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Sugar, Soil and Sequencing: Studies in *Fusarium venenatum*

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Abstract

Studies were undertaken to gain insights into the biology and genetics of the industrial microorganism *Fusarium venenatum* with the aim of improving biotechnological aspects of the Quorn™ mycoprotein industrial process. This was addressed first by exploring previously unexamined biodiversity in the species and second by trialling alternatives to the current high-cost glucose feedstock.

Since it's discovery in the 1960s only a single isolate, *F. venenatum* A3/5. had been implemented in the Quorn™ industrial process. Initial work involved establishing an isolate collection of *F. venenatum* with the aim of identifying isolates that might show improvements in biotechnologically relevant parameters compared to A3/5. Surveys of global culture collections and research contacts yielded 24 strains. Archival research at Quorn Foods revealed that several of these strains were not unique but instead A3/5-derived mutants. In parallel, environmental sampling was undertaken using *Fusarium* spp. selective media. Samples were collected from a variety of locations across the U.K. including the original site of isolation of A3/5. Work included development of an *F. venenatum*-specific PCR-based molecular diagnostic. Despite these efforts, no isolates of *F. venenatum* were retrieved, indicating that the species is relatively rare rather than a ubiquitous soil microorganism as originally thought. The genomes of 21 putative *F. venenatum* strains from the isolate collection were sequenced and assembled for use in comparative genomics. This provided insights into the potential for sexual reproduction in *F. venenatum.* Before this work, only A3/5 had been genome sequenced and shown to be of *MAT1-1* mating type. Using molecular and bioinformatic analysis, four *MAT1-2* strains were identified and the first structural description of the *F. venenatum MAT1-2* locus is provided. This work provides a foundation for future work aimed at using the sexual cycle for strain improvement of *F. venenatum* and also provides a valuable resource for future *F. venenatum* research, both industrial and academic, such as genome analysis of mycotoxin production.

The second aim of this thesis was to characterise *F. venenatum* growth on alternative carbon substrates, with the goal of identifying an alternative(s) to the current high-cost glucose syrup feedstock. A high-throughput optical density-based system was developed for rapid screening of growth on potential candidate substrates. *F. venenatum* A3/5 was shown to exhibit metabolic flexibility, with growth supported by a variety of sugars including D-xylose, D-galactose and lactose. It was also shown that low-cost substrates such as high-fructose syrups and lignocellulosic breakdown products were also effective carbon substrates for *F. venenatum*. Sucrose was identified as the most suitable replacement feedstock, supporting A3/5 growth rates of equal magnitude to glucose. Alternative strains of *F. venenatum* from the isolate collection were also screened for growth on glucose and sucrose, but no significant differences in growth were evident from A3/5. Further studies were made of the effect of sucrose on additional characteristics such as germination, substrate utilisation and mycotoxin biosynthesis using both small-scale and larger 5 L and 10 L batch or steady-state fermentations. Sucrose had previously been identified as a potent inducer of mycotoxin biosynthesis in the fusaria, with production of the Type A trichothecene, diacetoxyscirpenol (DAS) previously reported in *F. venenatum*. Therefore, investigations were made into the effect of the use of glucose and sucrose as feedstocks on mycotoxigenesis in A3/5 and the 24 alternative *F. venenatum* strains. A considerable strain-specific effect on DAS production was observed, with most strains presenting increased DAS biosynthesis at the stationary phase when supplied with sucrose as compared to glucose. However, a number of strains demonstrated lower levels of DAS biosynthesis compared to A3/5, suggesting that these might be potential industrial replacements. Of particular significance was the discovery of a putative non-DAS producing strain 114-23, which failed to synthesise DAS when fed glucose or sucrose. This strain when used in combination with sucrose may provide a worthy successor to the A3/5 and glucose paradigm currently established at Quorn™.

Acknowledgements

It's often said that it takes a village to raise a child, and I believe that is true of a PhD student too. I am fortunate to say that many hands built my village, supporting me throughout my PhD journey, especially during the COVID-19 pandemic.

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1

Introduction

1.1 Applications of Fungal Biotechnology

The origins of fungal biotechnology are rooted in the fermentation of food and beverages, used unknowingly by human society for millennia. Ancient descriptions of fermented beverages have been unearthed by archaeologists in China, Iran and Egypt, emerging alongside agriculture as a technology to ensure reliable food sources (McGovern et al., 2004; McGovern et al., 1997; Cavalieri et al. 2003). Scientific elucidation of the mechanisms governing fermentation were first performed in 1864, when cell-free extracts from *Saccharomyces cerevisiae* were able to convert sugar to ethanol (Buchholz and Poulsen, 2000). The work of Louis Pasteur established the role of microorganisms in fermentation of sugars to ethanol in 1876 (Barnett, 2003). Axenic cultures of *S. cerevisiae* and other biotechnologically relevant yeasts were established by breweries in the late 1800s, such as the Carlsberg brewery in Copenhagen (Steensels and Verstrepen, 2014).

The research and microbiological techniques developed using yeast species paved the way for subsequent work with filamentous fungi in the early 20th Century. This era of biotechnology saw filamentous fungi increasingly implemented to address problems facing modern human society. Over a century ago, James Currie discovered that fermentation of the mould *Aspergillus niger* efficiently synthesised citric acid, an important preservative which until then was extracted from lemons (Cairns et al. 2018). 1921, two years after Currie's discovery, saw the industrial production of citric acid by *A. niger*, jumpstarting the biotechnology revolution. Later that decade, Alexander Fleming serendipitously discovered penicillin following contamination of *Streptococcus* spp. growing on agar plates (Fleming, 1929). Upscaling

of penicillin production was demanded to combat wartime infection in the Second World War, driving innovations in the technologies required to generate microbial metabolites (Wells, 1991).

The route of evolution for filamentous fungi has uniquely equipped them with two critical features which make them ideal candidates for biotechnology. First, filamentous fungi demonstrate considerable capacity to hydrolyse organic matter and plant-based polymeric substrates using a varied arsenal of enzymes such as cellulases, pectinases, amylases etc. The number of predicted hydrolytic genes in these fungi ranges between 100 and 250, as opposed to the 30 found in *S. cerevisiae* (van den Brink and de Vries, 2011). This hydrolytic capacity is coupled with high secretion of enzymes, which has facilitated the development of efficient biorefineries such as *Trichoderma reesei* and *A. niger* (Ferreira et al., 2016). The combination of these factors has resulted in fermentations with filamentous fungi able to convert plant biomass to produce a wide variety of products such as pharmaceuticals, commodity and specialty chemicals and increasingly structural materials. This method of production is increasingly an attractive prospect, replacing traditional chemical synthesis pathways often based on petrochemicals and instead establishing a renewable, bio-based economy (Meyer at al. 2020).

1.1.1 Fungal Pharmaceuticals

Natural products from fungi, particularly fruiting bodies of so-called higher fungi (e.g. *Ganoderma lucidum*, *Cordyceps sinensis* and *Psilocybe caerulescens*) have been used in traditional medicine for centuries (Tang and Zhong, 2004; Das et al. 2021; Nichols, 2020). More recently, metabolites synthesised by mould species have provided modern medicine with revolutionary compounds for treatment of infection and disease (Figure 1.1).

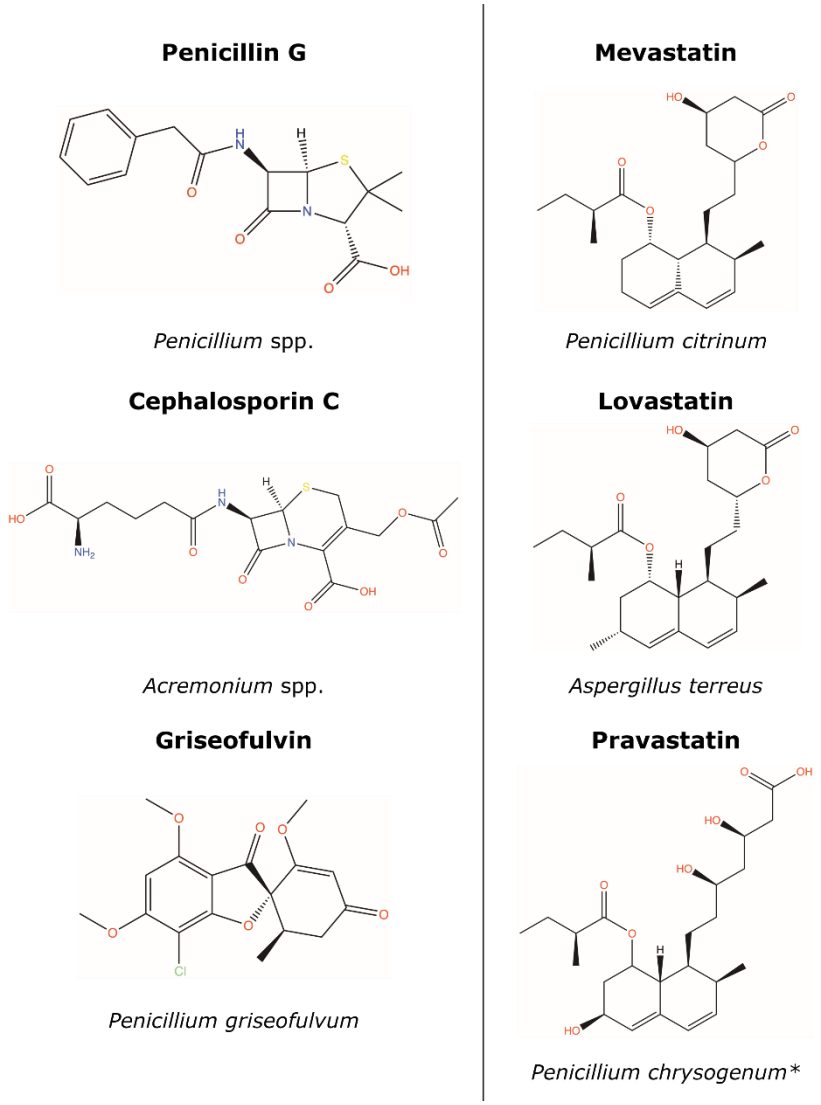


Figure 1.1. Examples of pharmaceuticals synthesised by mould species. Benzylpenicillin, cephalosporin C and griseofulvin are all examples of antimicrobials. Griseofulvin represents a rare example of an antifungal synthesised by a fungus. Mevastatin, lovastatin and pravastatin are representative of statins, compounds which inhibit HMG-CoA reductase enzymes. Pravastatin is synthesised by engineered *Penicillium chrysogenum* following introduction of the mevastatin biosynthesis pathway. Drawn using Chemdraw 20.1.

Arguably the best-known example of modern fungal biotechnology is the discovery of penicillin by Alexander Fleming more than 90 years ago (Fleming, 1929). This finding, along with the discovery of metabolites from soil-dwelling bacteria, jumpstarted the golden age of antibiotic discovery (da Cunha et al., 2019). In addition to penicillin, certain fungi synthesise important antimicrobial compounds such as the cephalosporins, griseofulvin and fusidic acid (Newton and Abraham, 1955; Grove et al., 1952; Godtfredsen et al. 1962). This period saw pharmaceutical companies endeavour to isolate and screen fungal strains from the environment for the discovery of new natural products with novel activities (Butler, 2004). These metabolites have also laid the groundwork for production of semi-synthetic derivatives as well as inspiring synthetic mimics (Aly et al. 2011; Leisner, 2020).

Statins were first discovered by Akira Endo in 1966, who was inspired by the elucidation of cholesterol biosynthesis by Konrad Bloch to discover a hypocholesterloemic natural product (Endo, 2010). This class of drugs inhibit the enzyme 3*β*-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, effectively reducing low-density lipid cholesterol levels and combating hypercholesterolemia (Pinal-Fernandez et al. 2018). Several of these drugs are produced by fermentation of filamentous fungi, for instance lovastatin and compactin are produced by *A. terreus* and *P. citrinum*, respectively (Manzoni and Rollini, 2002). Industrial fermentation of pravastatin is currently achieved in a two-step fermentation pathway. The first stage requires compactin synthesis from *P. citrinum*, which is then purified, and the lactone ring opened. The ring-opened compactin is then converted to pravastatin by the bacterium *Streptomyces carbophilus*. This process is plagued by low activity of the *S. carbophilus* cytochrome P450 enzyme, toxicity of compactin and low yield of pravastatin. To streamline this process, a β-lactam deficient strain of P. chrysogenum was engineered with the complete compactin biosynthesis pathway and a compactin hydroxylase. This facilitated a consolidated production process for pravastatin, synthesising more than 6 g/L at a pilot production scale (McLean et al., 2015).

1.1.2 Commodity & Specialty Chemicals

Filamentous fungi are increasingly implemented as biorefineries to convert waste substrates into commodity and speciality chemicals to replace current petrochemical-derived substances. *A. niger* launched human society into the modern era of biotechnology following the industrialisation of citric acid production, yet other members of this genus also synthesise organic acids with interesting prospects. For example, *A. terreus* synthesises itaconic acid (IA), although produced at substantially lower levels relative to citric acid, was identified by the US Department of Energy as one of the top 12 most promising platform chemicals for a bio-based economy (Werpy and Petersen, 2004). Fungal IA can be implemented as a substitute for petrochemically-produced products such as acrylic acid, paints and various biodegradable polymers through crosslinking (Teleky and Vodnar, 2019). Recent work at the University of Nottingham has shown that IA might be produced commercially using waste sorghum as a feedstock for fermentations with the production organism *Aspergillus terreus* (Ahmed El-Imam et al. 2022).

In some cases, low efficiency of these biorefineries in producing the desired metabolite limits the economic viability of these products. To that end, metabolic engineering strategies have been implemented to improve the final yield of the chemical. The thermophilic fungus *Myceliophthora thermophila* has been identified as a biorefinery for the conversion of lignocellulosic material to the C-4 diacids malic and succinic acid (Li et al., 2020). Gu et al. (2018) engineered the fungus to produce fumaric acid from glucose fermentation, an important feedstock for commodity biopolymers, synthetic resins and plasticisers. Rational metabolic engineering of several genes enabled the introduction of an export system and increased the precursor pool, ultimately generating a strain with an improved fumaric acid titre of 17 g/L, up from the original 7 g/L (Gu et al., 2018).

1.1.3 Industrial Enzymes

Many industrial processes implement catalysts to mitigate requirement for high temperatures, solvents or extreme reaction pH values. Enzymes can offer great advantages over chemical catalysts, namely significant specificity to substrates, are biodegradable and sustainably produced and often operate at mild temperatures and pH values. Hundreds of industrial enzymes have been implemented for diverse applications; proteases and cellulases in laundry detergents, amylases and pectinases in the textile industry, lipases in bread-making and so on (Meyer et al., 2020). The global enzymes market was worth $6 billion in 2017 and is expected to continue growing (Arnau et al., 2020).

Hydrolysis of starch to release glucose was traditionally performed using hydrochloric acid followed by a neutralisation step. This process had a number of drawbacks, namely significant salt formation and synthesis of several by-products, some of them potentially inhibitory to downstream processes. Implementation of commercial glucoamylases from *Aspergillus* spp. avoided by-product formation, generating a final product of greater purity and did not require the use of a strong acid (Cairns et al. 2018). Similarly, transesterification of oils is traditionally catalysed by one of two highly toxic and explosive compounds, sodium methylate (methoxide) or sodium ethylate (ethoxylate). The reaction hazards are compounded by the formation of dialkyl ketone by-products, generating an undesirable colour transition to a dark red/brown which then requires downstream processing to remove, reducing final yield (Santoro et al., 2018). Implementation of commercial enzymes negates the use of hazardous chemicals, improves product quality and yield, and avoids by-product formation thus reducing cost by mitigating downstream processing (Ferreira-Dias et al., 2013). Several commercial enzymes produced by heterologous fungal host platforms are now used for this application. For instance, the Lipozyme® series by Novozyme features *Candida antarctica* lipase B manufactured by *A. niger* or *Thermomyces languinosus*, or *Rhizomucor miehei* lipase synthesised in *Aspergillus oryzae* (Arnau et al., 2020).

Trichoderma reesei and *Talaromyces thermophilus* are both prodigious fabricators of cellulase and hemicellulase enzymes, which can be used in processing of lignocellulosic wastes for biofuel applications. Substantial strain improvement work has been undertaken with *T. reesei*, realising cellulase titres of 100 g/L or more (Cherry and Fidantsef, 2003). *T. thermophilus* is noted for the thermostability of its enzymes, as current processing methods utilise high temperatures to increase the limited solubility of lignocellulosic feedstocks and decrease viscosity of fermentation broth (Blumer-Schuette et al., 2014). Despite substantial cellulase activity, *T. reesei* lacks sufficient native β-glucosidase activity, and thus breakdown of lignocellulosic feedstocks often leads to an accumulation of cellobiose (Ellilä et al., 2017). Strain engineering has solved this issue through overexpression of native β-glucosidase or introduced heterologous enzyme activity (Zhang et al., 2010; Ma et al., 2011).

1.1.4 Fungi as Biomaterials

As a filamentous fungus colonises a solid substrate, the mycelial network expands, forming many hyphal filaments which extend across the substrate binding it together. The strength inherent to the hyphal cell wall, granted by chitin microfibrils, facilitates the formation of a natural composite material between the mycelium and substrate (Islam et al., 2018). Interrupting mycelial colonisation (e.g. by heat inactivation) before total substrate consumption can stabilise these matrices and thereby lead to low-cost and sustainable mycelium composites with interesting applications (Attias et al. 2020). For many bio-based composites, low-cost lignocellulosic wastes are used as substrates to enable waste cycling as part of establishing a circular bioeconomy (Jones et al. 2018).

Further research has shown that composite properties can be modulated by alterations to the substrate, fungal species, growth conditions and post-processing of the substrate:mycelium matrix (Figure 1.2**;** Appels et al. 2019). By controlling these manufacturing parameters, mycelium-

based materials can be implemented as sustainable alternatives to plastic films, foams (such as polystyrene) and semi-structural materials for use in flooring and furniture applications (Haneef et al., 2017; Pelletier et al., 2019; Jiang et al., 2017).

The textile and fashion industries have featured some of the first commercial products of fungal mycelium following increasing consumer demand for alternatives to animal-derived products. For example, the company MycoWorks has developed mycelial composites under the trade name Reishi™, a product marketed as demonstrating the suppleness and texture of traditional leather (Meyer et al. 2020). 47 patents and patent applications concerning fungal materials were filed between 2009 and 2018, including 9 applications from the automobile manufacturer Ford™ (reviewed in Cerimi et al. 2019). As more applications are envisioned, the opportunities for mycelium-derived materials to replace less sustainable, traditional alternatives will continue to grow.

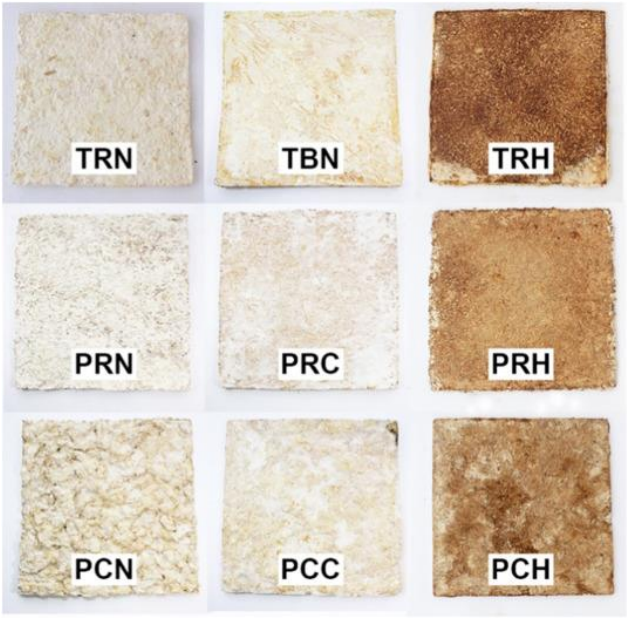


Figure 1.2. Diverse materials produced by varying treatment processes of mycelium of *Tramates mulitcolor* and *Pleurotus ostreatus*. Growth of *T. multicolor* on sawdust (TBN) or straw with or without heat pressing (TRH and TRN respectively). Growth of *P. ostreatus* on cotton with heat, cold or no pressing (PCH, PCC or PCN). PRH, PRC and PRN refer to *P. ostreatus* growth on straw with heat, cold or no pressing respectively. Figure taken from Appels et al. (2019).

1.2 Use of Fungi in the Food and Feed Industries

As the Earth's human population continues to grow, demands for many resources will similarly increase. Of primary concern is the availability of many essential, yet ultimately finite resources: clean water, crude oil, land for settlement and food. The final category is complex; sufficient provision of food is required to avoid malnutrition, yet there is a need for maintaining quality to avoid the detriments to health caused by an imbalanced diet. Essential to all life are elements such as carbon and nitrogen, the capture and incorporation of which is important for growth and reproduction of the organism in question. Of great importance to humans is the provision of high-quality protein, traditionally in the form of animal tissue.

Here, 'quality' refers to the amount and variety of the amino acids that constitute the protein source in question. Nine of the twenty standard amino acids are considered essential for the human diet (Table 1.1), as mammals generally lack the metabolic pathways required for their synthesis (Reeds 2000; Fürst and Stehle 2004). If adequate amino acid variety and/or quantity is not available, growth and development of the organism can be impaired, leading to disease and eventually death if sustained for too long. 'Complete' and 'incomplete' proteins describe sources that contain the necessary quantity and balance of amino acids or lack thereof respectively. Complete proteins are often those farmed from animals – meat, fish, eggs, milk etc. Incomplete proteins typically lack one or more essential amino acids and usually describe those found in plants, such as beans or grains. A third category, 'conditionally non-essential', are those amino acids which are non-essential unless the person in question is experiencing a period of acute stress or illness.

Table 1.1. Essential and non-essential amino acids in humans. * = Amino acids considered 'conditionally non-essential'.

