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Final year university research report

Knock-out of *SUC2* Gene in *Saccharomyces Cerevisiae*

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This experiment aimed to generate a gene knockout of the invertase gene (*SUC2*) in *Saccharomyces cerevisiae* and replace it with a selective marker: the gene for hygromycin resistance. Transformation was induced using lithium acetate and polyethylene glycol (PEG). The plasmid with the DNA cassette encoding the selective marker was introduced into the yeast cell to obtain antibiotic resistance. It was hypothesized that homologous recombination would occur, knocking out the *SUC2* gene, which could be proven using PCR to amplify the recombinant DNA. However, the knockout did not occur. An invertase assay was then used to check if the knockout was functionally successful. This too was not achieved as invertase activity was identified. However, this was not a surprise as the PCR results already showed that the knockout had not occurred. The results showed that although the transformation was successful, homologous recombination did not occur and therefore, our hypothesis was not confirmed.

Introduction

Genetic transformation is a process in which cells take up exogenous DNA sequences from their environment which can then recombine with the chromosomal DNA[1]. Genetic transformation has helped with many major genetic discoveries including determining DNA as the genetic and transforming material[2]. It is uncommon for cells to be naturally competent for transformation. Often, they need to be induced by a chemical treatment; lithium ions and polyethylene glycol (PEG) are commonly used. Lithium ions neutralize the negative charge on DNA allowing transformation to occur. It has also been suggested that the ions may generate small holes in the plasma membrane, allowing for the passage of the DNA into the cell. PEG promotes membrane fusion and may also change the water structure around plasma membranes. Once the DNA has been transformed into the cell, homologous recombination can take place if the correct primers are used.

Including a selective marker in a transformation DNA cassette allows for tests to ensure the transformation was successful. Antibiotic resistance is a good selective marker as it can easily be tested for; if the post-transformation yeast sample is resistant to the antibiotic, transformation has occurred.

Saccharomyces cerevisiae is commonly used as a model for research in gene function due to its similar basic cellular function to higher eukaryotes, yet it has a more simply organized and haploid genome making it easier to use. *S. cerevisiae* is also known for its efficient homologous recombination. A process called gene targeting takes advantage of the efficient homologous recombination. Gene targeting involves two sequences that are in close proximity to each other in the genome around a marker gene and homologous recombination almost always occurs at the site homologous to the cloned sequences[1].

The SUC2 gene encodes the enzyme invertase. Invertase catalyses the cleavage of sucrose into glucose and fructose[3,4]. An individual haploid strain of *S. cerevisiae* can have zero, one, or several *SUC* genes in its genome, with each of these genes encoding two forms of invertase[4]. Expression of invertase is repressed in high glucose environments as it is not necessary to make more glucose from available sucrose. Research on this topic is important as yeast is a great model for experimenting on the genetic interactions in eukaryotes. Findings from

research in yeast cells can then be related to genetics in other eukaryotes, such as humans.

Single-stranded salmon sperm was used as our carrier DNA since a high concentration of carrier DNA is required and salmon sperm DNA is rather inexpensive.

In this experiment, we hypothesized that by using carrier DNA from salmon sperm in combination with lithium acetate and PEG we could induce the transformation of the plasmid pFA6-kanMX4 and homologous recombination would occur using the knockout forward and reverse primers for the *SUC2* gene. PFA6-kanMX4 contains the DNA cassette encoding the gene for hygromycin resistance in *S. cerevisiae*.
In other words, we hope to knock out the *SUC2* gene in *S. cerevisiae* and replace it with a selective marker: the gene for hygromycin resistance.

This has been achieved many times before,[5] thus, the aim of this experiment was to further confirm the validity of and theory behind this method.

Method

The method was not required as part of this report.

For reference, the method was similar to that explained by J. Hageman[3].

