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Evolutionary-obtained *Saccharomyces cerevisiae*, resistant to DMEM cultured with prostate cancer cells

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Abstract

The complexity of the cell is a major problem to study on cancer treatment. In order to find an accurate solution to cancer, it is necessary to know the cell metabolism in detail, but the available knowledge and technology are inadequate. Herein, there is a need for alternative approaches to suggest a treatment way against cancer instead of trying to understand the cell metabolism in details. Therefore, this study aimed to obtain the desired phenotype of Saccharomyces cerevisiae which can easily grow in the DMEM pre-cultured with PC3 (PCM).

Evolutionary engineering was successfully applied to wild type (WT) yeast population to randomly generate a variety of genetic phenotypes. The resistant mutants against PCM were randomly selected from an agar plate. Furthermore, the best individual mutant being resistant in PCM was determined with genetic stability tests.

In the current study, the resistant yeasts were obtained by evolutionary engineering against PCM restricting the growth of WT. The growth fitness of selected mutants dramatically increased in the PCM, when compared to WT. The best mutant, MY2, is an example to be resistant in extreme environment with directing the instinct of survival of organisms. To suggest as a cancer treatment, the secondary metabolites of MY2 on cancer cells have to be tested.

Keywords: *Saccharomyces cerevisiae*, cancer, evolutionary engineering, PC3, yeast

Evrimsel mühendislikle elde edilmiş, prostat kanser hücrelerinin kültürlendiği DMEM ortamına dirençli *Saccharomyces cerevisiae*

Özet

Hücrenin karmaşıklığı kanser tedavisi geliştirmek için büyük bir sorundur. Kansere karşı doğru bir çözüm bulmak için, hücre metabolizmasını ayrıntılı olarak bilmek gerekir, ancak mevcut bilgi ve teknoloji bunun için yetersizdir. Bu noktada, kanser tedavisi için alternatif yaklaşımlara ihtiyaç duyulmaktadır. Bu nedenle, bu çalışma, PC3 (PCM) ile önceden kültürlenmiş DMEM'de kolaylıkla büyüyebilen mutant *Saccharomyces cerevisiae* fenotipinin elde edilmesini amaçlamıştır.

Evrimsel mühendislik yöntemi başarılı bir şekilde uygulanarak, doğal tip maya (WT) popülasyonunda çeşitli genetik fenotipler rasgele üretildi. PCM'ye karşı dirençli mutantlar bir agar plakasından rastgele olacak şekilde seçildi. Ayrıca, Seçilen mutant feneotipler içinden PCM'de dirençli en iyi bireysel mutant genetik kararlılık testleri ile belirlendi.

Mevcut çalışmada, WT'nin büyümesini sınırlayan PCM'ye karşı evrimsel mühendislik yöntemiyle dirençli muatantlar elde edildi. Seçilen mutantların PCM içindeki büyüme kapasitesi, WT'ye kıyasla anlamlı biçimde arttı. En iyi mutant olan MY2, organizmaların hayatta kalma içgüdüsünü yönlendiren uç koşullarda dirençli olmanın bir örneğidir. Kanser hücresi büyütülen ortamda rahatlıkla büyüye bilen MY2, üretiği ikincil metabolitlerin kanser hücreleri üzerinde test edilmesi gerekir. Elde edilen sonuçlara göre kanser tedavisinde kullanılabilirliği değerlendirilmelidir.

Anahtar Kelimeler: *Saccharomyces cerevisiae*, kanser, evrimsel mühendislik, PC3, maya

1. Introduction

Although cancer is often described as an uncontrolled cell division [1], it is actually a communication disorder between a maverick single cell and tissues in an advanced organism. In a living system, both internal and external environmental factors are important for communication continuity and tissue integrity. Therefore, there is no standard canceration process, because cells have various metabolic pathways that are open to faults leading to different types of cancer formation. Any cumulative defect occurs in any pathway of the cell can induce cancer by escaping from the cell checkpoints [2]. In complex organisms, the cell diversity is responsible for the various types of cancer because of the expressed genes and thus the active pathways are different due to cell function in every cell [2,3]. The presence of more than one metabolic pathway in the same cell may also promote different forms of the tumorigenesis.