Essential	Non-essential
Histidine	Alanine
Isoleucine	Arginine*
Leucine	Asparagine*
Lysine	Aspartic Acid
Methionine	Cysteine
Phenylalanine	Glutamic Acid
Threonine	Glutamine*
Tryptophan	Glycine*
Valine	Proline*
	Serine*
	Tyrosine*

Since the inception of agriculture ~11,000 years ago, human society has relied on animal husbandry to reliably incorporate protein into its diet, providing a sustainable supply for most of human existence. The explosive population growth witnessed in the 20th century forced the global community to consider whether these traditional methods would continue to be viable and justifiable. The challenge to supply adequate protein was met with the intensification of livestock cultivation and the development of techniques such as battery farming, antibiotic and hormone treatment etc. This has led to a host of environmental and animal welfare issues, signposted by disease outbreaks such as bovine spongiform encephalopathy (BSE), foot-and-mouth disease, antibiotic resistance, and several examples of zoonotic influenza (Wilesmith 1994; Gibbens et al. 2001; Reid et al. 1999; Lam et al. 2015). In addition, economic development has been strongly correlated with an increase in meat consumption (Fiala, 2008). The combination of continued population growth and global economic development has led to predictions of global meat consumption increasing by 75-80% by 2050 (Wellesley et al., 2015). Traditional methods of protein cultivation are unsustainable, and thus alternatives must be considered and developed before the concerns of the WHO in the 20th century become reality.

It is therefore promising that societal attitudes are increasingly trending towards reducing meat consumption. In Europe, meat-substitute sales saw an increase of 451% between 2014 and 2018 (Maaß, 2018). In the UK, the Food and You Survey observed a trend in consumers switching to reduced meat consumption, with a 246% increase in respondents identifying themselves as 'partly vegetarian' (Figure 1.3). Furthermore, the same survey found that since 2012, there has been an overall decline in meat consumption, particularly beef, lamb and pork. In 2012, 75% of adults surveyed had consumed a cut of one of these meats at least once a week, but this figure had dropped to 55% by 2018 (Benson et al., 2019). This market trend is likely to continue, as attitudes continue to turn away from traditional protein sources as a result of increasing animal welfare and environmental concerns.

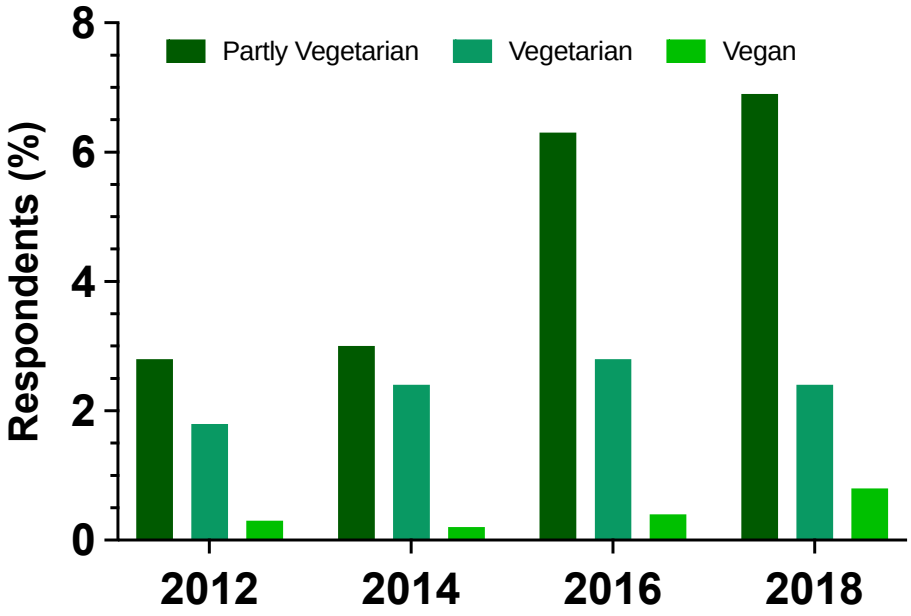


Figure 1.3. The trend towards reducing meat consumption is increasing in the U.K. Respondents identifying as partly vegetarian has tripled since the 2012 survey, while vegan attitudes also continue to grow in popularity. Data retrieved from the biennial Food and You Survey performed by the Food Standards Agency.

1.2.1 Historical Use of Fungi

Use of fungi in food has been a feature of human society for millennia. Many cultures across the globe have recorded the ingestion of mushroom fruiting bodies, often as medicine, for thousands of years (Stamets and Zwickey 2014). The influence of the domestication of *Saccharomyces cerevisiae* by human society is well-documented, providing us with foodstuffs such as bread, wine, beer and so on (Lahue et al., 2020). Similarly, mould species such as *Aspergillus oryzae* and *A. sojae* have been equally important in the development of fermented foods such as koji and soy sauce (Bamforth and Cook, 2019). The last century has added to these traditions via the direct manipulation of microbial cells for use as feed and food.

1.2.2 Single-Cell Protein

“Cellular agriculture” is a relatively modern term describing methods of producing agricultural goods without involvement of animals or crops (Chriki and Hocquette 2020; Nyssölä et al. 2022). It is often seen as a remedy to the increasing need for food provision while addressing limited agrarian space for its production. Most recent developments have implemented cell culture of animal or plant cells, involving sophisticated technologies such as 3D-printing (Davies and Deroles, 2014; Vanderburgh et al., 2017).

Here too, biotechnology already provided an answer in the 20th century– the cultivation of microorganisms to produce edible biomass, a subject area which has recently experienced a renaissance. This is referred to as ‘single-cell protein’ (SCP), despite numerous organisms used in its production of a multicellular nature (e.g. filamentous fungi and algae). In past decades, such products developed from microorganisms were referred to as ‘microbial’, ‘bacterial’ or even ‘petro-’ protein. The latter moniker refers to an era where the cost of hydrocarbon-based substrates was low enough to make their use in SCP fermentations

commercially feasible (Cooper et al. 1975). The term ‘single-cell protein’ was coined by the Massachusetts Institute of Technology to avoid negative connotations of “bacterial” or “petro-” protein which might lead to a lack of appeal with the general consumer (Trinci 1992).

SCP is usually manufactured to be utilised in one of two ways; either direct consumption as processed biomass, or as a supplement to increase the protein content of other food products. When direct-consumption products are considered, SCP has historically been produced and sold as feed for livestock rather than for human consumption. Important examples of feed products include Pruteen™ and protein made in the Pekilo process (Marstrand, 1981; Koivurinta et al. 1979). Pruteen™ was an important early product in SCP history, a feed product developed for pigs by Imperial Chemical Industries Ltd. using the bacterium *Methylophilus methylotrophus* grown on methanol and ammonium (Waterworth and Heath, 1981). Another early pioneer of SCP products was the Pekilo process, developed to make use of the sulphite wood pulping wastes generated in the Swedish pulp and paper industry (Ugalde and Castrillo, 2002). This process utilised the fungus *Paecilomyces variotii* to generate biomass for sale as supplements to pig and poultry feed formulations before the final plant closed in 1992 (Ritala et al. 2017).

When conceptualising the design of these commercial processes, several basic requirements need to be considered if the end-product is destined for human consumption. First, if the product is going to be a replacement for conventional protein sources, it follows that it must be high in protein and provide several essential amino acids. To achieve the high levels of protein demanded by the human diet, SCP is preferably produced from organisms which readily produce at least 30% of their biomass as protein. Indeed, the initial screening process at Quorn™ demanded a microorganism that produced at least 45% of its biomass as protein (Moore et al. 2011). Consequently, production of SCP is restricted to a relatively small number of species. The average protein

content of beef steak, chicken breast and pork chop are 31.0, 31.6 and 32.0 g per 100g of weight respectively (Anon, 2021). The microbial organisms used as production organisms in SCP (algae, fungi and bacteria) can contain 30-83% protein of their total weight, suggesting that in this regard, SCP can match if not surpass the protein quantity available in traditional sources (Anupama and Ravindra, 2000).

Next, the process of SCP manufacture must be economically competitive enough that the product can survive not only against traditional protein sources but also the increasingly crowded meat-analogues market. A meat-substitute is a product designed to replicate the aesthetic, chemical and physical properties of meat products yet originate from alternative biomass sources (Joshi and Kumar, 2016). Meat-substitutes now include plant-based products, often made with pea or soy proteins, cultured meat and increasing numbers of SCP-derived products.

Current large-scale manufacturing methods that produce SCP for human consumption use food-grade substrates (Ritala et al. 2017). In an effort to keep costs low and increase profit margins, novel processes which use alternative, inexpensive materials have been researched. An obvious and plentiful source of carbon is industrial waste generated from production of food, cellulosic ethanol, and paper. A key historical example, as already mentioned above, was the Pekilo® process developed in Finland to manufacture animal feed using sugars present in sulphite liquor paper mill effluents, paving the way for the use of waste substrates in SCP generation. In this system, the filamentous fungus *Paecilomyces variotii* KCL-24 was incubated in continuous fermentation, transforming sulphite liquor waste (32 g/L sugars) into biomass with a protein yield of 55% (w/w) (Ugalde and Castrillo 2002). Pekilo® SCP was initially developed and sold as animal feed but was also investigated as a protein additive in commercial meat products (Koivurinta et al. 1979). The first plant opened in 1974, with a second built in 1985 before both were closed in 1991 after the pulp industry was no longer producing

suitable sidestreams. This process has recently undergone a revival following the emergence of eniferBio, a biotech company aiming to re-establish Pekilo® as a modern biorefinery system for use in aquaculture.

Indeed, much of the research into SCP production has been focused on the use of waste substrates. Examples of such substrates include, lignin and lignocellulosic waste (in fermentations with *Chrysonilia sitophila*, Rodrìguez et al. 1997; *Candida utilis* and *Rhizopus oligosporus*, Yunus et al. 2015), cheese whey (*Candida krusei* and *Kluyveromyces marxianus*, Yadav et al. 2014), agricultural waste (*Candida utilis*, Jalasutram et al. 2013), industrial wastewaters (purple bacteria/microalgae, Hülsen et al. 2018) as well as common food industry wastes such as apple pomace (*Aspergillus niger*, Bhalla and Joshi 1994), banana wastes (*A. niger*, Baldensperger et al. 1985) and remarkably, prawn shells (marine yeast, Rhishipal and Philip 1998). These examples show the flexibility of using microorganisms to produce proteinaceous biomass from a diverse array of substrates and organisms. However, each of these substrates presents two problems: affordability and scalability. It is likely that many of these substrates will require hydrolysis to extract fermentable sugars, either by the industrial organism or as a pre-processing step before fermentation.

SCP products have witnessed a recent resurgence in popularity, the landscape of which is thoroughly reviewed in Ritala et al. (2017). As mentioned previously, the landmark Pekilo® process has been reinvigorated by eniferBio, exploiting the original strain used 30 years ago. Mycorena AB is a Swedish-based biotechnology which produces mycoprotein using food industry waste. Their flagship product, Promyc™ is marketed as a protein supplement for other food industry applications, rather than direct consumption. Mycorena has recently been awarded $26.8 million in a fundraising round, aiming to move Promyc™ from prototype to commercial product (Marston, 2022). ENOUGH (previously 3FBio Ltd.) is an example of a UK-based SCP company which produces meat-analogue Abunda® for direct consumption. In 2021 ENOUGH

raised €42 million in capital investment alongside establishing partnerships with Unilever and retailer Marks and Spencer to bring their products to market. Existing before this renaissance of interest in SCP, Quorn™ mycoprotein has been the market leading SCP product intended for human consumption for the past 35 years, despite a long period of development, as will now be described.

1.3 A History of Quorn™ Mycoprotein

1.3.1 A Historical Perspective

Quorn™ mycoprotein had a long gestation period before its first appearance on supermarket shelves in the UK in 1985 – a range of savoury pies appearing in Sainsbury's (Trinci, 1992). Explosive population growth in the 20th century led to predictions of global protein famine by the 1980s if human society continued to rely on traditional protein sources (Paddock and Paddock 1967). The United Nations produced a report (Anon 1968) which, among other solutions, considered how protein produced by microorganisms might prevent such a crisis. Much of the subsequent research was aimed at manufacturing bacterial-derived protein supplements for animal feed as a way of improving yields from traditional livestock farming. Development of Pruteen™ by Imperial Chemical Industries (ICI) was a key step in the commercialisation of SCP. This product was a pig feed manufactured produced using the bacterium *Methylophilus methylotrophus* fermented on methanol (Marstrand 1981).

In contrast to Pruteen™, Lord Arthur Rank of Rank-Hovis-McDougall (RHM) decided that it was of greater benefit — both economic and societal — to manufacture protein suitable for human consumption. RHM was a major producer of cereals and derived products and therefore produced great quantities of starch that could be exploited (Finnigan, 2012). Consequently, Lord Rank aimed to discover and

exploit a microorganism that would efficiently convert starch into edible biomass, rich in protein and suitable for human consumption.

With these goals in mind, filamentous fungi presented some valuable options. As discussed previously, fungi have been well-established as foodstuffs, either intentionally or unintentionally, throughout human history. Secondly, it was important to Lord Rank that a meat-substitute for human consumption should mimic the organoleptic properties of animal muscle and would therefore have greater appeal to consumers. To that end, it was hypothesised that the mycelial filaments generated during fungal growth would make a suitable analogue to animal muscle fibres. Finally, the filamentous nature of fungal growth would make downstream filtration much simpler compared to single-celled bacteria.

The initial search for a suitable microorganism began in a field adjacent to an RHM-owned wheat starch plant in Ashford, Kent (Angold et al. 1989). The field was regularly sprayed with starch to drive selection of organisms that preferentially metabolised starch as a carbon source. This method proved successful, yielding a *Penicillium notatum* strain designated ‘C1’, an isolate which exhibited a protein profile similar to that of casein. For 3 years, research and development focused on improving the fermentation process for C1. Unfortunately, *P. notatum* demonstrated unsuitable growth in continuous culture, rapidly colonising the internal surfaces of fermentation vessels, making extraction difficult (Finnigan, 2012). Once again, *P. notatum* won the runner-up prize in the annals of biotechnology following its replacement decades previous for use in penicillin production.

Following these initial trials with *P. notatum*, the search was broadened. Between 1967 and 1972, over 3,000 fungal isolates were collected by RHM from soil samples across the globe. These isolates were subsequently screened for several parameters: protein content and quality (i.e. nitrogen and α-amino nitrogen levels), growth in continuous culture, pigment production, lack of mycotoxin production, pathogenicity among others (Anderson et al. 1975; Anderson and Solomons 1984;

Moore et al. 2011). Following these preliminary screens, 20 fungal isolates were chosen for feeding trials with small animals, used as preliminary toxicology screens (Moore et al. 2011). This shortlist of fungal isolates comprised a number of *Aspergillus* species and *Neurospora sitophila*, together with eight *Fusarium* species including *F. culmorum*, *F. solani* and *F. graminearum* (Anderson et al. 1975; Anderson and Solomons 1984).

1.3.2 The Genus Fusarium

The genus *Fusarium* is a ubiquitous filamentous fungus taxon, demonstrating worldwide distribution with greater than 300 distinct species discovered to-date, comprising 20 species complexes (Figure 1.6) (O'Donnell et al., 2015). Members of the genus are often saprotrophic or associated with other fungi, plants and animals, many potentially as pathogens. *Fusarium* species have been isolated across the globe, for instance in peat soil (Malaysia; Karim et al., 2016), in various agricultural soils often associated with plant matter (e.g. India, tomato - Joshi et al., 2013; France, wheat – Karlsson et al., 2016; Ethiopia and Ghana, soybean – Hartman et al., 2019) and also from river and sea water (e.g. Spain, Palmero et al., 2009).

As discussed previously, the fusaria are most commonly known as plant pathogens, rated as one of principal plant pathogenic fungi globally (Dean et al. 2012). Fusarium head blight (FHB), foot, crown and root rot and fusarium wilt are all common examples of plant disease in which *Fusarium* species are the causative agents (Morgavi and Riley, 2007). Plant pathogenic species can be further delineated by describing races capable of infecting specific host plant cultivars. For instance, *Fusarium oxysporum* f. sp. c*ubense* has a number of races which are able to infect certain banana cultivars. Race 1 of this species was responsible for eradicating plantations of the 'Gros Michel' variety in the mid-20th Century (Kema et al., 2021). In response, this susceptible cultivar was replaced with the resistant 'Cavendish' variety, now grown in monocultures worldwide. However, the emergent tropical race 4 (TR4)

is now decimating Cavendish plantations, including recent outbreaks in Queensland, Australia (Pegg et al., 2019). While renowned as plant pathogens, non-pathogenic *Fusarium* isolates are often isolated from the roots of plant crops, but fail to cause disease symptoms (Sajeena et al., 2020). In some cases, both pathogenic and non-pathogenic isolates can cohabit the same host plant, as witnessed in cocoa *Thoebroma cocoa* (Castillo et al., 2016) In addition, several *Fusarium* species have also been recorded as opportunistic pathogens of humans, causing infections in immunocompromised patients (Namboothri et al., 2014).

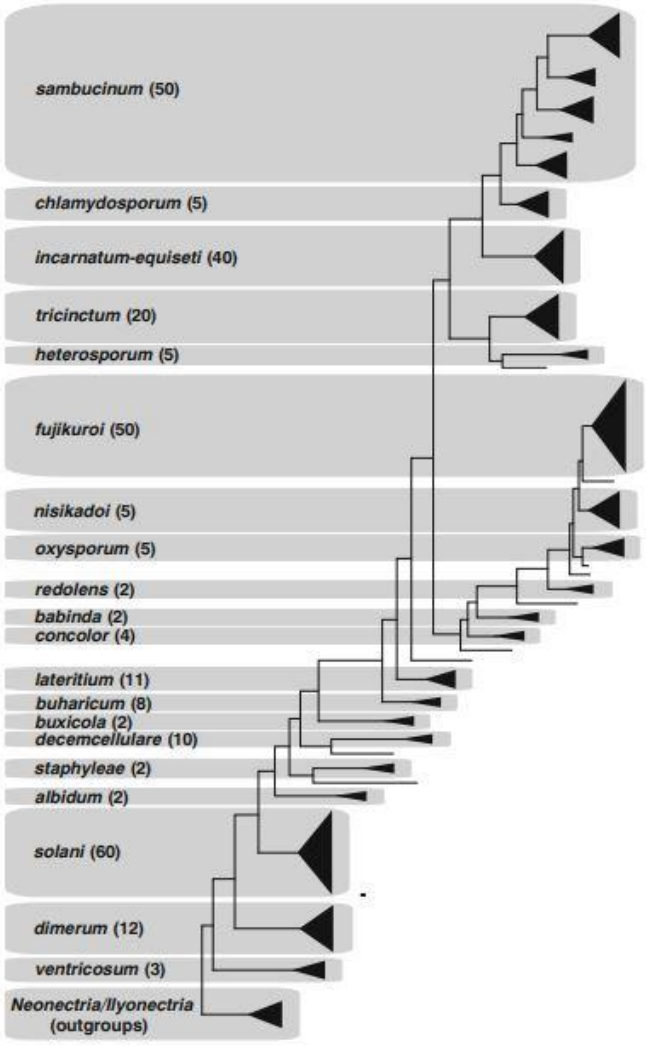


Figure 1.4. Phylogeny of the *Fusarium* genus as determined by a combined *RPB1* and *RPB2* dataset. Each species complex is grouped in a grey shaded box with the approximate number of species contained in brackets. Of these 4 complexes comprise more than two-thirds of the species; *solani*, *sambucinum*, *fujikuroi* and *incarnatum-equiseti*. RPB = DNA-directed RNA polymerase II largest (*RPB1*) and second largest (*RPB2*) subunits. Figure taken from O'Donnell et al. (2015).

1.3.3 The Discovery of F. venenatum

Ultimately, one strain of *F. graminearum* was taken forward based on the best combination of favourable protein production, suitability for bioreactor growth, low odour and low toxicity (Anderson and Solomons 1984). Ironically, despite the global sampling, the ancestor to the current production strain—then termed *F. graminearum* A3/5—was collected and isolated from a compost heap sample in Marlow Bottom just 4 miles from the Lord Rank Research Centre in Marlow, Buckinghamshire, UK, on 1 April 1968.

The fungus was thereafter deposited as *F. graminearum* at the International Mycological Institute (IMI), UK, in 1969 (A3/5/3; IMI 145425). Derivatives of A3/5/3 from lyophilised stocks have since been used for commercial Quorn™ mycoprotein production. A further strain was deposited by RHM in 1971 in the American Type Culture Collection (ATCC 20334), although this strain was later shown to exhibit markedly different mycotoxin levels to the original A3/5/3 strain (Miller and MacKenzie 2000). The precise lineage relationship of ATCC 20334 to A3/5/3 is unclear. In 1998, the Quorn™ production strain was re-identified as *F. venenatum* based on phylogenetic, morphological and mycotoxin analyses (O'Donnell et al. 1998). Importantly, *F. venenatum* was unable to synthesise the Type A trichothecene deoxynivalenol (DON), a characteristic of the phytopathogenic *F. graminearum*. This finding was confirmed by parallel RAPD analysis of ATCC 20334 compared to closely-related *Fusarium* species (Yoder and Christianson, 1998). More recently, comparative genome analysis together with pathogenicity trials confirmed that *F. venenatum* possesses significantly reduced plant pathogenicity compared to *F. graminearum*, incapable of colonising wheat plants and only producing a superficial infection of tomato after an extended incubation time (King et al. 2018).

Following the isolation and subsequent suitability trialling of *F. venenatum* (then referred to as *F. graminearum*), a decade-long study was performed between 1970 and 1980 to evaluate the safety of *F.*

venenatum mycelia for human consumption. Mycotoxins were a key concern, given the reputation of the *Fusarium* genus as mycotoxin producers in plant infection. Substantial toxicology screening was performed including feeding trails on several animal species such as pigs and cows. The results suggested that *F. venenatum* mycelium produced no adverse effects on either the animals or their offspring. The commercial product generated from the mycelium of *F. venenatum* was named 'mycoprotein' by the Food Standards Agency in the UK (Moore et al., 2011), referring to the high-protein content derived from fungi. Interestingly, this is not the original use of 'mycoprotein', but a reintroduction of a term first recorded in the nineteenth century which referred to protein extracted from microbial biomass (Nencki and Schaffer 1880, reported by Anon 1880). Trials with 2,500 human volunteers followed, concluding that there were no immunological manifestations or other symptoms following mycoprotein consumption (Moore et al. 2011). These results were presented in a report submitted to the now-defunct Ministry of Agriculture, Fisheries and Food (MAFF, UK) in 1983, leading to the approval for mycoprotein sales in 1985. Products were marketed under the brand name 'Quorn™' which at the time was a name (derived from a village in Leicestershire, UK) owned by RH for a range of packet sauce mixes but which tested well in consumer research for the new mycoprotein-based products. In 1994, further toxicity tests were performed and submitted to the United States Food and Drug Administration (FDA), the approval of which was required before sale of Quorn™ products was granted in the United States (Miller and Dywer, 2001).

