Oxidative Stress Reduction By Yeast Transporters

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Abstract

Oxidative stress poses a serious problem to the production of biologics via industrial cell lines, with reactive oxygen species (ROS) being the most common oxidant found in cells as it a natural byproduct of aerobic metabolism. The sulfur-containing amino acid methionine has the ability to act as a ROS scavenger, protecting crucial protein residues within cells. This project looked to decrease oxidative stress within *S. cerevisiae* via the overexpression of the methionine transporter, SLC7A5. The experiment would have served as a proof of concept to be tested in commonly used industrial cell lines, such as CHO. No experimental results were able to be obtained due to unsuccessful gene transformation into *S. cerevisiae*. This was attributed to issues with the construction of the plasmid containing the SLC7A5 gene. A review of the experimental procedure revealed that a different approach to plasmid construction should be explored if further work were to be done.

Table of Contents

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Introduction

In today's society, many common diseases are treated through the use of recombinant protein therapeutics. Products include monoclonal antibodies (mAbs), vaccines, hormones, and other biologics. These therapeutics are produced via the large-scale production of genetically engineered cell lines transformed with genes coding for proteins of interest. Microbial hosts, such as *Escherichia coli* (*E. coli*), lack the required cellular machinery to express recombinant proteins for human use. Therefore, the most commonly used cell lines are of mammalian nature: those from humans (HEK293, HT-1080), hamsters (CHO, BHK), and mice (NS0, Sp2/0).[1, 2]

Today, the most commonly used cell line for recombinant protein production are Chinese Hamster Ovary (CHO) cells. This is due to their ability to grow high densities in suspension culture, maintain high viability in large-scale bioreactors, and produce recombinant products in the 1-10 g/L range.[3]

With an increase in demand for recombinant protein therapeutics growing worldwide, the need for increased production has become necessary. Survey data taken between January 2014 and July 2018 has shown that cumulative sales for the biopharmaceutical industry reached a value of $651 billion. Since 2017, 87% of new biopharmaceutical active ingredients were classified as recombinant proteins, with 84% being produced from mammalian cell lines. Of these, CHO cells continued to prevail as the most common, with little eagerness being shown to investigate new cell lines for gene expression.[4] These trends highlight the importance of optimizing and improving the production of CHO cells.

Currently, it is presumed that media formulation, internal metabolic pathways, and secretion hinder CHO cell production. Therefore, most if not all efforts in increasing CHO cell productivity is being focused on improving media formulation and the overexpression of optimized enzymes.

However, the work being done to increase CHO cell production has potential drawbacks as well. When industrial cell lines are engineered to overexpress a protein of choice, it leads to metabolic adaptations that revolve around increasing energy production and improving secretory capacity.[5] This increased metabolic state can lead to an increase in cellular oxidative stress, which

can be described as the imbalance between the production of free radicals and the antioxidant response within cells.[6, 7]

When sustained levels of oxidative stress are experienced by cells, apoptosis, or programmed cell death, will occur to prevent necrosis, which is uncontrolled cell death. Triggering apoptosis to prevent necrosis is an organic defense mechanism for cells, however, in the context of bioprocesses and recombinant protein production, is not desirable.[6]

Evidence suggests that certain amino acids (AA), such as methionine, have the ability to act as an antioxidant, capable of reducing cellular oxidative stress. This paper describes a proof of concept experiment designed to reduce the cellular oxidative stress experienced by *Saccharomyces cerevisiae* (*S. cerevisiae*) when exposed to hydrogen peroxide by overexpressing the SLC amino acid transporter SLC7A5, which is responsible for the transport of methionine into the cell.

