRT-PCR analysis. Data are presented by means $\pm$ SEM ($n = 3$)

4.7. DNA Fragmentation Assay

The genomic DNA fragmentation in PC3 cells treated with WT-DM, MP-DM, and MY-DM was tested by agarose gel electrophoresis. At the first, PC3 cells were cultured and treated according to experimental grouping part. The harvested cells were centrifuged at 2500 rpm for 3 min and washed in PBS to isolate DNA by using G-Dex extraction kit protocol. Genomic DNAs were run on 1% agarose gel and stained with Ethidium Bromide. The DNA fragmentation was imagined by Image Lab (GelDoc EZ).

DNA fragmentation is a potential candidate for apoptosis (Takaki et al. 2014). The DNA fragmentation in PC3 cells treated with WT-DM, MP-DM, and MY-DM was assayed by gel electrophoresis. Figure 4.10 shows that there is no DNA fragmentation in the cultivation of PC3 cells with WT-DM, MP-DM, and MY-DM.

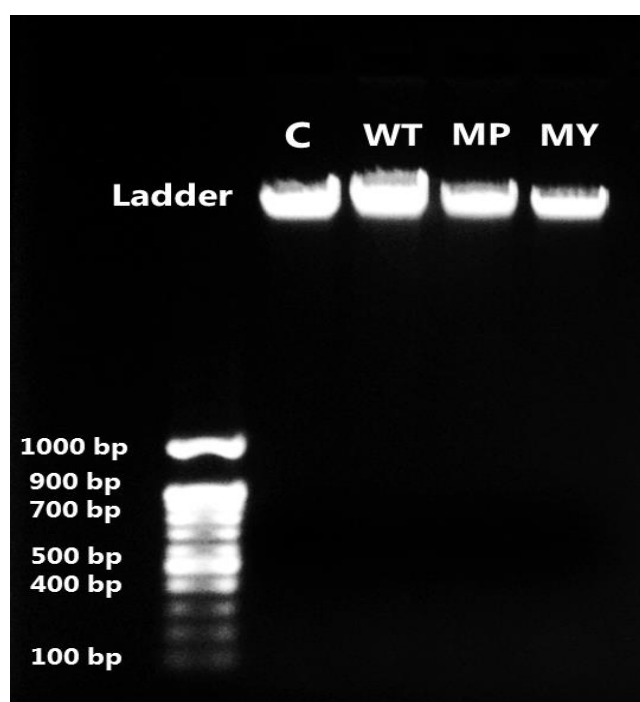


Figure 4.10. The consequences of WT-DM, MP-DM and MY-DM treatment on genomic DNA in PC3 cells. DNAs were run up with ethidium bromide stained 1.5% agarose gel electrophoresis. The gel was imagined in the GelDocEZ with Image Lab 5.2.1 software

4.8. Total Protein Isolation

Total protein was extracted to determine protein levels of Bax, procaspase-3, and GAPDH. The PC3 cells were cultured and treated with WT-DM, MP-DM, and MY-DM for 12 h, and then cells were harvested and washed up in the cold PBS. The ice-cold lysis buffer (Table 3.7) was added all samples due to cell number to obtain total cellular protein lysates. Then, the samples with lysis buffer were incubated on ice for 1h. After that, they were centrifuged at 14,000 rpm for 10 min at 4°C and, the supernatants were carefully collected in micro-centrifuge. Total protein concentration was measured with Bradford Assay (Kruger 1994). The Laemmli sample buffer 5X (Table 3.7) was added to all protein samples and heated at 95°C for 5 min. Finally, they were stored at -20°C for next steps.

Total protein was isolated and measured with UV-Spectrophotometer (Shimadzu, JAPAN) at 595 nm as mentioned in Bradford Assay (Kruger 1994). The total protein concentration was listed in Table 4.3.

Table 4.3. Total protein concentration of PC3 cell treated with WT-DM, MP-DM, and MY-DM

Sample	Total Protein Concentration (µg/ml)
C	3,91
WT-DM	3,81
MP-DM	4,80
MY-DM	3,24