Whilst the project began as a solution to an impending protein crisis, by the 1980s, it became apparent that no such crisis was on the immediate horizon. Instead, Quorn™ mycoprotein began to supply a market that was looking for healthy new proteins with a low environmental impact. Quorn Foods now supply over 100 different products to over 17 countries worldwide including much of Europe as well as the USA and Australia and more recently into new Asian markets.

1.3.4 Development of the Quorn™ Production Process

Once a satisfactory industrial organism had been identified, RHM required large-scale fermentation expertise to develop a cost-effective production system. A 3-year collaboration began in 1973 with DuPont (Delaware, USA) providing the necessary groundwork and knowledge to initiate the project. Several fermentation systems were tested in initial trials to produce mycoprotein; batch, fed-batch and continuous processes. The production efficiencies conferred by continuous fermentation allowed biomass to be generated over a long period of time, with fewer fermentation initiation events required.

The desire for filamentous morphology during the industrial process is a characteristic which distinguishes Quorn™ mycoprotein from other submerged fungal processes, and informed the development of the *F. venenatum* fermentation process. In the case of citric and itaconic acid production by *Aspergillus niger* and *A. terreus* respectively, “pellet” formation is crucial to increased productivities (Papagianni and Mattey, 2006). Rather than growing as long filaments with low branching, pellets are formed as short, swollen filaments with enlarged hyphal tips (Snell and Schweiger, 1949). Importantly, pelleted growth has distinctly different characteristics to filamentous growth, namely cell physiology, growth kinetics, broth rheology among others. A crucial concern for pelleted growth is the heterogeneous environment experienced across the cells within the pellet. Cells positioned on the surface of the pellet show active growth and metabolism due to adequate exposure to dissolved oxygen and nutrients contained in the medium (Figure 1.5, Espinosa-Ortis, 2016). Towards the centre of the pellet, cells grow in an environment starved of these important components (Papagianni, 2004). Despite this difficult environment, pelleted growth demonstrates significantly increased product biosynthesis (Gómez et al. 1988).

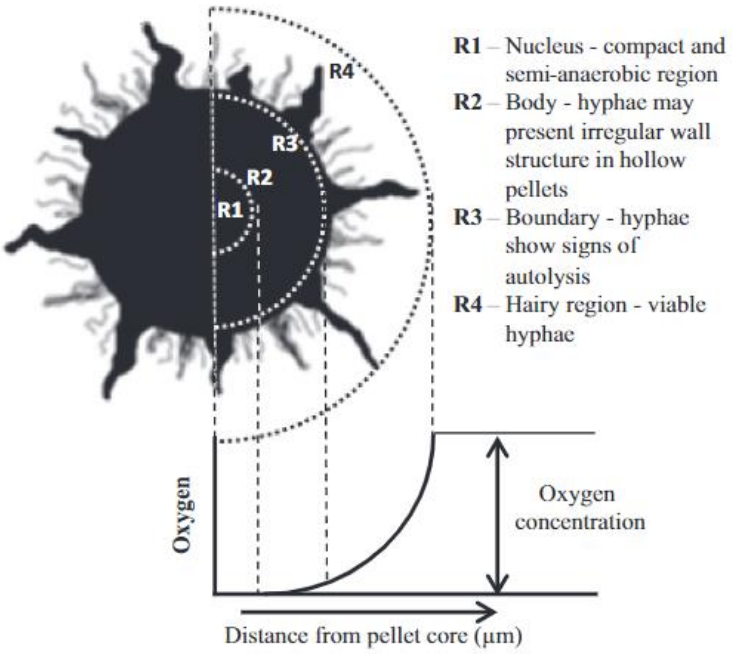
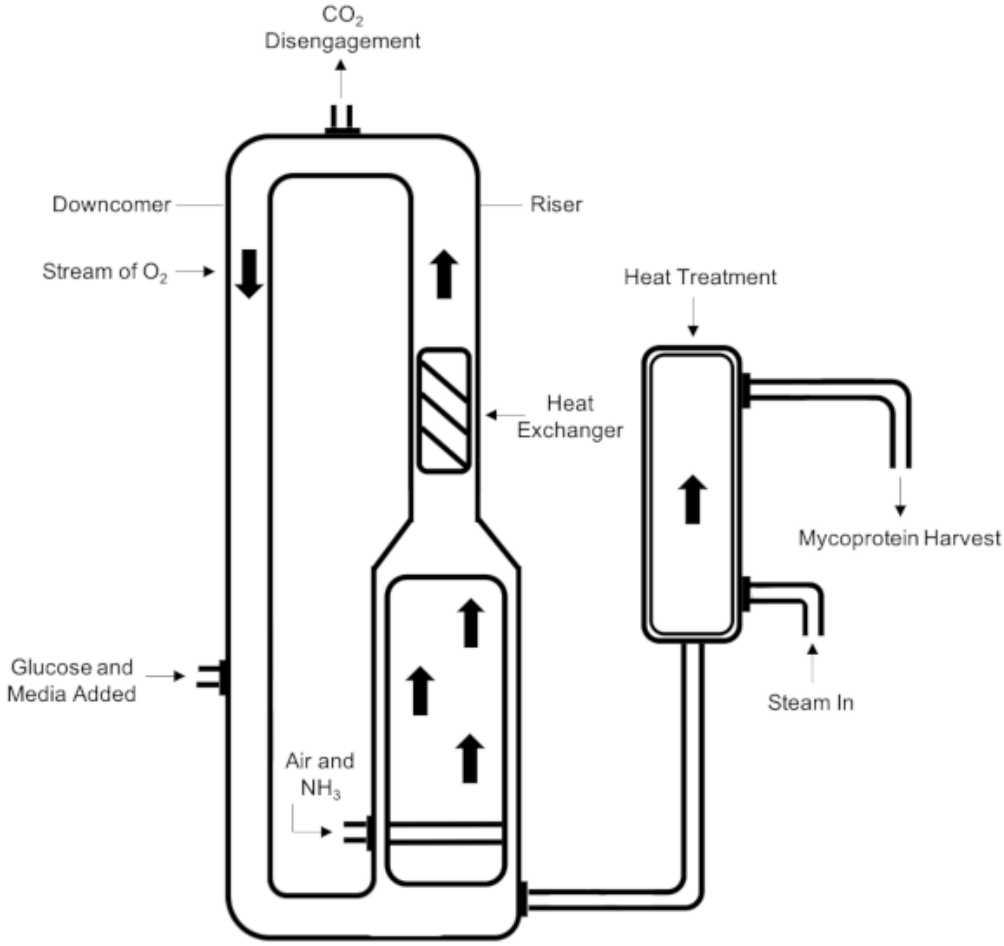


Figure 1.5 Cross-section of fungal pellet and subregions with oxygen gradient. Taken from Espinosa-Ortiz et al. (2016).

By contrast, Quorn™ mycoprotein aims to achieve maximum growth to allow for maximal hyphal length and limited branching. This requires mass transfer of oxygen and nutrient availability to be well-distributed in the culture and not experienced by few cells only. Initial upscaling projects utilised stirred tank reactors in continuous fermentation. However, it was a collaboration with ICI in 1984 that led to the development of the current airlift fermentation technology, following test production in a pressure loop fermenter originally used for the Pruteen™ pilot plant (Finnigan 2012; Seviour et al. 2013). Only this 40 m^3 pilot-scale fermenter (BQ1) was used to for production until 1994, producing 1,000 tonnes of mycoprotein per year. Following increased commercial demand, 1994 saw a new 155 m^3 airlift fermenter commissioned (BQ2) swiftly followed by another of the same design in 1996 (BQ3; Figure 1.4). These three fermenters had a combined output of 10-14,000 tonnes of mycoprotein per annum, with sales reaching £74 million/annum in 1997. Production was further boosted by construction of an additional BQ4 in 2015, bringing total capacity on site to 480,000 L. The production capacity continues to expand, with the construction of BQ5 and planning

of BQ6 by the end of 2024.

Figure 1.6. **Schematic of modern Quorn™ air-lift fermenters for production of mycoprotein.** F. venenatum culture is grown at a constant dilution rate, relying on partial pressure differentials to circulate the riser and downcomer. Growth of culture is monitored by online CO_2 generation. Mycelium is harvested using an outlet, feeding the mycelium directly into a separate heat treatment tank before harvest by centrifugation. Figure taken from Whittaker et al. (2020).



A key limiting factor in the fermentation of aerobic microorganisms is the poor solubility of oxygen in water (1.22×10^{-3} mol dm^{-3} at 25 °C, 1 atm.). This has implications for respiration, the stoichiometry of glucose consumption is as follows:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

At 25 °C, 8.10 mg/L of oxygen is dissolved in culture medium. For the consumption of 5 g of glucose, 5.35 g of oxygen is required and thus

less than 0.2% of the oxygen required is available for respiration. For efficient utilisation of substrate to enable maximum growth rate, fermentation strategies have to incorporate a method of increasing mass transfer of oxygen into the culture liquid.

Examples include sparge pipes which inject air directly in to the medium, baffled tanks to increase agitation and bubble generation as well as direct agitation by equipment such as impellers. Stirred-tank reactors (STRs) use mechanically operated impellers to increase mass transfer of oxygen into culture medium, but this increases the shear rate experienced by the culture (Sánchez Pérez et al., 2006). The mycoprotein product necessitates hyphal filaments to be long and lack branching to replicate animal muscle fibres most closely. By avoiding stirring, the Quorn™ fermentation has low rates of shearing and thus preventing breakage of the hyphal filaments.

Quorn™ fermenters are the largest, continuous-flow culture systems developed to-date, using airlift technology as opposed to more traditional STRs. Airlift technologies achieve mass transfer of oxygen into media by injecting sterile air bubbles directly into the culture medium at the base of the riser. Injection of air at the base introduces substantial hydrostatic pressure, which combined with the turbulence generated by the pumped air, provides excellent conditions for oxygen transfer from the gaseous to the liquid phase. This system generates a microenvironment within the riser where air bubbles constitute up to 50% of the volume. As distance from the riser increases, dissolved air constitutes approximately 10% of the volume resulting in a lower rate of oxygen transfer and lower relative pressure. Reduced pressure at the top of the riser drives remaining nitrogen, oxygen and CO_2 generated from the culture to be ejected from the culture through the gas disengage. The culture then enters the remaining part of the loop, the oxygen-depleted downcomer. To avoid culture death due to anoxic conditions in this region, low levels of oxygen are injected. Once the culture passes through the downcomer, it is then directed back into the

riser to be charged with air once again, completing the cycle. The relative difference in specific gravity between the oxygen-rich riser and the oxygen-depleted downcomer ensures that the hyphal filaments within the culture circulate continuously around the fermenter loop, completing one loop every 2 min for fermenters BQ2 and BQ3. It has been calculated that mycelium travels at an approximate speed of 4 m per second, making *F. venenatum* one of the 'fastest fungi' in the world (Whittaker et al. 2020).

The fermenter is operated as a glucostat—an environment where glucose is always in excess—with *F. venenatum* growing continuously at a specific growth rate nearing its maximum specific growth rate, to drive maximum biomass production. Once the fungus has reached a steady state approximately 4 days' post-inoculation, hyphal biomass is continually harvested and undergoes further processing. Culture medium is fed into the vessel at a dilution rate between 0.14 to 0.20 h^{-1}, whilst mycoprotein is harvested at the same rate, resulting in a combined output of 30 tonnes h^{-1} for BQ2 and BQ3 (Moore et al. 2011). The culture is incubated at 28 °C by a heat exchanger that is incorporated into the riser. Ammonia is used as the nitrogen supply for growth, which is fed into the fermenter along with the sterile compressed air at the base of the riser. The supply of ammonia into the vessel is controlled by a pH monitor set to maintain the culture at pH 6.0.

The raw mycelium harvested from the fermenter is heated to 68 °C in a separate tank, for approximately 15 min, to reduce nucleic acid levels. At this stage, the mycelium displays the desirable characteristics inherent to mycoprotein, with a minimum hyphal length of approximately 400–700 µm (Whittaker et al. 2020). Final products made with an increasing proportion of shorter hyphal filaments will possess reduced structural integrity, with increased crumbliness (Finnigan 2012; Finnigan et al. 2017). While the fibrous nature of mycoprotein does successfully emulate fibres found in meat, the hyphae lack the cross-linking structures prominent in muscle (Thrane 2007). To mimic this, egg

albumin is often used as a binding agent for final product formulation. However, increasing market trends towards vegan products has driven the search for other alternatives with agents such as agar, potato protein and locust bean (*Ceratonia siliqua*) gum potentially used in vegan Quorn™ products. The final step in producing the meat-like texture occurs by freeze texturisation of the fibre-binding complex, creating hyphal laminations replicating the organoleptic properties of cooked animal muscle. For further discussion of factors which contribute to final texture realisation such as hyphal morphology, interactions between hyphae, orientation and dispersion of hyphae and fibre alignment, readers are directed to the review by Finnigan et al. (2017).

Several studies have investigated the benefits of mycoprotein consumption, suggesting it can lower blood cholesterol, stimulate satiety (and thus reduce overall energy intakes) and help to control blood sugar levels. Denny et al. (2008) provides comprehensive details of the macro- and micronutrients present in mycoprotein. Energy density of mycoprotein is low (86 kcal/100 g), while protein-rich (11.5 g/100 g). Furthermore, it shows a favourable fatty acid profile low in saturates (0.6 g/100 g), is high in fibre (6 g/100 g) and low in sodium (4.0 mg/ 100 g). A key failing of mycoprotein nutrition is the quantity of iron and vitamin B12 – nutrients which are readily present in the meat which mycoprotein aims to replace. The iron level is low at 0.5 mg/100 g and will be less bioavailable than meat alternatives due to non-haem iron. *F. venenatum* mycoprotein does not contain vitamin B12, a vitamin present in beef and pork at approximately 2 µg/100 g (Denny et al., 2008). A comparison of metabolic markers of health in respondents consuming meat- or mycoprotein-based diets showed no significant difference, and the unique prospect of a glucan/chitin fibre-rich protein source continues to interest nutrition researchers (Coelho et al. 2018). Other benefits of mycoprotein consumption include a positive impact on appetite and insulinaemia, although no causal mechanism is established to-date, and remains a focus of ongoing research (Bottin et al. 2016).

Finally, Quorn™ mycoprotein has successfully provided a meat-analogue with reduced environmental impact. Lifecycle analysis of the production of mycoprotein has shown significant benefits when compared with that of animal proteins. Such analyses have shown that greenhouse gas emissions from mycoprotein production equate to 1.6 kg/kg, compared to beef farming which show values approximately 10-fold greater (Finnigan et al. 2017; Poore and Neemak 2018).

1.3.5 Biotechnology Issues of the Modern Industrial Process

Throughout the development history of Quorn™ mycoprotein, substantial progress has been made in the improvement and optimisation of the fermentation process. Nevertheless, there remain several areas which hinder production, and improvement of these requires a greater focus on and understanding of the biology of the industrial organism, *F. venenatum*.

1.3.5.1 Expensive Carbon Feedstock

For a given commercial process, the input materials are a necessary expenditure and thus attempts will be made to minimise the cost of such materials if possible. As previously discussed, demand for Quorn™ mycoprotein continues to increase and thus it follows that a greater supply of media components will be required to meet demand. Of particular importance to a microbial fermentation is the carbon substrate, the choice of which can have considerable impacts on both the growth rate and metabolite profile (Jiao et al., 2008). Current production of Quorn™ mycoprotein uses a highly purified glucose syrup derived by enzymatic hydrolysis of wheat or maize starch using an *A. niger* glucoamylase enzyme. Utilisation of this syrup has two significant logistical problems: price and supply. Concerning the latter, glucose syrup is delivered to the production site in Billingham (County Durham, UK) in heated liquid tankers, with several deliveries required each day to maintain production. Limited substrate supplies are maintained on-site, so should a supply be delayed for any significant amount of time, production will be stalled. Supplying glucose syrup in a heated liquid container also has inherent risks and subsequent safety-related costs, thus driving substrate costs higher.

As reviewed in Ritala et al. (2017), carbon substrates for SCP processes can originate from varied sources. For the Quorn™ fermentation process, glucose is exploited as a readily metabolised soluble sugar.

However, alternatives monosaccharides or disaccharides might be utilised. Furthermore, these soluble sugars can be derived from waste substrates, relinquished from polymeric sources through enzymatic, chemical and/or physical pre-processing strategies. As discussed previously, biotechnological processes are increasingly utilising waste substrates to reduce substrate costs but the heterogenous nature of these substrates can make control of fermentation product difficult. Furthermore, when polymeric carbon sources such as starch are implemented directly, the rate-limiting step is no longer the rate of substrate uptake but instead the activity of the necessary hydrolytic enzymes. As a consequence, utilising substrates of increased polymerisation is likely to result in a reduction of growth rate, as was indeed shown by Anderson et al. (1975). Increasing the level of substrate glucose polymerisation fed to *F. venenatum* cultures showed decreasing growth rates in maltose and maltotriose relative to glucose.

Thus, there are several options available for investigation to replace the current Quorn™ glucose substrate, potentially reducing overhead costs while maintaining fermentation output and quality.

1.3.5.2 Amino Acid Content

A fundamental selling point of SCP products, including Quorn™ mycoprotein, is that they contain a high amount of non-animal-derived protein. In general, fungi can contain up to 63% protein, but this has been shown to vary depending on the carbon substrate used and the selected organism. Rice bran was used by Valentino et al. (2016) to generate SCP from 9 fungal species: *Aspergillus ochraceus*, *A. niger*, *A. flavus*, *Penicillium citrinum*, *Monascus ruber*, *Fusarium semeticum* and two unclassified *Fusarium* species. The crude protein content of these solid-state fermentations was improved best by the aspergilli and *F. semeticum*, with approximately a 10% increase in protein content.

F. venenatum mycelium is protein-rich, with levels up to 44% (dry weight) when grown in glucose-fed liquid cultures as shown by Wiebe et al.

(2002). Quorn™ mycoprotein has been demonstrated to be a high-quality protein, with a protein digestibility corrected amino acid (PDCAAS) value in excess of many animal proteins (0.99 vs. 0.92 in beef; Edmund and Cummins, 2010). Recent research has built on this foundation, suggesting that ingestion of mycoprotein showed an equivalent response to milk protein for muscle-building in human respondents (Dunlop et al., 2017). Furthermore, *F. venenatum* mycoprotein shows greater abundance of each of the 9 essential amino acids than cow's milk, yet this is still lower than that in beef (Table 1.2).

Table 1.2. Essential amino acid content of Quorn™ mycoprotein, cow milk and beef. Numbers represent grams of each amino acid per 100 g of protein source. Data taken from "Amino Acid Lowdown", Anon (www.quornnutrition.com/essential-amino-acids).

Essential AAs	Quorn™ mycoprotein	Cow milk	Beef
Histidine	0.39	0.09	0.66
Isoleucine	0.57	0.20	0.87
Leucine	0.95	0.32	1.53
Lysine	0.91	0.26	1.60
Methionine	0.23	0.08	0.50
Phenylalanine	0.54	0.16	0.76
Threonine	0.61	0.15	0.84
Tryptophan	0.18	0.05	0.22
Valine	0.60	0.22	0.76

Nutritional changes have been shown to modulate the quantity and quality of amino acids. For instance, there is evidence to suggest that the use of a different sugar source can alter the amino acid content in *Fusarium* species. Anderson and Solomons (1984) compared total amino acid content in *F. venenatum* (*F. graminearum* in text) when grown on glucose and ribose. Ribose-fed cultures showed a reduction in total amino acid content in comparison to those grown on glucose (394 mg/g and 494 mg/g, respectively). While it is unlikely that ribose would be incorporated as a replacement for glucose, the example here highlights the importance of examining biological elements which might

change due to alternative carbon source usage.

Indeed, differences in *S. cerevisiae* protein expression was observed when cultures were fed glucose, galactose, maltose or trehalose (Garcia-Albornoz et al. 2020). In total, 892 proteins were determined to show significant differential abundance in pairwise comparisons with glucose. Of these, a total of 85 (69 up- and 16 down-regulated) proteins were common across all three carbon substrates. Those proteins showing the most significant increases in upregulation were associated with carbohydrate metabolic processes while those downregulated were involved in amino acid biosynthesis. This is another parameter that is necessary to observe, as a decline in amino acid content would be undesirable should this be an effect of the carbon source used.

1.3.5.3 Evolution of Morphological Mutants

Following early collaborations with DuPont, it was established that continuous fermentation offered substantial economic advantages for mycoprotein production from *F. venenatum*. Theoretically, the fermentations would proceed uninterrupted, with fungal mycelia harvested continuously throughout. In practice, the process must be terminated after approximately 1,000 hours (~160 generations) after the initiation of continuous conditions. Production is halted due to the occurrence of highly-branched mutants (often termed C-variants; Figure 1.7) which exhibit a competitive growth advantage over the wild type and rapidly overtake the wild-type culture (Trinci, 1992; Finnigan et al. 2017). Evolution of morphological variants is not unusual in chemostat cultures of filamentous fungi, with instances of variants arising in

continuous cultures of *Aspergillus nidulans* and *A. niger* (Swift et al. 2000), as well as *A. oryzae* (Withers et al. 1994).