Background

As stated in the introduction, cell lines engineered to overexpress proteins suffer from increased levels of oxidative stress, meaning there is an imbalance between the production of free radicals and the antioxidant response within the cell. The most common free radicals found in industrial cell lines, such as CHO, are reactive oxygen species (ROS), a natural byproduct of aerobic metabolism.[6]

The accumulation of ROS can disrupt regular cellular activities and alter cellular components. Specifically, most ROS have a high affinity to react with proteins, lipids, RNA, and DNA, which are some of the most important components in a cell. Sustained high levels of ROS within cells lead to irreparable damage to the aforementioned cellular components and with recombinant protein production being the main goal of genetically engineered cell lines, high levels of ROS within the cell need to be avoided at all costs.[6, 8]

All cells have two types of defense against cellular oxidative stress, enzymatic and non-enzymatic. The latter generally consists of small soluble molecules that act as free radical scavengers.[9, 10]

However, as previously stated, research has also been done that shows some amino acids also have antioxidant capabilities as well. In particular, the sulfur-containing amino acid methionine. This amino acid is essential to cells and is known to be hydrophobic. Because of this, most methionine residues are found in the interior of folded proteins. Yet, a small percentage of methionine residues are surface exposed.[11]

These surface methionine residues act as “molecular bodyguards” as they can be reversibly oxidized by the addition of oxygen to its sulfur atom, forming methionine sulfoxide (MetO). This reaction introduces chirality to the molecule and as a result, there exist two epimers of MetO, S-MetO and R-MetO. Both can be reduced back to methionine via two distinct methionine sulfoxide reductases, MsrA and MsrB, both of which are virtually universal among aerobic organisms.[12]

Methionine residue oxidation has the potential to remove hazardous substances such as hydroperoxide, hypochlorous, ozone, and lipid peroxide. This leads to methionine to be known as an innate antioxidant and natural scavenging system, positioned extremely well to intercept any

ROS species that could cause potential damage to a cell and protecting crucial residues within a protein.[12, 13]

For example, a 1994 study done by Reddy et al. showed that when exposed to chloramine, α-2-macroglobulin, an antiproteinase, lost activity only after the oxidation of eight methionine residues via the consumption of eight equivalent chloramine molecules. Continued exposure resulted in the oxidation of an additional six methionine residues and a single tryptophan residue. Reddy et al. found that the fractional deactivation of α-2-macroglobulin matched the loss of the tryptophan residue, indicating that α-2-macroglobulin did not lose its functionality until all surface methionine residues were oxidized.[14]

More recently, In 2008, Shen Luo and Rodney L. Levine performed an experiment that saw them replace 40% of the methionine residues found in the glutamine synthetase enzyme in Escherichia coli with norleucine, the carbon-containing analog of methionine. It should be noted that the intracellular free methionine was not altered. Luo and Levine saw that when unstressed, both control and norleucine substituted cells survived equally well for a minimum of 32 hours in the stationary phase, but oxidative stress was more damaging to the norleucine-substituted cells. These cells died faster than the control cells when exposed to either hypochlorite, hydrogen peroxide, or ionizing radiation.[15]

Knowing this, one can hypothesize that if two cells were exposed to the same oxidative stress-inducing environment, one with an increased uptake of methionine and the other with a normal uptake of methionine, the former would survive longer than the latter, due to a decrease in cellular oxidative stress. This could potentially lead to higher yields of recombinant protein.

To increase the uptake of amino acids into a cell, one can look at transport proteins, which are responsible for cellular transport. These proteins are categorized into four main superfamilies: ATP - binding cassette (ABC) transporters, ATPases, Ion Channels, and solute carrier proteins (SLC).[16]

SLCs are a superfamily of transport proteins totaling 458 transporters, categorized into 65 families that transport a wide variety of substances across the cell membrane. Transporters within this family are categorized such that proteins share a minimum of 20-25% amino acid sequence

similarity with at least one other protein in its family. Typical substrates for SLC proteins include sugars, amino acids, vitamins, nucleotides, metals, inorganic/organic ions, and oligopeptides.[16]

SLC families 1, 3, 7, 6, 38, and 43 all drive the transport of amino acids into and out of the cell. Specifically, the SLC3 and SLC7 family of transport proteins form a dimer that facilitates a wide range of amino acid transport. Both SLC3 and SLC7 proteins are bonded via a disulfide bridge and the resulting dimer transport proteins are known as Heteromeric amino acid transporters (HATs), each consisting of a light chain subunit (SLC7) and a heavy chain subunit (SLC3). HATs are most commonly referred to by their light chain subunit.[16]

The SLC7A5 HAT, comprised of SLC7A5 and SLC3A2 is most commonly referred to as LAT1 and is responsible for the transport of large hydrophobic neutral amino acids, such as methionine.[16] When discussing novel methods to increase methionine uptake, the overexpression of SLC7A5 should be considered. Knowing this, an experiment was designed in which the SLC7A5 transporter would be overexpressed in *S. cerevisiae*. These cells would then have been exposed to hydrogen peroxide, inducing oxidative stress, and their cell growth and density would have been measured and compared to a control group of *S. cerevisiae* with no overexpression of the SLC7A5 gene.

