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Evolutionary adaptation of engineered yeast strains

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Abstract

Industrial biosynthesis processes provide an excellent alternative to oil-based chemical production, as they could be used to convert renewable resources to a large variety of compounds. These biosynthesis processes, however, commonly use microbial cells expressing engineered production pathways. The engineered production pathways tend to compromise the fitness of cells. Thus, the population of cells is prone to losing the production efficiency in long term cultivation, as natural selection favours high fitness individuals. Therefore, tracking and characterizing the evolutionary stability of an engineered pathways is especially important for long-term industrial applications, where the unexpected loss of production efficiency can cripple the whole production process.

The aim of this work was to assess the evolutionary stability of production traits engineered in yeast *Saccharomyces cerevisiae* strains. The strains were exposed to adaptive laboratory evolution (ALE). The yeast strains included haploid and diploid indigoidine pigment producing strains, as well as their corresponding wild type strains. Additionally, haploid bikaverin pigment producing strain was also used as a comparison point to the indigoidine strains. These strains were adaptively evolved in four lineages in both rich and synthetic defined media. During the ALE, the haploid lineages were adapted for approximately ~200 generations, while the diploid lineages were adapted for ~120 generations. The growth rates and pigment production of the *S. cerevisiae* lineages were tracked during the ALE, as the data they provided could be used to assess the stability of the engineered production traits.

Growth rates of all yeast strains were determined at the start of the ALE and the growth rates of the adapted lineages were assessed at the end of the ALE. The growth rates of the adapted lineages were generally found to improve notably during the ALE, as on average, the adapted lineages experienced growth rate increase of 30%. The pigment production of the engineered lineages was tracked by plating a set amount of their diluted broth cultures onto solid rich media plates at predetermined time points. These plates were then used to count the proportion of pigment producing clones for each of the producing lineages. All the haploid indigoidine lineages lost their pigmentation completely after ~200 generations of adaptation, while the diploid indigoidine lineages lost over 80% of their pigmentation after ~120 generations. The haploid bikaverin lineages were surprisingly robust, as their rich media grown lineages lost only 63% of their pigmentation after ~200 generations, while two of the synthetic defined medium grown lineages did not lose any pigmentation during the ALE.

Knowledge on the evolutionary stability of an engineered pathway is important, as it can directly affect the production yield of biosynthesis processes. Stabilizing production yields can improve the economic feasibility of these processes, which is important when we are aiming for more sustainable and oil-free future.

Keywords Adaptive laboratory evolution, Heterologous expression stability, Microbial evolution, *Saccharomyces cerevisiae*

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Tiivistelmä

Teolliset biosynteesiprosessit tarjoavat erinomaisen vaihtoehdon öljypohjaiselle kemikaalien tuotannolle, johtuen mahdollisuudesta tuottaa monenlaisia yhdisteitä uusiutuvista resursseista. Näissä biosynteesiprosesseissa käytetään kuitenkin usein mikrobisoluja, joihin on viety heteroloogisia tuotantoreittejä. Heteroloogiset tuotantoreitit saattavat merkittävästi heikentää solujen elinvoimaisuutta, mikä voi johtaa odottamattomiin muutoksiin pitkäkestoisissa tuotantoprosesseissa. Luonnonvalinnan takia muunnellut mikrobikannat, ovat alttiita menettämään niihin kehitettyjen tuotantoreittien tehokkuutta. Siksi tuotantoreittien evoluutionäärisen stabiiliuden seuraaminen ja arvioiminen on tärkeää etenkin pitkänajan teollisissa käyttökohteissa, joissa odottamaton tuotantoreitin heikentyminen voi rampauttaa koko prosessin.

Tämän työn tavoitteena oli arvioida muunneltujen *Saccharomyces cerevisiae*-hiivakantojen tuotanto-ominaisuuksien evoluutionääristä stabiiliutta. Hiivakannat altistettiin adaptiiviselle laboratorioevoluutiolle (ALE). Hiivakannat sisälsivät indigoidine pigmenttiä tuottavat haploidi- ja diploidikannat, sekä näiden villityypit. Näiden ohella, bikaverin pigmenttiä tuottavaa haploidikantaa käytettiin vertailukohteena indigoidinea tuottaville kannoille. Näitä hiivakantoja kasvatettiin, sekä rikkaassa, että synteettisessä määritellyssä kasvatusalustassa, joissa molemmissa kaikilla hiivakannoilla oli neljä sukulinjaa. ALE:ssa haploidisukulinjoja kasvatettiin arviolta ~200 sukupolven ajan, kun taas diploidisukulinjoja adaptoitiin noin ~120 sukupolven ajan. Sukulinjojen kasvunopeuksia ja pigmenttien tuotantoa seurattiin ALE:n aikana, sillä näitä seuraamalla kantoihin kehitettyjen tuotanto-ominaisuuksien stabiiliutta pystyttiin arvioimaan.

Kaikkien hiivakantojen kasvunopeudet määritettiin sekä ALE:n alussa, että lopussa. Evolvoitujen kantojen kasvunopeudet kehittyivät keskimäärin 30% ALE:n aikana. Muunneltujen kantojen pigmenttituotantoa seurattiin levittämällä sukulinjojen laimennettua viljelmää petrimaljoille ennalta määrättyinä ajankohtina. Petrimaljojen inkubaation jälkeen, maljoilta pystyttiin laskemaan pigmenttiä tuottavien kloonien osuus jokaisessa sukulinjassa. Kaikki indigoidine pigmenttiä tuottavat haploidisukulinjat menettivät värityksensä kokonaan ~200 sukupolven kasvatuksen jälkeen. Diploidisukulinjat puolestaan menettivät yli 80% niiden värityksestä ~120 sukupolven jälkeen. Haploidit bikaverin pigmenttiä tuottavat sukulinjat kykenivät säilyttämään värityksensä yllättävän hyvin. Rikkaassa kasvatusalustassa kasvaneet sukulinjat menettivät vain 63% niiden värityksestä ~200 sukupolven kasvatuksen jälkeen. Puolet synteettisessä määritellyssä kasvatusalustassa kasvaneista sukulinjoista eivät menettäneet väritystä ollenkaan ALE:n aikana.

Tieto muunneltujen hiivakantojen tuotantoreittien evoluutionäärisestä stabiiliudesta on tärkeää, sillä se vaikuttaa suoraan biosynteesiprosessien tuotannon saantoihin. Tuotannon stabiloiminen parantaa biosynteesiprosessien taloudellista kannattavuutta, mikä on tärkeää kestävän ja öljyvapaan tulevaisuuden saavuttamiseksi.

Avainsanat Adaptiivinen laboratorio evoluutio, Heteroloogisen ilmentämisen stabiilisuus, Mikrobien evoluutio, *Saccharomyces cerevisiae*

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Preface

I want to thank my thesis supervisor Professor Paula Jouhten for supervising and guiding me through the thesis project, and overall, for making this whole project possible. I also want to thank Diogo de Lucena Antunes for showing me the ropes of laboratory work. Additionally, I also want to thank the whole Microbial physiology research group for relaxed and great work environment. Natalia Kakko, Celma Mekki, Linda Porri, An Nguyen, Laura Niemelä, Hoa Chu and Tomas Strucko I want to thank for support, encouragement, and great moments in the lab. Finally, I want to thank my family and friends for their support throughout the thesis project.

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Abbreviations

ALE	Adaptive laboratory evolution
BpsA	Blue pigment synthetase
cpm	Cycles per minute
G418	Geneticin
Mutagenesis	Production of mutations
NRPS	Non-ribosomal peptide synthetase
NTC	Nourseothricin
OD	Optical density
PHAs	Polyhydroxyalkanoates
PLA	Poly lactic acid
PPTase	4′-phosphopantetheinyl transferase
TCA	Tricarboxylic acid
VG	Vanillin-β-glucoside
YNB	Yeast nitrogen base without amino acids
YNBG	Yeast nitrogen base without amino acids + Galactose
YP	Yeast extract and bacteriological peptone
YPD	Yeast extract and bacteriological peptone + Glucose
YPG	Yeast extract and bacteriological peptone + Galactose

1 Introduction

Genomes of microbial species are said to have been evolutionarily streamlined. As these genomes are engineered, their natural optimization faces sudden changes, which result in the beginning of new streamlining processes. Engineered microbial species used in industrial biosynthesis processes may generate substantial number of generations throughout their production processes. During the formation of these cellular generations, the engineered genomes inevitably undergo cycles of mutations, adaptation, and selection.

Production routes for a large number of compounds have been engineered in both laboratory and industrial scale (Wehrs *et al.*, 2019; Carbonell *et al.*, 2018; Zhang *et al.*, 2016; Beller *et al.*, 2015), and the potential integration loci of these routes have been assessed for stable expression (Bai Flagfeldt *et al.*, 2009; Amen and Kaganovich, 2017). Stable expression at designated integration loci, however, does not guarantee the evolutionary stability of fitness compromising production routes. This evolutionary stability has not been tested extensively, and for this reason, this study aims to provide insight into the evolutionary stability of fitness compromising production routes.

In this study, indigoidine producing *Saccharomyces cerevisiae* strain(s) were exposed to adaptive laboratory evolution (ALE) for assessing the evolutionary stability of the engineered trait. The indigoidine production route requires the expression of blue pigment synthetase (BpsA), which is a non-ribosomal peptide synthetase (NRPS), and a 4′-phosphopantetheinyl transferase (PPTase), which is required to activate the BpsA from its apoform to its holo- form (Wehrs *et al.*, 2018). The activated NRPS holo-BpsA condenses two L-glutamine residues into a single indigoidine molecule in a single step process. Indigoidine synthesis pathway has been noted to benefit from respiratory environment that promotes the tricarboxylic acid (TCA) cycle (Wehrs *et al.*, 2018). In addition to indigoidine producing *S. cerevisiae*, a bikaverin producing strain was also adapted in similar conditions. Bikaverin synthesis occurs through a 13-step process that requires the heterologous integration of six different genes (Zhao *et al.*, 2020). These genes are Bik1, Bik2, Bik3, Bik6, npgA, and ppt1 (Zhao *et al.*, 2020).

S. cerevisiae has previously been adaptively evolved under various conditions to improve the species desirable traits, such as, high production yields, and high tolerance to extreme environments. As an example, Dhar *et al.* (2011) adaptively evolved *S. cerevisiae* to have improved saline tolerance, while Kutuna *et al.* (2011) improved the glycerol production of a wine yeast. In this study, the ALE was performed for ~100 - ~200 generations, depending on the strain, to mimic the potential long term industrial production conditions. Production stability is highly relevant for the feasibility of large-scale biotechnological production, that aims to replace oil-based

production. High production stability allows the biosynthesis processes to reach higher economic stability, as it allows the continuous production of bioproducts without the fear of losing production efficiency. This highlights the importance of understanding the evolutionary stability of a chosen production route, as stabile production of bioproducts enables us to reach a more sustainable future.

2 Literature review

2.1 Engineered microbial cells as production hosts

2.1.1 Selection of production host

In engineered microbial cells, the heterologous synthesis of a multitude of different compounds has been demonstrated in laboratory scale. These demonstrated compounds range from fuels and pharmaceuticals to bulk and fine chemicals (Wehrs *et al.*, 2019; Carbonell *et al.*, 2018; Zhang *et al.*, 2016; Beller *et al.*, 2015). For example, García-Franco *et al.* (2024) successfully biosynthesized styrene, a bulk chemical, in *Pseudomonas putilda*, while Deng *et al.* (2023) were able to biosynthesize antibacterial phenazine-1,6-dicarboxylic acid in *Streptomyces coelicolor* host strain. Important physiological host characteristics include growth rate, production yield, safety, and tolerance and adaptability to chosen physiochemical and nutritional conditions (Beller *et al.*, 2015; Fischer *et al.*, 2008; Rainha *et al.*, 2020; Zhang *et al.*, 2016). Readily available genetic tools and knowledge on the native metabolic networks are also essential for developing strains for heterologous production (Zhang *et al.*, 2016). Model host organisms, such as, bacterium *Escherichia coli* and yeast *S. cerevisiae*, are also typical heterologous production hosts, as they are fast growing, adaptable, and they have extensive metabolic models and genetic engineering strategies available for them (Heaver *et al.*, 2015; Lu *et al.*, 2019; Monk *et al.*, 2017; Raiha *et al.*, 2020; Siewers *et al.*, 2014; Wehrs *et al.*, 2019; Zhao *et al.*, 2016).