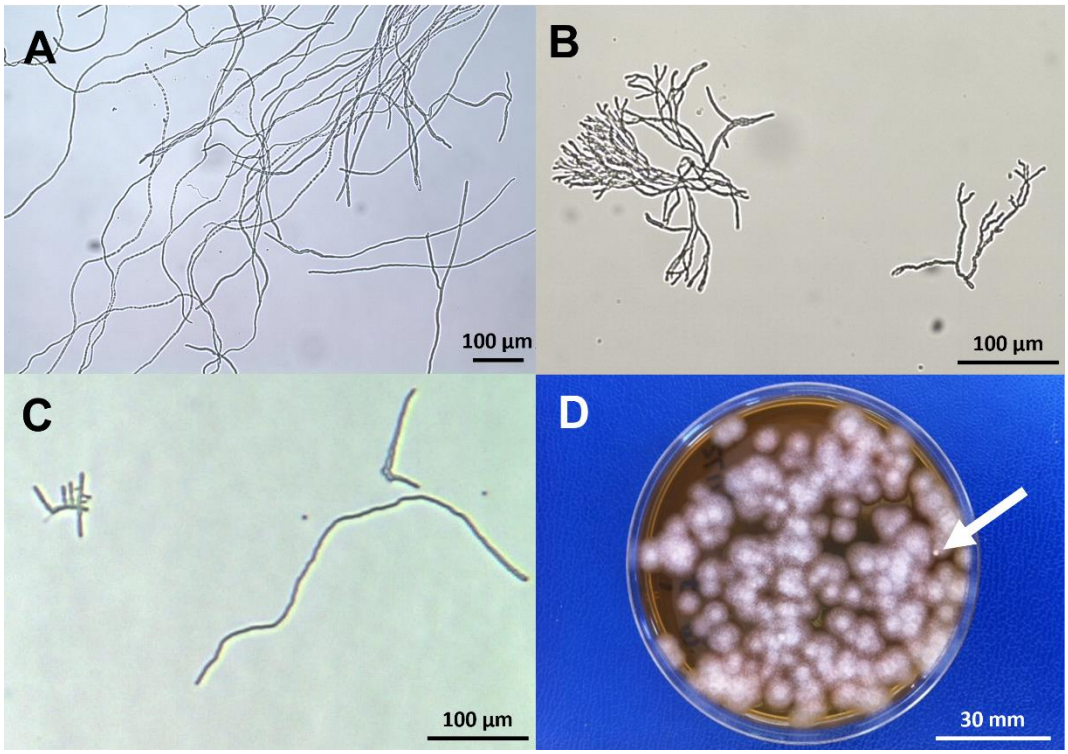


Figure 1.7. Morphological variants of *F. venenatum* as seen under the microscope (A-C) or on agar (D). A = Desired morphology of *F. venenatum* (A-variant). B = Morphological mutant (C-variant) with decreased hyphal and internodal length, increased branching frequency. C = direct comparison between C-variant (left) and A-variant (right). D = 90 mm plate following incubation at 25 °C for 48 hours, showing wild type colonies of *F. venenatum* in addition to the morphological mutant indicated by the white arrow. Taken from Whittaker et al. (2020).

Similarly, morphological mutants have been observed in the model organism *Neurospora crassa* (Garnjobst and Tatum, 1967). To understand the relationship between genotype and phenotype, spores from the morphological mutants and the wild-type were mixed in various ratios to create heterokaryons (Barratt and Garnjobst, 1949). The result demonstrated that the mutations controlling the morphological change were recessive, and that the ratio nuclear types was key to heterokaryon growth rate on solid agar.

Similarly, these genetic techniques revealed that the same was true in *F. venenatum*. Wiebe et al. (1992) created heterokaryons between 20 highly-branched morphological variants of *F. venenatum* in addition to

the wild-type strain (A3/5). In each case, heterokaryons formed between wild-type and mutant nuclei exhibited wild-type morphology.

Intriguingly, Simpson et al. (1998) showed that the C-variant is not the characteristic that drives mutation but is rather a consequence of a metabolic difference which confers a general growth rate advantage in continuous culture. which then produces a pleiotropic effect on hyphal morphology. Mixed cultures of A3/5 and one of sixteen morphological mutants were grown in glucostats limited by ammonium, magnesium or sulphate. Only one mutant showed a growth advantage over A3/5 in all culture conditions, whereas each of the other strains showed a disadvantage in at least one culture condition.

While these morphological mutants are similar in both chemical composition and nutritional quality as the wild-type culture, the problem posed by c-variant occurrence is an effect in the final product composition (Trinci 1994). C-variant morphology fails to produce fibrous muscle-like lattices instead creating a crumbly texture (Whittaker et al. 2020). Consequently, the quality of the final product cannot be maintained if C-variant is allowed to propagate within the fermenter. The current production solution requires the interruption of the fermentation process around 1,000 hours, maximising mycoprotein yield before C-variant accumulation has an adverse impact on the quality of the mycoprotein. The production process is then resumed several days later after cleaning and sterilisation of the relevant fermenter, leading to a loss of productivity (TJA Finnigan and RI Johnson, pers. comm.). Prevention or delay of morphological mutant evolution would enhance productivity of the fermentation process, allowing production to run for longer and thereby increasing yield.

Trinci (1994) proposed three strategies to reduce the occurrence of C-variants to extend the period of productivity. First, reducing the dilution rate would reduce selection pressure for certain morphological mutants which arise in these conditions. There is a significant trade-off, in that benefits of longer production times are offset by decreased biomass

production within the fermenter, rendering the adjustment non-viable from an economic viewpoint. The second strategy involves a rotational programme of selection pressures within the fermenter, producing an environment where no single mutant would be always favoured. This solution also poses a problem; not only does it complicate the production procedure but may also lead to a change in the final product. Finally, it may be possible to isolate or generate sparsely-branched strains of *F. venenatum* which are more stable than A3/5 with regards to mycelial morphology. Diploid strains of A3/5 used in laboratory-scale fermentations did not produce colonial mutants until 1,957 and 2,028 hours after the onset of continuous flow, extending fermentations by approximately 2-fold (Naylor et al. 1999; Moore et al. 2011). In two diploid strains, morphological mutants did not emerge for an 5- to 6-fold more generations compared to haploid A3/5 (Wiebe 2002). The use of such strains could potentially greatly improve the efficiency of the mycoprotein production process, assuming no other unwanted effects.

1.3.5.4 Mycotoxin Biosynthesis

The *Fusarium* genus is perhaps best-known for comprising many plant-pathogenic species which are often implicated in destructive epidemics worldwide. *F. oxysporum* f. sp *cubense* is the causal agent of Panama disease of bananas, destroying plantations in Latin America and increasingly worldwide (Xue et al., 2015). In the USA, *Fusarium* head blight (FHB) caused economic losses of $2.49 billion due to spoilage of wheat between 1993 and 2001 (Nganye et al., 2004). FHB caused further losses during an epidemic in 2003, devastating the premilling crop in the south-eastern states of the USA ($13.6 million; Cowger and Sutton, 2005). FHB is driven by two main agents *F. graminearum* and *F. culmorum*, causing both direct loss of crop but also in postharvest contamination by mycotoxins.

Mycotoxins are secondary metabolites produced by fungi which often contaminate food consumed by humans and animals. Under certain conditions, such as the detection of competing microorganisms or the

presence of a plant host, a fungus may begin mycotoxin biosynthesis to initiate biochemical warfare. The term mycotoxin represents a great number of compounds (300 – 400 are recognised) which demonstrate great variety in their chemical structure and activity. Initially, it was hypothesized that mycotoxin synthesis helps fungi to compete with other organisms for nutrient sources like fruits or seeds (Janzen, 1977). Mycotoxins are now also known to act as chemical signals between representatives of different kingdoms, e.g., as inhibitors of quorum sensing (QS), virulence factors in pathogens, or as a defence mechanism such as protecting sclerotia from insect predation (Ciegler, 1983; Wicklow et al. 1994; Desjardins and Hohn, 1997; Rasmussen et al. 2005; Rohlfs et al. 2010).

Mycotoxin contamination of food crops is a major threat to global food security and one which is predicted to worsen due to effects of climate change on temperature and water availability (Magan et al. 2011; Verheecke-Vaessen et al. 2019). Recent predictions suggest 25% of food crops worldwide are affected by mycotoxin contamination, posing significant risk to global food supply (Eskola et. al, 2019). Given their importance for food security, great efforts have been undertaken to identify strategies which will limit mycotoxin biosynthesis both pre- and post- harvest (Mylona et al. 2019).

As described, one of the main contaminating species is *Fusarium graminearum*. *F. graminearum* primarily synthesises deoxynivalenol (DON), a compound belonging to the trichothecene family of mycotoxins. Several fungal genera synthesise trichothecenes; sesquiterpenoid epoxides of low molecular weight (200 – 500 Da) that demonstrate potent inhibition of protein synthesis in eukaryotes, binding to ribosomes with significant affinity (Ji et al. 2019).

This large family of mycotoxins can be subcategorised in to four major groups, namely the A, B, C and D types which together form a collection of over 200 mycotoxins (McCormick et al. 2011). These groups are distinguished by the substitution pattern of the characteristic tricyclic

12,13-epoxytrichothec-9-ene (EPT) nucleus at the R positions. Types A, B and C can be differentiated by the nature of substitution at the C-8 position (R_5). Type A trichothecenes such as the T-2 and HT-2 toxins as well as diacetoxyscirpneol (DAS) lack a carbonyl group at this C-8 position. In the major type A trichothecenes (T-2 and HT-2) this position is filled by an isovalerate group, while there is no functional group at this position in DAS. Conversely, Type B trichothecenes like deoxynivalenol (DON; a virulence factor of the potent phytopathogen *Fusarium graminearum*) and nivalenol (NIV), are distinguished by a carbonyl group at the C-8 position. Type C trichothecenes contain an additional C-7, C-8 epoxidation such as that in crotocin. The Type D trichothecenes (e.g. satratoxin and roridin), contain a macrocyclic ring between C-4 and C-5 on the EPT nucleus, a feature which terms them as the macrocyclic trichothecenes.

Types A and B pose the greatest threat to global food security due to their greater toxicity than Type C and D trichothecenes. In addition, types A and B exhibit increased prevalence as contaminants in food and feed. The type A and B mycotoxins DON, NIV, T-2 and HT-2 are widely-reported as the most significant contaminants of food and feed globally (Foroud & Eudes, 2009).

Members of the genus *Fusarium* are well known for their ability to synthesise a wide variety of Type A and Type B trichothecenes (Ji et al. 2019). Species such *F. sporotrichioides*, *F. crookwellense*, *F. culmorum* and *F. langsethiae* among others demonstrate significant trichothecene production. Some mycotoxigenic *Fusarium* species are unable to synthesise trichothecenes, as is the case with *F. verticillioides*, instead producing the mutagenic compound fusarin C in addition to fumonisins (Savard and Miller, 1992). Genome analysis of *F. venenatum* revealed that the species contains a complete suite of trichothecene biosynthetic genes (King et al. 2018). In addition, a number of strains of *F. venenatum*, including the Quorn™ industrial organism, have been shown to synthesise the type A trichothecene diacetoxyscirpenol (DAS) under certain laboratory growth conditions (Laraba et al., 2021). Strains

of *F. venenatum* have been shown to produce the mycotoxins scirpentriol, 15-acetoxyscirpenol and 4-monoacetoxyscirpenol in addition to DAS under *in vitro* conditions with nitrogen limitation and deregulation of carbon metabolism (Altomare et al. 1995; O'Donnell et al. 1998; Miller and MacKenzie, 2000). However, to date Quorn™ mycoprotein has failed to demonstrate mycotoxin contamination, with the final product undergoing routine quality control processes. Indeed, before permission for sale was granted, Quorn™ mycoprotein was subjected to a decade-long period of safety trials. Permission was granted only after sufficient evidence that the production system was both non-toxic and non-allergenic following feeding trials in a number of animal species and subsequently humans. The lack of mycotoxin production in industrial fermentation is likely due to the growth conditions, with both nutrient supply and growth in the exponential phase thought to minimise expression of the mycotoxin biosynthetic genes. Nevertheless, any future development or optimisation of the industrial process must ensure limited impact on mycotoxin biosynthesis to avoid loss of final product due to contamination by mycotoxin(s).

1.4 Thesis Aims

Despite the economic importance of *F. venenatum* used for the production of Quorn™ mycoprotein, there has been surprisingly little research into the biology and genetics of the species, with just one strain (A3/5) exploited since the inception of Quorn™ production. The overall aim of this thesis was therefore to gain a greater understanding of the biology and genetics of *F. venenatum*, with the aim of gaining knowledge that would provide both applied and fundamental insights into the Quorn™ industrial paradigm. To that end, the specific aims of this study were as follows.

1. To examine biodiversity in *F. venenatum,* with the goal to identify novel isolates of *F. venenatum* which might show beneficial characteristics for mycoprotein production better than the existing A3/5 strain. This work involved the development of a molecular

diagnostic for retrieval of environmental strains.

2. To screen the industrial strain and novel isolates for growth on alternative carbon substrates, focusing on alternatives which are lower-cost or have lesser environmental impact than the use of the current glucose syrup.
3. To explore the effect of carbon substrate and/or fungal strain on trichothecene biosynthesis, with the goal to identify low or minimal mycotoxin producing strains.
4. To use comparative genomics to identify key differences between global *F. venenatum* strains, specifically at the trichothecene biosynthetic cluster(s) and mating-type (*MAT*) loci. Characterisation of the latter *MAT* loci would offer the exciting potential for sexual reproduction in *F. venenatum* as a means of strain improvement.

2

General Methods

All chemicals listed were supplied by Sigma-Aldrich (Poole, Dorset, UK) or Fischer Scientific, unless stated otherwise. All chemicals were of analytical or molecular biology grade.

Unless stated otherwise, sterilisation was achieved by autoclaving at 121 °C for 20 minutes.

2.1 General Strain Maintenance

The isolates used in this project were routinely cultured on 9 cm plates of potato dextrose agar (see, PDA; Oxoid) at 24 °C.

Long-term stocks of *F. venenatum* were prepared from spore suspensions stored in 10% glycerol both at -80 °C and under liquid nitrogen.

2.1.1 Preparation of Spore Suspensions

Spore suspensions for experiments were prepared by harvesting macroconidia from potato dextrose agar plates (see below) by adding 0.1% Tween 80 to the surface of the agar before brushing with a sterile spreader. This solution was then diluted using 0.1% tween 80 before filtration by sterile miracloth filter gauze (Merck/Calbiochem, Germany) attached to a funnel to remove any hyphal fragments or other large structures such as sporodochia.

Macroconidial concentration of each suspension was determined by haemocytometer microscope slide (Improved Neubauer; Weber Scientific, USA). The number of macroconidia in each of the 4 large corner squares (composed of 16 smaller squares) were counted and the

average calculated. The value was then multiplied by 10^4 to give the number of macroconidia/mL of suspension.

2.1.2 Fusarium venenatum *Isolates*

Several isolates of *Fusarium venenatum* were used throughout the study, listed in Table 2.1. The origin of these strains is detailed in Chapter 3, but of note are strains 114-14 and 114-25 were obtained as gifts from John Clarkson (Warwick University) and Kevin King (Rothamsted Research) respectively.

Table 2.1. Strains of *F. venenatum* obtained throughout this project.

Strain	Obtained as	Source of Isolation
114-1	N/A	Compost, Marlow
114-2	NRRL 22196	*Zea mays*, Germany
114-3	NRRL 22198	Unknown, Finland
114-4	NRRL 25413	Quorn Deposit
114-5	NRRL 25415	Quorn Deposit
114-6	NRRL 25416	Quorn Deposit
114-7	NRRL 25417	Quorn Deposit
114-8	NRRL 26139	Illinois, USA
114-9	NRRL 26228	*Triticum sativum*, Austria
114-10	NRRL 28571	*Cucurbita maxima*, New Zealand
114-11	NRRL 28572	*Spinacia oleracea*, Sweden
114-12	NRRL 36230	*Solanum tuberosum*, Finland
114-13	NRRL 66329	*Triticum sativum*, Austria
114-14	John Clarkson, Warwick University	Unknown
114-15	ITEM 831	Unknown
114-16	ITEM 834	Unknown
114-17	DSMZ 21779	Unknown
114-18	DMSZ 23358	Unknown
114-19	FRC R-2273	*Zea mays*, Australia
114-20	FRC R-8978	Soil, Turkey
114-21	NRRL 31989	Soil, Australia
114-22	NRRL 32015	Soil, Australia
114-23	NRRL 36234	*Triticum sativum*, Austria
114-24	NRRL 36260	Unknown
114-25	Kevin King, Rothamsted Research	Wheat or barley roots, Rothamsted, UK (1997)

2.2 General Culture Media

2.2.1 Potato Dextrose Medium

Potato dextrose medium is a rich culture medium used routinely for filamentous fungal culture. It was prepared as both broth for liquid culture (potato dextrose broth, PDB) or as agar (potato dextrose agar, PDA). In each case, 24 g of potato dextrose powder (Oxoid) is dissolved in distilled water per litre of medium. For agar, 15 g of bacteriological agar is added to the broth per litre of medium.

2.2.2 Rank-Hovis-McDougal (RHM) Medium

Rank-Hovis-McDougal media is used for the inoculum flasks at the Quorn™ Billingham site and was therefore used as the growth medium in this project's sugar growth assay.

1 litre of media contains; 20 g potassium dihydrogen phosphate (KH_2PO_4), 4.4 g ammonium chloride (NH_4Cl), 0.3 g potassium sulphate (K_2SO_4), 0.25 g magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), 5 mL of RHM trace elements, 1 mL of biotin solution (0.03 g/100 mL) and made up to 1 L with deionised water. When the media has dissolved, the pH is adjusted to 6.0 with NaOH.

RHM Trace Element Solution

1 L of this trace element solution was made using; 2.8 g ferrous (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), 1 g zinc chloride ($ZnCl_2$), 1 g manganese chloride heptahydrate ($MnCl_2 \cdot 7H_2O$), 0.2 g copper chloride ($CuCl_2$), 0.37 g cobaltous chloride ($CoCl_2 \cdot 6H_2O$), 0.2 g sodium molybdate (Na_2MoO_4), 2 g calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 1.5 mL 0.5 M citric acid and made up to 1 L with deionised water.

Biotin Solution

100 mL of this solution was made by adding 0.03 g of biotin to 100 mL of deionised water. Vacuum filtration was used to sterilise the solution.

2.2.3 Buffers

2.2.3.1 Tris Acetate EDTA (TAE) Buffer

One litre of 50X solution was prepared as follows; 242 g of tris, 57.1 mL of glacial acetic acid and 100 mL of 0.5M EDTA were mixed and made up to 1 L with deionised water.

2.2.3.2 0.1% Tween 80

Add 1 mL Tween 80 and make up to 1 L with SDW.

2.2.3.3 Phosphate Buffered Saline (PBS)

Prepared by adding 1 preformed tablet (Oxoid) to 100 mL distilled water.

2.3 Mycelial Genomic DNA Extraction

First, cultures of the desired isolates were prepared in 250 mL Erlenmeyer flasks containing 50 mL of PDB. These flasks were inoculated using stock suspensions as described in **Section 2.1**. Culture flasks were then incubated at 28 °C for 72 hours on an orbital shaker set to 150 rpm.

Once cultures showed sufficient growth, the resulting mycelium was then individually filtered through miracloth and ground in a pestle and mortar under liquid nitrogen. DNA extraction was performed using modified solution volumes for the Wizard® Genomic DNA Purification Kit (Promega, UK), otherwise performed to the manufacturer's instructions.

Concentration of DNA was measured using a Nanophotometer P330 (Implen, Germany). Purified gDNA was kept at 4 °C for long-term storage.

2.4 Gel Electrophoresis

Where discussed, the assessment of results of polymerase chain reactions, gel electrophoresis was used. In most cases, 1.0% agarose gels were used and prepared using TAE buffer and the addition of ethidium bromide (1 µg/mL final concentrationc) as staining solution.

Once gels had set, 8 µL of each PCR product was then combined with 2 µL 5X loading dye and pipetted in to a well. In addition to the samples, a DNA ladder was added to the gel to provide a size reference (Sigma Aldrich). The ladder used will be detailed where appropriate. Once loaded, gels were then subjected to an electrical current to facilitate separation. Once complete, DNA was visualised using a ChemiDoc™ XRS system (Bio-Rad, USA).

2.5 Data Visualisation and Statistical Analysis

Data visualisation and analysis were carried out using GraphPad Prism 9.3.1. for Windows (Graphpad Software, San Diego, California USA, www.graphpad.com).

3

Needles in a Haystack:

The Search for Novel Isolates of *Fusarium venenatum*

3.1 Introduction

Historically, many fungal species now recognised as either biotechnologically or medically important have their isolation origins rooted in anecdote. The discovery of penicillin is perhaps the most famous of these, where agar plates of *Streptococcus* spp. were "necessarily exposed to the air and became contaminated with various microorganisms" (Fleming, 1929). One contaminating microorganism was the mould *Penicillium rubens* (known by several monikers since the original classification as *P. rubrum* in Fleming's original paper; Houbraken et al., 2011), from which the discovery of penicillin was made. The unearthing of the compound would go mainly unacted upon until interest flared up during the Second World War due to interest in limiting wartime infection. Fleming's strain was unable to produce penicillin in submerged culture, and so production of the compound was difficult to upscale past shake flasks. Fermentation industries regarded the mass production of penicillin too risky given the low production by Fleming's strain, so new *Penicillium* strains were required to attract such interest (Wells, 1991). The continued efforts of Ken Raper at the NRRL in Peoria resulted in the isolation of a *Penicillium* species from a mouldy cantaloupe that had been donated by a member of the public (Spencer 1946). This isolate was *P. chrysogenum* (now *P. rubens*) NRRL 1951, which could produce a relatively high titre of penicillin in submerged fermentation and that was then subjected to strain improvement regimes to greatly amplify penicillin production by the end of the war (see General

Introduction **section 1.6.2**). Liberal application of penicillin and similar antibiotics in human society (dubbed the “era of abuse”) drove significant occurrence of antimicrobial resistance to β-lactam antibiotics, through evolution of bacterial β-lactamases and penicillin-binding proteins (PBPs) (Lobanovska and Pilla, 2017). Attempts to introduce biodiversity through isolation of novel *Penicillium* spp. from the environment are common. Indeed, Dayalan et al. (2011) and Ur-Rhaman et al. (2012) both isolated several *Penicillium* spp. from India and Pakistan respectively, testing each isolate for their capacity to synthesise penicillin. Discovery of novel fungal isolates allowed them to be introduced to strain improvement programmes through breeding with industrial strains. Such a strategy was employed by exploiting the newly discovered sexual cycle of *P. chrysogenum* (*P. rubens*) to abolish the biosynthesis of chrysogenin, a yellow pigment which otherwise had to be extracted to make pure product, while maintaining high titres of penicillin (Böhm et al. 2013).