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Tumours are maverick and able to quickly turn environmental conditions in favour of themselves by altering their metabolic pathways according to their progression requirements [4]. The tumour microenvironment (TME) inducing hypoxia (less oxygen), high lactate and energy deprivation plays a major role in cancer pathogenesis. Furthermore, TME contains chemokines secreted by cancer cells to regulate environmental homeostasis [5]. The recent evidence suggests that the tumour behaviour might be better understood with determination the effects of TME in cancer progression [6,7]. It is concluded that tumours have specific environments enabling advantages for cancer progression while causing stress for other cells.

According to current data, it is clearly understood that cancer treatment approaches should be specific to the cancer development process, as the classical methods and general drug treatments are insufficient to present an effective remedy for complex diseases [8]. It is required that humankind has to find more natural, practical and quick strategies against serious diseases.

Nature is a network balance of all living things establishing a commensal, symbiotic or pathogenic relationship with each other to survive, which can offer solutions against serious illness. The organism can deal with extreme conditions by constantly adapting their habits to the new environment. For example, they may take a position in habitat according to physical, social and psychological conditions of the environment [9]. Recent studies have shown that each advanced organism lives with a wide variety of microorganisms called "microbiota" [9,10]. Furthermore, the changes in microbial integrity are thought to play an important role in the development of modern diseases such as cancer, diabetes and neurodegenerative [11,12]. If the microbiome is sufficiently diverse, healthy and beneficial for the advanced organism, both sides have a chance to live well. It is well-understood that microbial metabolites are essential to fix environmental habitat [13,14]. The metabolite production of the microbiota is most important for the host to prevent harmful and undesirable microorganism and also reshape homeostasis [10,15]. Taken together these findings of microbial production with the extreme conditions of TME, the utilizing nature to improve new approaches is important for cancer-like diseases.

A detailed observation of organisms shows that they are able to make surround habitable with the instinct of survival when there are changes in the internal or external environment. In particular, the adaptation of microorganism, due to environmental changes, can be easily followed by scientists for their short life cycle. The best known examples of adaptation are antibiotic resistance lately worried the world [16] and the rapid mutations of influenza-type viruses to escape from the vaccines [17]. The rapid adaptation ability of microorganisms to chaotic and restricted environmental conditions can be used as a treatment approach for complicated disorders such as cancer, diabetes [8,9].

In the treatment of multidimensional diseases, it is necessary to understand the entire molecular metabolism of the cell, but both incomplete data and the complexity of cell metabolism make it difficult to find a definitive solution. Therefore, the robustness of microorganisms under stress conditions can be suggested to find a solution against complex diseases. On the other hand, every single piece of all molecular mechanisms should be studied to find a treatment. Although there is not yet a biologically modified microorganism for therapeutic purpose, several studies shown that fermented nutrients by microorganisms regulate metabolism [15,18].

The desired organism is mostly obtained by classic metabolic engineering method [19] manipulating cell component such as genes, enzymes, pathways [20]. However, to know entire molecular metabolism of the cell is a limitation of classical method, thus an alternative strategy is required to find specific characteristics. In this case, the most important point in a study is determining the right method. The inverse metabolic engineering method is an extremely effective and successful method to select individuals with specific properties [21]. To be master of all constituent parts of cell metabolism and know their relationship with each other is necessary to obtain a desired phenotype with rational metabolic engineering method [22]. On the other hand, evolutionary engineering as an approach in inverse metabolic engineering provides advantages to study with population rather than the single individual as the creating random mutations in each individual facilitates the finding of special strain [23, 24].

The commonly used evolutionary engineering was successfully applied in various microorganisms [25–27]. Moreover, the evolutionary engineering was especially used to improve *Saccharomyces cerevisiae* physiologically and productively [20], [28,29]. *S. cerevisiae* is the most commonly preferred model organism in scientific researches and also in industrial processes such as bread, beer and wine production [30]. It is generally recognized as safe (GRAS) and enables various advantages in research, such as short life cycle, rapidly growth and representing advanced organism as eukaryotic structure.

