PRODUCTION OF ARYL ALCOHOL OXIDASE UNDER GROWTH-LIMITED CONDITIONS USING AN *ASPERGILLUS NIDULANS* CELL FACTORY

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Abstract:

Trickle bed reactors (TBRs) have potential to be used for enzyme production due to reduced energy demands and easier product recovery compared to traditional submerged fermentations. The ligninolytic consortium is a group of enzymes found in microorganisms capable of degrading lignin. The identification and production of these enzymes could enable new pretreatment processes that compete with the harsh and costly chemical and/or physical methods for lignin removal from biomass in biorefineries. In the present study one of the enzymes from the ligninolytic consortium, an aryl alcohol oxidase, was produced using an *Aspergillus nidulans* cell factory. The strain was constructed using DNA mediated transformation and it contained a pyridoxine auxotrophic marker to regulate growth. Growth limitation can prevent clogging issues in the TBR system associated with excessive growth. The most important challenge observed during growth limitation conditions was the production of melanin, which was more prominent when the cell factory was cultured in the TBR than in shaken flasks, probably due to higher oxygen availability in the TBR. Different approaches were evaluated to reduce or suppress melanization of the *A. nidulans* cultures. Reducing copper concentrations and including pigmentation inhibitors in the media, such as tropolone or ascorbic acid, reduced melanin formation and enhanced aryl alcohol oxidase activity in submerged cultures. When continuous aryl alcohol oxidase production was established in the TBR, the use of media with no copper and 5 g/L ascorbic acid paired with 110 mL/min air flow rate reduced melanization and increased enzyme activities as opposed to other conditions. Aryl alcohol oxidase productivities were increased 20 to 140% when melanization was prevented, reaching a maximum of 2.4 U/mL*h. Three different random packing materials were evaluated as inert support in the TBR, two made of HDPE plastic and one of stainless steel. Additionally, three dilution rates (0.034, 0.038, 0.048 h^{-1}) were used. All packing materials were suitable for *A. nidulans* culture in a TBR and no significant differences were observed among them. Increasing the dilution rate favored melanin and acid formation, lowering enzyme production.

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CHAPTER I

INTRODUCTION

Depletion of non-renewable resources has raised concerns about issues such as environmental pollution, climate change, and national security. Discussion about oil and gas availability, among other resources, has dominated the conversation because of their critical role as energy sources. According to the US Energy Information Administration, 63% of the energy consumed in the country in 2013 was produced from petroleum and natural gas (EIA, 2015). The concept of "Peak Oil", the point at which the maximum level of production of conventional oil is reached, has been consequently discussed during the last decades (Chapman, 2014). A horizon in which non-renewable resources are scarce has stimulated interest in biotechnology. In 2012, the White House issued the National Bioeconomy Blueprint, a document that describes strategic objectives to expand the US bioeconomy (USWH, 2012). The language of this document included any sector with biological-based activities, from genetic engineering to biofuel production. Although energy (or bioenergy) is expected to continue representing a key portion of the bioeconomy, the term bioeconomy includes all processing, marketing, transportation and consumption of biologically-derived products (Johnson & Altman, 2014). The interest for fostering a biobased economy also has strong support in the European Union, where the European Commission launched the Bioeconomy Strategy in 2012 to

invest in research and innovation, promote policy interaction and stakeholder engagement and enhance markets and competitiveness (EC, 2016) .

Industrially, a bioeconomy develops when enzymes and microorganisms are used to obtain biobased products for a number of applications, including food, specialty chemicals, bioenergy, textiles, and paper (McCormick & Kautto, 2013). Other aspects of the bioeconomy include improvements in agriculture through genetic modifications and health-related products like pharmaceuticals (McCormick & Kautto, 2013). As stated above, many of the products to be developed rely on the existence or future availability of biocatalysts, either in the form of enzymes or as microbes. Enzymes such as proteases, amylases and cellulases are already produced for industrial applications (DiCosimo et al., 2013) Not surprisingly, the global market for enzymes has grown from $2.2 billion in 2004 (BCC-Research, 2004) to $4.8 billion in 2013 with expectations of a continuous annual growth of 8.2% until 2018 (BCC-Research, 2014). Figure 1.1 shows the global market size through 2013 and the 2018 projection, all figures released by BCC Research, a market research company that covers changes related to science and technology (BCC-Research, 2004; BCC-Research, 2014; BCC-Research, 2012).

The use of enzymes is critical for the second generation of biofuels, which are those derived from biomass feedstocks. Cellulase and hemicellulase enzymes are used to break down cellulose and hemicellulose in these feedstocks into monomeric sugars that can be fermented; however, enzymes not only have applications in bioenergy. For example, glucose isomerase is used to produce high-fructose syrup in the food industry (Miguel et al., 2013), laccase is used in the textile industry to treat denim without chlorine, and proteases have been used in detergent production (Novozymes, 2013). Figure 1.2 shows the distribution of sales by region and by sector of one of the biggest manufacturers of enzymes in the world, Novozymes.

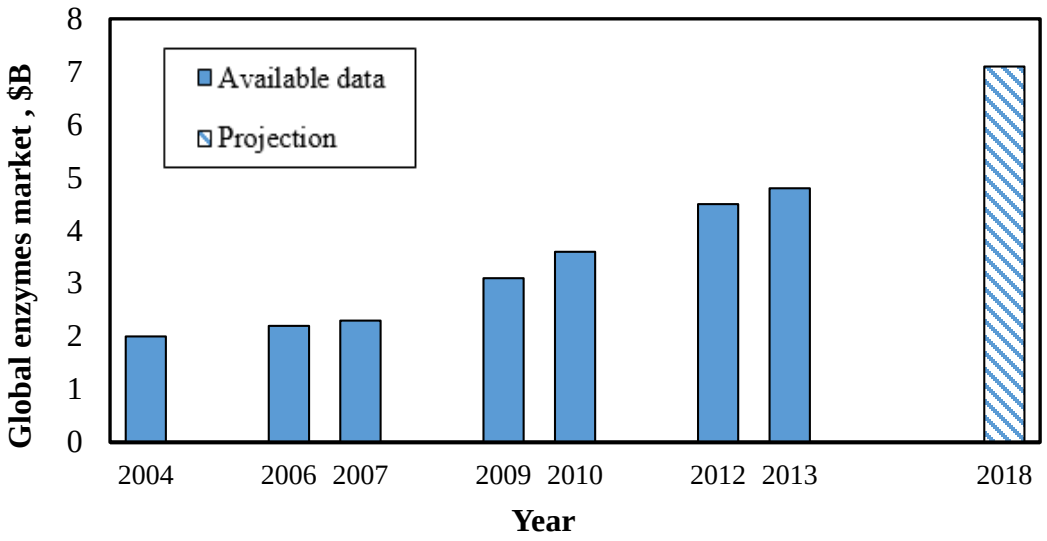

Figure 1.1. Global market for enzymes since 2004 (BCC-Research, 2004; BCC-Research, 2014; BCC-Research, 2012)

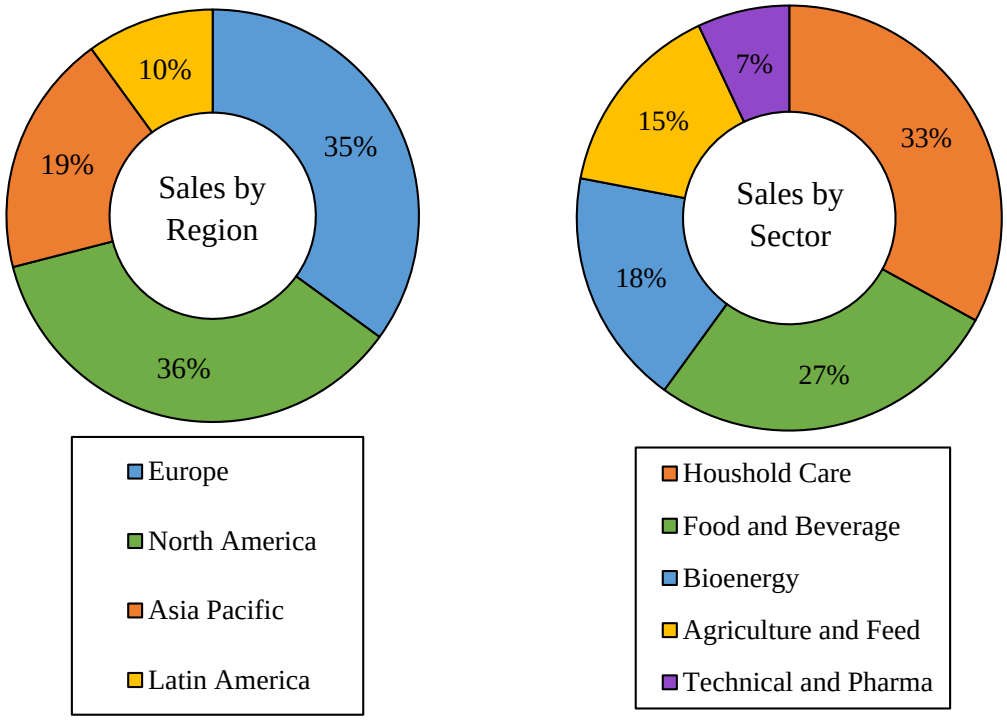

Figure 1.2. Market share of enzyme sales of Novozymes in 2015 by region and by sector (Novozymes, 2015)

While some enzymes are still obtained from animal or plant tissues, most current enzyme production is achieved by culturing bacterial or fungal strains (Leisola et al., 2001). Particularly, most enzymes are derived from *Aspergillus*, *Bacillus*, and *Kluyveromyces* species (Sarrouh et al., 2012). The following stages have been described for an enzyme production process (Leisola et al., 2001):

1. Identification of the enzyme
2. Selection of a production strain
3. Construction of an overproducing strain by genetic engineering
4. Optimization of culture medium and production conditions
5. Optimization of recovery process
6. Formulation of a stable enzyme product

Each of the steps described above has implications on the final performance and price of the product. For example, during the identification stage it is important to understand the requirements of pH and temperature for the specific enzyme in order to determine if those conditions exist during the desired application. When constructing a mutant overproducing strain, the yield of enzyme obtained per substrate consumed is essential to determine the economic feasibility of the process. Similarly, optimization of production conditions can significantly increase the product yields and favor the overall process feasibility. Finally, the recovery process used impacts the cost of the product; for example, if the protein is excreted into a liquid medium with a very low solids concentration its purification will be much easier (and cheaper) than when present in a highly viscous solution that will require additional energy towards separation steps. In summary, the enzyme production process needs to be well designed and each step should be planned according to the others so the cost of the final product can be optimized.

Traditionally, enzymes have been produced using the technology called submerged fermentation (SmF), in which the microorganism is grown in liquid medium where it releases the

protein (Couto & Toca-Herrera, 2007). The enzyme has to be recovered by separating solids (cell mass) and liquid, and subsequently concentrating it by ultrafiltration and/or using chromatographic methods. This methodology is well established and it has advantages such as easy process control and available continuous production technology (Pandey, 2003). However, there are issues related to mass transfer of oxygen in the liquid medium, shear stress to the cells, uncontrolled growth, and a more difficult separation of the product from the typically viscous cell-medium mixture (Müller et al., 2015). An alternative technology called solid state fermentation (SSF) uses little or no free water, and has been praised for low capital and operational costs, potential higher productivities, and lower downstream costs due to the cell-free liquid product obtained (Diaz-Godinez et al., 2001; Krishna, 2005; Viniegra-González et al., 2003). On the other hand, difficulties in process control and continuous operation have been also highlighted (Pandey, 2003).

In between these two options, a third technology that combines characteristics of both SmF and SSF using a trickle bed reactor (TBR) has been proposed (Müller et al., 2015). In this case, free liquid is present in the system, but in lower amounts than in SmF. The medium is pumped to the top of a column filled with an inert packing material where the cells grow, and then it trickles down the bed. Process control is easier than in SSF and continuous operation is possible. Downstream costs are expected to be less expensive than in SmF because the cell mass is retained in the reactor system. However, excessive cell growth can cause clogging issues both in the column and the pipelines. This problem can be potentially solved by operating the reactor at near zero growth conditions. Recent studies have proposed using mutant fungal strains with a genetic marker that prevents growth when an essential vitamin is removed from the medium (Müller et al., 2014). This concept was proven using a TBR to produce xylanase under growth limitation conditions using a pyridoxine marker (Müller et al., 2015).

Enzyme cost has been one of the bottlenecks of biofuel production from biomass. Currently, cellulases and xylanases are used to break down the polymeric structure of biomass into monomeric

sugars. Even though literature estimates of the contribution of the cost of enzymes to the cost of the overall process have varied between 0.10 and 0.40 $/gal ethanol, a detailed study stated that the real price contribution would be between 0.68 and 1.47 $/gal ethanol (Klein-Marcuschamer et al., 2012). The National Renewable Energy Laboratory (NREL), however, estimated that the cost of enzyme was 0.34 $/gal ethanol in a technoeconomic analysis of ethanol from corn stover in 2011 (Humbird et al., 2011). A more recent study that modelled the cost of cellulase for ethanol production concluded that the cost of enzymes could be reduced from 0.78 $/gal ethanol to 0.23 $/gal ethanol when implementing an on-site and integrated cellulase production process compared to off-site production (Johnson, 2016). Optimization of the enzyme production process based on the six steps mentioned earlier could continue to reduce the cost of enzymes.

Another bottleneck of the biomass to biofuel process is pretreatment of the feedstock. This step is necessary prior to enzymatic hydrolysis, in order to remove lignin and reduce the crystallinity of the material (Scheper et al., 2008). This is typically achieved by using harsh conditions (temperature and/or pressure) and chemicals. A Department of Energy (DOE) report estimated that about one third of the total cost of the process was contributed by the cost of pretreatment alone (DOE, 2011). If milder conditions could be used during this step, it is logical to think that this cost would be reduced. One potential solution could be identifying and producing a new range of enzymes that are able to attack the lignocellulosic structure. It is now known that some microorganisms in nature secrete enzymes that can degrade lignin (Brown & Chang, 2014). Based on their ability to break down a polymer that is considered very recalcitrant, this group of enzymes has been referred to as the ligninolytic consortium (Alcalde, 2015). Some enzymes included in the consortium are lignin peroxidases, manganese peroxidases, versatile peroxidases, hydrogen peroxide producing oxidases, among other proteins (Alcalde, 2015). If their synergistic action could be replicated in vitro, a new generation of pretreatment would emerge. This pretreatment could be performed at low temperatures and pressures without the need for harsh chemicals, and this step could be combined with enzymatic

hydrolysis in order to simplify the overall process. It is clear that research in the areas of reactor design and development of novel enzymes could lead to a decrease in the costs of production of cellulosic biofuels. Some of these advances in the field could positively impact other processes that require cheap enzymes in order to become economically feasible.

This dissertation presents the results of three studies that encompass the two aspects mentioned above: reactor operation and production of a novel enzyme that could have applications in the bioenergy industry. One aspect of this dissertation was to advance the knowledge on using TBRs for enzyme production. Instead of focusing on the commercial SmF or on the alternative technology SSF, the hybrid TBR technology was investigated. In order to regulate the growth of the *Aspergillus nidulans* cell factory to avoid clogging issues inside the reactor, a pyridoxine marker was included in the genotype of the microorganism, similar to what had been done in a previous study (Müller et al., 2015). The objectives were to obtain a more stable fermentation process by suppressing the production of secondary metabolites, such as melanin, that are initiated when the culture is submitted to growth limitation and to evaluate the reactor parameters dilution rate and type of packing material in the TBR for a better understanding of the technology. In addition to addressing reactor operation for enzyme production, this dissertation investigated the production of one of the enzymes from the ligninolytic consortium: aryl alcohol oxidase. This enzyme has been found to participate in the first steps of lignin degradation by ligninolytic microorganisms, by converting aryl alcohols to the corresponding aldehydes and releasing hydrogen peroxide, which can be then used by other enzymes in the consortium to continue the degradation process. The scarcity of research studies related to production and use of aryl alcohol oxidase for biomass conversion warrants this line of research.

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CHAPTER II

OBJECTIVES

1. Construct and evaluate an *A. nidulans* cell factory with a pyridoxine marker that overexpresses an aryl alcohol oxidase (MtGloA) enzyme from *Myceliophthora thermophila*.

2. Develop methods to mitigate melanization of *A. nidulans* cultures triggered by growth-limited conditions in order to favor aryl alcohol oxidase expression.

3. Establish a stable continuous aryl alcohol oxidase production using a growth-limited culture of the *A. nidulans* cell factory in a trickle bed reactor.

CHAPTER III

REVIEW OF LITERATURE

3.1 Reactors for enzyme production: submerged versus solid state fermentation

Most microorganisms –including filamentous fungi- tend to adhere to solid supports rather than live as suspended free cultures (Couto & Toca-Herrera, 2007; Hölker et al., 2004). Fungi are good candidates for biofilm formation due to their secretion of exoenzymes for absorptive digestion of nutrients and their apical hyphal growth (Harding et al., 2009). A biofilm has been defined as a "microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan & Costerton, 2002). Biofilm development involves the following stages: adsorption of single cells to a surface, active attachment to it, formation of microcolonies by cell aggregation, and/or multiplication, maturation, and dispersal (Harding et al., 2009). Several authors have reported advantages of immobilized communities of microorganisms over dispersed cells. These benefits have been reviewed by Couto and Toca-Herrera (2007) and include higher resistance to antimicrobial agents, environmental changes (e.g.

pH or toxic chemical concentrations) and shear stress. Moreover, immobilized cultures of recombinants showed fewer genetic instability issues than non-immobilized cells.

In bioprocess engineering, the use of immobilized fungal cultures for production of enzymes or other products is associated with solid state fermentation (SSF) technology, in contraposition to submerged fermentation (SmF). The main difference between these two technologies is that in SSF microorganisms grow on a solid support material with absence or small amounts of free liquid (Krishna, 2005); whereas, in SmF microorganisms grow inside a rich liquid medium (Couto & Toca-Herrera, 2007) with air supply when aerobic conditions are a requirement.

Industrial production of enzymes has been carried out mostly using SmF technology (Viniegra-González et al., 2003). The global market of enzymes is of great importance in biotechnology, as it was estimated to represent $4.5 billion in 2012, $4.8 billion in 2013, and with expectations of 8.2% annual growth rate until 2018 (BCC-Research, 2014). Due to many potential advantages of SSF versus SmF, there is an increasing interest in research for transitioning towards industrial solid-state production of enzymes. Table 3.1 summarizes some advantages and disadvantages of both SSF and SmF technologies. The main benefits listed for SmF are related to the fact that this technology has been adopted and developed in western countries for more than seventy years (Pandey, 2003), thereby making it available at commercial scale. However, several challenges exist in SmF, mostly related to the increased apparent viscosity of the broth due to dispersed mycelia growth. This increase in liquid viscosity causes limitations on nutrients and oxygen transfer. In addition, product recovery is more difficult because of its coexistence with the microorganisms inside the medium (Mueller et al., 2015). On the other hand, SSF is a promising technology because of potential lower costs associated with lower required fermentation volumes, and lower energy and water usage. Moreover, higher volumetric productivities and concentrations of products have been claimed (Krishna, 2005). For

Table 3.1. Advantages and disadvantages of SSF and SmF technologies (Couto & Toca-Herrera, 2007; Krishna, 2005; Mueller, 2012).

Technology	Advantages	Disadvantages
SSF	-Use of raw materials as support and substrate -Low capital cost (lower volume reactors) -Low energy expenses -Lower cost downstream processing (cells are easily separated) -Potential higher volumetric productivity -Higher concentration of products -Easier control of contamination	-Heterogeneous physiological, physical and chemical environments -Difficulties in process control (heat, moisture, aeration) -No continuous production in traditional SSF
SmF	-Well established at commercial scale -Well-known models exist -Good process control -Continuous production	- High apparent viscosity because of mycelia growth -Blockage/clogging issues due to uncontrolled growth -Limitation of oxygen mass transfer -Shear stress to mycelia -Lower productivities -Separation of product more challenging

example, higher titers and productivities of exo-pectinase were achieved with SSF compared to SmF using *A. niger* (Diaz-Godinez et al., 2001). Similarly, enhanced invertase production with SSF compared to SmF has been reported (Romero-Gomez et al., 2000). On the disadvantages side, SSF is not able to provide complete homogeneous environmental conditions due to physical and chemical gradients. As a consequence, SSF presents a more challenging process control situation than SmF. The disadvantages associated with traditional SSF can be palliated or even eliminated by using hybrid approaches such as trickle-bed reactors (TBRs). This reactor configuration combines features of SmF and SSF, and will be described and reviewed in a separate section.

3.1.1 Reactors for SmF

SmF consists of fermentations containing excess water. The microorganisms, e.g. filamentous fungi, grow inside a nutrient-rich liquid media with high oxygen concentrations (Couto & Toca-Herrera, 2007). There are mainly two different morphological forms of fungal growth in submerged cultures: growth of dispersed mycelia and pellet formation. The dominance of one or another morphology is determined by different factors such as strain type, media composition, agitation, medium temperature, and viscosity (Liao et al., 2007; Metz & Kossen, 1977). When the dominant morphology is the growth of dispersed mycelia, there is an increase of the apparent viscosity of the broth that leads to challenges such as increased mass transfer limitations. When fungi grow in this manner, the rheological behavior of the broth is non-Newtonian, and more specifically, these solutions behave as pseudoplastic fluids (the apparent viscosity decreases when shear force increases) (Gibbs et al., 2000). The other reported morphological form of filamentous fungi is growth as pellets. Pellet formation may be due to different mechanisms such as agglomeration of hyphae, agglomeration of spores and hyphae, or agglomeration of solid particles and hyphae (Metz & Kossen, 1977). This morphology is generally considered more beneficial for bioprocessing, since the fermentation broth tends to

behave as a Newtonian fluid, therefore reducing mass and oxygen transfer limitations (Liao et al., 2007). Currently, most commercial enzyme production is being carried out using SmF techniques. For example, the website of the largest enzyme manufacturer in the world, Novozymes (Denmark, European Union), mentions SmF as its enzyme production technology (Novozymes, 2013).

Different reactor configurations are available for SmF. The simplest one is the stirred tank reactor (STR) or similar configurations. This is a well-known culturing technique in bioprocessing where liquid medium inside a reactor vessel is continuously agitated and oxygen is sparged in the medium so it can be delivered to the microorganisms. The process can be carefully controlled by monitoring and manipulating parameters such as pH, temperature, dissolved oxygen concentration, carbon dioxide release, cell concentration, or enzyme activity. Novozymes claims to operate reactors as large as 1,000 cubic meters (Novozymes, 2013). A parameter of major importance in STR fermentation is the agitation rate, which relates to shear stress, mixing rate, and broth rheology. Rushton impellers traditionally have been used; although, they are known to provide high shear stress (Gibbs et al., 2000), which led to investigation of other stirring systems such as Effigas impellers (Berovic & Popovic, 2001) or two-speed dual impellers (Solomons, 1980). However, all these alternatives rely on mechanical agitation, which requires a significant energy input and, therefore, high costs. Hence, reactor configurations with no mechanical stirring are desired.

Examples of configurations with no mechanical stirring are bubble column reactors, and airlift reactors. Bubble columns are cylindrical, vertical reactors containing liquid media in which oxygen or air is sparged at the bottom and flows upwards. The medium can be recirculated in co-current or counter-current directions with respect to the direction of gas flow. Airlift column reactors are essentially modified bubble columns. They include an inner cylinder in which oxygen is gassed, and then oxygen moves to the outer cylinder. In this two-region configuration, mixing

is achieved by difference in densities, improving mass transfer and reducing shear forces (Chisti & Moo-Young, 1987). Sometimes, this non-mechanical mixing system is achieved by an external loop. The use of bubble and airlift reactors was studied for production of citric acid using *Aspergillus niger* in a broth with pseudoplastic behavior (Berovic & Popovic, 2001). The external loop airlift reactor was selected as more suitable for this application than the bubble column, and it showed comparable results to fermentation in stirred tanks. Stable operation was also achieved for lacasse production by *Trametes versicolor* using an airlift bioreactor (Rancaño et al., 2003). Another study compared the performance of a stirred tank reactor and an air lift reactor for production of lacasse and Mn-peroxidase using the white-rot fungus *Panus tigrinus* (Fenice et al., 2003). The stirred reactor was superior in terms of maximum enzymatic activity of laccase (4,600 versus 4,300 U/L), but Mn-peroxidase activity was higher in the airlift bioreactor (410 versus 360 U/L). In terms of volumetric productivity, the airlift configuration achieved better results for both enzymes.

3.1.2. Reactors for SSF

In SSF, fermentation is carried out in the absence of or with very limited amounts of free water. The main reactor types that have been researched for SSF are tray fermenters, packed bed bioreactors, and continuously and discontinuously rotating drum bioreactors. Other configurations exist, such as fluidized bed reactors, intermittently stirred beds, or more specific or uncommon designs (Krishna, 2005; Thomas et al., 2013). Each of these reactor configurations presents advantages and disadvantages for operation (Table 3.2). Factors like type of substrate and ease of operation and control have been identified as essential for selection of reactor configuration (Krishna, 2005). Since challenges in SSF especially exist in terms of mass and heat transfer through the bed, an appropriate bioreactor design for a given process would be able to overcome these issues, as well as facilitate recovery of target metabolites (Pandey, 2003).

Table 3.2. Advantages and disadvantages of different SSF reactor configurations (Krishna, 2005; Thomas et al., 2013).

Reactor Configuration	Advantages	Disadvantages
Tray Fermenter	-Controlled environment of temperature and humidity -Simplicity of operation -Proven laboratory, pilot and large scale operation -Appropriate for low volume production	-Large area -Low substrate loading -Labor intensive -Slow air diffusion -Uneven substrate temperature -Scale up only achieved by increased area of operation
Packed-Bed Bioreactor	-Simple design with additional process control -High substrate loading per volume -Closed system, better aseptic conditions -Significant modeling and experimental attention	-Reduced air flow rate and channeling due to pressure drop -Axial temperature gradients impossible to prevent
Rotating Drum Bioreactors	-Control over air flow rate, temperature, humidity, rotation. -In discontinuous rotation schemes, control on frequency, duration and intensity of rotation	-Agglomeration of particles at low rotation speeds -Rotation speed limited by mycelia sensitivity to shear forces

Tray fermenters made of wood, plastic, or metal are the simplest reactor configuration available for SSF. They are located in a temperature and humidity controlled environment where air is circulated around the tray. The substrate is placed on open trays that are perforated to allow a better air circulation. When the fermentation is finished, the substrate is collected and product recovery operations take place. Even though the simplicity of this reactor configuration makes it available at laboratory, pilot, and large scale, the trays present drawbacks such as poor air diffusion or uneven substrate temperature. In addition, the nature of the design leads to high area

utilization, and depending on the country of operation, high labor costs associated with operation may be detrimental (Krishna, 2005). Tray fermentation has received wide attention in the literature. For example, cellulolytic enzymes were produced using SSF in trays by a mixed culture of *Trichorderma reesei* and *A. oryzae* with soybean hull as a substrate (Brijwani et al., 2010). This research applied response surface method (RSM) statistical analysis for optimizing the SSF conditions and achieved a crude enzyme mixture with a similar activity to that achieved by flask fermentation (cellulase 10.7 FPU/g ds and xylanase 505 IU/g ds), which was then used for enzymatic hydrolysis of wheat straw. Another study with tray fermenters was able to achieve cellulase and xylanase activities of 35.8 FPU/(g ds) and 3,106.3 IU/(g ds) using a mixed culture of *A. niger* and *T. reesei* in wheat bran and rice straw medium (Dhillon et al., 2011).

Packed-bed reactors are columns packed with either the substrate or an inert material as support for growth (Krishna, 2005; Thomas et al., 2013). The material is usually loosely packed in the column and the air is forced from the bottom. A variation of this design is the TBR, which combines SSF and SmF characteristics. In TBRs, liquid media trickles down the bed where microorganisms grow while air is still provided from the bottom of the fermenter (Couto & Toca-Herrera, 2007). This particular reactor design will be discussed in a separate section due to its importance to the present study. Packed-bed reactors present advantages such as good process control, a high substrate density, and reduced chance of contamination. On the other hand, pressure drops of 0.1-0.5 cm water per cm of bed can cause channeling and reduced air flow rates, and the axial gradients in the column affecting temperature and oxygen are not preventable (Krishna, 2005). Packed-bed fermenters have been identified as more promising than tray or drum fermenters in terms of process economics (Pandey, 2003). *P. chrysosporium* was used in packed bed bioreactors operated both in discontinuous and continuous mode using different packing materials (nylon sponge and polyurethane foam cubes, and chopped corncob) (Rodríguez Couto et al., 2000). Maximum activity of manganese-dependent peroxidase (MnP) of 1,593 U/L

was achieved using nylon as packing material, while for lignin peroxidase (LiP) similar activities of 229 and 232 U/L were obtained by nylon and polyurethane support, respectively. These activities were achieved in discontinuous operation of the reactor, which reached higher activities than continuous mode. Examples of larger scale experiments can be found as well. Kumar et al. (2014) used a 1,200 L packed reactor (wheat bran and straw as a substrate) for the production of lovastatin by *A. terreus*. The experiments resulted in a lovastatin yield of 2.13 mg g^{-1} dry substrate, which was slightly lower than that achieved in a packed bed 7 L bioreactor, possibly due to poor thermal distribution in the larger reactor.

In rotating drum bioreactors, the substrate particles where the microorganisms grow occupy 10-40% of the reactor volume (Krishna, 2005). The air is not blown through the solid support, but through the headspace between substrate and drum walls. The ability to remove metabolic heat generated limits the reactor performance. Moreover, the rotation is problematic. A high rotation speed may damage mycelia, and slow rotation is associated with clogging issues. These problems can be confronted by applying intermittent instead of continuous rotation (Krishna, 2005). Many examples of rotating drum bioreactors are found in literature from decades back. An example of older work is the study done by Silman (1980), who produced galactosidase and invertase using *Aspergillus awamori* using wheat bran as substrate. Domínguez et al. (2001) also used this reactor configuration to produce ligninolytic enzymes by *P. chrysosporium* grown on nylon sponge. This research achieved activities of manganese-dependent peroxidase (MnP) and lignin peroxidase (LiP) of 1350 and 364 U/L, respectively, operating in batch modes, which were similar levels to those achieved previously by SmF research. In addition, it was the first successful published attempt of continuous production of LiP.

3.2 Trickle Bed Reactors

Trickle bed reactors (TBRs) have been widely used in the last few decades in chemical and biochemical engineering for applications that require good mass transfer between gas and liquid phases. TBRs are column reactors filled with a fixed bed of solid catalyst where the reaction takes place. Gas and liquid can flow co-currently downwards, or in a counter-current mode with the gas flowing upwards. The liquid, as the name of the reactor indicates, always trickles down the solid bed forming films, rivulets or droplets (Ranade et al., 2011). Figure 3.1 depicts the configuration of a counter-current TBR. The reactor height can be as tall as 30 m for large scale applications (Gianetto & Specchia, 1992).

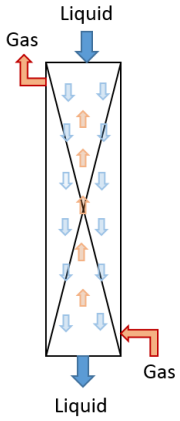

Figure 3.1. Schematic of counter-current TBR

TBRs were first used in wastewater treatment engineering, although usually referred to as trickling filters or biofilters. Stones usually served as solid support for the aerobic bacteria to grow on. These microorganisms would remove the organic matter from the wastewater stream as it trickled down the bed (Satterfield, 1975). In chemical engineering, TBRs have been especially used at a large scale in petroleum processing (e.g catalytic hydrocracking and dewaxing), but also in oxidation reactions such as oxidation of ethanol and phenol, in hydrogenation reactions, and Fisher-Tropsch synthesis (Ranade et al., 2011). In bioengineering, TBRs have been studied for various applications, such as air biofiltration (Hekmat et al., 2004), simultaneous acetone-

butanol-ethanol fermentation and product recovery (Park et al., 1991), or ethanol production via synthesis gas fermentation (Devarapalli et al., 2016). These are examples of TBRs where biofilms were established with both aerobic and anaerobic bacteria as a mono- or mixed culture.

However, TBRs have been also used with filamentous fungi for production of extracellular enzymes. Table 3.3 captures the main parameters from the few examples found in the literature. For example, lacasse was produced using *P. ostreatus* in a laboratory scale TBR packed with sugarcane bagasse (Lenz & Hölker, 2004). Operation using TBR maintained a productivity of 600 U/L*d for 9 weeks, whereas the same microorganism only produced 20 U/L*d when traditional SmF techniques were applied under otherwise similar conditions. In addition, these authors reported a higher productivity of lipase in a TBR using *Neurospora intermedia* and polyurethane cubes as a solid support than in SmF culture (1,800 versus 230 U/L*d, respectively) (Lenz & Hölker, 2004). Another lab-scale study focused on the influence of superficial liquid velocity on production of lignin peroxidases using *Phanerochaete chrysosporium* (Bosco et al., 1999). The study reported a higher productivity (133 U/L*d) than those achieved in previous literature using different reactor configurations such as stirred tank, bubble column, or rotating drum.

A TBR has also been used in our laboratories with a mutant strain of *A. nidulans* (Müller et al., 2015). The mutant expressed xylanase B (XynB) as a client protein and had a pyridoxine marker. The reactor consisted of a glass column with 10.5 cm internal diameter and 60 cm height filled with lava rocks that served as inert solid support for the fungi to grow on. Initially, the authors observed operational problems related to clogging of lines due to excessive microbial growth. This issue was solved by limiting the growth of the strain via pyridoxine removal from the media. The TBR reached a XynB output of 41 U/mL using glucose and maltose as substrate, which was 1.4 times higher than tray culture and also higher than other SSF enzyme production from literature (Mueller, 2012).

Table 3.3. Summary of main characteristics of research studies using filamentous fungi on TBRs

Fungal strain	Desired Enzyme	Reactor scale	Packing material	Mode of operation	Maximum productivity achieved	Reference
Pleurotus ostreatus	Lacasse	Lab scale (400 mL)	Sugarcane bagasse	Semi-continuous	600 U/Ld	(Lenz & Hölker, 2004)
Neurospora intermedia	Lipase	Lab scale (500 mL)	Polyurethane froth	Semi-continuous	1,800 U/Ld	(Lenz & Hölker, 2004)
Phanerochaete chrysosporium	Lignin peroxidases (LiP)	Lab scale (1.6 L reactor)	½″ Berl saddles coated with Ca-alginate	Semi-continuous	133 U/Ld	(Bosco et al., 1999)
Irpex lacteus	Manganese-dependent peroxidases (MnP) and lacasse	Lab scale (250 mL reactor)	1 cm Polyurethane Foam cubes	Semi-continuous	10.9[a] and 0.8[a] U/ Ld MnP and lacasse	(Tavčar et al., 2006)
Irpex lacteus	Manganese-dependent peroxidases (MnP) and lacasse	Lab scale (2.4 L reactor)	2 cm Polyurethane Foam cubes	Semi-continuous	2.9[a] and 0.5[a] U/Ld MnP and lacasse	(Tavčar et al., 2006)
Aspergillus nidulans	Xylanase B	Lab scale (4.5 L reactor)	4-5 cm lava rocks	Continuous	705.35 U/L d	(Mueller, 2012)

[a]Values calculated from data given in original publication

TBRs used for bioprocessing do not strictly fall in either of the definitions for SmF and SSF, but rather combine characteristics of both. Particularly, TBRs combine the benefits of SmF regarding a better process control and continuous production and those from SSF concerning cell immobilization on solid support and large gas-liquid interface. In addition, TBRs present a lower water usage than SmF and are easier to scale-up than traditional SSF reactors (Couto & Toca-Herrera, 2007). In fact, there is evidence in the literature of a TBR with a size of 60,000 L for vinegar production (Qureshi et al., 2005).

The use of TBRs for bioprocessing poses challenges that have to be addressed during the design and operation stages. The performance of TBRs is affected by factors such as gas-liquid and fluid-particle mass transfer, homogeneity of the bed, flow distribution, mixing, recycling streams, and wetting of catalyst (Ranade et al., 2011). Major design and operation characteristics to be manipulated include packing media inside the column, liquid distribution system, gas-liquid flow direction mode, and liquid and gas flow rates.

