BIOSYNTHETIC YEAST UTILIZED FOR HIGHER YIELDS OF AGRICULTURE ON MARS MISSION

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Abstract

This research proposes to evaluate the feasibility of modifying yeast for agriculture in a nutrient lacking environment. The project aim is to identify the critical enzyme coding genes that play an essential role in the auxin biosynthesis pathway in *S. cerevisiae*. To identify critical genes, knocking out the gene of interest and following up on the subsequent auxin production is necessary. This study compares two methods for gene knockout in *Saccharomyces cerevisiae*. Initially, the delittio perfetto method[1] was utilized but proved inefficient due to toxic effects and metabolic burden caused by the integration of antibiotic-resistant genes. Subsequently, a novel approach employing CRISPR/Cas9 technology based on the MoClo Yeast Toolkit[2] was adopted. By streamlining the construction of CRISPR/Cas9 multi-gene assembly cassettes, this approach facilitated the simultaneous knockout of single and multiple genes, offering a user-friendly and efficient method for genetic manipulation in yeast. The development of auxin-producing yeast in this study suggests astronauts could autonomously grow nutrient-rich crops in space, utilizing metabolically engineered yeasts to regulate plant growth and contribute to ecological balance beyond Earth's atmosphere.

Introduction

Plants and many microbes synthesize and perceive auxin, particularly Indole-3-acetic acid (IAA), the most prevalent natural auxin, to regulate nearly every aspect of growth and development. *S. cerevisiae* has been confirmed to synthesize auxin during its metabolic growth phases and several auxin biosynthetic pathways have been explored[3]. While significant variation in auxin biosynthesis levels exists among strains of *S. cerevisiae*, the causal mechanism for this variation remains unknown. Auxin-mediated interaction between yeast and plants can help increase nutrient availability and enhance stress resilience in plants[4]. Apart from the ability to synthesize auxin, yeast, or fungi can transform nutrients to plants. They play another significant role in the ecological system by breaking down organic matter for nutrients, gases, and energy into the soil and the atmosphere that are necessary for life[5]. Notably, certain yeast can use liquid and solid human waste as a food source[6], helping to reduce growth media costs. They also have a very fast regenerative rate, with their doubling time being approximately 90 minutes[7]. The speed of reproduction is important for research because results from offspring and growth can be obtained within days.

In this study, two different approaches for gene knockout in Saccharomyces cerevisiae were evaluated. The perfetto delittio method was initially chosen for gene knockout due to the familiarity of the protocol and the availability of resources in the laboratory. However, this approach presented challenges, including the integration and replacement of an antibiotic-resistant gene, leading to toxic effects and metabolic burdens. Additionally, the delitto perfetto method required multiple cloning steps, and decreased transformation efficiency. To overcome these limitations, we implemented a novel gene editing approach utilizing CRISPR/Cas9 technology based on the MoClo Yeast Toolkit. This approach

allowed for efficient gene knockout with reduced toxicity and metabolic burden. Additionally, by cloning Cas9-sgRNA multi-gene plasmids, we developed a streamlined protocol for constructing CRISPR/Cas9 multi-gene assembly cassettes in S. cerevisiae. This protocol enables the simultaneous knockout of single and multiple genes, providing a user-friendly and efficient method for genetic manipulation in yeast. Our findings highlight the benefits of MoClo cloning and CRISPR/Cas9 technology for gene knockout applications, offering improved efficiency and precision in future genetic engineering studies.

In the auxin metabolic pathway, each enzyme involved is encoded by a specific gene, which directs the synthesis of the corresponding enzyme through protein synthesis. Notably, these genes share the same name as the enzymes they encode.

Genes involved in auxin production

This experiment is aimed to uncover the critical genes involved with the auxin biosynthetic pathway in *S.cerevisiae*. Plasmids containing multiple gene knockouts have successfully been transformed into the *S.cerevisiae* strain which also carries a genetically encoded biosensor, capable of detecting endogenously produced auxin[8]. In this regard, the biosensor serves as a tool for detecting and quantifying auxin in a yeast cell. Using the In vivo site mutagenesis protocol, four different genes were successfully knocked out in yeast: Aldehyde dehydrogenase 2, Aldehyde dehydrogenase 3, Amidase, and Nitrilase. After the determination that the analysis of these genetic knockouts provided different results than the wildtype in auxin production, more genes were added for the subsequential aspect of the scientific examination. The new genes involved in the auxin biosynthesis pathway were determined to be viable knockouts for further investigation based on the KEGG database seen in figure 2. This study delivers a series of mutant yeast strains for later use and defines the pathways of auxin biosynthesis. These genes that regulate the enzymes are: Hydroxymethylbilane synthase, Aromatic amino acid aminotransferase 8, and Aldehyde dehydrogenase 5. An additional knockout identified in yeast with the possibility for auxin regulation is the transcription factor, Yeast AP1-like protein 1 (Yap1). Yap1 is a transcription factor that is currently being studied in the process of modulating auxin biosynthesis indirectly by regulating the expression of genes in response to environmental cues and stress[9].