2.1.2 Production optimization

In addition to host selection and increasing product variety, genetic engineering has also enabled the optimization and overexpression of targeted production pathways. This optimization can be done in multiple ways by utilizing the basic modification methods. Deletions, insertions, and replacements can be, for example, used to knockout genes that actively inhibit the production pathway of the targeted compound. One such knockout was performed by Montenegro-Silva *et al.* (2024) when they aimed to evaluate the effect of knocking out genes coding for different pyrroloquinolinequinone-dependent methanol dehydrogenases that affect bacteriological cellulose synthesis in *Komagataeibacter sucrofermentans*. In this study, the gene knockout was found to increase bacterial cellulose production when accompanied by another gene deletion. In addition to gene knockouts, gene insertions can also be utilized in the optimization of biotechnical production. For example, Li *et al.* (2024) overexpressed the genes of two crucial enzymes of D-arabitol synthesis pathway in *Zygosaccharomyces rouxii* by inserting

multiple copies of said genes, to make the production of D-arabitol more economically viable. As a result of this overexpression, Li *et al.* (2024) noted that the synthesis of D-arabitol did increase noticeably.

2.1.3 Replacing oil-based chemistry

One major application of engineered microbial cells is in the sustainable biosynthesis of petroleum-based products. Products that use petroleum are numerous and they include items such as fuels, plastics, resins, rubbers, solvents, textiles, paints, inks, and personal care items (Mori, 2023; Robinson *et al.*, 2017). The traditional manufacturing of these products is done by using non-renewable petroleum resources that have a finite supply, which have been estimated to last for the next 50 – 100 years more with the currently known natural deposits (Robinson *et al.*, 2017). In addition to the limited nature of their raw material, the production of petroleum-based compounds contributes massively to global greenhouse gas emissions. It has been estimated that the petroleum industry is responsible for up to 37% of global greenhouse gas emissions (Fethi and Rahuma, 2020). Additionally, many petroleum products are challenging to recycle, and have long degradation times, which has significantly contributed into the global microplastic issue (Hale *et al.*, 2020; Naser *et al.*, 2021). These challenges with petroleum-based compounds highlight the need for alternative and sustainable products that can be produced through environmentally friendly processes. With engineered microbial cells, the production of various petroleum-based compounds could be done with biosynthesis processes that uses renewable biomaterials as their main feedstocks (Wang *et al.*, 2018). Additionally, biosynthesis processes allow the production of many new biodegradable compounds that could be used to replace non-degradable petroleum-based compounds. For example, engineered microbial cells can be used to biosynthesize the basic components of bio-based polymers, such as poly lactic acid (PLA) and polyhydroxyalkanoates (PHAs), which can then be used to replace synthetic non-degradable polymers, such as polyethylene (Naser *et al.*, 2021). In addition to allowing the production of environmentally friendly compounds, biosynthesis also provides additional benefits. Biosynthesis can produce enantiopure compounds in mild reaction conditions, which can reduce the amount of needed purification processes when compared to traditional chemical synthesis (Zhang *et al.*, 2016).

2.1.4 Scaling-up bioprocesses

Although biosynthesis is a very attractive alternative to pure chemical synthesis, its successful and reliable implementation in large scale industrial production is very challenging (Rugbjeg and Sommer, 2019). The largest challenge is the overall economic viability of the designed biosynthesis

process at an industrial scale, as the whole scaling-up process and implementation can cost between 100 million to 1 billion USD (Crater and Lievese, 2018). Various compounds have been produced in proof-of-concept style in laboratory scale, but only a fraction of those have been successfully scaled-up into industrial processes, which are capable of manufacturing marketed products (Wehrs *et al.*, 2019). This is mainly caused by the unpredictable nature of the production hosts' performance on these two scales. This unpredictability is mainly due to the differing reaction conditions that preside in laboratory scale and industrial scale, and the overall stability of the engineered pathway (Humphrey, 1998; Rugbjeg and Olsson, 2020). For example, the operating pressures of industrial scale bioreactors are much higher when compared to the low pressures of laboratory scale flask cultures (Wehrs *et al.*, 2019). This increase in pressure can, for example, affect the gas exchange of microbial cells, thus affecting the potential biosynthesis efficiency of the process. Additionally, large scale bioreactors are prone to uneven mixing, which may cause the production host cells to experience variation in temperature, pH, dissolved oxygen concentrations, and substrate concentrations, all of which can heavily affect the biosynthesis performance (Neubauer and Junne, 2010; Wehrs *et al.*, 2019). These issues highlight the importance of testing chosen production host strains in a simulated environment that has the properties of an industrial bioreactor, since testing in real scale can be very expensive and somewhat inefficient (Neubauer and Junne, 2010; Wehrs *et al.*, 2019). The use of these simulated environments allows for a more accurate selection of production host and pathway combination that fulfills all needed requirements.

2.2 Compromised fitness of production hosts

2.2.1 Overexpression of engineered pathways can compromise the production host's fitness

Engineering microbial cells to reach economically attractive biosynthesis production levels inevitably draws resources from cell growth and therefore compromises their overall fitness. Fitness itself can be defined as the cell's ability to thrive in its current environment (Gregorio *et al.*, 2016). This ability can be tracked through multiple parameters that include transcriptional output, metabolic rate, growth rate, and signaling activity (Gregorio *et al.*, 2016). Overexpression of native or heterologous pathways can burden cells significantly due to the increased resource and co-factor requirements (D'Ambrosio *et al.*, 2020). The cells respond to this overexpression with decreased growth and unpredictable physiological changes that usually reduce the performance of the engineered cells (Ceroni *et al.*, 2018). Overexpression of pathways can also trigger protein burden, which is caused by the overproduction

of harmless proteins to a level which overloads the cells protein synthesis process (Kintaka *et al.*, 2020). The fitness of the cells can also be further reduced by high product and by-product concentrations, which may be toxic to the cells in economically attractive production levels (Wehrs *et al.*, 2019). Due to the compromised fitness of engineered strains, individual clones that experience fitness increasing mutations can become enriched in populations during long-term processes (Rugbjeg and Sommer, 2019). These fitness increasing mutations can potentially break the cells engineered properties, thus reducing the stability of engineered pathways. The compromised production stability of the engineered strains is often a limiting factor in the implementation of industrial scale bioprocesses, as in prolonged (e.g., > 40 generations) industrial scale bioprocesses the risk for substantial heterogeneity in production is high (D'Ambrosio *et al.*, 2020; Rugbjerg and Olsson, 2020). For this reason, understanding the underlying mechanisms of production stability and how loss of production can be alleviated are relevant topics in bioprocess development.

2.2.2 Heterogeneity in production host

Generally, heterogeneity affecting production can be of two types, either genetic heterogeneity, caused by gene- and strain-specific mutation types (Rugbjeg and Sommer, 2019; Rugbjerg and Olsson, 2020), or phenotypic heterogeneity, caused by stochastic gene and protein regulation, expression, and distribution to daughter cells (Binder *et al.*, 2017; Gasperotti *et al.*, 2020; Rugbjerg and Olsson, 2020). Genetic heterogeneity of the target engineered cells can be detected by using advanced DNA sequencing and protein analytics, which allow for the specific characterization of mutation rates, types, and targets (Lin *et al.*, 2017; Rugbjerg and Olsson, 2020). Phenotypic heterogeneity can be characterized by flow cytometry, microfluidic methods, microengraving, and biosensors (Binder *et al.*, 2017; D'Ambrosio *et al.*, 2020; Love *et al.*, 2010; Rugbjerg and Olsson, 2020). Impact of cell heterogeneity on long-term industrial bioprocesses can be reduced by coupling the product formation to growth (Jensen *et al.*, 2019; Wehrs *et al.*, 2019). One way this coupling can be achieved is by metabolically linking the flux of an engineered pathway to the cell's own growth essential pathway. For example, the engineered pathway can be made to recycle essential cofactors, such as NAD^+ (Shepelin *et al.*, 2018). This linking results in the engineered production pathway becoming growth essential and the product formation fitness improving. For example, Fong *et al.* (2005) in their study, managed to couple lactic acid production to growth by restricting the formation of alternative fermentation products (Fong *et al.*, 2005). If the coupling of an engineered pathway and an essential pathway has been successful, the new system can then be improved further by subjecting it to ALE, in which the phenotypes with improved fitness and the production trait, if it is coupled to fitness, are

selected for (Fong *et al.*, 2005, Shepelin *et al.*, 2018). Intuitively designing a growth coupled production systems can be challenging. However, modeling algorithms that utilize genome-scale metabolic models, such as OptGene, OptKnock, and Robustknock, can identify gene insertions and knockouts that can be combined to growth-coupled production pathways (Burgard *et al.*, 2003; Jensen *et al.*, 2019; Patil *et al.*, 2005; Tepper and Shlomi, 2010). Additionally, these algorithms can optimize the growth conditions of the designed coupled pathway, by suggesting necessary medium supplements, if the designed growth-coupling has resulted in auxotrophies (Jensen *et al.*, 2019).

2.2.3 Linking the production to growth via product addiction

The linking of engineered pathway to growth can also be established by creating a product addiction via biosensors that are either product or pathway sensitive (Rugbjerg and Olsson, 2020). These biosensors can regulate the growth of the engineered cells by promoting or repressing the transcription of constitutive essential genes depending on the concentration of chosen pathway metabolites or product (D'Ambrosio *et al.*, 2020). This use of biosensors results in a system that, for example, actively inhibits the growth and proliferation of low producing cell variants, as their fitness is decreased by biosensors that are activated by low product concentration. For example, D'Ambrosio *et al.* (2020) successfully built and tested regulatory control circuit in yeast *S. cerevisiae*, that stabilized vanillin-β-glucoside (VG) production in long term product formation. The regulatory control circuit utilized two separate biosensors, that actively inhibited the growth of yeast mutants that did not produce intermediate molecules that were associated with the implemented VG pathway (D'Ambrosio *et al.*, 2020). D'Ambrosio *et al.* (2020) managed to reach a 2-fold higher VG production, and 5-fold increase in pathway metabolite accumulation. This highlights the potential of using biosensors to extend the productive lifespan of a population.

2.2.4 Assessing the stability of the engineered strain

The productive lifespan and stability of the developed strain can be assessed with production load measurement and serial passaging (Rugbjerg *et al.*, 2018a; Rugbjerg *et al.*, 2018b). Production load measurement can be completed by measuring the specific growth rate of the engineered strain and comparing the resulting values to the specific growth rate values of a precursor strain that contains less modifications (Rugbjerg and Olsson, 2020). Serial passaging of the engineered strain can be used to simulate the effects of long-term cultivation. For example, Rugbjerg *et al.* (2018a) simulated large-scale mevalonic acid production in *E. coli* by serially passaging the cell cultures every eight hours. This passaging was repeated for a total of nine times,

resulting in the formation of approximately 80 generations. As a result of this serial passaging, the *E. coli* strain was noted to have lost its engineered pathway completely within just 70 generations. In return to this production loss, the *E. coli* strain had gained higher overall fitness, as the adapted *E. coli* populations were reported to have 28% higher growth rate than the initial populations (Rugbjerg *et al.*, 2018a). This example highlights the benefits of assessing the productive lifespan of an engineered strain, as proper assessment can give good general insight into the overall fitness of the strain and how prone it is to lose the production trait. In both production load measurement and serial passaging, the actual bioprocess conditions must be present in the testing environment to receive relevant data.

2.3 Evolution of microbial cells

2.3.1 Evolution relies on natural selection and genetic drift

Evolution can be defined as a change in allele frequencies in a population over time (Page and Holmes, 1998). For microbial cells used in industrial purposes, this change in allele frequencies is dictated by evolutionary forces of natural selection and genetic drift. Natural selection is a process in which individuals with certain heritable traits are more likely to survive and reproduce than individuals with less beneficial traits (Campbell *et al.*, 2017). Over time, this allows the clones with higher fitness in their surrounding environment to enrich in the population. Genetic drift can be defined as random fluctuations in the allele frequencies from one generation to another caused by chance events (Campbell *et al.*, 2017). Genetic drift is highly significant in small populations, as chance events have a higher probability to cause alleles to be disproportionately overrepresented or underrepresented. This can lead to situations where genetic drift eliminates certain alleles from population completely or causes slightly harmful alleles to become more common, which both can substantially affect the adaptability and survival of a population (Campbell *et al.*, 2017). Genetic variation is required for microbial evolution. Due to their fast growth rates and short generation times, microbial populations evolve quickly through molecular evolution that results from mutations. These mutations generate genetic variation in the form of new alleles, which fuel the evolution of microbial cells.