3.2.1 *Packing material*

Packing media type and size are very important parameters in TBR design and operation. Solid support for cell growth can be broadly classified in two main groups, inert or non-inert, depending on the ability of the support to provide the microorganisms with nutrients. Using a substrate for growth as a solid support in a packed column is interesting from an economical point of view (agricultural waste can be used as an inexpensive nutrient source), but is associated with challenges derived from the degradation of the biomass as the reactions take place (Couto & Toca-Herrera, 2007). This degradation of the support modifies the flow patterns in the column and can lead to channeling and creation of gradients, making continuous operation of the reactor difficult to achieve. Therefore, inert supports for commercial application of bioreactors seem to be a more viable alternative. The selection of packing material should focus on a number of characteristics such as high specific surface area (volume of exposed surface per volume of bulk

volume), sufficient bed porosity to avoid clogging while allowing the necessary gas supply, low cost and durability, and low pressure drops (Ranade et al., 2011; Tchobanoglous & Burton, 1991). Packing materials can be random or dumped, which include the traditional Berl saddles, Raschig rings and more recent and advanced designs (Figure 3.2); or structured packings, which are arranged modular structures. Generally, random packing fillings are significantly less expensive, but structured packing develop lower pressure drops and higher mass transfer efficiencies (Seader & Henley, 2006). When using random packing material, the size of the particles used is important in terms of liquid distribution. Different recommendations have been given on what should be the appropriate ratio between the column diameter (D) and the

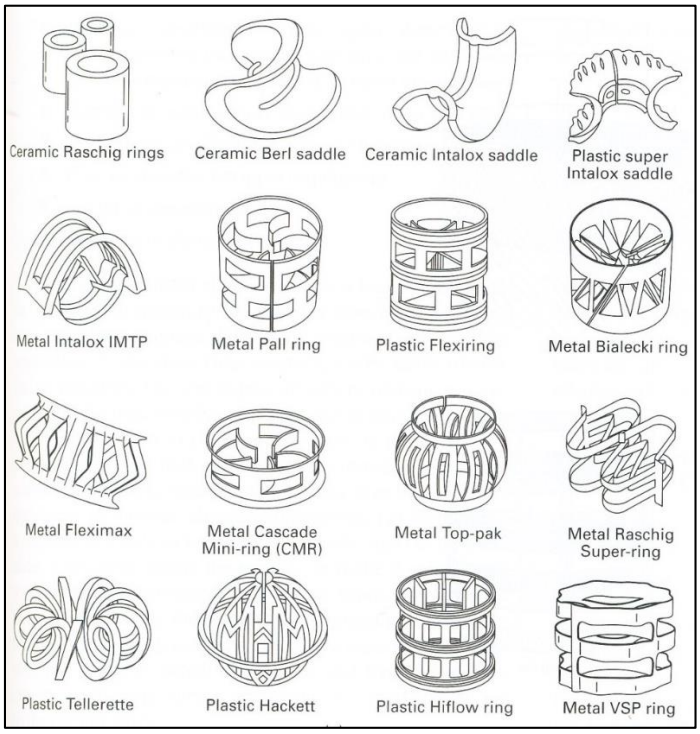

Figure 3.2. Typical random packing shapes for packed columns. Figure reprinted from Seader et al. (2006) with permission from the publisher. Copyright© 2011, 2006, 1998 JohnWiley & Sons, Inc. All rights reserved.

Equivalent diameter of the packing particles (d_p). In order to reduce channeling issues, some authors suggest a D/d_p ratio greater than 8 (Seader & Henley, 2006), while others recommend ratios of 12 and even 20 to 25 (Saroha et al., 1998).

3.2.2 Liquid Distributor

One of the major concerns during operation of TBRs is homogeneity throughout the column, which is greatly dependent on an even liquid distribution (Maiti & Nigam, 2007; Ranade et al., 2011). Hence, a key part of the reactor is the distribution system that delivers the liquid on top of the packing material. In commercial reactors the liquid distribution system can be driven by gravity or pressure (GEA, 2014). When only gravity is used as the driving force for flow, the liquid may be distributed as overflow across a weir or an outflow through one or more holes (GEA, 2014). Pressure systems, such as spray nozzles, can achieve a more even distribution although the cost is significantly higher. According to one of the manufacturers of liquid distribution systems (GEA Group, Germany, European Union), the operational cost of using nozzles in a particular application can be 13 times more expensive that outflow systems (GEA, 2014). In addition, liquid distribution systems in bioprocessing should be easy to maintain and clean and not be subjected to clogging due to microbial growth. When the packing height is greater than 20 ft., redistribution systems should be installed to avoid channeling (Seader & Henley, 2006).

3.2.3 ***Mode of operation***

Another important decision that needs to be addressed when operating TBRs is the mode of operation: co-current or counter-current. Operating a column in counter-current mode (liquid flowing downwards and gas flowing upwards) is generally preferred from a point of view of mass-transfer, since a higher driving force is available. However, it has been claimed in the literature that when irreversible reactions take place between dissolved gas and solid catalyst, the

mean concentration driving force is the same for co- or counter-current modes (Klasson et al., 1990). In that case, operating in co-current mode would be beneficial since it reduces the pressure drop and flooding issues. Flooding happens when there is too much entrainment of liquid droplets in the gas phase, causing the liquid to flow upwards flooding the column and interrupting normal operation of the reactor (Seader & Henley, 2006).

3.3 Prevention of clogging in reactor systems

Microbial clogging of the conduits, lines, or the biocatalyst bed itself, is a concern when operating bioreactors. This problem is especially relevant in filamentous fungi bioreactors, particularly using packed bed columns. Clogging can increase the operational costs due to increased pressure drop, decrease the efficiency of the system due to channeling, and eventually, terminate operation when total obstruction occurs. Prevention of clogging can be achieved by removing portions of the biofilm or by controlling the growth rate of the microorganisms.

3.3.1 Biomass removal techniques

Among biofilm cleaning procedures, physical and chemical methodologies have been investigated. Physical methods are based on increased shear forces, and examples of techniques used in air biofilters are stirring and backwashing (Delhoménie et al., 2003). Similarly, removal of inactive syngas-fermenting cells from a membrane biofilm was achieved by a temporary increase of the liquid recirculation rate (Tsai et al., 2012). Chemical treatments use compounds that can degrade the biofilm and assist on detaching it from the solid support. An example of this is the biomass control in an air biofilter by diluted NaOH washing (Weber & Hartmans, 1996).

3.3.2 Controlled cell growth – Carbon/energy limitation

Another available alternative, rather than removing excess biomass from the system, is to control cell growth so clogging cannot be possible. Two options are available in order to achieve

this zero-growth condition: carbon and/or energy limitation, or coenzyme limitation. Once the necessary cell biomass is formed, the consumption of carbon and energy to build up additional biomass can be considered a waste of resources as those nutrients and energy could be used in product formation instead. An example of this would be the use of a retentostat, a reactor that does not allow biomass to exit the vessel until a certain concentration is reached, and then limits the nutrient supply to provide energy equal to the maintenance energy of the microorganism (zero growth stage) (Jørgensen et al., 2010). Results with submerged retentostat cultures of *A. niger* showed that the system approached zero growth conditions (μ dropped from μmax 0.24 h^{-1} to 0.02 h^{-1}) although the growth yield (g_x/g_S) remained at 0.2-0.27 compared to 0.58 at exponential growth conditions and respiration of carbon increased two-fold (Jørgensen et al., 2010). Conidiation (asexual reproduction) was promoted under carbon and energy limited conditions. A different study showed that aerial phenotypes become expressed in *A. niger* mutants when carbon and energy limitations are imposed, and this affected production of extracellular proteins and secondary metabolites (Jørgensen et al., 2011). It is difficult to conclude, based on the studies published in the literature, if sustained product formation under carbon and energy limited conditions is feasible. In addition, there is a lack of reports on experiments with immobilized filamentous fungi in the literature (Müller et al., 2014).

3.3.3 Controlled cell growth – Coenzyme limitation

Another approach for enhancing product formation while interrupting cell growth is the limitation of essential nutrients that are not carbon or energy providers to the microorganism, for example vitamins or coenzymes that are required for certain functions. The first reported experiments on zero-growth via coenzyme limitation in filamentous fungi used a mutant of *A. nidulans* to produce xylanase B (Müller et al., 2014). The recombinant strain had a marker with pyridoxine deficiency and a genetic modification to secrete xylanase B as a client protein. Pyridoxine is a vitamin that is necessary for cell growth and is involved in many reactions of

amino acid metabolism. This study found that cell growth was prevented when pyridoxine was not present in the medium, while the client enzyme was still produced. However, a prolonged pyridoxine limitation led to a decrease in pH and promoted conidiation. These issues could be mitigated by periodical replenishment of the vitamin (Müller et al., 2014). The pyridoxine limitation technique was applied to a continuous enzyme production experiment using a TBR (Müller et al., 2015) . The methodology solved clogging issues observed in the TBR using medium containing pyridoxine, and the production yields obtained were higher than in tray cultures and those reported in the literature for SSF (Müller et al., 2015). On the negative side, there were challenges associated to enzyme production under growth-limited conditions. A prolonged pyridoxine limitation led to a decrease in pH and promoted conidiation paired with a halt on enzyme production. During this stage the liquid broth turned very dark, probably due to production of pigments such as melanin. These issues were mitigated by periodical replenishment of the vitamin (Müller et al., 2014).

3.3.4 Melanin production during controlled cell growth

One of the changes observed during *A. nidulans* experiments near zero growth conditions was the darkening of mycelia and liquid broth (Müller et al., 2014). This has been attributed to melanin production and it has been observed in different studies when growth was limited using various techniques; including carbon and nitrogen limitation (Horowitz & Shen, 1952; Rowley & Pirt, 1972) and pyridoxine limitation (Müller et al., 2014) in *A. nidulans* cultures, sulfur deficiency in *Neurospora sitophila* cultures (Horowitz & Shen, 1952) and even using fungal inhibitors such as cycloheximide or actinomycin in *N. crassa* cultures (Prade et al., 1984).

Melanins are black or brown pigments found in many organisms with various roles. In fungi, these pigments are secondary metabolites with a non-essential role in growth and product development (Goncalves & Pombeiro-Sponchiado, 2005). Melanin has been reported to have a

protective function in fungi. For example, melanized fungi were found to survive extreme environments such as Antarctica or inside nuclear reactors (Eisenman & Casadevall, 2012). Its role as an extracellular redox buffer improves the chances of survival because of the capacity to neutralize oxidants produced by environmental stress such as HOCl or H_2O_2 (Goncalves & Pombeiro-Sponchiado, 2005). In addition, it was shown that *A. nidulans* presented enhanced resistance to fungal lysis with increasing presence of melanin in the fungal walls (Kuo & Alexander, 1967). This could be due to the polyaromatic structure of the pigment that could act as a shield, or due to inhibition of enzymes that are involved in fungal lysis, such as chitinase, glucanase, or protease (Kuo & Alexander, 1967). The exact structure of melanin is unknown. It was noted in the literature that the current analytical methods are not sufficient to describe the polymer with exactitude and only structural models have been proposed (Eisenman & Casadevall, 2012).

There are two pathways for melanin production in fungal strains. Many species synthesize it through the 1,8-dihydroxnapthalene (DHN) pathway that involves the enzyme polyketide synthase (Eisenman & Casadevall, 2012). Fewer fungi produce melanin using the L-3,4-dihydroxyphenylalanine (L-DOPA) pathway. The enzyme tyrosinase is key in this process, because it catalyzes the oxidation of tyrosine to L-DOPA, which then spontaneously polymerizes into melanin (Held & Kutzner, 1990). Melanin produced via the DHN pathway contains carbon and oxygen, while melanin produced via L-DOPA pathway also contains nitrogen (Eisenman & Casadevall, 2012). A study determined that the composition of melanin produced by *A. nidulans* had a high percentage of nitrogen, and pigmentation was eliminated when tyrosinase inhibitors were present, indicating that this strain produces melanin via the L-DOPA pathway and not the DHN pathway (Gonçalves et al., 2012).

Even though there are positive aspects of melanin production for fungal protection, there are also drawbacks when melanin is produced in a bioreactor system with controlled growth.

First, melanin production uses part of the substrate for synthesizing this polymer that could be otherwise used for production of the desired protein or metabolite. Therefore, melanin production reduces the availability of nutrients for "useful" purposes. Second, melanin production creates a challenge for cell mass determination. In a study that used *A. nidulans*, melanin represented 5% of the dry weight of the fungus when the growth rate was 0.072 h^{-1} compared to 28% when the growth rate was reduced to 0.02 h^{-1} via glucose limitation (Carter et al., 1971). This could easily lead to overestimation of fungal biomass in a reactor that operates at low growth rates, which would distort any conclusion that could be drawn when studying those kinds of systems. Third, melanin changes the overall porosity of the fungus cell wall. Specifically, it has been found that melanin modifies the absorption properties of the mycelium and reduces the size of the pores on the wall (Eisenman et al., 2005). This pore reduction could impact the rate of transfer of nutrients and metabolites through the cell wall. Finally, melanin formation has been related to extensive foaming in bioreactors (Jørgensen et al., 2011), which can lead to serious operational issues, including incorrect monitoring and control due to foam deposition on sensors and ports, reduction of reactor working volume and blockage of outlet filters (Vardar-Sukan, 1998). For these reasons, reducing or eliminating melanin production in near-zero growth fungal systems in desired.

3.3.5. Prevention of melanin formation

As stated above, *A. nidulans* melanin formation is via the DOPA pathway, which is controlled by the enzyme tyrosinase. A method to reduce melanin production could be to inhibit the activity of this enzyme. There are a number of tyrosinase inhibitors, such as L-mimosine, tropolone, kojic acid, or 4-substituted resorcinols (Jiménez et al., 2001). Tropolone has been highlighted as one of the most powerful inhibitors of fungal tyrosinase (Kahn & Andrawis, 1985), but it also has been catalogued as a potent fungal inhibitor lethal to various fungal strains (Lindberg, 1981) as well as an antibacterial agent for both gram positive and negative cells (Trust, 1975). The mechanism proposed for tropolone inhibition of tyrosinase is the chelation of

copper (Goldstein et al., 1964). Tyrosinase contains copper in its active site (Held & Kutzner, 1990), which makes this trace element usually present in fungal media required for melanin formation. In a study using bacterial cultures of *Streptomyces michiganensis*, the authors demonstrated how copper is not only essential for tyrosinase, but also for the induction of the gene at the level of transcription (Held & Kutzner, 1990). In that study, when the media was copper-free the tyrosinase activities detected in the broth were almost zero. This was also observed in a different study using various fungal strains grown in different potato dextrose agar media. The authors reported different levels of pigmentation among the different media, with lower pigmentation when media was deficient in copper (Griffith et al., 2007). Copper is, therefore, an element in fungal media that could be key for controlling or eliminating the production of melanin, but copper's effects on protein expression and metabolite production are not known.

Other metals besides copper have been reported to affect pigmentation in fungi. Zinc and iron have been pointed as responsible for pigmentation in different fungal strains (Foster, 1939). Zinc defficiency inhibited mycelial pigmentation of *Macrosporium* and reduced pigmentation of *A. niger* spores (Foster, 1939). In addition, zinc is present in dopachrome tautomerase, or tyrosinase-related protein 2 (Tyrp2), which displays homology to tyrosinase (Solano & García-Borrón, 2006). It catalyzes the tautomerization of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to dopachrome, two of the intermediate compounds in the melanin pathway (Solano & García-Borrón, 2006). Extensive literature about this enzyme in mammals was found but no information regarding its existence in fungal strains. Iron also has been reported as a contributor to melanization and was found in the pigment of *A. niger* spores (Foster, 1939). In light of these observations, it is possible that the composition of the media (particularly the trace metal composition) can be optimized to reduce pigmentation. However, it is important to note that it is commonly accepted that iron is an absolute requirement of *Aspergillus* strains

(Agnihotri, 1967). A study analyzing the effect of trace metals in different aspergilli observed that removing zinc or iron from the media resulted in a significant reduction of growth, while copper did not affect growth of the strain (Agnihotri, 1967).

An alternative approach to reduce pigmentation is adding a compound that switches the pathway away from melanin production. Ascorbic acid, for example, reduces the intermediate o-quinones to their o-phenol precursors (Lozano de Gonzalez et al., 1993) preventing the formation of the final melanin product. In addition, ascorbic acid can act as a chelating agent and as a competitive inhibitor of tyrosinase (Lozano de Gonzalez et al., 1993). A study using fungal tyrosinase found that ascorbic acid inhibited tyrosinase at a concentration of 0.04 mM using in vitro tests (Hsu et al., 1988). It has also been extensively used in the food industry to avoid browning of fresh products, such as vegetables and fruits. An example of this is a study that proved the effectiveness of ascorbic acid preventing browning in fresh and dried apple rings (Lozano de Gonzalez et al., 1993) due to its reducing capacity. Similarly, this compound was reported to inhibit melanin formation in human skin (Denton et al., 1952), and instead of a dark pigment it formed a lighter colored water-soluble substance (Rothman, 1940) . Ascorbic acid was also used to inhibit tyrosinase from fungus. The addition of this compound resulted in a lag period in the activity of tyrosinase proportional to the concentration of ascorbic acid (Golan-Goldhirsh & Whitaker, 1984). Other reducing agents tested showed higher inhibition, including dithiothreitol, sodium bisulfite, and glutatione (Golan-Goldhirsh & Whitaker, 1984). In another study of an in vitro tyrosine-tyrosinase system, ascorbic acid was the most potent inhibitor at a concentration of 1 mM compared to similar concentrations of cysteine, glutathione, thiocarbamide and thiouracil (Paschkis et al., 1944). Therefore, the addition of reducing agents such as ascorbic acid in the liquid media may be beneficial to avoid or reduce melanization of cultures.

3.4 The ligninolytic consortium

Biomass conversion for production of biofuels or bioproducts require overcoming the lignin barrier. In contrast to cellulose and hemicellulose (the other two major biomass components), lignin is not a sugar polymer and it is recalcitrant to degradation by most microorganisms. However, a few microbes have been found to degrade lignin; for example fungal strains that use oxidative and reductive reactions (Ander & Marzullo, 1997). The enzymes involved in this process have been referred to as the ligninolytic consortium, and this consortium includes manganese, versatile or lignin peroxidases, lacasses, hydrogen peroxide supplying oxidases, and ferric-ion reducing enzymes (Alcalde, 2015). From a perspective of industrial bioconversion of biomass, pretreatment of the raw materials to remove lignin has been identified as a major contributor to overall bioconversions costs, as it requires the use of chemicals and high pressures and/or temperatures (Yang & Wyman, 2008). According to a report from the Department of Energy (DOE), the contribution of pretreatment to the cost of the overall biomass to ethanol process was 35% (DOE, 2011). The use of chemicals (acids or bases) during pretreatment causes an increase in costs due to the need for corrosion-resistant equipment and treatment of process and waste streams (Pang et al., 2008; Yang & Wyman, 2008). Harsh conditions also cause loss of sugars and generate fermentation inhibitors that have to be removed using detoxification processes (Liu et al., 2015a; Liu et al., 2015b; Yang & Wyman, 2008). Therefore, it is clear that a reduction of the overall cost of biomass conversion could be achieved by reducing or eliminating the use of chemicals and other harsh conditions during pretreatment. This milder pretreatment could be achieved by identifying, producing, purifying, and using the enzymes participating in the so-called ligninolytic consortium. The aim is to reproduce in the laboratory (and later in industrial scale plants) that which occurs in nature, namely microorganisms breaking down the biomass structure, including lignin. A combination of

synthetic biology and metabolic engineering has been pointed as the methodology to use in the advancement of this field of study (Alcalde, 2015).

3.4.1 Aryl alcohol oxidase

One of the enzymes that participates in the ligninolytic consortium is aryl alcohol oxidase. Aryl alcohol oxidases are flavoproteins that catalyze the oxidative reaction of aryl α- and α-β-unsaturated γ-alcohols to their corresponding aldehydes with simultaneous reduction of oxygen to hydrogen peroxide (Alcalde, 2015; Ander & Marzullo, 1997). After hydrogen peroxide is generated, it can either be used enzymatically in reaction catalyzed by peroxidases or peroxygenases (Alcalde, 2015) or it can be used non-enzymatically as part of Fenton reagent to degrade lignin (Ander & Marzullo, 1997). The Fenton reaction has been recognized as a route for wood decay where hydrogen peroxide is reduced to a molecule of water and the radical •OH, while iron is oxidized from Fe^{2+} to Fe^{3+}. •OH is a highly reactive radical considered to be the strongest oxidant agent in aqueous solutions. The attack of this radical on polymers such as cellulose or lignin has been studied and it has been proven that the radical can cleave these compounds, although it remains unclear if this radical is responsible for fungal wood decay or if it is predominantly caused by enzymatic reactions (Hammel et al., 2002). It was not possible to find in the current literature examples of research studies that use aryl alcohol oxidases on real biomass materials (and not model compounds).

Other proposed uses of aryl alcohol oxidases beyond bioenergy include the production of vanillin from vanillyl alcohol, a flavor agent for food industry (Hernández-Ortega et al., 2012), and its application in decolorization of textile dyes and dye containing textile waste water (Phugare et al., 2011)

There are a limited number of studies reporting production of aryl alcohol oxidases. *Bjerkandera adusta*, a white-rot fungus able to degrade lignin was used to produce aryl alcohol

oxidase using agitated cultures in carbon-limited medium (Muheim et al., 1990). The batch experiments yielded almost 40 U/L after 100 h, and the enzyme was purified using chromatographic techniques. Another study expressed aryl alcohol oxidase from *P. eryngii* using an *A. nidulans* strain (Varela et al., 2001). The maximum activity obtained in batch agitated cultures was almost 500 U/L. A different study presented the results of heterologous expression of aryl alcohol oxidase from *P. eryngii* using *A. nidulans* and *Escherichia coli* (Ruiz-Dueñas et al., 2006). The authors concluded that the expression in *E. coli* cultures enhanced protein production 100-fold compared to their *A. nidulans* cell factory. However, the enzyme produced by the bacterium was inactive and accumulated in inclusion bodies, which required additional refolding steps for enzyme and activity recovery. The total concentrations of protein obtained were 3.4 and 45-500 mg/L for *A. nidulans* and *E.coli* recombinants, respectively. Both values were higher than 0.8 mg/L obtained with the wild *P. eryngii* strain (Ruiz-Dueñas et al., 2006). Finally, a study using a *P. pulmonarius* strain claimed an aryl alcohol oxidase production of 257.5 mg/L with a maximum activity of 2,500 U/L after 10 days of batch agitated culture (Varela et al., 2000).

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CHAPTER IV

HIGH YIELD PRODUCTION OF ARYL ALCOHOL OXIDASE UNDER LIMITED GROWTH CONDITIONS IN SMALL SCALE SYSTEMS USING A MUTANT *ASPERGILLUS NIDULANS* STRAIN

4.1 Abstract

Aryl alcohol oxidase (MtGloA) is an enzyme that belongs to the ligninolytic consortium and can play an important role in the bioenergy industry. This study investigated production of an MtGloA client enzyme by a mutant strain of *Aspergillus nidulans* unable to synthesize its own pyridoxine. Pyridoxine limitation can be used to control cell growth, diverting substrate to protein production. In agitated cultures, enzyme production was similar when using media with and without pyridoxine (26.64±6.14 U/mg mycelia and 26.14±8.39 U/mg mycelia using media with and without pyridoxine, respectively). However, the treatment lacking pyridoxine had to be supplemented with 1 mg/L pyridoxine after 156 h of fermentation to sustain continued enzyme production. Use of extremely diluted pyridoxine levels allowed reduced fungal growth while maintaining steady enzyme production. Concentrations of 9 and 13.5 µg/L pyridoxine allowed MtGloA production with a growth rate of only 5% of that observed when using the standard 1 mg/L pyridoxine media.

4.2. Introduction

The global market for enzymes is of great importance in biotechnology, as it was estimated to represent $4.5 billion in 2012 and $4.8 billion in 2013 with expectations of an 8.2% annual growth rate until 2018 (BCC-Research, 2014). Enzymes are frequently produced using filamentous fungi, which grow as mycelia. These microorganisms tend to adhere to surfaces creating biofilms though the secretion of exoenzymes used for absorptive digestion of nutrients and for apical hyphal growth (Harding et al., 2009). The aggressive growth of filamentous fungi combined with the ability to easily form biofilms can lead to operating problems derived from clogging of pipes and tubing in a bioreactor system. This has been described as one of the biggest challenges of using trickle bed reactors (TBRs) to grow filamentous fungi (Yang et al., 2010). This problem can be addressed by controlling the growth rate of the fungus used. Another benefit of controlling growth is that carbon and energy used for growth can be redirected towards the formation of products. Two different strategies are available to achieve this goal of controlled cell growth.

One approach is to achieve carbon and energy limitation by the use of a retentostat, a reactor that does not allow biomass to exit the vessel until a certain concentration is reached, and then limits the nutrient supply to provide energy equal to the maintenance energy of the microorganism (zero growth stage) (Jørgensen et al., 2010). It was observed that submerged retentostat cultures of *Aspergillus niger* approached zero growth conditions (μ dropped from μ_{max} 0.24 h^{-1} to 0.02 h^{-1}) with growth yields (g_x/g_S) of 0.2-0.27 compared to 0.58 at exponential growth conditions (Jørgensen et al., 2010). Also, respiration of carbon in the retentostat increased two-fold over exponential growth. In addition, conidiation (asexual reproduction) is promoted under carbon and energy limited conditions because starvation stress induces the transcription of *brlA*, a regulator of conidiation (Prade & Timberlake, 1993). It is difficult to conclude, based on the studies previously published, if sustained product formation under carbon and energy limited conditions is feasible. For example, a study used a retentostat to evaluate the performance of an *A. niger*

transformant that produced glucoamylase (Schrickx et al., 1993). In this study, the authors claimed to be able to obtain similar enzyme production rates in a retentostat and in non-limited shaken cultures. However, they concluded that the production of enzyme when biomass concentration in the reactor was stable may have been due to dead mycelia providing an extra carbon source for growth (Schrickx et al., 1993). Another study using *A. niger* mutant cultures in retentostats showed how aerial phenotypes are expressed under carbon and energy limitation and affect production of both extracellular protein and secondary metabolites (Jørgensen et al., 2011).

A different approach for enhancing product formation while interrupting cell growth is limiting essential nutrients that are not carbon or energy providers to the microorganism. Examples of such essential nutrients are vitamins or coenzymes that are required for various cellular functions. The first reported study on zero-growth via coenzyme limitation in filamentous fungi used a mutant of *A. nidulans* to produce xylanase B (Müller et al., 2014). The recombinant strain had a marker with pyridoxine deficiency and a genetic modification to secrete xylanase B as a client protein. This study found that cell growth was prevented when pyridoxine was not present in the medium, while the client enzyme was still produced. However, prolonged pyridoxine limitation led to a decrease in pH and promoted conidiation paired with cessation of enzyme production. These issues were mitigated by periodic replenishment of pyridoxine when decreased pH was observed (Müller et al., 2014). The pyridoxine limitation technique was applied to a continuous enzyme production experiment using a TBR (Müller et al., 2015). The methodology solved clogging issues observed in the TBR using medium containing pyridoxine, and the production yields obtained were higher than yields in tray cultures and yields reported for solid state fermentation (Azin et al., 2007; Gessesse & Mamo, 1999). However, sudden additions of pyridoxine caused rapid changes in the fermentation process, including increases in pH and enzyme activity and, although not determined in the study, a likely increase in fungal biomass as a result of restoring heterotrophic conditions. In a continuous process like a TBR, it is desired to have a steady operation of the reactor that would

allow a better understanding and tuning of parameters like dilution rate and liquid recirculation rate, and a proper comparison among different packing materials to be used. A potential solution for this problem could be replacing the periodic additions of pyridoxine that disturb the process by the continuous use of medium containing highly diluted concentrations of this vitamin. This could limit cell growth while allowing continued enzyme production.

Overcoming the lignin barrier through pretreatment processes has been a major challenge and cost contributor to biofuel production processes (Yang & Wyman, 2008). In nature, some fungal strains are able to degrade complex biomass structures by secreting a group of enzymes (including lignin peroxidase, lacasse, or oxidases) referred to as the "ligninolytic consortium" because of their ability to attack lignin (Alcalde, 2015). The identification and application of these enzymes could be a promising alternative to costly and complex pretreatment technologies that use heat, chemicals and/or pressure. Aryl alcohol oxidase (MtGloA) (E.C. 1.1.3.7) is one of the enzymes that belong to the so-called ligninolytic consortium. This flavoprotein oxidizes aryl alcohols (present in the lignin structure) to their corresponding aldehydes. During this reaction, hydrogen peroxide is released, which can be used by other enzymes or can contribute to the Fenton chemistry that degrades lignin via formation of highly reactive $^{\bullet}OH$ radicals (Alcalde, 2015; Ander & Marzullo, 1997).

The goal of this study was to construct a mutant fungal *A. nidulans* strain that overexpresses the target enzyme (MtGloA) from *Myceliophthora thermophila* and has a pyridoxine marker to limit its growth. After successfully constructing the mutant strain, the objective was to confirm that it was able to produce the target enzyme under auxotrophic conditions (pyridoxine limitation). This was done by using small scale reactors under both static and agitated culture configurations. Finally, the possibility of using extremely diluted pyridoxine levels in the media was investigated as a method to obtain a more stable fermentation process.

4.3. Materials and Methods

Strain and spore production

A mutant *A. nidulans* strain was obtained from the Fungal Genetic Stock Culture (FGSC, Manhattan, Kansas, USA). The strain is deposited in the FGSC with the identifier A773 and presents the following genotype: *pyrG89*; *wA3*; *pyroA4*. This genotype includes a marker (*pyroA4*) that prevents the strain from producing its own pyridoxine, thus limiting growth whenever pyridoxine is not provided externally. The strain obtained from the FGSC was further genetically modified by DNA mediated transformation of a plasmid (pEXPYR), which contains a wild-type *pyrG* complementing gene and a construct that contains the glucoamylase promoter fused to the *MtGloA* ORF (including its own signal peptide) as described elsewhere (Segato et al., 2012). The resulting *A. nidulans* strain overexpresses and secretes *M. thermophila* MtGloA in media containing maltose. As a result of this procedure, several transformants were produced. Samples from culturing each transformant were analyzed using SDS-PAGE to confirm the production of the targeted MtGloA protein. The transformant showing the largest band for MtGloA (located between 66 and 97 kDa) was selected as the final *A. nidulans* strain used for all the experiments.

Spores for the experiments were produced by growing the resulting strain on solid medium containing (per L) 9.0 g glucose, 50 mL of 20x Clutterbuck salt solution (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4$, 30.4 g/L KH_2PO_4), 1 mL 1,000x trace element solution (22 g/L $ZnSO_4 \cdot 7H_2O$,11 g/L H_3BO_3, 5.0 g/L $MnCl_2 \cdot 7H_2O$, 5.0 g/L $FeSO_4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 5H_2O$, 1.6 g/L, $CuSO_4 \cdot 5H_2O$, 1.1 g/L $Na_2MoO_4 \cdot 4H_2O$, 50 g/L Na_2-EDTA), 0.001 g pyridoxine, and 15% agar. The pH was adjusted to between 6 and 6.5 using a 6N NaOH solution. The medium was sterilized by autoclaving at 121ºC for 20 min (PRIMUS, Sterilizer Co., Inc., Omaha, NE, USA). Twenty-five µL of spore stock solution were pipetted onto the solid medium and spread homogeneously under aseptic conditions. The plates were then incubated at 37ºC for 48 h until the surface of the solid media was covered by white spores. A control plate with medium lacking pyridoxine was

inoculated in a similar fashion to verify the stability of the strain. Observation of growth on this control plate served as an indicator of contamination or issues with the mutant strain. The plates with spores were kept refrigerated (4ºC) until used.

Composition of media used in experiments

Experimental medium contained (per L) 50 mL of 20x Clutterbuck salt solution and 1 mL 1,000x trace element solution. The resulting solution was sterilized by autoclaving at 121ºC for 20 min (PRIMUS, Sterilizer Co., Inc., Omaha, NE, USA). Adjustment of pH with sterile 6N NaOH solution was done after autoclaving the medium in order to avoid formation of precipitate. The medium for all experiments also contained 47.6 g/L maltose and 10.0 g/L glucose. Maltose is required to promote protein expression and the ratio between maltose and glucose has been described as the optimum for enzyme production (Mueller, 2012). A 10x sugar stock solution was sterilized using 0.20 µm PES sterile filters. In addition, pyridoxine was added for the pyridoxine-containing media at different levels. The control level of pyridoxine was obtained by adding 1 mL of 1g/L sterile pyridoxine stock solution per L of media.

Enzyme production in static cultures: effect of pyridoxine in the media

Static cultures were prepared by aseptically transferring 7 mL of liquid media with or without pyridoxine into pre-sterilized petri dishes. Spores were harvested from solid media plates and were aseptically transferred to a microcentrifuge tube containing 1.5 mL of sterile distilled water. Aliquots of 100 µL of this spore solution were pipetted into each petri dish to aim for equal inocula size among plates. Then, the plates were incubated at 37ºC with no agitation. Mycelia developed on top of the liquid media. During the first 48 h, media containing 1 mg/L pyridoxine was used in both treatments to allow mycelia growth. The liquid broth was then replaced by fresh media every 24 h to allow the experiment to proceed. Two treatments were run with two media compositions. The first treatment used media containing 1 mg/L pyridoxine and the second

treatment used media without pyridoxine. Both treatments were done in duplicate. Liquid samples were periodically withdrawn, centrifuged at 13,000 rpm for 10 min, and used for pH analysis, determination of sugars concentrations, protein concentration and MtGloA activity as described below. The dry weight of mycelia was determined at the end of the experiment by vacuum filtering the content of each plate on Whatman #1 filter paper, washing with distilled water and drying overnight at 105ºC.

Enzyme production in agitated cultures: effect of pyridoxine in the media

Agitated cultures were prepared in sterile baffled flasks containing 40 mL media. A procedure for inoculation similar to the one used for static cultures was applied. The baffled flasks were then incubated at 37ºC and agitated at 225 RPM in an orbital shaker. Initially, all flasks contained media with 1 mg/L pyridoxine to allow pellet growth. After 12 h, media was replaced with media with or without pyridoxine according to each of the two treatments. In order to reduce carryover of pyridoxine to the fresh media, cell pellets were washed prior to the first transfer. The contents of each baffled flask were poured into a 50 mL sterile centrifuge tube and centrifuged at 3,750 rpm for 15 min. The supernatant was removed and a similar amount of sterile 0.89% NaCl solution was added. The resulting suspension was well mixed and centrifuged again and then the supernatant was discarded. Finally, the washed pellets were aseptically transferred to the flasks containing fresh media. After this, the transfer of pellets to fresh media was done every 48 h, but without a washing step since any residual pyridoxine that was present in culture in pyridoxine-free media was removed in the first washing step. . Liquid samples were periodically withdrawn and used for pH analysis, determination of sugars concentrations, protein concentration and MtGloA activity as described below. Additional 1 mL liquid samples were periodically taken for dry mass determination. These samples were filtered through a 0.45 µm nylon filter and washed with distilled water. Then, the filter with solids was dried overnight at 105ºC for mass determination. The final dry weight of mycelia in each flask was determined at the end of the experiment by vacuum

filtering the content of each flask on Whatman #1 filter paper, washing with distilled water and drying overnight at 105ºC.

Enzyme production in agitated cultures: use of extremely diluted pyridoxine levels in the media

Plates with solid media were prepared containing different concentrations of pyridoxine ranging from 0 to 1,000 µg/L. Spores were inoculated in the center of the surface of the media and the plates were incubated at 37ºC for 72 h. After the incubation time, the diameters of the colonies formed were measured. Each treatment was done in duplicate. The information from this experiment was later used in a submerged fermentation. Submerged fermentation with dilute pyridoxine concentrations were performed with agitated cultures with 40 mL working volume, similar to the experiment described in the previous subsection. A control treatment used media with no pyridoxine, and treatments 1 and 2 used media with very low pyridoxine levels. The initial pyridoxine levels were 1.0 and 1.5 µg/L for treatments 1 and 2, respectively, which were increased to 3.0 and 4.5 µg/L at 144 h and to 9.0 and 13.5 µg/L at 192 h. In addition to the analysis done for the previous experiment, color of the liquid broth was monitored by measuring the absorbance at 425 nm (UV-2100, Cole Parmer, Vernon Hills, IL, USA). This wavelength was used elsewhere to follow formation of soluble melanin in *A. nidulans* fermentations (Rowley & Pirt, 1972). This new measurement was helpful to determine the extent of melanin formation, an undesired process that competes with enzyme synthesis for substrate.

Determination of protein concentration, MtGloA activity, and concentration of sugars

Protein concentration was measured using the Bradford assay. One-hundred-sixty µL of Bradford Commassie solution were transferred to a 96-well microplate. Thirty µL of fermentation broth were then added and the absorbance was measured at 595 nm using a UV-Vis microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). A calibration curve to relate protein content

to absorbance was prepared using different levels of purified bovine serum albumin (100x BSA, BioLabs, MA, USA).

MtGloA activity was analyzed using a spectrophotometric assay. The assay was based on the oxidation of the substrate, veratryl alcohol, and the reduction of the electron acceptor, 2,6-dichlorophenol indophenol (DCPIP). The reaction was monitored by measuring the change in absorbance of the reaction mixture at 600 nm over time using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). Absorbance decreases as DCPIP (blue) is reduced to $DCPIPH_2$ (colorless). Each reaction well contained 10 µL of 2 mM DCPIP, 10 µL of 250 mM veratryl alcohol, 10 µL of 50 mM phosphate buffer of pH 7.0 and 70 µL of sample. The reagents were equilibrated at 50°C for 5 min in a thermal cycler (PTC-200, Bio-Rad, CA, USA) before the reaction was started and the absorbance was monitored for 2 min. One unit of activity per mL was defined as the decrease of $4.2*10^{-3}$ absorbance units (at 600 nm) per min.

Concentrations of maltose and glucose were determined by high performance liquid chromatography (HPLC 1100 Series, Agilent, Santa Clara, CA) using an HPX-87P analytical column (BioRad, Hercules, CA) and a refractive index detector with distilled water as the mobile phase at a flow rate of 0.6 mL/min and a temperature of 80ºC. Concentrations of organic acids were determined by HPLC using an HPX-87H analytical column (BioRad, Hercules, CA) and a refractive index detector with 0.005M sulfuric acid as the mobile phase at a flow rate of 0.6 mL/min and a temperature of 60ºC. Concentrations were quantified based on a five level calibration curve of known standards.

4.4. Results and Discussion

Enzyme production in static cultures: effect of pyridoxine in the medium

The purpose of this experiment was to evaluate the effect of removing pyridoxine from the medium using static cultures. It was hypothesized that enzyme production in medium with or

without pyridoxine would be similar, but medium lacking pyridoxine would have to be supplemented with the vitamin at a certain point to sustain protein production. This hypothesis is based on previous work with another *A. nidulans* strain with the *pyroA4* marker producing a different enzyme (Müller et al., 2014).