In the present study, it is aimed to determine and 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. The outputs of this study will probably be helpful to give an idea of whether the survival instinct of microorganisms can be a potential treatment for complex diseases.

2. Materials and methods

2.1. Chemicals and media

Ethyl methyl sulfonate (EMS, Acros), Nickel chloride (NiCI2, Sigma-Aldrich), Ethanol (Sigma-Aldrich). Yeast Peptone Dextrose ‘YPD’ [1% w/v peptone, 1% w/v yeast extract 2% w/v glucose (Sigma-Aldrich)], Yeast Minimal Medium ‘YMM’ [2% w/v glucose, 0.67% w/v yeast nitrogen base without amino acids (Sigma-Aldrich)], Endothelium Growing Medium (EGM, Lonza), Dulbecco's Modified Eagle Medium (DMEM, marka), phosphate buffer saline (PBS), penicillin-streptomycin and endothelium growth factors; such as Insulin-like Growth Factor-1 (R3-IGF-1, Lonza), ascorbic acid (Lonza), fatal bovine serum (FBS, Lonza), human Fibroblast Growth Factor-Beta (hFGF-, Lonza), vascular endothelium growth factor (VEGF, Lonza) were used for culturing process.

2.2. Strain, storage conditions, and cultivation process

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 spectrophotometer (Shimadzu, JAPAN). The stock cultures were prepared in 1 mL in YMM containing 30% (v/v) glycerol and stored at - 80 °C.

2.3. Cells and culturing conditions

PC3 (ATCC® CRL1435™) and 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, USA). PC3 cells were grown in DMEM and HUVECs were cultured in endothelial complete growth media, including growth supplements [31] at 37° C in %5 CO_2 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% penicilin-streptomycin solution. The supernatants of PC3, MCF-7, and HUVECs cultures 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 ºC until used.

2.4. The growth ability of *S. cerevisiae* in cancerous environment

To determine the growth fitness of *S. cerevisiae* in the cancerous environment, yeast was cultivated in PC3-cultured-medium (PCM), MCF-7-cultured-medium (MCM) and HUVEC-cultured-medium (HCM) at 30 °C for 24 h. At the beginning, PC3, MC-7 and HUVEC cells (approximately 70% confluence) were cultured in their specific medium for 24 h. Then, the cells were harvested by centrifugation and the supernatant of each medium was taken to leach in three times with 0.22 µm filter. The filtered media were stored at 4 °C for next steps. After that, the pH values of PCM, MCM and HCM were measured to adjust according to optimum growth conditions of *S. cerevisiae.* In addition, to determine major stress factor on the growth fitness of S. cerevisiae, the glucose concentration in each PCM, MCM, and HCM was measured with glucose colorimetric assay (Cayman, USA). Finally, the growth curves of S. cerevisiae into 500 µL PCM, MCM, HCM and YMM (control medium) were analysed with iCELLigence analyser (ACEA Bioscience, USA) for 24 h at 30 °C. There is wifi connection between device and iPad tablet to share periodically measured data of cell intensity in each well to plot a growth curve.

2.5. Evolutionary engineering strategy to obtain PCM - resistant yeast mutants

The evolutionary engineering approach was used to obtain favoured strains with random EMS mutagenesis. To select desired colonies, EMS-treated yeast population inoculated in solid selective media containing stress factors. The yeast population was randomly mutated with EMS to find the aimed phenotype. Then, the best mutant was determined with several cross-stress tests. To select PCM-resistant *S. cerevisiae* mutants, evolutionary engineering strategy was directly performed with slight modifications as described previously [19,32]. The wild type (WT) yeast population was exposed to EMS to increase the genetic diversity [33]. Initially, *S.cerevisiae* was pre-cultured in 10 mL YPD medium overnight at 30°C and 150 rpm. After that, about $5x10^7$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 tube except for control. Tubes were vortexed and placed in a 30°C incubator at 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 3000 g. 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 30ºC, 150 rpm OD600 of the cultures was measured.

2.6. Direct selection mutants phenotypes

At the first, the growth fitness of EMS-mutagenized yeast population and WT strain were assessed in PCM for 24h with the iCELLigence analyzer. After that, to select special colonies, the mutant population was directly inoculated to a solid PCM-agar plate held at 30 °C until the colonies formed on the surface. Finally, the four independent mutant colonies (MYn) were randomly taken from solid PCM-agar plate to culture in YMM for further analysis (Table 1).