4.8.1. Western Blotting Assay

In addition to gene expression, the apoptosis related protein levels of PC3 cells treated with fermented DMEM were also assayed by Western Blotting. At first, total protein is isolated and measured with Bradford Assay. Then, total protein is run with SDS-PAGE gel electrophoresis. The separated proteins are transferred to a membrane for blotting process. The specific protein is tagged with primary antibody on the membrane and then

the secondary antibody is used to get a result. The fold changes in protein levels were normalized to the intensity of referenced protein (GAPDH) according to with the following article (Degasperi et al. 2014). Figure 4.11 BII displays that the level of procaspase-3 (a key protein of apoptotic pathway) was significantly decreased in PC3 cells treated with MY-DM and MP-DM, compared to WT-DM and control medium. On the other hand, there is no a significant difference in the pro-apoptotic Bax level of PC3 cells cultured with a variety of DMEMs. (Fig.4.11 BI)

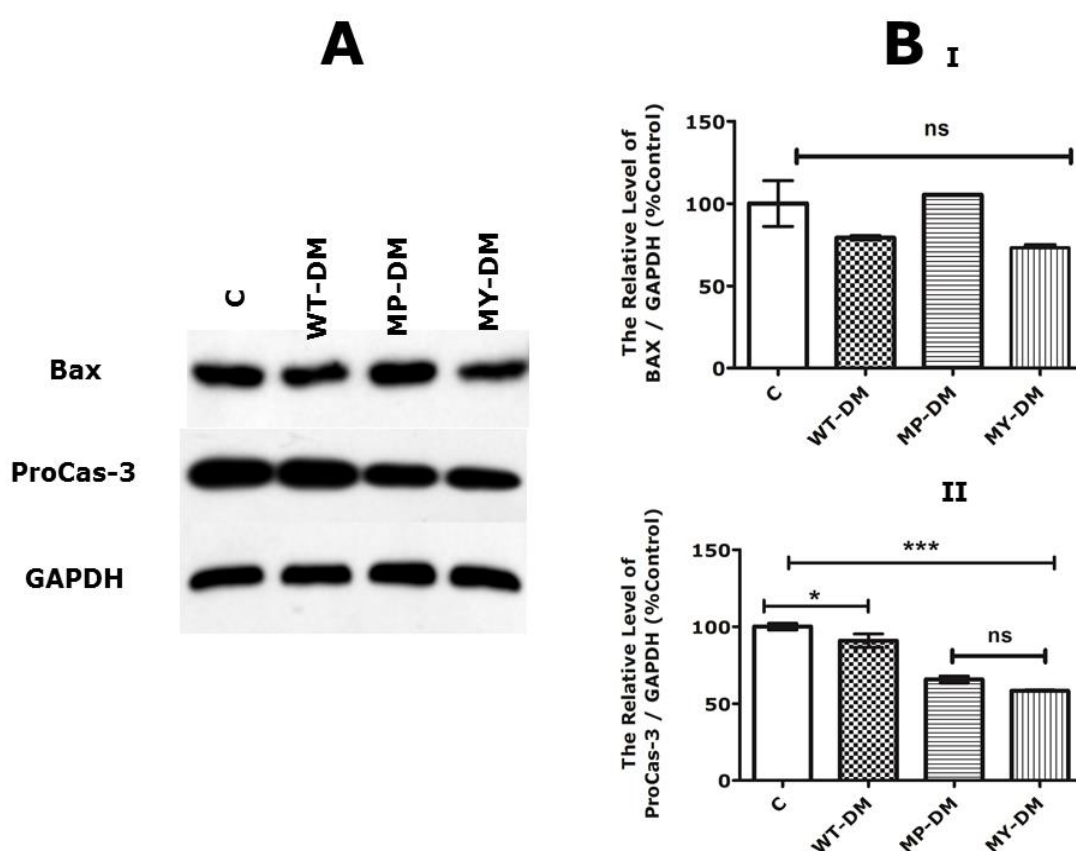


Figure 4.11. The apoptosis-related protein level in PC3 cells treated with DMEM cultured by yeast strains. Cells were treated with WT-DM, MP-DM, and MY-DM. (A) The expression of Bax (48 kDa) and ProCas-3 (34 kDa) protein levels were measured by Western blot analysis. GAPDH was used as loading control. (B) Densitometric analysis of proteins was quantified using Image Lab 5.2.1. Procaspase 3 was significantly decreased in PC3 cells by MP-DM and MY-DM treatment. There are no significant differences in Bax level of control PC3 cells and cells treated with WT-DM, MP-DM, and MY-DM. Data are presented by means $\pm$ SEM (n = 3), *p < 0.05 WT-DM vs C *** p < 0.001 MP-DM, MY-DM vs C

5. DISCUSSION

The complexity of cancer is a well-known major problem for the treatment of disease. The cell variety and the multiplicity of intracellular pathways make it difficult to find a single and definitive solution to cancer (Berridge et al. 2010; Jang, et al. 2013). Although the normal cells are controlled by many checkpoints to prevent cancer (Vogelstein and Kinzler 2004), it is hard to control a cell after to be cancer (Bertram 2001), because there are countless factors in cell being responsible from canceration (Meacham and Morrison 2013), so any approach is not singly sufficient to cope with this chaos.