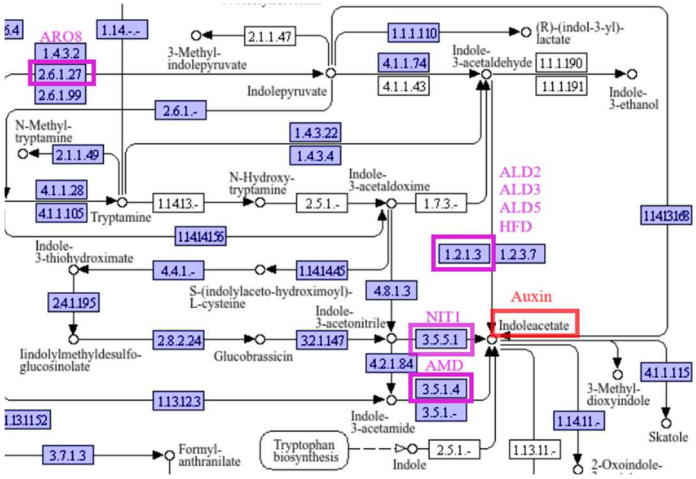

Figure 1. The auxin biosynthesis pathways in S.cerevisiae based on the KEGG database[10]. The target genes/enzymes of this project are shown in the labeled purple outlined boxes. Aldehyde dehydrogenase 2 ALD2 (1.2.1.3), Aldehyde dehydrogenase 3 ALD3 (1.2.1.3), Amidase AMD (3.5.1.4), and Nitrilase NIT (3.5.5.1), Hydroxymethylbilane synthase HFD (1.2.1.3), Aromatic amino acid aminotransferase 8 ARO8 (2.6.1.27), and Aldehyde dehydrogenase 5 ALD5 (1.2.1.3).

Results and Discussion

Delitto Perfetto method of site specific mutagenesis

The delittio perfetto method, coming from the phrase "perfect crime" in Italian[11], is a site-specific mutagenesis method that introduces precise changes in the genome of an organism. The objective of the In Vivo site mutagenesis is to produce single knockouts of genes that coded for enzymes that were responsible for converting substrates into auxin products. This aspect of the study focuses on four genes encoding enzymes, namely Aldehyde dehydrogenase 2 (ALD2), Aldehyde dehydrogenase 3 (ALD3), Amidase (AMD), and Nitrilase (NIT).

The common yeast expression vectors, pGSKU and pGHSU plasmids, were used as the PCR template to amplify the Counter selectable Reporter (CORE). Upstream and downstream DNA sequences of the target gene were designed to flank either side of the PCR products for integration into the genome by homologous recombination. The CORE cassettes with the tails of homology were introduced into yeast carrying the developed biosensor for the specific gene mutagenesis. Yeast transformation followed the protocol from Gietz R, Schiestl R. [12]

To measure and quantify the auxin production by the yeast mutants, the genetically encoded biosensor was integrated into the genome of the strains. DNA sequences both upstream and downstream of the targe gene were designed to allow for homologous integration of the PCR product into the genome. These cassettes were transformed into a strain of yeast carrying the biosensor. In order to check for accurate integration into the genome, yeast colony PCR was successfully run, and the results indicated that the genetic knockouts were successfully done. The data from Figure 3 shows the results of an auxin-induced degradation time-course assay of the 4 mutant yeast strains and the wild type YPH499 parent yeast strain, carrying the same AFB2 biosensor as the specific gene knockout strains.

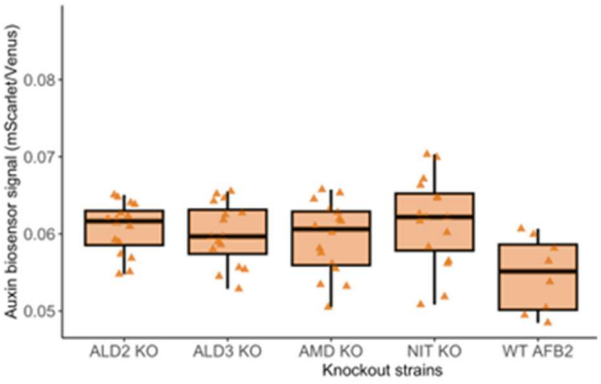

Figure 3. Auxin production in the knockouts. The ald2, ald3, amd, and nit knockout strains containing an AFB2 auxin biosensor show higher auxin production compared to the wild type biosensor strain (WT AFB2) at the exponential phase. X-axis is knockout strains, and Y-axis is the mScarlet-I/Venus ratio which reports relative auxin accumulation as the biosensor signal in response to auxin. Box plots are shown summarizing individual measurements (triangles) from four hours of cytometry measurements.