2.3.2 Mutations provide genetic variation

Mutations can be defined as permanent alterations in DNA sequences, and they can occur in both small and large scale (Page and Holmes, 1998; Riddle *et al* 1997). Small scale mutations occur on the sequence level of the organism's genome, and they consist of single nucleotide replacements, insertions,

and deletions (Page and Holmes, 1998; Riddle *et al* 1997). These single nucleotide mutations can have substantial impact on the fitness of the organism, if they occur in a coding sequence of a protein (Campbell *et al.*, 2017). These mutations can change the amino acid composition of the encoded protein, which may increase or decrease the proteins' function. These mutations can also be neutral in their effect, since multiple codons can encode for the same amino acid (Cutter *et al.*, 2006). Additionally, some genetic sequences can be non-coding, and a change in their sequence may not influence the fitness of the organism. Large scale mutations happen on the chromosomal level, and these mutations can affect the copy number and structure of the chromosomes present in the organism. The structure effecting mutations include chromosome breaks, inversions, translocations, and duplications (Page and Holmes, 1998; Riddle *et al* 1997). Most chromosome breaks are successfully repaired by joining the broken ends together, but in some cases, genetic material can be accidentally erased in the joining process. Chromosome breaks can also lead to chromosome inversions, where a broken off chromosome fragment is accidentally rejoined with the rest of its chromosome in a flipped configuration. In chromosome translocation, the broken chromosome fragment may join a completely different chromosome. Chromosome breaks, inversions and translocations can be harmless to the normal function of the cell, if they leave the genes of the organism intact, but in many cases, these changes are usually harmful (Campbell *et al.*, 2017).

2.3.3 Spontaneous and induced mutagenesis

The production of mutations (mutagenesis) can be spontaneous or induced (Riddle *et al.*, 1997). Spontaneous mutagenesis can commonly result from DNA replication errors that produce random mutations (Riddle *et al.,* 1997; Maki, 2002). In addition to replication errors, mutagenic nucleotide substrates, endogenous DNA lesions, and damaging background radiation can all contribute to spontaneous mutagenesis. The rate of spontaneous single nucleotide mutations is estimated to be approximately 10^{-10} to 10^{-9} per nucleotide per generation in bacteria and yeasts (Schroeder *et al.*, 2017; Liu and Zhang, 2019). These small mutation rates result in the generated cell mutants being the extreme minority in their surrounding populations. Induced mutagenesis occurs when the microbial cells are intentionally exposed to abnormal amounts of chemical or physical mutagens (Kodym and Afza, 2003). Chemical mutagens include alkylating agents and azides, while physical mutagens consist of electromagnetic radiation, such as γ-rays, X-rays, ultraviolet light, and particle radiation. (Kodym and Afza, 2003). The benefit of induced mutagenesis is that it allows the fast creation of a wide range of mutagen specific mutations that can be screened for their effects on the host organism metabolism and overall fitness (Singer and Grunberger, 1983).

2.4 Experimental adaptive laboratory evolution (ALE)

2.4.1 ALE is a highly versatile method

ALE is a versatile and operationally simple method in biological studies and strain optimization for biotechnical production. It provides effective means for obtaining strains with improved fitness such as tolerance to adverse growth conditions that are relevant in industrial environments (Wang *et al.*, 2023). In ALE, the population of a selected organism is subjected to long term selection under specified environment and growth conditions (Dragosits and Mattanovich, 2013; LaCroix *et al.*, 2017). This results in the selected population undergoing accumulating adaptive changes that increase its overall fitness under specified growth conditions, as natural selection only allows for the most suitable and highest fitness having individual cells to persist through multiple generations (Dragosits and Mattanovich, 2013). Additionally, ALE experiments can also give understanding to the mechanisms of molecular evolution that influence the strain phenotypes, performance, and stability (Dragosits and Mattanovich, 2013; Sandberg *et al.*, 2019). The ALE experiments vary in length, from weeks to years, and a fast-growing microorganism usually produces hundreds or thousands of generations throughout the experiment (Dragosits and Mattanovich, 2013). The most famous example of a long-lasting ALE experiment has been performed at Michigan State University by Professor Lenski and his research group. In this experiment that started in 1988, 12 initially identical populations of *E. coli* bacteria have been grown for 75 000 generations in well-controlled environment as of February 2023 (Lenski, 2023). The aim of the experiment was to assess the dynamics and repeatability of phenotypic and genetic evolution (Lenski, 2023).

2.4.2 ALE can be performed in batch and chemostat cultivations

ALE experiments are highly compatible with microorganisms due to their ease of handling and large population sizes (10^8 - 10^{10} cells) that provide large amounts of genetic diversity, which allows for mutants with beneficial traits to emerge during a long-term experiments that produce large number of cells (Sandberg *et al.*, 2019; Wang *et al.*, 2023). Generally, ALE experiments are performed in batch cultivations or continuous cultivations, depending on the aim of the experiment (LaCroix *et al.*, 2017). Both cultivation methods have their benefits and limitations. In batch cultivations, the first round of growth begins, when a small number of cells are inoculated into a fresh medium of choice. After physiologically and metabolically adjusting to the medium, the inoculated cells start growing and dividing (Gresham and Hong, 2014). Batch cultivation experiments can be completed in, for example, shake flasks or in deep well plates, that allow the creation of parallel serial cultures (Dragosits

and Mattanovich, 2013). At predetermined and regular intervals, a part of the grown culture is transferred into fresh media in another container for a new round of growth. The transfer volume that is used when inoculating the fresh media can vary significantly depending on the aim of the ALE experiment. The passage size can affect the success of the ALE experiment significantly, as too small of a passage can create bottlenecks that reduce genetic diversity, while too large passage sizes can slow down the emergence of new beneficial phenotypes by limiting their growth potential (LaCroix *et al.*, 2017). The experiment can then be continued indefinitely, or until enough generations have passed.

The largest benefit of batch cultivations is their ease of expansion through the addition of new flasks or plates as needed, and low costs since the use of complex machinery is not mandatory (Dragosits and Mattanovich, 2013; LaCroix *et al.*, 2017). Additionally, batch cultures allow the grown cells to experience all phases of microbial growth, including lag, exponential, and stationary phases, which all contribute to the selection pressure that the cells experience (Bertrand, 2019; LaCroix *et al.*, 2017). The batch cultures also allow the precise control of temperature and spatial culture homogeneity through constant mixing (Dragosits and Mattanovich, 2013). Additionally, the precise control of nutrient supply, pH, and oxygenation is possible in fed-batch cultures (Huang *et al.*, 2020). The limitations of batch cultures become apparent after the population reaches sufficient density. These limitations include varying population densities, accumulation of waste products, and fluctuations in growth rate (Dragosits and Mattanovich, 2013; Gresham and Hong, 2014). In many experiments, these variabilities are not of great importance, but when simulating complex environments, these limitations can be detrimental. Additionally, the effect of genetic drift can be significant in batch cultivations, where the serial passaging can create genetic bottlenecks through sheer coincidence (Gresham and Hong, 2014).

Continuous cultivation experiments have a similar beginning as batch cultivation experiments, where the initial culture, in a fresh medium of choice, is first started from a small number of cells. The population growth in a continuous bioreactor culture is fast at the beginning of the experiment. After the population has grown to a desired density, a flow of fresh medium is continuously supplied to the bioreactor, while an equal amount of both cells and spent medium is removed (Gresham and Hong, 2014). This supply of fresh medium allows the number of cells to increase at the same rate as they are withdrawn. This allows for constant growth rates and population densities (Dragosits and Mattanovich, 2013). Additionally, continuous bioreactor cultivation allows the precise control of nutrient supply, pH, and oxygenation (Dragosits and Mattanovich, 2013; Gresham and Hong, 2014). Typically, one essential nutrient is kept at growth-limiting concentration to maintain the correct cell density during the cultivation (Gresham and Hong, 2014). An additional benefit of using continuous bioreactor cultivation in

ALE experiments is its potential to reduce the effect of random genetic drift on the experiment, due to the lack of genetic bottlenecks that are preset during continuous cultivation process. Continuous release of cells from the used bioreactor does have a chance to dictate which genotypes are maintained in the culture, but this can be adjusted with proper flow rate adjustments (Gresham and Hong, 2014). The largest drawback of a continuous cultivation process is its large operation costs, which can significantly limit its implementation, scalability, and the number of available replicate microbial cultures. (Dragosits and Mattanovich, 2013). Additionally, in continuous cultivation processes, the grown cells are unable to experience all phases of microbial growth, which can cause growth trade-offs and reduced fitness in other phases of microbial growth (Wenger et al, 2011).

2.4.3 The selection pressure within ALE directs the attributes of the adapted strains

During the ALE, the sources of genetic variety and the following microbial evolution, are natural spontaneous mutations and artificially induced mutations (Wang *et al.*, 2023). The main attribute of all ALE experiments is the selection pressure that is imposed on the cell culture (LaCroix *et al.*, 2017). Selection pressure is responsible for directing the evolution of microbes. The selection pressure can be complex in ALE experiments, due to the various alternating environmental conditions that are present in batch cultures (LaCroix *et al.*, 2017). In batch cultures, the microbes are first subjected to nutrient rich environments that can turn to nutrient deficient environments if the fast-growing microbes are allowed to reach the carrying capacity limit of the used medium. This forces the microbes to go through various phases of growth, such as, lag phase, exponential growth, and stationary phase, which all have different dynamics between cells and the environment and thus different selection pressures (LaCroix *et al.*, 2017). The differing selection pressures also affect the way cells gain fitness, as in addition to changes in growth rate and yield, lag phase duration and stationary phase fitness also must be considered (LaCroix *et al.*, 2017). This issue is usually minimized and circumvented by transferring the batch cultures before the culture reaches stationary phase, meaning that the cells are grown mainly in exponential phase cultures that focus on fitness gained through improved growth rates (Dragosits and Mattanovich, 2013; LaCroix *et al.*, 2017). Growing cultures in constant exponential phase for prolonged times may require adjustments to the passage frequency and passage volumes, as improved growth rates reduce the time required to reach stationary phase (Sandberg *et al.*, 2019). In continuous cultivation, the growth rate of the cells and overall selection pressure can be kept constant more easily by supplying enough nutrients to achieve continuous growth (Dragosits and Mattanovich, 2013). The desired outcome of the ALE experiment dictates the optimal selection pressure that should be

imposed upon the grown cell cultures, thus highlighting the importance of experimental design. The experimental design can be complemented and assisted by using various model software and algorithms, such as ALEsim (LaCroix *et al.*, 2017) and EvolveX (Jouhten *et al.*, 2022), which allow for *in silico* testing and experimentation of various ALE parameters that lead to targeted adapted evolution. After the completion of an ALE experiment, high-throughput sequencing, bioinformatics, and genome editing technologies can be used to provide data for genomic analysis, mechanistic studies, and rational metabolic engineering (Sandberg *et al.*, 2019; Wang *et al.*, 2023).

2.4.4 ALE's role in increasing robustness and productive lifespan of engineered strains

ALE is a powerful tool that can used in combination with genomic analysis, mechanistic studies, and rational metabolic engineering (Wang *et al.*, 2023). It also has the potential of optimizing even the most complex metabolic systems through the power of natural selection, and it can be applied to almost any microbial species. Increasing the robustness of microbial strains is a common application of ALE, as large-scale industrial production can bring forth adverse growth conditions. For example, Dhar *et al.* (2011) utilized ALE to adapt *S. cerevisiae* strain to saline stress by growing three replica lineages of yeast under saline stress for 300 generations. This experiment proved to be successful, as the evolved lineages showed increased growth rates in high saline conditions when compared to their common ancestor (Dhar *et al.*, 2011). In this study, modest expression changes in several genes were observed, which were accompanied by ploidy increase in all three cell lineages, which suggests that adaptation to salt stress is associated with genome size (Dhar *et al.*, 2011). In addition to adaptation to adverse environments, ALE has also been used to increase the tolerance of microbial strains towards toxic products. For example, Mundhada *et al.* (2017) in their study, managed to increase the L-serine tolerance of *E. coli* by over tenfold by using ALE as a key method. This laboratory evolution was completed in 45 days, and the increased L-serine tolerance was created by increasing the amount of L-serine in the growth medium at regular intervals (Mundhada *et al.*, 2017).

In addition to increasing strain robustness, ALE has also been used to find novel solutions to pathway optimization. For example, Tokuyama *et al.* (2018) were able to create a knockout mutant *E. coli* strain that produced growth-coupled succinate from glycerol. This mutant strain was not able to reach the predicted production yield, and as a result, ALE was successfully used to improve the yields. ALE was noted to introduce novel mutations in the phosphoenolpyryvate carboxylate gene to reduce its kinetic limitations, which led to the improvement of succinate production (Tokuyama *et al.*, 2018). ALE has also been applied to improve the substrate

usage and the general fitness of engineered strains that suffer from defects that limit their regular metabolic functions (Wang *et al.*, 2023).

3 Research material and methods

3.1 Strains

Multiple haploid and diploid wild type and engineered strains of yeast *S. cerevisiae* were used in the ALE experiment. The strains included CEN.PK113-7D haploid wild type, CEN.PK113-7D haploid indigoidine producing, CEN.PK113-1Ax7D diploid wild type, and CEN.PK113-1Ax7D diploid indigoidine producing. Additionally, *S. cerevisiae* S288c haploid bikaverin producing strain was also used. *S. cerevisiae* CEN.PK113-7D haploid indigoidine producing and *S. cerevisiae* S288c haploid bikaverin producing strains were kindly provided by Prof. Uffe Hasbro Mårtenssen (Technical University of Denmark, Denmark). Pathway for the bikaverin synthesis had been assembled out of the previously published genes (Zhao et al., 2020). The CEN.PK113-7D haploid indigoidine producing and S288c haploid bikaverin producing strains had integrated antibiotic resistance marker for nourseothricin (NTC), while the other strains had Cas9 expressing plasmids containing the antibiotic resistance marker for G418 (Geneticin).

