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Green tea catechin extract enhances growth differences between wild-type and minimal glycolysis strains of *S. cerevisiae*.

ABSTRACT

Glycolysis is a conserved series of reactions ensuring organisms, including yeast cells, to extract energy from carbohydrates. These processes can only happen in the presence of enzymes specifically designed to execute a particular step in this metabolic pathway. Interestingly, due to whole-genome duplication, yeast cells *S. cerevisiae* contain multiple enzymes performing the same reaction, which from the energy utilization point of view seems inefficient. However, it could be the case that duplicated enzymes provide these organisms an advantage in stress conditions. This was investigated by comparing the growth of wild-type strain containing duplicated enzymes and minimal glycolysis strain containing only 1 copy of each enzyme. In this study wild-type (WT) and minimal glycolysis (MG) strains of yeast cells were exposed to green tea catechins extract (GTCE) and differences in growth and survivability were assessed by analyzing a growth curve and making a spot assay. According to the results, GTCE caused an increased growth rate in both WT and MG strains when growing on glucose and decreased growth rate when growing on ethanol.

INTRODUCTION

Glycolysis in *S. cerevisiae*

Glycolysis is the first and central metabolic pathway enabling organisms to process various carbon sources and metabolites. It is highly preserved in all living cells and ensures the synthesis of two ATP and two NADH molecules used as an energy source and as a reductant in further reactions, respectively. Glycolysis is independent of oxygen and therefore proceeds both in aerobic and anaerobic conditions. The product of anaerobic glycolysis is lactate or ethanol and CO_2. In the case of aerobic glycolysis, the final products are two pyruvate molecules proceeding to the TCA cycle (Akram M., 2013).

Similarly, as in many biochemical processes, glycolysis is also regulated by specific enzymes catalyzing metabolic reactions. Glycolysis has 10 steps divided into 3 phases. In the first phase, glucose is irreversibly phosphorylated and converted into fructose 1,6-bisphosphate (FBP). In the next phase, FBP is split into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate which is converted into another molecule of glyceraldehyde 3-phosphate. Thus, the substrates entering the third phase are two glyceraldehyde 3-phosphate molecules. Subsequently, these two molecules undergo several reactions resulting in the formation of 2 pyruvates yielding 2 ATPs and 2 NADH molecules (Akram M., 2013).

Yeasts are no exception to this pathway and therefore they have been used in many studies investigating this part of metabolism. Yeasts are probably most well-known for their ability to ferment, thanks to which they have become a stable element of our food industry. Fermentation is a process occurring usually in the absence of an external electron acceptor resulting in lactate or ethanol synthesis. However, in yeast cells fermentation occurs both in aerobic and anaerobic conditions. This ability is thought to have evolved due to high amounts of sugars in the environment. Rapid conversion of sugars to ethanol, even in presence of oxygen, seems to have granted these organisms a benefit others lacked. Because this metabolic pathway is found in today's yeasts it is highly probable that it provided higher efficiency in energy management (Dashko et al., 2014).

Another event associated with yeasts is whole-genome duplication (WGD). Both gene and genome duplications are important evolutionary events creating an opportunity for the duplicated genes to gain different functions and therefore provide an advantage for the organism. Although most of the genes are later deleted, the ones that remain usually evolve via mutations to have an altered role in the metabolism. According to a study by Kellis et al. (2004), *Saccharomyces cerevisiae* are descendants of genome duplication. The genome of *S. cerevisiae* contains approximately 8% of duplicated genes and for most of them, the specific function and reason why they are kept in the genome remains unknown (Kellis et al., 2004).

This is also the case of enzymes involved in glycolysis in yeasts. Although 90% of the duplicated genes were deleted, the other 10%, among which also the glycolytic enzymes are encoded, remained in the genome for an unknown reason. There are 12 steps needed to convert glucose to ethanol, which are facilitated by glycolytic enzymes. Technically 14 enzymes are sufficient to successfully accomplish glycolysis. However, there are 27 glycolytic enzymes encoded in the genome of *S. cerevisiae.* The purpose of these abundant enzymes has been investigated by subjecting the yeast cells to different conditions and analyzing their ability to grow. These yeast cells were genetically manipulated to only contain a minimal set of genes encoding glycolytic enzymes. In other words, there were no additional paralogs for glycolytic genes. This yeast strain is called the minimal glycolysis strain (MG). It was thought that paralogs of enzymes ensure better nutrient management in different conditions. However, in the study by Escalante et al. (2015), there were no differences observed between MG and wild-type species.

