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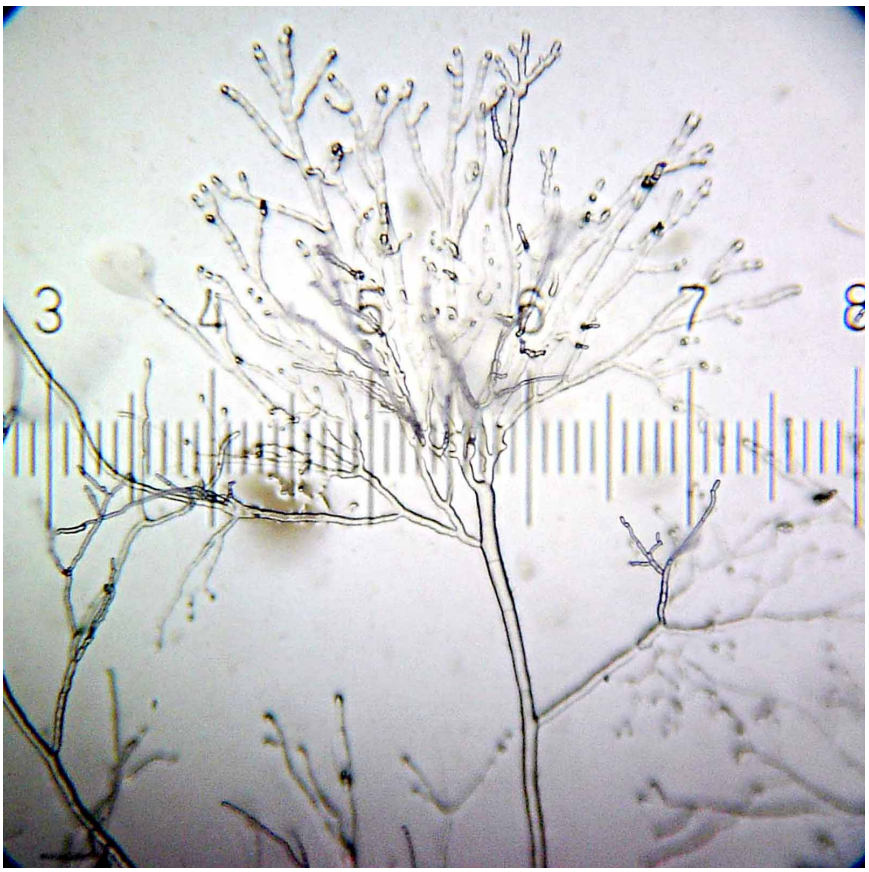

Creation and Evaluation of Fungal Mutants

ALE, Mutagenesis and High Throughput Screening

Master's thesis in Biotechnology

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Abstract

Climate change is becoming an ever-approaching issue and there has never been a better time for various industries to radically alter their production processes to reduce environmental pollutants. One of these industries that is among the most polluting food sources is the dairy industry. Where an unorthodox alternative to cattle could be to use filamentous fungi to produce the components of milk, such as casein, and mixing them together. Thereby creating a vegan milk alternative with the same nutrients content as the animal derived version. To further lessen the environmental impact the fungi could be cultivated on an industrial by-product. Increasing fermentation yields would require strain development to raise substrate tolerance, change morphology and remove casein degradation. The aim was to develop strains from two fungi using random mutagenesis to increase substrate tolerance and reduce the ability to degrade casein and adaptive laboratory evolution to increase substrate tolerance while at the same time changing morphology towards a more dispersed morphology. Random mutagenesis showed that an exposure time of at least 9 and 8 min of exposure to UV, respectively caused a survival rate of less than 1 % and resulted in changes in growth on industrial media as well as on casein. Adaptive laboratory evolution resulted in an increase in media tolerance of 25 % and 100 % for the respective fungi, while also changing the morphology to smaller mycelial clumps. Due to issues related to producing spores an incomplete screening was made of the mutants generated during this experiment. These results clearly indicate that random mutagenesis and adaptive laboratory evolution show great potential as methods for further developing the industrial use of filamentous fungi.

Keywords: Random, Mutagenesis, ALE, High, Throughput, Screening, Morphology, Filamentous, Fungi

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Introduction

As a majority of Europeans are gaining a growing awareness of the changing climate and realising that the impacts of such a grand scale shift would be bad [2], there has never been a better time for commercial sectors to consider alternative methods of production. These novel methods should aim to improve, innovate or radically change the way products, services and food are manufactured as the European Union is struggling to live up to its environmental and sustainability goals set for 2050 [8]. The dairy industry is one area where radical new methods of production are being developed, as the industry needs to innovate to lower its carbon emissions in time to reach the UN Climate Change Conference of Parties in Paris [21] [51]. Even though the dairy industry has taken strides to effectivize production and reduce carbon emissions, the global increase in the number of cattle offsets any environmental gain by lowering emissions resulting in an overall increase in greenhouse gas emissions [21]. Many plant-based alternatives to milk exist on the market already in the form of soy, wheat, almond milk etc. These still lack many of the compounds in dairy-based milk that give animal milk its properties and taste necessary for making other dairy products such as cheese, yoghurt and butter. This is where radical new production methods such as fermentation using microorganisms can be applied to produce dairy milk without the need for cattle. By introducing genes that encode milk proteins into microorganisms and then feeding them with energy-rich by-products generated from other industries, the chosen microorganisms can produce the individual compounds of milk that can then be used to synthesise mammalian milk. Looking to the future, this could decrease carbon emissions generated from cattle, fertilizer and large grazing areas as well as eliminate many of the ethical concerns surrounding the international dairy industry.

1.1 Environment and Ethical Considerations

As the world continues to struggle with reaching the targets set by the UN Climate Change Conference of Parties in Paris it is becoming clear that much of the industries such as dairy needs to evolve and reduce the harmful effects they have on the environment. Key areas that would be improved using a fermentation-based milk production strategy are the reduction of methane gas emission, diminishing acidification of land and a decrease in land use [19].

1.1.1 Current environment and Challenges of Protein Production

The mean global temperature of the Earth has increased by 0,85 degrees Celsius since 1880 [1]. Coupled with the ever increasing demand for milk, one of the most polluting types of food sources with regards to green house gas emissions [21], this means means that examining the environmental effects of using cattle as a protein source is highly necessary. In 2015, global emissions from the dairy sector increased by 18 percent or 256 million tonnes of CO_2 eq, of which 169 million tonnes of CO_2 eq were the result of CH_4 emissions [21]. Methane gas emitted from cattle is the greenhouse gas emission that has the largest impact on the climate, responsible for roughly 48-65 % of the total impact [19]. Replacing milk-producing cattle with fermenters will help reduce much of the methane emitted, although fermentation will still generate emissions such as carbon dioxide due to aerobic fermentation and will therefore not eliminate emissions completely. However, as methane has a carbon dioxide equivalent of 25 [3], meaning that 1 tonne of methane is equivalent to 25 tonnes of carbon dioxide, any reduction in methane emissions will still decrease the global warming potential of the industry, as long as carbon dioxide emissions do not increase more than 25-fold. This is not anticipated to occur, as emissions are overall expected to decrease [12]. In addition, the replacement of cattle will greatly reduce the acidification potential arising from milk production. As the use of volatile ammonia, which is part of the fertilizer used for making cattle feed, is responsible for 78-97 % of the acidification potential [19]. Removing the need for feed production by utilizing microorganisms that can grow on alternative carbon sources frees up land to be utilized for growing crops that can feed humans directly. This would reduce the energy losses occurring as energy is passed through multiple trophic levels [36].

Emissions are however not divided evenly on a global scale. If normalized, the emissions per amount of produced milk shows that areas with a less developed industry, such as Africa, South and West Asia have a higher impact ranging from 4,1 to 6,7 kg CO_2 eq. per kg fat-and-protein. In comparison, regions with a more developed industry have emissions ranging from 1,3 to 1,4 kg CO_2 eq. per kg fat-and-protein [21]. As can be seen developing industries can do much to reduce their carbon footprint and therefor the solution of adopting fermentation-based production method for dairy production is not a fix-all solution. Since different regions have varying degrees of developed dairy production this means that a switch to fermentation technology could require a technological transition that would be very large and therefor can be very costly [28]. Thus the alternative methods presented here is mostly targeted towards the large, developed industries of West Europe and North America that can pave the way for developing areas to greatly decrease their emissions in a much shorter time span.

1.2 Alternatives to Animal Sources Proteins

Alternatives to animal proteins, commonly derived from legumes, have been utilised in some way or another for millennia, often in a religious or ethical context such

as Hinduism or ethical veganism. While plant-based diets can have multiple health benefits, such as promoting an increase the overall health of the gut microbiome [49], there are still some important macro and micro nutrients that are missing or present in too low levels in plant-based diets. These include essential proteins in general, saturated fat and zinc with a particular lack of vitamin B_{12} and calcium [26]. Some of these can be easily added to the diet using supplements. However, as many supplements tend to come in a "one size fits all" dosage of pills it can be hard to properly dose the nutrients [44]. Therefore, being able to consume a vegan alternative with the same nutrient content as milk could bring an increase in quality of life from a nutritional perspective. Thus exploring the production of milk components, such as casein, using fermentation could be of interest for people that choose vegan or vegetarian diets as it contains all the nutrients and removes the ethics of animal husbandry.

One way of producing proteins such as casein would be by utilizing the fermentation ability of fungi. Already fungi are eaten as meat substitute with many species having descriptive nicknames such as the chicken or the beefsteak of the woods [41]. Eating fungi is not the only way of taking advantage their ability to synthesize protein at large scale [20]. Many species of fungi are used to produce additives for food such as *Aspergillus niger* is used for creating citric acid, *Mortierella alpina* used for fatty acid production or *Blakeslea trispora* that can produce β-carotene [41]. If it is possible to increase the list of products and include milk components such as casein it might result in a more ethical milk production, with reduced environmental effects and result in a secure food source for the future.

2
Aims

The aim of this project was to, using random mutagenesis, lower the lag phase and increase the growth rate of two species of filamentous fungi on an industrially relevant by-product containing sugars and biopolymers. The project also aimed to decrease or eliminate the ability of the fungi to degrade the protein casein.

Finally, the project aimed to alter the growth morphology of the fungal species to better suit the production of proteins in liquid reactors. In addition, the project also aims to lower the lag phase of the same species of filamentous fungi in the same industrially relevant by-product containing sugars and polymers using adaptive laboratory evolution.

3

Theory

There are many types of microorganisms that could theoretically be genetically modified to produce casein, such as bacteria, yeast and filamentous fungi. Out of all of these candidates filamentous fungi are among the most promising candidates due to there already being commercially available products based on milk protein beta-lactoglobulin produced by the genus *Trichoderma* since 2019 [42].

3.1 Fungal Biology

The kingdom of fungi contains an enormous variety of species with an enormous diversity in morphology, niches and industrial applications. There are monocellular fungi such as yeasts that can only be seen in microscopes, multicellular fungi with long threadlike filaments called hyphens as well as fungi that can change from monocellular growth to hyphal growth depending on the conditions [16]. Collectively, all true fungi have the ability to absorb nutrients from outside their cell wall from either living or dead organic matter. For a fungus to be able to ingest large, complex or insoluble compounds it needs to be able to secrete enzymes that can break down these compounds. Multicellular fungi secrete these enzymes from the ends of the long hyphens were it will absorb the nutrients and grow even further [16].