Table 1. Nomenclature of the mutants selected as single colony in selective media

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

2.7. Growth analysis of the wild type and the mutants

All yeast strains (WT and MYn mutants) were precultured in YMM until late logarithmic phase, at 30ºC and 150 rpm on a rotary shaker. They were subsequently cultured in E-plate at 30ºC with PCM to analyse growth properties of each strain with periodically measuring by the iCELLigence analyser.

2.8. Stress resistance estimation

The each selected mutant colony was tested by a high-throughput, most probable number (MPN) [34] and spotting assays to understand the sensitivity and resistance of it against PCM and also determine its cross resistance to several stress conditions. The previously described spotting assay [35] is tested with slightly changes based on serial dilutions on solid culture media. The overnight precultured yeast cells were centrifuged at 10000 g for 5 min and the supernatants were discarded. Afterwards, the resuspended pellets were diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, and two µL of each dilution were dropped onto the solid PCM-agar media plate. The same process was also repeated on YMM plates which contained 1.5 mM $NiCl_2$, and 8% ‘v/v’ethanol.

The cell viability of individual mutants was also tested with MPN assay. The MPN assay is based on counting serially diluted survival numbers in each-well after the 72h. The each precultured yeast strain was inoculated in each well containing 180 µL of PCM or YMM (with or without stressor) according to serial dilution from 10^{-1} to 10^{-8}. The assay was parallelly repeated five times for each yeast strain. Moreover, the survival rate of all samples was calculated in PCM according to MPN tables. The survival rate of each mutant strain was calculated by dividing the viable cell number of treated sample to that of non-treated one [32].

2.9. Statistical analysis

All results were repeated in triplicate. GraphPad Prism 5.01 software was preferred for statistical analysis and comparable data groups were evaluated by one-way ANOVA Newman-Keuls Post-Hoc Test; $p<0.05$ was considered significant.

3. Results

3.1. The cultivation of *S.cerevisiae* in cancerous environments

To investigate the restriction of the cultured medium on *S.cerevisiae*, the yeast growth curve was determined in YMM, DMEM, HCM, PCM, and MCM. Fig.1 indicates that the growth fitness of *S.cerevisiae* in PCM and MCM significantly 4-2-fold declined in comparison to YMM and DMEM ($p<0.05$). Moreover, HCM also 1-fold decreased yeast proliferation compared to control media (YMM and DMEM). The cancerous environments (MCM and especially PCM) were more effective to inhibit yeast growth.

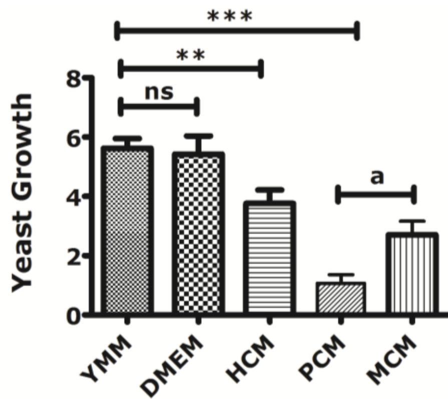

Figure 1. The yeast growth in YMM, DMEM, HCM, PCM, and MCM. The growth rate of S.cerevisiae was independently measured three times with iCELLigence Real Time Cell Analyzer. The data were expressed by mean ± SEM ($n \geq 3$), *** $P < 0.0001$ PCM, MCM vs DMEM, YMM, a $P < 0.0001$ MCM vs PCM.

3.2. The detection of the major stress factors

The pH value and glucose concentration [36] of PCM were measured to adjust medium conditions according to optimum yeast growth. To understand the main stress factor in PCM restricting yeast growth, *S.cerevisiae* was further cultivated in the various media containing different glucose concentrations (Table 2). Fig 2 shows an equivalent yeast growth in all media containing different glucose amount. The pH value and glucose concentration of PCM are not main stress factor on yeast growth rate reduced.