Catechins

Exposure of MG strain to different types of compounds could promote stress conditions and therefore clarify the purpose of duplicated enzymes. Green tea is one of many natural compounds praised for its anti-inflammatory and anticancer attributes. Antioxidants ensuring these properties are catechins, which belong to the group of flavonols . A characteristic trait of flavonols is their polyphenolic structure. In the case of catechins, the antioxidant properties are enhanced by multiple hydroxyl groups attached to the flavonoid backbone. There are 4 types of catechin molecules in

green tea, namely the epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epicatechin (EC) (figure 1) (Musial et al., 2020).

Thanks to these health-promoting qualities green tea has been subjected to various experiments to study its effect on glycolysis, metabolism, respiration, proliferation, and apoptosis. One of the effects monitored by several studies (Gao et al., 2015) (Wei et al., 2018), was the inhibition of glycolysis observed as lower glucose uptake and lactic acid production in the human tongue and breast cancer cells. By further investigation by Li et al. (2016), it was found that this was caused by altered PFK activity which was decreased by 55% after treatment with 50 μM of EGCG. Downregulation of PFK, as one of the key enzymes regulating glycolysis, therefore, leads to altered glycolytic rate. PFK is not the only enzyme affected by EGCG. Other studies showed that enzymes such as hexokinase or pyruvate kinase are influenced by EGCG and catechins in general (figure 2) (Wei et al., 2018).

Besides catechins, there are many other compounds affecting glycolysis by inducing stress in cells. One of the coping mechanisms in yeasts could be the presence of different isozymes encoded by gene paralogs ensuring proper function even in an unfavorable environment. In this study, this hypothesis will be tested by subjecting both WT and MG yeast strains to 0.2 mg/ml green tea catechin extract and observing the effects on glycolysis and growth.

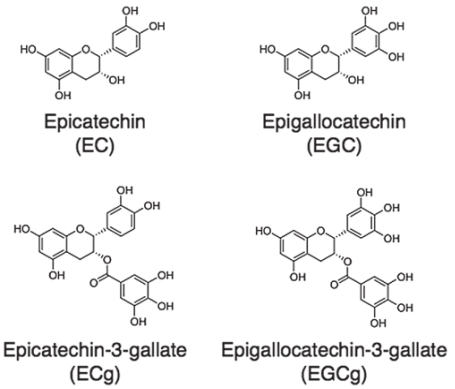

Figure 1: *Four types of catechin molecules found in green tea. Numerous hydroxyl groups attached to polyphenolic flavonoid backbone amplifies the antioxidant properties of these molecules. (Musial, C., (2020).*

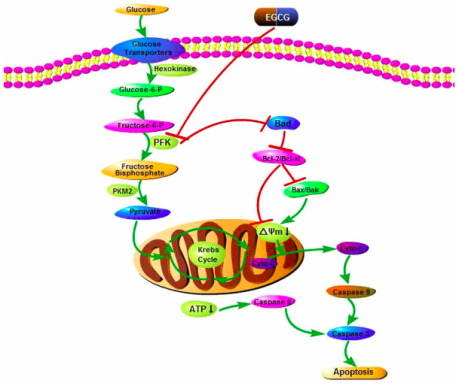

Figure 2: *The effect of EGCG on PFK and the following steps in the molecular cascade. EGCG suppresses the function of PFK leading to inhibition of the whole glycolysis pathway. (Li, S., (2016)).*

MATERIALS & METHODS

Control

For the control experiment both wild-type and MG yeasts (grown to OD 1 overnight) were 25x diluted with 1x YNB medium with 100mM glucose to a final volume of 1mL. Subsequently, the dilutions were loaded to 48 wells plate and put into a plate reader. The samples were being shaken at 700 rpm at a temperature of 30°C. Every 10 minutes the OD_{600} was measured.