It is always required a new research to remedy cancer. In order to find a definite solution to cancer, it is necessary to know the entire metabolism in detail (Meacham and Morrison 2013), but the current level of knowledge and technology is not sufficient. Therefore, it needs to find a natural solution that presents another way to struggle with the disease. The present study is based on a natural approach to deal with cancer; that is using the metabolite of an organism produced against cancer environment.

To test the natural approach hypothesis based on using the metabolite of a microorganism produced against the medium cultured with cancer cells, *S. cerevisiae* was initially seeded in PCM. However, Figure 4.1 shows that PCM significantly restricted yeast growth. Accordingly, the possible stress factors of that restriction on yeast were studied to determine stress source. The pH of PCM had been already arranged to 4-6 according to the optimum growth of *S. cerevisiae* (Salari and Salari 2017) before culturing process, so it cannot be a stressor. Additionally, yeast was cultured in different media including various glucose concentrations to compare with PCM. Figure 4.2 displays that yeast can grow in lower glucose level than measured in PCM. Therefore it is concluded that the glucose level is possibly not the main stress factor to prevent yeast growth in PCM. It might be thought that there is another stress factors in PCM to restrict yeast growth.

According to our findings, the main aim of the study was determined to attain *S.cerevisiae* strains having able to grow in PCM, and to that end, evolutionary engineering strategy (Cakar 2009) was successfully used to obtain the desired phenotype. In the present study, we applied two different ways to achieve PCM-resistant *S.cerevisiae* mutant strain by evolutionary engineering. In the first one, YPD-precultured *S.cerevisiae* cell population was treated with EMS to randomly mutagenize and then the mutant colonies were determined by direct selection on solid media supplemented with PCM. In the second strategy, EMS was exposed to PCM-precultured *S.cerevisiae* cell population to understand the contribution of the stress condition to the direction of mutation (Melkikh and Khrennikov 2017). According to quantum biology research, the stress factors can direct mutations in microorganism genome to bring them in positive functions (Lambert et al. 2012).

The desired colonies for both strategies were selected on solid media supplemented with PCM (Figure 4.3B). The individual mutants derived from both strategies are more resistant to PCM than WT (Fig. 4.4). Accordingly, the evolutionary engineering was a suitable strategy to obtain the desired phenotype, compared to metabolism engineering. Generating countless mutants with randomly EMS-mutagenized population has a better chance to obtain the desired phenotype, in comparison with the metabolism engineering that requires knowing the entire metabolism to create the desired one (Tilloy et al. 2015). Evolutionary engineering is successfully applied to obtain various stress-resistant mutants, for example, a nickel-resistant *S.cerevisiae* (Küçükgoze et al., 2013), an acetic acid tolerant *Spathaspora passalidarum* (Morales et al., 2017) and more (Patnaik, 2008).

The best mutants among the selected colonies obtained with evolutionary engineering were determined by cross-resistant to nickel and ethanol (Fig. 4.5). The cross-stress provides a growth fitness advantage to the microorganism in a stressful environment (Dragosits et al., 2013). Thus, MY2 and MP2 have a better chance to grow in PCM rather than WT and other selected colonies (Fig. 4.4). PCM is a restrictive environment for *S.cerevisiae* to grow, therefore MY2 and MP2 obtained by evolutionary engineering to be resistant to medium.

In the current study, the hypothesis is based on using a microorganism that might make cancerous environment habitable for itself with various metabolites production to treat cancer-like diseases. We had a combined purpose because of using two different ways. Firstly, we aimed to obtain the desired phenotype of *S.cerevisiae*, which can smoothly grow in PCM. Following this event, MY2 and MP2 were determined as special phenotypes that might produce metabolites during growth cycle to make the stressful environment of PCM habitable. As a second goal, the selected mutants and WT were cultured in DMEM to test their metabolites on PC3 cells. The microbial fermented media might regulate the metabolism of another organism (Phonnok et al. 2010; Smith et al. 2013) because the most of the organisms produce various metabolites to modify environment habitable. Many organisms firstly response to the altered environmental conditions with behavioural adjustments and also find the many ways to be adapted to extreme conditions (Brooks et al. 2011; Wong and Candolin, 2013).