All fungi are eukaryotes with organelles similar to plant or animal cells. However, their nuclei are particularly small with a diameter of less than 2 µm [15], housing one of the shortest genomes of any eukaryotes. Their genomes range from 8,5 megabase pair (Mb) to around 400 Mb, or about 1 % of the size of the human genome [15]. The small nuclei become an asset during reproduction for fungi that produce spores, as the nuclei are able to move from cell to cell in multi-cellular filamentous fungi and thereby move to developing spores [15]. These spores can either be sexual, from the conjugation of two different haploid nuclei that have undergone meiotic division, or asexual undergoing other mechanisms for recombination of hereditary properties [16]. Irregardless of the type of spore they may each contain a varying amount and type of mutations. Meaning that to obtain a colony with the same mutations they have to be isolated from a single spore. An issue that monocellular fungi can does not share as they can be isolated without having to induce sporulation.

Monocellular fungi have other advantages as well when used for genetic modification. For example, having only a single nucleus per cell allowing for easier gene editing, the process of budding will produce clones retaining the same gene editing as performed

on the mother cell, and there already exists a large toolbox for genetic manipulation. Filamentous fungi in large lack an extensive toolbox to utilize for gene editing. This is due to the time consuming and low yield work with transformation, the late development of genome sequencing for filamentous fungi and the fact that the researchers working within the fungi are in small and fragmented groups [41].
However, filamentous fungi have three aspects that make them interesting for the purposes of using them as protein factories. First, a high potential for protein secretion. Second, potential for posttranscriptional modification of proteins. And finally, there are a number of species that are considered Generally Regarded As Safe (GRAS) [45]. This means that exploring the capabilities of filamentous fungi is of great interest for industrial applications despite the difficulties associated with working with them. Thus focusing on this type of fungal microorganism would help to further the field of lab-produced dairy and food biology since it is a proven avenue of interest that can be further expanded upon.

3.1.1 Filamentous Fungi

Filamentous fungi are an indispensable asset for producing many of the compounds that are used in everyday life, such as amylases for removing stains, glucose oxidases for food preservation and cellulases that are used as fabric softeners [45]. Many of these compounds are perhaps not known to the wider public as being derived from fungal fermentation. Other more specialised compounds, such as penicillin, are probably more known, as the story of how Alexander Fleming discovered the antibiotic is one of biotechnology's most famous breakthroughs.

Seeing as so many proteins are produced using filamentous fungi, the efficiency of the production becomes a very important topic as maximising product is a simple fact for any industry. As mentioned, proteins are mainly secreted from the hyphal tips, the long filaments that make up the mycelia and the main biomass of any multi-cellular fungus [15]. The hypha consists of tubes made of cell walls surrounding the fungal cells inside. Once a fungal spore germinates it forms a hyphal element consisting of a main hyphal that will then branch out into sub-branches that will in turn branch out, forming an ever-growing web. The total hyphal biomass is in turn called mycelium [45]. As the hyphal tips are key for protein production, it becomes clear that how they are growing is an important aspect. Hyphal growth is usually distinguished by the length of the main hyphae, the total length of all the hyphae, the number of hyphal tips and the length of the hyphal growing unit. These characteristics are what defines the morphology of the fungi.

In nature, filamentous fungi grow on solid surfaces of soil, wood or even living tissue [15]. However, in industrial processes liquid media is used during fermentation for numerous reasons. This means that the morphology will be very different in in industrial processes as the main growth occurs in liquid phase. The two main types of morphologies are pelleted and dispersed [45]. The first is characterised by clumps of mycelia intertwined within itself that can range from several millimetres down to a couple of micrometres. The second type is characterised by the mycelia growing

as freely dispersed hyphens [45]. An ideal morphology is impossible to determine as many factors are important for extracting maximum yield, such as growth conditions, downstream processing and mixing capabilities.

The pelletized morphology is often preferred when working with very large quantities, due to the compact pellets being easier to separate in downstream processing than dispersed mycelia. The dispersed mycelia also have the effect of giving the liquid media non-Newtonian properties [45] exasperating the already difficult task of stirring large reactors [43]. However, there are also problems with using pelletized morphology. Primarily, there will be a nutrient gradient within the pellet, hindering growth and hyphal formation. Additionally, as a result of the densely packed mycelia, there will also be a product gradient forming [45], which could be troubling if the product has inhibitory effects. Morphology plays a large role in productivity and if a species of fungi that grows in a pelletized morphology would yield a higher yield of proteins if it instead grew in more dispersed clumps. Working on changing the morphology of industrial strains could therefore lead to a future where the microorganism that is best suited for producing a certain protein can be optimised with an ideal morphology, thereby increasing the yield. Experimenting with changing growth morphology can also lead to a better understanding of what genes are associated with the shape that fungi grow.

3.1.2 Previous work with Fungi

While growth morphology is important for product yield, the utility of fungi as industrial microorganisms is only as good as the products they can produce. One of the textbook examples is the production of penicillin, an antibiotic derived from the mold *Penicillium* and one of the first antibiotics used to treat infections in humans.Another very important product derived from fungal fermentation is citric acid. The commercial introduction of fermentation-derived citric acid was over a hundred years ago in 1919 when it was used as a food additive as well as part of the pharma and chemical industry. Today the basics are the same, citric acid is still produced by the same fungus *Aspergillus niger* and the industry now has a turnover of multiple billions of Euros [41]. Other fungi are also being used in the food industry, such as *Mortierella alpina* that produces polyunsaturated fatty acids, *Penicillium roqueforti* as part of cheese production and *Rhizopus oligosporus* as part of tempeh production [41]. These are only a select few examples of the utilization of fungi in food production and new species are constantly being investigated by academia. Attempts to expand the number of organic acids produced by *Aspergillus niger* are being investigated, with a focus on itaconate and galactarate, molecules of high interest in the biopolymer and bioplastic industry [25][29]. Another *Aspergillus* species, *A. oryzae* has also been investigated for the production of malate that can be used as a food additive among other applications [17].

3.2 Classical Strain Engineering of Fungi

In the process of creating microorganisms with new properties such as higher tolerance for inhibitors, shorter growth rates or changed morphologies the new strains that are created are considered mutants if their genome has been mutated [7]. Being considered a mutant means that there is an extensive set of EU rules that apply for these mutants in industrial settings that can be both time consuming and costly to get approval. These rules are mainly applied to newer methods for mutant generation, such as for the use of CRISPR-Cas9 or other methods that can perform targeted mutagenesis. Other classical strain engineering methods that instead utilise directed evolution are exempted due to their long proven safety as well as they so closely mimic the natural process of evolution [7]. As such, using directed evolution is preferred from a legislative point of view. Directed evolution can also be a favoured method when working with microorganisms that have a limited toolbox for gene editing [32]. This is the case for many filamentous fungi, which while able to be genetically edited using CRISPR-CAS9, still lack the extensive knowledge and tools of microorganisms such as *S. cerevisiae*. Directed evolution can also be a preferred method when the phenotype that is to be improved upon is dependent on many types of genes such as growth, as targeted gene editing can be very difficult due to the complex systems that regulate growth. Therefore directed evolution simply focuses on generating random mutants and by applying a selection pressure the mutants that have developed advantageous mutations will take over the population and become the dominant strain [32]. The two mutation methods, Random Mutagenesis (RM) and Adaptive Laboratory Evolution (ALE), used for generating mutants in this thesis are both considered directed evolution and have been used in previous work to change phenotypes of filamentous fungi.

3.2.1 Random Mutagenesis

Random mutagenesis is at its core a trial-and-error method for inducing changes in a microorganism's phenotype. The method is based around inducing randomised mutations by exposing organisms to DNA damaging mutagens. That will in turn cause point mutations or deletions at random points in the DNA [54]. This can change the gene expression of the created mutants since protein function will be affected should the mutations occur in protein coding sequences of the DNA.
In practice random mutagenesis is performed by exposing a microorganism culture to a physical and/or a chemical mutagen. These mutagens tend to be UV light at 254 nm as a physical mutagen, or DNA alkylating NTG (N'-methyl-N'-nitro-N'-nitrosoguanidine) as chemical mutagens [54]. The exposed culture will take damage to their DNA from the mutagen and many cells will die due to irreversible damage. The surviving cells will in turn be cultivated as pure cultures and be allowed to recover. As they recover the cells are repairing the damages done to the DNA and during this process mistakes will happen. These mistakes are the mutations as mismatched base pairs or deletions of base pairs can have an effect on protein structure should they happen inside protein coding sequence of DNA [54]. Deletions can even effect protein structure more as the reading frame will shift for protein expression.

In theory mutations can have an effect even outside of coding sequences as changes to promoter sequences can affect protein expression as RNA-polymerases affinity can change. To induce mutations the dosage of either type of mutagen needs to be kept just below lethal dosage for the culture being exposed [10], as a higher exposure rate will yield more mutants, thus increasing the chance of mutations occurring [48]. Depending on what type of microorganism is used the dosage will vary. However, filamentous fungi have proven to be very tolerant towards mutagenic effects, since they can still grow well and continue protein production even after serious alterations to their chromosomal DNA [54].

The randomised nature of random mutagenesis means that many of the mutants generated will have no or worse change in phenotype. Thus many mutants need to be generated to increase the chance of positive mutations occurring. Mutant generation is followed by screening for improved mutant phenotype. The mutant showing the most improvement is then cultivated and used as the basis for the next iteration of random mutagenesis [54]. By then repeating the loop of mutant generation with follow-up screening improvements will slowly accumulate over time as more and more mutations happen. Although as positive mutations accumulate so will negative that can affect stress resistance, morphology or protein secretion. This can lead to improvements of the strain stopping as new mutants are no longer viable [54]. Thus it becomes vital to save mutants generated during each generation of random mutagenesis to have points to return to should there be an aggregation of undesired mutants. As positive mutants accumulate the phenotype improvements will start to decrease over time as there is a limit for random mutagenesis and it becomes no longer useful. This is called the technological limit and for fungi happens around generation 10-50 [54].

3.2.2 Adaptive Laboratory Evolution

Adaptive laboratory evolution is a well-used method that has been implemented for over 35 years and heavily utilises the basic adaptational ability of microorganisms [20]. By cultivating the desired microorganism, such as a filamentous fungus, in a stressful environment the struggle for survival will lead to spontaneous mutations that increase fitness gets passed down to new generations [32]. As spontaneous mutations occur naturally during every new generation, microorganisms are ideal for ALE due to their short generation time that can be as low as 1,5 hours for filamentous fungi such as *Aspergillus niger* [52]. This means that during the weeks, months or even years that an ALE experiment is conducted hundreds or thousands of generations will be created. At first, many phenotypes will arise that all compete for dominance in the mixed culture. Over time, mutations that allow for a better growth such as shorter lag phase or higher growth rates will dominate the culture and the culture will stabilise [20]. As the culture stabilises a higher stress factor can be applied and the selection pressure increases, thereby driving the culture to further adapt to its environment.