Modular Cloning

Given the increasing number of gene knockouts desired, a more resilient mutagenesis approach was essential. Modular cloning enables the assembly of extensive cassette plasmids capable of accommodating multiple knockouts simultaneously. By employing this method, all possible knockout combinations can be examined within the time frame typically needed to create a single knockout utilizing an antibiotic resistance marker. This method speeds up the process of making multiple cassettes and can be used for a more efficient method to identify key genes involved in auxin synthesis.

Modular cloning is a variant of the Golden Gate Assembly method that is used to

fuse the assembly of DNA fragments into the larger DNA cassettes. This method works by using a Type IIS restriction enzyme, which cleaves the DNA at specific sequences outside their recognition sites, resulting in defined overhangs[13]. These overhangs are designed to be complimentary to each other and allow the DNA Fragments with compatible overhangs to attach and form a circular cassette at precise locations. These fragments were designed using Snapgene. The 2 Type IIS restriction enzymes used were BsmBI which cleaves at the 5'-CGTCTC(N1)↓(N4)-3' [14] and Bsa1 recognizes 5'-GGTCTC(N1)↓ (N4)-3'15 [16].

Based on initial findings from in vivo mutagenesis, it was determined that specific gene knockouts exert a substantial influence on auxin biosynthesis. These findings prompted the continuation of investigation into these four genes and the inclusion of additional targets to broaden the dataset. Employing the modular cloning technique enables the simultaneous creation of multiple knockouts, facilitated by a multi-gene plasmid containing four gRNAs.

Modular Cloning Methods and Materials

The CRISPR/Cas9 gene editing method is a streamlined for genetic engineering in yeast. This process requires three crucial consecutive Golden Gate assembly reactions.

Original ytk plasmids from the kit (site moclo paper) were struck out on their distinct antibiotic Lysogeny Broth (LB) Media plates with 1.5% agar. The LB media consists of1% Bacto–Tryptone (10 g/L), 0.5% Bacto–Yeast Extract (5 g/L), 0.5% NaCl (5 g/L), and autoclaved for sterilization. These antibiotics included Chloramphenicol, Ampicillin, and Kanamycin. Overnight cultures were grown in 5mL LB Media with corresponding antibiotics used. DNA Extraction was completed using the protocol From New England Biolabs[17]

In the initial Golden Gate assembly step, partially overlapping oligos containing the sgRNA sequence are annealed together. In annealing the oligos, 2 µLof each stock primer was added to 46uL of ethylenediaminetetraacetic acid (EDTA) and placed in the thermocycler under the conditions shown in Figure A6. The annealed oligos, now forming our target gRNAs are cloned with the BsmBI restriction enzyme into the Chloramphenicol resistant entry vector. The preparation of the Golden Gate assembly cloning mixture proceeded as follows: A 1:2 molar ratio of antibiotic-resistant vector to insert, along with 1 µL of T4 DNA Ligase buffer (NEB), 0.5 µL of T7 DNA Ligase (NEB)[18], 0.5 µL of a restriction enzyme, and molecular water were combined to achieve a total reaction volume of 10 µL. The reaction mixtures underwent incubation in a thermocycler following a specific program: 30 cycles of digestion and ligation (42 °C for 2 min, 16 °C for 5 min), succeeded by a final digestion step (65 °C for 10 min) and a heat inactivation step (80 °C for 10 min), before being held at 4°C[19].

The Golden gate reaction mixture was then Cleaned & Concentrated to remove salts and purify the DNA using the Zymolab DNA Cleanup and Concentration Kit[20], and following the protocol with a buffer ratio of 2:1 binding buffer to Golden gate mixture. The DNA was eluted to 7uL using molecular grade water. Next, the formed plasmid was transferred into E.coli Top 10 Competent cells, used due to their high transformation efficiency[21]. The cells were held on ice for 30 minutes, heat shocked at 42°C and mixed with 250 µL of SOC media consisting of 2% tryptone, 10 mM NaCl, 0.5% yeast extract, 10 mM $MgSO_4$, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose[22]. The following cell culture was incubated for 1 hour at 37°C with a shaking speed of 250 rpm. Plating was conducted on LB media antibiotic-selective plates. The process involved plating 20 µL of media supplemented with 80 µL of LB media on one plate, alongside another plate with 100 µL of culture. The plating volume varied proportional to the plasmid vector size. With

the larger plasmids transformed less efficiently, and therefore adjustments were made to the amount of E.coli volume plated, accordingly. Plates are incubated overnight at 37°C.