There are a variety of microorganism, also, studied by astrobiologists (Rampelotto 2010) that have abilities to adapt and survive under extreme conditions such as pressure, high temperature, vacuums, ultraviolet, salinity and radiation (Morozkina et al. 2010; Rampelotto, 2013). Especially, microorganisms are flexible to evolve in extreme conditions. This evolution helps both to survive in the extreme environment and to gain a function providing an ability to produce special metabolites to restrict environment for other creatures. Furthermore, we further cultured MY2, MP2 and WT in DMEM to get media with metabolites which may restrict the environment for PC3 cells. WT-DM, MY-DM, and MP-DM have microbially fermented media tested on PC3 cell culture to assess their treatment potential.

Furthermore, we have examined the anti-proliferation effects of WT-DM, MY-DM, and MP-DM in PC3 cells, which decreased cell growth (Fig. 4.6). Furthermore, the metabolites of both mutant strains have been more effective than WT's to decline cell proliferation. To eliminate the possible stress factors such pH and glucose concentration, we adjusted the pH of media according to physiological conditions (Perkel et al. 1990) and also measured the glucose concentration of each medium for critic level of PC3 (not lower than 1g/ml). In addition, Liu et al., (2010) suggests that prostate cells dominantly

uptake fatty acid over glucose. WT-DM, MY-DM, and MP-DM are also a kind of fermented medium including more fatty acid (Taylor and Kirsop 1977). Thus, PC3 cell growth was possibly reduced by secreted metabolites in WT-DM, MY-DM, and MP-DM except for pH and glucose concentration. Moreover, MY-DM and MP-DM derived from mutant strain obtained with evolutionary engineering were better than WT-DM to decrease PC3 cell index. The secondary metabolites of both WT and an individual mutant of *S.cerevisiae* induced a decrease in prostate cancer cell number.

We also investigated the pro-oxidant activity of WT-DM, MY-DM, and MP-DM in PC3 cells, which significantly increased the ROS production with WT-DM and MY-DM, however no significant differences in MP-DM. The ROS rate is increased in all cancer types comparing to a normal cell, which contributes to tumour progression (Zhou et al. 2014). However, cancer tissues also express more antioxidant molecules to partly detoxify oxidative damage (Liou and Storz 2010). The treatment of cancer cell lines with the secondary microbial production enhances ROS level (Beloborodova et al. 2012; Baez and Shiloach, 2014). WT-DM and MY-DM may cause dysfunction of antioxidant defence systems in by increasing ROS production in PC3 cells.

It is clear that pro-oxidants are main factors to activate oxidative injury in a cell with generating free radical, downregulation of GSH, SOD, and GSH-Px (Birben et al. 2012). Moreover, it is previously reported that microbial production may cause a rapid increase in the level of ROS (Jones et al. 2012). The oxidative damage has a critical role in the fate of cell viability and apoptosis. For example, ROS accumulation in cell induces blocking of antioxidant defence systems and increasing the oxidative stress, leading apoptosis.

In addition, we obviously showed that treatment of PC3 cells with WT-DM, MY-DM, and MP-DM decreased cell growth and also WT-DM and MY-DM provoked ROS generation. According to our findings, it might be suggested that there is a relation between decreased PC3 cell proliferation and increased intracellular ROS production.

The relation of microbial community with the host is definitely known that is able to affect cell metabolism by secondary metabolites in different ways. The secreted metabolites can reduce oxidative damage in normal tissues to suppress cancer progression and also activate the various apoptosis-related pathways in cancer cells to decrease tumorigenesis (Louis, Hold, and Flint 2014). Furthermore, the microbiome has an essential role with a variety of its degradation and production properties to maintain homeostasis and regulate metabolic balance (Garrett, Gordon, and Glimcher 2010). It might be proposed that the microbial secretion in culturing medium has a potential to regulate environmental conditions. Therefore, WT-DM, MY-DM and MP-DM, the examples of the medium including microbial metabolites, might lead the several changes in PC3 cells.

Additionally, we also analysed the effects of WT-DM, MY-DM and MP-DM treatment in invasive PC3 cells. The number of migrated cells in fermented media is lower than control one. The starved PC3 cells mostly kept away MY-DM whereas this avoidance was less to WT-DM and MP-DM. The results are clear that all fermented media declined the cell migration compared to DMEM.