S. cerevisiae, a valuable and common tool used for eukaryotic research was planned on being used instead of CHO due to its ease of use in a laboratory setting. Its use to study the effects of certain genes on the metabolism of eukaryotic cells has become common practice in molecular biology due to its unicellular nature, short life cycle, and that nearly all eukaryotic biological functions are present and conserved in *S. cerevisiae*. Additionally, genetic manipulation of *S. cerevisiae* has been made easier because its entire genome has been sequenced, making processes such as gene discovery and characterization extremely easy.[17, 18]

Materials and Methodology

The general workflow that would enable successful transformation of the SLC7A5 gene into *S. cerevisiae* can be seen below in Figure 1. As seen, the gene, along with a promoter (TDH3), terminator (ADH1), and backbone (PY127) had to be stitched together via a Type IIS reaction to create a plasmid containing the SLC7A5 gene. Upon completion, the plasmid would be cloned and replicated via bacterial transformation into competent NEB 5-α *E. coli* cells. Finally, the plasmid would be purified and transformed into the S288C strain of *S. cerevisiae*. The information below goes into further detail about the preparatory steps that had to be taken in order to follow the general workflow outlined in Figure 1.

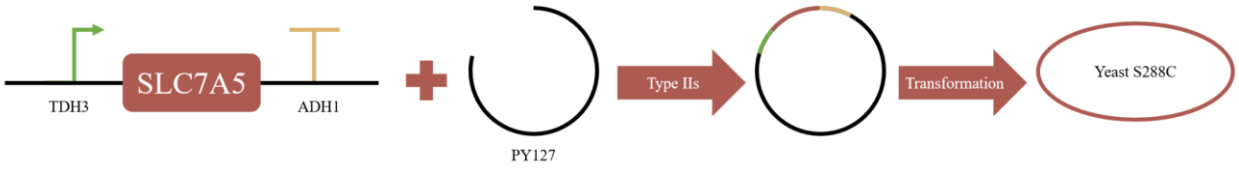

Figure 1. The above graphic depicts the workflow for how the amino acid transporter SLC7A5 would be engineered into the S288C strain of *S. cerevisiae*.

Inoculation

Before performing the Type IIS reaction, all four DNA components had to be inoculated. Both TDH3 and ADH1 were inoculated with 5µL of the antibiotic ampicillin in 5mL of LB media. Both PY127 and SLC7A5 were inoculated with 5µL of the antibiotic chloramphenicol in 5mL of LB media. These were then incubated at 37℃ for 24 hours.

Miniprep

The next step was to miniprep each inoculated DNA component. To do so, the Qiagen QIAprep® Spin Miniprep Kit was utilized. This procedure saw the aforementioned DNA components centrifuged at 5,000 RPM for approximately 3-5 minutes to obtain pelleted cells. Once this was achieved, 250µL of Qiagen P1 Buffer was used to resuspend the cells which were then transferred into a microcentrifuge tube. This was followed by adding 250µL of Qiagen P2 Buffer and vortexing the resulting solution. Within five minutes, 350µL of Qiagen N3 Buffer was added and the resulting solution was vortexed again. All DNA components were then centrifuged at 13,000 RPM for 10 minutes. After 10 minutes, 800µL of the supernatant for each component was

collected and pipetted into a QIAprep® 2.0 spin column. These spin columns were then centrifuged at 13,000 RPM for one minute. They were then washed with 0.5mL of Qiagen PB Buffer and centrifuged again at 13,000 RPM for one minute. The column was washed again using 0.75mL of Qiagen PE Buffer and then centrifuged twice for one minute to remove any residual buffer. The inner tube of the QIAprep® 2.0 spin columns were then placed into a new microcentrifuge tube and 50μL of Qiagen EB Buffer was added to elute the DNA components from the spin column into the microcentrifuge tube. After elution, the DNA components were appropriately labeled and stored at 5℃.