In the second Golden Gate assembly, the sgRNA part is assembled with appropriate connectors in a second vector (AmpR-ColE1) using BsaI digestion. The result is a sgRNA cassette plasmid which has replaced the Green fluorescent protein, in which colonies appear green, see figure 5. The white colonies are selected for on Ampicillin plates. The subsequent culturing and transformations are done according to the protocol above to extract the DNA from the Level 2 (Ampicillin resistant plasmids). In the third Golden Gate assembly, the sgRNA cassettes are assembled with the Cas9 cassette in a yeast expression vector with Kanamycin resistance. The replacement of the GFP Dropout gene by the desired sequence can be seen on the plating of plasmid containing E. coli. The green fluorescence is easy to distinguish from the white colonies with the help of a black light, as seen depicted in Figure 5. This method was used as a distinguishment between the desired white plasmids and incorrectly assembled neon colonies.

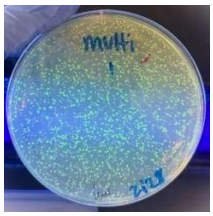

Figure 5: Assembled Multigene cassette plasmid. A visual check to ensure the replacement of the Green fluorescent protein coding gene.

Colony PCR was run on all colonies plated to check the correct integration via the plasmid size. The PCR reaction mixture contains: 0.5µL Forward Primer, 0.5µL Reverse Primer, 12.5µL OneTaq® 2X Master Mix with Standard Buffer (NEB), 11 µL molecular H_2O, and a small swab of colony from struck out LB Plate and inoculated into the solution. The sequencing is sent out for Sanger sequencing to ensure the backbone and inserts are integrated correctly. See Sanger Sequencing results in Appendix Figures A2, Figure A4 and Figure A5.

The results of the intermediary work conducted on the modular cloning aspect of this project are provided in the appendix, showcasing successful colony PCR and Sanger sequencing results. Presently, assembling Multi-gene cassette 1 presents the greatest challenge due to its larger size and inclusion of four gRNA inserts. Precision in stitching is crucial, and with additional time and luck, the Multi-gene plasmid containing knockouts for ALD2, ALD3, AMD, and NIT genes will soon be completed. Multi-gene plasmids 2, containing HFD, ARO8, and ALD5, and multi-gene plasmid 3, containing two Yap1 gRNAs, have been successfully integrated, with results detailed in the Appendix. While much work remains to quantify the investigation's results on auxin, the success of modular cloning represents a positive step forward in determining auxins biosynthetic pathway.