3.2 Cultivation media

Two different liquid media were used. The rich medium contained 10 g/L of yeast extract (Neogen), and 20 g/L of bacteriological peptone (Neogen) (YP), and synthetic defined medium contained of 6.7 g/L yeast nitrogen base without amino acids (YNB, Sigma-Aldrich). The composition of the yeast nitrogen base without amino acids was 5.0 g/L of ammonium sulfate as its nitrogen source, 2.0 µg/L of biotin, 400 µg/L of calcium pantothenate, 2.0 µg/L of folic acid, 2.0 mg/L of inositol, 400 µg/L of nicotinic acid, 200 µg/L of p-aminobenzoic acid, 400 µg/L of pyridoxine HCl, 200 µg/L of riboflavin, and 400 µg/L of thiamine HCL, as its vitamins. The trace elements consisted of 500 µg/L of boric acid, 40 µg/L of copper sulfate, 100 µg/L of potassium iodide, 200 µg/L of ferric chloride, 400 µg/L of manganese sulfate, 200 µg/L of sodium molybdate, and 400 µg/L of zinc sulfate. The salts consisted of 1.0 g/L of potassium phosphate monobasic, 0.5 g/L of magnesium sulfate, 0.1 g/L of sodium chloride, and 0.1 g/L of calcium chloride. The carbon source in both media was 30 g/L D-(+)-galactose (Sigma-Aldrich). In addition to the liquid media, solid growth media was also used. This solid growth media consisted of 10 g/L of yeast extract (Neogen), and 20 g/L of bacteriological peptone (Neogen), 20 g/L of d-glucose (VWR Chemicals), and 20 g/L of bacteriological agar (VWR Chemicals). The solid growth media used 20 g/L of d-glucose as its main carbon source to reduce the potential effect of phenotypical adaptation on the results.

3.3 Cas9 removal

Cas9 expressing plasmids were removed prior to the ALE experiment. First, the Cas9 expressing plasmid containing strains were grown overnight in 12 mL round bottom tubes containing 4 mL of YP + 20 g/L D-(+)-glucose (YPD). The cultures were inoculated with cell mass taken from multiple colonies. In the case of pigment producing strains, only pigmented colonies were taken. The caps of the 12 mL round bottom tubes were in aeration mode. In aeration mode, the tube's cap is loosely but securely attached to the tube allowing for gas exchange between the inside and the outside of the tube. The cap can also be used in closed mode, where gas exchange is highly limited or completely restricted. The broth culture incubation was done at 30 °C with 220 rpm shaking in an Innova 44 Incubator Shaker (New Brunswick). After the overnight growth, the strains were passed on to new 12 mL round bottom tubes containing 4 mL of a fresh batch of YPD media. The passage size was 4 % (160 µL), and the cultures were left to incubate from morning to evening (~ 8 h). In the evening, serial dilutions were made for each of the cultures. The used dilution factor was 1/100 000. 100 µL of the final dilution was pipetted and spread to a fresh YPD-Agar plate. The spreading was completed by using sterile L-shaped cell spreaders. These YPD-Agar plates were then incubated for 48 h at 30 °C, after which they contained 50 – 200 medium sized individual colonies. These plates were then used in replica plating as master plates. In replica plating, the individual colonies of a master plate were first inoculated into a fresh YPD-Agar plate, and then to a selection plate consisting of YPD-Agar + 200 µg/L G418. A replica plating tool manufactured by SP Bel-Art (Catalog No. F37848-0000) was used. The freshly inoculated plates were then incubated for 48 h at 30 °C. The colonies that still contained the Cas9 expressing plasmid could grow on both plates, but the colonies that have lost the plasmid could only grow on the YPD-Agar plate. Thus, the colonies that had lost the Cas9 expressing plasmid could be selected from the master plate. Approximately 30 – 50% of colonies had lost the Cas9 expressing plasmid after two transfers. Cryostocks were prepared of the Cas9 free strains in 15% glycerol and stored at -80 °C.

3.4 Adaptive laboratory evolution

The ALE experiment was performed for all the *S. cerevisiae* strains on both the rich and the synthetic defined media. At the start of the experiment, initial cultures of each strain (transfer 0) were made on both rich and synthetic defined media (i.e., a total of 10 cultures). 4 mL of rich or synthetic defined medium in 12 mL round bottom tubes was inoculated with cell mass taken from YPD-Agar plates. For the indigoidine and bikaverin producing strains,

only pigment producing colonies were used for the inoculation. Cell mass was taken from multiple different colonies to increase the initial genetic variation. The initial cultures were incubated with the tube caps in aeration mode at 30 °C with 220 rpm shaking in an Innova 44 Incubator Shaker (New Brunswick) for 2 days. Then, they were used to inoculate the transfer 1 cultures at OD 0.1 in quadruplicate. The ALE cultures from transfer 1 onwards were incubated with the tube caps in aeration mode at 30 °C with 220 rpm shaking in an Innova 44 Incubator Shaker (New Brunswick) for 2-3 days before the subsequent transfer. From the second transfer (transfer 2) onwards, the transfer volumes were 1% (40 µL) for rich media cultures, and 2% (80 µL) synthetic media cultures. For transfers 8 and 9 of haploid CEN.PK113-7D indigoidine in both yeast extract and bacteriological peptone + galactose (YPG) and yeast nitrogen base without amino acids + galactose (YNBG) media 100 mg/L ampicillin was added to remove bacterial contamination. Bacterial contamination was not observed afterwards or in any other cultures. Haploid CEN.PK113-7D wild type ALE lineages in both media, haploid CEN.PK113-7D indigoidine lineages in both media, and haploid S288c bikaverin lineages in both media were transferred 25 times in total. Diploid CEN.PK113-1Ax7D indigoidine lineages in both media were transferred 15 times in total, and diploid CEN.PK113-1Ax7D wild type lineages in both media were transferred 14 times in total.

3.5 Plate counting

The tracking color production during the ALE experiment was done by plating the pigment producing strains on YPD-Agar plates and counting the pigmented and all colonies. The plate counting was made for each replicate culture in transfers between zero to ten, and for each replicate culture in transfers 15, 20, and 25. In transfers zero to ten, and 15 the plate counting was made in single replicate, while in transfers 20 and 25 the plate counting was made in four replicates. To prepare plates where individual colonies could be counted separately, dilution series were made of each of the ALE cultures of the pigment producing strains in sterile deionized water. A dilution of 1/10 000 or 1/100 000 was used, depending on the growth medium. A lower dilution was used for some cultures on synthetic media, while the higher dilution was used for the rest of the cultures. The 1/10 000 dilution factor was used in the beginning of the ALE experiment, and it was completely replaced by the 1/100 000 dilution factor after a couple of transfers to obtain suitable numbers of colonies in plating. 100 µL of the chosen dilution was spread to an individual YPD-Agar plate. The spreading was completed by using sterile L-shaped cell spreaders. The plates were then incubated at 30 °C for 2 – 3 days depending on the growth rates of the strains. The plated dilutions resulted in 40 – 700 individual colonies depending on the strain and the

dilution used. Two – three days of incubation was not usually enough for the pigmentation to appear in case of some of the strains. For this reason, after their incubation, the plates were placed in a fridge for at least one week and a maximum of three weeks before counting pigmented and non-pigmented colonies.

3.6 Growth curve determination

Growth curve determination for all the strains in both types of media was completed by using a Synergy H1 plate reader. The pre-inoculums and the final transfer for each of the strains were chosen for this growth curve determination. The samples used in this determination were started from previously prepared cryostock, and they were grown in 12 mL round bottom tubes at 30 °C with 220 rpm shaking in an Innova 44 Incubator Shaker (New Brunswick). The 12 mL tubes contained 4 mL of either rich or synthetic media depending on the sample. The caps of the tubes were in aeration mode. The samples made from the pre-inoculum cryostock were grown for 48 h, after which they were used to prepare the necessary 96 well plate for the growth curve determination. For the final transfers, they were grown for 16 h until they were used in 96 well plate preparation. In the 96 well plate preparation, all the samples of interest were started at 0.05 OD. The 96 well plates had round bottom wells, and they were filled with 100 µL of media. Additionally, the plates were covered with "Microseal 'B' PCR Plate sealing film" manufactured by Bio-Rad, instead of using the standard hard plastic plate covers. The Synergy H1 plate reader program comprised of the following settings. The incubation temperature was 30 °C. Runtime of the program was 72 h for the pre-inoculums and 24 h for the final transfers. Absorbance measurements were taken every 15 minutes at a wavelength of 600 nm. The samples were shaken in a continuous double orbital motion, and the shaking frequency was at 807 cycles per minute (cpm). The OD600 measurements that the Synergy H1 produced were converted to corresponding spectrophotometer values by using standard curves.

3.7 Statistical significance of specific growth rates

The statistical significance of the specific growth rate changes was tested by using a *t*-test, which compared the growth rates of each final transfer to the growth rates of their respective pre-inoculum and its six replicates. The *t*-test was two-tailed and the α was set to 0.01. Additionally, equal variance was assumed between the two sets of values.

4 Results

4.1 Wild type and engineered strains adaptively evolved to grow on respirative media

An ALE experiment was performed in which the engineered yeast strains were adaptively evolved for approximately ~200 generations. More specifically, the maximum number of generations for each lineage with 25 transfers was approximated to be ~175 for synthetic media cultures, and ~200 for rich media cultures. This difference originates from the different carrying capacities of the media. The rich media cultures, on average, had a final optical density (OD) value of ~20 for yeast cells, while the synthetic media cultures had a final OD value of ~10. Maximum number of generations for lineage with 15 transfers was approximated to be ~ 105 for synthetic media cultures, and ~ 120 for rich media cultures, while the maximum number of generations for lineage with 14 transfers was approximated to be ~ 98 for rich media cultures and ~ 112 for synthetic media cultures.

In the ALE experiment, 30 g/L D-(+)-galactose was used as the main carbon source. On galactose the metabolism of *S. cerevisiae* is respirative which promotes the production of indigoidine in yeast cells (Wehrs et al., 2018). The *S. cerevisiae* CEN.PK113 strains were able to grow on galactose as their main carbon source efficiently from the very beginning of the ALE experiment, while the S288c strain required more time to adapt. *S. cerevisiae* S288c strains are gal2-, which is why they are unable to use galactose anaerobically (S288c, Yeastgenome.org). This may have resulted in the S288c strain requiring more time to adapt to the new carbon source in the beginning of the ALE experiment. Additionally, the aeration efficiency of the used incubation tubes may also have limited the aerobic galactose consumption since the exact aeration efficiency is not known. This adjustment to the new carbon source was especially noticeable in the synthetic media pre-inoculum of the S288c bikaverin producing strain. After two days of incubation, this strain had an OD value of 0.5, and after five days an OD value of 3.2, which was then used in the first transfer. From the first transfer onward, this strain grew at the same pace as the other studied strains. This quick adaptation suggested that *S. cerevisiae* S288c growth on galactose was initially improved through phenotypic mechanisms.

Growth rates and pigment production of the *S. cerevisiae* lineages were tracked during the ALE experiment. Plate counting of pigmented and wild type kind of colonies was used as the main method to follow pigmentation loss, and it was done to transfers zero to ten, 15, 20, and 25. Growth rate measurements were performed for the pre-inoculums, and the last transfers for each of the yeast lineages. This gave a concise look at how

the strains have adapted and evolved during the ALE experiment. The workflow of the ALE experiment is presented in Figure 1.