Fig. 4.1 shows the main fermentation parameters for this experiment. Unlike what had been hypothesized based on previous work, the treatment lacking pyridoxine did not suffer a halt of enzyme production coupled with a decrease in pH. In this case, the pH trends for both treatments (Fig. 4.1b) were similar except for a diverging point at 120 h. Moreover, the enzyme activities for both treatments (Fig. 4.1a) were similar and only start diverging after 168 h of fermentation. Sugar consumption was also similar for both treatments (Fig. 4.1c and 4.1d), but it can be seen that maltose consumption slightly decreased after 168 h when no pyridoxine was added. This is in agreement with a decreased MtGloA activity on that period. The cumulative MtGloA activity obtained was 163.99±2.49 and 140.53±1.56 U/mL for the treatments with and without pyridoxine, respectively. Therefore, even though there was not a complete cessation of enzyme production when pyridoxine was removed, there was a decrease in the activity near the end of the fermentation that resulted in a lower cumulative value. However, it was not necessary to supplement the medium with pyridoxine like what was observed for a similar strain in a previous study (Müller et al., 2014).

The dry mass of the mycelium for each treatment was 1.093±0.003 g for medium with pyridoxine and 0.923±0.015 g for medium without pyridoxine at the end of fermentation. There is a significant difference in cell mass between treatments, ($p=0.0084$). In addition, the color of the mycelium for the treatment with no pyridoxine turned darker as did the liquid broth (Fig. 4.2). This is in agreement with the observations of a previous study that stated that when *A. nidulans* is subject to pyridoxine limitation, melanin could be formed as the stress induces the transcription of the factor *brlA* (Mueller, 2012) . Melanin is likely to be the contributor to pigmentation of mycelia and broths (Jørgensen et al., 2010). This modest difference in mass and the different visual aspect of the

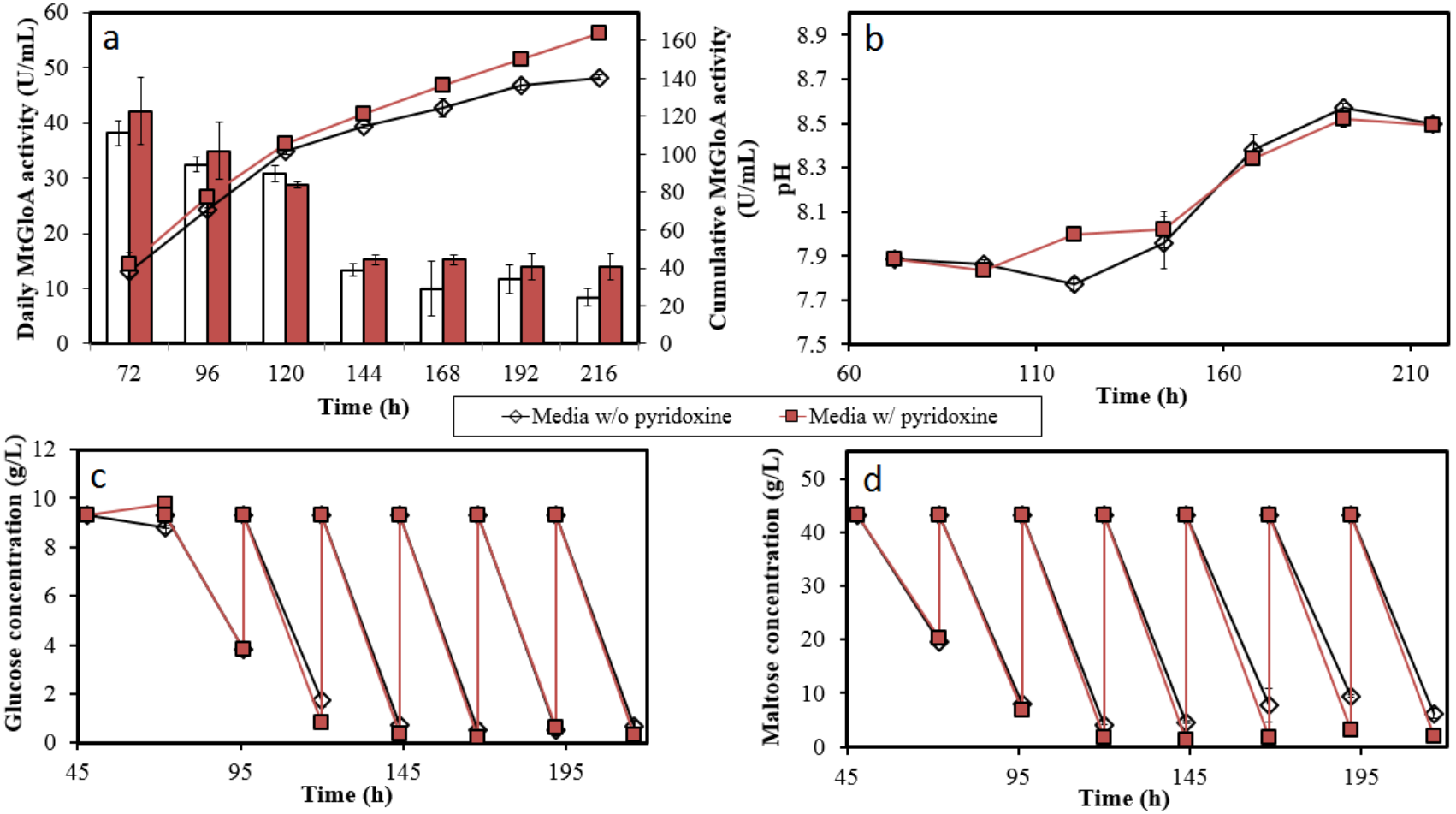

Figure 4.1 (a) MtGloA daily (bars) and cumulative (symbols) activity (b) pH (c) Glucose concentration (d) Maltose concentration profiles during static culture of mutant *A. nidulans* using media with and without pyridoxine. Error bars represent one standard deviation (n=2)

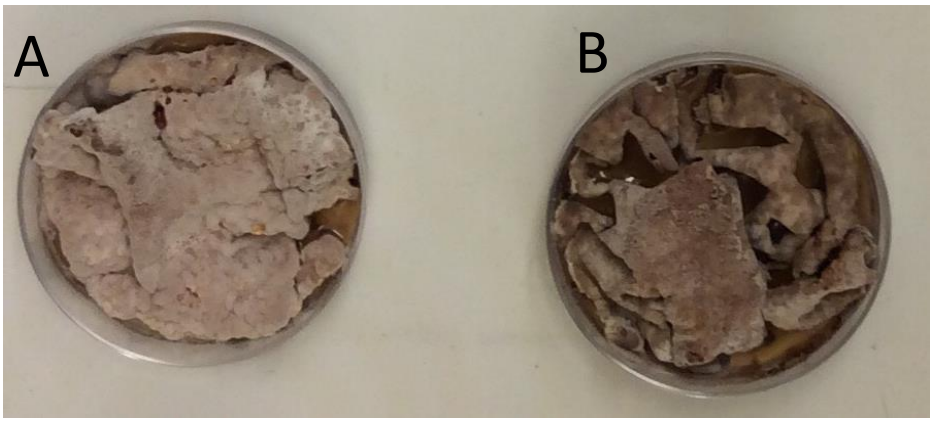

Figure 4.2 Plates at the end of fermentation. (a) Treatment of media with pyridoxine resulted in mycelia of lighter color and clearer fermentation broth (b) Treatment of media without pyridoxine resulted in darker mycelia and liquid broth

mycelia which did not halt enzyme production suggests that auxotrophic conditions may have been reached only partially, perhaps due to carry-over of pyridoxine during mycelia transfers.

Enzyme production in agitated cultures: effect of pyridoxine in the medium

In order to avoid potential interferences associated with carryover of pyridoxine into fresh media, agitated cultures were used to investigate the effect of pyridoxine in the media. Under these conditions, filamentous fungi tend to grow in the form of pellets that can be easily washed during cell mass transplantation to fresh media. Fig. 4.3 and Fig. 4.4 show the main fermentation parameters for both the treatment with pyridoxine and the treatment without pyridoxine. Fig. 4.3a and 4.3b provide daily MtGloA activity data (daily and cumulative) in terms of units per volume and also units per gram of fungus. The latter units are needed to establish a comparison between treatments due to the large difference in fungal mass observed between them. It can be seen how MtGloA activity was similar for both treatments during the first 48 h when compared on an activity per mass basis (Fig. 4.3b), but then activity started decreasing for the treatment with no pyridoxine as a result of vitamin depletion. This decrease is illustrated by the cumulative MtGloA activity curve flattening out at 156 h (Fig. 4.3d). This decrease in MtGloA activity was encompassed with a

steady decrease in pH, from a maximum of 7 at 36 h to near 5.2 at 156 h (Fig. 4.4a). A previous study stated that decrease in pH could be related to succinic acid production (Mueller, 2012). While in the present study, the concentration of succinic acid was slightly lower for the treatment without pyridoxine, other organic acids were found in higher concentrations. In the present study, the concentration of succinic acid in the treatment with pyridoxine varied from 0.25 to 0.67 g/L, while it ranged from 0.13 to 0.45 g/L for the treatment with no pyridoxine during the first 156 h. However, citric acid concentration was higher for the treatment lacking pyridoxine (1.26 to 1.55 g/L) than for the treatment with pyridoxine (0.71 to 0.83 g/L). Similarly, lactic acid concentration ranged from 0.09 to 0.22 g/L for the treatment with no pyridoxine and 0.05 to 0.08 g/L for the treatment with pyridoxine. During the first 156 h, the suspended fungal dry mass in the treatment with pyridoxine reached a maximum concentration of 24.90±1.64 g/L while the treatment lacking pyridoxine only reached 2.93±0.34 g/L (Fig. 4.5). There were more than eight times more suspended biomass in the treatment with pyridoxine than the treatment without pyridoxine. This finding confirms that auxotrophic conditions were reached for the treatment with no pyridoxine. It must be noted that the suspended biomass data underestimates the total biomass for the treatment with pyridoxine, since part of the fungus grew attached to the walls of the baffled flask. The appearances of the treatments were similar to that observed in static cultures: the treatment without pyridoxine had broth and pellets with a much darker color than the treatment with pyridoxine (Fig. 4.6). If this information is taken into account, the previously stated concentrations of organic acids reveal that the treatment without pyridoxine produced between 11 and 16 times more citric acid, between 2 and 6 times more succinic acid, and between 10 and 27 times more lactic acid on a gram of organic acid per gram of mycelium basis. Therefore, the auxotrophic conditions favored organic acid production.

At 168 h pyridoxine (1 mg/L) was added to the treatment that previously did not contain pyridoxine to resume MtGloA production that had been lost between 132 and 156 h. The pellets

were washed with 0.89% NaCl solution after 12h of the addition to avoid excessive growth due to pyrodixine carryover. The addition of the vitamin had an impact on all parameters measured. Media pH stopped decreasing and quickly recovered to levels similar to the ones observed in the treatment with pyridoxine (Fig. 4.4a). Protein concentration increased from below 0.2 g/L to a range of 0.4 to 0.8 g/L, together with a rapid increase in MtGloA activity. This increase in activity resulted in similar cumulative values for both treatments at the end of fermentation: 26.64±6.14 U/mg suspended mycelia and 26.14±8.39 U/mg suspended mycelia for the treatments with and without pyridoxine, respectively. The cell mass concentration of suspended mycelia (pellets) also increased from 2.73±0.53 g/L at 168 h to 9.87±0.93 g/L at 180 h and reached 12.93±2.01 g/L at the end of fermentation (276 h) in the treatment with no pyridoxine (Fig. 4.5). The color of the pellets and broth also turned from dark brown to clear yellow when pyridoxine was added to this treatment. These results show that it is possible to sustain MtGloA production using auxotrophic conditions coupled with periodical heterotrophic conditions (addition of pyridoxine) that are similar to full heterotrophic conditions. In summary, these findings suggest that it would be possible to maintain a low cell mass and sustained enzyme production when good control of periodic pyridoxine supplementations is used.

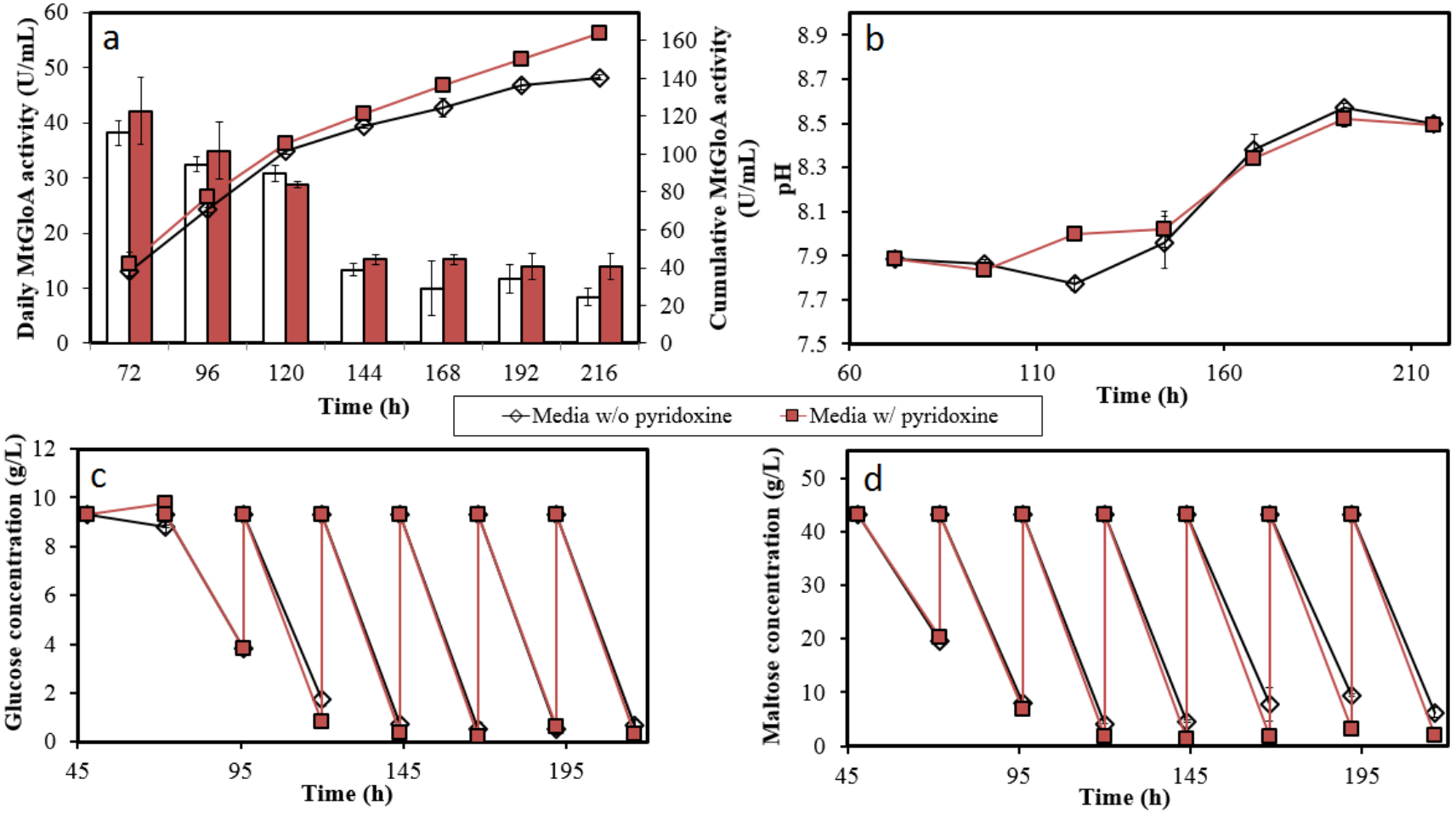

Figure 4.3 (a) Daily MtGloA activity (U/mL) (b) Daily MtGloA activity (U/mg mycelia) (c) Cumulative MtGloA activity (U/mL) (d) Cumulative MtGloA activity (U/g mycelia) profiles during agitated culture of mutant *A. nidulans* using media with and without pyridoxine. Error bars represent one standard deviation (n=3). An arrow points at the sample before media was supplemented with 1 mg/L pyridoxine (168 h)

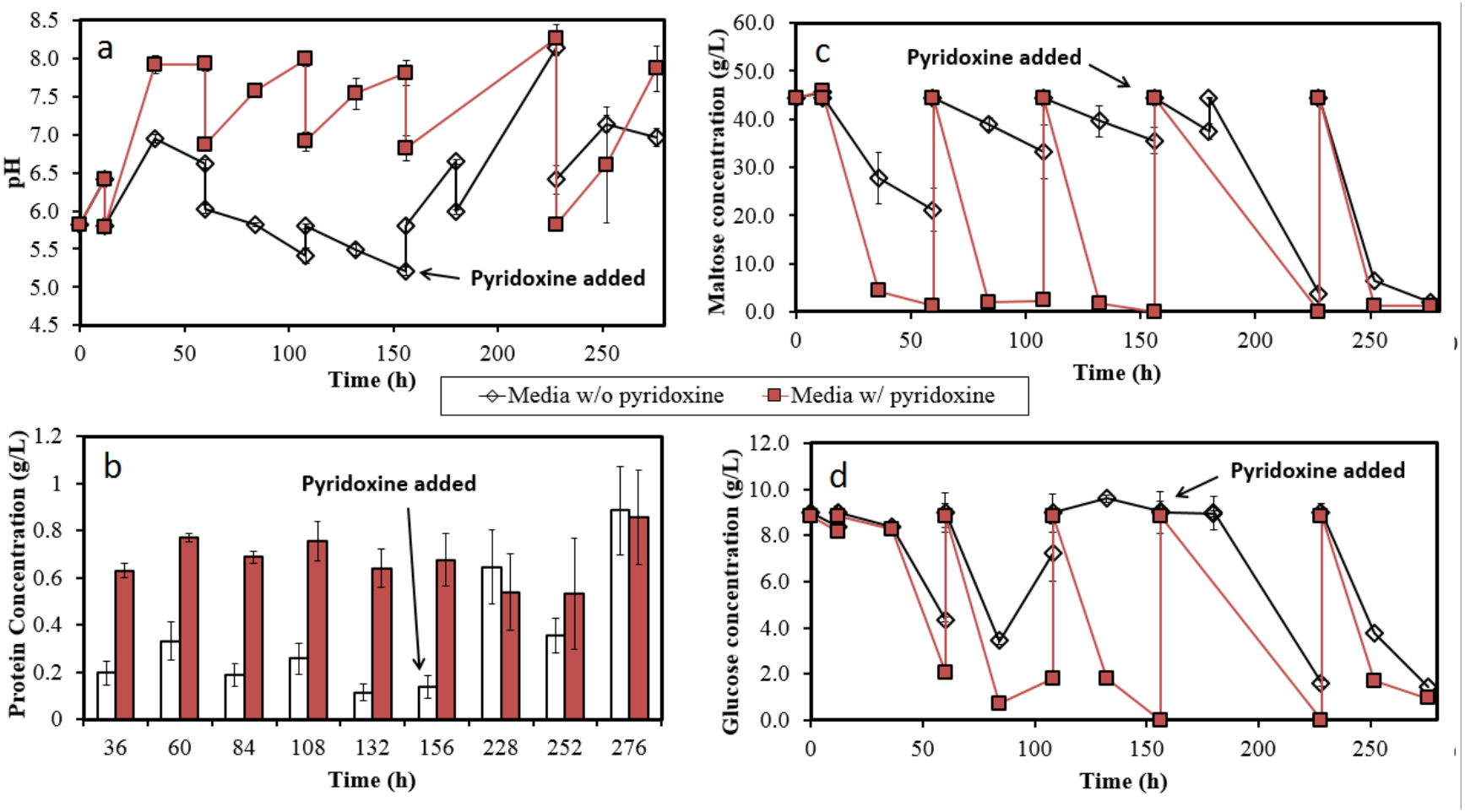

Figure 4.4 (a) pH (b) Protein concentration (g/L) (c) Maltose concentration (g/L) (d) Glucose concentration (g/L) profiles during agitated culture of mutant *A. nidulans* using media with and without pyridoxine. Error bars represent one standard deviation (n=3). An arrow points at the sample before media was supplemented with 1 mg/L pyridoxine (168 h)

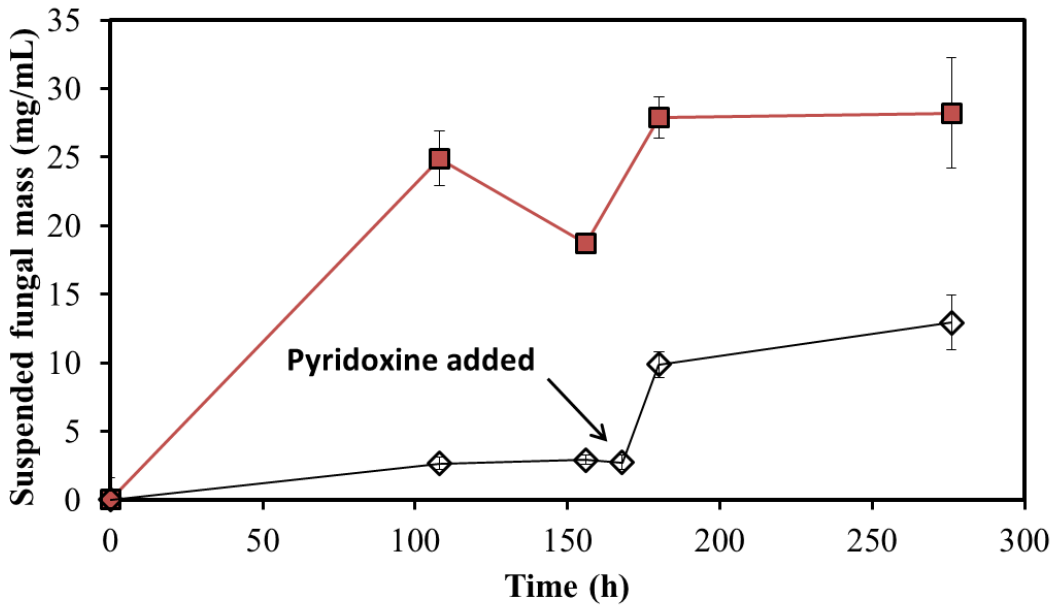

Figure 4.5 Suspended dry fungal mass (pellets) during agitated culture of mutant *A. nidulans* using media with pyridoxine (■) and media without pyridoxine (♦). Error bars represent one standard deviation (n=3). An arrow points at the time when media was supplemented with 1 mg/L pyridoxine (168 h)

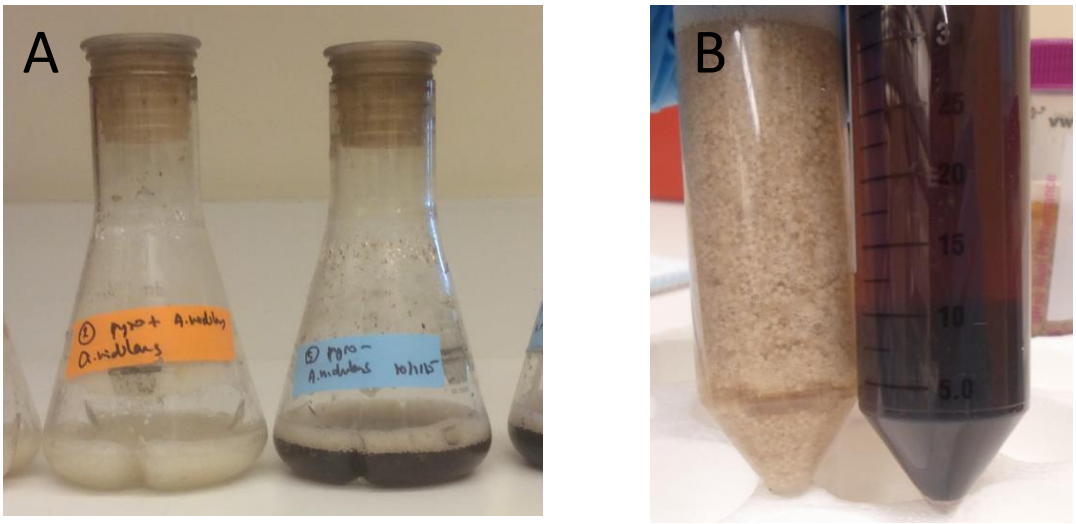

Figure 4.6 Picture of flasks during the experiment (a) Flasks during fermentation, on the left side a flask with media with pyridoxine and on the right side a flask with media without pyridoxine (b) A closer look at the pellets from each treatment, in the same order

Enzyme production in agitated cultures: use of extremely diluted pyridoxine levels in the media

It was hypothesized that growth of the mutant *A. nidulans* can be controlled at a very slow rate while sustaining enzyme production by using very low pyridoxine concentrations. Reduced growth of an auxotrophic *A. nidulans* strain with a riboflavin marker has been shown previously in the literature (Zheng et al., 2015). In order to confirm that it is possible to control the growth rate of the MtGloA mutant *A. nidulans* strain, an experiment with solid media was designed. Fig. 4.7 shows the growth of the MtGloA *A. nidulans* mutant with media containing various concentrations of pyridoxine. Levels of pyridoxine lower than 1.0 µg/L resulted in no growth and concentrations equal to or higher than 4 µg/L resulted in growth similar to the standard pyridoxine-containing medium (1,000 µg/L). Therefore, the range of 1.0 to 4.0 µg/L pyridoxine was selected as a starting point for an agitated, submerged fermentation experiment that studied the possibility of sustaining enzyme production at reduced growth rates by using dilute pyridoxine concentrations.

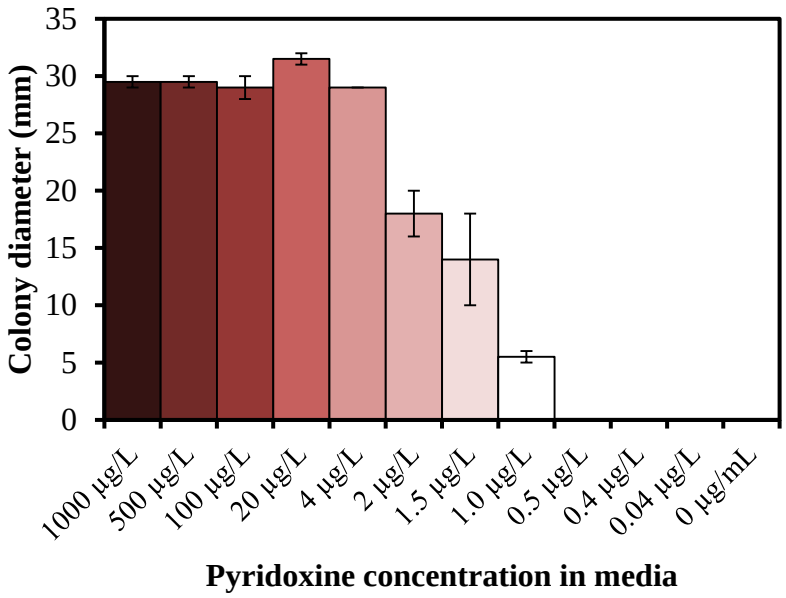

Figure 4.7 Diameter of colonies after incubation for 72 h of mutant *A. nidulans* using agar media with different concentrations of pyridoxine. Error bars represent standard deviation (n=2)

Fig. 4.8 and Fig. 4.9 show the main fermentation parameters for an experiment investigating extremely diluted levels of pyridoxine in the media of agitated cultures. The control treatment had no pyridoxine, and two treatments used media with very low concentrations of pyridoxine based on the findings revealed in the preliminary experiment (Fig. 4.7). At time zero the concentrations used were 1.0 and 1.5 µg/L for treatments 1 and 2, respectively, and then were increased to 3.0 and 4.5 µg/L at 144 h and to 9.0 and 13.5 µg/L at 192 h. Until the last change in media composition (192 h), no significant changes were observed among treatments, except in terms of protein concentration (Fig. 4.8b), which quickly decreased to zero at 196 h for the control treatment and remained between 0.12 and 0.3 g/L for the other two treatments. Media pH and MtGloA activity were very similar among treatments until 196 h. After that point, which corresponds with the last change in media composition for treatments 1 and 2 (9.0 and 13.5 µg/L, respectively), these two treatments resumed enzyme production (Fig. 4.8c and 4.8d), sugar consumption (Fig. 4.9) and suffered pH changes (Fig. 4.8a). These pH changes were different from those observed when media with pyridoxine (1,000 µg/L) was used (increase of pH up to 8.0). In this case, the resumption of enzyme production was coupled with a decrease in pH, especially for the treatment with the lowest pyridoxine concentration (Treatment 1), which decreased below 6.0. The final cumulative MtGloA activities for each treatment were 12.30±3.22, 43.68±16.29, and 34.25±3.89 U/mL for the control and treatments 1 and 2, respectively (Fig. 4.8d). It can be seen that treatments 1 and 2 resulted in similar enzyme production due to the large variability observed among replicates for one of the data points of treatment 1. Therefore, it was concluded that a pyridoxine concentration between 9 and 13.5 µg/L can allow sustained MtGloA production with a slow growth rate of the strain. These concentrations, however, may have to be increased when more fungal biomass is available, in order to guarantee a minimum supply of the vitamin to all cells present.

A potential method to decide whether to maintain or increase the concentration of pyridoxine in the media may be the color of the broth. It has been seen in previous experiments that the broth turns darker as the fungus is stressed by pyridoxine starvation. These changes in coloration were monitored in this experiment by spectrophotometry at 425 nm (Fig. 4.10). It was observed that in the control treatment absorbance decrease was paired with loss of MtGloA activity until the absorbance was close to zero from 192 h. For the other two treatments, however, absorbance remained high (1.5-1.9 units) until the last change in pyridoxine concentration (192 h). After that point, which coincided with the recovery of enzyme production, a sudden decrease in absorbance to levels ranging from 0.5 to 1.0 units was observed. A trend can be observed when all the absorbance data points are plotted together versus MtGloA activity (Fig. 4.10b). Absorbance of the broth at 425 nm appears to be related to the activity obtained. When absorbance was very low (below 0.5 units), MtGloA activity was close to zero (less than 2 U/mL), which corresponds with the last data points for the control treatment where no fungal activity was observed. In the same manner, MtGloA activity was very low when the color of the liquid broth was very dark (absorbance above 1.8 units). This corresponds with stressed fungal cells due to the unavailability of pyridoxine. The region between 0.5 and 1.5 units of absorbance at 425 nm correlated well with the highest MtGloA activities, which matches with the absorbance observed for treatments 1 and 2 after the last change in pyridoxine concentrations that lead to the resumption of enzyme production. These results suggest that spectrophotometric analyses can be used to monitor future continuous fermentations, thus enabling decisions on the concentration of pyridoxine in the liquid media that should be selected.

The ability to control fungal growth using very diluted concentrations of pyridoxine was also assessed based on measurements of the suspended mycelia mass as pellets throughout the fermentation (Fig. 4.11**)**. It can be seen how the last change in media composition (192 h) caused an increase in biomass formation that coincided with resumption of MtGloA production.

Pyridoxine concentrations used at 192 h were 9.0 and 13.5 µg/L for treatments 1 and 2, respectively. Growth resumed in a slow fashion compared to what had been observed when adding pyridoxine at 1,000 µg/L. In this case (9-13.5 µg/L pyridoxine), the dry mass concentration of pellets increased from 2.0 to 6.5 g/L in 144 h compared to a higher increase (2.7 to 9.9 g/L) in only 12 h when pyridoxine was added at 1,000 µg/L (Fig. 4.5)**.** This means that the growth rate of the strain using extremely diluted pyridoxine concentrations was 0.03 g mycelia/L*h; only 5% of that observed for the media with 1,000 µg/L pyridoxine (0.60 g mycelia/L*h).

A concentration of 9.0 µg/L pyridoxine could be a good candidate to be used in continuous TBR fermentations, since it resulted in reduced cell growth while allowing enzyme production.

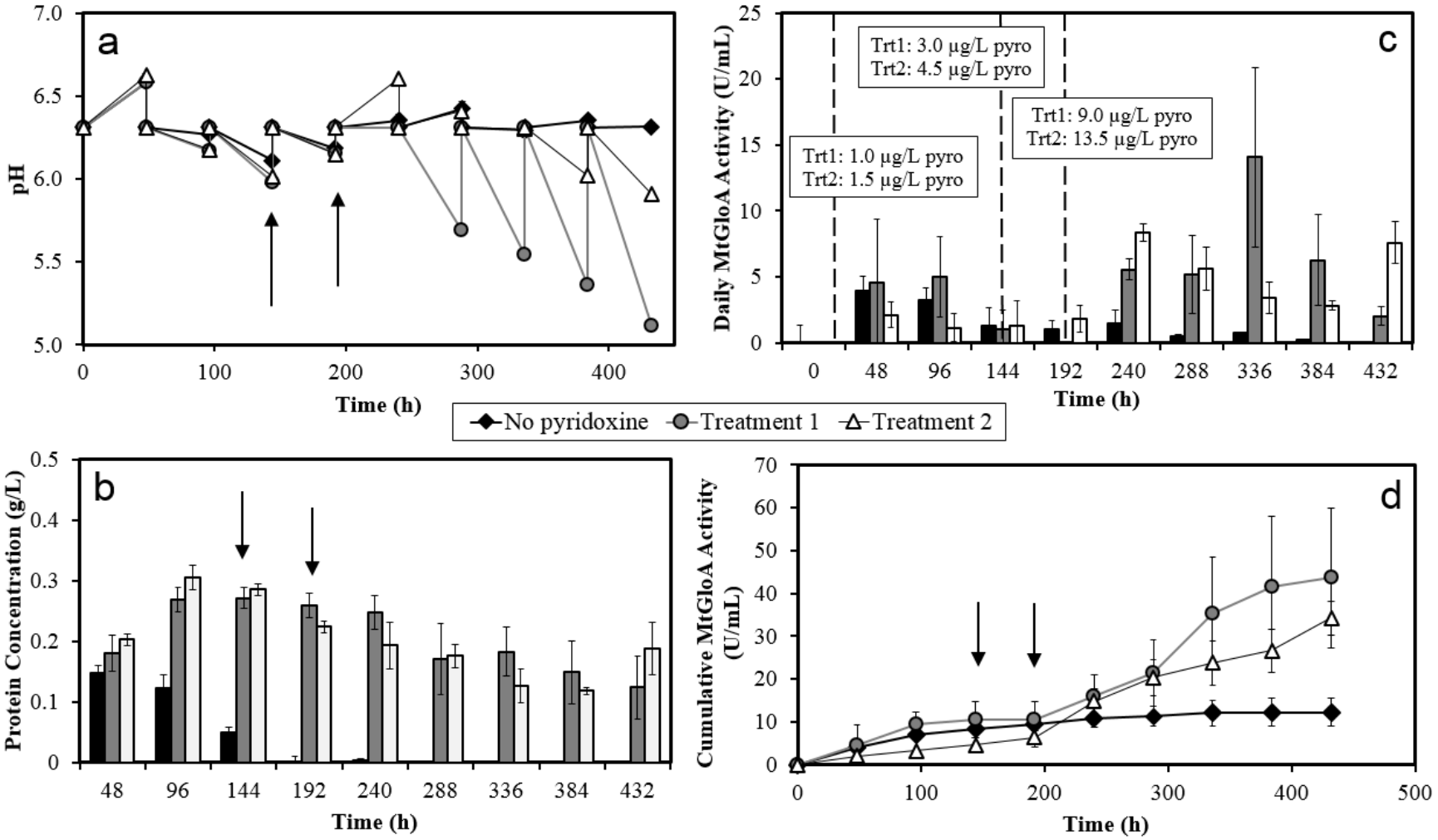

Figure 4.8 (a) pH (b) Protein concentration (g/L) (c) Daily MtGloA activity (U/mL) (d) Cumulative MtGloA activity (U/mL) profiles during agitated culture of mutant *A. nidulans* using media with extremely diluted pyridoxine levels. Concentrations of pyridoxine levels in treatments 1 and 2 are shown in (c). The arrows point at the times when pyridoxine concentrations were changed. Error bars represent one standard deviation (n=3)