Catechin

In order to investigate the inhibitory effect of green tea catechin extract two experiments were conducted - a growth experiment and spot assay. For both experiments, a 10x stock compound was prepared by combining 1.16x YNB medium, glucose, and GTCE resulting in a solution with 100mM glucose and 2mg/ml GTCE. Besides that, both wild-type and MG strains of yeasts were inoculated in 1x YNB medium with 4mM glucose and put into a shaker at 30°C to grow overnight. The next day cells were diluted to OD_{600} of 0.01 to minimize any inequalities made during inoculation.

The growth experiment

For the growth experiment, cells were diluted 25x with the prepared 10x stock compound (100mM glucose, 2mg/mL GTCE). The final solution had 10mM glucose and 0.2mg/mL GTCE. After dilution, samples were loaded into a 48 wells-plate and put into a Clariostar plate reader. OD_{600} was measured every 10 minutes and samples were shaken at 700 rpm at 30°C. Next, the data from the Clariostar plate reader were used to analyze the differences in growth between WT and MG strain. For more accurate results and a larger amount of data, this experiment was conducted twice. Data analysis was performed in Excel and conclusions were drawn after analyzing the growth rate from the growth curve and comparing the final ODs of two different strains.

The spot assay

In order to perform the spot assay experiment, YNB agarose gel plates were treated with the prepared 10x stock compound and left to dry before adding yeast cultures. Before the addition of yeast cells, a dilution series was made by combining standard medium without glucose with inoculated yeast cells. 6 dilutions were made ranging from undiluted to 10^5x diluted samples from which small amounts were placed on the agar plate in a form of a droplet and left to dry before putting the petri dish into an incubator with 30°C. Lastly, the data obtained from the spot assay were used for calculating the number of cells in the undiluted sample, and results were used to compare metabolic and growth differences between WT and MG strain.

RESULTS

Control

A control experiment for measuring the growth without the catechins was performed using a Clariostar plate reader. The final OD for the WT strain was 0.60 and the final OD for the MG strain was 0.56. The growth rates of the curves were also determined. The growth rate for the WT strain on glucose was 0.23 dlnOD/h and on ethanol, it was found to be 0.11dlnOD/h. The growth rate for the MG

strain on glucose was 0.24 dlnOD/h and on ethanol, it was 0.05 dlnOD/h (figure 3 and 5).

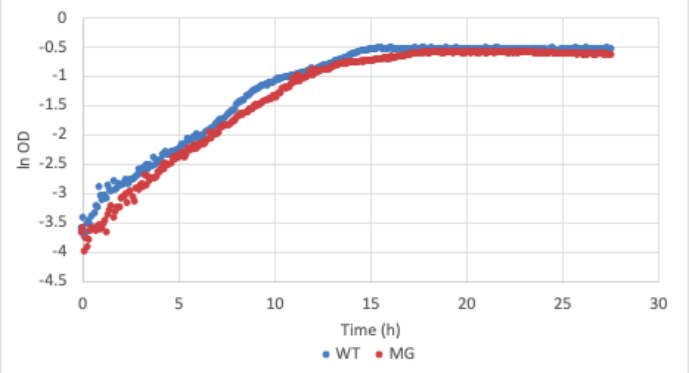

Figure 3: *Control growth curves. Growth curves of both the WT strain and MG strain without catechin added. The y-axis shows the ln OD, which is a measure of the light extinction. The x-axis shows the time in hours. The curves show the growth of yeast cells in change in light extinction over time. The dots in the graph represent the extinction after every 5 minutes from the start of the measurement to the end. The growth rate of the WT strain is 0.23 dlnOD/h for glucose and 0.11dlnOD/h for ethanol. The growth rate of the MG strain is 0.24 dlnOD/h for glucose and 0.05 dlnOD/h for ethanol.*

Catechin

The growth experiment

In order to test how well the two yeast strains grew when they were exposed to catechins, their growth was measured and the results were plotted in figure 4. The growth of the WT cells and MG cells was measured twice and an average growth was determined (figure 4.). The final OD for the WT strain was 1.3 and the final OD for the MG strain was 0.89. The difference between the two strains is approximately 46%. The growth rate for the WT strain on glucose was 0.26 dlnOD/h and on ethanol, it was 0.06 dlnOD/h. The growth rate for the MG strain on glucose was 0.32 dlnOD/h and on ethanol, it was 0.04 dlnOD/h (figure 5).