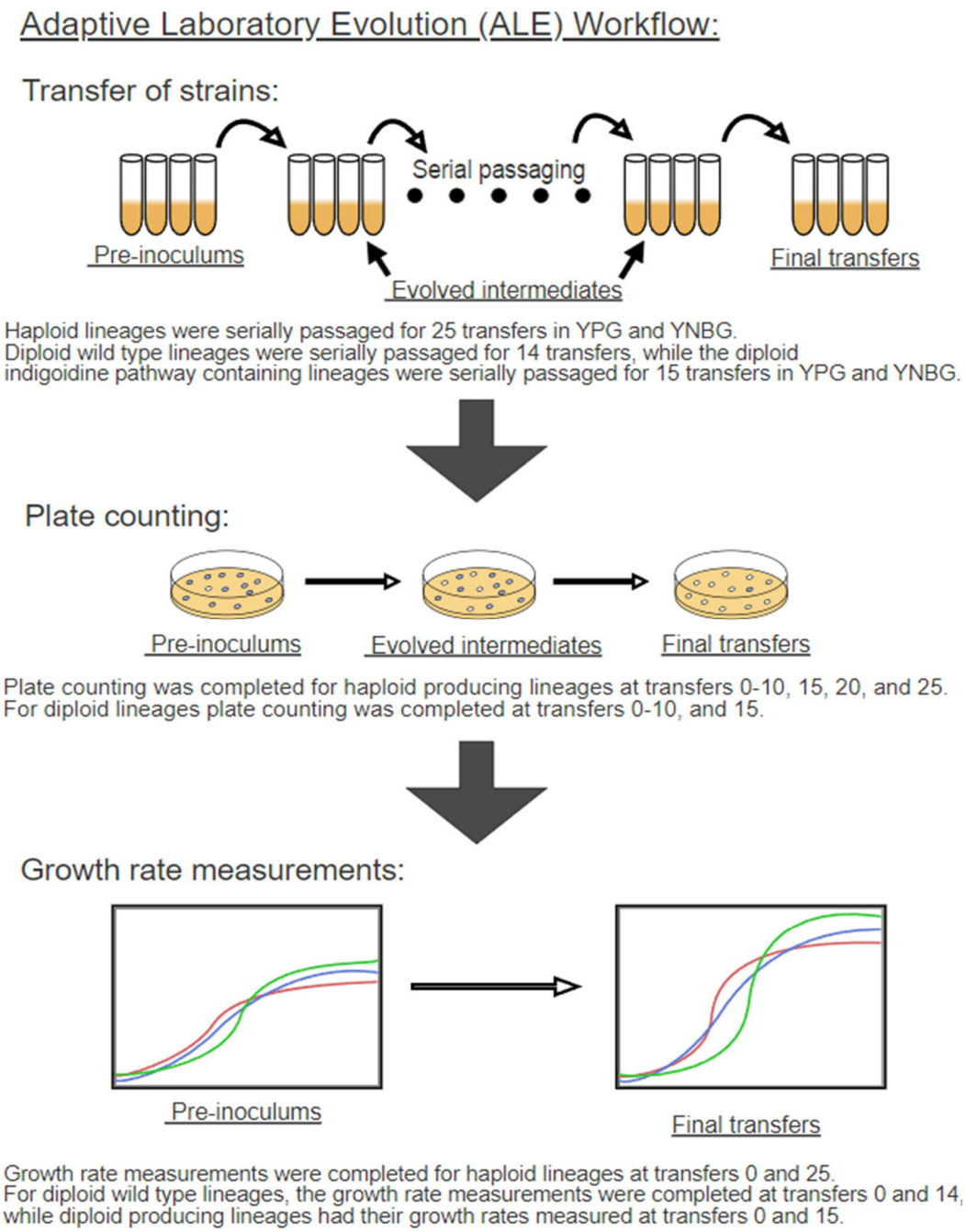

Figure 1. Adaptive laboratory evolution workflow. Wild type and producing haploid lineages were serially passaged for 25 transfers. Diploid wild type lineages were passaged for 14 transfers, while the diploid indigoidine producing lineages were passaged for 15 transfers. Plate counting samples from the producing lineages were taken at predetermined time points (transfers zero to ten, 15, 20, and 25). The growth rate measurements were completed for the pre-inoculums and the final transfers of each lineage.

4.2 Adaptation of growth rates

Growth rates of the engineered yeast strains generally improved during the adaptive evolution experiment. The changes in growth rates were observed by subjecting the pre-inoculums (transfer 0) and the final transfer of each cell lineage to growth curve determination by plate reader measurements. The OD600 values measured by the plate reader were converted to corresponding OD600 spectrophotometer values, by using standard curves that were specific for each strain and media combination. The pre-inoculums had six duplicate samples, while the final transfers had four duplicate samples. For each of the lineages, growth curves were formed for all their duplicates. From the produced growth curves, five datapoints were selected and used to calculate the highest exponential growth rate for each of the cell lineages by fitting the datapoints to an exponential curve. Both wild type and pathway containing strains had their average growth rates measured (Tables 1 - 5). In addition to growth rates, the growth curve determination also provided insight to the lag phases of the cell lineages. Generally, the lag phases were observed to be shorter in the adapted lineages. This reduction in lag phase was especially apparent in synthetic media (YNBG) grown lineages.

4.2.1 Haploid wild type and indigoidine producing strains

The specific growth rates in adaptively evolving lineages for the *S. cerevisiae* CEN.PK113-7D haploid wild type strain and CEN.PK113-7D haploid indigoidine pathway containing strain saw varying degrees of change during the ALE. Surprisingly, half of the haploid wild type lineages grown on YPG had reduced growth rates, while the remaining half had a minimal increase in their growth rates (Table 1). The wild type lineages grown on YNBG saw improvement of at least 23% in their growth rates (Table 2). The lineages of indigoidine pathway containing strain saw growth rate increase in both types of media. All the YPG grown lineages saw at least 34% increase (Table 1), while the YNBG grown lineages had a minimum of 58% increase (Table 2).

Table 1. Specific growth rates of YPG grown *S. cerevisiae* CEN.PK113-7D haploid wild type lineages (WT-H-YPG) and CEN.PK113-7D haploid indigoidine pathway containing lineages (IND-H-YPG). The percentual change value represents the difference between the pre-inoculum growth rate and the adapted lineages growth rates. Asterisk indicates the statistical significance (* = *P*-value ≤ 0.01)

Lineage	Growth rate (h^{-1})	Change (%)
WT-H-YPG-0	0.517 ± 0.011	n/a
WT-H-YPG-25-1	0.313 ± 0.008	-39.3
WT-H-YPG-25-2	0.460 ± 0.007	-10.9
WT-H-YPG-25-3	0.522 ± 0.011	1.1
WT-H-YPG-25-4	0.549 ± 0.008	6.3
IND-H-YPG-0*	0.195 ± 0.009	n/a
IND-H-YPG-25-1*	0.263 ± 0.010	34.5
IND-H-YPG-25-2*	0.266 ± 0.003	36.2
IND-H-YPG-25-3*	0.271 ± 0.018	38.7
IND-H-YPG-25-4*	0.287 ± 0.018	46.9

The growth rates of the adapted *S. cerevisiae* CEN.PK113-7D haploid wild type strain saw varying degrees of change in all four replica lineages that were grown on YPG (Table 1). The first replica lineage saw unexpectedly high decrease of -39% in its growth rate, which is highly unusual, as in ALE experiments, the specific growth rate of the cells tends to increase with fitness as the experiment progresses. In addition to the first lineage, the second lineage also had quite noticeable decrease of -10% in its growth rate. The third and fourth replica lineages had minimal increases in their respective growth rates. The lag phases of these lineages were observed to decrease during the ALE. The pre-inoculum sample had a lag phase of approximately 6.5 h, while the final transfers had lag phase lengths that ranged from 3.5 h to 6 h. The statistical significance of the specific growth rate results was tested by using a two-tailed *t*-test, which assumed equal variance. The haploid *S. cerevisiae* CEN.PK113-7D wild type strain grown on YPG was the only strain that was unable to reject the null hypothesis (*P*-value 0.34 > 0.01).

The growth rates of the adapted *S. cerevisiae* CEN.PK113-7D haploid indigoidine pathway containing strain saw increase in all four replica lineages that were grown on YPG (Table 1). The highest increase in growth rate was measured in the fourth replica lineage that had a 46% increase when compared to the growth rate of the pre-inoculum. The lag phases of the final transfer lineages were observed to be shorter than the pre-inoculum's lag phase. The pre-inoculum sample had a lag phase of approximately 8.5 h, while the final transfers had lag phase lengths that ranged from 7 h to 8 h.

Table 2. Specific growth rates of YNBG grown *S. cerevisiae* CEN.PK113-7D haploid wild type lineages (WT-H-YNBG) and CEN.PK113-7D haploid indigoidine pathway containing lineages (IND-H-YNBG). The percentual change value represents the difference between the pre-inoculum growth rate and the adapted lineages growth rates. Asterisk indicates the statistical significance (* = *P*-value ≤ 0.01)

Lineage	Growth rate (h^{-1})	Change (%)
WT-H-YNBG-0*	0.140 ± 0.008	n/a
WT-H-YNBG-25-1*	0.208 ± 0.012	48.9
WT-H-YNBG-25-2*	0.172 ± 0.013	22.9
WT-H-YNBG-25-3*	0.191 ± 0.011	36.4
WT-H-YNBG-25-4*	0.172 ± 0.007	23.1
IND-H-YNBG-0*	0.123 ± 0.020	n/a
IND-H-YNBG-25-1*	0.195 ± 0.012	58.9
IND-H-YNBG-25-2*	0.203 ± 0.030	65.3
IND-H-YNBG-25-3*	0.237 ± 0.009	92.8
IND-H-YNBG-25-4*	0.198 ± 0.010	61.4

The growth rate of the adapted *S. cerevisiae* CEN.PK113-7D haploid wild type strain and CEN.PK113-7D haploid indigoidine pathway containing strain saw an increase in all lineages grown on YNBG (Table 2). For the wild type strain, the highest increase in growth rate was observed in the first replica lineage, which saw an increase of 48% when compared to the growth rate of the wild type pre-inoculum. In addition to the increased growth rates, the final transfer lineages also had reduced lag phase lengths when compared to the pre-inoculum's lag phase. The pre-inoculum had a lag phase of approximately 9 h, while the final transfers had lag phase lengths that ranged from 7 h to 8 h. For the indigoidine pathway containing strain, the highest growth rate increase was observed in the third replica lineage, which had a 92.8% increase in its growth rate when compared to the pre-inoculum's growth rate. In addition to the fourth lineage, the other lineages also had increase in their growth rates. The final transfer lineages had decrease in their lag phase lengths when compared to the pre-inoculum's lag phase. The pre-inoculum had a lag phase of approximately 15 h, while the final transfers had lag phase lengths that ranged from 7.5 h to 8.5 h.

4.2.2 Diploid wild type and indigoidine producing strains

The growth rates in adaptively evolving lineages for the *S. cerevisiae* CEN.PK113-1Ax7D diploid wild type strain and CEN.PK113-1Ax7D diploid indigoidine pathway containing strain increased notably during the ALE. The diploid wild type lineages experienced high growth rate increase in both YPG

media (Table 3), and YNBG media (Table 4). This was the same for the indigoidine pathway containing lineages, which had a minimum of 27% increase in YPG (Table 3), and 26% increase in YNBG (Table 4).

Table 3. Specific growth rates of YPG grown *S. cerevisiae* CEN.PK113-1Ax7D diploid wild type lineages (WT-D-YPG) and CEN.PK113-1Ax7D diploid indigoidine pathway containing lineages (IND-D-YPG). The percentual change value represents the difference between the pre-inoculum growth rate and the adapted lineages growth rates. Asterisk indicates the statistical significance (* = *P*-value ≤ 0.01)

Lineage	Growth rate (h^{-1})	Change (%)
WT-D-YPG-0*	0.207 ± 0.021	n/a
WT-D-YPG-14-1*	0.312 ± 0.007	51.1
WT-D-YPG-14-2*	0.316 ± 0.009	53.1
WT-D-YPG-14-3*	0.320 ± 0.009	54.8
WT-D-YPG-14-4*	0.277 ±0.023	34.3
IND-D-YPG-0*	0.220 ± 0.012	n/a
IND-D-YPG-15-1*	0.293 ± 0.005	32.8
IND-D-YPG-15-2*	0.289 ± 0.008	30.9
IND-D-YPG-15-3*	0.303 ± 0.005	37.5
IND-D-YPG-15-4*	0.280 ± 0.006	27.1

The growth rates of the adapted *S. cerevisiae* CEN.PK113-1Ax7D diploid wild type strain increased in all four replica lineages that were grown on YPG (Table 3). The fourth replica lineage had the lowest increase of 34%, while the other lineages had over 50% increase in their growth rates. The lag phase length of these adapted lineages was the same as the pre-inoculum's lag phase length of 7 h. For the YPG grown indigoidine pathway containing diploid strain, the growth rates increased in a more moderate manner (Table 3). The lowest increase of 27% was measured in the fourth replica lineage, while the highest increase of 37% was measured in the third lineage. In addition to growth rate improvements, the lag phase length of the adapted lineages had shortened during the ALE. The pre-inoculum had a lag phase length of 8.5 h, while the final transfers had a lag phase length of only 7 h.