When cultivating microorganisms during ALE there are several factors that are important. First is the mode of cultivation. There are two main modes of cultivation, either in a continuous culture or in a batch culture. Each operational mode has its distinct advantages however, batch cultures are the most common ones [32]. In flask batch cultures, many parallel cultures can be grown in series where the main limiting factor is space and manpower. The cultivation method is very simple, on regular intervals the media is changed for the microorganisms by transferring an aliquot of the culture into flasks containing fresh media. This medium change should preferably be made before the culture reaches stationary phase to avoid adaptations not linked to growth [20]. However, although a cheap and low maintenance method the conditions of the batch culture is always changing. The conditions that change are nutrients concentrating, pH, changing growth rates etc. and will vary during the growth in the shake flasks. These conditions are not always relevant due to the simple nature of batch culture experiments leading to many of these conditions simply being ignored. Should these conditions be of interest, another type of ALE can be performed using a continuous reactor. Then factors such as pH, dissolved oxygen or even growth rate can be kept constant or changed depending on the nature of the experiment. Further on, continuous cultures can be cultivated at maximum specific growth rate. This increased control does come with a trade-off of a much higher running cost as media consumption is increased dramatically and the complexity of the chemostat requires much more care and manpower [20].

The second factor that is of high importance is the time spent on ALE. As a standard, ALE experiments are performed for a period of 100 to 2000 generations and the time needed for adaptations is closely linked to the generation time of the microorganism. Thus an appropriate amount of generations is usually reached after a couple of weeks to a few months [20]. The reliance on spontaneous mutations to occur naturally and compete within the culture means that time becomes important for the adaptability of the microorganism. Another factor that is also closely linked to experiment time length is the degree of which fitness can be increased [20]. This is important as because a micro organism that can not adapt to the stress condition is useless for an ALE experiment. Since no adaptations mean that no increase in fitness can be achieved [20]. The final factor presented is the selection pressure applied throughout ALE, is an aspect easily assumed to be simple yet deceivingly difficult. During ALE it is important to remember that one is only selecting for the mutations that allow for the most proliferation for each generation. As an example one can imagine that a chemostat experiment designed to increase growth rate by slowly building up dilution rate can also select for cell stickiness since sticky cells will also multiply and might even take over the culture. Regular screening of mutants can be performed to ensure that the targeted phenotypes are being selected for. However, due to the mixed nature of ALE mutants it can never be assumed that the culture is pure and individual mutants need to be isolated [20]. One such way of isolating individual mutants would be to sporulate the fungi and isolate individual colonies once the spores have been germinated. Each individual colony would then be derived from a single nucleus and each cell then has the same mutations [16].

3.3 Mutant Strain Evaluation

Since countless mutants are generated using random mutagenesis and ALE it becomes vital to evaluate and select mutants that show favourable adaptations. However, cultivating each mutant one at a time and evaluating them sequentially quickly becomes a never-ending struggle as the time spent on mutant evaluation will rapidly outweigh the time spent generating mutants. Instead a more time and cost-efficient method for mutant evaluation is needed to rapidly assess the phenotype of all the generated mutants [31]. This is where high-throughput screening becomes a useful tool, a method for quickly scanning and evaluating numerous samples and then step by step reducing the number of samples but increasing the sensitivity of the tests.

High-throughput screening is a method first pioneered in the 1990s [39] and has since then been adapted to numerous different scientific fields such as drug screening [9], catalyst testing [37] and mutant evaluation [11]. Within the biological field it is often used to reproduce culture conditions of lab scale experiments on a micro scale screening plate. Thus increasing the throughput of cultures being evaluated. Due to this much development has been spent to control experimental parameters such as oxygenation, feed rate etc [39]. Since screening cultures in conditions that do not represent the experimental conditions are hardly useful.
Generally, there is a trade-off between the throughput of the system and the sensitivity of the tests. Therefore the basic structure of high-throughput screening is a tiering system that first screens numerous mutants for improved phenotype, identifies them and moves those mutants to the next tier. This time with lower throughput and instead more sensitive tests [39].

The method for screening biological cultures has often been to do spectroscopic tests of e.g. optical density, absorbance or fluorescence [39]. These screening methods work well when the improvement screened for is expected to increase substantially. However, as phenotypic improvements stagnate more sensitive methods are needed to see improvements and minimise noise. Although outside the scope of this thesis, mass spectrometer is a useful analytical tool that can even detect changes in the molecular composition of culture broth [39]. As automation also becomes an integrated part of high-throughput screening the current bottleneck of limited testing ability will be alleviated and leading to even larger pools of mutants able to be evaluated [39]. Another screening alternative is to use high performance liquid or gas chromatography to analyse media composition. This alternative is useful if the degradation or production of certain compounds can be linked to improved phenotype.

4
Material and Methods

In this project two different species of filamentous fungi were used to create and evaluate mutants, due to being covered by a confidentiality agreement they were denoted as fungi A and fungi B respectively. Further on, the industrial by-product that mutants were selected for increased tolerance, is also covered by the same confidentiality agreement. The medium, denoted as BL, is an industrial relevant by-product that contains sugars and polymers and further questions regarding confidential details should be directed to Yvonne Nygård.

4.1 Media and Cultivation

Fungi A and B were both consistently cultivated on four different types of solid media, depending on the purposes of the cultivation. Recipes for media, salt solutions and trace element solution can be found in Appendix A.
- Potato Dextrose Agar (PDA), for general growth and as positive controls during screening, is an all-purpose complex medium consisting of dehydrated potato infusion, dextrose and 2 % agar [5].
- Malt Extract Agar (MEA), for inducing sporulation of the filamentous fungi, is a complex medium consisting of dehydrated malt extract, peptone and 2 % agar [4].
- BL-Agar 40 %, used to screen for growth, a complex medium containing 40 % of an industrial by-product, added salt solutions, trace element solution and 2 % agar.
- Casein-agar, used to screen for growth, a defined medium containing 1 % casein, added salt solutions, trace element solution and 2 % agar.

Fungi A and B were also cultivated on two types of liquid media during ALE. Recipes for media, salt solutions and trace element solution can be found in Appendix A.
- BL 30, 40, 50 and 60 %, used to apply selection pressure on fungi A and B, complex medium containing 30, 40, 50 and 60 % of an industrial by-product, added salt solutions and trace element solution.
- Potato Dextrose Broth, used to recover colony health during ALE, a complex medium identical to PDA, but lacking agar [6].

4.2 Fungal Spore Generation

When generating spores, used for inoculation or mutant generation, an initial inoculation of wild type spores for fungi A and B were made on petri dishes with 5 ml MEA per plate. These were incubated at 30°C until a clear colour change

occurred due to the number of pigmented spores. Wild type spores were harvested by adding 0,05 % Tween 80 solution to the MEA plates. Then, using a T-spreader, light mechanical force was applied to dislodge spores and suspend them in the 0,05 % Tween 80 solution. The spore suspensions were poured through a double layer of miracloth (Millipore) placed in a funnel into sterile falcon tubes. Part of the spore suspension was then diluted 100 times and 10 µl of the spore solution was added to a hemacytometer and used to count the concentration of spores in million spores per ml, counting at least two 4x4 squares. Spores were stored at 4°C.

4.3 Generation of Fungal Strains

The two methods used for mutant generation follow very different approaches. Random mutagenesis utilises a physical mutagen, in the form of UV-light, to induce DNA damage and thereby mutations, while adaptive laboratory evolution uses the already present ability to adapt to selection pressures that drives evolution. These two approaches therefore require different workflows to generate mutants and the intensity of the work will vary substantially.

4.3.1 Strain Generation With Random Mutagenesis

The flowchart for how mutants were generated using random mutagenesis, shown in figure 4.1 was at its core identical for both fungus A and fungus B, with the only difference being the time of UV-light exposure. First petri dishes with 5 ml MEA per plate were inoculated with a set amount of 2500 spores per plate. Wild type control plates, that were not to be UV irradiated, were produced containing 250 spores per plate to calculate the germination rate of the fungi. Inoculation of the plates was done by adding 250 µl of the generated wild type spore solution and then spreading the solution evenly over the MEA plates using a T-spreader. The plates were allowed to dry, closed and sealed with parafilm. The plates were divided in groups of three plates for each time point tested, including a triplicate of the wild type control plates. The plates were placed on a High Performance UV Transilluminator (Analytik Jena), irradiating at a wavelength of 302 nm, with the plate cover pointing down. The Transilluminator was set to High intensity and turned on for the determined exposure time. The time range used was between 6 to 10 min as presented in section 5.1.1. After UV exposure the plates were incubated at 30°C overnight. After one night, colonies on the wild type control plates were visible and could be counted. The number of colonies on the control plate were used to calculate the germination rate of the fungi according to equation 4.1.

$$g = \frac{N}{N_{control}} \tag{4.1}$$

$$g = Germination\ rate$$
$$N = Number\ of\ colonies$$
$$N_{control} = Number\ of\ spores\ on\ control\ plate$$

As the stress of the UV exposure affected the time needed for the mutants to germinate, the irradiated plates needed another night until visible colonies were formed. On the next day the number of colonies that had germinated on the UV irradiated plates could be counted and the survival rate was calculated by using equation 4.2

$$s = \frac{N}{N_{tot} * g} \tag{4.2}$$

$$\begin{aligned} s &= Survival\ rate \\ N_{tot} &= Number\ of\ spores\ on\ plate \\ g &= Germination\ rate \end{aligned}$$

Once the survival rate had been calculated for each time point, individual colonies could be collected using toothpicks from plates with a survival rate of less than 1 %. The colonies were collected in 96-well plates, with each well containing 200 µl of MEA, covered with Breathe-EASIER membranes (Diversified Biotech) and incubated at 30°C until spore formation. Once spores were produced 175 µl of MilliQ water was added and used to resuspend the spores. 15 µl of spore solution was used to inoculate, as a triplicate, three other 96-well plates containing PDA, casein-agar and BL-agar 40 % and used to evaluate growth rate, μ_{MAX} and lag phase. See section 4.4 for details on evaluation. Once a mutant had been selected after evaluation it was cultivated on MEA until spores were formed. These mutant spores were used to inoculate MEA plates with roughly 2500 spores per plate. A wild type control plate and a mutant control plate containing 250 spores per plate were also produced. The first plate was used to control fungal growth on MEA plates. The second control was used to calculate the germination rate. The process was repeated for each mutant generation.