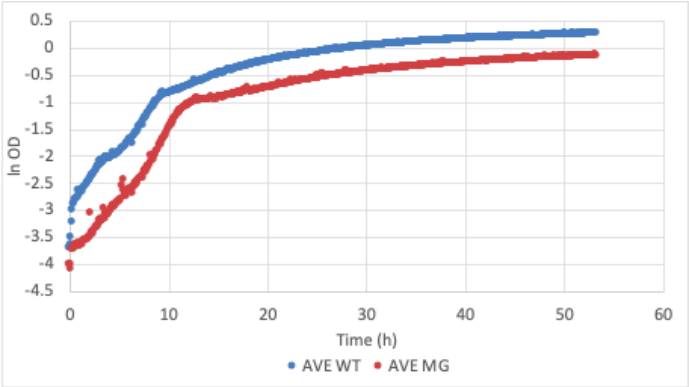

Figure 4: *The average growth (AVE) curves of both the two WT samples and the two MG samples. The y-axis shows the ln OD, which is a measure of the light extinction. The x-axis shows the time in hours. The curves show the growth of yeast cells in change in light extinction over time. The dots in the graph represent the extinction after every 5 minutes from the start of the measurement to the end. The growth rate of the WT strain is 0.26 dlnOD/h for glucose and 0.06 dlnOD/h for ethanol. The growth rate of the MG strain is 0.32 dlnOD/h for glucose and 0.04 dlnOD/h for ethanol.*

	Glucose	Ethanol
Control		
WT	0.23	0.11
MG	0.24	0.05
Catechin		
WT	0.26	0.06
MG	0.32	0.04

Figure 5: *Slopes of the growth curves on glucose and ethanol expressed in dlnOD/h for the control and catechin experiment.*

The spot assay

In order to see if there is a difference between the number of viable cells of the WT and MG strain that grew in the presence of catechins, a spot assay was performed (figure 6). The spots on the agarose gel were counted and using these results, the amount of viable cells in 1 mL could be determined. Results from the first growth assay showed that for the WT strain the original viable cell concentration was $25*10^6$ cells/mL. For the MG strain, this was $17*10^6$ cells/mL. This means there were 47% more viable cells of the WT strain than of the MG strain. The results from the second spot assay showed that the concentration for the WT strain was $2.5*10^6$ cells/mL and for the MG strain it was $0.4*10^6$ cells/mL. This means there were 525% more viable cells of the WT strain than of the MG strain in the original concentration.

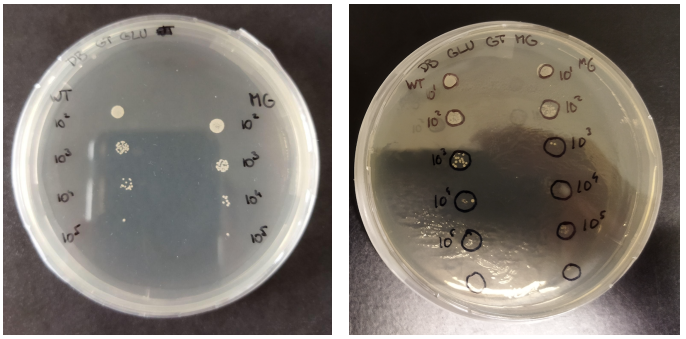

Figure 6*: Two spot assays displaying colonies of WT and MG S. cerevisiae on agar plates infused with GTCE. Numbers next to colonies represent the level of dilution of the original solution. The WT strain in the left colony had an original viable cell concentration of $25*10^6$ cells/mL and the MG strain had an original concentration of viable cells of $17*10^6$ cells/mL. The WT strain on the right had an original viable cell concentration of $2.5*10^6$ cells/mL and the MG strain had an original concentration of viable cells of $0.4*10^6$ cells/mL. The spots on the plate represent the number of cells in 5.0 µL of the dilutions, which are written next to the spots.*

DISCUSSION

The aim of this study was to find out if there is a difference in growth between the wild-type yeast strain and the minimal glycolysis yeast strain in the presence of a green tea catechin extract. In order to see if there is a difference, two experiments were performed, a growth experiment and a spot assay. The findings of the experiments are discussed below. From the growth experiment, it could be observed that the final OD's were higher in the presence of catechins than without the catechins. It was also found that there was a 46% difference in final OD between the WT and MG strain in the experimental group. The results from the spot assays also showed differences between the MG and WT strain in original viable cell concentration. The first experiment found that there were 47% more viable cells in the original concentration for the WT strain in comparison to the MG strain. The second experiment found that there were 525% more viable cells in the original concentration for the WT strain in comparison to the MG strain.