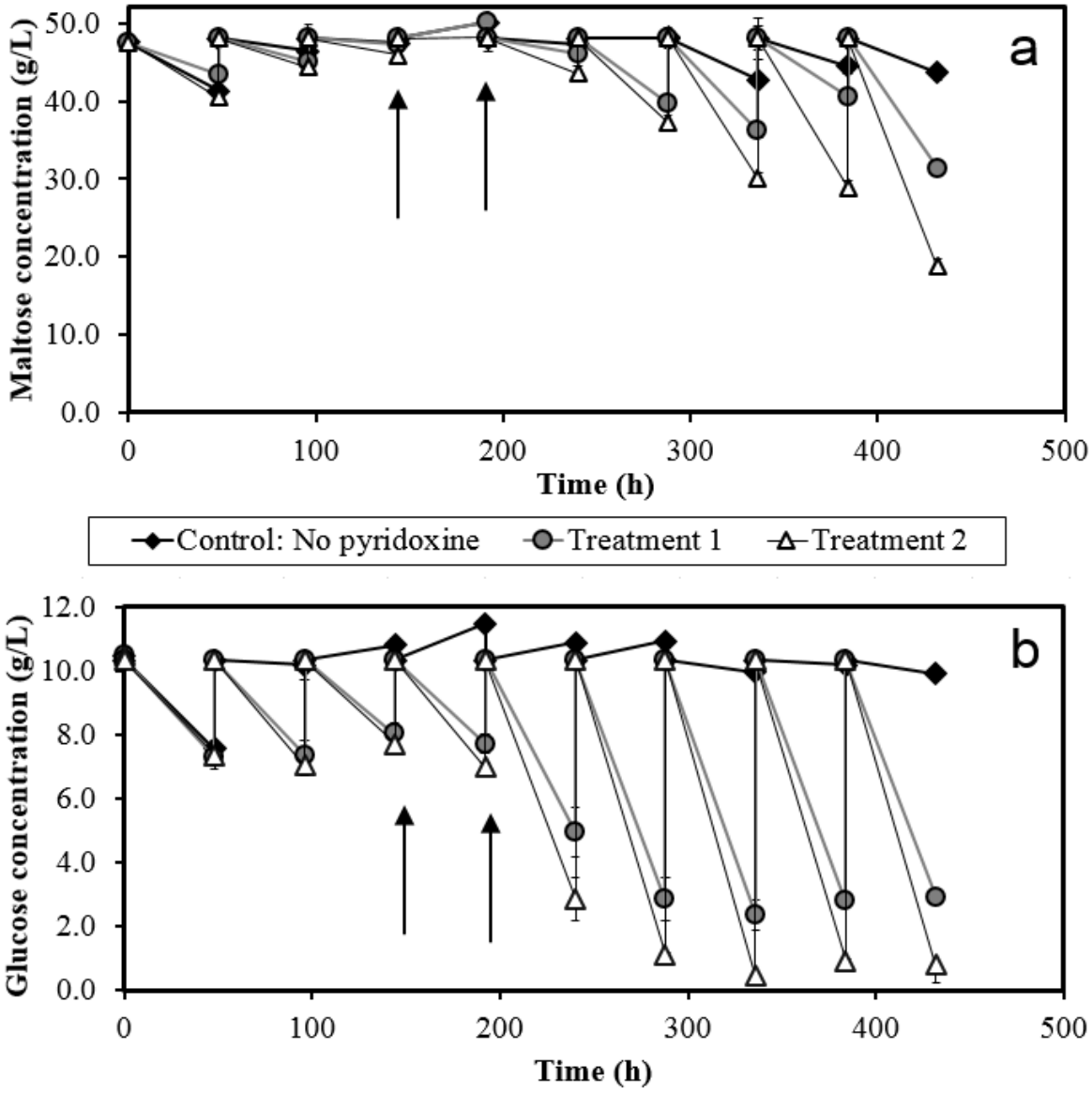

Figure 4.9 (a) Concentration of maltose and (b) concentration of glucose during agitated culture of mutant *A. nidulans* using media with extremely diluted pyridoxine levels. Concentrations of pyridoxine in treatments 1 and 2 were changed at 0, 144 and 192 h, as shown in Fig. 4.7c. The arrows point at the times when pyridoxine concentrations were changed. Error bars represent one standard deviation (n=3)

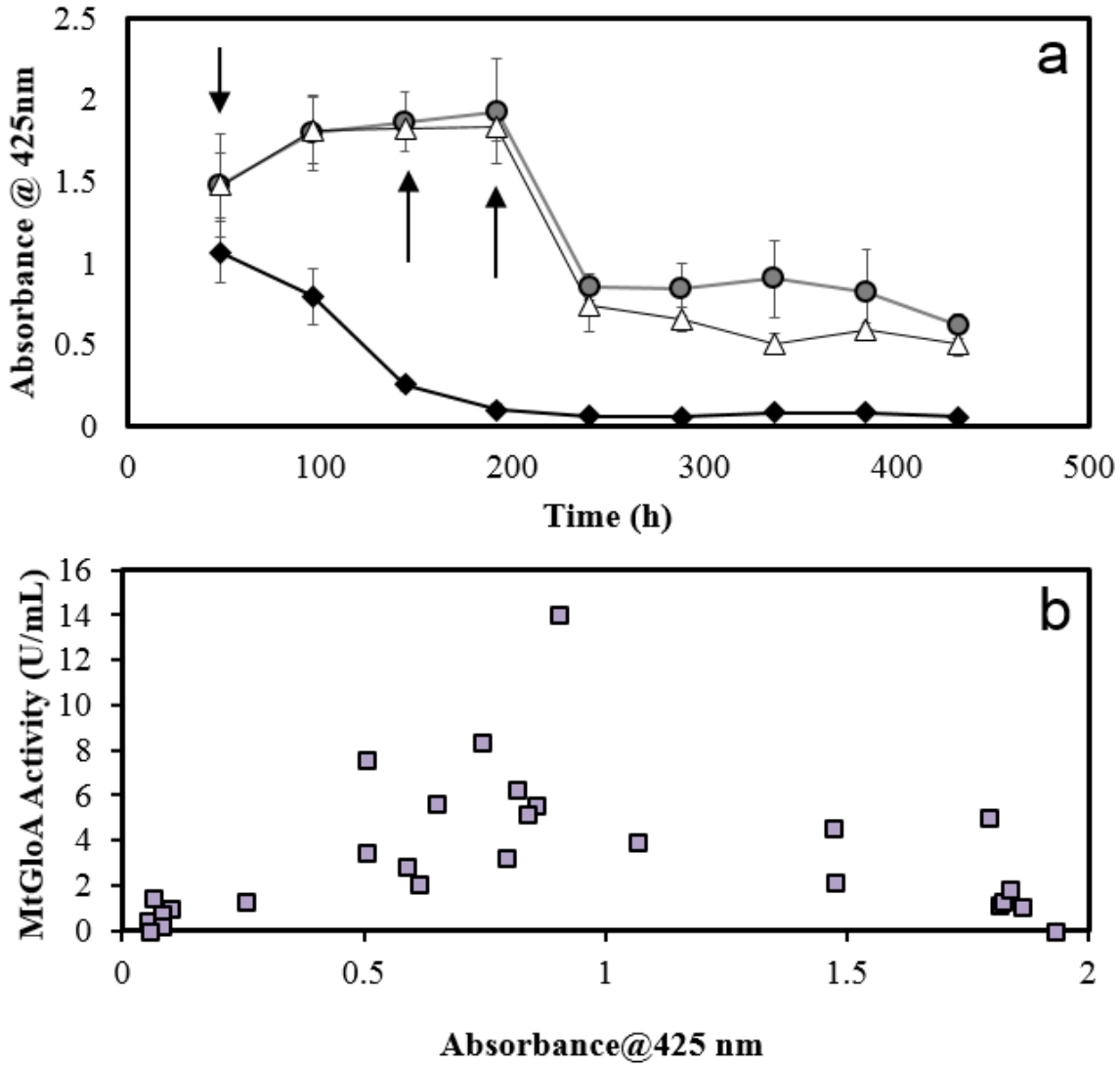

Figure 4.10 (a) Absorbance at 425 nm in control treatment (♦), treatment 1 (●), and treatment 2 (▲) (b) Absorbance at 425 nm versus *MtGloA* activity in the liquid broth of agitated cultures of mutant *A. nidulans* using media with extremely diluted pyridoxine levels. The arrows point at the times when pyridoxine concentrations were changed as indicated in Fig. 4.7c. Error bars represent one standard deviation (n=3)

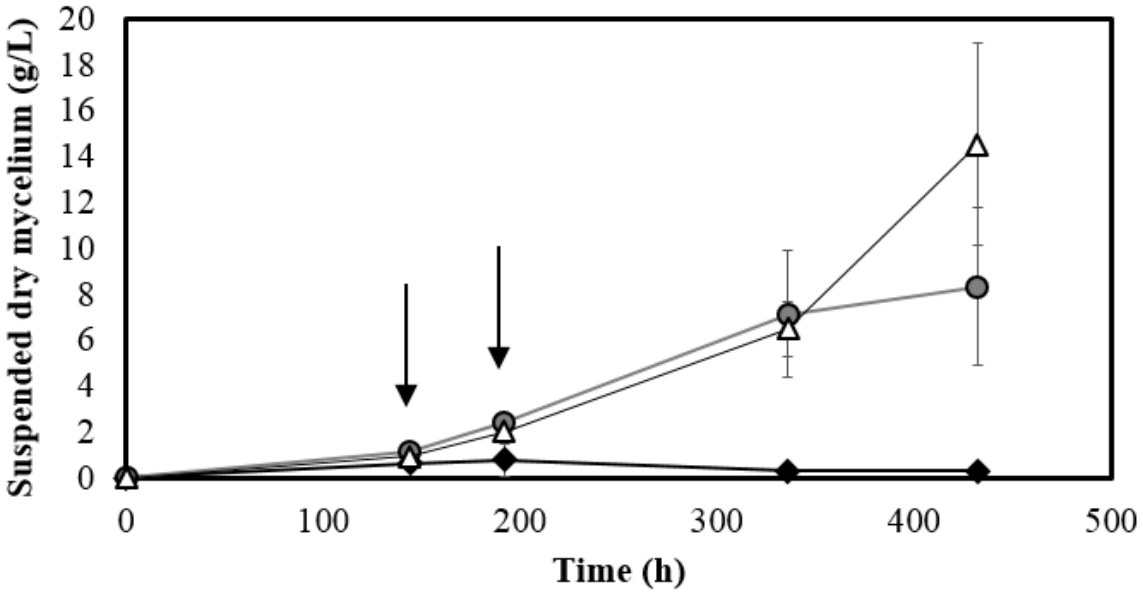

Figure 4.11 Suspended dry fungal mass (pellets) during agitated culture of mutant *A. nidulans* in control treatment (♦), treatment 1 (●), and treatment 2 (▲). The arrows point at the times when pyridoxine concentrations were changed. Error bars represent one standard deviation (n=3)

4.5 Conclusions

In this study, a mutant *A. nidulans* strain that overexpressed an MtGloA enzyme was constructed. The strain had a pyridoxine marker therefore requiring this vitamin for growth. Auxotrophic conditions favored melanin and organic acid formation and reduced protein secretion. Supplementation of media with extremely diluted pyridoxine concentrations allowed enzyme production to be resumed while controlling the growth rate of the strain at 5% of the rate observed with standard 1 mg/L pyridoxine media. This strategy can be used in the future to operate bioreactors without clogging issues.

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CHAPTER V

PREVENTION OF MELANIN FORMATION DURING ARYL ALCOHOL OXIDASE PRODUCTION UNDER GROWTH-LIMITED CONDITIONS USING AN *ASPERGILLUS NIDULANS* CELL FACTORY

5.1 Abstract

An *A. nidulans* cell factory was genetically engineered to produce an aryl alcohol oxidase (MtGloA). The cell factory initiated the production of melanin when limited growth conditions were established using stationary plates and shaken flasks. This phenomenon was more pronounced when the strain was cultured in a trickle bed reactor (TBR). This study investigated different approaches to reduce melanization of the fungal mycelia and liquid medium in order to increase the yield of enzyme production. Removal of copper from the medium recipe reduced melanization in agar cultures, and increased enzyme activities by 48% in agitated liquid cultures. Copper has been reported as a key element for tyrosinase, an enzyme responsible for melanin production. Additionally, two pigmentation inhibitors were tested in agitated liquid cultures. Both tropolone and ascorbic acid reduced melanization, but the former inhibited the fungus when used at high concentrations (20 mg/L). Ascorbic acid (0.44 g/L) abolished melanin accumulation, did not affect growth parameters and resulted in enhanced aryl alcohol oxidase activity: more than two-fold higher compared to a control treatment with no ascorbic acid.

5.2 Introduction

Filamentous fungi, including aspergilli, are widely used for enzyme production. Commercial production of enzymes had a market of $4.5 billion in 2012, $4.8 billion in 2013 and expectations of annual growth rate over 8% until 2018 (BCC-Research, 2014). Most industrial processes use the technology called submerged fermentation (SmF) in which fungi is grown in liquid medium with nutrients and oxygen availability (Couto & Toca-Herrera, 2007; Pandey, 2003). Solid state fermentation (SSF) technology has gained attention because of higher enzyme activities obtained (Diaz-Godinez et al., 2001; Krishna, 2005) and potential lower costs due to the absence of free water in the reactor. However, challenges such as process control or difficulty to establish continuous operation remain (Pandey, 2003). A hybrid technology that uses a trickle bed reactor (TBR) has been proposed, and it combines characteristics of both SmF and SSF (Müller et al., 2015). The fungus is grown on an inert solid material inside a column reactor and liquid medium with nutrients is pumped to the top of the column and then trickles down the bed. This system allows continuous operation and it presents an easier enzyme recovery than SmF because the mycelia remains inside the reactor. However, excessive growth of the fungus can lead to clogging in the column and pipelines. This has been recently addressed by including a pyridoxine marker in the genotype of an *Aspergillus nidulans* strain that overexpresses xylanase (Müller et al., 2015). When pyridoxine was not provided externally, growth of the strain could be controlled while enzyme production continued. However, growth limitation in aspergilli has been observed to trigger pigmentation processes such as melanin production (Horowitz & Shen, 1952; Müller et al., 2014; Rowley & Pirt, 1972).

Melanins are secondary metabolites with non-essential roles in product development or growth in fungi (Goncalves & Pombeiro-Sponchiado, 2005). They are brown or black pigments that play a protective function in the microorganism. As an extracellular redox buffer, melanin neutralizes oxidants such as HOCl or H_2O_2 (Goncalves & Pombeiro-Sponchiado, 2005). In addition, the presence of melanin in the fungal wall increased the resistance of *A. nidulans* to cell lysis (Kuo & Alexander,

1967). Melanized fungal strains have been reported to survive in extreme conditions such as in Antarctica or inside nuclear reactors (Eisenman & Casadevall, 2012). Unfortunately, pigmentation redirects the use of substrate towards melanin formation and it reduces the potential for enzyme production. It also generates insoluble material inside the system, which could result in clogging issues upon accumulation, and it promotes undesirable foaming in the reactor (Jørgensen et al., 2011). One of the key steps in melanin production in *A. nidulans* involves enzymatic reactions catalyzed by tyrosinase, an enzyme that contains two copper atoms in its active site (Held & Kutzner, 1990). Previous literature studies suggest that melanin production can be reduced or suppressed by using inhibitors of tyrosinase. Some of these inhibitors, such as tropolone, act as copper chelators (Kahn & Andrawis, 1985). Others, such as ascorbic acid, switch the pathway away from melanin production by consuming intermediate compounds. Melanin has been described in the literature as a product of an oxidation-reduction system, in which a reducing agent can shift the potential away from the optimum for melanogenesis (Figge, 1940). Specifically, the mechanism used is the reduction of o-quinones to their o-phenol precursors (Lozano de Gonzalez et al., 1993). A number of other reducing agents, such as cysteine or glutathione have been reported to inhibit pigmentation (Paschkis et al., 1944). Many of the studies investigating inhibition of tyrosinase were done in vitro using the purified enzyme (Golan-Goldhirsh & Whitaker, 1984; Paschkis et al., 1944) instead of living microorganisms, so their effect on the other cell functions has not been evaluated.

To the best of our knowledge, there are no published studies that investigate the possibility of reducing melanin production in limited growth fungal cultures for enzyme production using pigmentation inhibitors. The present study addresses this research gap by evaluating methods to mitigate the melanization of an *A. nidulans* engineered strain that produces an aryl alcohol oxidase under growth limited conditions. Two strategies were studied including changes in the trace element composition of the medium, and use of two inhibitors: tropolone and ascorbic acid.

5.3 Materials and Methods

Strain and spore production

An *A. nidulans* cell factory was obtained from the Fungal Genetic Stock Culture (FGSC, Manhattan, Kansas, USA). The strain is deposited in the FGSC with the identifier A773 and presents the following genotype: *pyrG89*; *wA3*; *pyroA4*. This genotype includes a marker (*pyroA4*) that limits growth of the strain when pyridoxine is not provided in the medium since the microorganism is not able to synthesize it on its own. The strain obtained from the FGSC was further genetically engineered by DNA mediated transformation of a plasmid (pEXPYR) that contains a wild-type *pyrG* complementing gene and a construct that contains the glucoamylase promoter fused to the *MtgloA* ORF (including its own signal peptide) as described elsewhere (Segato et al., 2012). The resulting *A. nidulans* strain overexpresses and secretes *Myceliophthora thermophila* MtGloA in media containing maltose.

Spores for the experiments were produced by growing the resulting strain on solid medium containing (per L) 9.0 g glucose, 50 mL of 20x Clutterbuck salt solution (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4$, 30.4 g/L KH_2PO_4), 1 mL 1,000x trace element solution (22 g/L $ZnSO_4 \cdot 7H_2O$,11 g/L H_3BO_3, 5.0 g/L $MnCl_2 \cdot 7H_2O$, 5.0 g/L $FeSO_4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 5H_2O$, 1.6 g/L, $CuSO_4 \cdot 5H_2O$, 1.1 g/L $Na_2MoO_4 \cdot 4H_2O$, 50 g/L Na_2-EDTA), 0.001 g pyridoxine, and 15% agar. The pH was adjusted to between 6 and 6.5 using a 6N NaOH solution. The medium was sterilized by autoclaving at 121ºC for 20 min (PRIMUS, Sterilizer Co., Inc., Omaha, NE, USA). Twenty-five µL of spore stock solution were pipetted onto the solid medium and spread homogeneously using aseptic conditions. The plates were then incubated at 37ºC for 48 h until the surface of the solid media was covered by white spores. A control plate with medium lacking pyridoxine was inoculated in a similar fashion to verify the stability of the strain. Observation of growth on this control plate served as an indicator of contamination or issues with the engineered strain. The plates with spores were kept refrigerated (4ºC) until used.

Composition of media used in experiments

Different media compositions were used during this study, which were variations from the medium named "Medium A" used in previous research shown in Chapter 4 and elsewhere (Müller et al., 2015; Müller et al., 2014) . All recipes included 50 mL/L Clutterbuck salt solution as described above and 47.6 g/L maltose and 10.0 g/L glucose. Maltose is required to promote protein expression and the ratio between maltose and glucose has been described as the optimum for enzyme production (Mueller, 2012). The media recipes differed in the composition of the 1000x trace element solution added at a concentration of 1 mL/L. Table 5.1 shows the concentrations of each trace element in the 1000x solutions used. The recipe of Medium B was adapted from a commonly used media recipe from the literature (Cove, 1966; Pontecorvo et al., 1953). Medium C was a modification of Medium B by removing all elements except boron, iron and Na-EDTA.

Table 5.1. Composition of trace metals in different 1000x Trace Element solution recipes

	Concentration, g/L (1000x)		
Trace Element	**Medium A**	**Medium B**	**Medium C**
H_3BO_3	11.0	0.04	0.04
$FeSO_4 \cdot 7H_2O$	5.0	0.8	0.8
Na_2-EDTA	50.0	50.0	50.0
$ZnSO_4 \cdot 7H_2O$	22	8	-
$MnCl_2 \cdot 7H_2O$	5.0	0.8	-
$Na_2MoO_4 \cdot 4H_2O$	1.1	0.8	-
$CuSO_4 \cdot 5H_2O$	1.6	-	-
$CoCl_2 \cdot 5H_2O$	1.6	-	-

Pyridoxine was added for the pyridoxine-containing media at different levels, to be presented later in this section. The growth media, for example, contained 1 mg/L pyridoxine. All media

components, except for sugars, were sterilized by autoclaving at 121ºC for 20 min. Adjustment of pH with sterile 6N NaOH solution was done after autoclaving the medium in order to avoid formation of precipitate. A concentrated 10x sugar stock solution was filter sterilized using 0.20 µm PES sterile filters. Whenever used, inhibitors of tyrosinase (tropolone and ascorbic acid) were also filter sterilized and added to the media at different concentrations.

Continuous enzyme production in TBR: reactor set up and operation

The custom built TBR system is depicted in Figure 5.1. The column was a 60 cm tall glass column with a diameter of 10.5 cm. The random packing material used in this experiment was comprised of 568 HDPE plastic rings (model BCN 020, GEA 2H Water Technologies GmbH, Germany, Europe) that filled 53 cm of the column. The rings had equal length and diameter (20 mm), a surface of 610 m^2/m^3 and a protected surface of 400 m^2/m^3. A recirculation loop recycled the medium from the sump back to the top of the packed bed using a peristaltic pump (Masterflex, Cole-Parmer, IL, USA). The liquid trickled down the packing material of the column where the mycelia developed. A sampling port in the recirculation loop allowed periodic sampling of the liquid broth. Two additional peristaltic pumps were used to add fresh media in the system and to remove product at the same rate to keep a constant total volume in the system. Air was pumped from the room at 1.15 L/min through a sterile 0.2 µm PTFE filter (Pall Corporation, NY, USA) and exited the reactor at the top through a 250 mL glass bottle that served as condensation vessel to prevent the exhaust line from being blocked by condensing liquid. The reactor was maintained at 37ºC using a heating tape wrapped around the column. The TBR column with the packing material was sterilized by autoclaving at 121ºC for 20 min. The tubing and fittings for the recirculation loop were autoclaved separately and assembled aseptically in a biosafety cabinet (Nuaire, Plymouth, MN, USA).

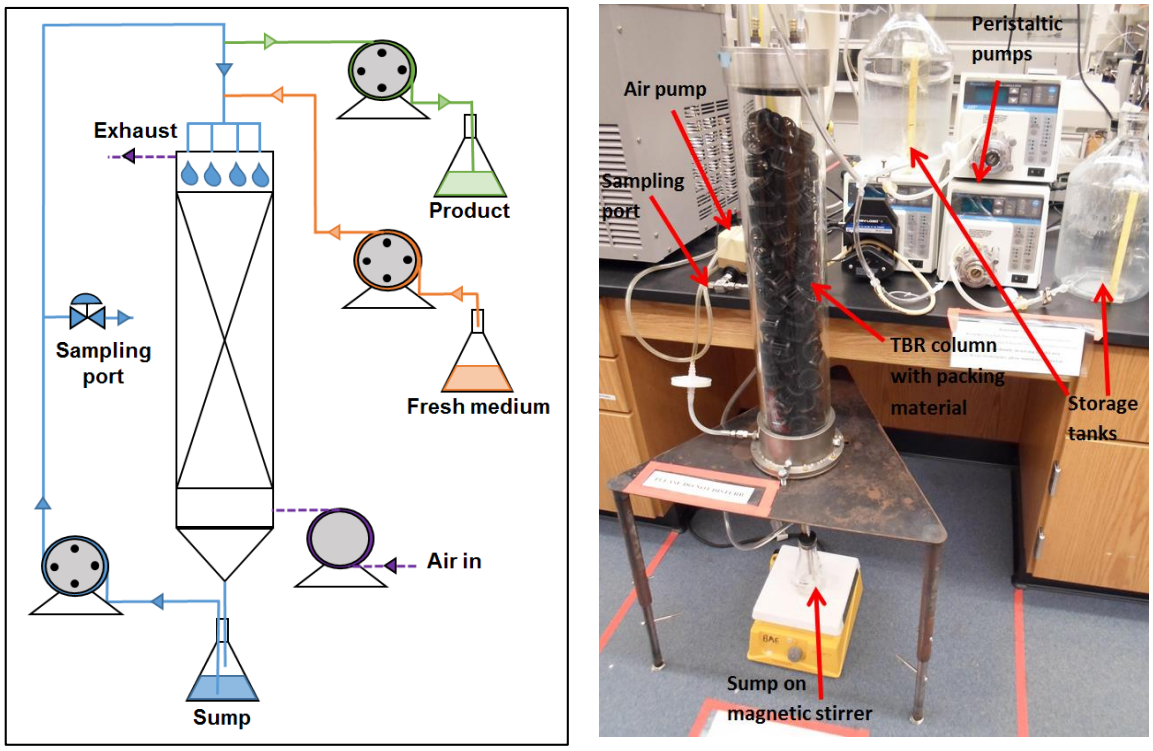

Figure 5.1. Diagram of the continuous TBR setup (left) and picture of the real system in the laboratory (right)

The reactor was initially filled with 800 mL medium A (Table 5.1) with 1 mg/L pyridoxine to allow fungal growth. The media was recirculated at 250 mL/min for 1 h prior to inoculation. Ten mL sterile DI water containing *A. nidulans* spores (10^7-10^8 spores/mL) were aseptically added through a port on top of the column. The mycelia was allowed to develop on the packing material for 39 h. Then, the remaining free liquid was collected and 800 mL of sterile 0.89% NaCl were added in the system. The solution was recirculated for about 2h to wash the system and reduce carryover of pyridoxine, and then the free liquid was removed. After the washing step fresh medium with reduced pyridoxine concentration (30 µg/L) was added to obtain a total volume of 1.1 L. Continuous fermentation was started by continuously adding fresh media and removing product at similar rates. The flow rates of media in and out determined the dilution rate used. When the dilution rate was increased, the concentration of pyridoxine was reduced accordingly during media preparation in order to maintain a constant pyridoxine supply of 1 µg/L*h. Liquid samples were aseptically taken every 7

to 10 h and were analyzed for pH, protein and sugar concentration and enzyme activity as described below. Pigmentation was assessed both by visual observation of the color of the mycelia covering the packing material and by measuring the absorbance of the liquid broth at 425 nm (UV-2100, Cole Parmer, Vernon Hills, IL, USA). This wavelength was used elsewhere to follow formation of soluble melanin in *A. nidulans* fermentations (Rowley & Pirt, 1972). When the experiment was terminated, the system was autoclaved and the packed bed was washed using 5L of water to remove remaining sugars and metabolites. Then, the packing material with the fungus was air dried inside a fume hood for determination of the final fungal mass by weight.

Evaluation of pigmentation using solid media and agitated flasks

A preliminary evaluation of pigmentation was done using solid media in petri dishes. Plates with 10 mL of different media with 15% agar and 1 mg/L pyridoxine were used. Spores of the fungus were inoculated in the center point of the dish and the cultures were incubated at 37ºC for 96 h. The medium in the center of the plates turned dark upon depletion of nutrients. This was used as a qualitative method of screening media recipes in relation to their pigmentation.

Quantification of pigmentation in different media was done using submerged fermentation in 40 mL agitated cultures with very dilute pyridoxine levels (10 µg/L). Spores were harvested from solid media plates and were aseptically transferred to a microcentrifuge tube containing 1.5 mL of sterile distilled water. Aliquots of 100 µL of this spore solution were pipetted into each baffled flask. The flasks were incubated at 37ºC and agitated at 225 RPM in an orbital shaker. Initially, all flasks contained media with 1 mg/L pyridoxine to allow pellet growth. After 12 h, media was replaced with media with only 10 µg/L pyridoxine to reduce growth while allowing protein production as observed in Chapter 4. In order to reduce carryover of pyridoxine to the fresh media, cell pellets were washed prior to the first transfer. The contents of each baffled flask were poured into a 50 mL sterile centrifuge tube and centrifuged at 3,750 rpm for 15 min. The supernatant was removed and a similar

amount of sterile 0.89% NaCl solution was added. The resulting suspension was well mixed and centrifuged again and then the supernatant was discarded. Finally, the washed pellets were aseptically transferred to the flasks containing fresh media. After this, the transfer of pellets to fresh media was done every 48 h without a washing step. Liquid samples were periodically withdrawn and used for absorbance reading at 425 nm (as indication of pigmentation), pH analysis, determination of sugars concentration by HPLC, protein concentration and MtGloA activity as described below. Each treatment was run in triplicate.

This type of study was done twice to evaluate the effect of two inhibitors of pigmentation: tropolone and ascorbic acid. The first experiment evaluated the effect of adding tropolone (a known inhibitor of tyrosinase) to media A. A control treatment had no tropolone, and three treatments included increasing levels of tropolone. At first, the levels selected were 0.1, 0.5, and 1.0 mg/L tropolone based on the concentrations used in a previous study (Gonçalves et al., 2012). After the first media change, the levels were increased twenty-fold to 2, 10, and 20 mg/L, respectively. The second experiment evaluated the effect of adding 0.44 g/L (2.5 mM) L-ascorbic acid to medium B.

Effect of media composition on fermentation parameters using agitated cultures under growth conditions

Different media recipes were tested to evaluate the effect of media composition on parameters such as growth, protein production, activity of enzyme produced, sugars consumption, and pH variation. The inoculation procedure was similar to the one described above. Cultures of 40 mL working volume containing 1 mg/L pyridoxine to allow growth were agitated at 225 RPM and incubated at 37ºC for 48h. At the end of the experiment liquid samples were taken and used for pH analysis, determination of sugars concentration by HPLC, protein concentration and MtGloA activity as described below. In addition, 1 mL was used for fungal mass determination. The samples were vacuum filtered through 0.45 µm nylon filters, washed with 40 mL DI water to remove sugars and

metabolites and dried at 105ºC for one day to determine the dry weight of the fungus. Each treatment was run in triplicate.

This type of study was done three times. The first experiment evaluated the effect of decreasing the concentration of copper in media A. Copper plays a fundamental role in DOPA-melanin production by tyrosinase (Griffith et al., 2007). The objective was to ascertain if reducing or even eliminating copper from the media would allow cell growth and protein production. The control treatment used medium A, which contains 407 ng/mL Cu^{+2}. Three other treatments used medium A with only 20.35 ng/mL Cu^{+2} (5% of control), 4.07 ng/mL Cu^{+2} (1% of control), and no copper added (0% of control). The reduced copper levels were selected based on a study that concluded that copper levels in the medium below 50 ng/mL resulted in deficient pigmentation (Griffith et al., 2007). Another experiment evaluated the effect of reducing the trace metals in the media. Treatment 1 was medium A with no copper added, Treatment 2 was medium B and Treatment 3 was medium C. Finally, a third experiment evaluated the effect of adding different levels of ascorbic acid in the media. The control treatment was medium B without ascorbic acid. Treatment 1 used media medium B with 0.44 g/L L-ascorbic acid (2.5 mM), treatment 2 used medium B with 0.88 g/L L-ascorbic acid (5 mM), and treatment 3 used medium B with 1.76 g/L L-ascorbic acid (10 mM).

Determination of protein concentration, MtGloA activity, and concentration of sugars and copper

Protein concentration was measured using the Bradford assay. One-hundred-sixty µL of Bradford Commassie solution were transferred to a 96-well microplate. Thirty µL of fermentation broth were then added and the absorbance was measured at 595 nm using a UV-Vis microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). A calibration curve to relate protein content to absorbance was prepared using different levels of purified bovine serum albumin (100x BSA, BioLabs, MA, USA).

MtGloA activity was analyzed using a spectrophotometric assay. The assay was based on the oxidation of the substrate, veratryl alcohol, and the reduction of the electron acceptor, 2,6-dichlorophenol indophenol (DCPIP). The reaction was monitored by measuring the change in absorbance of the reaction mixture at 600 nm over time using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). Absorbance decreases as DCPIP (blue) is reduced to $DCPIPH_2$ (colorless). Each reaction well contained 10 µL of 2 mM DCPIP, 10 µL of 250 mM veratryl alcohol, 10 µL of 50 mM phosphate buffer of pH 7.0 and 70 µL of sample. The reagents were equilibrated at 50°C for 5 min in a thermal cycler (PTC-200, Bio-Rad, CA, USA) before the reaction was started and the absorbance was monitored for 2 min. One unit of activity per mL was defined as the decrease of $4.2*10^{-3}$ absorbance units (at 600 nm) per min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for further characterization of the protein mix and verification of AAO production. A 12% separation gel was prepared by mixing 1.7 mL H_2O, 1.25 mL 1.5 M Tris, 50 µL 10% SDS, 2.0 mL 30% acryl, 25 µL 10% ammonium per sulfite (APS) and 25 µL tetramethylethylendiamin (TEMED). The stacking gel comprises 2.5 mL H_2O, 0.38 mL 0.5 M Tris, 0.03 mL 10% SDS, 0.5 mL 30% acryl, 0.03 mL APS and 3.0 µL TEMED. Protein concentrated by ultrafiltration was used for the SDS-PAGE analysis. An equivalent of 10 µg of protein was mixed with 2x Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl) and boiled for 5 min in a thermal cycler (PTC-200, Bio-Rad, CA, USA). The solution was then pipetted into the gel and run at 180V for 1.5 h (Mini Protean TetraCell, Bio-Rad, CA, USA). Then, the gels were stained with Commassie stain (Bio-Rad, CA, USA) for 20 min and de-stained with a 10% acetic acid solution overnight.

Concentrations of maltose and glucose were determined by high performance liquid chromatography (HPLC 1100 Series, Agilent, Santa Clara, CA) using an HPX-87P analytical column (BioRad, Hercules, CA) and a refractive index detector with distilled water as the mobile phase at a

flow rate of 0.6 mL/min and a temperature of 80ºC. Concentrations were quantified based on a five level calibration curve of known standards.

Determination of copper in medium B was analyzed externally by the Soil, Water and Forage Analytical Laboratory at Oklahoma State University using inductively coupled plasma atomic emission spectroscopy (ICP-AES Spectro Blue, Mahwah, NJ, US).

Statistical analysis

The results of the experiments evaluating the effect of media composition on fermentation parameters were analyzed using SAS release 9.4 (SAS, Cary, NC, USA). The generalized linear model (GLM) was used to conduct analysis of variance (ANOVA). Post-hoc analyses to determine the differences among treatments were done using Tukey's honestly significant different test at a 95% confidence interval.

5.4 Results and Discussion

Enzyme production in TBR

The TBR was run for 350 hours using four dilution rates and three different recirculation rates (Fig. 5.3- 5.6). It was expected that decreasing the recirculation rate would enhance substrate utilization paired with increased enzyme production, since the liquid spends a longer time in contact with the fungal mycelia. This was observed in a previous study using a TBR for production of xylanase using an *A. nidulans* cell factory with a pyridoxine marker (Müller et al., 2015). However, in the present study, the enzyme activities obtained decreased when recirculation rate was decreased from 250 to 150 and to 50 mL/min while maintaining a fixed dilution rate of 0.034 h^{-1} in the time interval 94-166 h (Fig. 5.3A). This could be either due to poor liquid distribution observed at lower

recirculation rates (parts of the packed bed may not enter in contact with the media) or a switch towards pigment production, which will be discussed later.

The protein concentration did increase during this time, which could mean that the enzyme produced is less active in terms of U/mg of protein. However, an SDS-PAGE gel of samples taken during the reactor run revealed that the size of the MtGloA band located between 66 and 97 kDa decreased considerably with time while no other bands emerged during this time in the protein size range of 14 to 200 kDa (Fig. 5.2). The increase in protein concentration could be, therefore, due to production of proteins not detected or due to interference of the Bradford analysis by melanin in the liquid broth. A previous study stated that "no satisfactory assay for protein in the presence of melanin could be found" (Rowley & Pirt, 1972). Therefore, caution should be advised when reading the protein concentration data (Fig. 5.3). The MtGloA activity was maintained fairly stable in the range of 15 to 20 U/mL during the three highest dilution rates (0.038, 0.048, 0.058 h^{-1}) (Fig. 5.3A) which led to a slight increase in enzyme productivity when increasing the dilution rate because of faster product removal (Fig. 5.3B). The concentration of sugars remained below 10 and 4 g/L (maltose and glucose, respectively) from 87h until the end of the experiment (350h) (Fig. 5.4). This translated into a maltose consumption higher than 75% during that time. The pH profile of the liquid broth was generally decreasing, from initial values over 8 to slightly below 6 at 308 h, and then it increased when the two highest dilution rates (0.048 and 0.058 h^{-1}) were used (Fig. 5.5).

A very important parameter monitored was the absorbance of the liquid broth measured at 425 nm (Fig. 5.6). During the experiment, the absorbance readings increased quickly from near zero at the beginning to around 3.5 units at 184h and 232h. The absorbance readings were taken in order to have a qualitative evaluation of the pigment production. Previous studies in this dissertation and elsewhere discussed the production of melanin in *A. nidulans* cultures submitted to stress. It was found that many types of nutrient starvation trigger melanin production, including glucose and nitrogen limitation (Horowitz & Shen, 1952; Rowley & Pirt, 1972), pyridoxine limitation (Müller et

al., 2014) in *A. nidulans*, and sulfur deficiency in *Neurospora sitophila* cultures (Horowitz & Shen, 1952). A previous experiment shown in Chapter 4 with the strain used in this study cultured in agitated flasks reported an increase in absorbance measured at 425 nm of almost 2 units. This was exceeded in the TBR configuration. This can be explained both by the higher fungal biomass concentration in the TBR (about 10-fold higher than in the agitated flask experiments in Chapter 4) and by the higher oxygen availability. In agitated cultures, oxygen has to dissolve from the headspace into the liquid medium and then from the liquid phase into the fungal pellets. Therefore, the mass transfer of oxygen in agitated cultures is expected to be lower than in a TBR, where oxygen and mycelia are in direct contact. A study found that the mass transfer coefficient in a trickle bed reactor using glass beads as packing material was 3.7 times higher than in a stirred tank reactor (Orgill et al., 2013). Since melanin production is an oxidative process, it seems reasonable that more available oxygen will favor the pigmentation process. In fact, a previous study found that reducing oxygen tension in *A. nidulans* cultures decreased melanin production (Rowley & Pirt, 1972). In the present study not only the liquid broth became melanized but the fungal mycelia gradually turned darker when the media was switched from growth media (containing 1 mg/L pyridoxine) to continuous fermentation media (with only 30 µg/L pyridoxine). The pigmentation not only changed the color of the fungus, but it also seemed to allow deposition of colored material on the glass column, both on the packed bed (Fig. 5.7) and inside the tubing used to recycle the liquid medium. This became an important issue for a number of reasons. First, as it was discussed above, the enzyme activity followed a decreasing trend that may be explained by the switch to melanin production observed. Even if substrate was being consumed, it seems possible that a great portion of the substrate was switched towards pigmentation of the culture. Additionally, melanin production caused operational problems because the space within the packed bed continued to decrease as insoluble melanin was deposited. Clogging problems were also observed in the tubing as a result of the accumulation of melanized biomass. Melanin deposition in the packed bed can also lead to overestimation of fungal growth. In this experiment, the final dry mass of mycelia was 171.1 g, or 155.5 g/L. However, a

portion of this weight could be insoluble melanin produced by the culture. For example, in a different study that used *A. nidulans*, melanin accounted for 5% of the total weight of the fungus when growth rate in the bioreactor was 0.072 h^{-1} compared to 28% when growth rate was limited to 0.02 h^{-1} using glucose starvation (Carter et al., 1971). Additionally, melanin production is associated with foaming issues in bioreactors (Jørgensen et al., 2011), which were also observed in the present study.