Table 2. 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

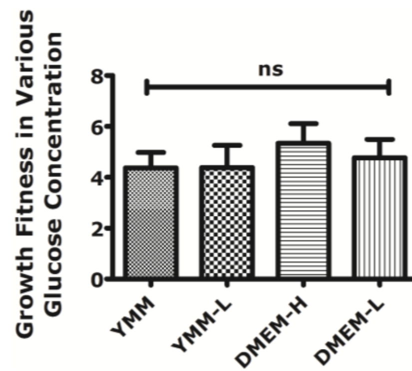

Figure 2. The yeast growth fitness in YMM, YMM-L, DMEM-H, and DMEM-L. The growth rate of S.cerevisiae was measured in various media containing different glucose concentration to determine glucose stress level. The data were expressed by mean ± SEM ($n \geq 3$), ns $P < 0.05$.

3.3. An evolutionary engineering strategy

To obtain a variety of genetic mutants of *S.cerevisiae*, the initial WT population was randomly mutagenized with EMS. The mutant population was initially cultured in YPD for overnight to control growth health. In the following step, the growth fitness of the mutant population (MYn) and WT were assessed in PCM with iCELLigence Real Time Cell Analyzer. MYn has better cell proliferation in PCM compared to WT (Fig. 3).The growth rate of MYn incomparably increased in PCM displays that there might be desired phenotypes in the mutant population.

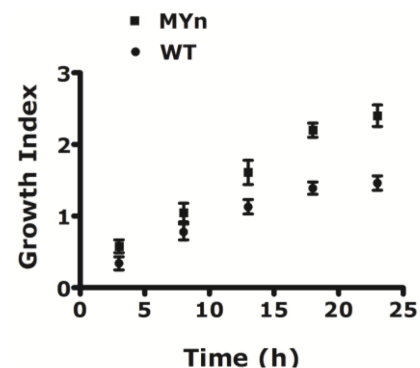

Figure 3. The growth curve of WT and MYn yeast population was measured in PCM by iCELLigence Real Time Cell Analyzer. The data were expressed by mean ± SEM ($n \geq 3$). There is a significant growth difference between MYn and WT. ($P < 0.05$).

3.4 Colony selection and determination of the best mutant

To determine individual mutants with aimed specific characteristics, MYn precultured in liquid YMM, was seeded solid PCM-agar plate with spreading technique. Table 1 lists the four individual colonies that were randomly selected. After that, the each of selected mutants (MY1, MY2, MY3 and MY4) previously cultured in YMM was inoculated in PCM with an equal number to analyse their growth curve. Fig. 4 demonstrates that all mutant strains have better growth fitness in PCM compared to WT, and also MY2 is the best one with 2-fold growth rate. In comparison with WT, all special mutants obtained by evolutionary engineering approach can grow without any difficulty in PCM.

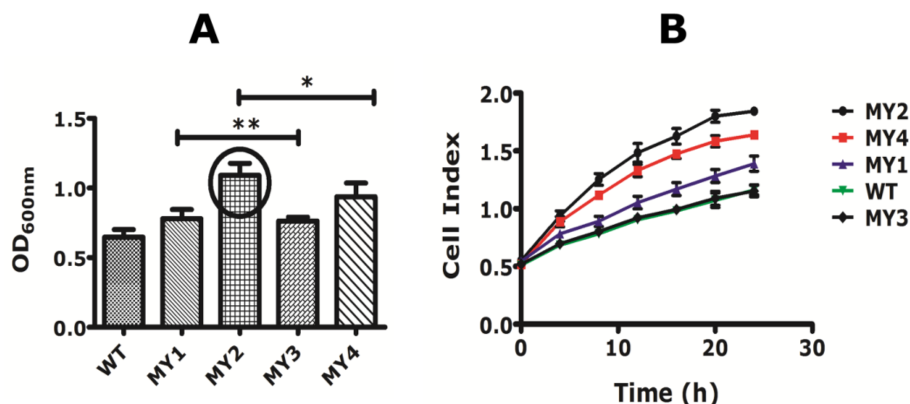

Figure 4. To determine the best mutant among selected colonies, A) The growth fitness of the selected mutants in PCM were analysed with OD600 measurement. The data were expressed by mean ± SEM (n ≥ 3), ** P < 0.01, MY2 vs MY1, MY3, WT; * P < 0.05, MY2 vs MY4 B) The growth curve of WT and mutants were measured in PCM by iCELLigence Real Time Cell Analyzer. The data were expressed by mean ± SEM (n ≥ 3)