Results

PCR Analysis and Gel Electrophoresis

After the transformation of the SUC2 knockout cassette, 34 colonies grew on the YPD + G418 plate, with a transformation efficiency of 8.5 x 10^3 transformants per μg of DNA. Thus, transformation occurred since the antibiotic-resistant phenotype is present in these cells. PCR analysis only showed bands for the 3 positive controls, using ITS forward and reverse primers, and not for either of the transformant yeast extracts or the wild-type (*Figure 1*).
As the DNS used in the invertase assay reacts with reducing sugars, the absorbances obtained are measures of the glucose present in the samples.

Invertase assay

All five samples showed evidence of invertase activity. No glucose or fructose was added to any of the samples, yet the invertase assay revealed that each sample (S1-S5) contained glucose. Therefore, the glucose is a result of invertase breaking down the sucrose (*Table 1*). The concentration of glucose produced in each extract was measured using a spectrophotometer (*Figure* 2). From sample 1 (S1) an absorbance of 0.9621 was obtained. The other transformant yeast extract (S2) had an even higher absorbance of 1.1366. Thus, there is activity of invertase in the transformant cell extracts as no glucose was added to these samples, only sucrose.
The wild-type yeast extracts (S3, S4, and S5) had absorbance readings of 0.2458, 0.9812, and 1.0646, respectively. This was expected as the absorbance increased when the amount of wild-type yeast in the sample increased. The more wild-type yeast present, the more invertase enzyme expected to be in the culture, therefore more sucrose will be broken into glucose and fructose.

Discussion

The presence of yeast colonies on the YPD + G418 plate confirms that transformation of the DNA cassette occurred as the yeast is now resistant to G418. Had transformation not occurred, there would be no growth on the G418 plate. The colony PCR and gel electrophoresis were then required to prove that homologous recombination occurred and therefore, the invertase gene will have been knocked out. If the knockout was successful, the gel electrophoresis would show bands for both of the transformant yeast cell extracts (lanes 6 and 8) as well as the positive controls since they all should contain amplified *SUC2* knock-out products. However, this did not occur. As can be seen in *figure 1*, there are bands for only the lanes with positive controls. This means that even though the transformation occurred, the invertase gene was not successfully knocked out. Yet, the presence of