For all the reasons described above, it is necessary to find a solution to reduce or prevent formation of melanin. The rest of this study describes how media composition or addition of inhibitors of pigmentation were used to reduce or eliminate melanin production.

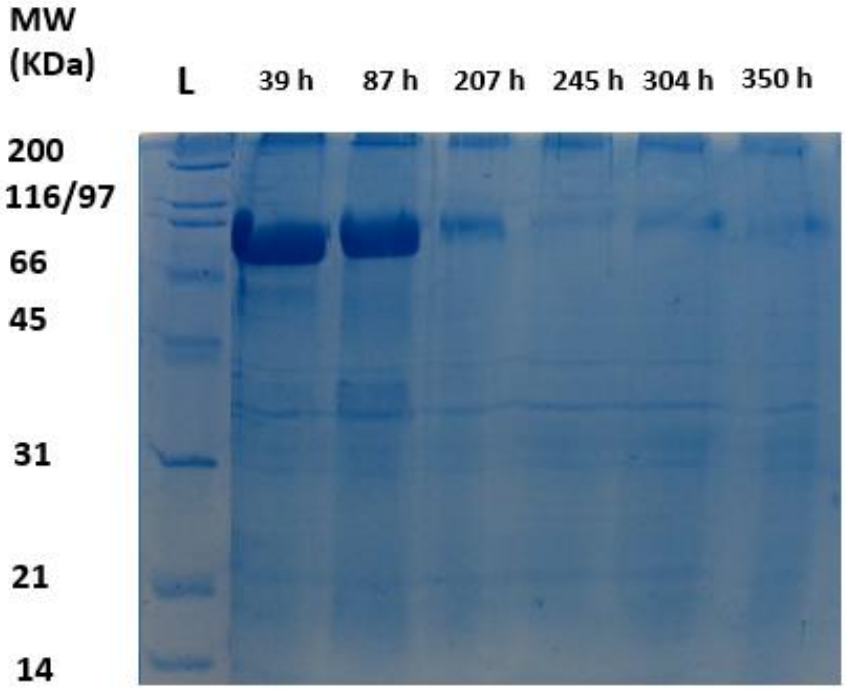

Figure 5.2. SDS-PAGE analysis of TBR run at different times (L denotes "ladder")

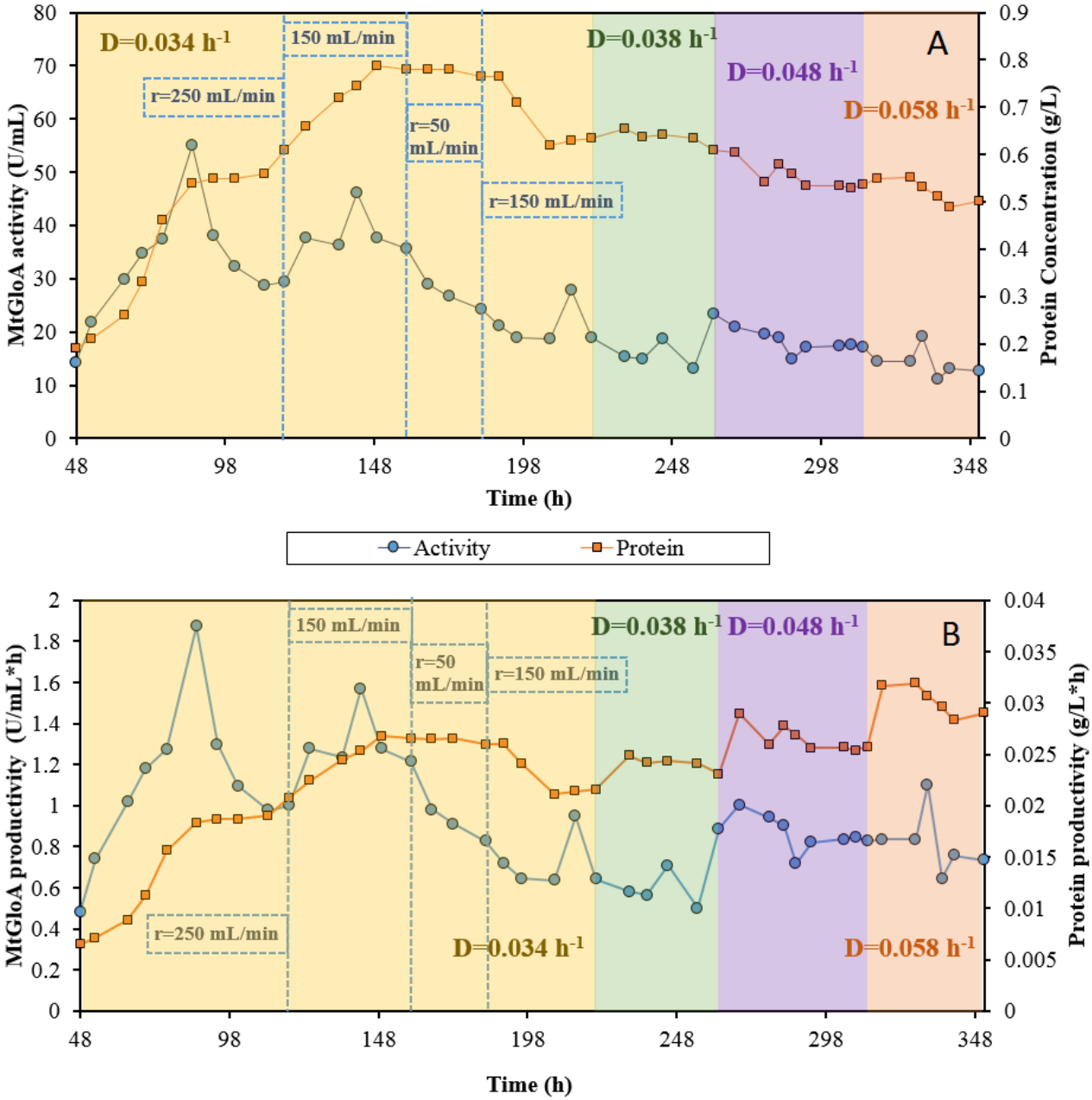

Figure 5.3. (A) MtGloA activity and protein concentration (B) MtGloA productivity and protein productivity profiles during TBR run. D denotes dilution rate and r denotes recirculation rate (0-48 h data for batch operation and washing step not shown)

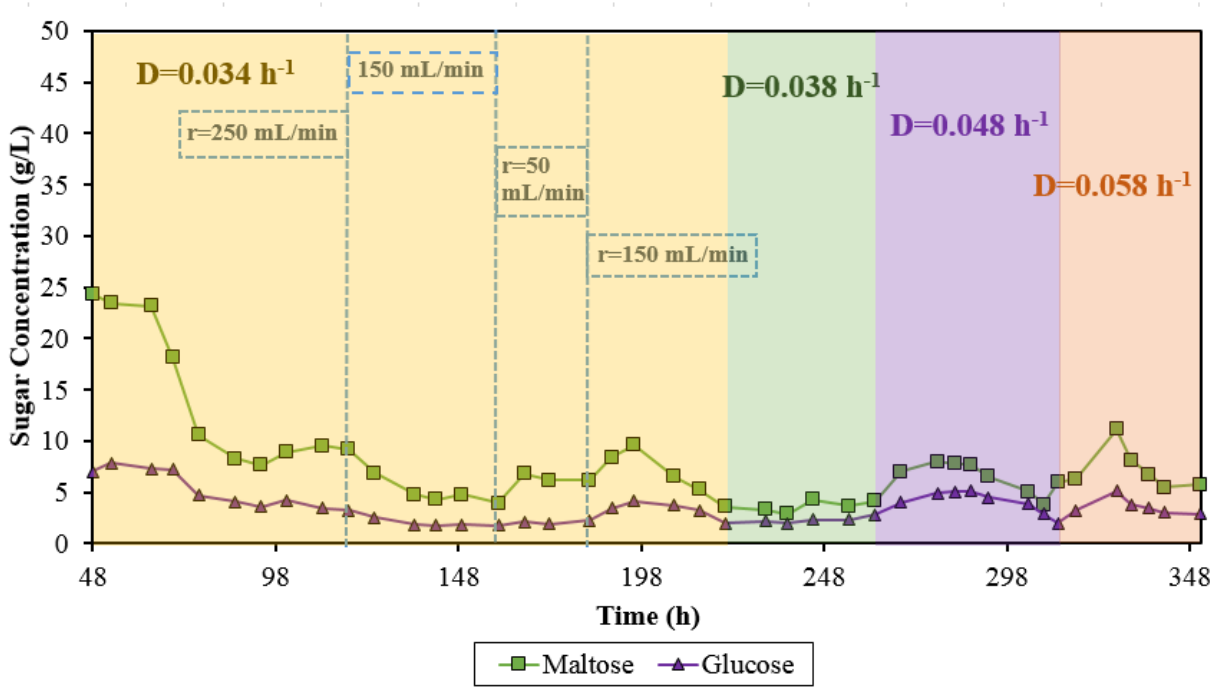

Figure 5.4. Concentration of maltose and glucose during TBR run. D denotes dilution rate and r denotes recirculation rate (0-48 h data for batch operation and washing step not shown)

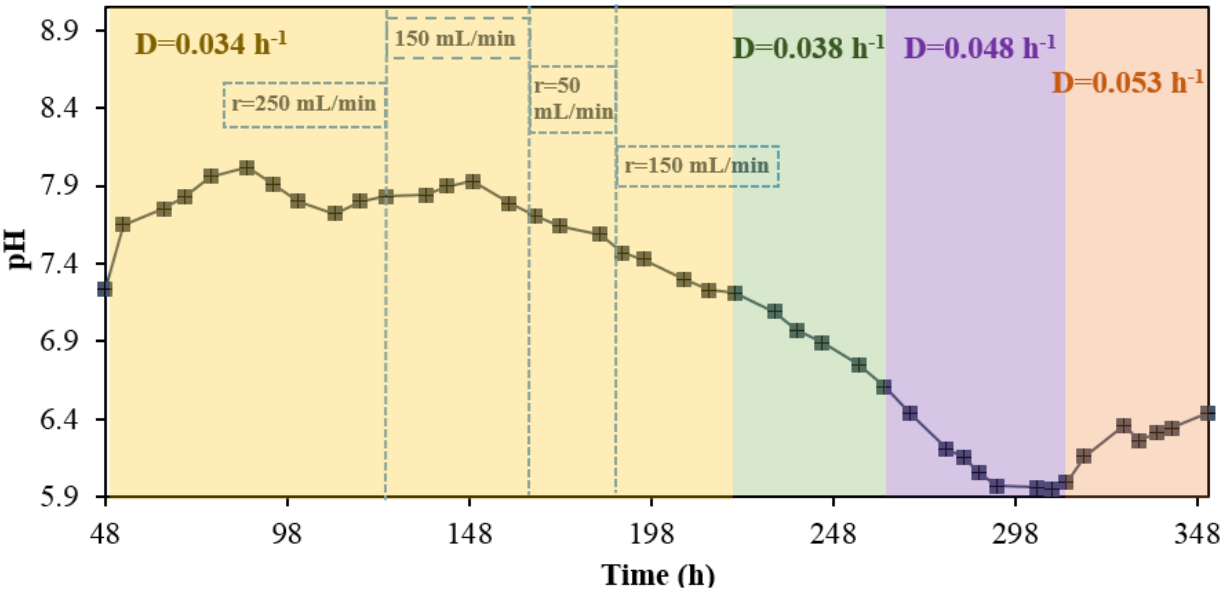

Figure 5.5. pH profile during TBR run. D denotes dilution rate and r denotes recirculation rate (0-48 h data for batch operation and washing step not shown)

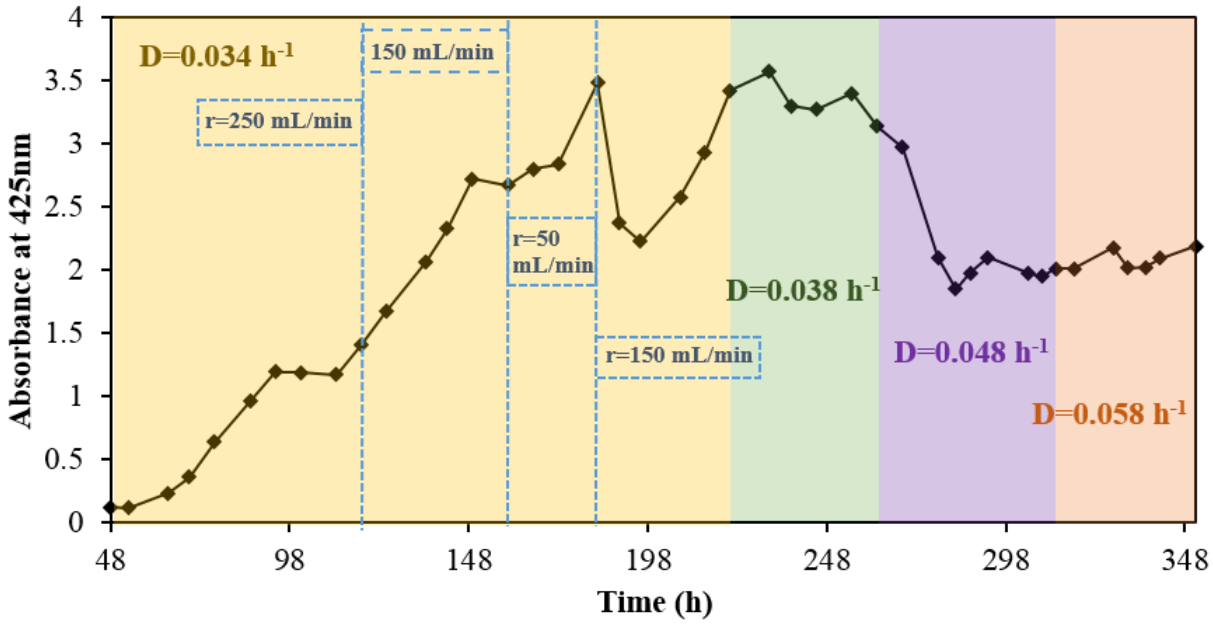

Figure 5.6. Absorbance of liquid broth measured at 425 nm during TBR run. D denotes dilution rate and r denotes recirculation rate (0-48 h data for batch operation and washing step not shown)

Figure 5.7. Picture of the bottom of the TBR column at different times during the experiment (A) 51h (B) 64h (C) 125h (D) 245h

Evaluation of pigmentation using solid media

Different media recipes were screened using solid agar media with a center point inoculation. Fig. 5.8 shows pictures of cell growth and pigmentation of the three media recipes used throughout this study. They differ in the composition of the trace element solution used, i.e. the trace metals

present in the medium (shown in Table 5.1). It can be observed how removing copper had a negative impact on pigmentation when using media recipes richer in metals (media A and B) (Fig. 5.8A-5.8D). However, it was expected that pigmentation would be completely suppressed by removing copper, since the enzyme responsible for melanin production (tyrosinase) has copper atoms in its active site (Held & Kutzner, 1990) . A later analysis of the medium by inductively coupled plasma atomic emission spectroscopy (ICP-AES) revealed that a significant concentration of copper (approximately 50 ng/L) was available in the medium even when copper salts were not added to it. The presence of this concentration of copper could be due to residual traces of this element in the chemicals used for the medium or due to traces of copper in the material used in the laboratory. Pigmentation completely ceased using medium C (which contains only iron and boron as trace metals) and adding copper to that medium recipe did not trigger color formation (Fig. 5.8E-5.8F). Given this evidence, it appears that not only copper, but also other metals play a role in pigmentation of this *A. nidulans* strain.

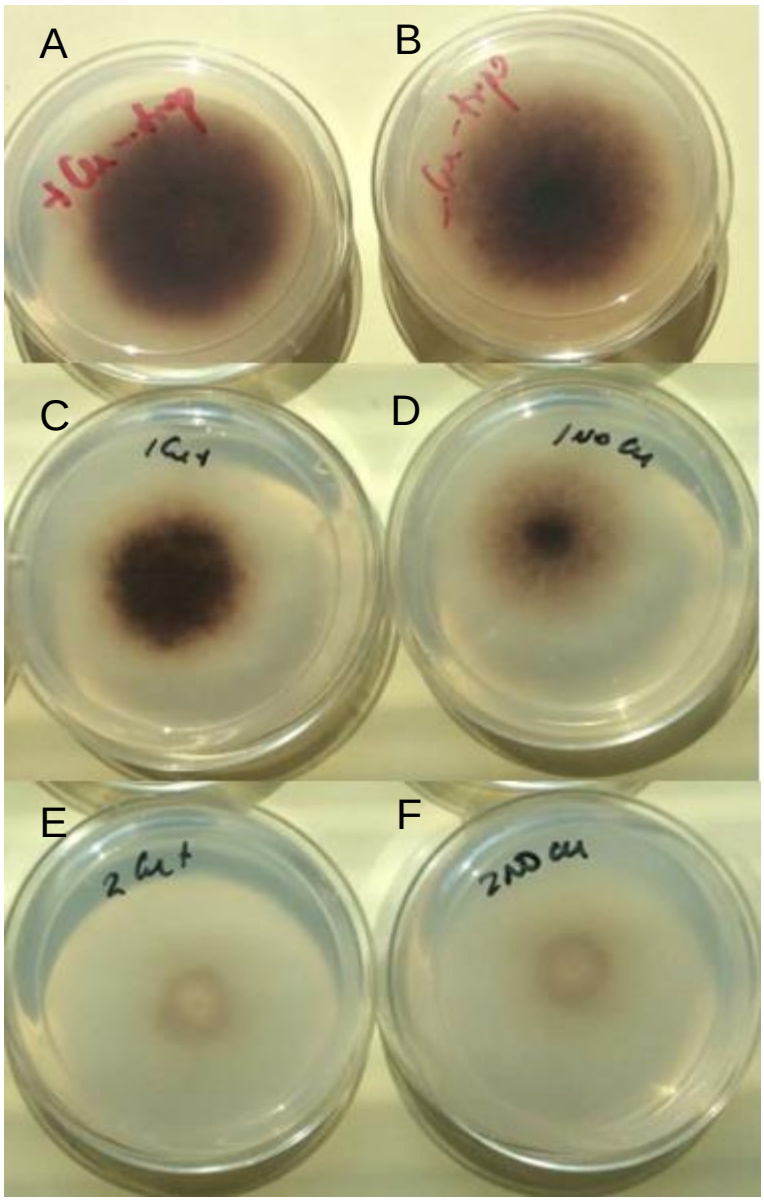

Figure 5.8. Pictures of plates after 96h incubation (A) Medium A with copper (B) Medium A without copper (C) Medium B medium with copper (D) Medium B without copper © Medium C with copper (F) Medium C without copper

The difference in composition between media B and C is that medium B contains zinc, manganese, and molybdenum and medium C does not (Table 5.1). Since medium C did not show pigmentation but medium B did, one or more of these three trace metals must enable the pigmentation process. Medium C was later individually supplemented with these metals and with combinations of them. It was observed that pigmentation occurred only when zinc was present in the medium (Fig.

5.9). Darkening of the agar medium still happened even if the amount of zinc was reduced 10-fold (Fig. 5.9) and 100-fold (not shown). Heavy metals have been associated with fungal pigmentation in other studies. Zinc, but also iron and copper to a lesser extent, have been identified as media components responsible for pigmentation (Foster, 1939; Saravanan et al., 2004). It is important to note that all plates showed growth of *A. nidulans*, but the cultures growing in media lacking zinc resulted in colonies with a cotton-like morphology compared to the cultures developed on all the other media recipes used. During the present study, there was no attempt to remove iron from the media since it is commonly accepted that iron is an absolute requirement for fungal development as it is essential for several enzymes (Agnihotri, 1967; Foster, 1939). Based on these results, removing copper and zinc from the media should be attempted in order to reduce pigmentation. The effect of removing these components on protein production and growth of the strain were assessed and discussed in the following section.

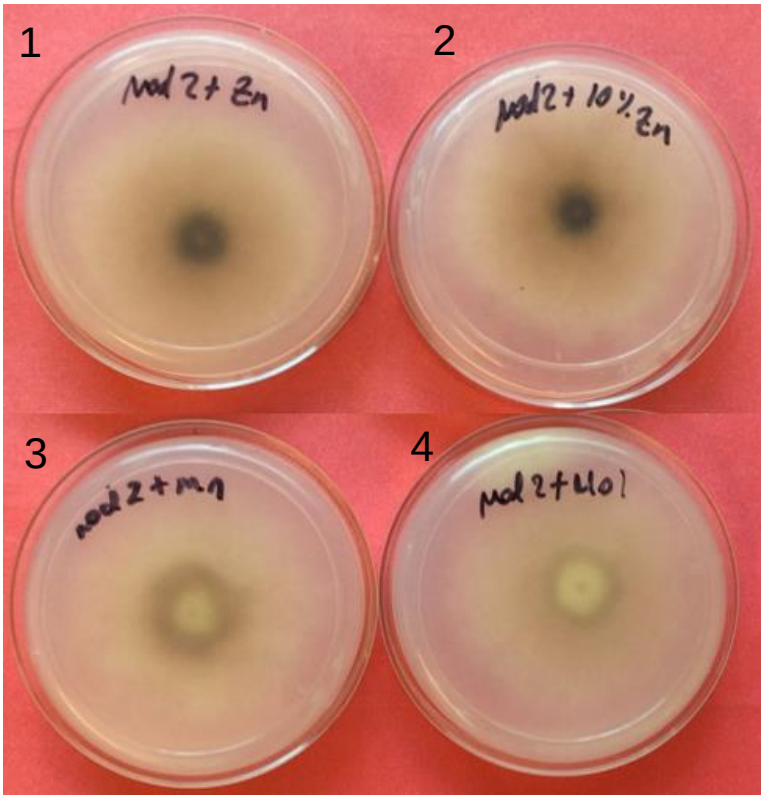

Figure 5.9. Pictures of plates after 96h of incubation (1) Medium C with zinc (2) Medium C with 10-fold zinc reduction (3) Medium C with manganese (4) Medium C with molybdenum

Effect of media composition on fermentation parameters using agitated cultures under growth conditions

Agitated culture experiments were performed in order to evaluate the effect of media changes on parameters like protein production and fungal growth. First, the effect of decreasing the concentration of copper in the medium was evaluated using medium A as control. Table 5.2 shows the main results of the experiment after 48h of incubation at 37ºC and 225RPM using growth media (1 mg/L pyridoxine added). The reduction or even removal of copper from the media did not significantly affect the final fungal mass ($p=0.1520$) or the final pH ($p=0.1271$). However, reducing the amount of copper in the medium had a significant effect on MtGloA activity, protein concentration, and sugars consumption. In general, less copper in the medium resulted in better performance of the culture because the enzyme activity obtained with no copper added to the medium (244.19±28.18 U/mL) was significantly higher than the rest of treatments ($p=0.0054$). Protein concentration was also significantly higher when copper was excluded ($p=0.0300$), reaching 0.80±0.03g/L compared to 0.69±0.06 g/L for control. Maltose and glucose consumption were significantly lower ($p=0.0175$ and 0.0007, respectively) when copper was removed from the media recipe. The difference in maltose consumption, although significant, was small (95.1±1.1 % versus 97.6±0.2 % in treatment 3 and control, respectively). Glucose consumption did suffer an important decrease when copper was not added to the media (51.7±4.4 % consumption without copper versus 81.0±4.9 % for control). It is hypothesized that a reduced availability of copper inactivates pathways catalyzed by copper enzymes that are not involved in MtGloA production, such as tyrosinase, lowering the demand for substrate invested in those pathways. Due to all the observations made above, it was concluded that copper should be excluded from the medium recipe, as it was seen in the preliminary plate screening that it contributes to pigmentation and the present experiment indicates that its removal does not affect growth and it enhances enzyme production. It has to be noted again

that ICP-AES analysis of medium without copper added indicated that approximately 50 ng/L of this element was still present when copper was not added to the media (Treatment 3).

Table 5.2. Effect of copper addition to original medium on agitated cultures of an *A. nidulans* cell factory after 48 h. Values listed in this table are means±standard deviation (n=3)

Treatment	Control: Original media 100% Cu (407 ng/mL Cu^{2+})	Treatment 1: Original media 5% Cu (20 ng/mL Cu^{2+})	Treatment 2: Original media 1% Cu (4 ng/mL Cu^{2+})	Treatment 3: Original media 0% Cu (0 ng/mL Cu^{2+})
MtGloA Activity (U/mL)	165.60 ± 23.72^{A}	132.55 ± 13.97^{A}	174.92 ± 18.06^{A}	244.19 ± 28.18^{B}
Protein concentration (g/L)	0.69 ± 0.06^{A}	0.68 ± 0.00^{A}	$0.73\pm0.02^{A,B}$	0.80 ± 0.03^{B}
Final pH	7.83 ± 0.12^{A}	7.65 ± 0.22^{A}	7.51 ± 0.03^{A}	7.51 ± 0.06^{A}
Maltose consumed (%)	97.6 ± 0.2^{A}	$95.8\pm0.7^{A,B}$	94.4 ± 0.8^{B}	95.1 ± 1.1^{B}
Glucose consumed (%)	81.0 ± 4.9^{A}	66.2 ± 3.6^{B}	54.0 ± 5.2^{C}	51.7 ± 4.4^{C}
Suspended mycelia (g/L)	15.23 ± 1.22^{A}	12.43 ± 0.97^{A}	15.53 ± 1.55^{A}	15.13 ± 1.53^{A}

^{A,B,C}Values listed in the same row with the same letter are not statistically different at 95% confidence intervals

The second experiment compared the performance of cultures using three different media recipes: medium A (with no copper), medium B, and medium C. As stated above, all three differ in the composition of the trace element solutions (Table 5.1, note that media A in this case had copper removed). Medium A, which was used in the TBR study, is richer in metals, and medium C only contains boron and iron. Both medium A and B include zinc, but medium C does not. Table 5.3 shows the results of the comparison. There was a significant difference among treatments for all the parameters studied. In all cases, cultures using medium C show an inferior performance in all aspects: less growth, and lower protein concentration, enzyme activity and consumption of sugars. These differences were statistically significant ($p>0.05$). The results for medium A and medium B were not significantly different for any of the parameters studied. It can be concluded that even though medium C showed no pigmentation in the preliminary screening experiments (Fig. 5.8), it is not appropriate to

be used for MtGloA production because of its poor performance in terms of limited growth and lower enzyme production. In addition, it is clear that medium B can be used instead of medium A, since it contains a lower amount of all trace metals (some of which contribute to pigmentation) and it results in similar growth of the strain and similar enzyme production.

Table 5.3. Effect of different media recipes on agitated cultures of an *A. nidulans* cell factory after 48 h. Values listed in this table are means±standard deviation (n=3)

Treatment	Medium A with no copper	Medium B	Medium C
MtGloA Activity (U/mL)	185.63±11.65^{A}	176.36±32.78^{A}	16.12±4.96^{B}
Protein concentration (g/L)	0.77±0.06^{A}	0.68±0.09^{A}	0.11±0.03^{B}
Final pH	7.63±0.22^{A}	7.60±0.04^{A}	7.10±0.06^{B}
Maltose consumed (%)	97.8±0.7^{A}	96.0±1.0^{A}	22.9±8.8^{B}
Glucose consumed (%)	89.2±1.7^{A}	81.3±2.3^{A}	10.4±5.8^{B}
Suspended mycelia (g/L)	19.12±2.70^{A}	22.03±3.21^{A}	6.13±3.23^{B}

^{A,B}Values listed in the same row with the same letter are not statistically different at 95% confidence intervals

Evaluation of pigmentation using tyrosinase inhibitors: effect of tropolone and ascorbic acid

Reducing pigmentation of *A. nidulans* cultures under growth-limited conditions can be achieved not only by modifying the composition of the medium used, but also through specific inhibitors. In this study, two of these inhibitors were tested: tropolone and L-ascorbic acid (vitamin C). Tropolone is a very powerful inhibitor of the enzyme responsible for melanin production in some fungal strains: tyrosinase (Kahn & Andrawis, 1985). The mechanism of action of tropolone is the chelation of copper (Goldstein et al., 1964), which is present in the active site of tyrosinase (Held & Kutzner, 1990). Fig. 5.10 shows the effect of different tropolone concentrations on the main fermentation parameters and Fig. 5.11 shows the absorbance readiings measured at 425 nm. The experiments were performed using a very diluted pyridoxine concentration (10 µg/L) to limit growth,

which generally promotes pigmentation. It has to be noted that the levels of tropolone used during the first 48 h were too low because no differences were observed among the treatments (including control) in terms of pigmentation or enzyme production (Figs. 5.10 and 5.11), so they were increased 20-fold during the media changes after that. In general, higher tropolone concentrations caused a reduction of pigmentation of the liquid media. Higher tropolone concentrations resulted in lower absorbance readings, which indicate lower pigmentation (Fig. 5.11). The absorbance readings are related to soluble melanin produced, but it could also be visually observed how the fungal pellets became more pigmented as tropolone concentrations were decreased (Fig. 5.12). The reduction of pigmentation in *A. nidulans* by tropolone was also observed in a previous study that characterized the pigment of *A. nidulans* as DOPA-melanin (Gonçalves et al., 2012). However, in the present study it was also observed that a high concentration of tropolone in the medium could damage the fungal culture. It can be noted how both the enzyme activity and the protein concentration achieved when using 20 mg/L tropolone (Treatment 3) reached a maximum at 96h and then rapidly decreased to values near zero at 192 h (Fig. 5.10A and 5.10C). The pH also decreased to values below 5 during that time (Fig. 5.10D). At 192 h the concentration of tropolone in the medium in Treatment 3 was reduced to 10 mg/L and the culture recovered as observed by resumption of enzyme production. This negative effect of tropolone at high concentrations can be explained because of its fungicidal nature (Lindberg, 1981). Treatment 2 used 10 mg/L tropolone after 96 h and it showed the best protein production results (Fig. 5.10A and 5.10B). While this treatment reached a cumulative MtGloA activity of 102.11±16.22 U/mL, the control treatment (no tropolone) only resulted in 73.05±8.34 U/mL (Fig. 5.10B). This is a 40% increase in activity when using tropolone. It is hypothesised that when melanization occurs part of the substrate is invested in this process instead of MtGloA production. In addition, it has been reported that melanin production results in a reduction of the cell wall pore size (Eisenman et al., 2005), which could slow down the rate of transfer of metabolites, such as extracellular enzymes, through the cell wall.

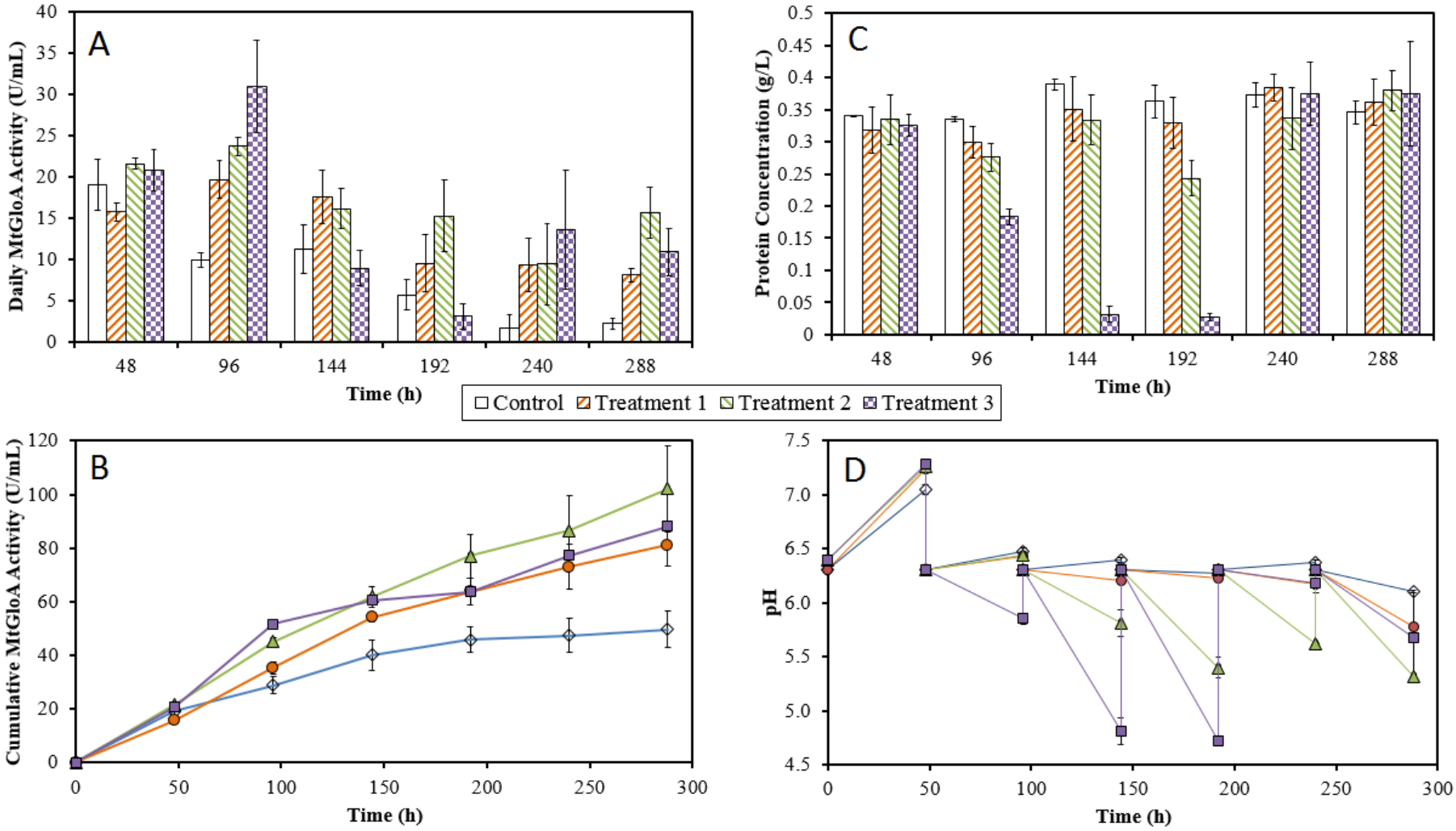

Figure 5.10. (A) Daily MtGloA activity (B) Cumulative MtGloA activity (C) Protein concentration (D) pH profiles during agitated culture of *A. nidulans* cell factory using media with very diluted pyridoxine concentration (10 µg/L) to evaluate effect of tropolone on pigmentation. Control (♦) had no tropolone, Treatment 1 (●) contained 0.1 mg/L tropolone until 48h and 2 mg/L onwards, Treatment 2 (▲) contained 0.5 mg/L tropolone until 48h and 10 mg/L onwards, Treatment 3 (■) had 1 mg/L pyridoxine until 48h and 20 mg/L until 196h, then it was reduced to 10 mg/L. Error bars represent one standard deviation (n=3)

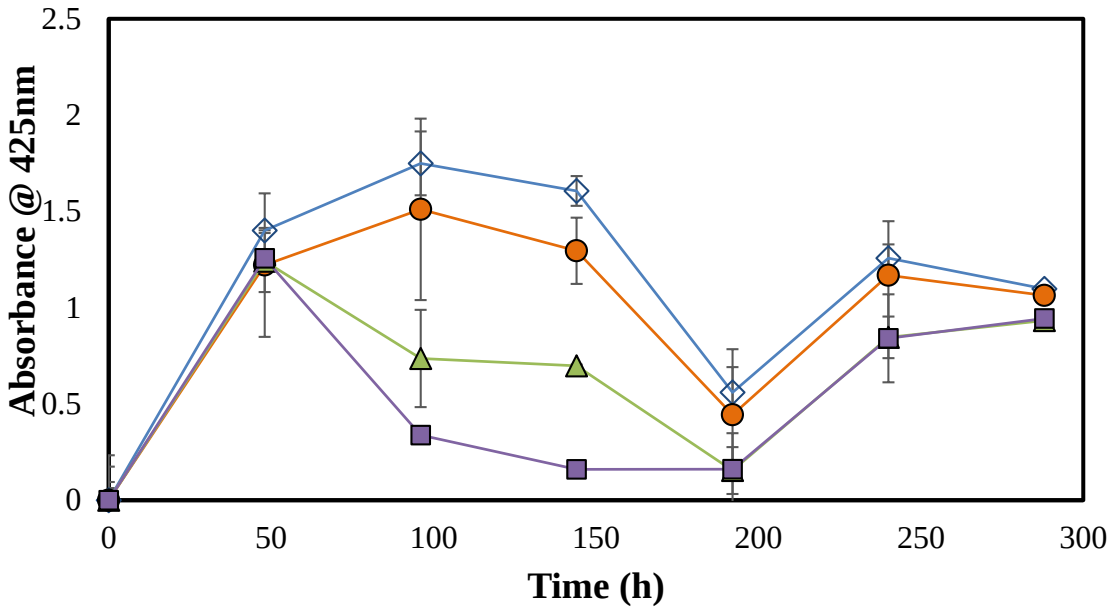

Figure 5.11. Absorbance of liquid broth measured at 425 nm in control treatment (♦), treatment 1 (●), treatment 2 (▲), and treatment 3 (■) during agitated culture of *A. nidulans* cell factory using media with very diluted pyridoxine concentration (10 µg/L) to evaluate effect of tropolone on pigmentation. Error bars represent one standard deviation (n=3)

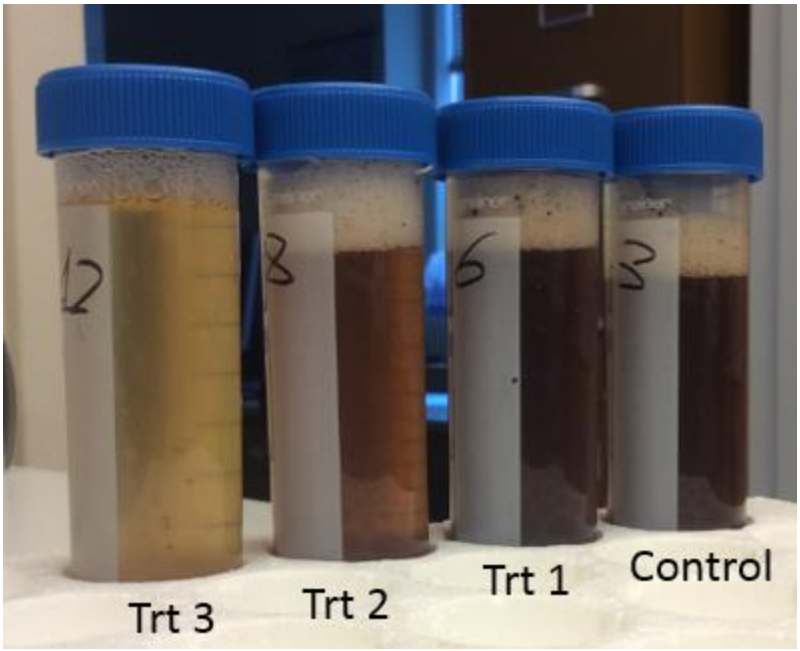

Figure 5.12. Picture of liquid broth from all treatments at 96h during agitated culture of *A. nidulans* cell factory using media with very diluted pyridoxine concentration (10 µg/L) to evaluate effect of tropolone on pigmentation.