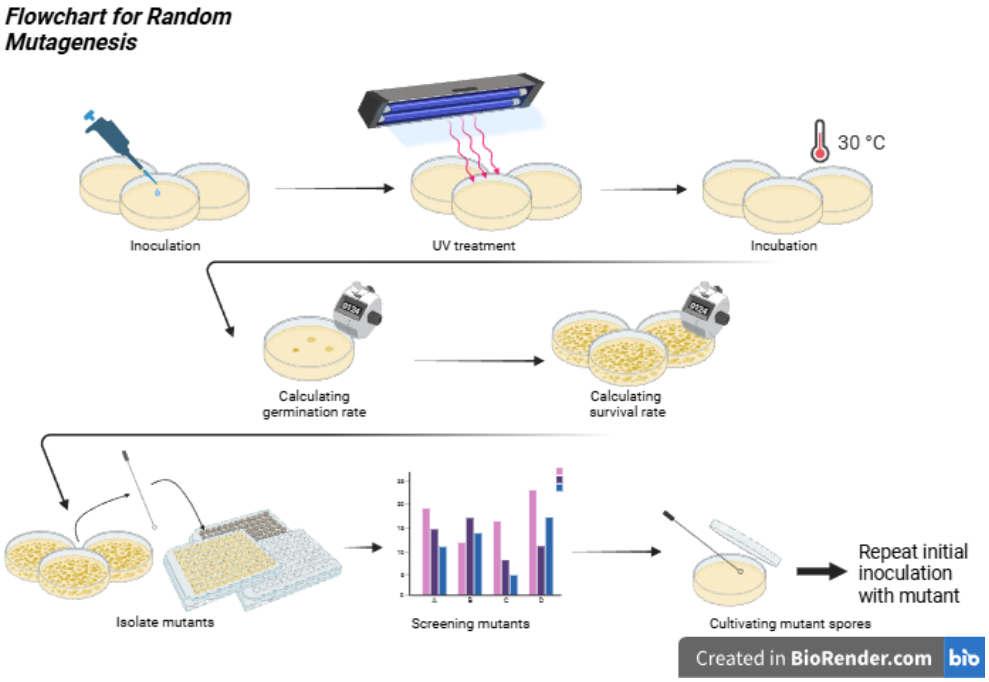


Figure 4.1: Workflow for Random Mutagenesis. Created with BioRender.com

4.3.2 Strain Generation With Adaptive Laboratory Evolution

As with random mutagenesis the workflow used for generating mutants using ALE, as can be seen in figure 4.2, was identical for both species of fungi. The workflow for ALE, as can be seen in figure 4.2, was performed by first inoculating 50 ml of a starting concentration of BL with fungal spores. The spores were grown in non-baffled shake flasks at 150 rpm and 30 °C until biomass formation, whereafter samples of ca 1,5 ml were taken and stored at -20 °C. An aliquot of the mixed culture was then used to make a new flask inoculation. Once a new set of non-baffled shake flasks with 50 ml liquid medium were inoculated they were cultivated at 150 rpm and 30 °C until biomass formation.

At first an initial experiment to determine the starting concentrations of BL for A and B was performed by growing triplicates of fungi A and B in non-baffled shake flasks containing 50 ml of BL 30 and 40 % respectively for seven days. The flasks were inoculated with a final concentration of 200 000 spores per ml and placed at 30°C on a shake incubator at 150 rpm. Samples of 1 ml for HPLC analysis were taken each day and after 166 hours of growth a final sample was taken and the flasks were autoclaved. The contents of the flasks of BL and mycelia were transferred to 50 ml falcon tubes, spun down and washed, dried at 65°C and the biomass was weighted. After comparing the fungal cultures for the largest amount of biomass, as can be seen in section 5.1.2, an initial starting concentration for A and B was set

to 40 and 30 % BL respectively.

Using the starting concentrations of 30 and 40 % BL determined by the initial experiment for each fungus. Triplicates of each fungi denoted as A1, A2, A3, B1, B2 and B3, were made by inoculating 6 non-baffled shake flasks, containing 50 ml of BL, with 200 000 spores per flask and cultivated according to the flowchart seen in image 4.2. After one week's growth a sample of 1,5 ml for HPLC was taken, as was an image of the flasks with mycelia and 1 ml sample for inoculating new liquid cultures. After 3 weeks of one week growth periods, the growth period was shortened to a non-fixed interval for A and B. New liquid cultures were made when biomass formation was observed, usually after approximately 3-4 days. The new liquid cultures were made by adding 50 ml of BL 30 or 40 % and then inoculating the new flasks with 1 ml of the liquid contents of the previous flask, filtered through miracloth to remove mycelial clumps. After 5 weeks of cultivation, the 1 ml inoculation liquid was no longer filtered through miracloth and was instead transferred directly from flasks to flask with a pipette avoiding large mycelial clumps. After 39 days of ALE the mixed culture of flask A3 ceased growing and was replaced by inoculating 300 μl of culture A1 and A2 creating flask A1+2.
During the ALE experiment the media composition was changed according to table 4.1 for A and B. The original flasks were stored at 4°C until confirmed growth in the new liquid cultures. All dates for new liquid inoculations, concentration increases and change of medium can be seen in table B.1 in Appendix B.

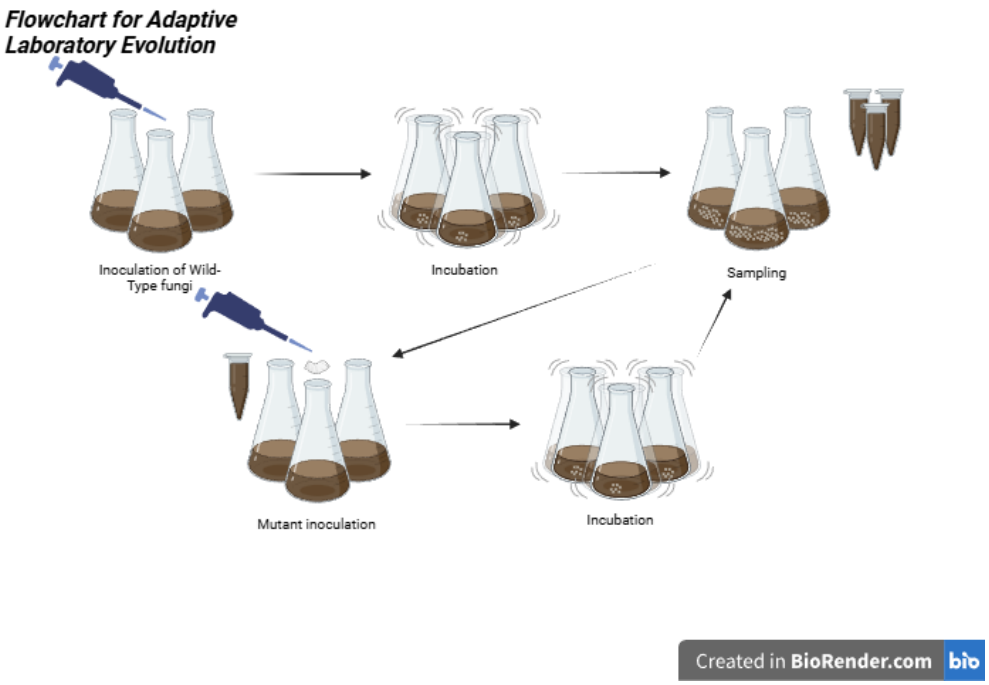


Figure 4.2: Workflow for Adaptive Laboratory Evolution. Created with BioRender.com

Table 4.1: Changes in medium composition shown as days since inoculation

Days	Change in medium composition
19	Concentration increase for B 30 % to 40 % BL
22	Concentration increase for A 40 % to 50 % BL
33	Concentration increase for B 40 % to 50 % BL
40	Medium change for A from 50 % BL to PDB
46	Concentration increase for B 50 % to 60 % BL
50	Medium change for B from 60 % BL to PDB
56	Medium change for A from PDB to 50 % BL
56	Medium change for B from PDB to 60 % BL

After 12 weeks of ALE the mutants' adaptations were evaluated by first taking images of the culture, 1,5 ml sample for HPLC and then filtering the contents of each flask through a double layer of miracloth into a falcon tube. The spore concentrations of the mixed cultures were determined and a part was diluted to 1000 spores per ml. 250 µl of the dilution was added to PDA plates, spread evenly and incubated at 30°C overnight. The next day individual colonies were isolated on a 96-well MEA plate with 15 colonies isolated from each flask. These were incubated at 30°C until sporulation. The mutant spores were re-suspended in MilliQ water and inoculated on PDA plates, spread evenly and incubated at 30°C overnight.

Individual colonies were isolated on a 96-well MEA plate and the isolated cultures were evaluated as described in section 4.4.

4.4 Selection of Mutant Strains

When evaluating the mutants generated through random mutagenesis or ALE the criteria being assessed were lag phase, growth rate and maximum specific growth rate. These were determined by analysing the mutants as triplicates using the Growth Profiler 960 ensuring that no triplicate had more than one replicate on the edge of the 96-well plate. For mutants generated through RM, the mutants were evaluated on BL 40 %, casein-agar and PDA. The 96-well plates were then placed in the Growth Profiler 960 at 30°C and 250 rpm for a 9-day growth period.
The green values collected by the Growth Profiler 960 were analysed for lag phase, growth rate and maximum growth rate using a modified version of a prewritten R-script [50]. The data was analysed to select for mutants that show no growth on casein, short lag phase as well as high growth rate on BL 40 % and better or equal growth on PDA. Using these selection criteria a mutant was selected for further developing using random mutagenesis as described in section 4.3.1.

The ALE mutants were evaluated on two different tiers. In the first tier each of the flasks were compared with each other, as well as with a triplicate of wild type. In the second evaluation tier each mutant from a single flask was analysed using the Growth Profiler 960 in a similar way as the mutants generated through RM.

When comparing the mixed cultures from each flask A1, A2, A3, B1, B2 and B3 with wild type A and B the setup was done in the following way. First, each flask of A or B was inoculated with the same number of spores for both mutants and wild type. These were left to incubate at 30°C and 150 rpm for 7 days. Each day an HPLC sample was taken from each flask and on the final day the contents of each flask was emptied into a falcon tube. Washed by centrifuging the contents at 5525 g for 10 min. Removing the supernatant and resuspending the pellet in MilliQ water. The process was repeated until the water was clear. The water was discarded and the pellet was dried at 65°C overnight. The falcon tubes were weighted and the biomass was calculated. A larger amount of mutant biomass compared to wild type indicates that adaptations have occurred that allow the ALE mutants to better grow in BL. By comparing the HPLC spectrum for each mutant and the wild type it will, with good enough separation of compounds, show how fast the sugars present in BL are consumed. When no sugar is present no growth is assumed, meaning that fast sugar consumption could be an indicator for fast growth. By selecting the mixed culture with the fastest sugar consumption on BL and the most biomass generated, three tier one evaluations were made for A-BL 50 %, B-BL 50 % and B-60 %. From the selected mixed culture pure cultures could be evaluated by performing a second screening using the Growth Profiler 960.

When evaluating the isolated colonies generated through ALE similar criteria were used for assessing the individual mutant using the Growth Profiler 960. The spores

from each isolate from section 4.3.2 were resuspended, added to 96-well plate as triplicates containing PDA and BL of the same concentration as the tier one evaluation. They are allowed to grow for a 9-day period at 30°C and 250 rpm. The green values were analysed for lag phase, growth rate and maximum growth rate using a modified version of a prewritten R-script [50]. The mutant showing the shortest lag phase and highest specific growth rate was considered to be the mutant with the best adaptation.

5
Results

Below are the results from both the random mutagenesis and adaptive laboratory evolution, including both the method development and screening results for identifying improved mutants.

5.1 Protocol Development

During method development an iterative process was used to determine many of the experimental details used for random mutagenesis and ALE. Here the individual results from the method development are presented, together with the measures taken based on the results.

5.1.1 Establishment of Random Mutagenesis Protocol

To calculate the survival rate for the random mutagenesis, the germination rate needed to be determined first. In table 5.1 it can be seen that the germination rate was higher for both fungus A and B on MEA, with an increase of 9,9 percentile units resulting in a 45 % increase of germinating cells for fungus A. For fungus B there was an increase of 85 percentile units from 0 % to 85 % when comparing PDA to MEA. Thus MEA was used to inoculate further random mutagenesis experiments.

Survival rates for five tested UV irradiation times are shown in table 5.2 for fungus A and in table 5.3 for fungus B. An exposure time of minimum 9 minutes for fungus A, and minimum 8 minutes for fungus B, resulted in a survival rate of <1 %. These mutants were then selected for screening, with the results shown in section 5.2.1.