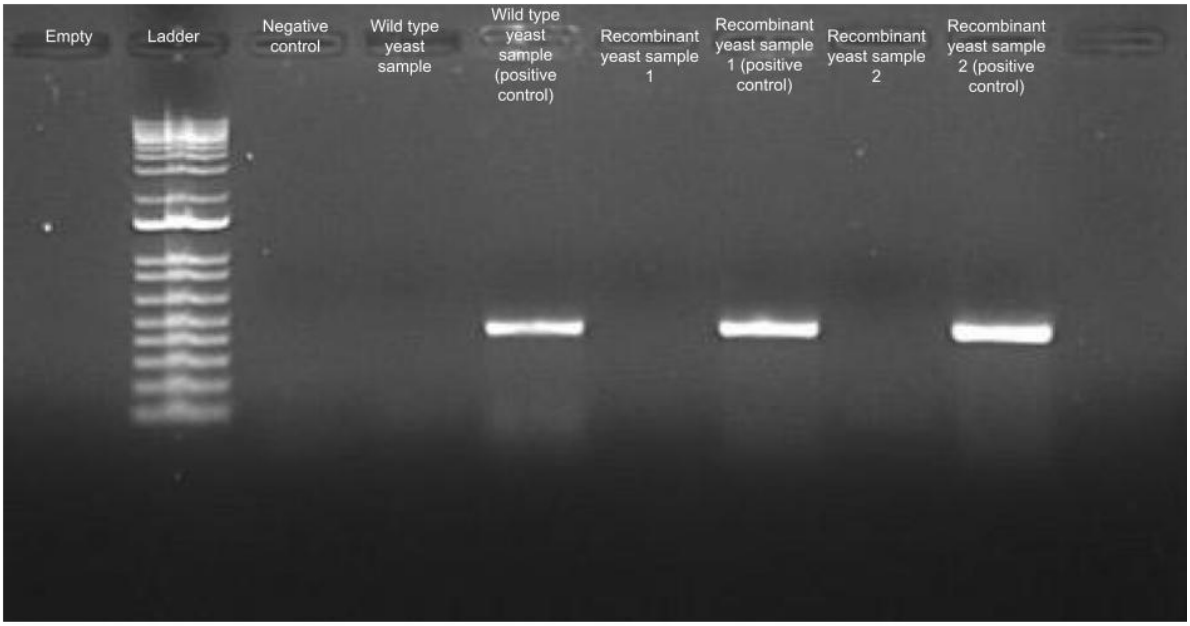

Figure 1 Colony PCR of recombinant yeast cell extracts. PCR product was only obtained from the 3 positive controls. Therefore, homologous recombination did not occur in either of the 'recombinant' yeast cell extracts. As such, the SUC2 gene has not been deleted.

Sample + µl of Yeast	Concentration of Glucose Produced (%)	Absorbance (OD540)
Negative control (0 µl)	0.0000	0.0000
S1 (150 µl of transformant yeast)	0.0550	0.9621
S2 (150 µl of other sample of transformant yeast)	0.0655	1.1366
S3 (10 µl wild-type yeast)	0.0119	0.2458
S4 (50 µl wild-type yeast)	0.0562	0.9812
S5 (150 µl wild-type yeast)	0.0612	1.0646
Positive control (0 µl yeast, glucose added instead of sucrose)	0.0625	1.0250

Table 1: Glucose concentration in yeast cell extract

PCR product in the positive controls and the absence of the PCR product in the negative control confirms that the results are accurate. Further, the results of the invertase assay confirm the results of the PCR. The invertase assay shows that there is glucose present in both of the samples containing transformant yeast cell extracts (S1 and S2). This should not have occurred if the homologous recombination was successful; if the *SUC2* gene was knocked out, there would be no invertase in the cell, so no sucrose could be converted into glucose.

Interestingly, the positive and negative controls in both the colony PCR and the invertase assay gave the expected results, so it can be determined that the failure of homologous recombination was an error on our part, rather than a larger-scale problem such as an issue with the equipment. Other than human errors, such as measuring incorrectly or adding the