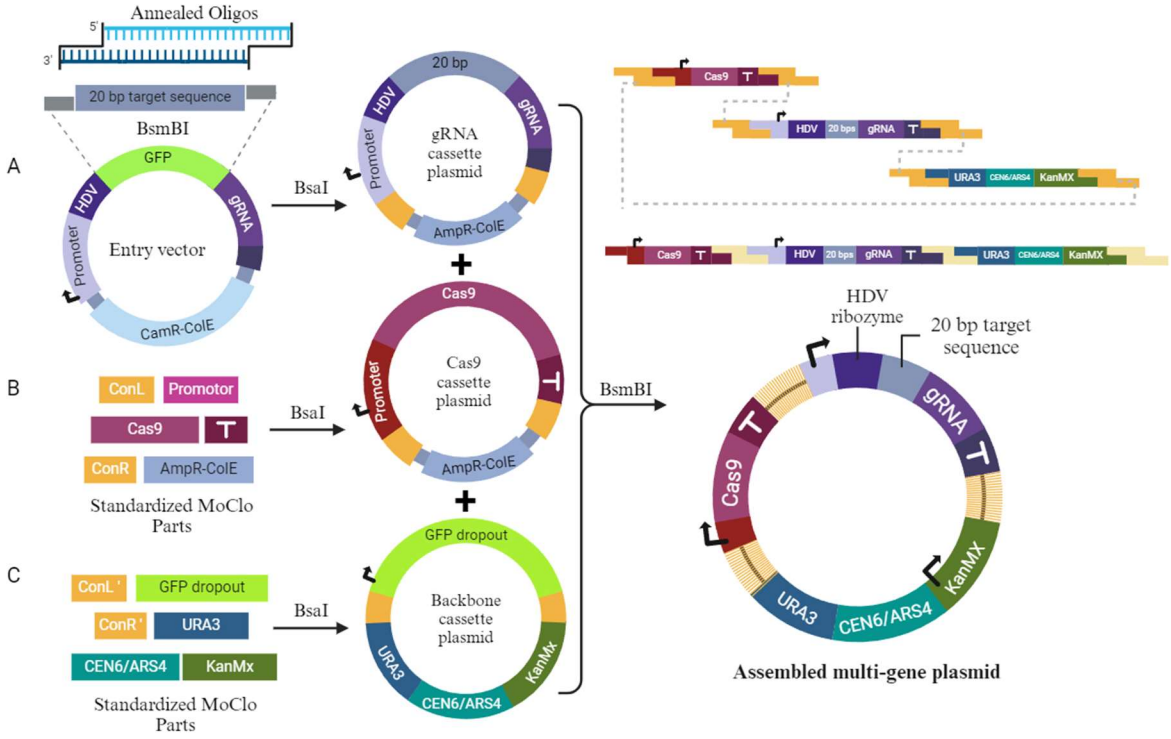

Figure 4. Specialized Modular Cloning workflow to produce Multi-gene Plasmid

Integration of the Biosensor

In the lab, a high throughput biosensor, seen in Figure 2, has already been developed to screen and select the heterologous chassis, the engineered host cell factory. Utilizing the engineered biosensor to quantify auxin production in different yeast strains will allow us to understand the optimal metabolic route and essential genes involved in the auxin biosynthesis pathway. With the success of this project using *Saccharomyces cerevisiae*, further research could be carried out by using the approach and strategy from this research to develop yeast strains as the tool to produce medicines, materials, or various goods for life on Earth, space and Mars.

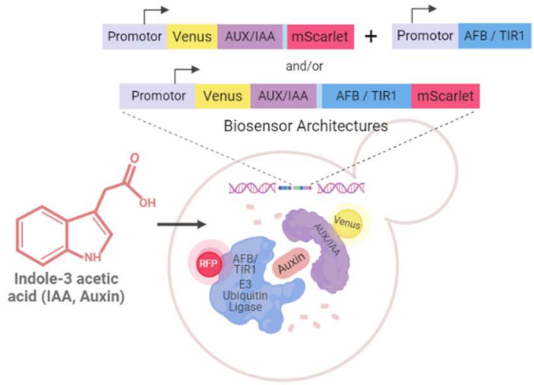

Figure 2: Developed Auxin Biosensor[23]. Used to quantify the amount of Indole-3 acetic acid present through the measurement of mScarlet red fluorescent protein and YPV-Venus fluorescent protein, measured through a cytometer. This then shows the degradation of Aux/IAA over time as a measurement of Auxin present.

Future work

Next Steps in Research

This study has provided valuable insights into genetic mutations specifically using modular cloning techniques. The next steps in the project are displayed in figure 6, beginning with transforming the positive *E. coli* DNA into yeast. positive plasmid DNA from *E. coli* into yeast. YPH499 is the chosen yeast strain which is derivative of S288C with a very well characterized genome critical for CRISPR/Cas9 targeting. Next, completing an auxin-induced degradation time-course assay previously developed in the lab, and used in the analysis of the Delitto Perfetto yeast mutant analysis, is crucial in quantifying auxin production in mutant yeast strains. This process includes isolating and regrowing yeast colonies to the active metabolic phases for auxin production. Their responses to different concentrations from each mutant strain detected by the biosensor will help identify important genes of the auxin biosynthesis pathways, help determine rational designs to optimize the metabolic pathway efficiently and improve auxin production in *S. Cerevisiae*. After having validated that the modular cloning technique works in yeast with no toxic effects of an antibiotic resistant gene, we can work towards maximizing the combinations of gene knockouts.

As a way of using multi-gene knockout cassettes as a starting template for all combinations of gene knockouts, genomic spacers must be integrated into the genomes. In testing on multiple gene cassettes containing knockouts for 4 genes (ALD2, ALD3, AMD, NIT), 3 genes (HFD, ARO8, ALD5), and 2 genes (yap1.1, yap1.2), there must be a way to mix and match the knockouts together regardless of which cassette they're in. The insertion of spacers allows for the knockout of one or two genes, and the combination of many. These spacers act as filler sequences within the cassette, bridging gaps in the assembly process. In the absence of a gRNA, the plasmid may incorrectly reassemble or fail to form a circular plasmid. The spacer, a non-functional DNA sequence, facilitates plasmid formation without interfering with its functionality.