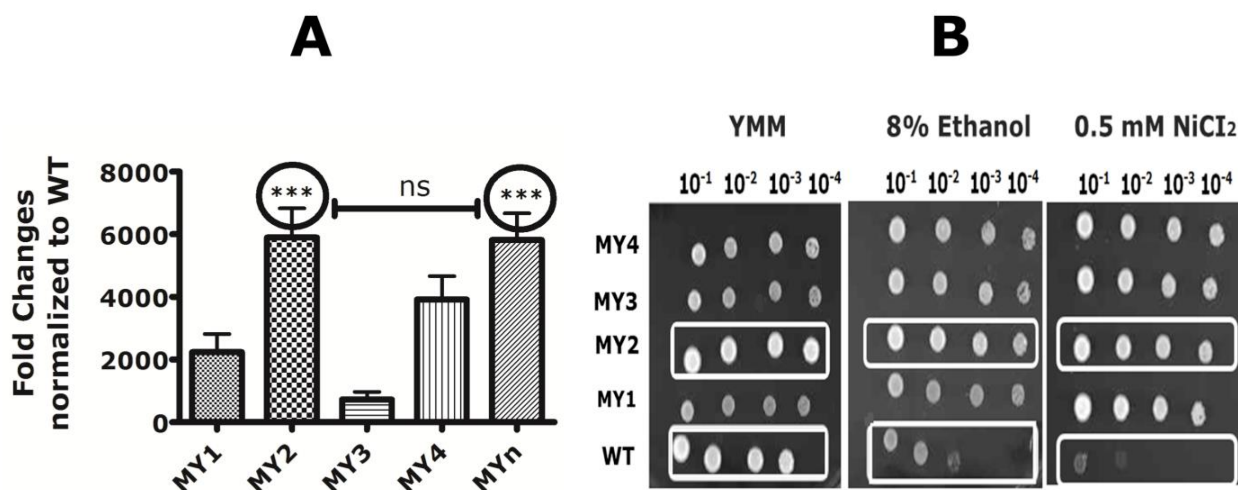

Figure 5. The direct selection strategy and determine cross-resistance of individual mutants, A) The normalized survival rate of selected mutants in PCM was determined by MPN assay. The data were expressed by mean ± SEM (n ≥ 3), *** P < 0.0001, MY2 vs MY1, MY3, MY4; MYn vs MY1, MY3, MY4. B) The spot test results of WT and individual mutants on YMM plates containing different stress factors to determine cross resistances.

In the present study, we further examined the best mutant in selected ones with the cross-resistant test assays. Therefore, the equal amount of all samples were serially diluted and spotted onto the YMM agar plates containing 0.5 mM NiCl2 and % 8 (v/v) ethanol. Fig.5 clearly shows that MY2 is the best mutant to have a cross-resistance among selected colonies. Moreover, MY2 had the better survival ratio according to MPN assay compared other mutants and WT (Fig.5). MY2 was determined the best individual mutant with specific properties, which presents stable growth in PCM and also better has cross-resistance in various stress factor.

4. Discussion

Cancer is a complex disease associated with entire metabolism, which makes it difficult to find a specific solution due to the cell diversity and the multiplicity of intracellular pathways [37,38]. Unlike normal cells [6], there is a rising chaos in cancer cells, which bypasses checkpoints [2]. Thus, it is not possible to talk about a single certain treatment for all cancer types, because cells have countless factors potentially being liable in carcinogenesis [39]. Although, the entire metabolism must be known in detail for the accurate solution in cancer treatment [39], the available technology and the level of knowledge is not enough for that. Therefore, it is required a different perspective to overcome cancer-like diseases. In the present study, we applied a natural approach based on improving the survival instinct of S. cerevisiae against the cancerous environment. This approach has been aimed to obtain the desired character of S. cerevisiae, which facilitates growing in PCM with evolutionary engineering strategy [20].