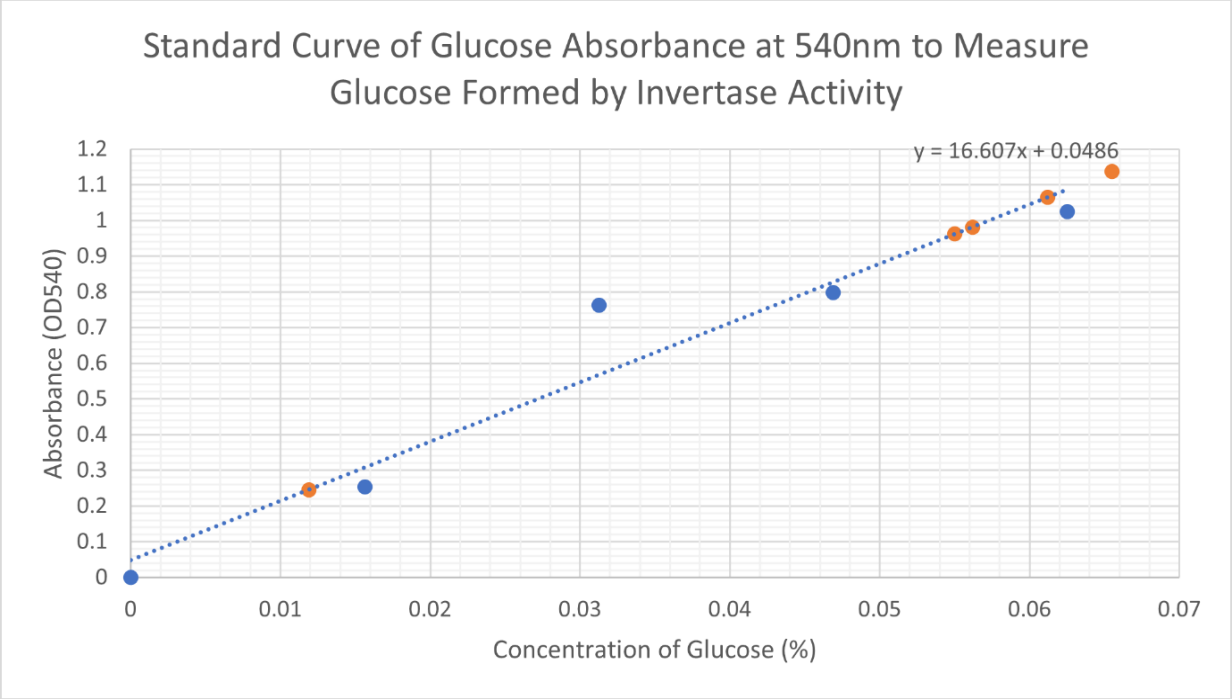

Figure 2 Standard curve of glucose absorbance. Blue represents the glucose controls used to make the standard curve. Orange represents the samples in which glucose has been produced from sucrose by invertase activity (samples S1 to S5 as in Table 1

wrong primers, there are a few possible explanations to explore as to why the homologous recombination did not occur correctly, or at all, leaving the transformant DNA as a plasmid in the cell.
As mentioned in the introduction, each haploid strain of *S. cerevisiae* may contain zero to several *SUC* genes in their genome and each of these encodes invertase[4]. Based on this information, it is possible that the *SUC2* gene was knocked out and another *SUC* gene was still producing invertase, allowing for the presence of glucose we saw in the invertase assay. However, this does not account for the result of the PCR. In this scenario, the transformant yeast extracts would have bands in the gel if the *SUC2* gene was deleted, which did not happen.
Another explanation could be that recombination occurred in a different place on the chromosome. This would occur if the primers did not allow the DNA cassette to be inserted in the right orientation in the open reading frame (ORF) of the invertase gene. It has been shown that the longer the region of homology in the plasmid, the higher the frequency of homologous recombination[6]. According to R. Rothstein[6], sequences with 125 nucleotides of homology do not integrate well, but sequences with 250 nucleotides of homology allow targeted integration. Perhaps our DNA cassette did not have enough nucleotides of homology with the invertase gene.
A final reason could be that ectopic integration occurred, which means recombination occurred at a site of non-homology. This, however, is rare[2] and since most experiments in our laboratory stream obtained similar results, this is unlikely.

As similar results were obtained by the other groups in our laboratory, it seems that the most likely explanation is a problem with the initial knockout primers, causing homologous recombination to not occur since transformation was successful but *SUC2* was not knocked out. The problem could have been a lack of homology between the knockout primers and the sequences flanking the ORF of the invertase gene or a human error in which the conditions for homologous recombination were not optimal.

As the literature shows that the *SUC2* gene can be knocked out by this method,[3,5,7] our findings

are not significant and are unreliable. However, had our results been as expected, they would serve as a confirmation that yeast can be used as a model organism for understanding gene function, genetic interactions, and gene-environment interactions[5].

If the results were reliable, they could be used to argue that the *SUC2* gene cannot be knocked out using the DNA cassette encoding the selective marker gene (in plasmid pFA6-kanMX4) and chromosomal sequence flanking the ORF of SUC2. However, we know that it can be done by this method, so our results cannot be relied upon for further investigation of this hypothesis. Unfortunately, this means that we did not prove our hypothesis to be true. But we cannot determine that our initial hypothesis was wrong as the literature says otherwise. As such we would need to conduct more experiments to know where we went wrong. The experiment should be repeated to try to either replicate these results or obtain the expected results.

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