Table 4. Specific growth rates of YNBG grown *S. cerevisiae* CEN.PK113-1Ax7D diploid wild type lineages (WT-D-YNBG) and CEN.PK113-1Ax7D diploid indigoidine pathway containing lineages (IND-D-YNBG). The percentual change value represents the difference between the pre-inoculum growth rate and the adapted lineages growth rates. Asterisk indicates the statistical significance (* = *P*-value ≤ 0.01)

Lineage	Growth rate (h^{-1})	Change (%)
WT-D-YNBG-0*	0.167 ± 0.018	n/a
WT-D-YNBG-14-1*	0.255 ± 0.006	53.2
WT-D-YNBG-14-2*	0.253 ± 0.026	52.7
WT-D-YNBG-14-3*	0.221 ± 0.014	32.7
WT-D-YNBG-14-4*	0.190 ± 0.012	13.7
IND-D-YNBG-0*	0.177 ± 0.010	n/a
IND-D-YNBG-15-1*	0.225 ± 0.018	26.9
IND-D-YNBG-15-2*	0.251 ± 0.016	41.8
IND-D-YNBG-15-3*	0.234 ± 0.013	32.1
IND-D-YNBG-15-4*	0.224 ± 0.008	26.1

The growth rates of the adapted *S. cerevisiae* CEN.PK113-1Ax7D diploid wild type strain increased in all four replica lineages that were grown on YNBG (Table 4). The growth rate increase had quite a large variation between the wild type lineages, as the lowest increase of 13% was in the fourth lineage, and the highest increase of 53% was in the first lineage. The diploid wild type lineages had shorter lag phase than the wild type pre-inoculum. The lag phase of these lineages varied from 6 h to 6.5 h, while the pre-inoculum had a lag phase of 7 h in length. For the diploid indigoidine pathway containing strain grown on YNBG, the replica lineages had increased growth rates when compared to the indigoidine pre-inoculum (Table 4). The growth rate increase was 26% in replica lineages one and four, while lineages two and three had over 32% and 41% increase, respectively. In addition to general growth rate improvements, the lag phase length of the adapted diploid indigoidine lineages had shortened during the ALE. The YNBG grown final transfers had 6.5 h lag phase, while the indigoidine pre-inoculum had a lag phase of 16 h.

4.2.3 Haploid bikaverin producing strain

The growth rates in adaptively evolving lineages for the *S. cerevisiae* S288c bikaverin pathway containing strain increased in both media types during the ALE (Table 5). The YPG grown bikaverin pathway containing lineages had their growth rates increase by at least 35% when compared to their pre-inoculum's growth rate. At the beginning of the ALE experiment, the YNBG grown bikaverin pathway containing strain had difficulties with the used

galactose carbon source, due to their gal2- nature. This resulted in the YNBG grown pre-inoculum having poor growth and extremely long lag phase, which made the formation of proper standard curves challenging. Due to this, the microplate reader OD600 values were not converted into their corresponding spectrophotometer values. Additionally, the measured growth rate of the YNBG grown bikaverin pre-inoculum was minimal, which is why the comparison between the growth rates of the adapted lineages and the growth rate of the pre-inoculum was not made. The YPG grown S288c bikaverin pathway containing strain did not have similar issues as the YNBG grown strain.

Table 5. Specific growth rates of YPG and YNBG grown *S. cerevisiae* S288c bikaverin pathway containing lineages (BIK-S288c-YPG and BIK-S288c-YNBG). The percentual change value represents the difference between the pre-inoculum growth rate and the adapted lineages growth rates.

Lineage	Growth rate (h^{-1})	Change (%)
BIK-S288c-YPG-0*	0.210 ± 0.005	n/a
BIK-S288c-YPG-25-1*	0.311 ± 0.017	47.6
BIK-S288c-YPG-25-2*	0.294 ± 0.008	39.7
BIK-S288c-YPG-25-3*	0.323 ± 0.010	53.6
BIK-S288c-YPG-25-4*	0.286 ± 0.009	35.9
BIK-S288c-YNBG-0*	0.009 ± 0.008	
BIK-S288c-YNBG-25-1*	0.149 ± 0.013	
BIK-S288c-YNBG-25-2*	0.148 ± 0.003	
BIK-S288c-YNBG-25-3*	0.188 ± 0.002	
BIK-S288c-YNBG-25-4*	0.178 ± 0.006	

The growth rates of the adapted *S. cerevisiae* S288c bikaverin pathway containing strain increased in all four replica lineages that were grown on YPG (Table 5). The maximum amount of increase was in the third replicate lineage, where the growth rate increased by 53%. The lag phase of the YPG grown lineages decreased during the ALE. The pre-inoculum of the YPG grown lineages had a lag phase of eight hours, while the adapted lineages had lag phases that were seven hours in duration. The YNBG grown S288c bikaverin pathway containing lineages also saw an increase in their growth rates as they adapted to using galactose during the ALE experiment. The lag phases of the YNBG grown lineages were between 8 and 11 hours in duration.

4.3 Proportion of pigment producing clones declined during the ALE experiment

The proportion of pigment producing clones was assessed by performing plate counting of pigmented colonies during the ALE experiment. The colonies were visually classified after one week of incubation as producing or non-producing when strongly pigmented or like wild type colonies, respectively (Figure 2). Generally, it was observed that the proportion of pigment producing clones was reduced during the ALE experiment. In just 15 transfers (~105 to 120 generations) all the lineages of indigoidine pathway containing strain had at least 47% reduction in the proportion of pigmented clones (Tables 6 - 9). In the same number of transfers, the YPG grown lineages of bikaverin pathway containing strain had a reduction of at least 29% (Table 10), while the YNBG grown lineages had almost no reduction (Table 11). This stark contrast between the YPG and YNBG grown lineages of bikaverin pathway containing strain suggested that the used growth medium had an influence on the loss of pigment production. In addition to the change in the proportion of producing clones, the color intensity of the producing colonies was also observed to reduce during the ALE experiment (Figures 2 - 6). The only outlier was the YNBG grown lineages of bikaverin pathway containing strain, which had more stable pigment intensity throughout the ALE experiment (Figure 7).

4.3.1 Proportion of indigoidine producing *S. cerevisiae* clones declined in ALE depending on the medium

The proportion of pigmented clones in adaptively evolving lineages for the *S. cerevisiae* CEN.PK113-7D haploid indigoidine producing strain and CEN.PK113-1Ax7D diploid indigoidine producing strain saw decline during the ALE in both YPG and YNBG. The indigoidine production loss of CEN.PK113-7D haploid indigoidine producing strain was more gradual in the YPG grown lineages (Table 6), while in YNBG lineages the production loss was more sudden (Table 7). In both media types, the individual lineages had noticeable variation when compared to each other. Some lost their pigment production capabilities faster than others. In both cases, the pigment production was completely lost at the end of the ALE experiment (i.e., in ~175 - 200 generations).

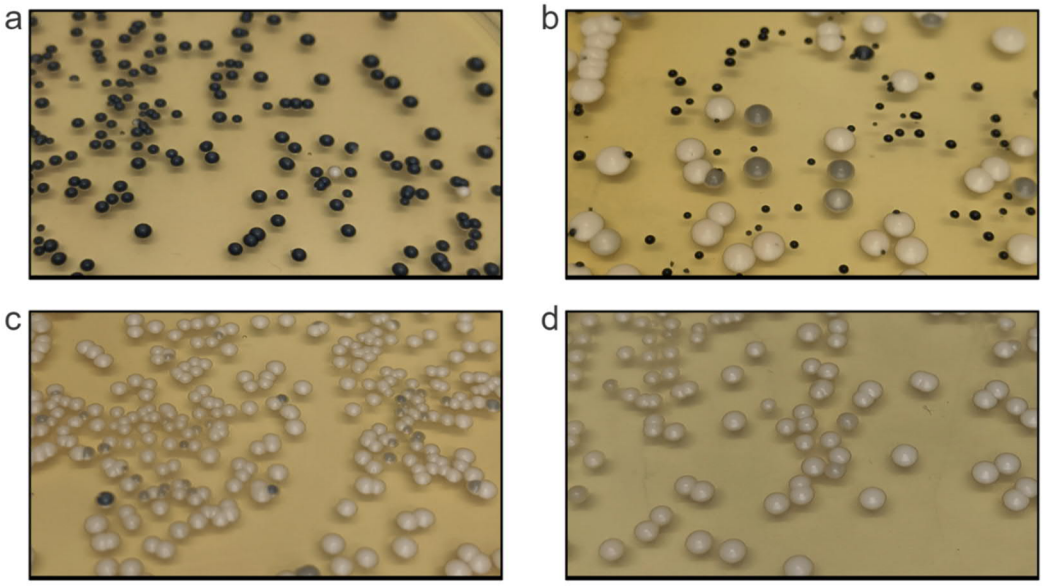

Figure 2. Representative examples of ALE lineages of YPG grown *S. cerevisiae* CEN.PK113-7D Indigoidine pathway containing strain plated on YPD at transfers a) 0, b) 10, C) 20, and d) 25. Examples for the transfers 10, 15, and 25 were taken from the first replicate lineage.

Transfer zero of the YPG grown haploid *S. cerevisiae* CEN.PK113-7D indigoidine pathway containing strain had two different clone phenotypes present of which most were pigment producing, and only a few were non-producing (Figure 2 (a)). For the first replicate lineage of this strain, the proportion of pigmented clones had changed drastically by the tenth transfer, where three major clone phenotypes were present (Figure 2 (b)). The most abundant clones were small and intensely pigmented, while the second most common clones were large and non-producing. The third phenotype consisted of large and lightly pigmented clones that had a limited presence. By transfer 20, the proportion of pigmented clones had changed notably when compared to previous transfers. Transfer 20 had two clone phenotypes present of which non-producing clones made up the majority by a considerable amount (Figure 2 (c)). The minority of the clones consisted only of lightly pigmented individuals, meaning that the intensely pigmented clones had completely ceased to exist. The trend of decline in the proportion of pigmented clones continued in the following transfers. By transfer 25, the population consisted completely of non-producing clones (Figure 2 (d)).

Table 6. Proportion of pigment producing clones in four replicate lineages of YPG grown haploid *S. cerevisiae* CEN.PK113-7D indigoidine pathway containing strain during the ALE.

Transfer	1. Replicate Producing %	2. Replicate Producing %	3. Replicate Producing %	4. Replicate Producing %
0	97.54	97.54	97.54	97.54
1	100.00	100.00	100.00	100.00
2	97.31	98.20	N/A	97.08
3	100.00	97.84	100.00	100.00
4	93.75	51.64	97.18	96.88
5	96.62	71.86	98.01	95.72
6	94.41	19.84	96.40	86.99
7	97.89	25.95	93.56	57.69
8	90.05	16.79	75.31	27.94
9	83.33	16.21	61.98	11.26
10	67.07	1.90	44.74	2.92
15	9.63	5.88	15.79	3.88
20*	9.25	3.78	9.48	1.67
25*	0	0	0	0

The replica lineages of YPG grown haploid *S. cerevisiae* CEN.PK113-7D Indigoidine pathway containing strain had high amounts of variation between their production stability during the ALE (Table 6). For the first three transfers, the lineages had over 90% proportion of pigmented clones. The first lineage to undergo drastic change in its proportion of pigmented clones was the second replica lineage. At transfer four, the second lineage had only 52% proportion of pigmented clones remaining. The next noticeable variation occurred in the fourth replica lineage, which had 58% proportion of pigmented clones remaining at transfer seven. Replica lineages one and three saw noticeable decline in their proportion of pigmented clones from transfer eight onwards. At transfer 25, all four replica lineages had completely lost their pigmented clones.

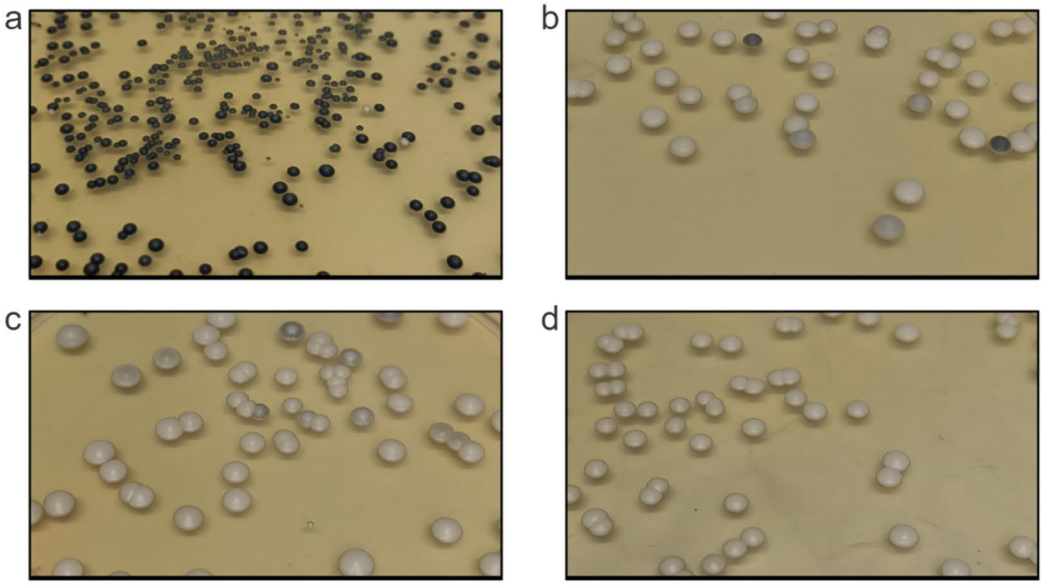

Figure 3. Representative examples of ALE lineages of YNBG grown *S. cerevisiae* CEN.PK113-7D Indigoidine pathway containing strain plated on YPD at transfers a) 0, b) 10, C) 20, and d) 25. Examples for the transfers 10, 20, and 25 were taken from the second replicate lineage.