Similarly, the isolation and biotechnological development of *Trichoderma reesei* provides an intriguing story. This mesophilic fungus was first isolated from the Solomon Islands during WWII when it was noticed that US Army canvas camp equipment was degrading at an accelerated pace (Reese 1976). The isolate was originally identified as *T. viride* and named QM6a as part of the collection at the US Army Quartermaster Research and Development Center, Natick Massachusetts. Unlike in penicillin production, all of the current industrial *T. reesei* strains are descended from this original QM6a strain, noted for its ability to attack crystalline cellulose. The hyper-cellulolytic descendent RUT-C30 was developed thanks to an efficient three-step strain improvement and subsequent screening programme developed at Rutgers University, New Jersey, involving UV mutagenesis, N-nitroguanidine treatment, and then further UV mutagenesis (Montenecourt and Eveleigh 1977a; Montenecourt and Eveleigh 1977b; Montenecourt and Eveleigh 1979). This process demonstrated the power of strain improvement, resulting in 15-20-fold increased cellulase

production than that of QM6a in shake flasks, in addition to some freedom from carbon catabolite repression (Montencourt and Everleigh, 1979). Further improvement has been investigated by implementing sexual reproduction using environmental isolates of *T. reesei* (teleomorph *Hypocrea jecorina*) (Seidl et al., 2009). *T. reesei* has continued to be used in the biotechnology industry due to its tremendous secretory capacity and prolific cellulolytic enzyme production. The fungus has been shown to metabolise cellulosic substrates with the resultant production of compounds including ethanol, suggesting that it is a promising candidate for use in consolidated bioprocessing (CBP) systems. Conversion of cellulosic feedstocks to bioalcohols by microorganisms could be an answer to the renewable energy crisis faced by humanity (addressed in more detail in Lynd et al. 2002). Unfortunately, the ethanologenic capacity of the fungus has not been as systematically improved as *T. reesei*'s cellulolytic ability, presenting an enticing target for strain improvement (Bonnaccorsi et al., 2006; Xu et al., 2009).

Like *P. rubrum* and *T. reesei*, *Fusarium venenatum* (then known as *F. graminearum*) was isolated serendipitously from a compost heap in Marlow, Buckinghamshire. Since this original isolation over 50 years ago, the same strain has enjoyed an uninterrupted run as the industrial strain for mycoprotein production (Whittaker et al., 2020). While investigations have been made into classical mutagenesis methods, there is some reluctance over their use, as the final product is destined for human consumption. Any undesirable mutations that arise due to non-specific UV or X-ray irradiation may manifest as adverse effects upon mycoprotein consumption. Therefore, isolation and screening of novel isolates from the environment is considered a safer approach as part of a strain improvement programme, both for the consumer and for the company. Such strains may display beneficial characteristics over the Quorn™ production strain (*F. venenatum* A3/5), with the possibility of later exploiting sexual reproduction for breeding purposes to produce further novel strains with combined traits of interest.

Following its original isolation, the apocryphal story that the Quorn™ production species is ubiquitous in soil is still reported in recent literature (e.g. King et al. 2018). However, several recent studies involving the isolation of *Fusarium* spp. from both soil and plant samples have instead alluded to the relative scarcity of *F. venenatum* (Table 3.1). Even considering studies which did isolate *F. venenatum*, there is an apparent lack of consensus with regards to the main environmental niche (e.g. whether soil based and/or linked to a particular plant host type), complicating understanding of the *F. venenatum* lifestyle in the environment.

A variety of techniques have been implemented to survey microbial diversity within agricultural soils. The genus *Fusarium* includes many high-profile phytopathogens resulting in many published studies focused on determining the composition of *Fusarium* populations within a crop field using metagenomic approaches (e.g. Karlsson et al. 2015, Abdelfattah et al. 2016, Kaushal et al. 2020). These have mainly utilised molecular techniques which amplify and sequence a fragment of a highly conserved gene e.g. Translation elongation factor 1-alpha (Edel-Hermann et al. 2015, Boutigny et al. 2019 etc). As a consequence, they were not designed for the isolation of specific fungal colonies and the necessary requirements for mass sequencing means that this approach is inefficient when screening many soil samples for a single, potentially rare species such as *F. venenatum*.

Table 3.1. A survey of literature between 2008-2021 concerning the worldwide isolation of *Fusarium* spp. from the environment. "Rare" incidences refer to studies where *F. venenatum* composed <2% of the total isolates.

Incidence of *F. venenatum*	Isolation Source	Location of Study	Reference
Rare	Maize kernels	Various, Switzerland	Dorn et al. (2009)
Rare	Soil	Dijon, France	Edel-Hermann et al. (2015)
Rare	Sugar beet	Germany, France, US, Poland, Italy, Sweden	Nitschke et al. (2009)
Rare	Brewing barley grains	Various, Brazil	Piacentini et al. (2019)
Rare	Plant debris	Slavonia, Croatia	Postic et al. (2011)
Rare	Potato tubers	Various, Poland	Stefańczyk et al. (2016)
Absent	Soil	Various, Sardinia	Balmas et al. (2010)
Absent	Barley kernels	Quebec, Canada	Bourdages et al. (2006)
Absent	Barley, durum & bread wheat	Various, France	Boutingny et al. (2019)
Absent	Oat	Various, Korea	Choi et al. (2019)
Absent	Proso millet	Jeju Island, Korea	Choi et al. (2021)
Absent	Maize kernels	Various, Poland	Czembor et al. (2015)
Absent	Wheat	Bavaria, Germany	Denschlag et al. (2014)
Absent	Seed potato tubers	Michigan, US	Gachango et al. (2012)
Absent	Soybean roots	Ethiopia & Ghana	Hartman et al. (2019)
Absent	Winter wheat	Flanders, Belgium	Isebaert et al. (2009)
Absent	Barley kernels	Various, Brazil	Iwase et al. (2020)
Absent	Soil & tomato rhizosphere	Uttar Pradesh, India	Joshi et al. (2013)
Absent	Peat forest soil	Perak and Pahang, Malaysia	Karim et al. (2016)
Absent	Soil and wheat kernels	Various, France	Karlsson et al. (2016)
Absent	Soil	Minnesota, US	LeBlanc et al. (2017)
Absent	Roots	Alicante, Spain	Maciá-Vicente et al. (2008)
Absent	River and sea water	Andarax River & Mediterranean Sea, Spain	Palmero et al. (2009)
Absent	Wheat and barley spikes	Paraná, Brazil	Pereira et al. (2021)
Absent	Soil	Various, Iran	Saremi & Saremi (2013)
Absent	Durum wheat	Various, Italy	Shah et al. (2005)
Absent	Cucurbitaceae-associated soil	Peninsular Malaysia	Siti Nordahliawate (2012)
Absent	Soil	Ansfelden, Austria	Steinkeller & Langer (2004)
Absent	Spring wheat, barley & oat	Ontario, Canada	Xue et al. (2019)

3.1.1 Chapter Aims

As previously mentioned, the Quorn™ industrial process has utilised a single strain (A3/5) of *F. venenatum* since its original isolation in the late 1960s (Whittaker et al. 2020). This chapter focuses on work whose aim was to:

1. Establish a culture collection of *F. venenatum* isolates for use in other areas of the project. This would ideally include novel strains of *F. venenatum* from the environment
2. In parallel, develop a high-throughput species-specific molecular diagnostic which would facilitate pre-screening of environmental samples (e.g. soil) before time-consuming, culture-based isolation methods were implemented, thereby expediting the isolation process.

3.2 Materials and Methods

3.2.1 Selective Media

To isolate fungal colonies from the environment, selective media was required. Application of such media impedes the growth of colonies, permitting extraction of slower-growing fungi without interference from faster-growing genera such as *Mucor* and *Aspergillus*. Sterilisation was by autoclaving (121 °C for 20 mins).

3.2.1.1 Malachite Green Agar (MGA)

Malachite green agar is a semi-selective medium used in the isolation of *Fusarium* (Thompson et al. 2013). A litre of this media is made by mixing 15.0 g peptone, 20.0 g agar, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2.5 mg malachite green oxalate and made up to 1 L with distilled water.

Post-autoclave, this media was amended with ampicillin and streptomycin to restrict bacterial contamination. MGA was supplemented with an aliquot of a filter-sterilised antibiotic stock solution (ampicillin and streptomycin each at 1 g/10 mL) to yield a final concentration of 100 mg/L of each antibiotic.

3.2.1.2 Rose Bengal Agar (RBA)

Like MGA, rose Bengal agar is a selective medium that is used for the enumeration of yeasts and fungi associated with food spoilage (Ottow 1972). One litre of this media is made by suspending 32.0 g of dehydrated rose Bengal agar (ThermoFisher Scientific) in distilled water which was then brought to the boil to dissolve before autoclaving. Chloramphenicol is added post-autoclave to the RBA base to a final concentration of 100 mg/L.

3.2.2 Suitability of Selective Media

It was necessary to understand *F. venenatum* growth behaviour on the two selective media types before soil sampling was attempted. Spore

suspensions of *F. venenatum* were prepared as described in **Section 2.1.1** and used to inoculate 9 cm diameter MGA, RBA, and PDA (control) plates in quadruplicate. Each media type was inoculated with 100 spores (100 µL of a 1 x 10^3 Spores per mL suspension) of each of one *F. venenatum* strain; A3/5, 114-2 and 114-3. Visible colonies were counted every 24 hours for a total of 120 hours. The results were recorded as a percentage of the total plated.

3.2.3 Isolate Acquisition from Global Collections

To widen the search for *F. venenatum* isolates beyond environmental sampling, numerous worldwide fungal collections were interrogated for the presence of *F. venenatum*, and personal contacts were approached, in addition to calls made for isolates at the 2019 Fungal Genetics Conference, Asilomar (USA). Furthermore, archival material at the Quorn™ Billingham site was examined to establish relationships that might exist between industrial isolates.

3.2.4 UK Environmental Sample Collection

Environmental soil samples were obtained from a variety of sites across the UK. Information about each site in is provided in Supplementary Table 3.1. Samples of approximately 50 g were collected from each site. To obtain samples, the topsoil was removed, and the underlying soil to a maximum depth of 10 cm was collected using a trowel before depositing in individual zip-lock bags. Where agricultural plots were sampled, soil was taken as close to the field crop as was possible. The trowel was cleaned using 70% isopropanol between samples to prevent cross-contamination. In a limited number of cases, samples of plant debris were also collected.

On return to the University samples were homogenised using a soil sieve with a 2 mm pore size, to ensure soil particles of similar sizes. Soil

samples were then stored at 4 °C until required.

3.2.5 Screening of Samples from UK Field Sites

3.2.5.1 Sampling by Soil Solution

Fungal cultures were isolated from soil using a serial dilution approach. 1 g of each soil sample was added to 9 mL of phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM sodium chloride, pH 7.3). Subsequent tenfold dilutions were prepared with PBS. For each sample, 100 µL of the 1 x 10^{-3} and 10^{-4} dilutions was used to inoculate individual 9 cm MGA plates in duplicate. Plates were incubated at 24 °C for 48 hours in light conditions.

3.2.5.2 Sampling from Plant Debris

In addition to soil samples, plant debris was also screened in an attempt to isolate fungal cultures. Plant debris was obtained from certain soil sampling sites and stored in a marked, resealable bag. These samples were washed in SDW to remove any soil, distributed on 9 cm MGA plates, and incubated in a similar manner to the soil plates as **section 3.2.5.1**.

3.2.5.3 Isolation of Fungal Colonies from MGA

Following incubation, visible fungal colonies were transferred from MGA to a 5 cm diameter PDA plate using a sterile toothpick. These plates were then incubated at 24 °C for 7 days to allow sufficient time for colonies to develop and conidiation to occur.

For plant debris samples, visible colonies were streaked onto 9 cm diameter PDA plates. Following 24–48 hours of incubation, individual colonies were then transferred to a 5 cm PDA plate using a sterile toothpick. A further 24 hours of incubation proceeded.

Once this second round of incubation was completed, isolated colonies were studied to determine whether they were *Fusarium* spp. Many colonies could be eliminated due to macro-morphological characteristics

on agar (e.g. development of conidiophore structures not seen in fusaria). Promising colonies were then observed using light microscopy (Motic BA310 Trinocular LED Microscope). A sterile swab was dipped in to 0.1% Tween 80 and the mycelial material was lightly scraped from the surface of the plate. Material was transferred to a water droplet on the surface of a microscope slide. If characteristic *Fusarium* macroconidia were observed (Leslie and Summerell, 2006), the isolate in question advanced to the next stage of analysis.

3.2.5.4 Identification of Isolated Fungal Colonies

A 250 mL Erlenmeyer flask containing 25 mL of PDB (**section 2.2.1**) was prepared for each suspected *Fusarium* isolate. The flasks were inoculated using a sterile swab, transferring fungal material directly from the agar plate to the liquid media. Cultures were incubated aerobically at 28 °C on a rotary shaker at 150 rpm for at least 72 hours. Genomic DNA extraction was carried out as per the method outlined in **Section 2.3**.

Molecular confirmation of fungal identity was facilitated by partial amplification of a fragment of *TEF-1α,* a gene which encodes the translation elongation factor 1α protein. The sequence of this gene shows sufficient intraspecies conservation for reliable identification of fungal species and has been successfully used to distinguish *Fusarium* species (Karlsson et al. 2016). Amplification was accomplished by PCR using Phusion® High Fidelity DNA Polymerase (NEB, USA) in a final reaction volume of 20 µL as described in **Section 2.2.5**. The oligonucleotides used for this purpose were forward and reverse oligonucleotides Fa+7 (5’ AAC GTC GTC GTC ATC GGC CAC GTC GAC TCT) and Ra+6 (5’ ACA TAC CAA TGA CGG TGA CAT AGT AGC G), respectively (Karlsson et al. 2016). The generated amplicon had an expected band size of approx 600 bp.

The Tm for this reaction was 64 °C. Following confirmation of a successful amplicon, the PCR product was purified and subsequently sequenced (**Section 2.2.7**). A nucleotide BLAST search

(https://blast.ncbi.nlm.nih.gov/Blast.cgi) was then performed to determine the identity of the isolated colony based on DNA sequence homology.

3.2.7 Development of a PCR-based Molecular Diagnostic

A high-throughput screening method was developed to assess the possible presence of *F. venenatum* in environmental samples. Species-specific oligonucleotides were designed which would be predicted to produce amplicons only when *F. venenatum* DNA was present in a sample. The stages of development are outlined in Sections 3.2.7.1-3.2.7.5 as follows.

3.2.7.1 Diagnostic Oligonucleotide Design

Oligonucleotides were designed from gene sequences identified as *F. venenatum*-specific in a comparative genome study with the *F. graminearum* type strain (PH-1) genome (King et al. 2018). Target genes were secondary metabolite cluster genes, as these were more likely to be species-specific. *In silico* assessment of specificity was facilitated using nucleotide BLAST searches (Madden et al. 2019). Each gene was screened for homologues in closely related species and once oligonucleotides were designed, these were tested for possible non-specific erroneous amplification in other fungal species. A variety of different oligonucleotide pairs were designed to try and ensure successful specificity of at least one primer set.

A summary of oligonucleotides assessed in this study can be found in Table 3.2.

Table 3.2. Oligonucleotides assessed during diagnostic development. Each row of the table shows the target locus tag and primer pairs designed for gene amplification (including predicted amplicon size). Where possible,

Name	Target Gene	Predicted Protein Domains	Sequence (5' → 3')	Product Size (bp)
ven1_for ven1_rev	FVRRES_11296	PAT1 Superfamily	TCA CCC GAA CCA CAA TCA CC AGG TAT GTT CTG GGC GTT TC	831
ven2_for ven2_rev	FVRRES_11296		AGG GAA CCA CCG ACA AGA TG TTG TGT GTT GGG CAA GAA CC	476
ven3_for ven3_rev	FVRRES_00849	-	TCA GTG GCT GGT TTC ATT TTC G GGA CTT GCG TGT AAC TCT GG	532
ven4_for ven4_rev	FVRRES_00849		CGG CGT TGA ACA TTC TCT TGG CCA TGT GTC GGC ATG TTT ACG	471
ven5_for ven5_rev	FVRRES_03949	PAN APPLE Superfamily	GGC TCC ATT GCC CTT TTT GC CCG GAA GGT GTT TAT CTG AAG C	432
ven6_for ven6_rev	FVRRES_03949		TCT CGG TTG ACC TTG CTT GC CAC ACC AAA GAC CGA AAG ACG	400
ven7_for ven7_rev	FVRRES_10539	HET Superfamily	CGC GTT GAG CTT GTC TTC TG CAC CGG ATT GAC CGA AAA CG	451
ven8_for ven8_rev	FVRRES_00229	-	TAC TGG CGC ATA CTG TCA GC AGA GGA TGT CCC ATG AAT CCG	408
ven9_for ven9_rev	FVVRES_00229		GCA ACG GAT TAC CCA TGC TG CCG ATG TTC TTC ATC GTC GC	433
ven10_for ven10_rev	FVRRES_00257	-	TGA TTC CAA AGG CGC TAA AGG TGT AGG TAC TCC AAC GCT AGG	485
ven11_for ven11_rev	FVRRES_09359	-	TCT GCT CTT CTG GAG TGT CG TAC TGC AAA CGG GGA GTG C	489
ven12_for ven12_rev	FVRRES_11058	NACHT N WD40 P-loop NTPase Superfamily	GCA TCT GAA CCA ACA ACT CAA CC GAC CTG CCA GTC GAT TCA CC	1609
ven13_for ven13_rev	FVRRES_11058	PRK05641 Superfamily	CGT CTG CCT TGG ACT AGA GG CTT TGC ATT GTC CAG TTT CTC C	1096
ven14_for ven14_rev	FVRRES_00023	MRP assoc pro Superfamily	GTG CTG GCT TTT CTC TGT GC TTC CGC TTC CTG TTC TAC CG	919
ven15_for ven15_rev	FVRRES_00023		CGC TTT TCC GAG TGT GTG G TTT CCG CTT CCT GTT CTA CCG	1029
ven16_for ven16_rev	FVRRES_12025	-	CAG GAA GGC TGA TGT TGA CG TCA GGA GTA GCA GCA ACA CC	566
ven17_for ven17_rev	FVRRES_12025		GAC GGC TGC ATC TTG TTC AGC AGC AAC ACC ATC ACA C	672
ven18_for ven18_rev	FVRRES_00025	PcbC Superfamily	TTC TTC GCC CTG ACT ACT GC ATC GGC TCT GTG AAC TTG GG	839
ven19_for ven19_rev	FVRRES_00025		ATT CTT CGC CCT GAC TAC TGC CAT CGG CTC TGT GAA CTT GG	841
ven20_for ven20_rev	FVRRES_12550	CYP503A1-like	TCG GCT TGT GTC GTC TTT CG GTA GGT CCG TCG TGG TAT GG	566
ven21_for ven21_rev	FVRRES_12550		ATC GGC TTG TGT CGT CTT TCG AGG TCC GTC GTG GTA TGG	918

3.2.7.2 Intragenus Test

Oligonucleotide pairs were first tested for their specificity among *Fusarium* spp. Genomic DNA of several phytopathogenic *Fusarium* species common to soil (*F. graminearum, F. oxysporum* and *F. solani*) was extracted. *F. oxysporum* and *F. solani* were regenerated from freeze-dried stocks available in the Fungal Biology and Genetics group in Nottingham, while *F. graminearum* was kindly provided by Dr Rumiana Ray (University of Nottingham). Generation of the expected amplicon in these species was assessed alongside *F. venenatum* A3/5.

Gradient PCRs were performed with each oligonucleotide pair to assess possible amplification of the gene fragment in each *Fusarium* species tested. The diagnostic was performed using Phusion® High Fidelity DNA Polymerase (NEB, USA) in a final reaction volume of 20 µL (12.2 µL SDW, 4 µL 5X high-fidelity buffer, 0.4 µL 10 mM dNTP mix, 1 µL 10 mM forward primer, 1 µL 10 mM reverse primer, 50 ng DNA template and 1 U (0.4 µL) Phusion polymerase). The PCR cycling conditions were as follows: an initial denaturation step of 98 °C of 15 seconds, followed by 30 cycles of 98 °C for 10 seconds, an annealing step with varied temperatures ranging from 59 – 67 °C incremented in 2 °C for 15 seconds and 72 °C for 20 seconds. A final elongation step of 72 °C for 5 minutes was performed. All reactions were performed using a Techne TC-5000 PCR Thermal Cycler. PCR products were subsequently analysed by gel electrophoresis with a 100 bp ladder used as reference for product length. Products were then analysed by gel electrophoresis (**Section 2.4**).

An oligonucleotide pair was deemed suitable if amplification was observed in *F. venenatum* but not in other *Fusarium* species at a given temperature.

3.2.7.3 Intergenus Test

If an oligonucleotide pair was successful in the intergenus test,

specificity investigations were carried out with other fungal taxa, chosen using two parameters. First, each of the taxa was considered to be a member of a fungal genus common in soil. Naturally-occurring members of these genera could contribute to confounding results if design of the diagnostic did not account for their presence. Second, genera were chosen to have a varied level of phylogenetic relatedness to *Fusarium*; *Trichoderma koningii* (both belong to the family Hypocreaceae), *Penicillium viridicatum* (belongs to the class Eurotiomycetes, not the Sordariomycetes), *Aspergillus niger* (same principle as *P. viridicatum*) and *Mucor hiemalis* (belongs to the Zygomycota, not the Ascomycota). These strains were each restored from freeze-dried cultures available in the Fungal Biology and Genetics group in Nottingham.

Genomic DNA of these species was extracted from liquid cultures as described in **Section 2.3**. The same test methodology strategy was used as for the intragenus test in **Section 3.2.7.2**. As before, success was regarded to be the lack of amplification from this the outgroup taxa.

3.2.7.4 Assessment of Sensitivity

The sensitivity of the molecular diagnostic was assessed using soil spiked with known quantities of *F. venenatum* A3/5 macroconidia, used as a proxy for amount of genetic material. The diagnostic was then applied to DNA extracted from each spiked soil sample.