Figure 5.13 shows the effect of ascorbic acid addition (0.44 g/L) to medium B under limited growth conditions (10 µg/L pyridoxine). The inhibition mechanism of L-ascorbic acid on pigmentation of cultures is different than that of tropolone. While ascorbic acid acts as a chelating agent like tropolone, it also reduces o-quinones to o-phenol precursors (Lozano de Gonzalez et al., 1993). Quinones are intermediate compounds in the melanin pathway, so by reducing them the pathway is blocked and melanin pigmentation does not occur. Similarly to what had been observed with tropolone, the addition of ascorbic acid resulted in a higher enzyme activity compared to the control treatment (Fig. 5.13A and 5.13B) . The final cumulative MtGloA activity for the treatment with ascorbic acid was 2-fold higher than in the control treatment (43.83±2.06 U/mL versus 18.18±5.21 U/mL, respectively) (Fig. 5.13B). The pH values of the ascorbic acid treatment were lower than those observed in the control treatment (Fig. 5.13C), although this did not result in a halt of enzyme production.

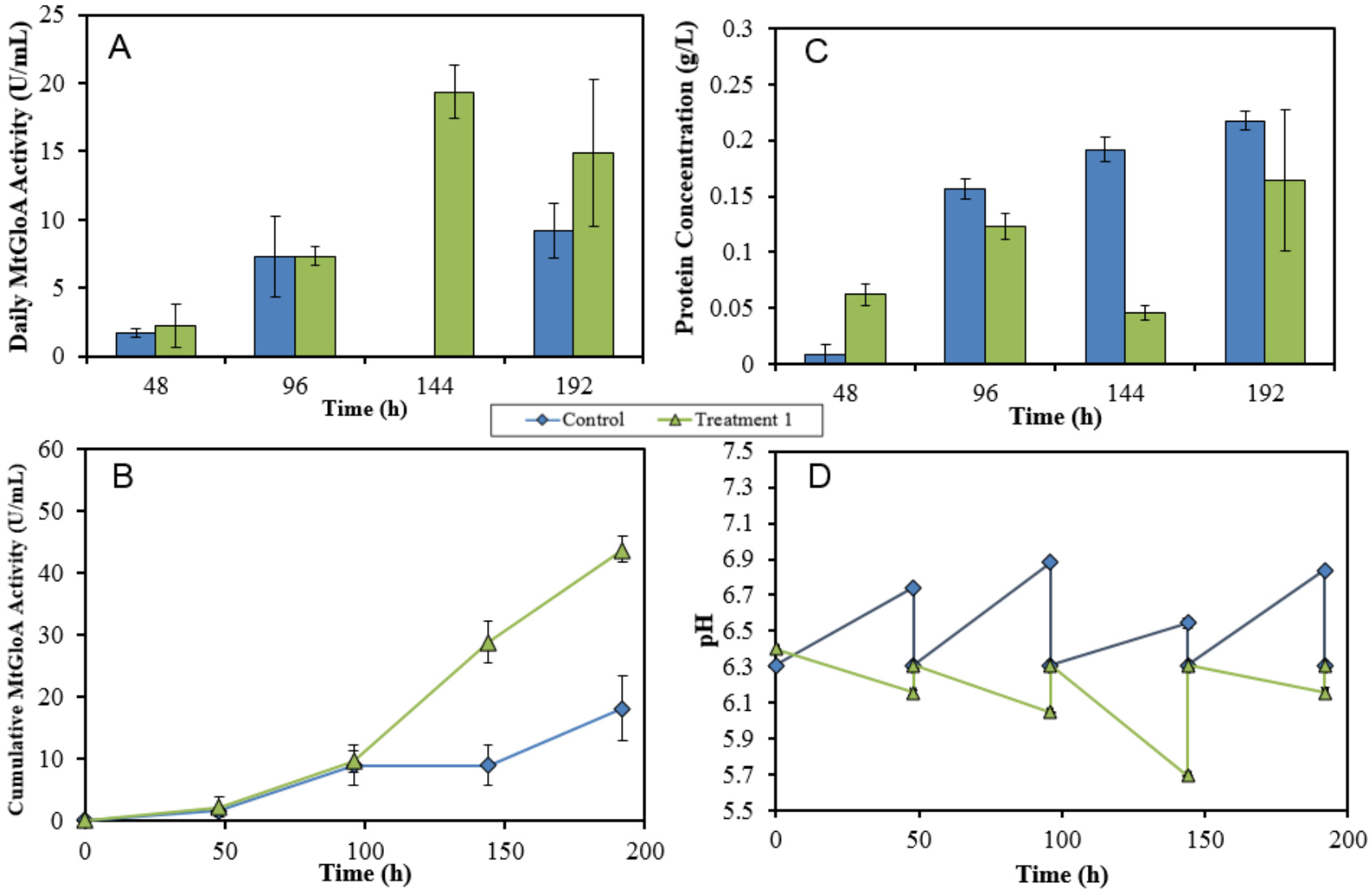

Figure 5.13. (A) Daily MtGloA activity (B) Cumulative MtGloA activity (C) Protein concentration (D) pH profiles during agitated culture of *A. nidulans* cell factory using media with very diluted pyridoxine concentration (10 µg/L) to evaluate effect of ascorbic acid on pigmentation. Control had no ascorbic acid, and Treatment 1 contained 0.44 g/L ascorbic acid. Error bars represent one standard deviation (n=3)

In terms of absorbance of the liquid broth, the treatment using ascorbic acid acheved lower absorbance readings than the control (Fig. 5.14). The difference between treatments was generally low (between 0.1 and 0.4 units of absorbance). However, it could be observed how the fungal pellets in the control treatment became much more melanized (dark and compact) than those in the treatment using ascorbic acid (Fig. 5.15).

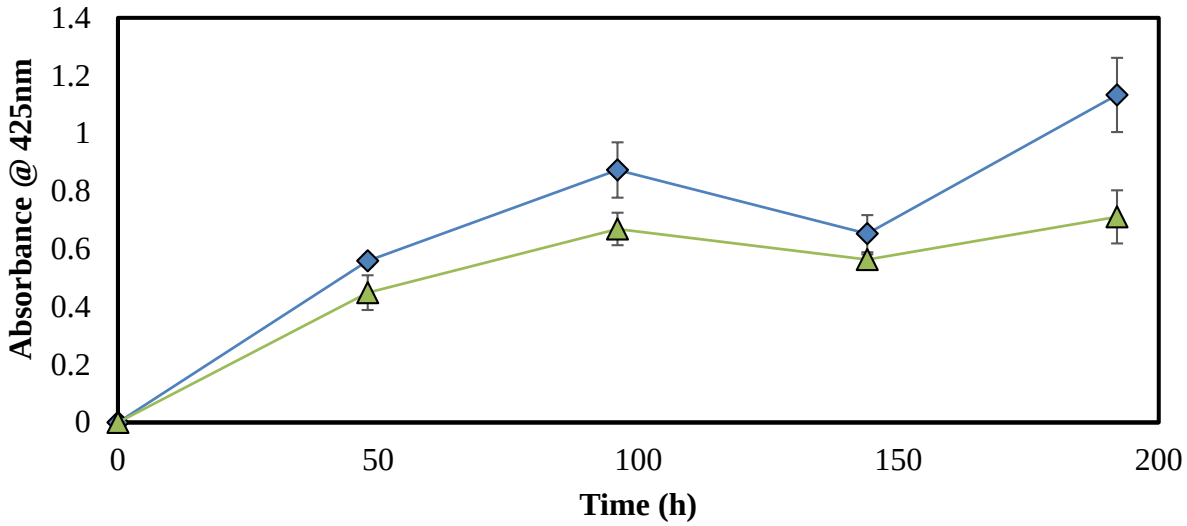

Figure 5.14. Absorbance of liquid broth measured at 425 nm in control treatment (♦), and treatment 1 (with 0.44 g/L ascorbic acid) (▲) during agitated culture of *A. nidulans* cell factory using media with very diluted pyridoxine concentration (10 µg/L) to evaluate effect of ascorbic acid on pigmentation. Error bars represent one standard deviation (n=3)

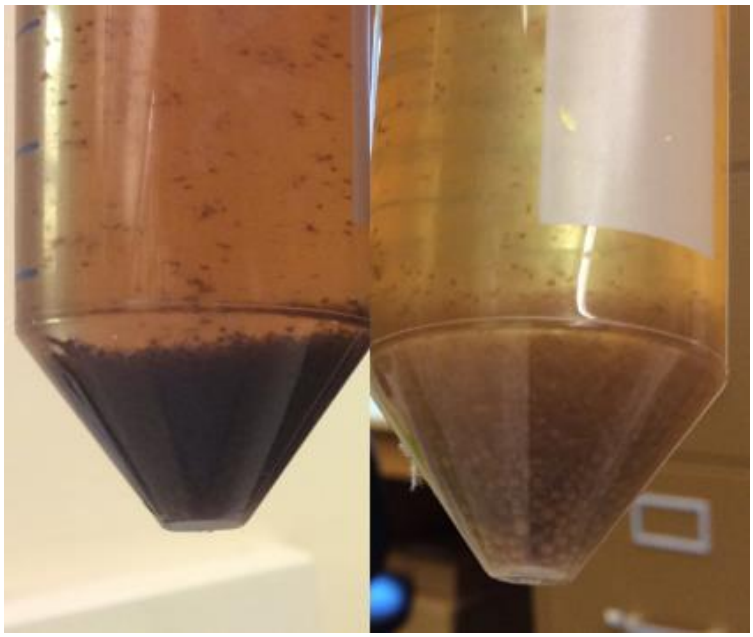

Figure 5.15. Picture of the pellets during agitated culture of *A. nidulans* cell factory using media with very diluted pyridoxine concentration (10 µg/L) to evaluate effect of ascorbic acid on pigmentation. The tube on the left side is the control treatment (no ascorbic acid) and the tube on the left is treatment 1 (ascorbic acid added).

An additional experiment was performed in order to assess if higher concentration of ascorbic acid could damage the culture, as was observed for tropolone. The effect of different concentrations of ascorbic acid (0, 0.44, 0.88, 1.76 g/L) on growth and enzyme production of *A. nidulans* cultures was evaluated under growth conditions (1 mg/L pyridoxine). Table 5.4 lists the main results for the experiment. It can be seen how there was no statistical difference among treatments regarding protein concentration, final pH, or suspended mycelia. However, there was a statistical difference for enzyme activities obtained ($p=0.0001$), as the control treatment (no ascorbic acid) resulted in lower activity than all the other treatments. The inclusion of ascorbic acid in the medium, therefore, resulted in a more active enzyme broth. Additionally, statistical differences were found in maltose consumption ($p=0.018$) and glucose consumption ($p=0.024$). The addition of ascorbic acid at different levels resulted in slightly higher maltose consumption (97 to 98%) than in the control treatment (93%). Glucose consumption was only significantly increased in treatments 1 and 2 (approximately 83%) but not in treatment 3 (69%) compared to

control (63%) (Table 5.4). Ascorbic acid appears to be a better candidate for reduction of pigmentation of *A. nidulans* cultures than tropolone, since it did not show detrimental effects when using concentrations up to 1.76 g/L in the medium.

Table 5.4. Effect of ascorbic acid on agitated cultures of an *A. nidulans* cell factory after 48 h. Values listed in this table are means±standard deviation (n=3)

Treatment	Control: Medium B with no ascorbic acid	Treatment 1: Medium B with 0.44 g/L ascorbic acid	Treatment 2: Medium B with 0.88 g/L ascorbic acid	Treatment 3: Medium B with 1.76 g/L ascorbic acid
MtGloA Activity (U/mL)	194.59±18.63^{A}	368.39±19.95^{B}	417.15±18.20^{B,C}	440.46±49.06^{B,C}
Protein concentration (g/L)	0.68±0.05^{A}	0.73±0.05^{A}	0.76±0.03^{A}	0.73±0.02^{A}
Final pH	7.69±0.12^{A}	7.49±0.18^{A}	7.42±0.29^{A}	6.59±0.38^{B}
Maltose consumed (%)	93.4±2.6^{A}	98.4±0.2^{B}	98.0±0.1^{B}	96.9±0.4^{B}
Glucose consumed (%)	62.4±10.0^{A}	82.9±2.7^{B}	83.5±4.3^{B}	69.1±5.8^{A,B}
Suspended mycelia (g/L)	15.57±2.13^{A}	15.70±2.16^{A}	14.10±1.45^{A}	16.00±2.57^{A}

^{A,B}Values listed in the same row with the same letter are not statistically different at 95% confidence intervals

5.5. Conclusions

Cultures of an engineered *A. nidulans* strain that produces an aryl alcohol oxidase (MtGloA) enzyme switched to melanin production under growth-limited conditions. This pigmentation process was accentuated when using a TBR, due to the higher availability of oxygen and fungal biomass. Higher concentrations of copper and zinc in the medium were found to favor melanin production. In addition, the use of two inhibitors of the pigmentation process were evaluated. The addition of either tropolone or ascorbic acid reduced the melanization of the cultures. However, at high concentration (20 mg/L) tropolone inhibited the fungus and caused a halt on enzyme production. Ascorbic acid did not affect the cultures adversely at concentrations up to 1.76 g/L. In addition, the MtGloA activity obtained was more than two times higher than the activity in the control treatment with no ascorbic acid. Therefore, future TBR studies should use medium with lower copper and zinc concentrations and should also include ascorbic acid.

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CHAPTER VI

CONTINOUS ARYL ALCOHOL OXIDASE PRODUCTION UNDER GROWTH LIMITED CONDITIONS USING A TRICKLE BED REACTOR

6.1 Abstract

Enzyme production using a trickle bed reactor (TBR) is a technology with potential due to reduced energy requirements and easier protein recovery compared to commercial submerged fermentations. In this study, an *A. nidulans* cell factory with a pyridoxine marker was used to establish continuous production of an aryl alcohol oxidase (MtGloA). The marker allowed limitation of growth of the cell factory to avoid clogging issues in the column and recirculation tubing. The use of modified medium with 5 g/L ascorbic acid reduced melanin production observed under growth limited conditions, increasing MtGloA productivities. Three different random packing materials were tested as a support for the fungus inside the TBR column, two of which were made of HDPE plastic and one of stainless steel. In addition, the fermentations were run at three different dilution rates. The results indicate that the packing material selection did not have a high impact on cell adhesion and MtGloA production. Increasing dilution rates favored melanin formation and organic acid accumulation, hurting the enzyme production process**.**

6.2 Introduction

Industrial enzyme production is currently carried out using the technology called submerged fermentation (SmF), in which microorganisms are grown in large agitated vessels containing liquid media (Couto & Toca-Herrera, 2007). Novozymes, one of the leading companies in the business, lists SmF as their main enzyme production methodology, using reactors with a size of up to 1,000 m^3 (Novozymes, 2013). SmF allows good process control, continuous production, and is well established as commercial scale technology. On the other hand, disadvantages include limitation of mass transfer due to increased apparent viscosity paired to mycelia growth, shear stress to microorganisms, and a challenging product recovery from the microorganism-broth mixture (Couto & Toca-Herrera, 2007; Krishna, 2005; Mueller, 2012). Solid state fermentation (SSF) is able to overcome these disadvantages by growing the microorganisms in an environment with little or no free water. In addition, SSF presents lower capital and operational costs due to lower volume reactors and water and energy demands. However, there are challenges associated with control of process conditions such as heat, moisture and aeration, and the absence of continuous production methods (Couto & Toca-Herrera, 2007; Krishna, 2005; Mueller, 2012).

A third approach that uses a trickle bed reactor (TBR) combines characteristics of both SmF and SSF. In TBRs, there is free liquid media but in lower amounts than in SmF and the energy requirement is lower because no agitation is needed: media is pumped to the top of the column and it trickles down the bed where the microorganism grows. Similar to SSF, the cells grow on solid surfaces as a biofilm, which is considered a more natural environment that results in higher resistance to antimicrobial agents, shear stress and environmental changes (Couto & Toca-Herrera, 2007). This technology, contrary to SSF, does permit continuous production, and the product stream is a cell-free broth, which reduces the complexity of recovery processes. Unfortunately, the aggressive growth of fungal strains that are often used for enzyme production causes clogging issues in the TBR system. A

solution to this problem is the inclusion of a marker in the fungal genotype to control growth of the strain. This technology was demonstrated using an *A. nidulans* cell factory with a pyridoxine marker in a TBR with lava rocks as the solid bed support for production of xylanase (Müller et al., 2015). When pyridoxine was not included in the media recipe, the cell factory was unable to grow and clogging issues were prevented (Müller et al., 2015). On the negative side, continued pyridoxine deprivation caused a pH drop, increased melanin production, and reduction of enzyme yields. Periodic additions of pyridoxine mitigated these issues (Müller et al., 2014). Another study used a similar *A. nidulans* cell factory with a pyridoxine marker for production of a different enzyme, an aryl alcohol oxidase (Chapter 5). In that instance, pyridoxine was continuously present in the medium at very low levels to limit growth without halting enzyme production, eliminating the need for periodic pyridoxine replenishment. This strain, however, resulted in much higher melanin production when cultured in a TBR than the xylanase-producing strain. This phenomenon hurt enzyme production due to lower substrate availability and increased operational problems related to melanin deposition in the column and tubing. Melanin formation in *A. nidulans* is regulated by the enzyme tyrosinase (Held & Kutzner, 1990). Reducing the concentration of trace elements (especially copper and zinc) in the medium, as well as adding ascorbic acid as a tyrosinase inhibitor, reduced melanin formation and have a positive impact on enzyme production (Chapter 5).

Several factors can affect the performance of TBRs in bioprocessing, some of which are specific to this type of reactor configuration, such as the selection of packing material or liquid recirculation rate, and others that are common to any bioreactor operation, such as dilution rate. The selection of the type of solid support in the packed bed aims to obtain a high surface area to allow growth of the microorganism and proper contact between liquid and gas phases, sufficient bed porosity, low cost and durability, and low pressure drops (Ranade et al., 2011; Tchobanoglous & Burton, 1991). The packed bed can be comprised of random rings or can be a continuous structure often referred to as structured packing. Random packing rings are usually less expensive, but offer

higher pressure drops and lower mass transfer efficiencies than structured packings (Seader & Henley, 2006). An important challenge of TBRs is to achieve a homogenous liquid distribution across the bed, ensuring proper wetting of the biocatalyst and avoiding channeling. The liquid recirculation rate can be crucial for reactions in which mass transfer is limiting, such as conversion of gas substrates by microorganisms. A study that evaluated the gas-liquid mass transfer in reactors for syngas fermentation concluded that the mass transfer in a TBR decreased with higher recirculation rates due to an increase in liquid hold-ups (Orgill et al., 2013). Dilution rates, on another hand, are important for any bioreactor configuration because they control the overall retention time of the liquid media in the system. Higher dilution rates lead to higher growth rates in systems without growth limitations due to the increased availability of substrate, and potentially higher productivities until the kinetic limitations of the culture are reached. A previous study using an *A. nidulans* cell factory with a pyridoxine marker for xylanase production investigated the effect of recirculation rate on the performance of a TBR (Müller et al., 2015). The authors concluded that lowering the recirculation rate had a positive effect on enzyme production due to increased contact time between the liquid media and the fungus (Müller et al., 2015). The effects of packing material selection and dilution rate on the process were not investigated.

The objective of the present study is to establish a stable aryl alcohol oxidase production process using a growth-limited culture of an *A. nidulans* cell factory in a TBR system. In order to do that, melanin production during growth limitation conditions were controlled by reducing the concentration of copper in the medium and including ascorbic acid as a tyrosinase inhibitor, as recommended by a previous study (Chapter 5). The suitability of different random packing materials, including plastic and stainless steel, will be assessed. The effect of increasing the dilution rate also was investigate**.**

6.3 Materials and Methods

Strain and spore production

An. *A. nidulans* cell factory was purchased from the Fungal Genetic Stock Center (FGSC, Manhattan, Kansas, USA). The strain, deposited under the identifier A773, includes a marker (*pyroA4*) for suppression of growth of the microorganism when pyridoxine is not supplied externally. This strain was further genetically modified by DNA mediated transformation of a plasmid (pEXPYR) that contains a wild-type *pyrG* complementing gene and a construct that contains the glucoamylase promoter fused to the *MtgloA* ORF (including its own signal peptide) as described elsewhere (Segato et al., 2012). The resulting *A. nidulans* cell factory overexpresses and secretes *Myceliophthora thermophila* aryl alcohol oxidase (MtGloA) in media containing maltose.

All the experiments described in this study were done using spores produced by growing the resulting strain on solid medium containing (per L) 9.0 g glucose, 50 mL of 20x Clutterbuck salt solution (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4$, 30.4 g/L KH_2PO_4), 1 mL 1,000x trace element solution (22 g/L $ZnSO_4 \cdot 7H_2O$,11 g/L H_3BO_3, 5.0 g/L $MnCl_2 \cdot 7H_2O$, 5.0 g/L $FeSO_4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 5H_2O$, 1.6 g/L, $CuSO_4 \cdot 5H_2O$, 1.1 g/L $Na_2MoO_4 \cdot 4H_2O$, 50 g/L Na_2-EDTA), 0.001 g pyridoxine, and 15% agar. The pH was adjusted to between 6 and 6.5 using a 6N NaOH solution. The medium was sterilized by autoclaving at 121ºC for 20 min (PRIMUS, Sterilizer Co., Inc., Omaha, NE, USA). Twenty-five µL of spore stock solution were pipetted onto the solid medium and spread homogeneously under aseptic conditions. The plates were then incubated at 37ºC for 48 h until the surface of the solid media was covered by white spores. A control plate with medium lacking pyridoxine was inoculated in a similar manner to verify the stability of the strain. Observation of growth on this control plate served as an indicator of contamination or issues with the engineered strain. The plates with spores were kept refrigerated (4ºC) until used.

Reactor setup

Figure 6.1 shows the scheme and a picture of the TBR system. The reactor was constructed in-house using a 60 cm tall glass column with an internal diameter of 10.5 cm. The inside of the column was filled with an inert packing material for the fungus to grow on. Three different random packing materials were used for the experiments; two made of high-density polyethylene plastic (HDPE) (GEA 2H Water Technologies, Dusseldorf, Germany, EU) and one made of stainless steel (Sulzer Chemtech, Tulsa, Oklahoma, USA). Table 6.1 summarizes the characteristics of each packing material, hereafter refer to as packing #1, 2, or 3 as indicated in Table 6.1. A picture of the different packing materials can be seen in Figure 6.2. The liquid medium was recycled from the sump to the top of the column through a recirculation loop using a peristaltic pump (Masterflex, Cole-Parmer, IL, USA). Four outlets on top of the column distributed the liquid media across the top of the packed bed. Two other pumps were used to add fresh medium and collect product at the same rates. A sampling port in the recirculation loop allowed samples to be taken periodically. Air (110 mL/min or 1.1 L/min) was pumped from the room through a sterile 0.2 µm PTFE filter into the bottom of the column, and exited the reactor at the top. The temperature was maintained at 37ºC by wrapping a heating tape around the column. The TBR with the packing material and the sump were sterilized by autoclaving at 121ºC for 20 min. The fittings and tubing parts of the recirculation loop were autoclaved separately and assembled aseptically in a biosafety cabinet (Nuaire, Plymouth, MN, USA).

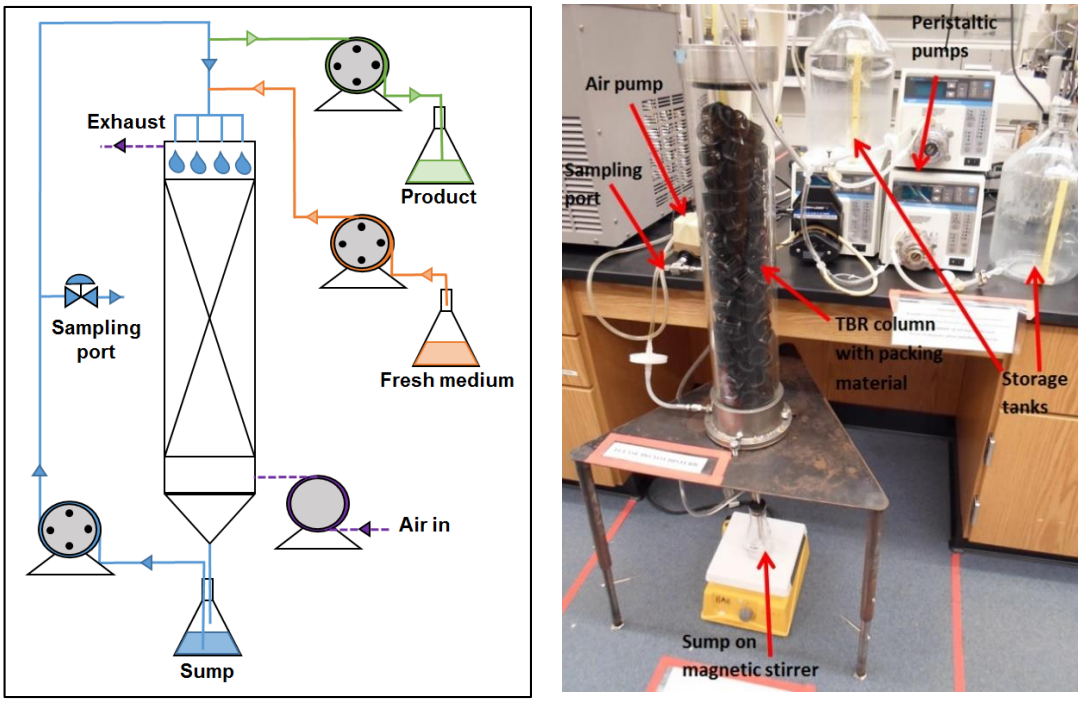

Figure 6.1. Diagram of the continuous TBR setup (left) and picture of the real system in the laboratory (right)

Table 6.1. Properties of the three random packing materials used during this study

Property	**Packing #1**	**Packing #2**	**Packing #3**
Company	GEA 2H Water Technologies	GEA 2H Water Technologies	Sulzer Chemtech
Model	BCN 030	BCN 020	NeXRing #0.6
Material	HDPE	HDPE	316 Stainless Steel
Hydrophobicity	Hydrophobic	Hydrophobic	Hydrophilic
Surface (m^2/m^3)	320	610	NA[a]
Protected surface (m^2/m^3)	259	400	NA[a]
Length (mm)	30	20	20
Diameter (mm)	30/36	20	21/22

[a]NA: Not available, information was kept confidential by the manufacturer

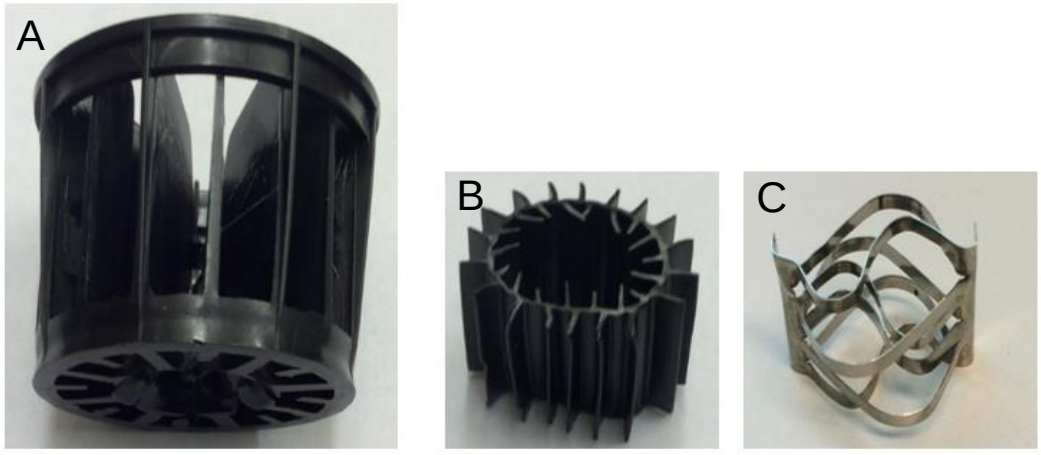

Figure 6.2. Picture of the three types of random packing materials used (A) Packing #1 (B) Packing #2 (C) Packing #3.

Analysis of bed void fraction and flow through the reactor

The dynamic hold-up and the void volume for each packing material was determined by using water at room temperature. The TBR column was filled up to 53 cm high with each of the three different packing materials (similar to the configuration used for fermentation experiments) and the bottom of the TBR was sealed to prevent liquid from exiting the system. Water was poured in the column until the volume of liquid covered the packed bed. This volume was recorded as V_1. Then, the column was drained and the volume of water collected was recorded as V_2. Finally, the column was emptied and it was filled with water up to 53 cm (bed height). This volume was recorded as V_3. The static hold-up was calculated as V_1-V_2. The void fraction of the bed is defined as the fraction of the volume not occupied by the rings. The void fraction (ε) can be calculated as

$$\varepsilon = \frac{V_3 - V_1}{V_3} \qquad \text{(Eq. 6.1)}$$

The dynamic hold-up is the amount of liquid retained in the packed bed while the recirculation pump is being used. It was observed that the gas flow rate did not affect the dynamic

hold-up, so no air was pumped in the system during the flow behavior experiments. This is in agreement to what was observed by another research group for a different TBR (Orgill et al., 2013), although gas flow rate may have an effect in other systems where moderate to high flow rates are used (Ranade et al., 2011). The system was initially filled with water and the recirculation loop tubing was filled by activating the pump for 5 min. The sump below the column was a graduated baffled flask. Initially, the pump was off and the volume of water in the sump was recorded (V_4). Then, the pump was turned on at a certain speed (between 20 and 300 mL/min) until the volume in the sump was stable (V_5). The dynamic hold-up was calculated as V_4-V_5. Finally, the contact time of liquid with the packing material (min) at each recirculation flow rate could be calculated by dividing the dynamic hold-up by the recycling rate (mL/min) at which it was measured (Mueller, 2012).

Reactor operation for continuous MtGloA production in TBR

Two different operation modes were used throughout this study. The trace element solution used for the medium was different in each case: mode 1 used "Medium A", while mode 2 used "Medium B", which contained lower amounts of all elements and copper was removed. The full recipes can be found in Chapter 5. Decreased melanization was observed in a previous study (Chapter 5) when zinc and copper concentrations in the medium were reduced. However, this factor alone did not abolish melanin production. Therefore, the medium used in mode 2 contained 5 g/L ascorbic acid. Ascorbic acid was found to reduce melanization of the engineered *A. nidulans* strain in a previous study (Chapter 5). An antifoam agent was included in the media to prevent foaming (Antifoam B, Sigma Aldrich, St. Luis, MO, USA) at concentrations of 0.06% and 0.02% (v/v) in mode 1 and mode 2, respectively. Higher antifoam concentration was required for mode 1 due to increased foaming observed. Mode 2 used only 10% of the air flow rate of mode 1 (110 mL/min versus 1.1 L/min, respectively). This corresponded to volume of air per volume of liquid media per minute (vvm) of 0.1

and 1.0 min^{-1}, respectively. It was hypothesized that higher air flow rates used in the reactor could oxidize ascorbic acid faster, decreasing its reducing power and therefore its ability to decrease melanization of the cultures. In addition, it was observed how lower air flow rates reduced issues associated with condensation in the exhaust line.

For both modes of operation, the reactor was run in batch mode for 39h using 800 mL medium with 1 g/L pyridoxine in order to allow growth of the fungus. Then, the remaining free liquid medium was collected and 800 mL of 0.89% NaCl solution was recirculated for about 2h as a washing step in order to reduce the carryover of pyridoxine to the next steps. Continuous fermentation was then started by adding fresh medium with reduced pyridoxine concentration (30 µg/L) and removing product at the same flow rates. These flow rates used determined the dilution rate tested. Whenever the dilution rate was increased, the concentration of pyridoxine in the medium was reduced accordingly to maintain a constant supply of pyridoxine of 1 µg/L*h. The total volume of liquid medium in the system was 1.1 L during continuous operation. Samples of the liquid broth were aseptically taken every 7 to 10 h and were analyzed for protein and sugar concentration, pH, and MtGloA activity as explained below. Pigmentation of mycelia was assessed visually, and darkening of the media was followed by spectrophotometry at 425 nm. This wavelength was used elsewhere to track production of soluble melanin in *A. nidulans* cultures (Rowley & Pirt, 1972). Upon termination of the experiment, the whole system was sterilized by autoclaving and the packed bed was washed with 5 L water in order to wash out remaining metabolites. Finally, the packing material with the fungus was air dried inside a fume hood and the final mass of mycelia was determined by weight.

Effect of air flow rate on reactor performance in mode 2

The effect of air flow rate was evaluated in an experiment using packing material #1 as solid support. The reactor was first operated in mode 2 as described above, and when the steady state was reached (at 135 h) the air flow rate was increased from 110 mL/min to 1.1 L/min. Samples were taken

throughout the experiment and analyzed as described below. The results of these analyses were used to evaluate the effect of air flow rate on the performance of aryl alcohol oxidase production in the TBR.

Determination of protein concentration, MtGloA activity, and concentration of sugars

Bradford assay was used to measure protein concentration. One-hundred-sixty µL of Bradford Commassie solution were transferred to a 96-well microplate. Thirty µL of fermentation broth were then added and the absorbance was measured at 595 nm using a UV-Vis microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). Purified bovine serum albumin (100x BSA, BioLabs, MA, USA) was used to prepare a calibration curve relating protein content to absorbance.

A spectrophotometric assay was used to measure MtGloA activity. The assay was based on the oxidation of the substrate, veratryl alcohol, and the reduction of the electron acceptor, 2,6-dichlorophenol indophenol (DCPIP). The change in absorbance of the reaction mixture at 600 nm was monitored using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). Absorbance decreases as DCPIP (blue) is reduced to $DCPIPH_2$ (colorless). Each reaction well contained 10 µL of 2 mM DCPIP, 10 µL of 250 mM veratryl alcohol, 10 µL of 50 mM phosphate buffer of pH 7.0 and 70 µL of sample. The reagents were equilibrated at 50°C for 5 min in a thermal cycler (PTC-200, Bio-Rad, CA, USA) before the reaction was started and the absorbance was monitored for 2 min. One unit of activity per mL was defined as the decrease of $4.2*10^{-3}$ absorbance units (at 600 nm) per min. MtGloA activity of the samples containing ascorbic acid (those generated when operating the reactor in mode 2) was determined after the samples were dialyzed against 50 mM phosphate buffer at pH 7.0. This was done in order to avoid assay interferences caused by the reducing power of ascorbic acid on DCPIP, which would result in overestimation of the activities. Five-hundred µL of sample of liquid broth were pipetted into a modified polyethersulfone ultrafiltration unit (Nanosep 30K Omega, Pall Corporation, New York, USA) and centrifuged for 10 min at 10,000 rpm until most of the initial

volume of sample passed through the membrane. Then, 500 µL of buffer were added to the concentrate and the ultrafiltration unit was centrifuged again for 10 min at 10,000 rpm. Finally, the volume of the resulting washed concentrate containing the enzyme was increased to 500 µL using phosphate buffer. The resulting dialyzed sample was used for the MtGloA assay described above.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for further characterization of the protein mix and verification of AAO production. A 12% separation gel was prepared by mixing 1.7 mL H_2O, 1.25 mL 1.5 M Tris, 50 µL 10% SDS, 2.0 mL 30% acryl, 25 µL 10% ammonium per sulfite (APS) and 25 µL tetramethylethylendiamin (TEMED). The stacking gel comprises 2.5 mL H_2O, 0.38 mL 0.5 M Tris, 0.03 mL 10% SDS, 0.5 mL 30% acryl, 0.03 mL APS and 3.0 µL TEMED. Protein concentrated by ultrafiltration was used for the SDS-PAGE analysis. An equivalent of 10 µg of protein was mixed with 2x Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl) and boiled for 5 min in a thermal cycler (PTC-200, Bio-Rad, CA, USA). The solution was then pipetted into the gel and run at 180V for 1.5 h (Mini Protean TetraCell, Bio-Rad, CA, USA). Then, the gels were stained with Commassie stain (Bio-Rad, CA, USA) for 20 min and de-stained with a 10% acetic acid solution overnight.