Table 5.1: The germination rate for combinations of fungus A and B on PDA and MEA.

Media + Fungus	Germination rate %
PDA+A	22 ± 2.62
PDA+B	0 ± 0.4
MEA+A	32 ± 6.12
MEA+B	85 ± 3.0

Table 5.2: UV exposure time for fungus A on MEA with colony count for each triplicate, average and survival rate.

Time (min)	Survival rate %
6	3.6 ± 0.92
7	1.5 ± 0.51
8	1.4 ± 0.32
9	0.5 ± 0.44
10	0.8 ± 0.81

Table 5.3: UV exposure time for fungus B on MEA with colony count for each triplicate, average and survival rate.

Time (min)	Survival rate %
6	3.0 ± 1.18
7	1.5 ± 0.38
8	0.6 ± 0.40
9	0.6 ± 0.41
10	0.4 ± 0.12

5.1.2 Establishment of the Adaptive laboratory Evolution Protocol

During the preparatory experiment for ALE, fungus A and B were cultivated on 30 and 40 % BL. The biomass weights after 8 days are shown in table 5.4 and indicate that A is able to grow well on both 30 and 40 % BL with similar amounts of biomass generated from both cultivations. Fungus B, meanwhile, generated a negligible amount of biomass on 40 % BL, with the only readable amount being 50 mg at 30 % BL. As such, the starting concentrations for the ALE experiment were set to 40 % for A and 30 % for B.

Table 5.4: Generated biomass, after 8 days of growth, of wild-type fungi A and B cultivated in 50 ml of 30 % and 40 % BL.

Fungus + % BL	Weight (g)
WTA 30%	0.098 ± 0.014
WTA 40%	0.112 ± 0.005
WTB 30%	0.050 ± 0.017
WTB 40 %	0.002 ± 0.003

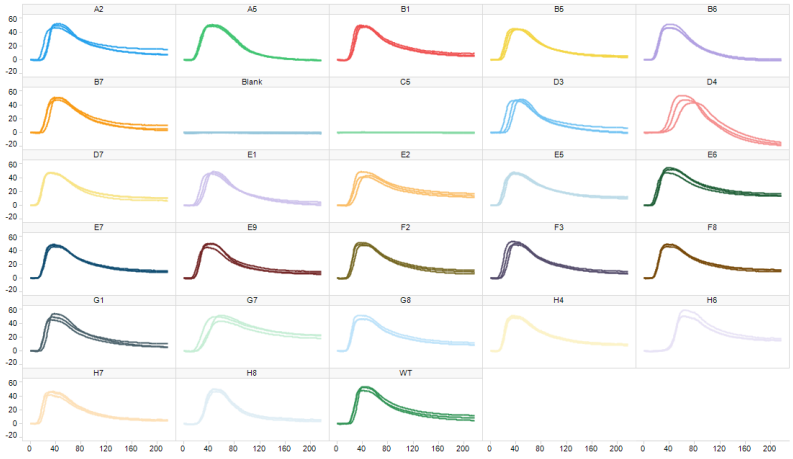
5.2 Mutant Generation and Screening

Analysing the data from the screening of mutants generated through the use of random mutagenesis or ALE indicated what mutants showed favourable adaptations and were the ones to be picked out for evaluation and further development.

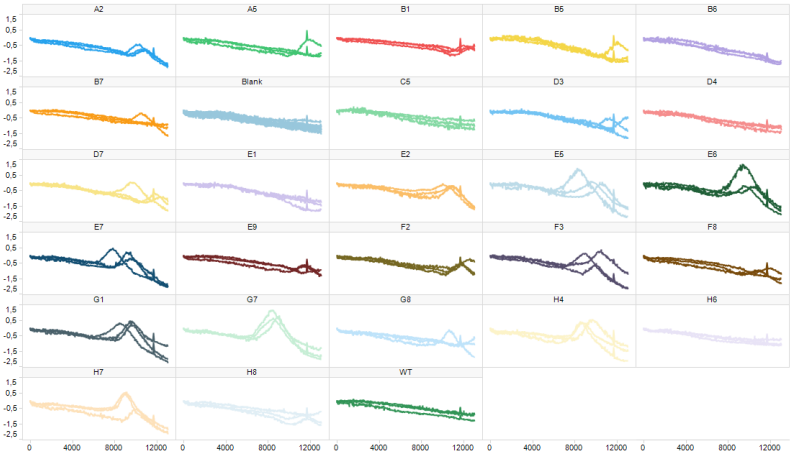
5.2.1 Mutants Generated by Random Mutagenesis

The growth, as measured with green values, gathered from analyzing the random mutagenesis mutants of fungus A in the Growth Profiler 960 are plotted against time in figure 5.1, showing that there was no growth for mutant C6 and no growth in the blank slot that had not been inoculated. The growth curves for PDA in figure 5.1a show slight differences in lag phase and μ_{MAX}, which is confirmed by figure 5.2 showing the μ_{MAX} and lag phase of each mutant on PDA. The top five highest μ_{MAX} and shortest lag phases of fungus A can be seen in table 5.5, indicating that random mutagenesis does result in differences in growth characteristics.

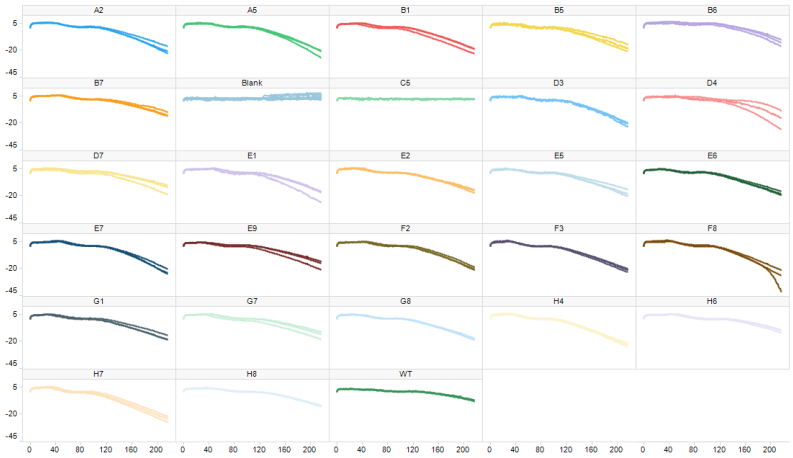
Figure 5.1b and 5.1c show that the green values changes for all mutants and wild type over time, due to the irregular changes in green values, no link between green values and growth characteristics could be established for BL or casein. However, comparing the blank slot and the non-viable mutant C6 with the other mutants in figure 5.1b and 5.1c seem to indicate that the changes in green values over time could be linked to growth. As there is a significant difference in the curve shape between the cells with and without confirmed growth from figure 5.1. An image taken by the Growth Profiler 960 after 5 days was used to qualitatively determine the mutants with the best growth on 40 % BL, shown in figure 5.3. The selection was based on the total change of color seen in the triplicates of each mutant. The four mutants selected and what wells they were in are shown in table 5.6.



(a) Growth curves of screened mutants of fungus A generated through random mutagenesis. Mutants were grown on PDA in MTPs in the Growth Profiler 960.

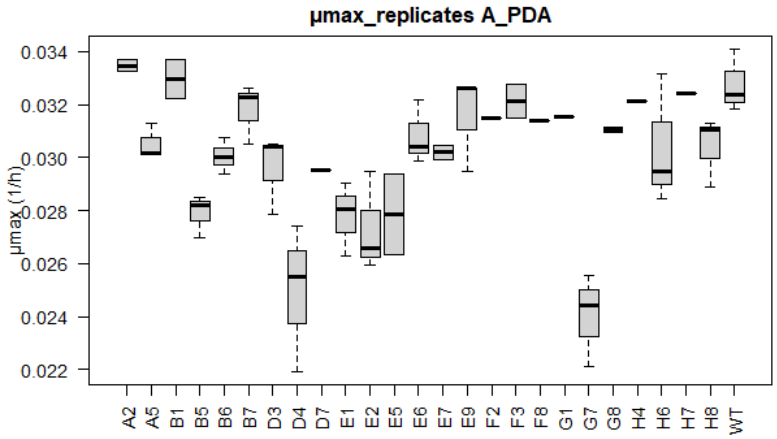


(b) Green values of screened mutants of fungus A generated through random mutagenesis. Mutants were grown on 40 % BL in MTPs in the Growth Profiler 960.

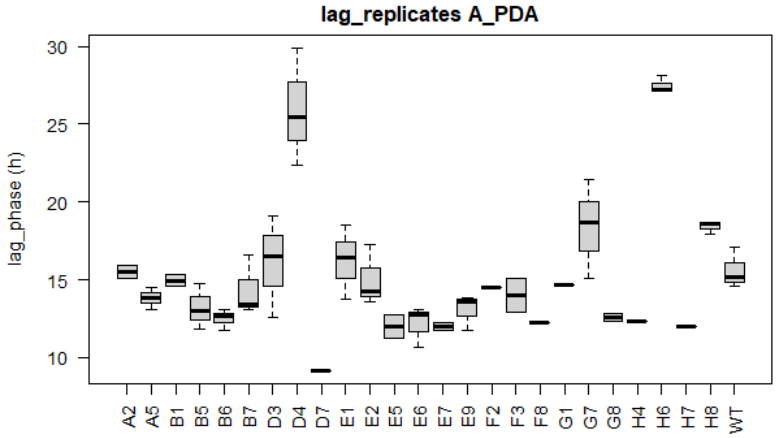


(c) Green values of screened mutants of fungus A generated through random mutagenesis. Mutants were grown on casein in MTPs in the Growth Profiler 960.

Figure 5.1: Growth curves on PDA as well as green values of 40 % BL and Casein. Showing growth of 26 isolated mutants, wild-type (dark green) and Blank (light blue). All as triplicates and normalized by subtracting the initial value from every subsequent time point



(a) Maximum specific growth rate for each random mutagenesis mutant for fungus A.



(b) Lag phase for each random mutagenesis mutant for fungus A on PDA.

Figure 5.2: Maximum specific growth rate and lag phase for the viable random mutagenesis mutants for fungus A on PDA. Mean results higher than the maximum specific growth rate and shorter than the lag phase of wild-type are sought after.

Table 5.5: Top 5 mutant strains of fungus A with highest mean μ_{MAX} or lowest mean lag phase in relation to wild-type.

Strain	μ_{MAX} $(\frac{1}{h})$	Strain	Lag phase (h)
A2	0.033	D7	9.2
B1	0.033	H7	12.0
WT	0.033	E5	12.0
H7	0.032	E7	12.0
F3	0.032	E6	12.1
H4	0.032	WT	15.6

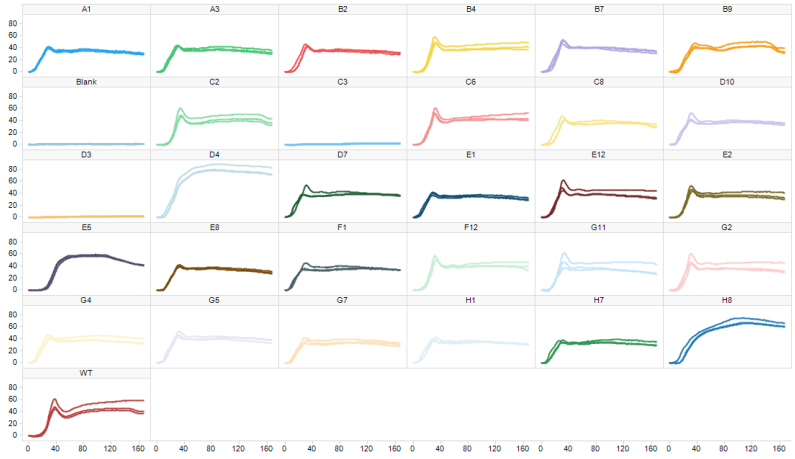
Figure 5.3: Image used for qualitative determination of the mutants of fungus A with the best growth on 40 % BL. Growth is determined by change in colour.