Soil collected from Bunny (University of Nottingham Farm, Nottinghamshire, England) was sterilised for the purposes of this experiment. First, fragments of large organic matter such as roots were removed from the samples. Next, the soil was first homogenised using a 2 mm soil sieve. 50 g was autoclaved at 121 °C for 20 minutes on two consecutive days to ensure sterilisation and degradation of DNA present in soil. Once sterile, the soil was separated into 6 aliquots weighing 0.25 g and deposited into sterile 2 mL microcentrifuge tubes. Each sample was inoculated with 50 µL of a *F. venenatum* macroconidial suspension from 10-fold serial dilutions containing between 1 x 10 to 10^6 total macroconidia – and briefly vortexed. The inoculated soil was air dried at

room temperature in a Class II microbiological safety cabinet for four hours before processing by the Dneasy PowerSoil Kit performed to manufacturer's instructions (Qiagen, Netherlands) to extract the DNA. The extracted DNA was stored at -20 °C, allowing the same DNA to be used in all sensitivity trials.

Pairs of oligonucleotide primers were then tested for their ability to amplify the DNA by PCR in each of these spore-spiked samples using PCR cycle conditions determined to be optimal for limiting aberrant amplification in the intra- and intergenus tests described in **Sections 3.2.7.2** and **3.2.7.3** above. Success was to be judged on amplification at least at the 10^2 level (representing 400 spores/g of soil), based on the suspected rarity of *F. venenatum*.

3.2.7.5 Intraspecies Compatibility Test

As a final stage in diagnostic development trials were undertaken with candidate oligonucleotide pairs to ensure that they were compatible with other *F. venenatum* strains (i.e. able to produce amplicons), as development had only used gDNA from a single strain (A3/5) up to this point. PCR amplification was therefore assessed in four additional strains (114-3, 114-7 and 114-14, 114-19) using gradient PCR methodology as described in **3.2.7.2** and **3.2.7.3**.

3.2.8 Screening of Samples from UK Field Sites via New Molecular Diagnostic

Once promising oligonucleotide pairs had been identified, trials with soil samples were performed. Genomic DNA was extracted from 32 soil samples using a Dneasy soil extraction kit (Qiagen) according to manufacturer's instructions. The soil samples comprised 16 samples randomly chosen from the entire sample set collected during the present project, 8 additional samples selected from soil samples from the UK collected in other projects ongoing at Nottingham and a further 8 samples obtained from a citizen science project surveying the presence

of fungi in UK compost soil samples (Shelton et al., 2022). In addition, soils from the original area of isolation of the A3/5 strain (Marlow, Buckinghamshire, UK) were used in this screen. These samples were randomly selected from a variety of different soil types, e.g. agricultural and allotment soils, composts etc.

3.2.9 Extractions from Mycoprotein Samples

The diagnostic was assayed in other analyses beyond that of surveying environmental samples. The Food Chemicals Codex (FCC; USA) required Quorn Foods to provide a definition of mycoprotein made from *F. venenatum* in order to distinguish it from other fungal foodstuffs, including mycoprotein from rival manufacturers. The diagnostic was therefore tested to see if it could provide a specific molecular definition of *F. venenatum* mycoprotein.

DNA extractions were performed on both mycoprotein paste and several Quorn™ food products (quarter pounder, mince, deli ham slices and pieces) using methods described in **Section 2.3**. The extracted DNA was then subjected to the newly developed diagnostic test using oligonucleotide pair ‘ven1’ to ascertain whether the diagnostic would be applicable.

3.3 Results

3.3.1 F. venenatum Acquisition from Global Culture Collections

In total it was only possible to obtain 25 potentially different strains of *F. venenatum* from worldwide culture collections (available online) and from private research contacts (Table 3.3), contradicting the supposed ubiquity of *F. venenatum*. Isolates were listed in only a handful of collections, namely the Leibniz Institute DMSZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany), ITEM (Institute of Sciences of Food Production, Italy) and NRRL (Agricultural Research Service Culture Collection, USA). All isolates were given a University of Nottingham BDUN culture code 114-x, denoting *F. venenatum* (Table 3.3). This included A3/5 given the code 114-1, but for expediency this isolate will continue to be referred to as A3/5 in this thesis rather than 114-1.

Indeed, research in the Quorn archives at Billingham, County Durham, revealed that several strains deposited in the NRRL collection (114-4 to 114-7) were not in fact truly unique isolates (Figure 3.1). Instead, internal reports detail that these strains all originated from A3/5 and were forays into strain improvement. NRRL 25417 (114-7) was fermented in glucostat conditions for 1100 hours with mycelial extracts then plated on malt extract plates (Patent GB1346061). A number of these were then selected and assessed for mycelial and conidial morphology, growth rate and other physical characteristics. Representative strains were then submitted by the Quorn™ company to the now defunct International Mycological Institute (IMI, Egham, England) before acquisition by the NRRL.114-7 was stated to be descended from the A3/5 industrial production strain but exact details of the nature of this relationship could not be found (Figure 3.1). For the purposes of this project, the discovery that 4 of the 25 strains collected were not truly unique was unfortunate as this limited the number of independent isolates available.

Table 3.3. Strains of *F. venenatum* obtained during this project.

BDUN Strain	Obtained from	Source of Isolation
114-1	N/A	Compost, Marlow
114-2	NRRL 22196	*Zea mays*, Germany
114-3	NRRL 22198	Unknown, Finland
114-4	NRRL 25413	Quorn Deposit
114-5	NRRL 25415	Quorn Deposit
114-6	NRRL 25416	Quorn Deposit
114-7	NRRL 25417	Quorn Deposit
114-8	NRRL 26139	Illinois, USA
114-9	NRRL 26228	*Triticum sativum*, Austria
114-10	NRRL 28571	*Cucurbita maxima*, New Zealand
114-11	NRRL 28572	*Spinacia oleracea*, Sweden
114-12	NRRL 36230	*Solanum tuberosum*, Finland
114-13	NRRL 66329	*Triticum sativum*, Austria
114-14	John Clarkson, Warwick University	Unknown
114-15	ITEM 831	Unknown
114-16	ITEM 834	Unknown
114-17	DSMZ 21779	Unknown
114-18	DMSZ 23358	Unknown
114-19	FRC R-2273	*Zea mays*, Australia
114-20	FRC R-8978	Soil, Turkey
114-21	NRRL 31989	Soil, Australia
114-22	NRRL 32015	Soil, Australia
114-23	NRRL 36234	*Triticum sativum*, Austria
114-24	NRRL 36260	Unknown
114-25	Kevin King, Rothamsted Research	Wheat or barley roots, Rothamsted, UK (1997)

Similarly, we clarified the origin of the original site of isolation for the Quorn™ strain. The history of this isolation claimed that isolation occurred from a field near the RHM production facility in Marlow. Interrogations of ex-staff revealed that isolation had occurred from an employee's garden compost heap, found close to the supposed site in Marlow Bottom, Buckinghamshire.

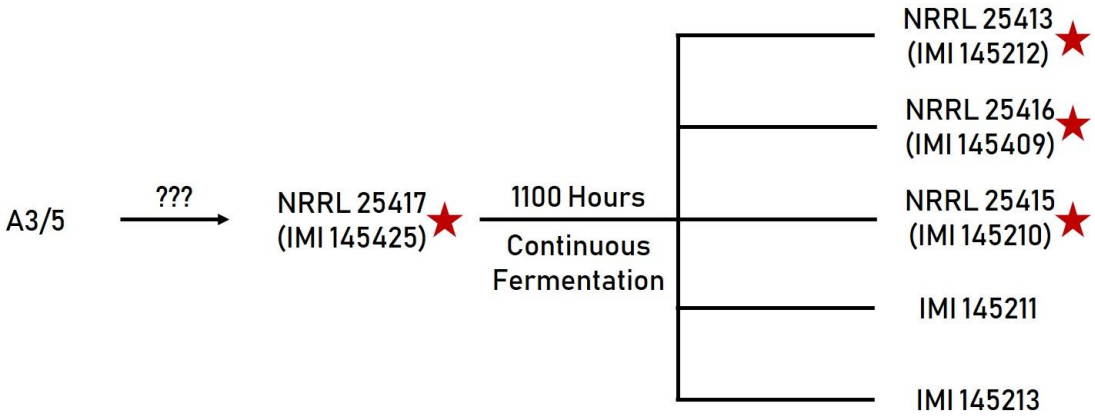


Figure 3.1 Summary of *F. venenatum* A3/5 descendants submitted to IMI by Marlow Foods. Red stars indicate strains acquired during this project.

3.3.2 Screening of Selective Media

F. venenatum growth up to 120 hr was characterised on the selective media malachite green agar (MGA) and rose Bengal agar (RBA), together with potato dextrose agar (PDA) as a control media (Figure 3.2).

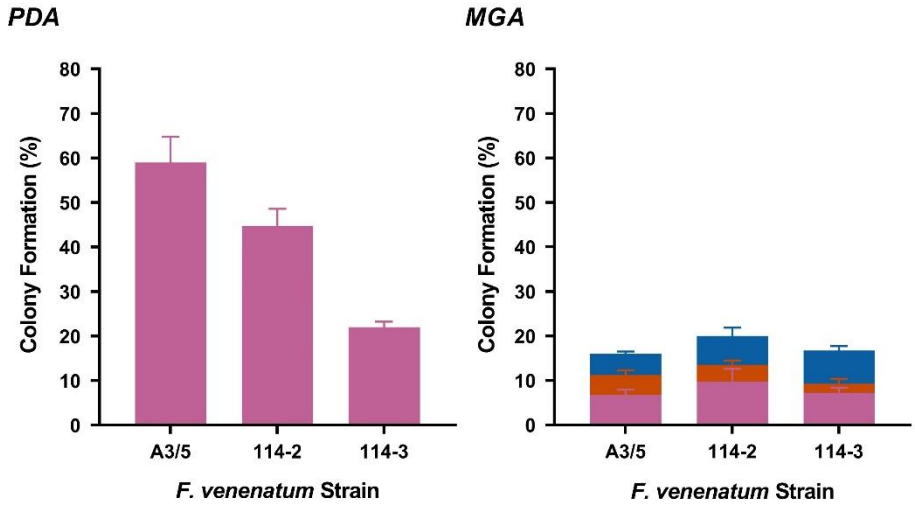


Figure 3.2. MGA is successful at limiting the growth of *F. venenatum*, with the window of isolation occurring 48 hours post-inoculation. Y-axis refers to the percentage of germinated spores of those applied to the plates. RBA is not represented, due to complete lack of germination from each of the strains. Pink = 48 hpi, Orange = 72h hpi, Blue = 96 hpi.

Germination rates on the PDA control varied significantly between the three test strains (Two-way ANOVA, $F(2,9) = 21.15$; $p = 0.0004$). A3/5 demonstrated the greatest germination efficiency (59.0% ± 5.8), followed by 114-14 (44.7 ± 3.8) and then 114-18 (22.0 ± 1.2). All three strains showed a significant reduction in germination rate when inoculated onto MGA, showing 16.0% (± 1.8), 20.0% (± 5.3) and 16.7% (± 2.1) germination of colonies after 96 hr incubation for strains A3/5, 114-14 and 114-18, respectively. RBA appeared to completely prevent germination of *F. venenatum* macroconidia, as no colonies were observed for any of the strains in the 120-hr growth period.

MGA was therefore chosen as the selective media. Furthermore, the 48-hour timepoint was selected to screen for growth of colonies from soil sampling because whilst greater germination rates might have been achieved by an extended incubation time, isolation of *F. venenatum* might then have been compromised by overgrowth of faster growing fungi.

3.3.3 Sample Screening from UK Field Sites

Environmental samples consisting of soil samples and plant debris were screened for the presence of novel *F. venenatum* strains with results as follows.

3.3.3.1 Soil Screening

Isolation was attempted from a total of 48 soil samples of varying compositions and geographic locations within the UK (Supplementary Table 3.1**)** using a culture approach with MGA acting as a selective medium. From these experiments, 32 *Fusarium* isolates were obtained and identified using *TEF-1α* sequences to distinguish isolates at the species level (Supplementary Table 3.2). Of these, the most commonly isolated species were *F. oxysporum* (12), *F. graminearum* (8) and *F. culmorum* (7) with *F. hostae* and *F. solani* appearing in low numbers (3 and 2 respectively). However, no isolates of *F. venenatum* were identified.

3.3.3.2 Plant Debris Screening

In addition to soil sampling, a limited sample of plant debris samples (6; diseased maize heads, assorted root samples, seed pod) were collected and screened as potential sources of fungal isolates. In total, 6 fungal colonies were retrieved from plant debris with 2 colonies from diseased maize heads determined to be *F. graminearum*.

3.3.4 Development of a PCR-based Molecular Diagnostic

The scarcity of *F. venenatum* in soil samples demanded a more targeted isolation approach. To complement the low-throughput culture-based method previously described, a molecular diagnostic was therefore devised with the aim of using *F. venenatum*-specific primers in PCR to identify the presence of the species in microbial DNA extracted from soil samples. A total of 20 primer pairs were designed to be *F. venenatum*-specific (Section 3.2.7.1, Table 3.2). To ensure that the diagnostic was both reliable and specific, several tests were performed for each prospective oligonucleotide pair. In each case, this results section will present a representative model outcome using the successful oligonucleotide pairs ven1 and ven2. An overall summary of the results is presented in Table 3.4.

Table 3.4. Assessment of oligonucleotide suitability for the PCR-based soil diagnostic for identification of *F. venenatum*. Numbers in brackets indicate the number of successful oligonucleotide pairs in that stage of those tested. + and – icons indicate an oligonucleotide set passing or failing the test respectively.

Oligonucleotide Pair Name	Target Gene	Intragenus (18/20)	Intergenus (15/18)	Sensitivity (4/15)	Intraspecies (4/4)
ven1	FVRRES_00023	+	+	+	+
ven2	FVRRES_00023	+	+	+	+
ven3	FVRRES_00849	-			
ven4	FVRRES_00849	-			
ven5	FVRRES_03949	+	+	-	
ven6	FVRRES_03949	+	+	-	
ven7	FVRRES_10539	+	-		
ven8	FVRRES_00229	+	+	-	
ven9	FVRRES_00229	+	+	-	
ven10	FVRRES_00257	+	+	-	
ven11	FVRRES_09359	+	+	-	
ven12	FVRRES_11058	+	-		
ven13	FVRRES_11058	+	-		
ven14	FVRRES_11296	+	+	+	+
ven15	FVRRES_11296	+	+	+	+
ven16	FVRRES_12025	+	-		
ven17	FVRRES_00025	+	+	-	
ven18	FVRRES_00025	+	+	-	
ven19	FVRRES_12550	+	+	-	
ven20	FVRRES_12550	+	+	-	

3.3.4.1 Intragenus Test

Oligonucleotide pairs were first trialled for specificity among *Fusarium*

spp. with amplification only desired in *F. venenatum.* Out of twenty oligonucleotide pairs assessed, a majority (18) showed sufficient specificity, failing to generate amplicons from genomic DNA from other test *Fusarium* species (*F. graminearum*, *F. oxysporum* and *F. solani*) (Table 3.4). For example, oligonucleotide pairs ven1 and ven2 showed strong amplicon production across all tested annealing temperatures (Figure 3.3). Successful oligonucleotide pairs advanced to the next stage of testing.

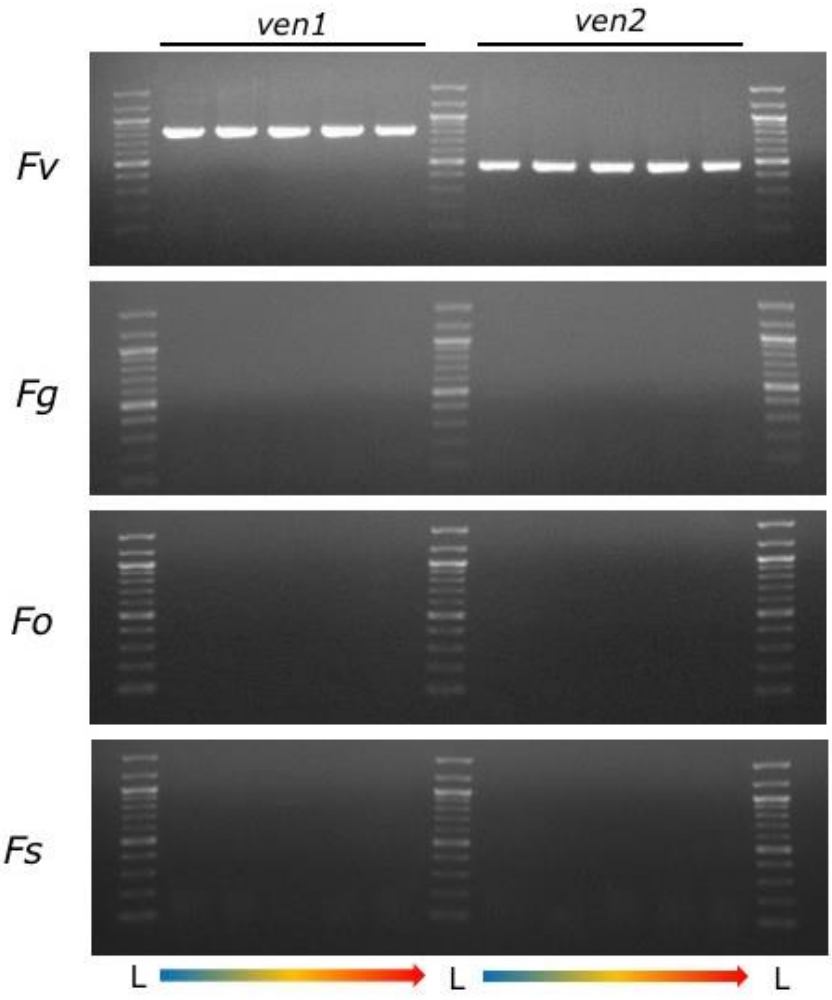


Figure 3.3. Intragenus test results using model primer pairs. Fv = *F. venenatum*; Fg = *F. graminearum*; Fo = *F. oxysporum*; Fs = *F. solani*. Arrows indicate 2 °C increases in annealing temperature between wells (59 - 67 °C). Amplicon sizes were 831 (ven1) and 476 bp (ven2). A 100 bp ladder (L) was loaded alongside samples.

3.3.4.2 Intergenus Test

Oligonucleotide pairs were next trialled for specificity against DNA extracted from filamentous fungal genera common in soil, namely *Mucor hiemalis*, *Penicillium viridicatum, Rhizopus oligosporus* and *Aspergillus niger*. Out of 18 oligonucleotide pairs tested, 15 were deemed appropriate for further testing due to lack of production of non-specific amplicons of the predicted size for *F. venenatum* (Table 3.4). However, some PCR products of different sizes were sometimes produced. For example, aberrant amplicons were generated from *T. koningii* and *P. viridicatum* using primer pair ven1, particularly at lower temperatures. However, their sizes (mainly over 1,000 bp) differed from the size (831 bp) expected for *F. venenatum* (Figure 3.4). Successful oligonucleotide pairs advanced to the next stage of testing.

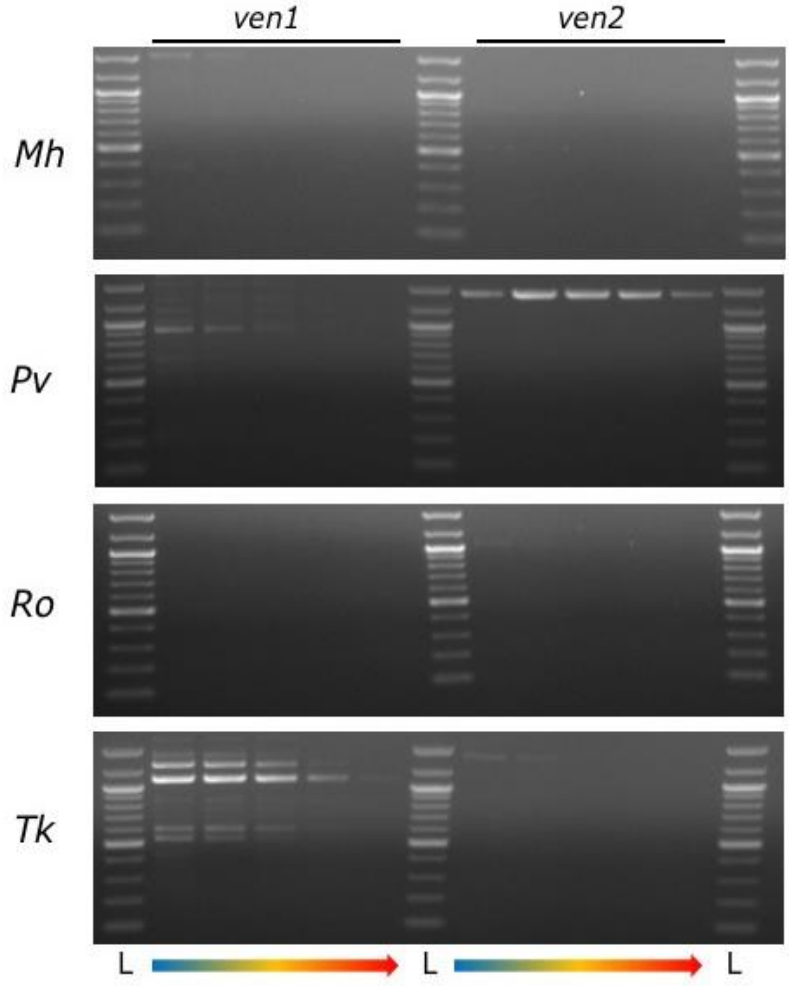


Figure 3.4. Intergenus test results using model primer pairs. Arrows indicate 2 °C increases in annealing temperature between wells (59 – 67 °C). Expected amplicon sizes for *F. venenatum* were 831 (ven1) and 476 bp (ven2). A 100 bp ladder (L) was loaded alongside samples.

3.3.4.3 Diagnostic Sensitivity Test

To ensure that the molecular diagnostic was able to detect low levels of *F. venenatum* DNA (given the apparent rarity of the species) the different oligonucleotide pairs were trialled for the ability to generate amplicons from DNA extractions from soil samples spiked with *F. venenatum* spores. This test proved to be the most revealing for oligonucleotide suitability, with only 4 of the 15 oligonucleotide pairs able to successfully amplify the lowest concentration of *F. venenatum* DNA in the samples (Table 3.4). For example, oligonucleotide primer pairs ven1 and ven2 were able to generate amplicons in samples spiked with only 10 macroconidia/0.25 g soil (Figure 3.5).