Type IIS

After miniprep, the next step was to perform a Type IIS reaction to stitch all respective DNA parts together. In order to do so, the concentration of each had to be obtained. This was done using a Thermo Scientific™ NanoDrop™ One UV-Vis Spectrophotometer. Each of the four components was measured twice for accuracy.

Once the concentrations of all DNA parts were known, a Type IIS reaction was attempted. In order to perform this reaction, 20fM stock solutions had to be created for each DNA component. To do this, the concentration and base pair length of each DNA part had to be known. As previously stated, the concentration of each was obtained via a Thermo Scientific™ NanoDrop™ machine. TDH3, SLC7A5, ADH1, and PY127 had base pair lengths of 1022, 1521, 101, and 4690 respectively. Using these values, 20fM stock solutions were created of each DNA component. Once this was accomplished, 1μL of each DNA 20fM stock, BbsI restriction enzyme, and ligase buffer along with 0.4μL of T4 DNA ligase buffer were added to a PCR tube. Added to this mixture was 3.9μL of deionized water to bring the total reaction volume to 10.3μL. The sample was placed in a thermocycler to perform the Type IIS reaction. It was run at 37℃ for 90 seconds before decreasing its temperature to 16℃ for three minutes. The samples were then heated at 50℃ for five minutes and then at 80℃ for an additional 10 minutes.

Bacterial Transformation

After the Type IIS reaction, the newly constructed plasmid was attempted to be transformed into competent *E. coli* cells. To do this, 10µL of thawed NEB 5-α *E. coli* cells were added to a clean PCR tube. The constructed plasmid was then added at a quantity of 2-2.5µL. The cells then sat on ice without mixing for 30 minutes. They were then taken off ice and heat shocked at 42℃ for 30 seconds in a thermocycler. They were then returned to the ice bath for an additional five minutes. After, 100µL of room temperature SOC Outgrowth Medium was added to the cells, which were then incubated at 37℃ for one hour in a thermocycler. After this step, approximately 50µL of the cells were plated on a chloramphenicol selection plate using 4.5mm sterile Rattler Plating Beads from Zymo Research. The plate was then left to incubate at 37℃ until colonies were visible.

Once colonies became visible, the two largest ones were colony picked from the plate. These were then sequenced to determine if the plasmid had been successfully cloned and replicated. First attempts at the procedure outlined above saw sequencing results come back negative. Thus, it was repeated for a second time but sequencing results came back negative again. The results and discussion section below highlights possible reasons for failure and the next steps that would have been taken if the bacterial transformation into *E. coli* was successful.

Results and Discussion

Unsuccessful Bacterial Transformation

One main reason for unsuccessful bacterial transformation can be due to reaction kinetics. Before undergoing a Type IIS reaction and after getting miniprepped, each DNA fragment underwent a polymerase chain (PCR) reaction to increase the number of their copies. These PCR products then went through a Type IIS reaction to construct the full plasmid.

Conventionally, before undergoing a PCR reaction, each DNA fragment is held within their own respective plasmid. These "level 0" plasmids then undergo a PCR and Type IIS reaction before being transformed into bacteria. For this experiment, the respective DNA fragments were not held within level 0 plasmids before undergoing a PCR reaction, they were kept in solution in PCR tubes.

Because the enzymes used to recognize DNA fragments in both the PCR and Type IIS reaction are temperature sensitive, having small fragments of DNA in solution instead of being held within plasmids disrupts what the reaction system should be experiencing as the smaller DNA fragments move faster during the reaction as opposed to if they were held within plasmids. This small change in experimental protocol could have led to unsuccessful bacterial transformation into *E. coli*. This can be seen in Figure 2 below.