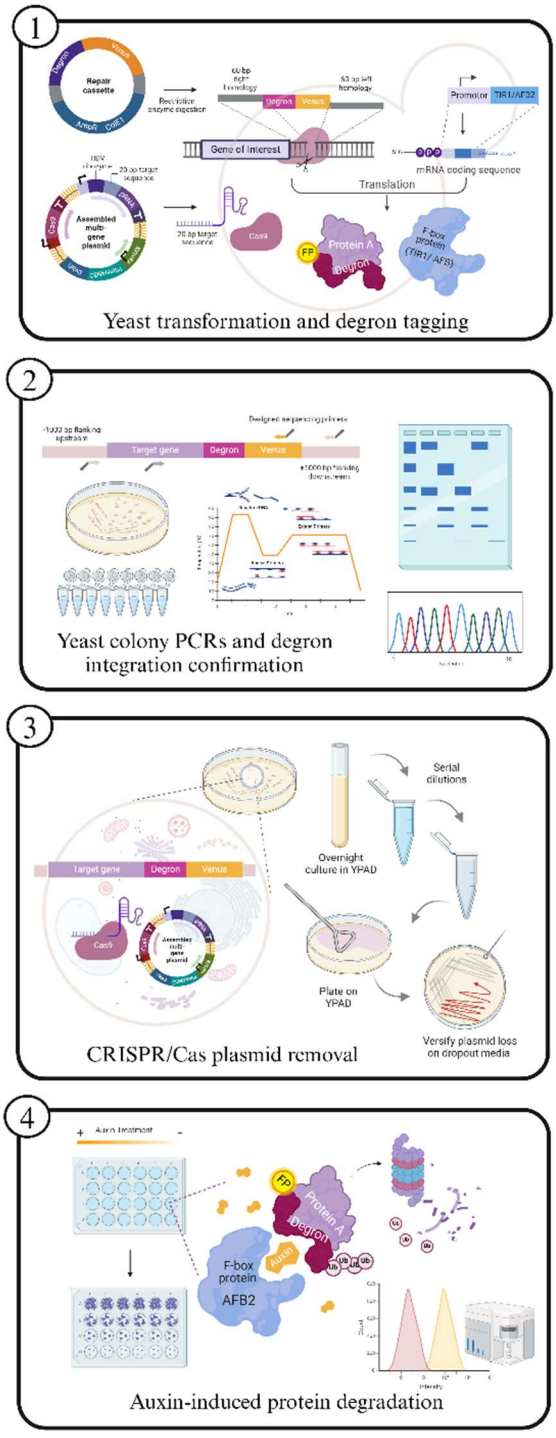

Figure 6: Future work workflow

Down the Line

The knowledge, findings and deliverables from this research will provide a proof-of-concept of the biomanufacturing pipeline to engineer microbes for chemical production and as a carbon source. In future work, I aim to engineer the biosynthetic pathways in other yeast strains such as the freeze resistant yeast strain, *Rhodotorula frigidialcoholis*[24], so it can produce auxin and other chemicals while living in harsh environment conditions like Mars.

Acknowledgements

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Appendix A

From left to right: colony 1, 2, 3, 21, 22

Figure A1: Colony PCR of Cas9 Cassette Plasmid. Left image is colony PCR using primer oWL 755 the ConL1 reverse sequence, the amplification length was expected to be 1127 base pairs. The picture on the right shows these same colonies but amplified the full Cas9 gene using primers oWL 753 and oWL 755. The amplification length was expected to be 5113 base pairs.