Table 5.6: The selected mutants from the qualitative screening. Image taken after 5 days.

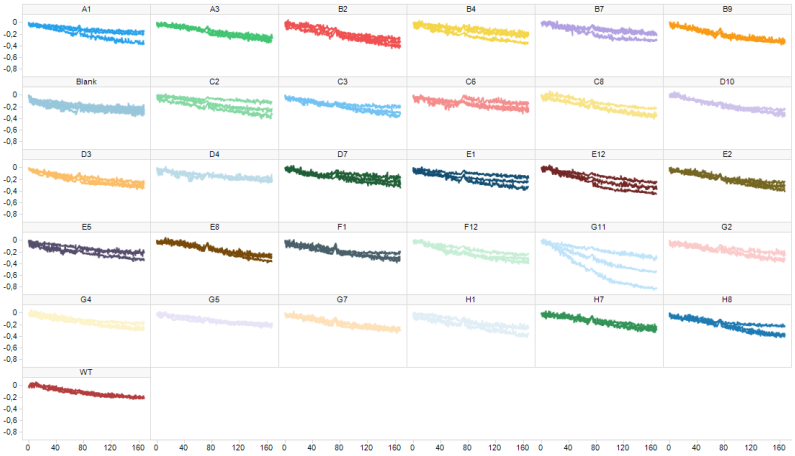
Mutant	Wells	Mutant	Wells
	A5		B5
G7	C5	G1	F5
	G5		H5
	A7		B3
E7	C7	H4	F3
	G7		H3

The growth, as measured with green values, gathered from analyzing the random mutagenesis mutants of fungus B in the Growth Profiler 960 are plotted against time in figure 5.4, showing no growth for mutants C3 and D3 nor in the blank slot with no inoculation. The growth curves of B on MEA are shown in figure 5.4a and indicate differences in both μ_{MAX} and lag phase. This is corroborated in figure 5.4, indicating that the μ_{MAX} is lower for most mutants than wild-type and that the lag phase is shorter for a majority of mutants with exception of mutant E5. Looking at table 5.7 it is clear that lag phase is shortened by more than 50 % as a result of RM.

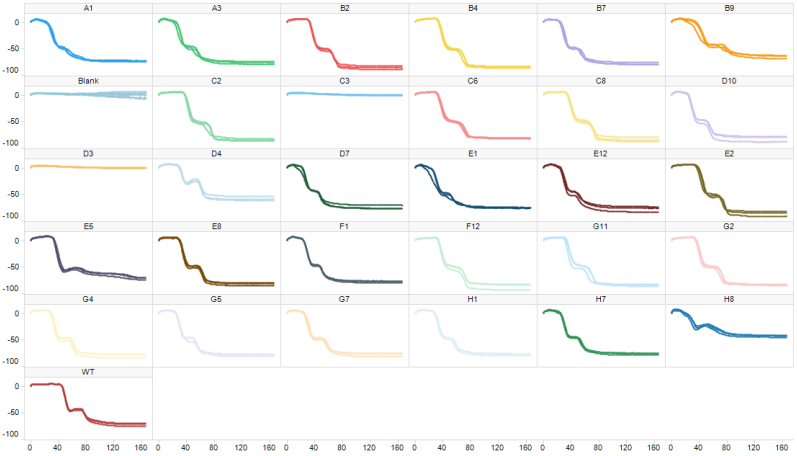
The green values generated by the mutants in figure 5.4b show that there was no growth of any kind on solid 40 % BL, indicating that the concentration of BL is too high and therefore inhibiting growth for fungus B. This result is consistent with what was previously shown in table 5.4 with growth on liquid 40 % BL.
In figure 5.4c the green values of fungus B growing on casein are presented. Over time, the green values of the mutants with confirmed growth, from figure 5.4a, are diminishing in a pattern resembling an inverse growth curve. This could indicate that a correlation between green values and fungal growth on casein could be established in future studies to qualitatively measure and select for limited growth on casein.



(a) Growth curves of screened mutants of fungus B generated through random mutagenesis. Mutants were grown on MEA in MTPs in the Growth Profiler 960.

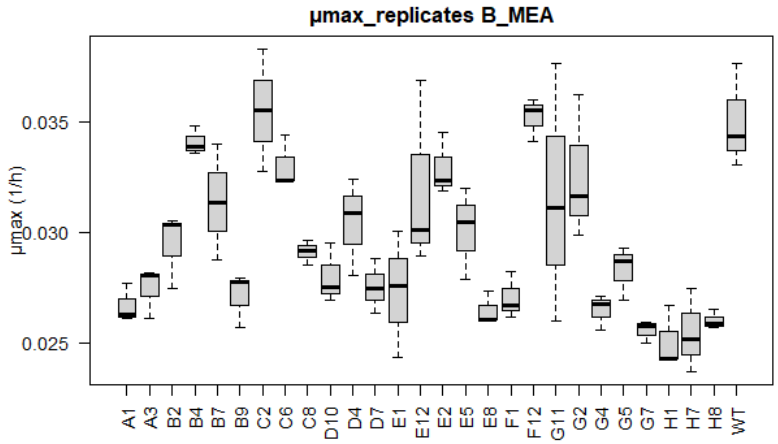


(b) Green values of screened mutants of fungus B generated through random mutagenesis. Mutants were grown on 40 % BL in MTPs in the Growth Profiler 960.

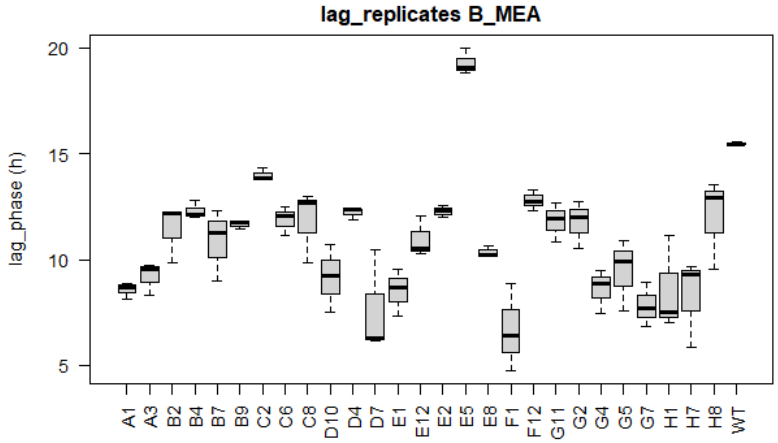


(c) Green values of screened mutants of fungus B generated through random mutagenesis. Mutants were grown on casein in MTPs in the Growth Profiler 960.

Figure 5.4: Growth curves on MEA as well as green values of 40 % BL and Casein. Showing growth of 29 isolated mutants, wild-type (burgundy) and Blank (light blue) wells. All as triplicates and normalized by subtracting the initial value from every subsequent time point.



(a) Maximum specific growth rate for each random mutagenesis mutant for fungus A on PDA



(b) Lag phase for each random mutagenesis mutant for fungus B on MEA

Figure 5.5: Maximum specific growth rate and lag phase for each random mutagenesis mutant for fungus B on MEA. Results higher than the maximum specific growth rate and shorter than the lag phase of wild-type are sought after

Table 5.7: Top 5 mutant strains of fungus B with highest mean μ_{MAX} or lowest mean lag phase in relation to wild-type on MEA

Strain	μ_{MAX} $(\frac{1}{h})$	Strain	Lag phase (h)
C2	0.036	F1	6.7
F12	0.035	D7	7.6
WT	0.035	G7	7.8
B4	0.034	H7	8.3
C6	0.033	E1	8.5
E2	0.033	WT (No.27)	15.5

5.2.2 Mutants Generated by Adaptive laboratory Evolution

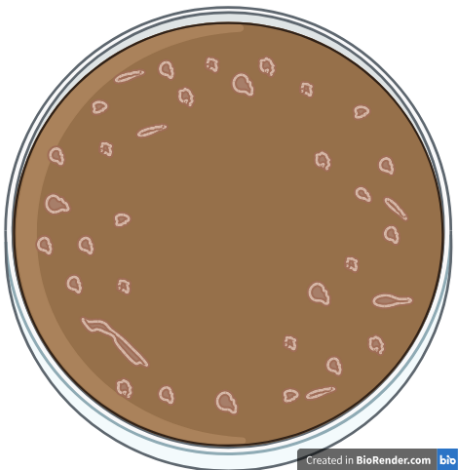
Fungus A managed to adapt to a concentration increase of 25 % from 40 to 50 % BL while also forming smaller mycelial clumps. That can be seen in the example images shown in figure 5.6, illustrating that a change in morphology occurred. Fungus B managed to survive and adapt to a concentration increase of 100 % from 30 to 60 % BL. In addition, B formed much smaller mycelial clumps than wild type, which can be seen in the example images shown in figure 5.7, indicating a morphological change.

(a) Schematic image of contents of shake flask containing culture A after 8 days of growth on 40 % BL

(b) Schematic image of contents of shake flask containing culture A after 8 days of growth on 50 % BL

Figure 5.6: Example images of culture A showing growth after 8 days. 5.6a shows after 2 weeks of ALE. 5.6b shows after 12 weeks of ALE. Created with BioRender.com

(a) Schematic image of contents of shake flask containing culture B after 8 days of growth on 30 % BL



(b) Schematic image of contents of shake flask containing culture B after 8 days of growth on 60 % BL

Figure 5.7: Example images of culture B showing growth after 8 days. 5.7a shows after 2 weeks of ALE. 5.7b shows after 12 weeks of ALE. Created using BioRender.com

The collected dried biomass from ALE mutants on 50 and 60 % BL can be seen in table 5.8, where the mutants can be compared to the biomass generated by wild type at the same concentration. Weight values that were negative have been set to zero. The comparison of A 50 % BL and B 60 % BL was made after twelve weeks at the end of the ALE run, with an extra comparison to fungus B on 50 % BL after seven weeks of ALE. As can be seen in table 5.8, the mutants showed an increased biomass formation in comparison with the wild type for every comparison. Growth was only detected for wild type B on 50 % BL, with no growth detected for wild type A on 50 % BL or wild type B on 60 % BL.