Moreover, the apoptosis index of PC3 cells treated with WT-DM, MY-DM, and MP-DM were determined by TUNEL assay, which is significantly induced by all fermented media. Especially, MY-DM indicated a powerful impact by forcing cells to trigger apoptosis. Our findings in good agreement with previous studies revealed that elevated oxidative damage has an important role in apoptosis (Kannan and Jain 2000). Apoptosis is a type of cell death, that can be regulated various pathways in cell metabolism. Accumulated ROS is just potential signal in the cell to induce apoptotic pathways. These findings plus our results strongly suggest that WT-DM and MY-DM increased ROS generation might be potential responsible to induce apoptosis in PC3 cells. However, there is probably another way for MP-DM to cause cell death in PC3.

Our results further demonstrated that there is no genomic DNA fragmentation in PC3 cells treated with WT-DM, MY-DM, and MP-DM. Although not in all cell types (Yuste et al. 2001), particularly fragmented DNA is considered as a marker for apoptotic cells

(Collins et al. 1997). Schulze-Osthoff and co-workers (1994) suggested that DNA degradation is not a necessity for induction of apoptotic cell death. Although we showed apoptosis by WT-DM, MY-DM and MP-DM treatment in PC3 cells, there is no proof for genomic DNA fragmentation; so we concluded that PC3 cell death might be caused by apoptosis lacked DNA degradation.

Herein, we also appraised molecular pathways by which WT-DM, MY-DM, and MP-DM might be effective to induce apoptosis in PC3 cells. There are the intrinsic and extrinsic pathways in apoptosis metabolism. The intrinsic pathway is most important, related to mitochondrial dysfunction promoting apoptosis by the change of membrane permeabilization causing the release of cytochrome c into the cytoplasm. There are two essential proteins in the mitochondrial pathway have the main role to determine the fate of cell apoptosis. Both of them are localized to mitochondria membrane and related to the intrinsic apoptotic pathway, Bcl-2 is anti-apoptotic to suppress apoptosis, the other one is proapoptotic Bax inducing programmed cell death (Tsujimoto 1998; Damitri and Yusof, 2010). The mitochondrial membrane permeability to cytochrome c is controlled by the mitochondria membrane permeability transition pore (MPTP) and the location of Bcl-2 proteins into mitochondria (Jurgensmeier et al. 1998). The release of cytochrome c into the cytoplasm is a crucial hallmark of the apoptotic process. The process begins with the translocation of Bcl-2 proteins into mitochondria, which is a signal to release cytochrome c into the cytoplasm. Then, cytochrome c combines with Apaf-1 in the cytosol to produce apoptosome activating procaspase 9 to induce procaspase 3 cleavage (Elmore 2007).

We also showed that the treatment of PC3 with WT-DM, MY-DM, and MP-DM altered Bax/Bcl-2 expression ratio, which has an important responsibility to decide apoptosis in a cell (Perlman et al. 1999). The Bax/Bcl-2 ratio was increased more than 20-fold changes in PC3 cells by cultured with WT-DM and MY-DM, whereas the cultivation with MP-DM almost 70-fold decreased it. Furthermore, our results suggest that the changes in Bax/Bcl-2 ratio lead to the release of cytochrome c which plays a crucial role to promote apoptosis. The expression of cytochrome c was upregulated in PC3 cells with treatment of MY-DM. However, WT-DM and MP-DM were mostly caused a downregulation of cytochrome c expression. Additionally, we observed that both caspase-3 and caspase-9

expressions had similar results to cytochrome c. The activation of caspase-3 and caspase-9 triggered by apoptosome formed due to the efflux of cytochrome c into the cytoplasm, have a critical role as an effector in the intrinsic pathway of apoptosis (Kannan and Jain 2000). The results of apoptosis-related genes expression have to be supported with the level of proteins associated with apoptotic pathways.

We further revealed that the protein level of procaspase3 down-regulated by MY-DM and MP-DM induction in PC3 cells whereas there is no difference with the treatment of WT-DM and control medium. The caspase cascade activation has an important role to finalize morphological and biochemical features of apoptosis (Salakou et al. 2007). There is a balance between caspase3 and its precursor, procaspase3 hence any change in the amount of procaspase will directly affect caspase level. For example, it might be suggested that a decrease in procaspase3 level can cause an increase in caspase3 level. Therefore, the decreased level of procaspase 3 in PC3 cells treated with MY-DM and MP-DM might be a signal of the up-regulation of caspase 3, which is a strong evidence for apoptosis (Elmore 2007). On the other hand, there is no a significant difference in the Bax level of PC3 cells cultured with WT-DM, MY-DM, and MP-DM. Bax is a proapoptotic protein localized into mitochondria, but there is a balance between Bax and Bcl-2 into there (Damitri and Yusof 2010). We previously mentioned a change in the BAX/BCL-2 ratio depend on the gene expression results. Thus we suggest that the stable level of Bax in PC3 cells might indirectly indicate a change in Bcl-2 level.