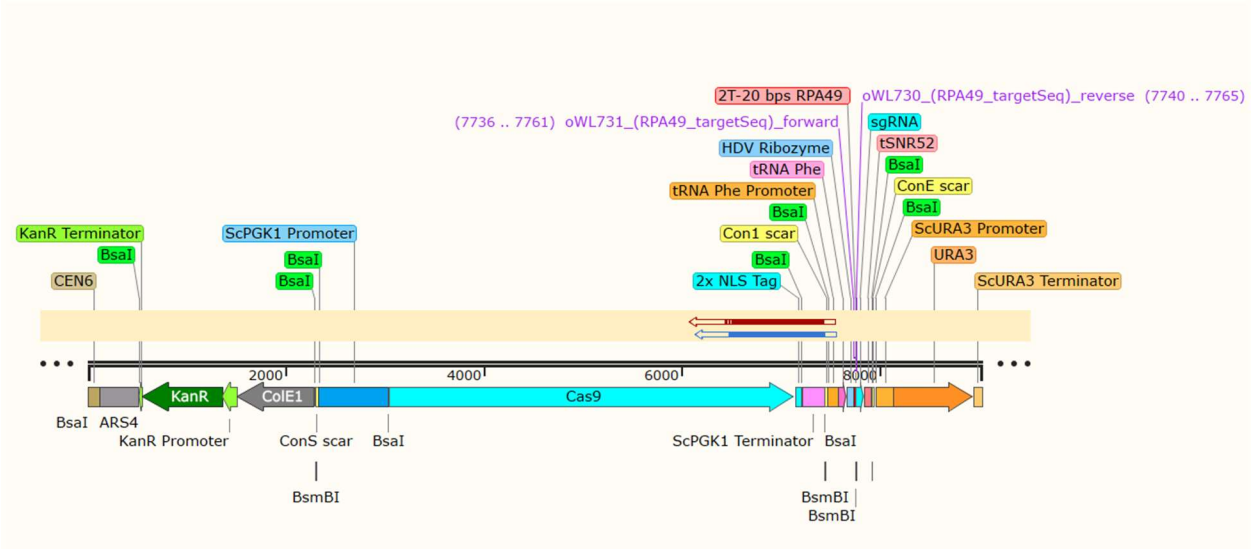

Figure A2: Cas 9 Cassette sequenced check of 2 colonies. Colony 2 and colony 21.

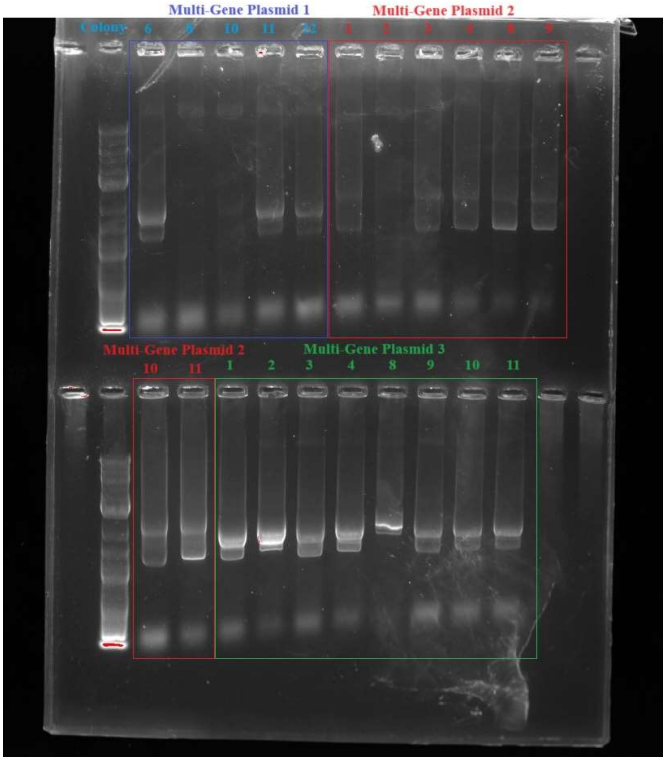

Figure A3: Colony PCR of Multi-gene cassette plasmid 1, 2, and 3. The primers used for amplification are oWL 720 and oWL 721. The region amplified was expected to be ~2600 bp.

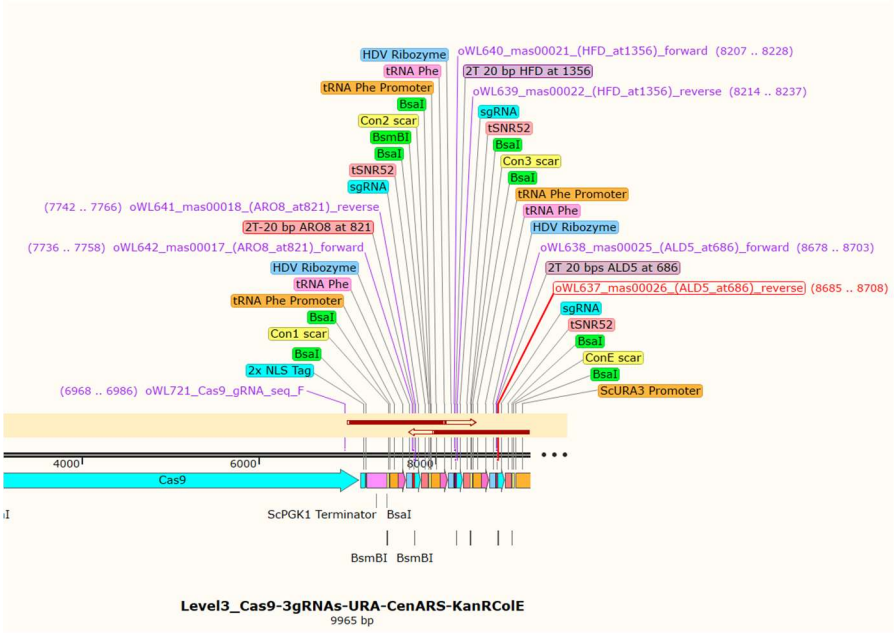

Figure A4: Multi Cassette 2 (Level 3) Positive Sequencing. Sequencing results of Colony 8, using forward and reverse primer ensuring that the integration occurred correctly.