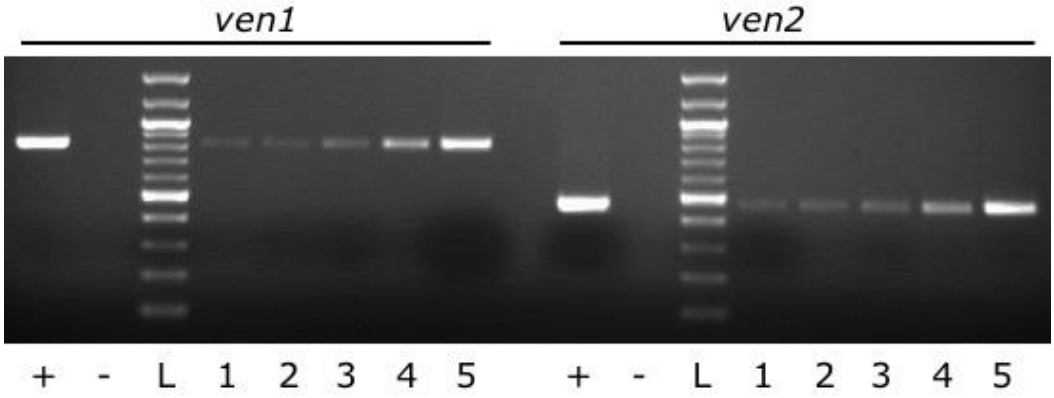


Figure 3.5. Sensitivity trial for soil molecular diagnostic test for *F. venenatum* DNA. Labels indicate number of macroconidia added per sample: 1 = 10 spores, 2 = 100 spores, 3 = 1000 spores, 4 = 10,000 spores, 5 = 100,000 spores. + = A3/5 genomic DNA; - = non-inoculated autoclaved soil. Predicted amplicon sizes were 831 (ven1) and 476 bp (ven2). A 100 bp ladder (L) was loaded alongside samples.

3.3.4.4 Intraspecies Compatibility Test

The diagnostic had thus far been assessed only using genomic DNA extracted from the *F. venenatum* production strain A3/5. Further trial PCR using DNA extracted from an additional four strains of *F. venenatum* revealed that all four remaining oligonucleotide pairs showed the predicted amplicon production (Table 3.4). For example, PCR with primer pairs ven1 and ven2 generated the predicted PCR products, although each additional strain except 114-3 showed some aberrant amplification not observed in A3/5 (Figure 3.6). Following this

assessment of the oligonucleotides, 67 °C was chosen as the annealing temperature most suitable for the diagnostic when using oligonucleotide pairs ven1 and ven2.

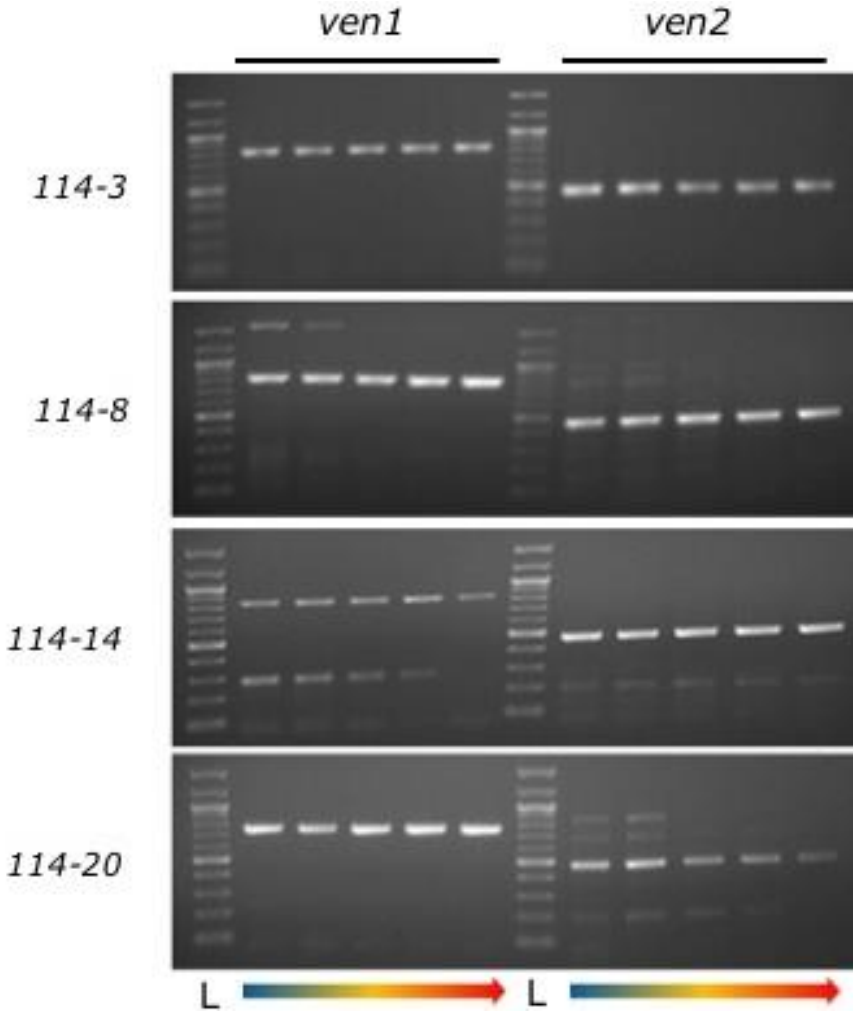


Figure 3.6. Intraspecies test of molecular diagnostic test for *F. venenatum* to ensure that primer pair ven1 and ven2 are not strain-specific. Arrows indicate 2 °C increases in annealing temperature between wells (59 - 67 °C). Predicted amplicon sizes were 831 (ven1) and 476 bp (ven2). A 100 bp ladder (L) was loaded alongside samples.

3.3.4.5 Screening Soil Samples by Molecular Diagnostic

DNA was extracted from 32 soil samples of varying origin and then screened by PCR using the ven1 diagnostic oligonucleotide pair that had proved successful in all development tests (Table 3.4). Almost all samples (31/32) failed to produced amplicons. However, a single soil sample demonstrated consistent generation of an amplicon of the correct size – soil 17 from Lincolnshire, UK (Figure 3.7).

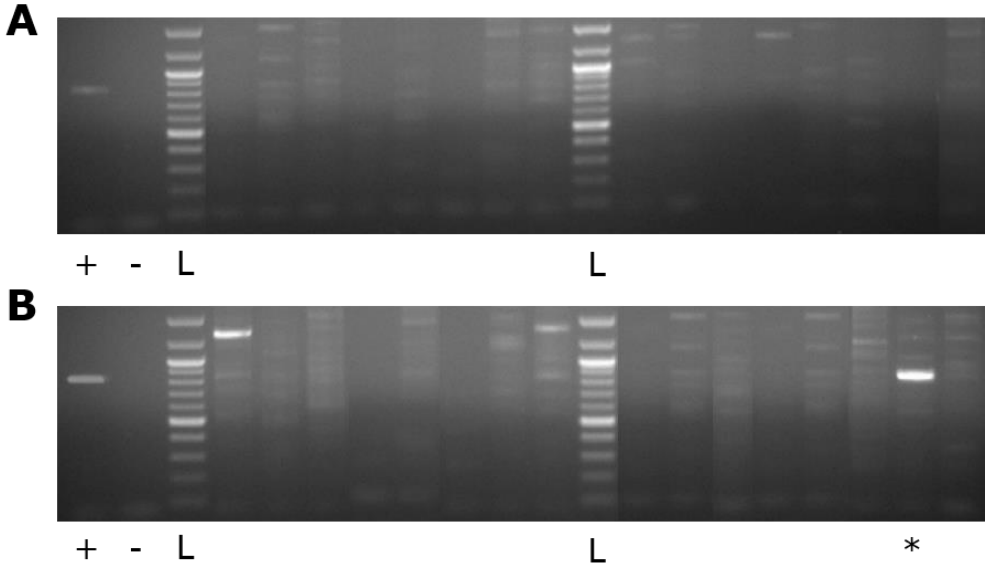


Figure 3.7. Results of screening of soil samples for presence of *F. venenatum* using primer pair ven1 as a molecular diagnostic. A = Soil samples collected throughout the project. B = Soil samples obtained from alternative sources. Expected amplicon size of positive sample was 831 bp (ven1). + and – indicate positive (A3/5 gDNA) and negative (SDW) controls respectively, and asterisk indicates soil sample 17. A 100 bp ladder (L) was loaded alongside samples.

3.3.4.6 Targeted Sampling Approach

Successful amplification from soil 17 required that further soil sampling was performed in the original area of isolation. 7 samples (SL_042 – SL_048; Supplementary Table 3.1) were obtained from North Kesteven, Lincolnshire, comprising mostly of agricultural soils. Each sample underwent DNA diagnostic analysis as described previously. In this case, sample SL_048 was the only sample which produced an amplicon when subjected to the diagnostic. Thorough isolation using classic cultural methods (**Sections 3.2.5.1, 3.2.5.3**) from this soil sample was

then undertaken, ultimately leading to the retrieval of 200 fungal isolates from the soil. Of these, 37 produced macroconidia and so were processed further at the molecular level (**Section 3.2.5.4**). Following sequencing of TEF1α fragments from these isolates, 34 isolates were revealed to be *F. redolens* while a single isolate of *F. culmorum* was also retrieved. However, no *F. venenatum* was isolated.

3.3.5 Applications of the Molecular Diagnostic

Two experiments were performed to test if the molecular diagnostic using primer pair ven1 could be used to define the specific presence of *F. venenatum* in Quorn food products. First, genomic DNA was extracted from raw *F. venenatum* mycoprotein paste and liquid cultures of *F. venenatum* as well as from fungi used in other fungal foodstuffs (*Neurospora* spp., *Rhizopus oligosporus*). Results confirmed that this diagnostic could be used to distinguish *F. venenatum* from other food fungi, with a distinctive major band in the Quorn™ paste sample and only minor nonspecific amplification witnessed in the *Neurospora* sp. (Figure 3.8). The amplicon derived from the paste sample was extracted, sequenced, and aligned with the amplicon from the mycelial control. Total alignment was observed, confirming that the source of Quorn™ mycoprotein was *F. venenatum*. The diagnostic could therefore be used for the identification purposes required by the FCC.

A further experiment was then conducted to determine whether *F. venenatum* DNA could be retrieved by PCR with the molecular diagnostic from various Quorn™ commercial products that would have undergone extensive and varied processing since the Quorn™ paste stage. Amplification using primer pair ven1 was successful for DNA samples from Quorn quarter pounders and mince, but not from Quorn pieces and smoky ham free slices (Figure 3.9).

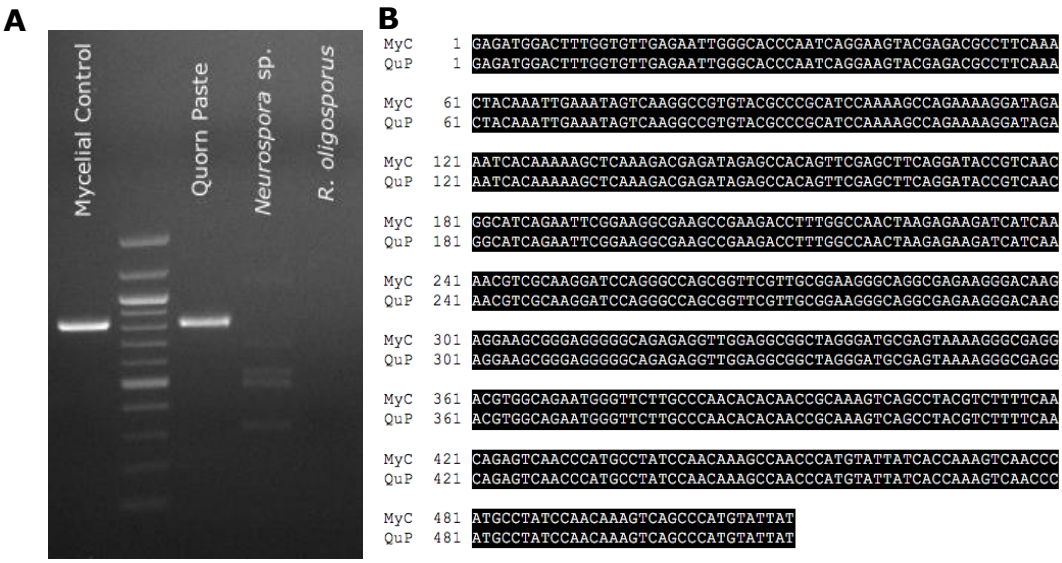


Figure 3.8. Use of molecular diagnostic to identify *F. venenatum* from other fungi used in food production. A. Paste-extracted DNA generates the same amplicon as that derived from culture-derived DNA of *F. venenatum*. B. Sequenced DNA amplicons are identical as determined by multiple sequence alignment. Expected amplicon size was 831 bp (ven1). A 100 bp ladder (L) was loaded alongside samples.

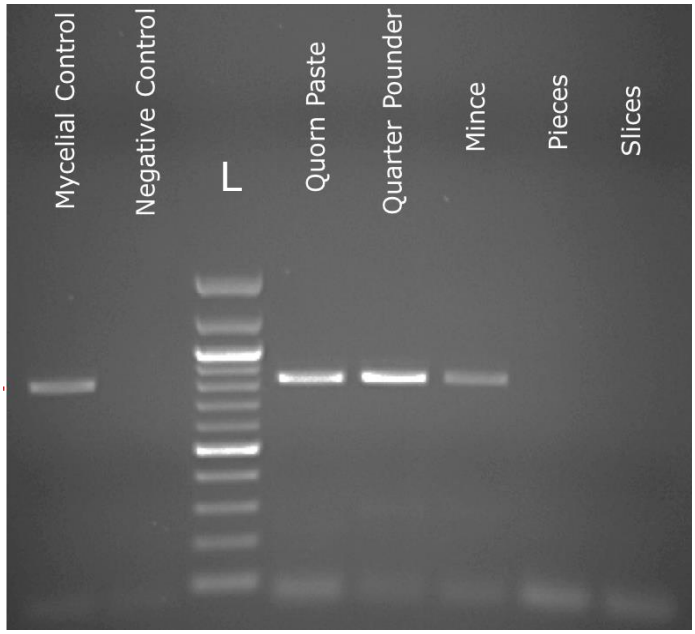


Figure 3.9. Use of molecular diagnostic to test for the presence of *F. venenatum* DNA in various commercial products manufactured by Quorn™. Expected amplicon size was 831 bp (ven1). A 100 bp ladder (L) was loaded alongside samples.

3.4 Discussion

The genus *Fusarium* is a prime example of a successful fungal lineage, widely distributed among different soil types worldwide. Many reports have confirmed that *Fusarium* is one of the most common filamentous fungal genera found in agricultural soils (Thorn, 1997; Nesci et al., 2006; Silvestro et al., 2013). Several species are particularly cosmopolitan, with *F. equiseti*, *F. oxysporum* and *F. solani* frequently isolated across the globe e.g. reports from South Africa (Marasas et al., 1988), the UK (Bateman and Murray, 2001), Kenya (Maina et al., 2009) and Argentina (Silvestro et al., 2013). Work in the present chapter aimed to establish a collection of *F. venenatum* isolates for subsequent use in PhD work, drawing on both established culture collections and novel environmental sampling.

3.4.1 Establishment of Culture Collection

The Quorn™ fungus was originally identified as *F. graminaerum* and was therefore said to be a relatively common soil fungus. However, since its reclassification by O'Donnell et al. (1998), *F. venenatum* has rarely been reported from studies investigating the composition of *Fusarium* communities in agricultural soils. Indeed, *F. venenatum* was more often associated with plant debris (Table 3.1; Dorn et al., 2009; Postic et al., 2012; Edel-Hermann et al., 2015; Stefańczyk et al., 2016). Despite this, it was possible to obtain 20 putative independent isolates of *F. venenatum* from international culture collections and personal research contacts (Table 3.3), achieving a key goal of this chapter.

By contrast, efforts to obtain novel isolates of *F. venenatum* from environmental sampling proved unsuccessful. Out of a total of 70 *Fusarium* isolates obtained from 48 soil samples, no *F. venenatum* strains were found despite sampling from a wide variety of environments and geographic sites – including from the region (Marlow, UK) where the Quorn strain of *F. venenatum* was originally isolated. This finding is consistent with the hypothesis that *F. venenatum* is in fact a relatively

rare, rather than ubiquitous, species. The fusaria that were obtained from these soil samples were representative of common *Fusarium* species seen in other soil community surveys. It is noted that there might have been some redundancy within this collection given that multiple isolates of the same strain might have been obtained from some sites e.g. the *F. redolens* isolates obtained from soil sample SL_048.

It is noted that identification of isolates was complicated by the nature of *Fusarium* phylogeny, whereby a single *Fusarium* taxon can represent a number of so-called species complexes. Given this convolution, it is likely that some *Fusarium* species described in classical studies have been misidentified and have maintained the incorrect species moniker, because studies were conducted before the emergence of rapid DNA sequencing methods. Indeed, Laraba et al. (2021) revealed that two *Fusarium* isolates previously identified as *F. culmorum* and *F. cerealis* were in fact *F. venenatum* based on genomic analysis. As with the original classification of the Quorn™ strain, both of these misidentified strains belonged to the *Graminearum* clade of the *Fusarium sambucinum* species complex (FSAMSC), suggesting that some erroneous classifications might still exist.

It is worth noting that *Fusarium* isolates comprised less than 5% of the ca. 1,500 fungal colonies retrieved from soil samples in the present study, an unexpected outcome considering the supposed ubiquity of the genus *Fusarium,* particularly in agricultural soils. Despite the application of MGA as a selective medium, common fungi such as *Mucor* spp. were isolated in great numbers. This suggests that the isolation protocol is suboptimal for the retrieval of *Fusarium* spp. from soil. Moreover, while many *Fusarium* species produce macroconidia, a number (e.g. *F. langsethiae*, *F. poae*) do not, instead producing unicellular microconidia. As the occurrence of macroconidia was a key screening step, isolates incapable of generating macroconidia will have been biased against during the screening process, reducing the total number of identified

isolates of *Fusarium*, which might have also included low-sporulating strains of *F. venenatum.*

Several hypotheses can be proposed regarding why efforts to isolate *F. venenatum* from soil samples proved unsuccessful. First concerns its supposed ubiquity. The fungus was previously classified as the phytopathogen *F. graminearum*, a cosmopolitan species commonly found in soil associated with wheat and other crops (Yang et al. 2013, Marburger et al. 2017). Erroneous classification of the fungus for 30 years would have meant characteristics associated with *F. graminearum*, such as ubiquity in soil, were also attributed to the Quorn fungus. Upon reclassification in the late 1990s, these traits that were now coupled with the Quorn fungus were also now assumed to be 'true' of *F. venenatum*. This would suggest that the reported commonality of *F. venenatum* in the environment is simply the result of a decades-long Telephone Game.

A second hypothesis posits that the fungus was widespread at the original time of isolation (late 1960s) but subsequent changes to the environment and/or soil microbiome composition have since seen *F. venenatum* communities decline in competitiveness. There have been many studies describing shifts in the soil microbiome as a consequence of changing agricultural practices/land use (Bevivino et al. 2014, Wakelin et al. 2008, Wehr et al. 2019 and Lacerda-Júnior et al 2019). In addition, the nature of flora found in the British Isles has shown changes since the original isolation of *F. venenatum* with the introduction of invasive plant species having notable effects on soil microbial communities (Manchester and Bullock, 2000). It is possible therefore, that the plant variety found in British gardens today are considerably different to those in the past. Given that *F. venenatum* was originally isolated in a compost heap, this may suggest that the Quorn™ fungus was associated with garden plant species more common in the 1960s than today.

The original isolation of *F. venenatum* from a compost heap poses questions about the ecological niche of the fungus in the environment.

The heterogeneous nature of the compost environment can sustain a variety of lifestyles, and thus an isolated organism could be a saprotroph, a phytopathogen (opportunistic or otherwise) infecting plant material deposited in the compost, or even a symbiont in association with a plant rhizosphere. Furthermore, the lack of a single consensus 'host' plant from which *F. venenatum* isolates have been retrieved continues to confound our understanding of the fungus and its environmental niche. This lends itself to a third potential hypothesis, that *F. venenatum* is a secondary invader, exploiting weaknesses caused by other more dedicated phytopathogenic species, which are more likely to be present in the environment than *F. venenatum* itself. Non-pathogenic *Fusarium* species have been found to be associated with infected banana crops displaying symptoms of Fusarium wilt, suggesting that *Fusarium* complexes of varying lifestyle can exist within a diseased plant (Maryani et al. 2019). While *F. venenatum* has shown limited pathogenicity on tomato and wheat crops, coinfection studies with other plant pathogens are yet to be performed (King et al. 2018). Validity of this secondary invader hypothesis could be tested through the coinfection of several plant crop species with *F. venenatum* together with other known phytopathogens, both within the *Fusarium* genus. and outside for example *Pyrenopeziza brassicae* and *Magnaporthe oryzae*.

This third hypothesis is strengthened by genetic evidence that *F. venenatum* is a latent phytopathogen or had this capability in the past. The genome of *F. venenatum* has been shown to have a predicted functional profile resembling floral cereal pathogens within the *Fusarium* genus (*F. graminearum, F. verticillioides* and *F. langsethiae;* King et al. 2018). Furthermore, direct comparison of the *F. graminearum* and *F. venenatum* genomes revealed that the two species possess an almost matching set of so-called PHI-base genes, identified as playing a role in pathogen-host interactions (King et al. 2018, Urban et al. 2020). The crucial difference between the two fungal species is that *F. venenatum* lacks two genes compared to *F. graminearum,* GzZC120 (FGRRES_08028_M) and GzZC305 (FGRRES_00147). Both genes are

putative transcription factors with a role in amplifying virulence. Indeed, when these genes are deleted in *F. graminearum*, the pathogen displayed reduced virulence towards wheat heads (Son et al. 2011). In addition, five of the seven secondary metabolite biosynthetic gene clusters expressed during asymptomatic establishment of *F. graminearum* wheat head infections were found to be absent or highly divergent within the *F. venenatum* genome. Reintroduction of these genes by genetic transformation with subsequent transcription and pathogenicity studies may reveal if these genes can restore dormant pathogenicity within *F. venenatum*, clarifying its function in the environment.