Transfer zero of the YNBG grown haploid *S. cerevisiae* CEN.PK113-7D indigoidine pathway containing strain had two different clone phenotypes present (Figure 3 (a)). Most of the clones were intensely producing, while few of the clones were non-producing. For the second lineage of this strain, the proportion of pigmented clones saw notable decline in only ten transfers (Figure 3 (b)). Transfer ten contained three different clone phenotypes, of which non-producing clones were the most numerous, while the rest of the clones were either lightly or intensely pigmented. By transfer 20, the number of phenotypes present had reduced to two (Figure 3 (c)). Out of these two phenotypes, the non-producing clones were the majority, while the lightly pigmented clones were the minority. The decline in the proportion of pigmented clones continued in the subsequent transfers, and by transfer 25, the population consisted completely of non-producing clones (Figure 3 (d)).

Table 7. Proportion of pigment producing clones in four replicate lineages of YNBG grown haploid *S. cerevisiae* CEN.PK113-7D indigoidine pathway containing strain during the ALE.

Transfer	1. Replicate Producing %	2. Replicate Producing %	3. Replicate Producing %	4. Replicate Producing %
0	99.04	99.04	99.04	99.04
1	100.00	99.52	100.00	98.70
2	93.15	100.00	98.90	98.32
3	97.47	98.35	90.57	99.15
4	77.78	37.50	21.43	73.68
5	62.41	89.25	15.56	50.00
6	12.57	5.64	6.02	7.35
7	0.00	0.00	0.00	11.11
8	7.59	3.66	4.94	2.86
9	4.11	6.25	3.41	2.90
10	0.00	1.39	2.15	0.00
15	1.12	2.78	1.43	6.32
20*	3.38	5.56	2.16	1.43
25*	0.00	0.00	0.00	0.00

Similarly to the YPG grown version, the replica lineages of YNBG grown haploid *S. cerevisiae* CEN.PK113-7D Indigoidine pathway containing strain had high amounts of variation between their production stability during the ALE (Table 7). At transfer three, all replicate lineages had over 90% proportion of pigmented clones remaining. This proportion saw a fast decline in the following transfers, and at transfer six, all replicates had under 13% proportion of pigmented clones remaining. After transfer six, the decline slowed down notably, and only at transfer 25, the proportion of pigmented clones was zero in all replica lineages.

The proportion of pigmented clones in adaptively evolving lineages of the diploid *S. cerevisiae* CEN.PK113-1Ax7D indigoidine producing strain saw notable decline in just 15 transfers. Generally, substantial pigmentation loss was observed in the YPG grown lineages during the later stages of the experiment (Table 8), while YNBG grown lineages experienced similar loss at earlier stages (Table 9). In both media, there was notable variation between the replicate lineages. Some lineages lost their pigmentation more suddenly, while others had a more gradual decline. At the end of the experiment, the indigoidine production was completely lost in only one replicate lineage, whilst most of the other lineages had over 90% reduction in pigmentation.

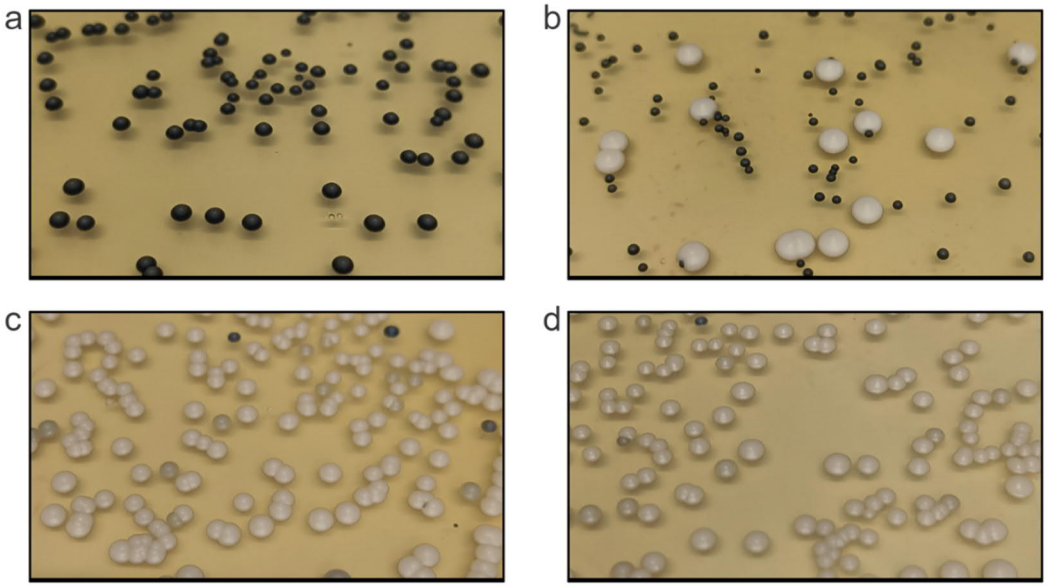

Figure 4. Representative examples of ALE lineages of YPG grown *S. cerevisiae* CEN.PK113-1Ax7D Indigoidine pathway containing strain plated on YPD at transfers a) 0, b) 5, C) 10, and d) 15. Examples for the transfers 5, 10, and 15 were taken from the second replicate lineage.

Initially at the beginning of the ALE, the transfer zero of the diploid *S. cerevisiae* CEN.PK113-1Ax7D indigoidine pathway containing strain had two clone phenotypes present (Figure 4 (a)). The majority of clones were intensely pigmented, while only a small fraction of the clones was non-producing. For the second replicate lineage of this strain, the proportion of pigmented clones had changed noticeably by the fifth transfer (Figure 4 (b)). In the fifth transfer, the non-producing clones had a notable rise in their number, while the proportion of pigmented clones was reduced. By transfer ten, the proportion of non-producing clones had continued to increase even further (Figure 4 (c)). In transfer ten, the non-producing clones were the majority phenotype, while lightly and intensely pigmented clones were in the minority. The trend of decline in the proportion of pigmented clones continued in the subsequent transfers, and by transfer 15, the pigmented phenotypes were present in even smaller proportions (Figure 4 (d)).

Table 8. Proportion of pigment producing clones in four replicate lineages of YPG grown diploid *S. cerevisiae* CEN.PK113-1Ax7D indigoidine pathway containing strain during the ALE.

Transfer	1. Replicate Producing %	2. Replicate Producing %	3. Replicate Producing %	4. Replicate Producing %
0	99.40	99.40	99.40	99.40
1	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00
3	100.00	100.00	100.00	100.00
4	100.00	98.48	100.00	87.22
5	100.00	86.99	100.00	20.00
6	100.00	58.14	100.00	12.44
7	100.00	17.62	98.39	13.57
8	98.99	14.38	98.15	7.45
9	98.19	8.66	96.25	7.30
10	97.42	8.26	94.74	10.50
15	2.13	1.22	52.47	1.99

The indigoidine production stability of the YPG grown diploid *S. cerevisiae* CEN.PK113-1Ax7D indigoidine pathway containing strain declined throughout the ALE (Table 8). Replica lineages one and three were more conservative in their loss of pigmented clones, while in comparison, lineages two and four saw faster and larger pigmentation loss. Replica lineage four was the first lineage to lose over half of its pigmented clones. At transfer four, the pigmented clones made up 87% of the whole population, while a transfer later, the proportion was only 20%. Replica lineage two was the second lineage to lose more than half of its pigmented clones and at transfer seven, the proportion of pigmented clones for it was under 18%. In contrast to lineages two and four, replica lineages one and three were able to hold their proportion of pigmented clones higher for far longer. At transfer ten, lineages one and three had over 94% proportion of pigmented clones remaining. At transfer 15, none of the replicate lineages had completely lost their pigmented clones. Lineages one, two, and four had their proportion of pigmented clones at approximately 2%, while lineage three had 52% proportion remaining.

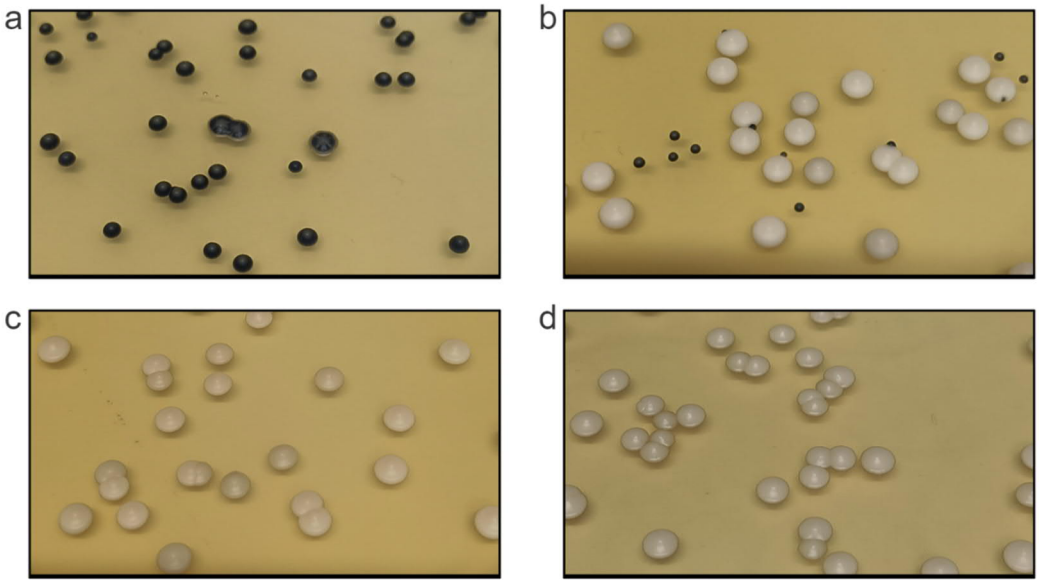

Figure 5. Representative examples of ALE lineages of YNBG grown *S. cerevisiae* CEN.PK113-1Ax7D Indigoidine pathway containing strain plated on YPD at transfers a) 0, b) 5, C) 10, and d) 15. Examples for the transfers 5, 10, and 15 were taken from the second replicate lineage.

The transfer zero of YNBG grown diploid *S. cerevisiae* CEN.PK113-1Ax7D Indigoidine pathway containing strain contained only intensely pigmented clones (Figure 5 (a)). The proportion of pigmented clones reduced considerably in only five transfers. The fifth transfer of the second lineage contained two clone phenotypes (Figure 5 (b)). Most of the clones were large and non-producing, while small and intensely pigmented clones made up the rest of the population. The decline in the proportion of pigmented clones continued in the subsequent transfers. At transfer ten, the clonal population consisted purely of non-pigmented clones (Figure 5 (c)). In addition to the tenth transfer, transfer 15 also contained only non-pigmented clones (Figure 5 (d)).

Table 9. Proportion of pigment producing clones in four replicate lineages of YNBG grown diploid *S. cerevisiae* CEN.PK113-1Ax7D Indigoidine pathway containing strain during the ALE.

Transfer	1. Replicate Producing %	2. Replicate Producing %	3. Replicate Producing %	4. Replicate Producing %
0	100.00	100.00	100.00	100.00
1	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00
3	97.50	97.96	100.00	97.30
4	84.09	81.82	85.11	67.74
5	32.56	44.07	70.73	4.44
6	25.58	17.24	43.33	2.04
7	10.17	9.26	3.77	6.25
8	7.69	4.35	12.50	16.13
9	8.82	3.23	5.13	2.17
10	9.80	0.00	10.42	0.00
15	14.52	0.00	3.70	5.00

For the first three transfers of the YNBG grown diploid *S. cerevisiae* CEN.PK113-1Ax7D Indigoidine pathway containing strain, all four replicate lineages had over 97% proportion of pigmented clones remaining (Table 9). The first lineage to see large change was lineage four, which by transfer four, had only a 68% proportion of producing clones remaining. By transfer five, the fourth lineage had only 4% proportion remaining. The next lineages to see large drop in their pigmented populations were lineages one and two, which had only 33% and 44% of their pigmented populations remaining by transfer five. By transfer six, the third replica lineage had also lost over 50% of its pigmented population. By transfer 15, all the lineages had under 15% proportion of pigmented clones remaining. The only lineage to reach 0% by the end of the ALE was the second replica lineage.