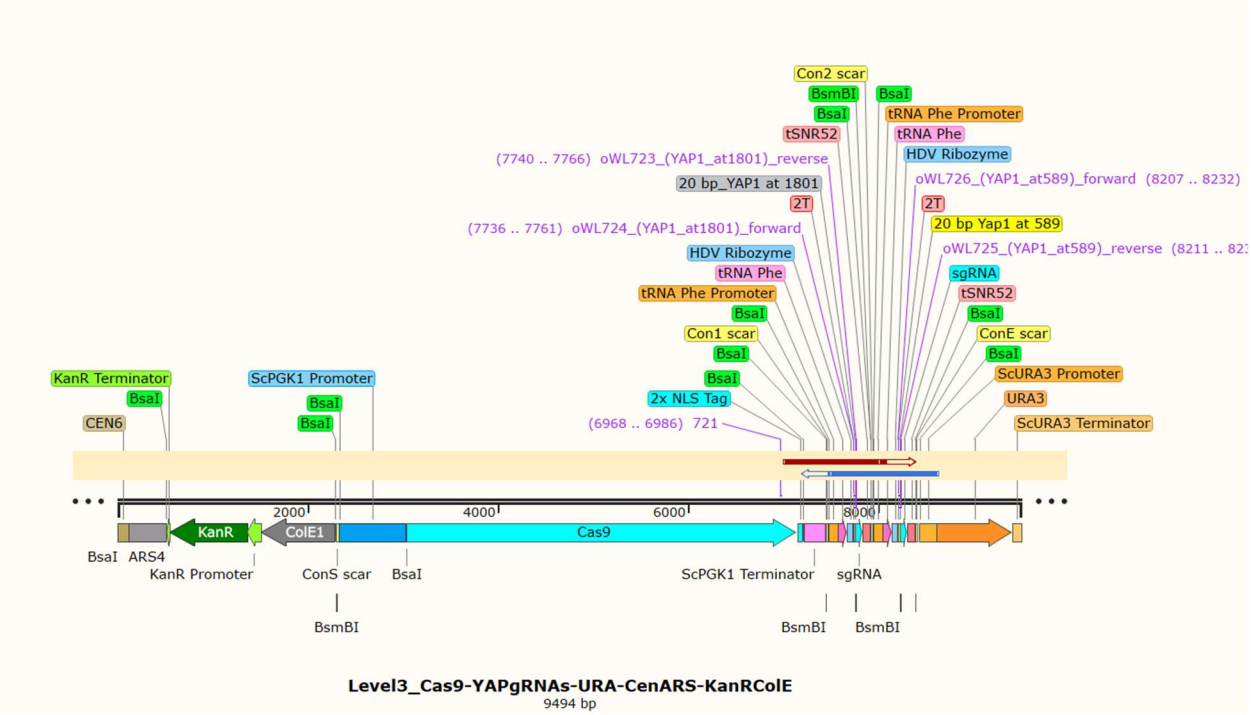

Figure A5: Multi Cassete 3 (Level 3) Positive sequencing. Colony 2 sequencing using forward primer oWL 721 and Reverse primer oWL 722.

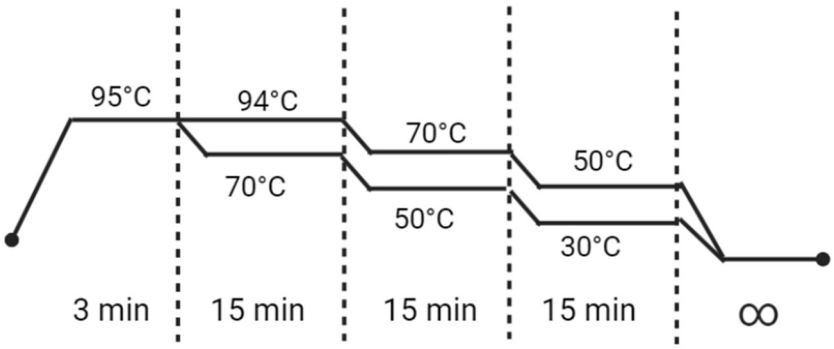

Figure A6: Thermocycler conditions for annealing oligos. Steps 2-4 induce a gradient on the samples.