Concentrations of maltose and glucose were determined by high performance liquid chromatography (HPLC 1100 Series, Agilent, Santa Clara, CA) using an HPX-87P analytical column (BioRad, Hercules, CA) and a refractive index detector with distilled water as the mobile phase at a flow rate of 0.6 mL/min and a temperature of 80ºC. Concentrations were quantified based on a five level calibration curve of known standards.

Calculation of productivities and yields

Evaluation of the reactor performance at each condition tested was done based on productivities and yields. The concentrations of total protein and MtGloA activity were converted to productivities as follows:

$$MtGloA\ productivity\ = MtGloA\ activity\ \cdot\ D \qquad \left[\frac{U}{mL\ h}\right] \qquad \text{(Eq. 6.2)}$$

$$Protein\ productivity\ = Protein\ concentration\ \cdot\ D \quad \left[\frac{mg}{mL\ h}\right] \qquad \text{(Eq. 6.3)}$$

Where MtGloA activity is given in U/mL and protein concentration in mg/L, and D is the dilution rate:

$$D\ = \frac{F}{V} \qquad [h^{-1}] \qquad \text{(Eq. 6.4)}$$

Where F is the inlet/outlet flow rate in mL/h and V the total liquid volume in the TBR system in mL.

The product from cell mass yield ($Y_{P/X}$) was calculated as

$$Y_{P/X} = \frac{MtGloA\ activity}{X_{final}} \qquad \left[\frac{U}{g_X}\right] \qquad \text{(Eq. 6.5)}$$

Where X_{final} is the final dry fungal biomass in g/mL.

The product to substrate yield ($Y_{P/S}$) was calculated as

$$Y_{P/S} = \frac{MtGloA\ activity}{S_{consumed}} \qquad \left[\frac{U}{g_{sugar}}\right] \qquad \text{(Eq. 6.6)}$$

Where $S_{consumed}$ is the total amount of sugar (maltose and glucose) consumed in g/mL**.**

6.4 Results and Discussion

Analysis of bed void fraction and flow through reactor

The static hold-ups (liquid that remained on the bed once drained) were similar for all three packing materials used: 88, 95, and 78 mL for materials 1, 2, and 3, respectively. Table 6.2 shows the void fractions for all three packing materials. Material #2 provided the lowest void fraction (84%),

followed by material #1 (90%) and material #3 (97%). The void fraction of the bed is important in terms of the internals of flow in the column because it determines the space that will be available for both the liquid to trickle down the bed and the air to flow upwards, and also the space available for the fungal mycelia to develop. Based on this factor alone, material #2 would provide a more compact bed with less space for fungal growth and lower contact between the liquid media and air, followed by materials 1 and 3. The availability of space for the fungus to grow is also related to the surface areas provided by the material used. Unfortunately, only the surface areas for materials 1 and 2 are known (the manufacturer of material #3 retained this information as confidential). The surface area of material #2 is nearly two-fold higher than that of material #1 (Table 6.1), which could compensate for the lower void fraction of the bed.

Figure 6.3 shows the contact times of the liquid on the packing material at each condition. The contact time of the liquid on the packing material was calculated by dividing the dynamic liquid hold up (mL) by the recycling rate (mL/min). A logarithmic relationship can be observed, which is similar to what was reported in a previous study using the same reactor with lava rocks as packing material (Mueller, 2012). In this case, the longer contact times obtained for both materials (up to 0.7 or 0.4 min at the minimum recycling rate for packing #1 and #2, respectively) were significantly lower than that observed for lava rocks (up to 4 min at the minimum recycling rate). This is probably due to the porous nature of the lava rocks used in that past study compared to the non-porous plastic and stainless steel rings currently used. More porous materials are able to retain more liquid in the bed because of the increased effective surface area and the interaction of the material with water.

Materials #2 and #3 had very similar residence times at all the flow rates tested, and they differed from those observed for material #1 only when very low recirculation flow rates were used (less than 50 mL/min). At these low flow rates, the residence time for material #1 was 50 to 75% higher (0.4 and 0.7 min at 50 and 20 mL/min, respectively) than for the other two materials. As

mentioned before, these contact times were much lower than those observed for porous lava rocks (Mueller, 2012). It is important to note that these residence times would apply only at the beginning of the fermentation process, because as cell biomass increases, the static hold-up, dynamic hold-up, and void fraction of the packed bed will gradually change. Therefore, the purpose of this comparison is merely to provide more information about the three different packing materials used, beyond the information provided by the manufacturer shown in Table 6.1.

Table 6.2. Void fraction of the packed bed for each type of random packing material used

Packing material #	Void Fraction
1	90%
2	84%
3	97%

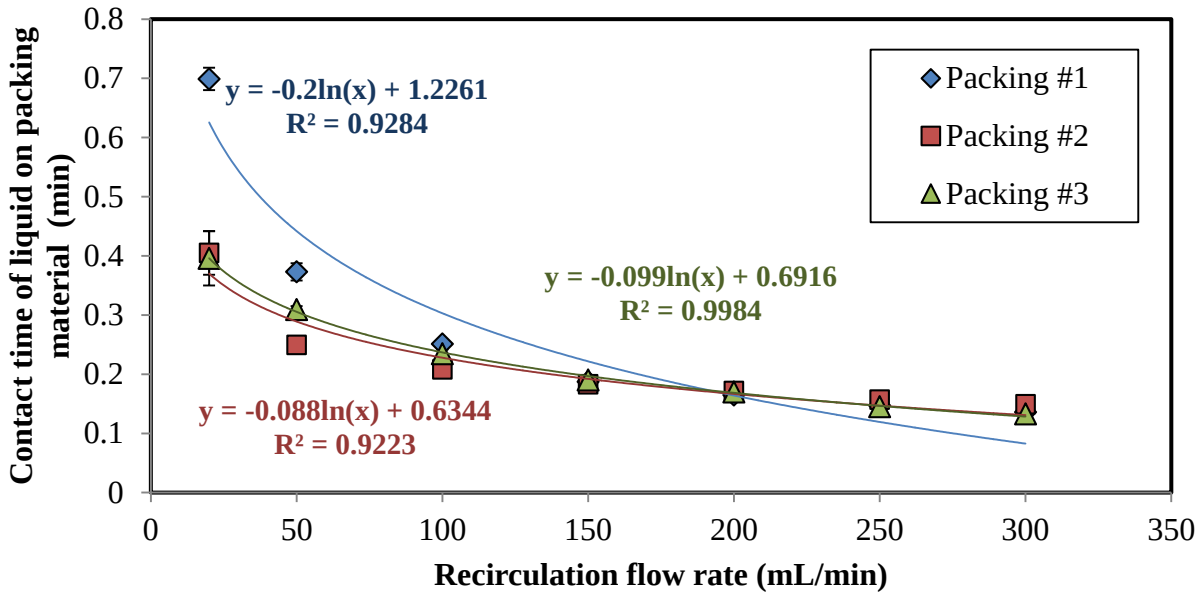

Figure 6.3. Contact times of liquid on the packing materials in the TBR at different recirculation flow rates

Continuous MtGloA production in TBR: effect of mode of operation

The mode in which fermentation was carried out had an evident effect on the results. Table 6.3 shows a summary of the results for each condition tested. Figures 6.4 to 6.9 show the profiles for MtGloA and protein productivities, pH, sugar concentration, and absorbance of the liquid broth at 425 nm for all three packing materials tested. For each packing material, the profiles for both modes of operation were plotted in the same graph for easy comparison. Running the fermentation in mode 2 resulted in an increase of MtGloA productivities. This effect was more pronounced when using packing materials #1 and #3 (Fig. 6.4A and 6.8A, respectively). In these instances, greater enhancements of productivity were obtained after 150 h, and resulted in average increases of 190 and 140% for materials #1 and #3, respectively, compared to mode 1. For packing material #2 this improvement was more modest, but still represented an increase of up to 70% in MtGloA productivities (Fig. 6.6A) compared to mode 1. For all three packing materials used, the total protein productivities obtained were generally lower for mode 2 (Fig. 6.4A, 6.5A, 6.6A), indicating that mode 2 resulted in a purer MtGloA product than mode 1. SDS-PAGE analysis revealed larger bands for the samples from mode 2 than those from mode 1 (Figure 6.10).

The pH of the liquid broth was always higher in mode 1 than in mode 2 (Fig. 6.4B, 6.6B, 6.8B) even though the initial pH of the media was slightly higher for mode 2 (7.2) than for mode 1 (6.5). This is in agreement to what was observed in a previous study using submerged cultures with media supplemented with ascorbic acid (Chapter 5). In that case, even when both the treatment with ascorbic acid and a control treatment with no ascorbic acid had similar initial pH values, the final pH was lower for the cultures with ascorbic acid. This decrease in pH could be due to degradation of ascorbic acid in presence of oxygen, enabling fragmentation reactions. Degradation products of ascorbic acid include threonic, oxalic, glyceric, glyoxylic acids, and other unknown compounds (Shin & Feather, 1990). In addition, the interaction of any of these species with the fungal strain, or with

other compounds secreted by the fungus could enable different reactions that are currently unknown and may have an effect on pH. Only citric, succinic, lactic, and acetic acid were quantified in this study, and their concentrations were similar in both modes of operation and will be discussed in a subsequent section. In terms of sugar consumption, both modes resulted in a similar maltose consumption of higher than 80% after the first 75 h of operation, but glucose consumption was lower for mode 2 than for mode 1 (Fig. 6.5A, 6.7A, 6.9A). Glucose consumption typically exceeded 50% in mode 1, but rarely did in mode 2. Higher glucose consumption in mode 1 probably is related to higher melanization of the culture, as this would increase the substrate requirement of the cells.

The absorbance of the liquid broth measured at 425 nm (a method used to estimate the soluble melanin produced by the culture) was generally lower for mode 2 than mode 1 (Fig. 6.5B, 6.7B, 6.9B). It was expected that operating the reactor in mode 2 would decrease melanin production due to the presence of ascorbic acid in the media, as was observed in Chapter 5. Ascorbic acid can reduce o-quinones (intermediates in melanogenesis) back to their o-phenol precursors, blocking the pathway to melanin production (Lozano de Gonzalez et al., 1993). The maximum absorbance readings for packing materials #1 and #2 were similar: 2.6 for mode 1 and 2.0 for mode 2. When using material #3, however, the maximum absorbance registered using mode 1 was much higher, reaching 5.2, and was only 2.0 when operating in mode 2. Material #3 provided the highest void fraction in the packed bed (Table 6.3), which translated to a higher availability of space for contact between the liquid media and the air. Since melanization reactions require oxygen, the internal configuration of the bed using this material could have increased the oxygen mass transfer to the mycelia, favoring melanin production. This could be a reason why the absorbance of the broth in mode 1 was twice as high as for the other packing materials providing lower void volumes.

The easiest way to evaluate the melanization process for each reactor was visual observation of the biomass on the packing material. Figure 6.11 shows pictures of the packed bed using mode 1

and 2. When using mode 1, the color of the mycelia covering the packing material quickly turned from white color (batch mode: 0-39h) to light brown with red tones (approximately until 85 h) to dark brown and even black color until the end of the fermentation process. For mode 2, however, the color of the mycelia changed from white (batch mode: 0 to 39 h) to yellow tones (approximately until 85 h) to light brown color until the end of the fermentation process. No dark brown or black pigments were observed using mode 2 of operation. This is a clear indication that mode 2 suppressed, at least partially, the melanization process. While soluble melanin production was reduced by mode 2 only moderately, insoluble melanin deposition was greatly affected by the mode of operation.

It was also observed that the melanized mycelia during mode 1 became harder while it remained soft in mode 2. This apparent change in the biomass hardness reduced operational problems associated to clogging in mode 2 compared to mode 1. In addition, this may have caused higher MtGloA productivities in mode 2 than in mode 1. Insoluble melanin formation decreases the porosity of the fungus cell wall, reducing the size of the pores (Eisenman et al., 2005). This could have negative implications on the delivery of substrate to the fungus and the release of product to the liquid broth. The overall lower sugar utilization observed for mode 2, especially for glucose, may be another consequence of lower melanization, which would require less substrate for this pigmentation process. Finally, the weight of total biomass in the packed bed at the end of the experiments was 11 to 14% lower for mode 2 than for mode 1, depending on the packing material used (Figure 6.12). This is in agreement to a previous study indicating that melanin production can lead to overestimation of fungal biomass (Carter et al., 1971). In that study, melanin represented only 5% of the dry weight of the *A. nidulans* culture without growth limitation compared to 28% when the same strain was carbon-limited and melanin formation was promoted.

Continuous MtGloA production in TBR: effect of dilution rate

Three dilution rates were tested for each packing material used in both modes of operation (Figures 6.4 to 6.9). In the graphs, the dilution rate (D) used is clearly indicated by a different background color. The first change of dilution rate, from 0.034 h^{-1} to 0.038 h^{-1} represented an 11% increase; while the second one, from 0.038 h^{-1} to 0.048 h^{-1}, was equivalent to a 26% increase in dilution rate. It was expected that increasing the dilution rate while controlling the growth of the cell factory by limiting pyridoxine would result in increased productivities until a kinetic limitation was reached. At that point, the rate of uptake of nutrients and production of enzyme would be maximum, and a further increase in dilution rate would only result in unconverted substrate and steady MtGloA productivities. However, it was observed for all packing materials that the highest MtGloA productivities were obtained in the first dilution rate (0.034 h^{-1}), and then decreased to similar levels for the other two dilution rates tested. For example, the experiments using packing material #1 in mode 2 resulted in MtGloA productivities of 2.3, 1.9, and 1.89 U/mL*h, at dilution rates of 0.034 h^{-1}, 0.038 h^{-1}, and 0.048 h^{-1}, respectively (Table 6.3). It could be hypothesized that the kinetic limitation of the cells was already reached using the lowest dilution rate, but in that case increasing D should result in constant productivities, and not lower.

Increasing the dilution rate caused a decrease in pH for all the packing materials and modes tested. While running the reactor at 0.034 h^{-1} resulted in stable pH levels above 7 for both modes of operation, increasing D resulted in all cases in a drop of pH. The lowest pH values were observed for mode of operation 2 during the third dilution rate tested (0.048 h^{-1}). For packing material #1, for example, the pH dropped as low as 5.25 at 237 h. Due to the declining MtGloA productivities observed when the pH dropped below 6 (Fig. 6.7), it was decided to adjust the pH of the liquid broth by injecting small amounts (1 to 4 mL) of 6N NaOH solution, as indicated by the arrows in the graphs (Fig. 6.4B, 6.6B, 6.8B). This adjustment of pH resulted in a recovery of the MtGloA productivity, and

was then applied to the remaining experiments using mode 2. Raising the pH reduces the level of undissociated acids in the liquid broth, which have been extensively reported to be inhibitory to microorganisms (Lambert & Stratford, 1999). For example, adjusting the pH of a *Clostridium acetobutylicum* culture resulted in a 4.3-fold increase in butanol concentration due to the reduction of undissociated acids concentration (Liu et al., 2015).

In the present study, the concentration of the measured organic acids increased during the fermentation as dilution rate was increased for both modes and all three packing materials. Concentration of citric acid increased from 0.5 to 1 g/L, succinic acid from 0.1 to up to 0.7 g/L, lactic acid from 0 to up to 0.5 g/L while acetic acid was generally below 0.1 g/L. This gradual increase of citric, succinic, and lactic acid throughout the fermentation suggests favored organic acid production at higher dilution rates. This increase in acid concentrations may be the reason why MtGloA productivities did not increase with increased dilution rates and instead remained constant at 0.038 and 0.048 h^{-1}. An increase in acid production was also observed in agitated cultures using the same cell factory when pyridoxine was removed from the medium (Chapter 4). In the current TBR study pyridoxine was limited to control growth, but it was not completely removed. Instead, it was continuously provided at very low concentrations and its supply was kept constant at 1 µg/L*h at the various dilution rates. However, it is possible that the steady state for pyridoxine concentration in the system is very slow to reach because of initial accumulation inside the cells, and the observed decrease in MtGloA productivities and favored acid productions are indications of this slow depletion of pyridoxine.

The sugar concentrations did not increase significantly when dilution rate was increased. This is another piece of evidence supporting the idea of favored organic acid production (and perhaps other unidentified metabolites) instead of enzyme production at higher substrate supply rates. For example, for packing #1 in mode 2, the sugar concentration in the liquid broth actually decreased from 12.78

g/L at D=0.034 h^{-1} to 7.46 at 0.048 h^{-1} (Table 6.3). Reduction of MtGloA productivity paired with higher substrate consumption resulted in lower product to substrate ($Y_{P/S}$) yields. For example, for packing material #1 in mode 2 the yield was 1,542 U/g sugar at 0.034 h^{-1} and only 779 U/g sugar for 0.048 h^{-1} (Table 6.3). Absorbance measurements at 425 nm were rather independent of the dilution rate used. For example, for packing material #3 in mode 2 the absorbances recorded were stable between 1.8 and 2.0 units from 112 h to the end of fermentation, at 256 h (Fig. 6.9B). This indicates that soluble melanin production was favored at higher dilution rates, which could contribute to the use of substrate for purposes other than MtGloA production, similar to the production of organic acids already discussed. In some instances, the absorbance decreased during the last dilution rate, probably paired to the low pH levels reached and the inhibitory effects observed on MtGloA production discussed earlier. An example of this decrease in absorbance during the third dilution rate was the experiment using packing material #1 at mode 2, until the pH was adjusted at 237 h and the absorbance values increased again (Fig. 6.5B).

Continuous MtGloA production in TBR: effect of packing material

The packing material used inside the column for culturing the *A. nidulans* cell factory did not have a great impact on the fermentation results. It was expected that the largest effect would be derived from the space available for cell growth during the initial batch phase. Packing materials with higher surface areas and void volumes can potentially offer more space for the fungus to grown. Among the hydrophobic HDPE materials used, material #2 provided a higher surface area than material #1 (610 versus 320 m^2/m^3), while the surface area of the stainless steel material #3 was undisclosed by the manufacturer (Table 6.1). Material #3 provided the highest void volume, followed by materials #1 and #2 (Table 6.3). In addition, it was hypothesized that the hydrophobicity of the support material could have an effect on the attachment of spores to it. Spores from *A. nidulans* are hydrophobic due to presence of hydrophobins DewA and RodA in the walls of conidia (Dynesen &

Nielsen, 2003). It was expected that using a hydrophobic material as support for growth would be beneficial for cell attachment, as demonstrated in the literature both for bacteria (Rönner et al., 1990) and fungus (Webb et al., 1999). In this study, materials #1 and #2 were more hydrophobic (HDPE) than material #3 (stainless steel). However, all these differences among packing materials did not seem to affect the final biomass collected after each experiment. In fact, the differences in final weight of biomass among different materials were less than 5% for the experiments run in mode 1 and less than 1% for mode 2 (Figure 6.12). Only the mode of operation, as discussed earlier, had an effect on the final biomass weight. The fungus grew easily in both materials (HDPE and stainless steel) tested. It also grew on the glass walls of the column and on the plastic tubing surfaces. Hence, the type of material used as support does not seem to be of great relevance for enabling *A. nidulans* growth. In the past, a similar cell factory for production of a different enzyme grew on lava rocks, a material that contrary to the ones tested in the present study, displays a high porosity (Müller et al., 2015)

Given the similarity in biomass availability in the reactor for different materials, it is not a surprise that the fermentation parameters, including enzyme activity and protein productivity, sugar concentration, and yields did not reflect large differences among materials tested. Use of materials #1 and #3 in mode 2 resulted in very similar MtGloA productivities, ranging from 1.6 to 2.4 U/mL/h at different dilution rates, slightly higher than those registered when using material #2: 1.5 to 1.9 U/mL*h (Table 6.3). Protein productivity and sugar concentrations were similar for all the packing materials tested when operating at similar conditions (Table 6.3).

Even if the fermentation results were not very different among packing materials, there were differences in operation. Specifically, use of packing material #2 resulting in accumulation of liquid on the top of the column for both modes 1 and 2. After approximately 180 h of operation, liquid broth flooded the top of the packed bed, probably due to insufficient space to trickle down the bed at an

appropriate rate. This phenomenon was not observed for materials #1 and #3. This difference in operation could be due to the unequal void volumes provided by the different materials. Packing material #2 provided a lower void volume fraction in the bed (84%) than the other two materials (90 and 97% for #1 and #3, respectively) (Table 6.3). In addition, the shape and size of the rings could have increased tortuosity when using material #2 compared to the other materials, favoring the pooling phenomenon. This feature could also have impacted the distribution of liquid media throughout the bed. It is reasonable to hypothesize that pooling of liquid on top of the column was related to a decreased availability of pathways for the liquid to trickle down, resulting in preferential routes that could favor liquid maldistribution. In consequence, different portions of the packed bed could have suffered from lack of substrate, and overall, the bed could have displayed undesired heterogeneous conditions across the radial and axial dimensions.

Table 6.3. Summary of main results for TBR experiments using three different packing materials and two different modes of reactor operation.

Conditions		MtGloA activity (U/mL)[b]	MtGloA productivity (U/mL*h)[b]	Protein concentration (g/L)[b]	Protein productivity (mg/L*h)[b]	Sugar concentration (g/L)[b]	$Y_{P/S}$ (U/g_{sugar})[b]	$Y_{P/X}$ (U/g_{cells})[b,c]
Dilution rate (h^{-1})	**Mode[a]**							
Packing Material #1								
0.034	1	54.23±11.35	1.83±0.38	0.47±0.03	16.04±1.04	8.44±1.66	1106.15±241.06	489.76±102.48
0.034	**2**	**69.02±6.56**	**2.33±0.22**	**0.64±0.01**	**21.60±0.41**	**12.78±0.58**	**1541.62±160.84**	**701.68±66.70**
0.038	1	18.28±1.46	0.71±0.06	0.56±0.01	21.59±0.52	3.53±0.85	337.83±24.39	165.06±13.19
0.038	**2**	**48.94±4.53**	**1.89±0.17**	**0.52±0.02**	**20.21±0.89**	**11.08±0.55**	**1052.91±106.85**	**497.50±46.02**
0.048	1	16.48±1.66	0.79±0.08	0.48±0.02	23.10±1.19	11.72±1.74	359.87±40.06	148.82±14.95
0.048	**2**	**39.05±1.94**	**1.87±0.09**	**0.34±0.01**	**16.50±0.65**	**7.46±0.08**	**778.87±37.77**	**397.05±19.75**
Packing Material #2								
0.034	1	39.20±4.24	1.32±0.14	0.53±0.01	18.03±0.49	12.09±3.53	866.98±115.93	341.97±36.97
0.034	**2**	**56.38±5.45**	**1.91±0.18**	**0.52±0.04**	**17.61±1.32**	**10.86±0.97**	**1208.43±134.65**	**574.80±55.56**
0.038	1	34.07±6.09	1.32±0.23	0.62±0.01	24.10±0.30	7.45±1.64	677.80±108.32	297.25±53.11
0.038	**2**	**41.45±2.57**	**1.60±0.10**	**0.54±0.01**	**21.11±0.28**	**10.44±0.44**	**879.00±55.38**	**422.53±26.20**
0.048	1	23.72±4.14	1.14±0.20	0.61±0.02	29.26±0.82	11.78±1.49	520.40±101.00	206.95±36.15
0.048	**2**	**31.32±2.50**	**1.50±0.12**	**0.44±0.02**	**21.27±0.99**	**13.01±0.69**	**702.84±58.98**	**319.33±25.49**
Packing Material #3								
0.034	1	45.83±9.26	1.55±0.31	0.56±0.03	18.94±0.93	5.46±0.80	878.52±173.15	396.95±80.25
0.034	**2**	**69.84±7.18**	**2.36±0.24**	**0.62±0.02**	**20.42±0.85**	**11.89±1.44**	**1533.84±199.90**	**701.73±73.02**
0.038	1	16.98±2.31	0.66±0.09	0.58±0.01	22.43±0.26	5.42±1.68	326.63±52.01	147.08±20.05
0.038	**2**	**40.40±5.31**	**1.56±0.20**	**0.51±0.03**	**19.72±0.97**	**12.19±1.18**	**889.27±114.57**	**411.07±54.01**
0.048	1	16.68±2.18	0.80±0.11	0.52±0.01	24.95±0.29	11.70±2.07	364.72±53.44	144.51±18.96
0.048	**2**	**33.91±1.64**	**1.63±0.08**	**0.47±0.01**	**22.57±0.26**	**13.76±1.85**	**773.86±30.00**	**345.02±16.72**

[a] Mode 1 used media containing copper but no ascorbic acid, and a flow rate of 1.1 L/min air. Mode 2 used media with no copper and 5 g/L ascorbic acid added, and a flow rate of 110 mL/min air

[b] Values represent mean±standard deviation of the last four data points for each condition tested

[c] The activity-to-biomass yields ($Y_{P/X}$) were calculated based on the final biomass weight.

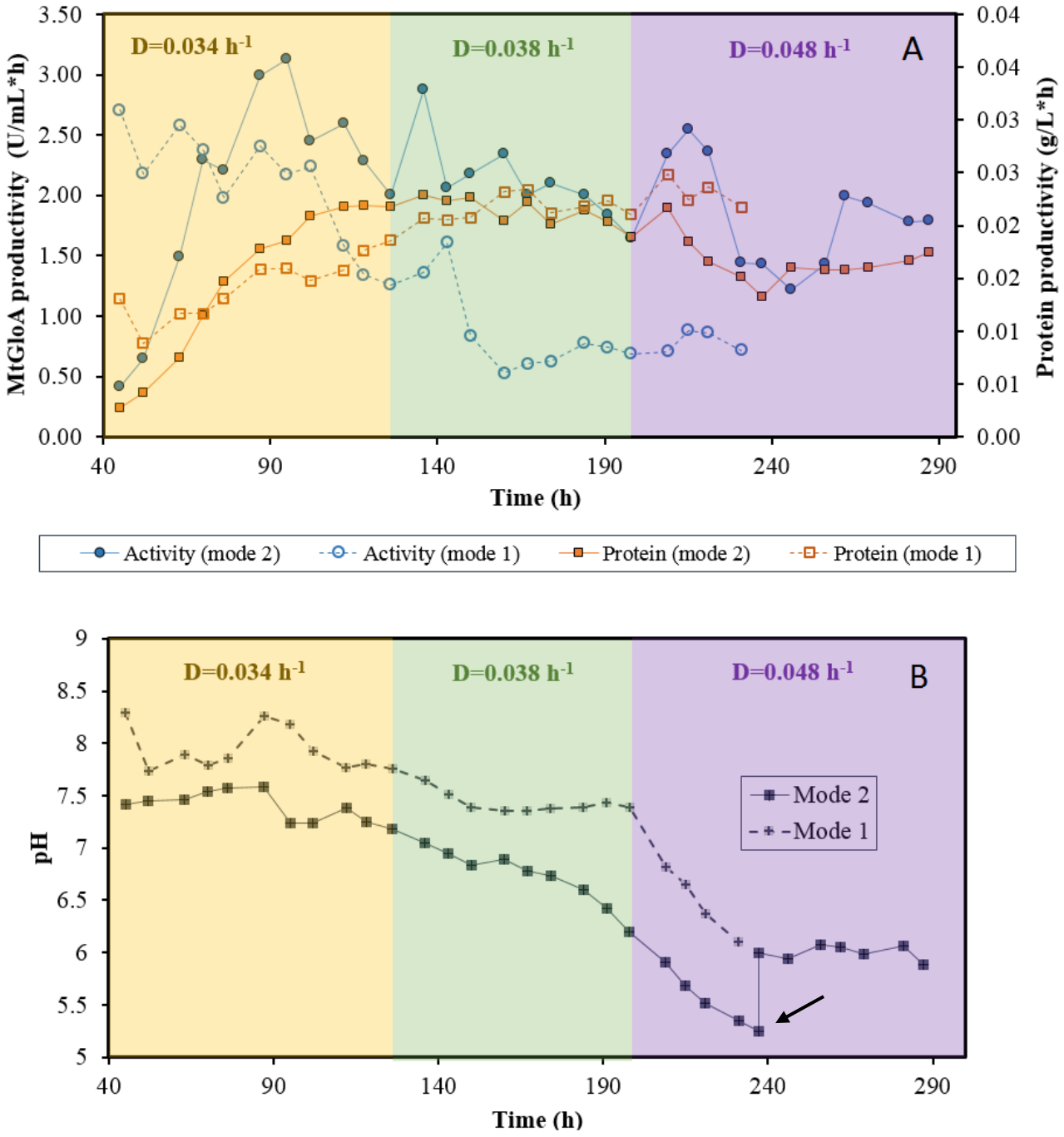

Figure 6.4. (A) MtGloA productivity and protein productivity for modes 1 and 2 using packing material #1 (B) pH profiles for modes 1 and 2 using packing material #1. An arrow indicates pH adjustment (0-45 h data for batch operation and washing step not shown)

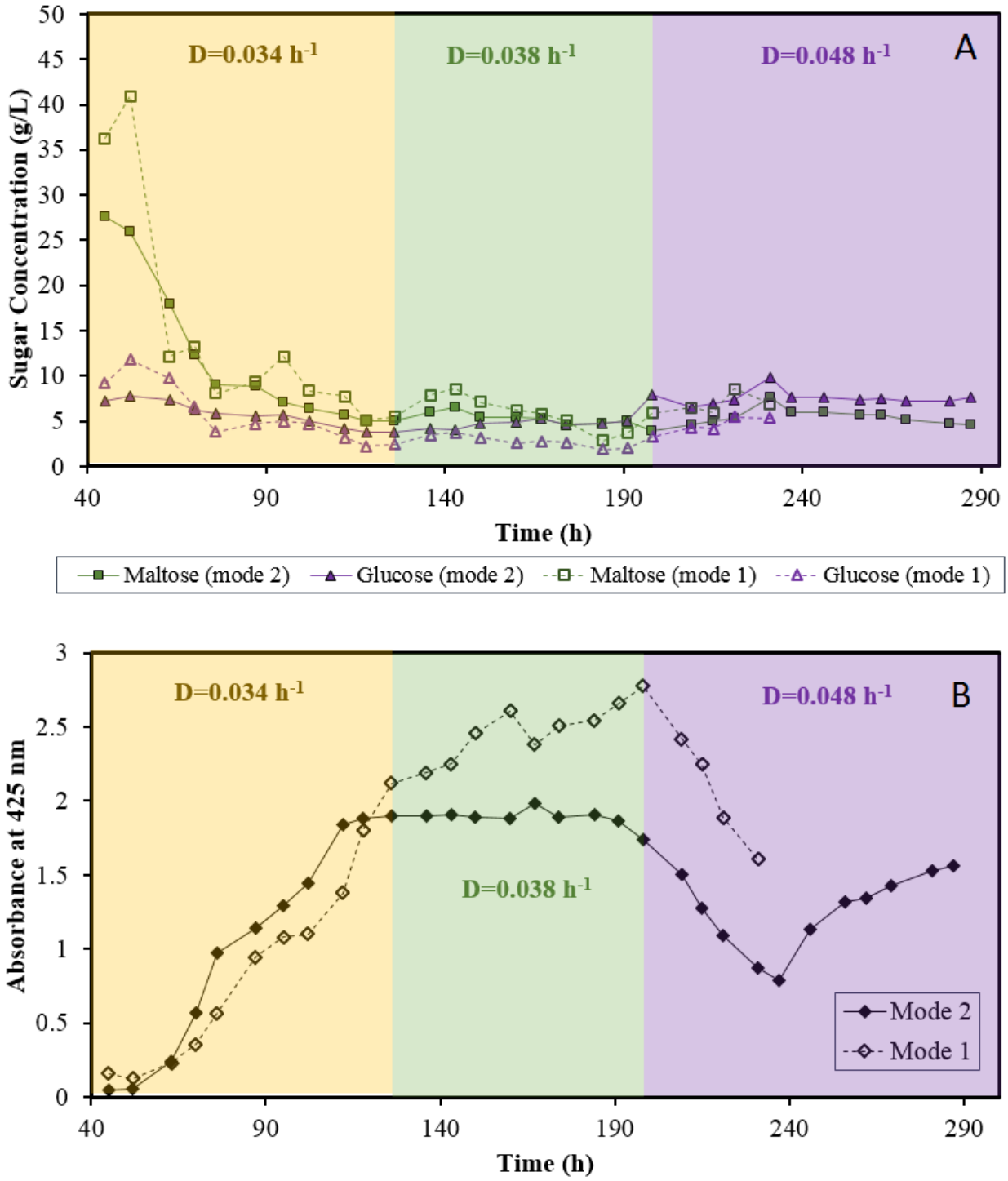

Figure 6.5. (A) Sugar concentration for modes 1 and 2 using packing material #1 (B) Absorbance of liquid broth measured at 425 nm for modes 1 and 2 using packing material #1 (0-45 h data for batch operation and washing step not shown)

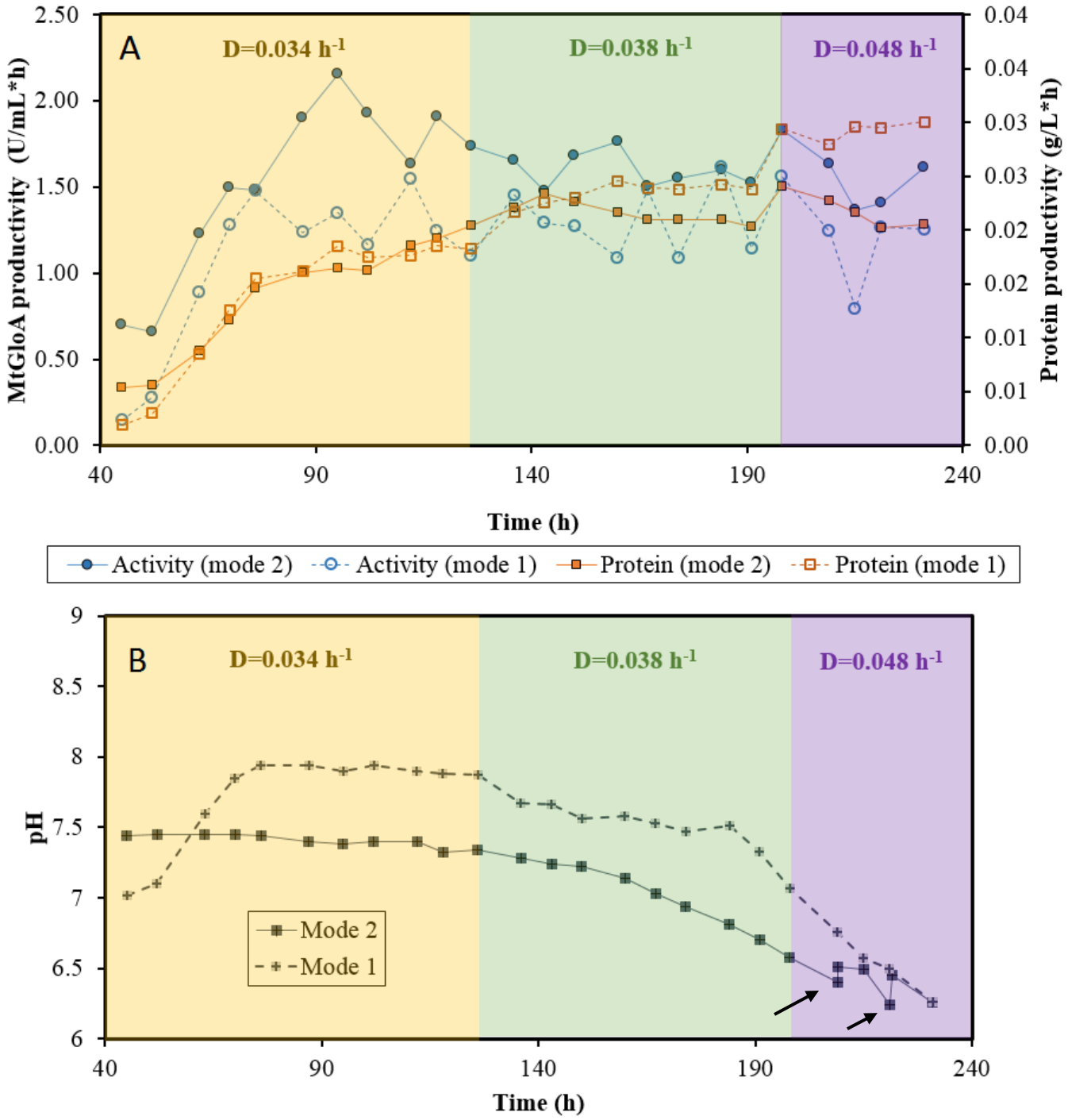

Figure 6.6. (A) MtGloA productivity and protein productivity for modes 1 and 2 using packing material #2 (B) pH profiles for modes 1 and 2 using packing material #2. Arrows indicate pH adjustment (0-45 h data for batch operation and washing step not shown)