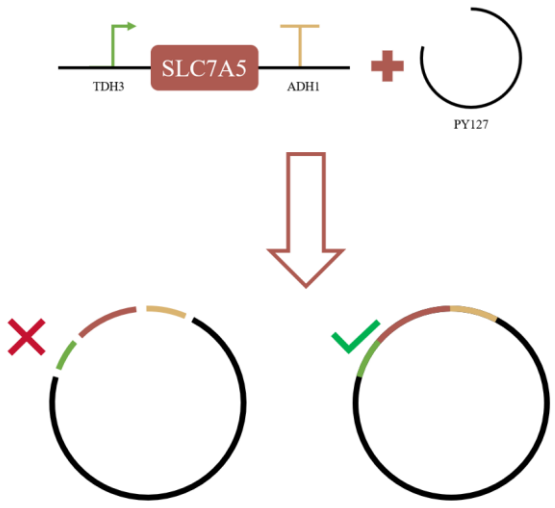

Figure 2. Graphic depicting incorrect vs. correct PY127 plasmid construction.

Future Experimentation

Without successful bacterial transformation, transformation of the SLC7A5 gene into *S. cerevisiae* was not possible. However, if the bacterial transformation was successful, and the resulting sequencing results were positive, ensuring the constructed plasmid had been replicated, the cells would have been miniprepped to isolate and purify the constructed plasmid from the *E. coli* cells.

Once miniprep was completed, an overnight yeast culture would have been grown using the S288C strain of *S. cerevisiae*. This culture would have been grown in appropriate media for approximately 5 hours before being incubated at 30℃ for six hours. During this time, a master mix would have been prepared containing the correctly sequenced plasmid. Once the master mix was made, the *S. cerevisiae* culture would have been centrifuged, resuspended, and combined with the master mix in a 1:3 ratio respectively.

The cells would have then been heat shocked in a water bath at 42℃ for approximately 42 minutes. The cells would have then been pelleted and incubated at 30℃ for two days. While the experimental *S. cerevisiae* culture was being grown, an unmanipulated *S. cerevisiae* culture would have also been grown to be used as a control group during experimentation.

Once both the experimental and control group of *S. cerevisiae* were grown, tests would have been performed in order to determine changes in cell count and density between both groups in response to induced oxidative stress. This would have been achieved by exposing both groups to hydrogen peroxide. Initial results would have been recorded at 50 hours after exposure with subsequent data points being taken every 25 hours for a total of 300 hours. Viable cell density would be obtained via the Tryphan Blue Exclusion Assay. This would allow a comparison to be done between the number of cells still living and those cells that died. Cell count would have been measured using a Cedex automated cell counter.

Theoretically, the team would have expected a higher cell count and density within the experimental group as compared to the control group of *S. cerevisiae*, as seen in Figure 3. This

would have indicated that the experimental group experienced lower levels of oxidative stress due to the overexpression of SLC7A5.

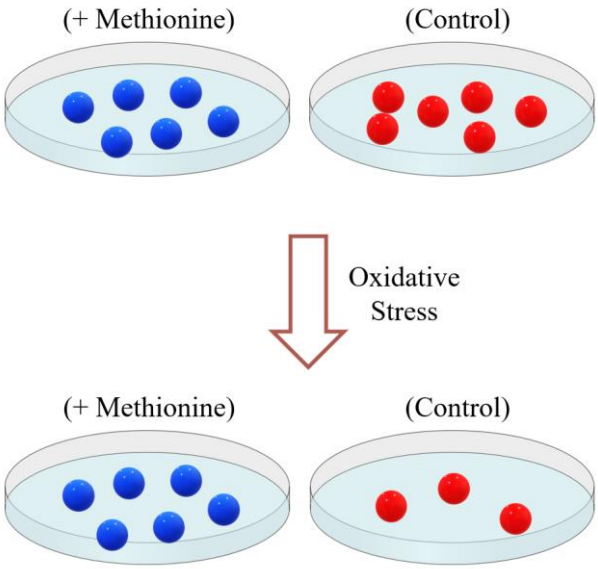

Figure 3. Expected results of both experimental and control group of *S. cerevisiae* after being exposed to oxidative stress.

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