The growth experiment

A difference can be observed between the growth curve of the WT strain and the growth curve of the MG strain that were exposed to the catechins (figure 4) in comparison to almost no difference in the control group (figure 3). The OD of the WT strain seems to be higher than that of the MG strain in the experimental group. The final OD in the control group of the WT strain was 0.60 while the OD of the MG strain was 0.56, which gives a difference of 0.04. The final OD value in the experimental group for the WT strain was 1.3 while the OD of the MG strain was 0.89, which gives a difference of 0.41.

Both strains seemed to grow better when they were exposed to catechins according to the differences in final OD between the control and experimental group, but the differences in growth between MG and WT was bigger in the catechin experiment than it was in the control experiment. The difference between the two strains is approximately 46% in the catechin experiment, in comparison to 7% in the control experiment, so it might be the case that the MG strain grows less well in the presence of catechins than the WT strain. The results from this study do not seem to align with the previous findings. It has been shown that catechins inhibit glycolysis in cancer cells and catechins do not seem to inhibit glycolysis in this study (Gao et al., 2015) (Wei et al., 2018). It was also discovered that EGCG downregulates PFK, which is a major glycolytic enzyme, so it was expected that the green tea catechin extract would inhibit glycolysis (Li et al. (2016)). In this study, it was found that both the WT and MG strain grew better in the presence of catechins because the final OD's in the experimental group were higher than in the control group. Both yeast strains also had higher growth rates on glucose when they were exposed to catechins. On the other hand the growth rates on ethanol were lower in the experimental group than in the control group (figure 5). This might mean that there are other effects of catechins on the yeast cells that might stimulate growth more.

The data of the experimental group is only based on two measurements and the results of the control group only on one, so it would be better to perform more of the growth experiments to see whether there really is a significant difference.

The spot assay

The original viable cell concentration in 1 mL was calculated from different dilutions. The results from the first and second spot assay differ substantially. The original concentration of the WT strain was 10x larger in the first experiment than in the second experiment while the original concentration of the MG strain was 42.5x larger in the first experiment than in the second experiment. Also, the difference in original viable cell concentration between the two strains differed a lot between experiments. The difference between the two strains was 47% in the first experiment and 525% in the second experiment. This might be the result of inaccurate pipetting or not resuspending the dilutions. Performing more spot assays could give a more reliable representation of how well the yeast cells grow in the presence of catechins. If similar results are found again, this might mean that the WT strain grows better in the presence of catechins than the MG strain.

Unfortunately, a control spot assay to compare the growth of the yeast cells without catechins in the medium is missing, so it is not possible to conclude whether the cells grew better or worse in the presence of catechins.

Final conclusion

The results from the growth experiment showed a difference in growth between the WT and MG strain. This could be caused by the presence of catechins, but in order to make the conclusion more reliable, the experiment should be performed again to collect more data. The results from the spot assay show a difference in the number of viable cells between the WT and MG strain. However, the results of the two experiments differ a lot, so the experiments should be performed again to conclude that there is an actual difference in growth between the two strains. Further research could look into the effect of catechins in other microorganisms or other yeast strains to see whether it could enhance their glycolysis. This could be interesting for the brewery industry.

Appendix

Yield calculation

The yield of the two growth experiments was calculated according to the following formula:

$$Y_S = \frac{produced\ biomass}{comsumed\ substrate} = \frac{\Delta OD}{\Delta S} = \frac{OD_{end} - OD_{start}}{S_{medium}}$$

Control

The yield for WT strain was $1.2*10^5$ cells/mol glucose and the yield for the MG strain was $1.1*10^5$ cells/mol glucose.

Catechin

The yield for both strains was determined and it was found that the WT strain had a yield of $2.6*10^5$ cells/mol glucose and the MG strain yield of $1.7*10^5$ cells/mol glucose

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