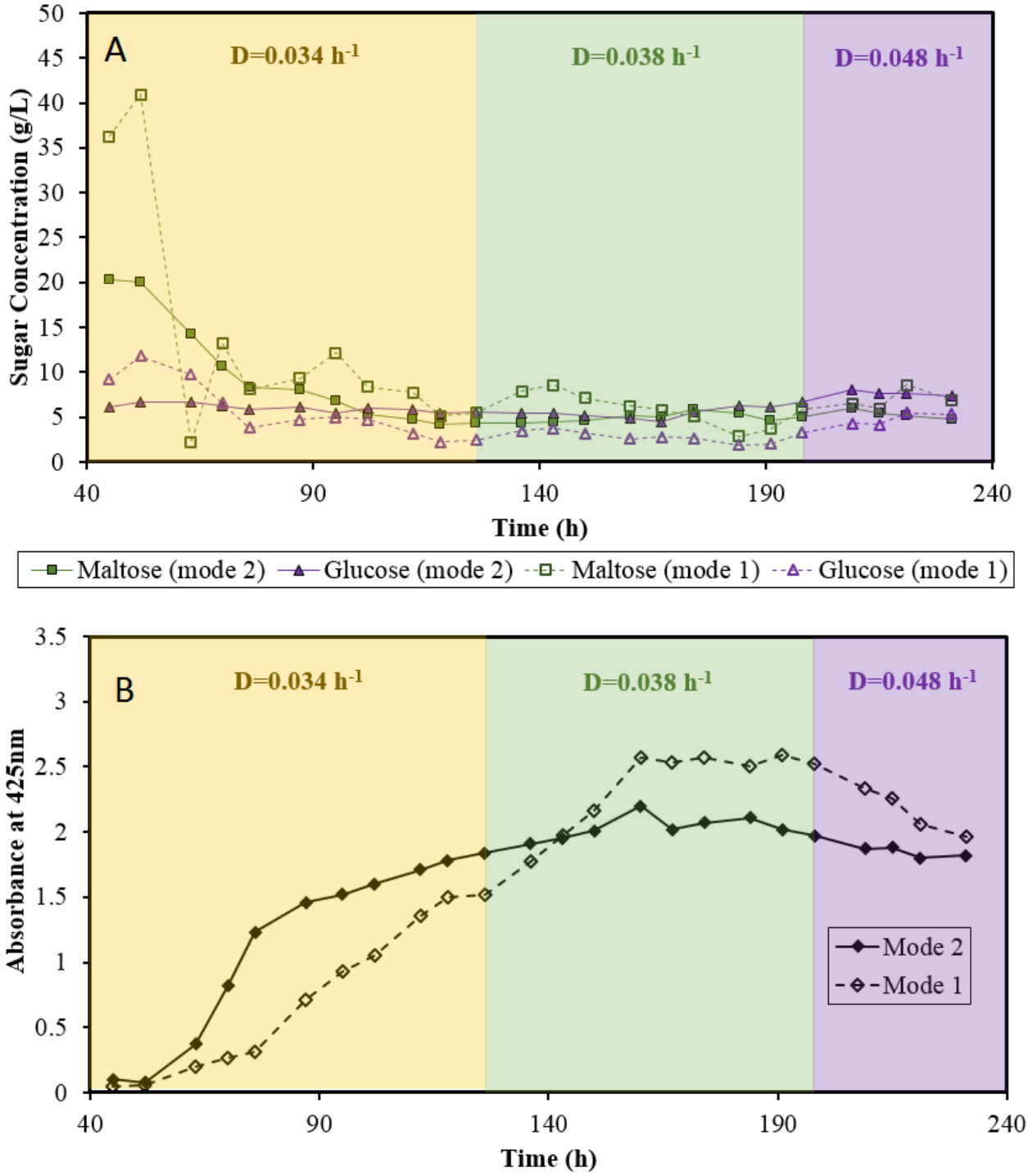

Figure 6.7. (A) Sugar concentration for modes 1 and 2 using packing material #2 (B) Absorbance of liquid broth measured at 425 nm for modes 1 and 2 using packing material #2 (0-45 h data for batch operation and washing step not shown)

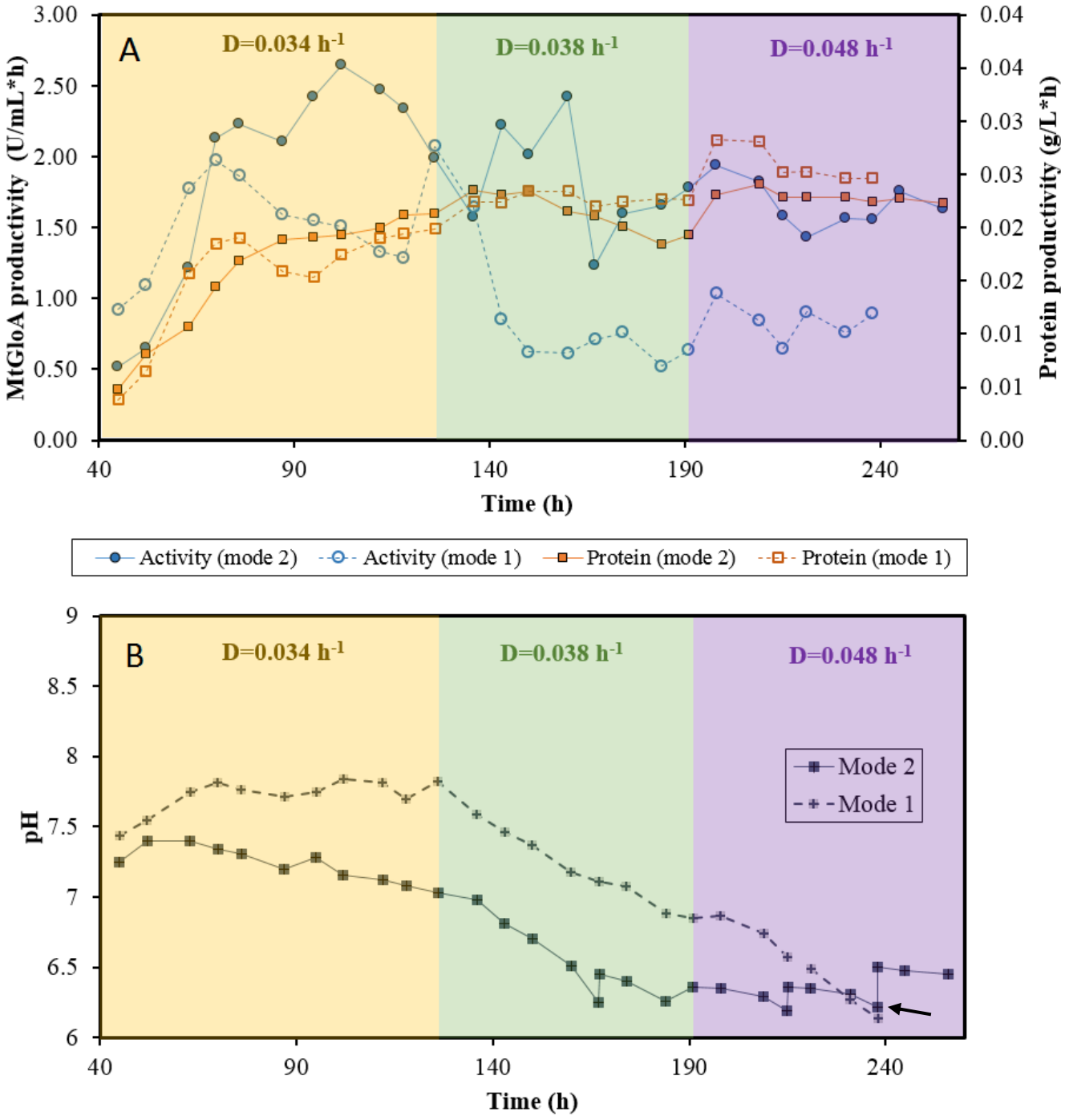

Figure 6.8. (A) MtGloA productivity and protein productivity for modes 1 and 2 using packing material #3 (B) pH profiles for modes 1 and 2 using packing material #3. An arrow indicate pH adjustment. (0-45 h data for batch operation and washing step not shown)

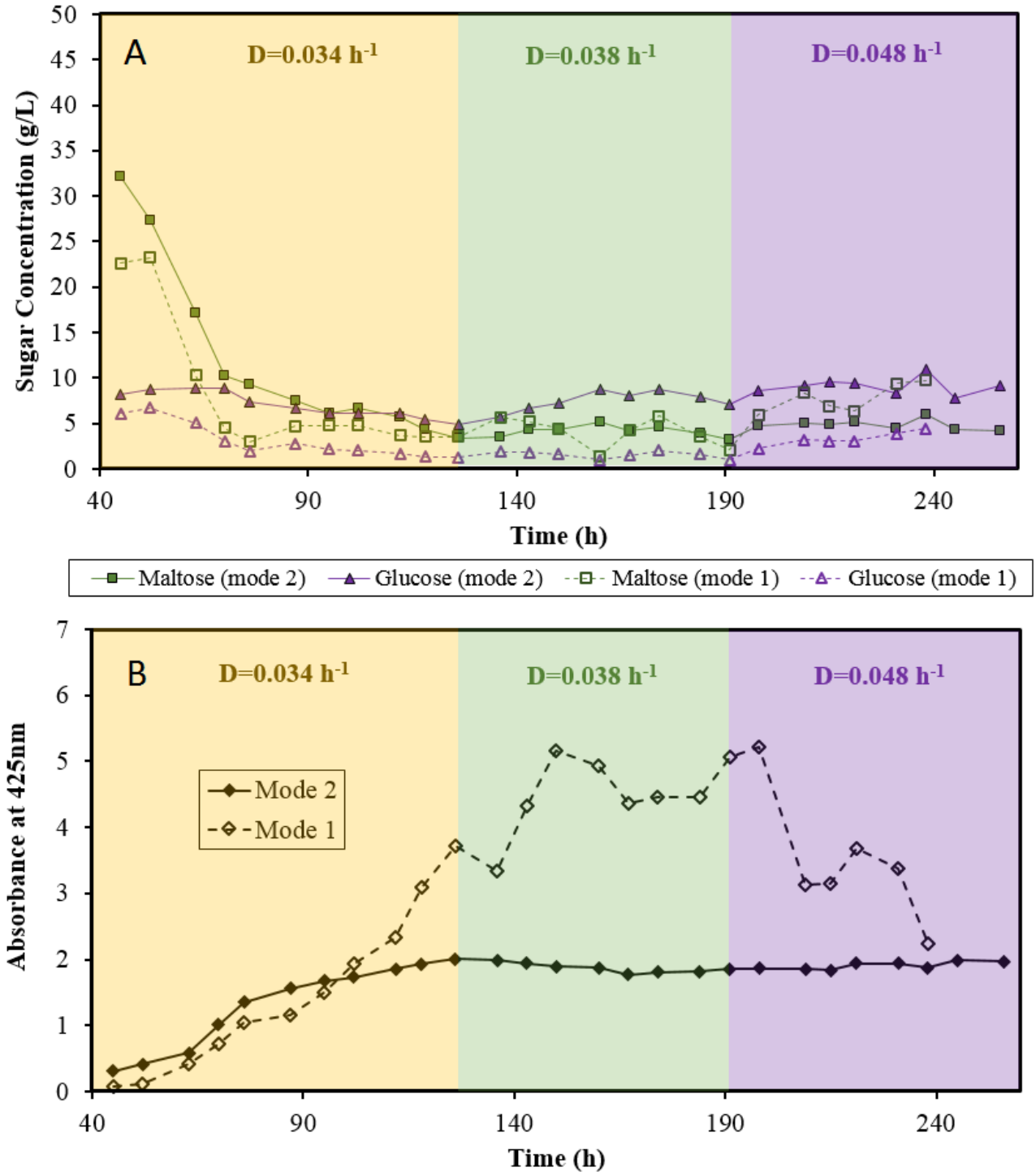

Figure 6.9. (A) Sugar concentration for modes 1 and 2 using packing material #3 (B) Absorbance of liquid broth measured at 425 nm for modes 1 and 2 using packing material #3 (0-45 h data for batch operation and washing step not shown)

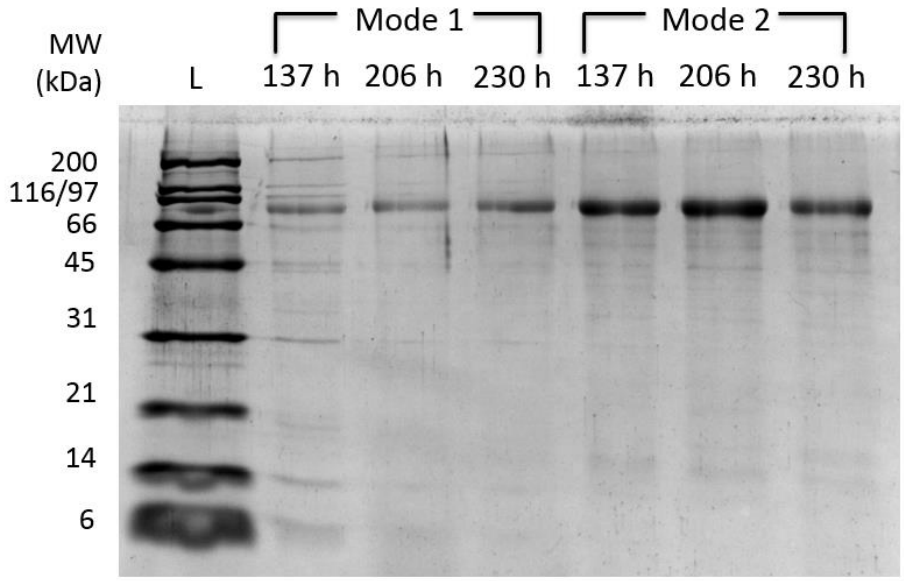

Figure 6.10. SDS-PAGE analysis of samples (137, 206, 230 h) from continuous operation of TBR using *A. nidulans* cell factory under modes 1 and 2 using packing #1. L denotes ladder

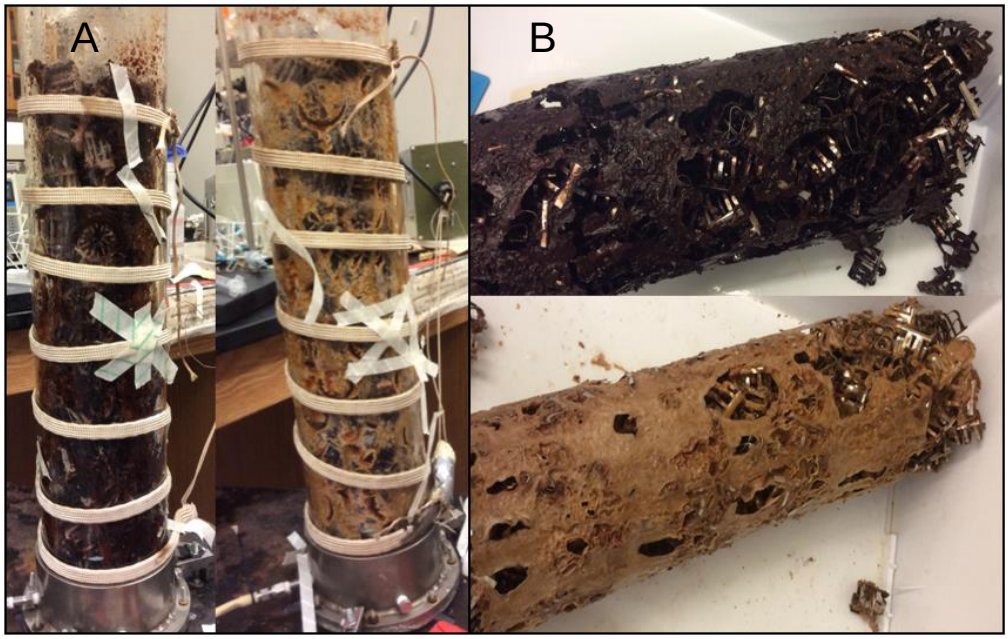

Figure 6.11. (A) Pictures of the TBR column at 126 h using packing material #1 on mode 1 (left) and mode 2 (right) (B) Pictures of the packing material #3 with biomass at the end of the fermentation using mode 1 (top) and mode 2 (bottom)

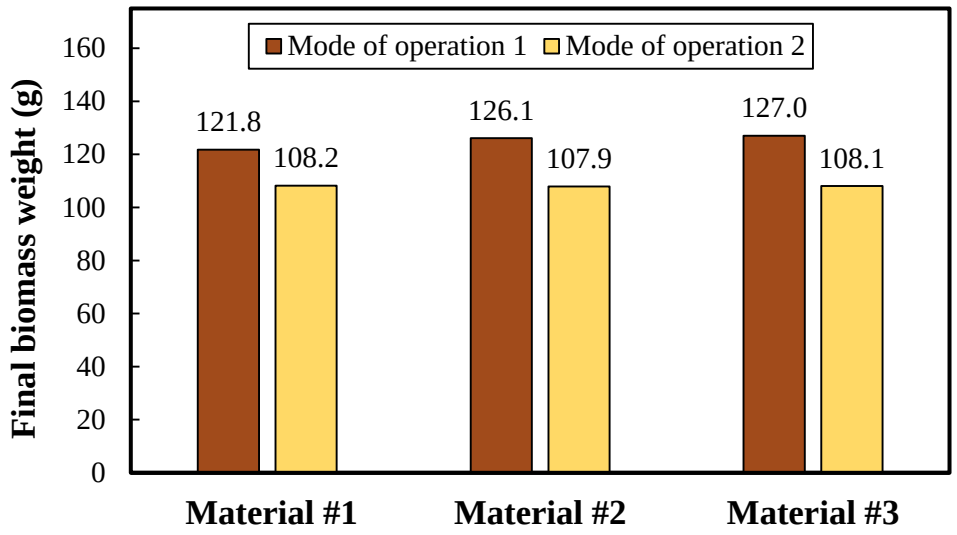

Figure 6.12. Final biomass weight on the different packing materials for both modes of operation

Effect of air flow rate on reactor performance in mode 2

Figures 6.13 and 6.14 show the profiles for MtGloA and protein productivities, pH, sugar concentration, and absorbance of the liquid broth at 425 nm for an experiment evaluating the effect of the air flow rate on the fermentation process. Continuous operation was established using mode 2, and at 135 h the air flow rate was increased from 110 mL/min to 1.1 L/min (the same flow rate used in mode 1). MtGloA productivities dropped after the air was increased, from values near 2 U/mL*h to below 1 U/mL*h (Fig. 6.13A). The pH slowly decreased from 7.4 at the beginning of continuous operation to 6.7, but raised after the change, reaching 7.7 at the end of the experiment (197 h) (Fig. 6.13B). The absorbance of the liquid broth had reached a steady state of 1.8 units before the air flow rate was increased, and increased up to 2.5 after the change (Fig. 6.14A). Sugar concentrations were stable at approximately 5 g/L for both maltose and glucose before increasing the air supply, and dropped to almost zero by the end of the experiment (197 h) (Fig. 6.14B).

All these changes in fermentation parameters suggest a switch to melanin production when the air flow rate was increased. As seen previously for mode 1, when melanization of the culture occurs MtGloA productivities are negatively affected. The higher consumption of sugars paired with lower aryl alcohol oxidase activities indicates that substrate utilization is being directed to formation of other products, such as melanin. The increase of absorbance of the liquid broth is an additional indication of the melanization process taking place. Insoluble melanin formation was evaluated visually and documented by taking pictures of the reactor at different times. Figure 6.15 shows pictures of the culture before and after increasing the air flow rate. It is evident that at the end of the fermentation (197 h) the mycelia displayed a much darker color than before the change in air flow rate was made. The color of the culture before the air flow rate was increased was similar to that displayed in previous experiments using mode 2 (Fig. 6.11).

Increased air flow rate could have promoted melanization through two mechanisms. First, higher availability of oxygen in the reactor may cause a faster degradation of the ascorbic acid supplied in the liquid medium. Ascorbic acid can be oxidized by oxygen to dehydroascorbic acid, which has five times less antioxidant activity than ascorbic acid (Van Bree et al., 2012). In the current experiment air flow rate was increased 10-fold at 135 h, which could have accelerated the degradation of ascorbic acid present in the liquid medium. Consequently, a lower concentration of ascorbic acid would result in decreased capacity to reduce o-quinones to their o-phenol precursors (Lozano de Gonzalez et al., 1993), allowing the melanin pathway to proceed to the formation of the pigment. Second, oxygen is required in this pathway, so its availability can favor melanin formation. Oxygen is needed in two keys steps: during the conversion of L-tyrosine to L-DOPA, and for its further transformation to dopaquinone, both reactions catalyzed by tyrosinase (Ates et al., 2007). A study that investigated the production of L-DOPA using tyrosinase reported a 5-fold increase in productivities when air was introduced in a packed bed reactor (Ates et al., 2007).

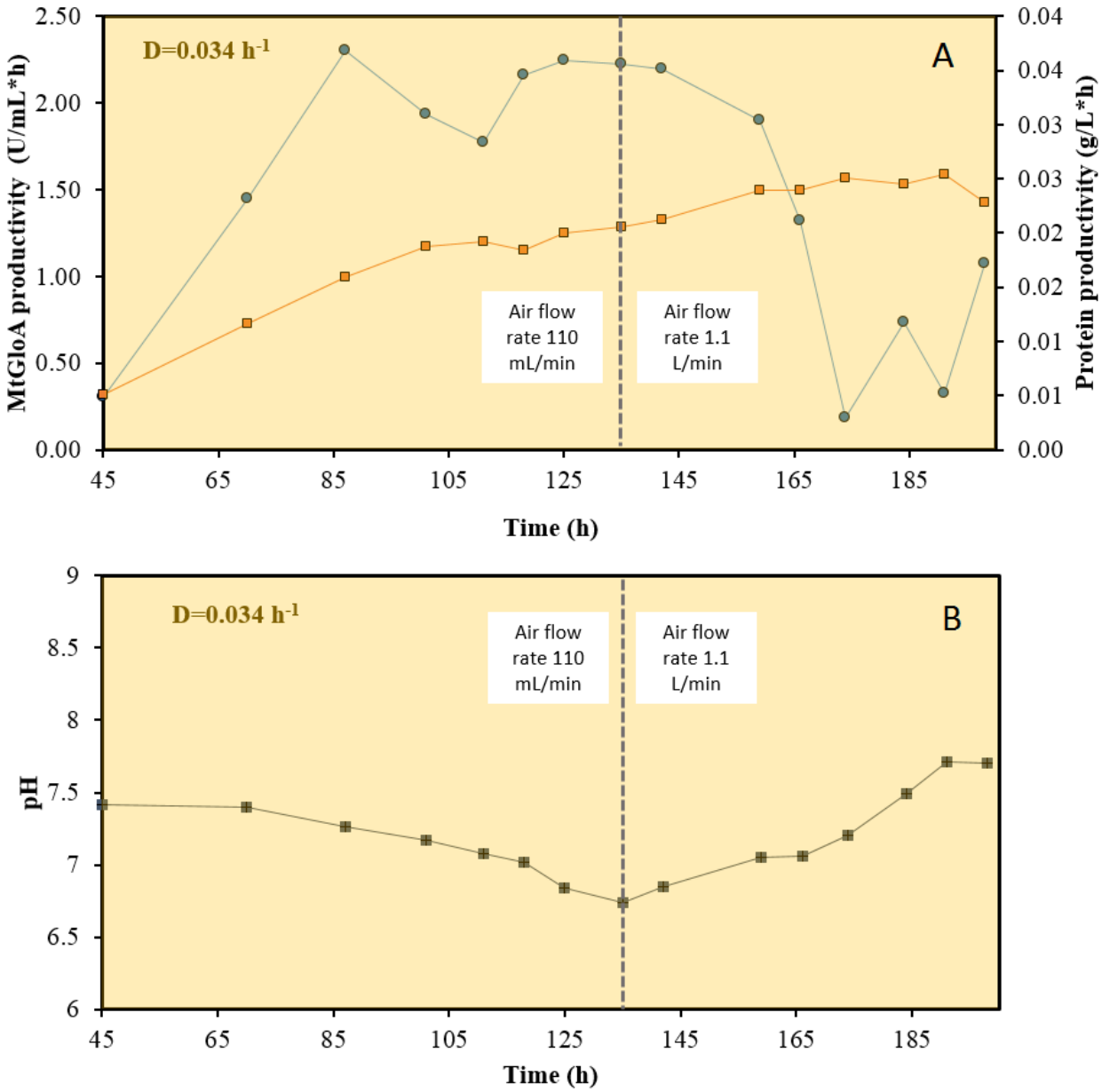

Figure 6.13. Effect of air flow rate on (A) MtGloA productivity (●) and protein productivity (■) and (B) pH for using packing material #1 (0-45 h data for batch operation and washing step not shown)

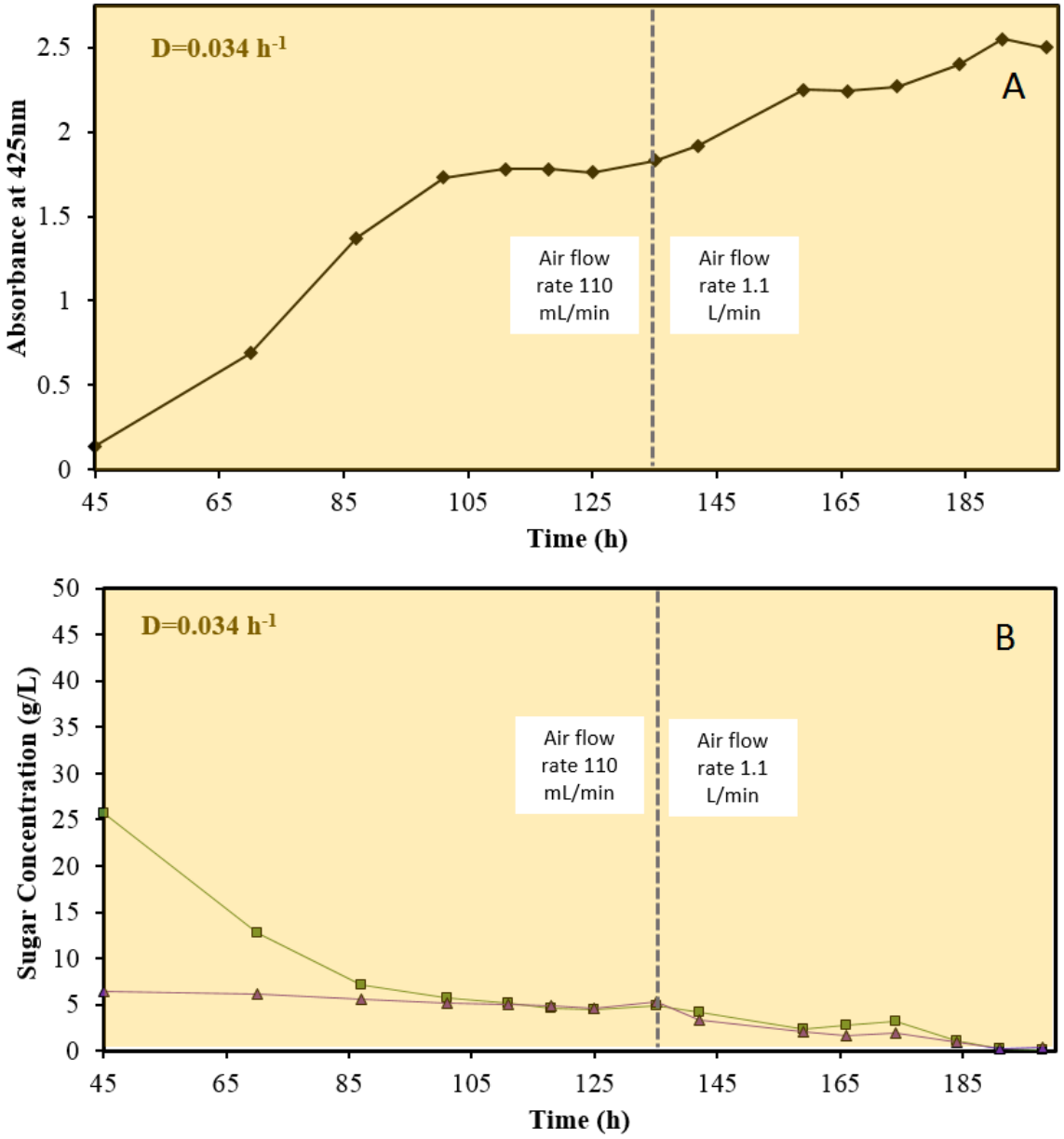

Figure 6.14. Effect of air flow rate on (A) absorbance of liquid broth measured at 425 nm and (B) maltose (■) and glucose (●) concentrations using packing material #1 (0-45 h data for batch operation and washing step not shown)

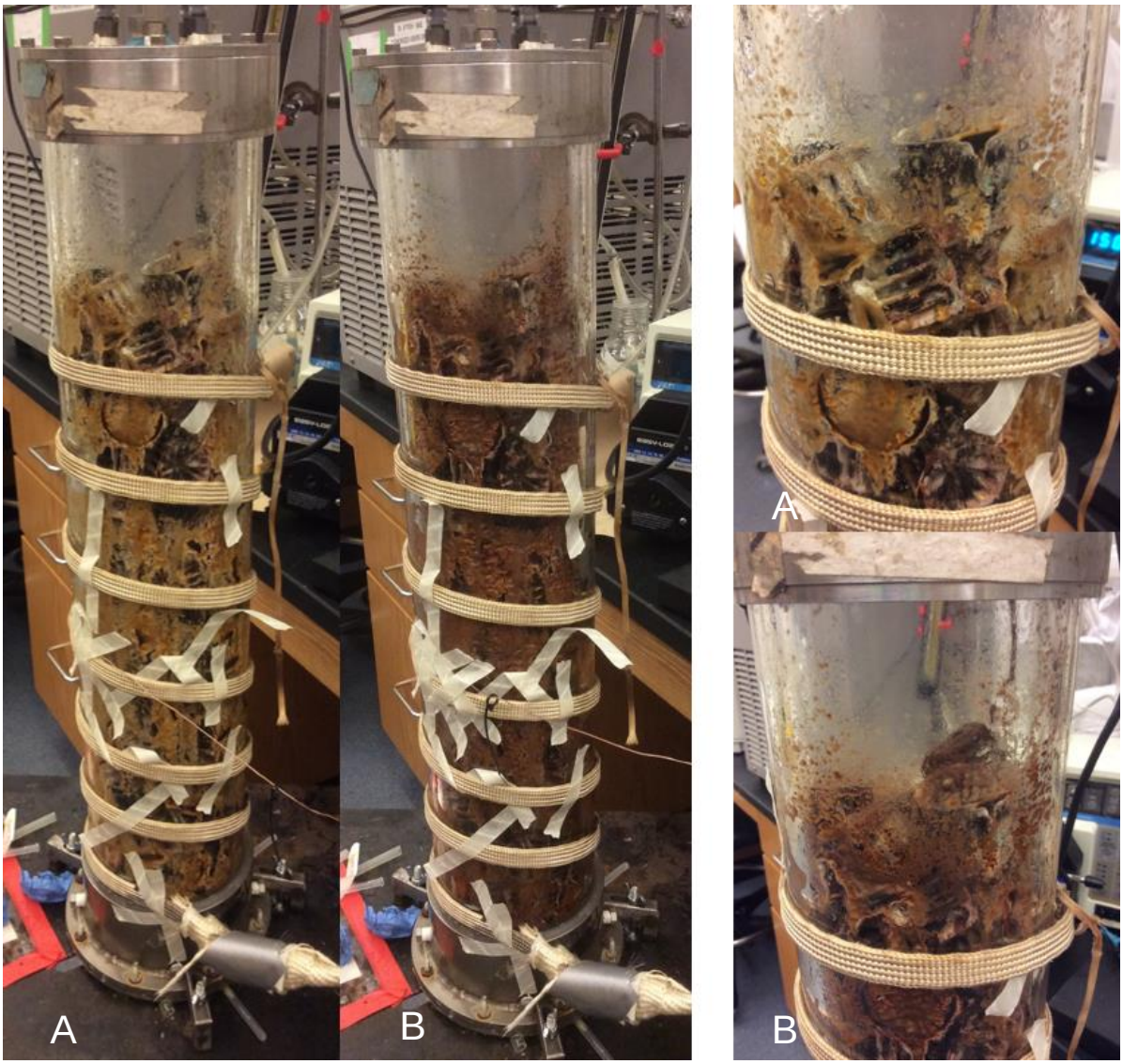

Figure 6.15. Pictures of the TBR (A) before (135 h) and (B) after (197 h) increasing the air flow rate

6.5 Conclusions

Continuous production of an aryl alcohol oxidase (MtGloA) enzyme from *M. thermophila* was demonstrated using an. *A. nidulans* cell factory with a pyridoxine marker. The marker was used to limit the growth of the microorganism to avoid clogging issues in the TBR system. Modified media with 5 g/L ascorbic acid reduced melanization of the culture, resulting in increased MtGloA productivities. Increasing the air flow rate supply from 110 mL/min to 1.1 L/min favored melanin production. Higher dilution rates also favored the pigmentation process and organic acid accumulation, reducing MtGloA productivities. The type of material used to fill the packed bed (HDPE or stainless steel) and the shape of the random rings had little or no effect on the fermentation results.

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CHAPTER VII

FUTURE WORK

The present chapter describes potential future steps to further advance the research field of enzyme production using growth-limited cell factories.

7. 1 Improvement of trickle bed reactor (TBR) dimensions

The reactor used in this study has a height (L) of 60 cm and a diameter (D) of 10.5 cm. This represents a height to diameter (L/D) ratio of approximately 5.7. Typical L/D ratios used in industrial scale TBRs range between 1.0 and 2.0 (Winterbottom & King, 1999). A narrow design was used in this project because the reactor had been constructed using the glass column from a bubble column reactor that was available in the laboratories. Much of the liquid pumped to the top of the column flows downward along the glass walls instead of trickling through the packing material promoting channeling and reducing the efficiency of the TBR. For future studies, a reactor that resembles industrial TBRs should be constructed.

Another feature that has an impact on liquid channeling is the column diameter to particle diameter (d_p) ratio (D/d_p). The D/d_p ratios used in the present study were between 2.9 and 5.3,

depending on the packing material selected. Some studies suggest ratios greater than 8 (Seader & Henley, 2006), while others recommend D/d_p of 12 (Saroha et al., 1998) and even higher than 25 (Maiti & Nigam, 2007) for reduced wall flow. The new design of the TBR used for future experiments should consider using proper L/D and D/d_p ratios.

7.2. Investigate different reactor configurations for the current cell factory

The *A. nidulans* cell factory used in this study was difficult to culture in a TBR due to the melanization phenomenon described in Chapters 5 and 6. The pigmentation process, which requires oxygen to proceed, was favored in the TBR compared to submerged fermentation, probably due to the direct contact between air and the mycelia in the TBR. Future studies could investigate the possibility of establishing continuous MtGloA production in a stirred tank reactor while controlling its growth with use of ascorbic acid to prevent melanin formation as done in Chapter 5. In this configuration, the strain grows as pellets, and shear stress on the mycelia aggregates would be a concern. However, the oxygen requirements for this engineered *A. nidulans* appear to be low, as the strain grew quickly in shaken flasks with only passive aeration, i.e. mass transfer provided only by agitation of the flasks (Chapters 4 and 5). Therefore, it is possible that a combination of air sparging and slow agitation of the liquid medium is enough to meet the oxygen demands of the fungus, since the mass transfer in stirred tank reactors is higher than in agitated flasks (Rhodes & Gaden, 1957). This would allow continuous submerged culture with reduced melanization compared to the TBR, which could lead to higher enzyme productivities. Furthermore, an investigation on the effect of dissolved oxygen on melanin production could help understanding the pigmentation process better. If the integrity of the pellets structure is compromised by agitation, a reactor design similar to a bubble column reactor (with the fungal pellets as fluidized bed) could be also tested. Another potential reactor configuration to be tested for this particular strain could be a packed bed reactor. First, the fungal pellets would be prepared in agitated cultures with medium with pyridoxine, and then aseptically transferred to a

column for their use as packing material and biocatalyst under pyridoxine limitation. All these alternative reactor configurations also allow good dissolved oxygen control.

7.3 Investigate the removal of pyridoxine from medium with ascorbic acid

The TBR experiments shown in Chapter 6 were performed using a very low constant supply of pyridoxine (1 µg/L*h) to limit growth without causing a halt on enzyme production. However, it is reasonable to hypothesize that pyridoxine can be removed completely from the medium containing ascorbic acid based on the results observed in Chapter 5. In an experiment using a reduced concentration of pyridoxine (10 µg/L), the control treatment with no ascorbic acid eventually stopped producing the MtGloA enzyme, indicating that the pyridoxine level was not enough to prevent melanin formation and to sustain enzyme production. However, the treatment with ascorbic acid was able to continue producing enzyme using the same pyridoxine concentration. This suggests that pyridoxine concentration could be reduced when ascorbic acid is used as inhibitor of the pigmentation process. Future studies could investigate the possibility of removing this vitamin from the medium completely. This could further decrease growth in a reactor system, theoretically allowing longer uninterrupted operation.

7. 4. Investigate the effect of ascorbic acid on other cell factories

The current MtGloA producing strain resulted in more melanin formation under growth-limited conditions compared to a similar strain used in a different study for production of xylanase (Müller et al., 2014). However, limiting growth of both cell factories resulted in pigmentation and reduced protein formation, which is undesired. In the present dissertation, inclusion of ascorbic acid in the medium reduced melanization and increased enzyme activities (Chapters 5 and 6). Higher enzyme activities were also obtained when the strain was not submitted to pyridoxine deprivation (Chapter 5), for reasons that remain unclear. Using ascorbic acid in the medium formulation of a

different cell factory could help elucidate the reasons behind this improvement.

7.5 Investigate the role of aryl alcohol oxidase in degradation of biomass

Aryl alcohol oxidases have been included in the so called ligninolytic consortium, a group of enzymes produced by certain microorganisms that are able to break down lignin in biomass material (Alcalde, 2015). The role of aryl alcohol oxidases in this process is the enzymatic generation of hydrogen peroxide that can be used by other lignin-degrading enzymes, such as peroxidases (Alcalde, 2015), or can participate in Fenton chemistry to further degrade lignin (Ander & Marzullo, 1997). Aryl alcohol oxidase was also found to prevent repolymerization of the phenolic compounds produced by the action of laccase on lignin, favoring the overall degradation process (Marzullo et al., 1995). Unfortunately, no studies investigating the function of aryl alcohol oxidase on degradation of real lignocellulosic materials have been found in the literature. Future research could study the mechanism of action of this enzyme and its synergistic effects with other enzymes in the ligninolytic consortium, including peroxidases and lacasses, on promising renewable feedstocks such as switchgrass.

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