To test our purpose, *S. cerevisiae* was initially cultivated in YMM, DMEM, HCM, PCM, and MCM. Fig. 1 displays that the cancerous environments dramatically inhibited yeast growth. PCM was the most effective medium restricting yeast proliferation. The possible major stress factor in PCM inhibiting yeast growth was investigated by adjustment of pH level and glucose concentration. All media in Fig.1 had been previously arranged to pH 4-6 according to the optimum growth conditions of *S. cerevisiae* [40]. However, to determine the effect of glucose level on yeast growth, *S. cerevisiae* was cultured in various media as given in Table 2 to compare with PCM. The glucose concentration has not been responsible to decrease the growth index of yeast in PCM

(Fig. 2). Moreover, the PC3-produced metabolites in PCM might suppress yeast growth as another stress factor. Thus, the special strain of *S. cerevisiae* has to be improved with resistance to PCM.

In the present study, EMS-mutagenized *S.cerevisiae* population demonstrated the better growth fitness in PCM compared to WT (Fig.3) The randomly mutagenized yeast population contains specific phenotypes with high probability [23]. Subsequently, to determine PCM-resistant yeast strains, we randomly selected individual mutant colonies grown on the solid PCM-agar plate (Table 1). The cell proliferation of nearly all mutants in Table 1 achieved by evolutionary engineering increased in PCM compared to WT (Fig.4). According to Fig.4, it is unnecessary turning back to colony selection, however, if the selected mutants do not normally grow in a stress environment, the colony selection had to be repeated again. Consequently, the evolutionary engineering is a useful method to create a specific character, compared to metabolism engineering. There are also many examples of stress-resistant mutants in literature successfully accessed by evolutionary engineering such as a nickel-resistant *S.cerevisiae* [23], an acetic acid tolerant *Spathaspora passalidarum* [27] and more [41]. Because, it is possible in evolutionary engineering to generate numerous mutants with random mutations that give a better chance to find an aimed phenotype, whereas, in metabolism engineering, the necessity of information about entire metabolism is a limitation to reveal a specific yeast strain [42]. Eventually, the evolutionary engineering method successfully performed to get MY1, MY2, MY3, and MY4 with aimed characteristics.

In the present study, we further determined the best mutant among the selected ones with cross-resistance tests and also examined their robustness with MPN. Fig.5 indicated that MY2 is the best yeast strain selected from EMS-mutagenized yeast population which can grow under stress of 0.5 mM $NiCl_2$ and % 8 (v/v) ethanol. Furthermore, in comparison with WT, we observed that the tested robustness of all mutant strains in PCM by MPN significantly increased. Any microorganism with cross-stress properties has advantages to better growth in the stressful environments [43]. PCM is a stressful environment for *S.cerevisiae* to grow, therefore MY2 collected by evolutionary engineering as a special phenotype with aimed properties to be resistant to PCM.

There are several limitations of the present study; which might be good to discuss for future studies. Firstly, the counter effects of MY2 metabolites on PC3 have to be investigated to determine the anticancer production of the mutant yeast strain. Because MY2 has resistance to grow in cancerous environment inhibiting WT yeast proliferation, thus it may secondarily produce various metabolites to restrict the growth of cancer cells. Secondly, the molecular characterization of MY2 should be done to well-known the impacts of randomly-mutations in yeast metabolism. The understanding of which kind of pathways reorganize by random mutations in MY2 may help to determine how they contribute to resistance mechanism in PCM. Lastly, the whole genome of MY2 might be sequenced to compare WT, hence the position of each mutation will be detected.

In summary, in vivo evolutionary engineering was successfully applied to attain specific yeast strain, MY2, which is resistance to PCM rather than WT. Therefore, we proved once more that evolutionary engineering is simple and useful to reach the desired phenotype compared to metabolism engineering method. Furthermore, we definitely showed that the cancerous environment is extreme for *S.cerevisiae,* and the instinct of survival in yeast can be directed by evolutionary engineering to find a special strain being resistance to this environment. Our findings have to be supported by testing the secondary metabolite of MY2 in cancer cells and also examining in detail its molecular characterization.

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