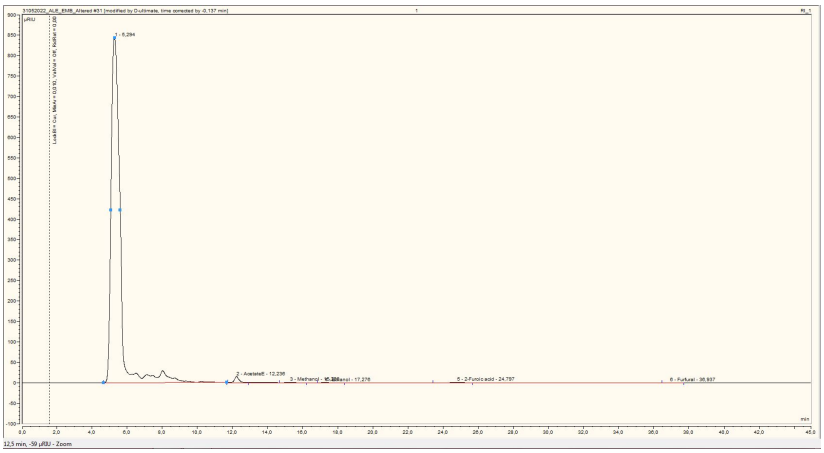
The HPLC analysis of daily samples for tier one evaluation showed inconclusive results as exemplified in figure 5.8, illustrating that the sugar content is coeluting and therefore cannot be quantified using the column HPX-87H Ion Exclusion Column by Aminex. The full chromatogram can be seen in figure 5.8a where only e.g. acetate, methanol and furfural have separated enough to form separate peaks. The coelution of sugars can be better observed in the zoomed in figure 5.8b showing the first 11 minutes of elution. Where no separation of sugars can be seen, making it impossible to draw conclusions on sugar consumption.
Because of the coelution the tier one evaluation was instead based on total amount of biomass produced, resulting in A2 and B1 being the cultures whose mutants were to be evaluated further in tier two.

Due to a loss in sporulation capacity from ALE mutants of fungus A or B tier two evaluation was not performed due to limited time.

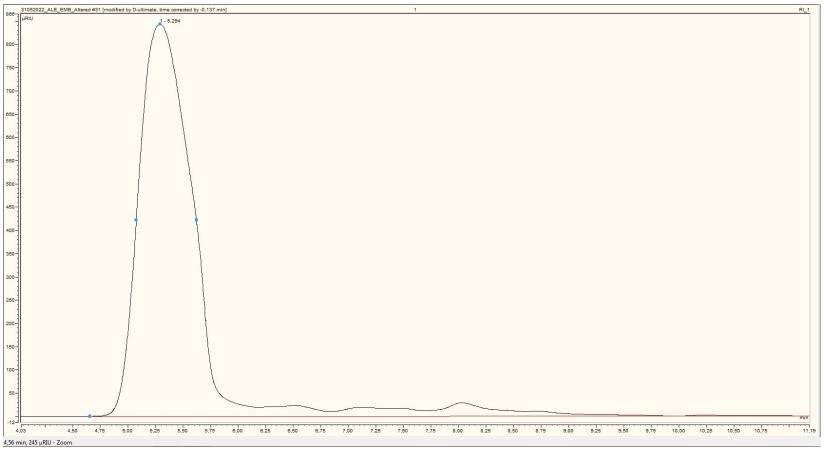
Table 5.8: Biomass and Dry Cell weight (DWC), of fungus A and wild-type A on 50 % BL and fungus B and wild-type B on 50 and 60 % BL, from tier one screening of ALE

Fungus A 50 % BL	Biomass (g)	DCW (g/l)
A1	0.10367	2.073
A2	0.10771	2.154
A1+2	0.0973	1.946
WTA	0 ± 0	0 ± 0

Fungus B 50 % BL	Biomass (g)	DCW (g/l)	Fungus B 60 % BL	Biomass	DCW (g/l)
B1	0.08607	1.721	B1	0,14523	2.905
B2	0.0922	1.844	B2	0,14371	2.874
B3	0.09007	1.801	B3	0,08918	1.784
WTB	0.043 ± 0.029	1 ± 1	WTB	0 ± 0	0 ± 0



(a) Full chromatogram spectra from culture B1 after 24 hours.



(b) Zoomed in chromatogram spectra of coeluting sugar peaks from culture B1 after 24 hours

Figure 5.8: Example chromatogram spectra taken of culture B1 after 24 hours. illustrating the coelution of sugars between t = 5,75 and 10 min

6
Discussion

6.1 Method Development

The method development for random mutagenesis showed that MEA is an ideal solid growth media for germinating spores. As Table 5.1 shows, MEA is the preferred medium for both types of fungi due to the increased germination rate compared to PDA. Especially fungi B showed a substantial increase in germination rate from 0 to 85 %. Meanwhile, fungi A showed a smaller increase in germination rate in MEA compared to PDA, from 22 to 32 %. This was unexpected, as multiple studies have shown that PDA and MEA result in similar germination rates [38][53][40]. A possible explanation could be that the concentration of copper in the media varies, resulting in differing germination rates, as the copper concentrations in differing brands of PDA have shown to affect the germination rates of multiple species of fungi [24].

To acquire a survival rate of less than 1 % a minimum UV irradiation time of 9 minutes for fungus A and 8 minutes for fungus B was needed according to table 5.2 and 5.3. These exposure times were similar to other studies working with fungus, although with higher survival rates of 10 % [27]. This low survival rate was semi-arbitrarily set to 1 % due to the high tolerance filamentous fungi have towards mutations in their DNA [54]. Since part of the aim of this study was to effect the degradation of casein it was assumed that major mutations had to be introduced to generate the desired effect. High exposure would lead to more chances of damaging the pathways used to degrade casein. The main issue with this assumption was that high exposure rate does not necessarily lead to more point mutations as can be seen in similar setups with bacteria [48]. While outside the scope of this study it would be of great interest to, given more time, relate the number of point mutations with exposure rate. This would be very helpful in confirming the assumption of higher exposure time yielding higher mutation rates and thereby creating a more varied pool of mutants to select from.

To determine growth, fungi A and B were screened using the Growth Profiler 960 on four types of solid media: PDA, MEA, 40 % BL and casein. On the potato-based media PDA, fungus A grew well, as seen in figure 5.1a. This figure could then be used to determine both lag phase and μ_{MAX}. The same conclusion could be drawn for fungus B growing on the malt based media MEA by looking at figure 5.4a. Both PDA and MEA are useful for selecting mutants with improved growth characteris-

tics as they provide clear and easily readable curves when using the Growth Profiler 960. This is further corroborated as these media have been used for tier one screening of mutants generated by random mutagenesis previous studies [33]. Screening on 40 % BL proved more difficult as the Growth Profiler 960 was unable to detect fungal growth on the dark solid media. In figure 5.1b, it can be seen that no growth is detected until much later, where an unexplained peak is seen for a majority of mutants generated from fungus A. Comparing these results with figure 5.3, showing biomass formation at a much earlier time point, indicates that the peaks were not correlated with mycelial biomass. Thus, other methods for analyzing growth should be implemented. Other studies on more opaque media utilize liquid cultures that are then harvested at different time points to determine biomass formation [22]. This alternative would be very laborious due to multiple replicates of the same mutant being cultivated for harvesting. Thus, it is impractical to use as a tier one screening method and is better suited to screen for higher tiers. The lack of real time data would also make it difficult to calculate lag phase and μ_{MAX}. Another potential alternative could be to dilute the BL media to 30 % and analyze mutants again. However, this would run the risk of not applying enough selection pressure to allow for favorable mutants to distinguish themselves.

6.1.1 Prospect of Random Mutagenesis

Mutants generated by UV random mutagenesis showed a reduced sporulation ability resulting in long waiting times for each evaluation, as a new generation of spores needed to form in-between each screening tier. Sporulation has been shown to decrease for numerous species of fungi due to UV exposure [46] and especially due to exposure to low wavelengths [14]. These sporulation inhibiting effects are unfortunate since they are especially prevalent when near lethal dosages of high energy UV-light are used [14]. Multiple methods have been used to induce sporulation, including limited nutrients media, temperature changes and differing levels of light exposure [47]. One of the more prevalent methods is exposing fungi to near UV-light for prolonged periods of time followed by darkness. This method mimics natural light exposure and has proved to induce sporulation for multiple species of fungi [46][14]. Continuous UV-light exposure is however not necessarily the best option as many protective measures against UV damage are polygenetic and can take damage during random mutagenesis, thereby reducing the tolerance against UV-exposure [14]. If UV induced sporulation would be applied, special care should be placed in the intensity of the light used.

6.1.2 Prospect of Adaptive Laboratory Evolution

The method developed for ALE shows promise, as the initial experiment for determining starting concentration resulted in biomass formation that was then improved upon. Changing media every week on a set basis was quickly determined to be ill-advised as the fungi would enter a stationary phase that could result in unwanted selection pressure. Thus, instead of set time intervals, the media should be changed

depending on availability of nutrients as the time needed to consume the nutrients available varied as the mixed cultures started to adapt to the BL. Taking care to avoid entering the stationary phase and continuing to remain in the exponential phase will hypothetically lead to a selection pressure for beneficial growth related mutations [20]. During the first five weeks of ALE the aliquots used for inoculation were filtered to remove all biomass and only allow for the passing of spores and smaller mycelial clumps. This was made in the hope of applying a selection pressure for faster germination rate in liquid media and thus reduce the lag phase for growth in new liquid inoculations. This, in turn, would be a useful feature as the fungi were preferably kept in the exponential phase to allow for new mutants to quickly germinate and start competing for viability. For the remaining weeks of ALE the inoculum aliquot was instead taken by transferring media without filtration. By avoiding larger biomass clumps, an intended selection pressure for smaller and more dispersed mycelial clumps was placed. However, as has been previously stated it is possible for unintended adaptations to emerge [32].

6.1.3 Change in Selection Pressure

Determining when to increase the concentration of BL was difficult to assess objectively. From literature research there were no studies found on any criteria used for when to increase the concentration of a toxic compound. Instead unique criteria had to be established for each ALE experiment based on what is being selected for and the microorganism used. As such, the growth for each flask was observed individually and a qualitative assessment for each of the two fungus species was made for when they were deemed ready for an increase in concentration. Future work should therefore develop a quantitative threshold for increases in media proportions. As an example of thresholds, spore concretions or amount of produced biomass over a predetermined time period are phenotypes that could be utilized. Media composition is also an interesting avenue to use for determining when media increase should be applied. For example, when sugar concentrations start to quickly diminish an increase of concentration can be applied. Although increases in stress factors should be applied with caution: the risk of killing the culture is always present and therefore saving spore samples is very important. In addition, it is recommended to make two new liquid inoculations when increasing concentrations. One with the increased concentration and one with the same concentration as previously, to ensure the ALE continues even if the culture does not survive in the new media. This also reduces the chance of losing cultures during ALE.

Finally, the media volumes of 50 ml proved to be a good amount for performing ALE, as the fungi were given ample room to grow and their morphologies were clearly visible. Future studies should also consider the media volumes as smaller volumes could result in more cultures being worked on simultaneously. Cultivating mutants in micro-tither plates would increase the potential for new mutants dramatically as the number of mixed cultures could be increased. Although the use of micro-tither plates would make it difficult to observe morphology [34][35].

6.2 Mutant Screening

Ideally the mutants would be screened in multiple tiers to isolate a pure culture with the best characteristics. However, due to time limitations the mutants were only able to be screened in one tier as mixed cultures. For the mutants generated by random mutagenesis in the Growth Profiler 960 the tier one screenings performed on fungus A and B had to be redone multiple times due to the Growth Profiler 960 having issues with detecting fungal growth. The ALE mutants were screened by HPLC and biomass formation to find which of the three mixed cultures had the highest growth rate. Due to not having time for a second tier of evaluation identifying a single best pure mutant strain was not achieved. However, promising candidates were identified for both random mutagenesis and ALE. These mixed cultures could in the future be isolated by repeatedly streak-plating mixed culture spores, cultivating them and collecting individual colonies of fungi.