4.3.2 Proportion of bikaverin producing *S. cerevisiae* clones remained more stable when compared to indigoidine producing clones during the ALE

The proportion of pigmented clones in adaptively evolving lineages for the *S. cerevisiae* S288c bikaverin producing strain saw higher stability than indigoidine producing clones during the ALE. The bikaverin production loss was more consistent and gradual in the YPG grown lineages (Table 10), while in the YNBG grown lineages, only half of the replicates lost pigmentation during the ALE (Table 11). Initially, the YNBG grown lineages had little variation between one another and the pigmentation remained highly stable in all

lineages (> 98 % pigmentation). However, at transfers 20 and 25, the pigmentation started to decline in two of the lineages, which resulted in over 93 % pigmentation loss. The replica lineages that were grown on YPG had some variation between each other, but it did not reach the level of the YNBG grown replicates. At the end of the experiment, only one YNBG replicate lineage had completely lost its bikaverin production. All the other replica lineages, in both media types, had at least some bikaverin production remaining.

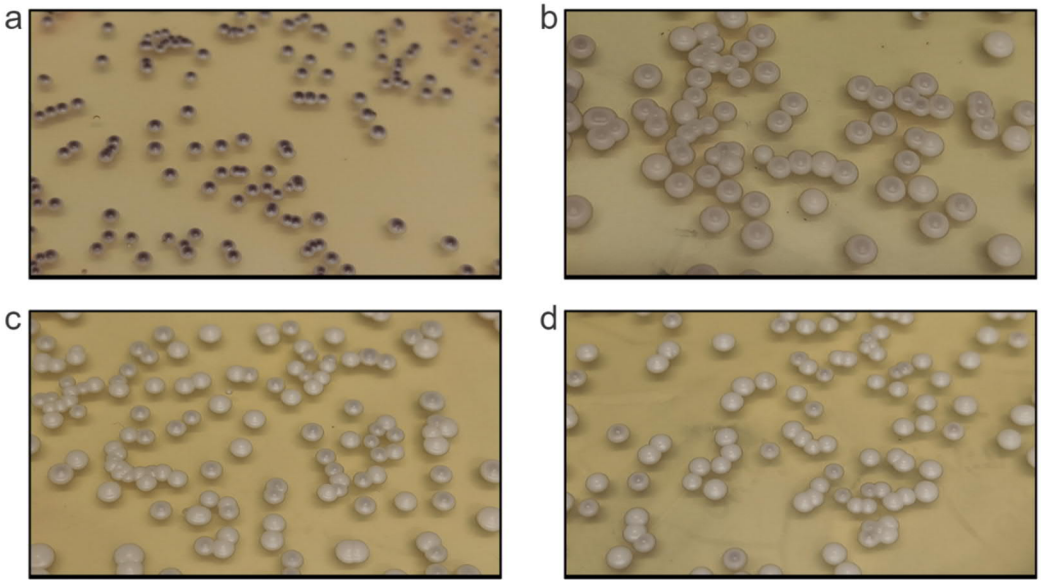

Figure 6. Representative examples of ALE lineages of YPG grown *S. cerevisiae* S288c Bikaverin pathway containing strain plated on YPD at transfers a) 0, b) 5, C) 15, and d) 25. Examples for the transfers 5, 15, and 25 were taken from the third replicate lineage.

The proportion of pigmented clones for the YPG grown *S. cerevisiae* S288c Bikaverin pathway containing strain saw noticeable change during the ALE (Figure 6). Intensely pigmented clones were the only phenotype present in the transfer zero sample (Figure 6 (a)), but in just few transfers, their proportion started to decline. By transfer five of the third replica lineage, the intensely producing phenotype had transformed into two main clone phenotypes (Figure 6 (b)). The more common phenotype of these two were lightly pigmented clones, while non-producing clones were present in smaller numbers. In the subsequent transfers, the proportion of pigmented clones had reduced even further. By transfer 15, the lightly pigmented clones and non-producing clones were almost evenly distributed throughout the sample (Figure 6 (c)). The proportion of pigmented clones continued to decline, and by transfer 25, they made up the minority of the population while non-producing clones were the majority (Figure 6 (d)).

Table 10. Proportion of pigment producing clones in four replicate lineages of YPG grown haploid *S. cerevisiae* S288c bikaverin pathway containing strain during the ALE.

Transfer	1. Replicate Producing %	2. Replicate Producing %	3. Replicate Producing %	4. Replicate Producing %
0	100.00	100.00	100.00	100.00
1	98.46	99.66	95.17	98.29
2	91.04	90.86	79.90	83.52
3	88.15	83.83	78.85	82.58
4	81.82	72.60	78.30	80.60
5	100.00	94.12	78.49	74.68
6	84.92	82.63	73.36	77.52
7	76.00	82.14	59.46	100.00
8	78.35	77.78	75.51	73.76
9	82.81	75.98	78.72	74.85
10	81.16	56.69	67.28	27.10
15	70.92	58.76	50.48	59.80
20*	58.88	52.64	42.82	45.47
25*	45.70	37.99	32.03	39.02

The YPG grown *S. cerevisiae* S288c Bikaverin pathway containing strain had its proportion of pigmented clones reduce gradually during the ALE (Table 10). By transfer nine, all four replica lineages had over 74% proportion of pigmented clones remaining. The proportion of pigmented clones remained quite stable, and by transfer 15, all the lineages still had over 50% proportion remaining. This stability and slow decline continued, and by transfer 25, all lineages had over 32% proportion of pigmented clones remaining.

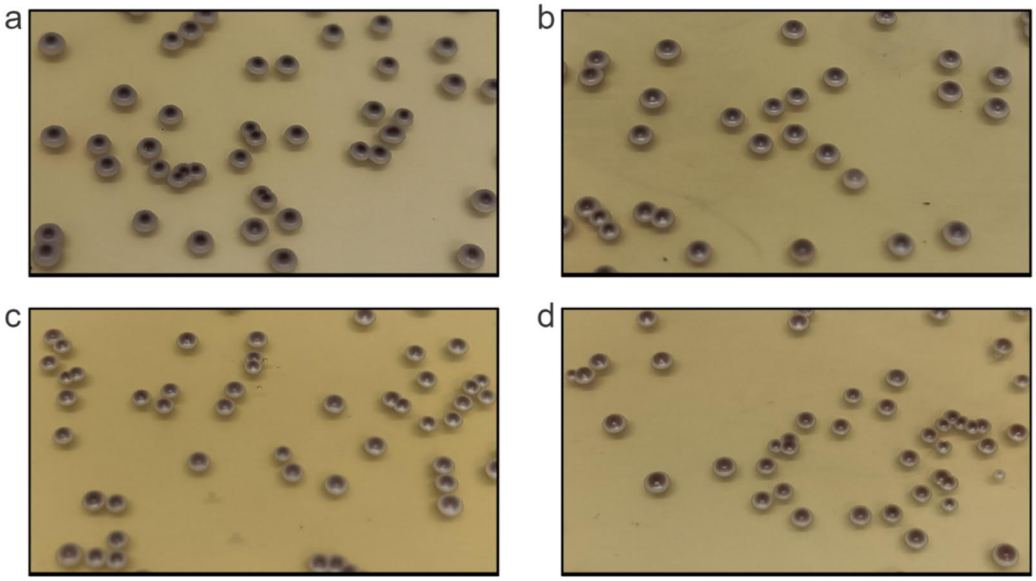

Figure 7. Representative examples of ALE lineages of YNBG grown *S. cerevisiae* S288c Bikaverin pathway containing strain plated on YPD at transfers a) 0, b) 10, C) 20, and d) 25. Examples for the transfers 10, 20, and 25 were taken from the first replicate lineage.

During the ALE, the proportion of pigmented clones remained quite stable for the YNBG grown *S. cerevisiae* S288c Bikaverin pathway containing strain (Figure 7). For the first replicate lineage, all the representative example transfers contained only pigmented clones (Figure 7 (a-d)). These pigmented clones were able to preserve their pigment intensity throughout the ALE, which was rare when compared to the other pigment producing strains.

Table 11. Proportion of pigment producing clones in four replicate lineages of YNBG grown *S. cerevisiae* S288c Bikaverin pathway containing strain during the ALE.

Transfer	1. Replicate Producing %	2. Replicate Producing %	3. Replicate Producing %	4. Replicate Producing %
0	100.00	100.00	100.00	100.00
1	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00
3	100.00	99.18	100.00	100.00
4	100.00	100.00	100.00	100.00
5	100.00	100.00	100.00	100.00
6	100.00	100.00	100.00	100.00
7	100.00	100.00	100.00	100.00
8	100.00	100.00	100.00	100.00
9	100.00	100.00	100.00	100.00
10	100.00	100.00	100.00	100.00
15	100.00	98.80	98.94	100.00
20*	100.00	100.00	0.00	86.28
25*	100.00	100.00	0.00	6.29

The YNBG grown *S. cerevisiae* S288c Bikaverin pathway containing strain had its proportion of pigmented clones remain remarkably stable during the ALE (Table 11). By transfer 15, all lineages still had over 98% proportion of pigmented clones remaining. By transfer 20, only two lineages saw noticeable decline. Lineage three had its proportion drop to 0%, while lineage four had 86% proportion of pigmented clones remaining. By transfer 25, lineages one and two had their proportions of pigmented clones at 100%, while lineage three had a 0% proportion, and lineage four had 6% proportion remaining.

5 Discussion

Nature has a way to streamline the genomes of microbial strains in a given environment through the mechanisms of evolution. ALE takes advantage of this optimization in biological studies and strain development. Generally, wild type strains have a relatively high fitness when compared to highly engineered strains that are metabolically burdened by their engineered pathways. In an ALE experiment, this leads to a situation where the wild type strains are not necessarily assumed to have as high fitness increase throughout the experiment, unlike the metabolically burdened engineered strains that can experience high fitness gains as their engineered pathway loses efficiency or other solution to counteract the burden arise.

Loss of production has been previously observed in bacterium *E. coli* (Rugbjerg *et al.* 2018a), and yeast *S. cerevisiae* (D'Ambrosio *et al.*, 2020; Lee *et al.*, (2021)). In the study by Rugbjerg *et al.* (2018a), large-scale mevalonic acid production was simulated in *E. coli* through serial passaging. Rugbjerg *et al.* (2018a) noted that the production loss in *E. coli* began after 40 generations, and it continued for the following 55 generations, until the end of the experiment. This resulted in the loss of over 95% of mevalonic acid production and in the notable increase of maximum growth rates for the studied strains. The loss of production in the *E. coli* strain proceeded at a pace comparable to the pigmentation loss observed in this work in YNBG grown indigoidine pathway containing lineages.

Similarly to this work, Lee *et al.*, (2021) had previously observed yeast *S. cerevisiae* CEN.PK2-1C to lose the N-acetylglucosamine (GlcNAc) production trait when grown on synthetic defined minimal medium containing glucose. GlcNAc overproducing *S. cerevisiae* had also a conditionally active synthetic product-addiction circuit (Lee *et al.*, 2021). Without the activation of the circuit, the engineered cells started to lose their production ability after 100 generations. The following production loss was quite fast as the studied lineages had lost their production completely after only 150 to 200 generations. The start of production loss in the study by Lee *et al.*, (2021), occurred more slowly than the production loss that was observed in this work, where the indigoidine producing lineages started to lose production after only ~40 generations. The time it took to reach complete production loss was quite similar, as the indigoidine producing lineages grown in YPG were noted to lose their production after ~160 generations, which is similar to the 150 to 200 generations observed in study by Lee *et al.*, (2021).

Loss of production in an engineered pathway was also observed in a study by D'Ambrosio *et al.*, (2020). They observed the stability of a fitness burdening vanillin-β-glucoside (VG) biosynthetic pathway in *S. cerevisiae* to reduce rapidly in serial passaging tests. Loss in VG production began after just 14 generations. After 35 generations, the production loss was noted to be over 91%. This production loss is much more drastic than the

indigoidine production loss that was observed in this work. In addition to production loss, D'Ambrosio *et al*., (2020) reported that the growth rates of the strains improved notably as they lost VG production. This observation aligns with the observations of this work, where the indigoidine production loss was accompanied by increase in the specific growth rates of the adapted lineages.

6 Conclusions

ALE is a versatile method that can be utilized in strain development and genomic analysis. This work aimed to characterize the evolutionary adaptation of engineered microbial cells, by subjecting them to long-term ALE. As a result, strains with engineered production pathways gained fitness while sacrificing the efficiency of their engineered production traits. Indigoidine pathway containing lineages were noted to have lost their production trait almost completely, while bikaverin pathway containing lineages were more stable. The loss of production trait was an expected result, due to the natural adaptation and evolution of microbial cells. Additionally, previous studies support this result, as production traits have been noted to decline in long-term experiments. Whole genome sequencing of the adapted lineages will reveal the specific genomic changes that have taken place, as the lineages have gained fitness throughout the ALE.

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