According to our results on the molecular level of PC3 cells treated with WT-DM, MY-DM, and MP-DM, it is clear that MY-DM includes various metabolites to induce apoptosis in PC3 with increasing the expression of apoptosis-related genes. On the other hand, there are no coherent findings between molecular and physiological level for WT-DM and MP-DM treatment to promote apoptosis in PC3 cells. The desired phenotypes obtained by the different strategy of evolutionary engineering might have totally diverse metabolites profile in cultured medium, which induce several alterations in PC3 cells. Actually, MP-DM might cause a special type of apoptosis called anoikis which changes anchorage-dependent cells morphology by detaching from the ECM or surrounding cells (Gilmore 2005). However, the both of them showed a specific impact in PC3 cells

different from WT. Eventually, the destiny of cells depends on the stability of Bax/Bcl-2 ratio and the activation of caspase-3 to enter the apoptosis path (Salakou et al. 2007).

Herein, we evidently proved that WT-DM and MY-DM increase oxidative stress in PC3 cells by promoting ROS generation, and also altered Bax/Bcl-2 ratio, which are keys for the intrinsic pathway of apoptosis. In addition, the cultivation of PC3 with MP-DM was not only lost on ROS production but also downregulated expression of apoptosis-related genes.

In summary, we clearly demonstrated that the instinct of survival of the organism in extreme environment can be directed to obtain the desired phenotype, and that might be used as a weapon to struggle with extreme conditions. However, we have successfully obtained the desired phenotypes by evolutionary engineering strategies (MY2 and MP2) to alter the situation that is the restricted growth of WT in PCM. Moreover, we showed that the cultured DMEM with MP2, MY2, and WT include effective metabolites to induce apoptosis in PC3 cells.

6. CONCLUSION

In this study, we used in vivo evolutionary engineering approach to attain a very resistant phenotype, able to easily grow in cancer environment, in *S.cerevisiae* cells. *S.cerevisiae* mutants were obtained by evolutionary engineering. For this purpose, the yeast population was randomly mutagenized with EMS to obtain a variety of mutants. After that, individual colonies were selected on a solid PCM-agar plate to determine the best mutant among them with cross-test assays. At the end of all this process, MY2 and MP2 have chosen the best mutant which can normally cultivate in PCM. According to our findings, the both MY2 and MP2 indicated that they have a great fitness to grow in PCM. Moreover, our results proved once more that evolutionary engineering is an easy and powerful method compared to metabolism engineering to obtain and identify mutant strains with desired characteristics.

We also assessed that the counter effect of DMEMs fermented with individual mutants and WT yeasts in PC3 cells. WT-DM, MP-DM, and MY-DM significantly decreased cell growth in PC3 cell culture. WT-DM and MP-DM displayed variable outcomes that cause several conflicts, for example, MP-DM increased apoptotic index whereas it was downregulated apoptotic genes expression. WT-DM also reduced cell proliferation and migration whereas it did not induce apoptotic pathways. Unlike WT-DM and MP-DM, MY-DM simultaneously activated many molecular pathways, for instance, elevated ROS production, suppressed cell migration and upregulation of apoptotic gene expressions, to promote apoptosis in PC3 cells. This evidence shows that an organism has the ability to make environment habitable by its metabolites production that modifies the metabolism of other creatures living in the same area. We further showed that a dominant and specific phenotype can be obtained with evolutionary engineering strategy to reorganize the extreme environment.

The aim of this study is to investigate a resistant yeast strain which is able to grow in the cancerous environment. It is also a unique research to examine the counter-effects of metabolites secreted by the mutant yeasts obtained with evolutionary engineering, in PC3 cells. In the current study, although we clearly found that the selected specific mutants seemed to produce important metabolites in DMEM to induce apoptosis in PC3 cells, the molecular characterization of desired phenotypes determined by evolutionary engineering is still not identified, which is mainly responsible for this special character. As a conclusion, the complex network of metabolic pathways is still a challenge to deeply understand the interactions between genes and gene regulations. For that reason, we propose several future strategies such as the proteomic analysis and whole genome sequencing to clarify the molecular mechanisms of PCM-resistant yeast.

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