6.2.1 Screening Random Mutagenesis Mutants

Due to the high noise levels when screening random mutagenesis mutants of fungus A on solid 40 % BL on the Growth Profiler 960, image manipulation was performed on all images taken to attempt to increase contrast and reanalyze the images. This gave unsatisfactory results as fungal growth was still not detected until late stage growth when a large amount of biomass had already formed. Therefore a qualitative assessment was made, looking at one of the images generated by the Growth Profiler 960, seen in figure 5.3. The fungal mutants with the most generated biomass were chosen as they were assumed to be the ones with the lowest lag phase and highest μ_{MAX}. As each mutant was cultivated as a triplicate the wells were evaluated as an average. These mutants should in future studies be evaluated for their growth on 40 % BL as to confirm the qualitative assessment. This could be done by measuring the growth using absorption at 630 nm instead of relying on green values as previous studies have successfully linked absorption with growth [30]. In addition, a better understanding of the growth of A mutants on casein is also needed to properly select an ideal mutant.

The mutants generated from fungus B were more successful in selecting an ideal mutant. Mutant B9 was shown to have the lowest μ_{MAX} and longest lag phase combination indicating that this mutant grows the worst on casein. Low casein consumption is a sought after trait. However, as no growth on solid 40 % BL was observed for fungus B mutant B9 future studies should focus on evaluation on BL. A lower concentration of BL is necessary and a recommended start would be 30 % BL as the ALE experiment has shown that wild type B can grow on liquid 30 % BL. However, the other fungus B mutants should also be evaluated on 30 % BL as it is possible that another mutant has better growth on BL and thus is still interesting for continued work.

Screening for lack of casein consumption for fungus A and B gave inconclusive results. A noticeable change in colour of casein over time was detected for fungus

B as can be seen in figure 5.4c, where an interesting decrease in green values can be seen, with a plateau between t = 40 and 60 hours. The plateau is difficult to explain and the causation can only be speculated on, due to limited previous research on the topic. A possible explanation could be that this change in color is caused by fungus B consuming all available nutrients and having to alter its protein secretion to access nutrients deeper inside the agar. Another reason could be that the solid casein media dries during the screening process, making it difficult for fungus B to access the casein as it has dried up and compacted. Since little research on change in casein color due to digestion is available, it can not be definitively said that this indicates growth. However, this could be of interest for further studies as comparing the blank wells without inoculation with inoculated wells shows a clear difference in green values. Would a link between casein color and fungal growth be established, this method of analysis could then be used to determine lag phase and μ_{MAX}. A potential way to establish a link would be to measure the color at certain time points and then extract the casein agar, then treat it before using reverse phase chromatography to determine casein content [18][13]. Alternatively, protease activity could be measured by cultivating the fungi in liquid media containing casein and then evaluating the amount of protease using SDS-PAGE [23].

6.2.2 Screening Adaptive Laboratory Evolution Mutants

Both ALE mutants have shown a clear improvement in morphology. As can be seen in the example images seen in figure 5.6 and 5.7, the fungi both grew in smaller clumps than in the beginning of the ALE. This might be a result of the decision to only transfer small mycelial clumps when doing new liquid inoculations. Alternatively, it could be an effect of the increase in stress resulting from growing in BL since the stressors could be reducing cell viability reducing the formation of long hyphens [45]. These smaller mycelial clumps are nevertheless advantageous when scaling production to larger bioreactors as the smaller clumps will create a more homogeneous reactor while avoiding the non-Newtonian properties that result from more dispersed mycelial growth [45]. Future work should continue to apply selection pressure by increasing the concentration of BL to an ideal 100 % for both fungus A and B. Reaching this ideal threshold would reduce the need for preprocessing the media when scaling the bioreactors to an industrial scale and allow the fungi to grow in higher concentration of nutrients. Future work should also continue to monitor mycelial clump size to determine if there is a correlation between size and stress increase. If no such correlation can be established further review of the method of transferring small mycelial clumps should be made to ensure the methods validity and ways to improve it.

By looking at the initial biomass growth in table 5.4 it is clear that the wild type of fungus A has better growth in both 30 and 40 % BL than the wild type of fungus B. This would indicate that fungus A is more well suited to grow on BL from the start. This changed during the ALE run, as table 5.8 shows, with the mutant cultures of fungus B vastly outperforming B wild type, while fungus A mutants also showed

clear improvements in comparison to wild type A. The fact that B was able to grow well on double the initial concentration of BL indicates that B has a very high potential for further improvements. As such, fungus B is of extra interest for further studies, with there being no indication that adaptations to higher concentrations of BL have stagnated and that B has reached its adaptive limit. Thus, the ALE work on the mixed cultures of B1, B2 and B3 could be continued in further studies.

It is important to note that it has not been determined that fungus A has reached its highest potential either. Although, since the culture A3 died out during the ALE experiment it appears that A might have a lower or at least slower potential for adaptations than fungus B. Thus further ALE experiments should either further develop the mixed cultures of fungus A.

As the HPLC data collected was unsatisfactory due to problems with coelution, as seen in figure 5.8, the basis for selecting the best culture was the produced biomass. With this criterion, culture A2 and B1 were selected as the two best mixed cultures developed during ALE. However, there are small differences in biomass between the three mixed cultures of fungus A and B making the selection practically arbitrary. Future work should therefore continue experimenting with HPLC-methods to eliminate coelution and separate the sugars in BL. This would allow the pure cultures isolated from the mixed cultures A1 and B2 to be evaluated on e.g. sugar consumption and possibly growth rate.

7
Conclusion

Using random mutagenesis to lower lag phase and increase growth rate of the two filamentous fungi A and B proved difficult to determine quantitatively. While fungus A showed promising growth curves on solid PDA, it was impossible to determine the growth curve on the industrial by-product denoted BL. Because no growth curve on BL could be established, fungus A had to be evaluated only on PDA where higher growth rate and lower lag phase could be identified for mutants generated by random mutagenesis. Similar issues were present for fungus B, with no quantitative way of determining growth on solid BL. Growth on solid MEA was used to evaluate growth characteristics instead. While many mutants showed a lower lag phase no mutant had any significantly higher growth rate than wild type. Due to the issues with evaluating fungus A and B on solid BL, future work should prioritize improving the evaluation methods to increase the contrast between biomass and the opaque colored BL.

Evaluating the degradation of casein in solid media proved challenging as no clear correlation with changes in casein color and fungal growth could be established. If this correlation can be made in future studies, fungus A and B can be evaluated on the basis of casein degradation as a prominent change in casein coloration was observed during cultivation on solid casein media.

Using adaptive laboratory evolution to increase tolerance to BL proved successful. A high increase in biomass formation was observed for both fungi A and B when increasing the concentration of BL stepwise. As wild type fungus was unable to grow in increased concentration of BL the mutants produced using ALE could be useful for industrial use. Especially if the tolerance of BL could be increased to 100 %. Morphology was also successfully changed during the ALE experiment, with smaller mycelial clumps being formed as concentrations were increased.

Lag phase was difficult to evaluate as the sporulation capability of fungi A and B was lost or severely reduced when growing in liquid BL at high concentrations. HPLC was attempted as a way of determining growth characteristics for fungus A and B. However, the complex media coeluted and inconclusive results were produced. Thus, no lag phase could be established. To be able to evaluate lag phase it is imperative to be able to further develop the HPLC protocol and study the consumption of sugars to determine when biomass formation begins.

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A

Media Recipes

All media recipes used

A.1 Solid Media recipes

Solid media recipes of PDA, MEA, solid BL media of X % and solid casein media

Table A.1: Recipe for solid media of PDA 2 % agar

Amount	Compound
39 g/l	PDA
5 g/l	Agar

Table A.2: Recipe for solid media of MEA 2 % agar

Amount	Compound
30 g/l	Malt extract
20 g/l	Agar

Table A.3: Recipe for media of solid X % BL with 1,5 % agar

Amount	Compound
x %	BL
15 g/l	Agar
4 g/l	KH_2PO_4
13,6 g/l	$(NH_4)_2SO_4$
0,8 g/l	$CaCl_2$
0,7 g/l	$MgSO_4$
10 mg/l	$FeSO_4$
3,2 mg/l	$MnSO_4$
2,8 mg/L	$ZnSO_4$
4 mg/l	$CoCl_2$
3,5 mg/l	$CuSO_4$

Table A.4: Recipe for media of solid casein media with 1,5 % agar

Amount	Compound
40 g/l	Casein
15 g/l	Agar
4 g/l	KH_2PO_4
13,6 g/l	$(NH_4)_2SO_4$
0,8 g/l	$CaCl_2$
0,7 g/l	$MgSO_4$
10 mg/l	$FeSO_4$
3,2 mg/l	$MnSO_4$
2,8 mg/L	$ZnSO_4$
4 mg/l	$CoCl_2$
3,5 mg/l	$CuSO_4$

A.2 Liquid Media

Liquid media recipes of liquid BL media of X % and PDB

Table A.5: Recipe for liquid media of X % BL

Amount	Compound
x %	BL
4 g/l	KH_2PO_4
13,6 g/l	$(NH_4)_2SO_4$
0,8 g/l	$CaCl_2$
0,7 g/l	$MgSO_4$
10 mg/l	$FeSO_4$
3,2 mg/l	$MnSO_4$
2,8 mg/L	$ZnSO_4$
4 mg/l	$CoCl_2$
3,5 mg/l	$CuSO_4$

Table A.6: Recipe for liquid media of PDB

Amount	Compound
39 g/l	Malt extract

B

ALE Logbook

This logbook chronicles the changes made during the ALE run with the dates of each change as well as what concentration the current bottle being inoculated has at the end of the given date.

Table B.1: Dates for new liquid inoculations, concentration increases and change of media during ALE

Date	Liquid inoculation	Conc. of BL for A	Conc. of BL for B
14-feb	First inoculation of ALE	40 %	30 %
21-feb	New liquid inoculation for A and B	40 %	30 %
28-feb	New liquid inoculation for A and B	40 %	30 %
4-mar	Concentration increase for B to 40 %	40 %	40 %
7-mar	Concentration increase for A to 50 %	50 %	40 %
9-mar	New liquid inoculation for B	50 %	40 %
14-mar	New liquid inoculation for A and B	50 %	40 %
17-mar	New liquid inoculation for B	50 %	40 %
18-mar	New liquid inoculation for A Concentration increase for B to 50 %	50 %	50 %
21-mar	New liquid inoculation for A and B	50 %	50 %
24-mar	New liquid inoculation for B	50 %	50 %
25-mar	Changed media for A to PDB	Na	50 %
28-mar	New liquid inoculation for B	Na	50 %
31-mar	Concentration increase for B to 60 %	Na	60 %
4-apr	Changed media for B to PDB	Na	Na
7-apr	Changed media for B to BL 60 %	Na	60 %
11-apr	Changed media for A to BL 50 % New liquid inoculation for B	50 %	60 %
14-apr	New liquid inoculation for B	50 %	60 %
19-apr	New liquid inoculation for B	50 %	60 %
20-apr	New liquid inoculation for A	50 %	60 %
22-apr	New liquid inoculation for B	50 %	60 %
26-apr	New liquid inoculation for A and B	50 %	60 %
2-may	Finished ALE	50 %	60 %

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