3.4.2 Establishment of Molecular Diagnostic

High-throughput molecular diagnostic strategies for the detection of fungal species have been implemented in a variety of applications, particularly for the early diagnosis of invasive fungal diseases (IFDs; Kidd et al. 2020) or phytopathogens (Hariharan & Prasannath 2021). These assays adopt one of two approaches; “panfungal” diagnostics which aim to detect the presence of “any fungus” or those which detect specific genera or species.

The failure in this project to isolate *F. venenatum* using classical cultural-based methods necessitated the development of an alternative approach given the high time demands of such culture-based methods. Therefore, it was decided to develop a more high-throughput molecular diagnostic capable of specifically detecting *F. venenatum* within soil samples. The development of this diagnostic faced a number of challenges from the outset, namely species specificity, reliability, sensitivity, and subsequent isolation of the desired strain via classical cultural means. To tackle the issue of species specificity, secondary metabolite genes belonging to unique clusters in *F. venenatum* (when compared to *F. graminearum)* were chosen as amplification targets (King et al. 2018). This strategy proved successful, with almost all primer sets only producing amplicons with *F. venenatum* rather than other test

Fusarium species (Table 3.4). The primer sets also showed good specificity to *F. venenatum* as opposed to other common soil fungi in the intergenus testing and were able to produce amplicons from all test strains of *F. venenatum.* The most discriminatory results for primer sets were obtained from the sensitivity testing. This led to the selection of primer pairs ven1 and ven 2 (both of which amplify different regions of the same gene FVRRES_00023) which were able to detect the presence of *F. venenatum* in soil samples spiked with as little as 10 macroconidia/0.25 g soil.

The molecular diagnostic using primer pair ven1 was then applied to DNA extracted from 32 soil samples from throughout the UK, including samples acquired from Marlow, Buckinghamshire (the original area of isolation of strain A3/5). Sadly, almost all samples failed to produce the desired amplicons. A putative *F. venenatum* signal was detected in a single soil sample from Lincolnshire, UK but despite intensive cultural efforts it was still not possible to isolate a novel strain of *F. venenatum.* In that sense the outcome of the development of the molecular diagnostic was disappointing. However, there were two positive outcomes. First, the failure to detect *F. venenatum* using the sensitive molecular tests confirmed the results of the culture-based studies that *F. venenatum* is a relatively rare fungus, at least in UK soils. Second, the molecular diagnostic was able to differentiate Quorn mycoprotein from other fungal foodstuffs, fulfilling a requirement of the Food Chemicals Codex (FCC) of the United States which had requested a test that would allow distinction of Quorn™ mycoprotein from other fungal foodstuffs. This outcome was of great benefit to the industrial sponsor partner of the present PhD.

3.4.3 Critique and Further Development of Molecular Diagnostic

First, a control “pan-fungal” primer was not implemented routinely in the study. Such a primer would have been designed to amplify a genomic

region present across fungi, and thus would demonstrate that DNA extraction from soil was successful. There are several candidates which could have been implemented, such as primers which target the internal transcribed space region (Bellemain et al. 2010). In most cases, PCR products were found when investigating soil samples suggesting that DNA extraction had occurred. This approach assumes that the DNA had originated from fungi, rather than other organisms in soil (e.g. bacteria) or were contaminations.

It is noted that the diagnostic itself relies on the sequencing of a single gene using conventional PCR amplification. While a cost-effective approach when compared to other diagnosis options, it is nevertheless cautioned that this method might still encounter difficulty in discriminating between closely-related fungal species beyond the three test species trialled. This is compounded by the diversity of species of the *Fusarium* genus and the convoluted nature of its phylogeny. Thus, comparing amplicon generation of four test *Fusarium* sp. may not have sufficiently tested whether the target gene is suitable for species-specific detection. This lack of species coverage might explain why the application of the diagnostic with the North Lincolnshire samples resulted in the isolation of 34 *F. redolens* colonies from the environment.

The Phusion DNA polymerase was the enzyme of choice for this diagnostic (New England Biolabs). Other enzymes, such as Phire II, are reputed to produce stronger PCR signals might have been used, but the 5'-3' proofreading activity of Phusion was desirable. Using Phusion, PCR products from environmental samples could be purified and subsequently sequenced to confirm the identity as *F. venenatum*. As a consequence, the development steps of the diagnostic also had to implement this enzyme to ensure compatibility.

Finally, there was varied success when extracting DNA from shop-bought Quorn™ products. Interestingly, it might have been assumed the pieces and slices were the least processed of the goods considered and thus amplification was more likely from those products. This might be

explained by a particular additive included in the formulation of these products which is inhibitory to the amplification of the sequence. Such limitations have been observed in studies aiming to amplify DNA from food samples suspected to be contaminated with pathogens (Rossen et al., 1992).

In spite of the limitations of using conventional PCR in this application, the target genes chosen for use in the final diagnostic (FVRRES_00023; primer pairs ven1) hold great promise for future further development of the diagnostic. BLASTn analyses showed that each gene was unique to *F. venenatum*, with related sequences in other fungi showing considerable divergence. Therefore, targeting of these genes by more sophisticated techniques is likely to improve diagnostic capability. For instance, the accuracy of detection of fungal isolates from the field has been greatly improved by utilising loop-mediated isothermal amplification (LAMP) methodology (Le & Vu 2017; King et al. 2018). LAMP reactions consist of an initial annealing step, cycling amplification, and then an elongation step. Rather than relying on a single primer pair, LAMP utilises four to six primers which recognise six unique sequences within the target nucleic acid. The activity of these primers facilitates the formation of loop structures within the DNA, allowing the amplification of a small gene fragment at a single temperature. LAMP assays have been implemented with great success for the rapid species-specific detection of numerous fungi, as well as seeing use the assessment of other genetic characteristics, such as fungicide resistance and mating-type determination (King et al. 2019).

4

We Are Family: Phylogenomic Analysis of *Fusarium venenatum*

4.1 Introduction

4.1.1 Genome Sequencing in Fungal Biotechnology

At the dawn of the genomics era, several fungal species paved the way for this important technology. Of these, important models with long histories of genetic and molecular research were the first, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Neurospora crassa* (Goffeau et al., 1996; Wood et al., 2002; Galagan et al. 2003). With the launching of the 1000 Fungal Genomes programme established by the US Department of Energy Joint Genome Institute, great efforts have been made to capture the metabolic diversity of fungal strains in medicine, agriculture and biotechnology (http://jgi.doe.gov.fungi).

Before the introduction of this technology, a number of biotechnological processes were already established at the industrial level. Genome sequences revealed information on the biosynthesis of important metabolites such as citric acid and penicillin, which allowed further development of these industrial processes beyond alterations to fermentation strategies and nutritional optimisation. For these processes, genome sequencing enables understanding and improvement from the level of the genotype. In addition, sequencing

novel genomes opens the possibility for discovery of novel metabolites for future industrial processes. Importantly, sequencing fungal genomes has revealed metabolites previously undiscovered due to “synthetic silence” during standard cultivation conditions (Brakhage 2013). *In silico* identification of a polyketide synthase (PKS) or non-ribosomal peptide synthetase (NRPS) gene and analysis of flanking genes can be used to hypothesise that a particular genome region may contain a biosynthetic cluster (Bergmann et al. 2007). For this, genomic data is processed using bioinformatic algorithms such as SMURF, antiSMASH and DEREPLICATOR+ to predict biosynthetic clusters (Khaldi et al. 2010; Sanchez et al. 2011; Mohimani et al. 2018). This information can then be implemented in genetic engineering strategies to unlock this hidden potential. Aspyridones are cytotoxic compounds that were previously described as the cryptic *apd* biosynthetic gene cluster in *A. nidulans*. Overexpression of the *apdR* transcription factor gene awoke this silent cluster and led to the discovery of aspyridones (Bergmann et al. 2007).

Industrial citric acid production was originally performed by extraction from lemon fruits, beginning in England around 1826 (Show et al. 2015). Under a century later, James Currie discovered that *Aspergillus niger* could produce the organic acid during batch fermentation when grown at low pH (Currie, 1917). Upscaling this process rapidly displaced the labour-intensive lemon extraction by the late 1920s. Citric acid is now one of the most pervasive additives in human society, used in a variety of industries with global production estimated to be approximately 2.1 million tonnes and a market valued at $3.6 billion in 2020 (Ciriminna et al. 2017; Börekçi et al. 2021). Since the publication of the *A. niger* genome (strain CBS 513.88) in 2007, considerable progress has been made in identifying and engineering the genes responsible for regulation and transport of this important metabolite (Pej et al., 2007; Niu et al., 2015; Steiger et al., 2019).

Similarly, the discovery of penicillin was a critical development during World War II providing an effective treatment against wartime infection.

Genomic analyses of Fleming's strain uncovered its species identity, which then revealed that strains with increased penicillin production featured a gene duplication event of the biosynthetic cluster (Pathak et al., 2020). Early penicillin fermentations with *Penicillium chrysogenum* were contaminated with yellow-coloured metabolites now known to be sorbicillionoids, with strain improvement strategies aimed at preventing their synthesis (Salo et al., 2015). Genomic mutational analysis identified the polyketide synthetase gene cluster responsible for the synthesis of these contaminants and later their characterisation (Salo et al., 2015; Guzmán-Chávez et al., 2017). These contaminants demonstrate interesting pharmacological activities themselves, for example in the treatment of HIV-1 and influenza A (Nicoletti and Trincone, 2016).

To date, the Quorn™ industrial organism *Fusarium venenatum* has had 4 genome assemblies published covering 3 strains, available in the NCBI database. By comparison, other fungi used in biotechnology have been more thoroughly investigated. For instance, *Trichoderma reesei* and *Aspergillus niger* have 11 and 23 genome assemblies available in the NCBI genome databases covering 6 and 23 strains respectively. Unlike many other fungi important to biotechnology, commercial interest in *Fusarium venenatum* is in the accumulation of proteinaceous biomass, rather than the production of metabolites. Nevertheless, there are several traits of great importance for future development of the industrial organism. Of particular interest to this study are the *MAT* identity in addition to the trichothecene biosynthetic clusters, both potential routes for strain improvement.

4.1.2 MAT Loci in Filamentous Fungi

Ascomycetous fungi can propagate themselves by undergoing asexual or sexual reproduction. While many species exhibit asexual reproduction for the generation of clonal offspring, fewer demonstrate sexual reproduction. In recent years, significant endeavours in the discovery of cryptic sexual cycles in previously “asexual” filamentous fungi has taken place (Dyer and O’Gorman, 2011). The sexual cycles of some biotechnologically important fungi have been realised, of particular note is *Trichoderma reesei* (teleomorph: *Hypocrea jecorina*) where the sexual cycle has been implemented to compound strain development programmes (Seidl et al. 2009). Additionally, the sexual lifestyle of the cell factory *A. niger* has continued to evade researchers, despite several publications describing features suggestive of a sexual cycle (Pej et al., 2007; de Vries et al., 2017).

Sexual identity in the Pezizomycotina is controlled by a regulatory locus termed the mating type (*MAT*) locus (; Turgeon 1998). Depending on the gene arrangement at these loci, fungal species may be termed heterothallic or homothallic. Heterothallic species can be described as “self-sterile”, owing to the requirement of a partner to undergo sexual reproduction. Compatible partners in heterothallic couplings comprise of an isolate carrying the *MAT1-1* locus and another carrying the *MAT1-2* locus. These loci, termed idiomorphs, are genetically distinct, containing a different set of genes yet are located at the same locus in the genome (Metzenberg and Glass, 1990). The structures of these loci are species-specific but, in each locus at least one transcriptional regulator gene is conserved across the Pezizomycotina. The *MAT1-1* sequence contains an alpha-box-containing gene named *MAT1-1-1* which encodes a protein homologous to the *S. cerevisiae* MATα1 protein (Mahoney and Broach, 1989). Conversely the *MAT1-2* structure encodes a *MAT1-2*-1 gene containing a high mobility group (HMG) DNA-binding domain encoding a MATa1 homologue. Homothallic species are “self-fertile” and thus can undergo sexual reproduction by themselves. As shown in

Figure 4.1, homothallic species contain both *MAT1-1* and *MAT1-2* loci within a single genome, either at a single locus or dispersed in separate locations.

As described, *MAT* loci structure is species-specific, yet *MAT1-1-1* and *MAT1-2-1* homologues are conserved. Other genes can be found within the *MAT* loci although the contribution of these auxiliary genes to sexual reproduction varies on the gene in question (Dyer et al., 2016). For instance, heterothallic *Fusarium* species, like *F. venenatum*, contain 3 genes in their *MAT1-1* locus; *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3* (King et al. 2018). The *MAT1-2* often only contains the conserved *MAT1-2-1* gene yet some fusaria such as members of the *Fusarium fujikuroi* species complex (e.g. *F. asiaticum*) contain a *MAT1-2-9* gene (Montoya-Martinez et al. 2019). These “secondary” genes display a range of functions and are hypothesised to have finer control of sexual reproduction than the conserved *MAT1-1-1* and *MAT1-2-1* genes (Yu et al., 2017).

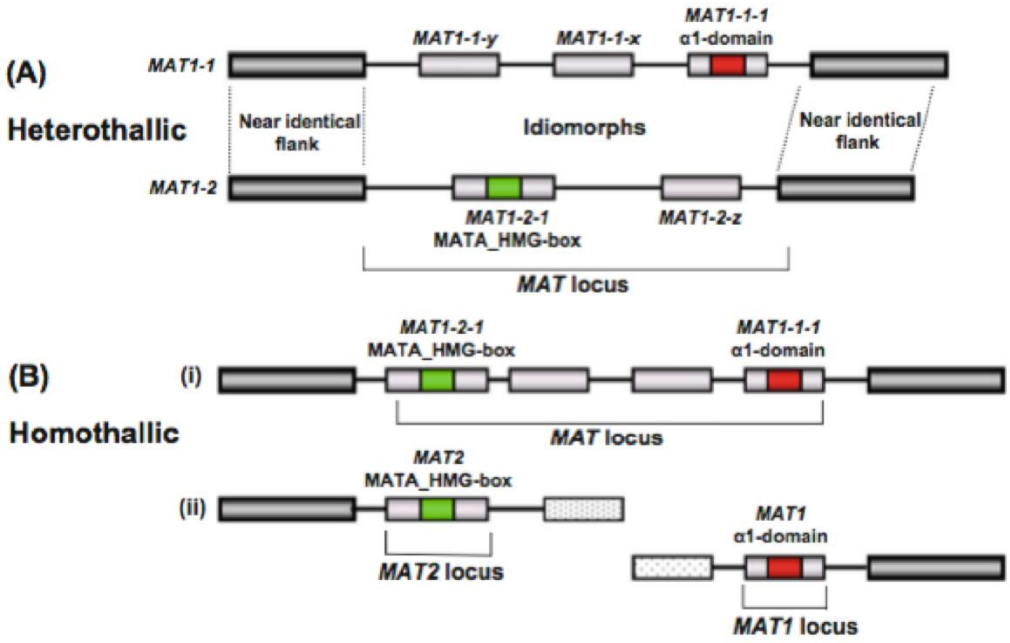


Figure 4.1. The structure of the varied *MAT* loci in the Pezizomycotina. The *MAT* regions are flanked by regions showing limited divergence. A. Canonical structure of *MAT* loci in heterothallic species. *MAT1-1* and *MAT1-2* loci each contain a conserved homologue, *MAT1-1-1* and *MAT1-2-1* respectively. In addition, other genes may feature in the *MAT* loci but these are often genus or species specific. B. Genomes of homothallic species feature both *MAT1-1-1* and *MAT1-2-1* genes. The *MAT* idiomorphs in these isolates may be located at a single locus (i) or arranged in separate positions (ii). Figure taken from Dyer et al. (2016).

4.1.3 Trichothecene Biosynthetic Clusters in Fusarium spp.

In *F. venenatum*, the trichothecene biosynthetic genes are dispersed between several loci (Figure 4.2). The main gene cluster is comprised of 12 genes encoding the fundamental biosynthetic pathway, efflux pumps and transcription factors (Table 4.1). There is a second minor cluster containing two genes, *Tri1* and *Tri16*, but the remaining genes are found in isolated positions across the genome.

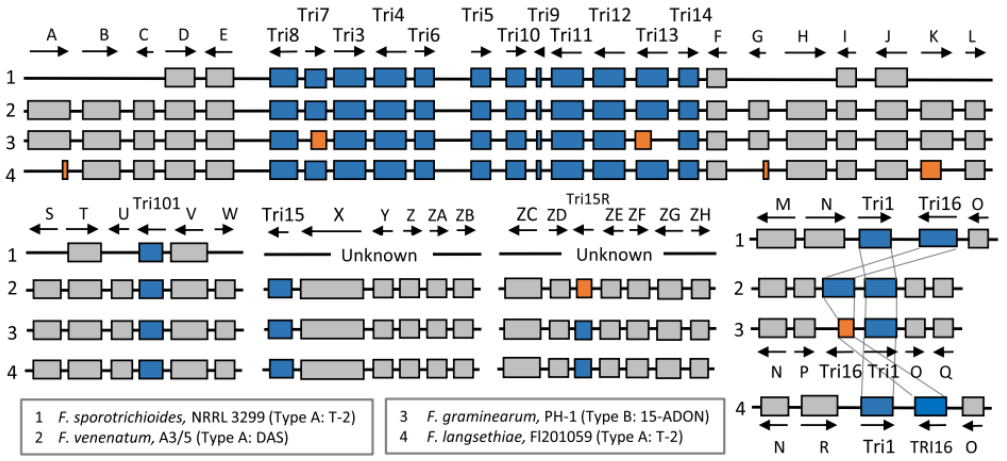


Figure 4.2. *Tri* gene arrangement in *F. sporotrichioides*, *F. venenatum*, *F. graminearum* and *F. langsethiae*. Trichothecene genes are indicated in blue boxes, whereas gene deletions or loss of function are orange. Grey boxes display conserved, flanking non-*tri* genes. Figure taken from King et al. (2018).

The synteny of the main cluster is considerably conserved across *Fusarium* species belonging to different chemotypes, shown above between the Type A subgroup *F. sporotrichioides*, *F. venenatum*, *F. langesthiae* and the Type B producer *F. graminearum*. The minor *tri* locus shows a translocation between *F. venenatum* and *F. graminearum* relative to the T-2 producing strains. Interestingly, *F. venenatum* contains a full suite of genes except for a truncated *tri15R* sequence. Despite *F. venenatum* presenting a *tri* gene identity identical to *F. sporotrichioides*, there has been no evidence that the species is able to synthesise T-2 or HT-2 toxin (Laraba et al. 2021). These mycotoxins exist downstream in the biosynthetic pathway of DAS, the principal mycotoxin synthesised by *F. venenatum*. In *F. sporotrichioides*, conversion of DAS to T-2 toxin requires the activity of 4 gene products; *tri7, tri1, tri16* and *tri8* (McCormick et al. 2011). To date, there has been no investigation to determine if there are any major differences between these gene homologues in *F. venenatum* and *F. sporotrichioides*. Similarly, there is substantial overlap of the *tri* genes possessed by *F. venenatum* and *F. graminearum*, yet the former is unable to synthesise deoxynivalenol (DON), a type B trichothecene.

A solution to these discrepancies can be answered in part by considering *F. graminearum* chemotypes. Strains belonging to *F. graminearum* can

be organised in to 3 chemotype groups which demonstrate different trichothecene biosynthetic profiles. Isolates can show the NIV chemotype in which nivalenol is produced, the 3-ADON chemotype which produces DON and 3-acetylDON or the 15-ADON chemotype which produces DON and 15-acetylDON. The acetylated chemotypes demonstrate varying global prominence, with the 15-ADON chemotype found frequently in Canada while 3-ADON strains are predominantly found in Europe and China (Miller et al. 1991). These differences were originally linked with sequence variations in the TRI8 enzyme, but it has become increasingly difficult to link genotype and chemotype of *F. graminearum* strains (Alexander et al. 2011; Crippin et al. 2019). One study discovered a population of *F. graminearum* which exhibited the 3-ADON genotype, yet was unable to produce 3ADON, 15ADON, or NIV fusarenone X (4ANIV) (Gale et al. 2010). Genomic investigation into this strain revealed sequence variation in the *tri1* sequence which yielded a novel Type A trichothecene known as the NX-2 toxin, which was structurally identical to 3ADON yet lacked the C-8 keto oxygen (Varga et al. 2015). Thus, elucidation of intraspecies variation in mycotoxin biosynthesis cannot only rely on the presence or absence of genes but also sequence variation.

Table 4.1. Trichothecene biosynthetic genes in *Fusarium* spp. discovered to date.

Gene	Activity	Reference
Tri1	Hydroxylation at C-7 and C-8; 3,4,5-TAS → 3-acetylneosolaniol.	McCormick et al. (2006)
Tri3	Acetylation at C-15; 15-decalonectrin → calonectrin.	Garvey et al. (2009)
Tri4	Oxygenation of C-2, C-3, C-11, C-12 and C-13.	McCormick et al. (2006)
Tri5	Cyclisation of farnesyl pyrophosphate → trichodiene.	Hohn et al. (1986 & 1989)
Tri6	Encodes Cys_2His_2 Zn finger DNA-binding transcription factor.	Seong et al. (2009)
Tri7	Acetylation at C-4; 3,15-DAS → 3,4,15-triacetoxyscirpenol.	Lee et al. (2002)
Tri8	Deacetylation at C-3; 3-acetyl T-2 toxin → T-2 toxin.	McCormick and Alexander (2002)
Tri9	Putative trichothecene biosynthetic gene.	
Tri10	Regulatory gene of *tri4, tri5, tri6* and *tri101*.	Seong et al. (2009)
Tri11	Hydroxyl addition at C-15; isotrichodermin → 15-decalonectrin.	Alexander et al. (1998)
Tri12	Trichothecene efflux pump.	Alexander et al. (1999)
Tri13	Hydroxyl addition at C-4; calonectrin → 3,15-DAS.	Lee et al. (2002)
Tri14	Unknown function.	Shentu et al. (2015)
Tri15	Proposed negative regulator.	Alexander et al. (2004)
Tri15R	Unknown function.	King et al. (2018)
Tri16	Isovaleryl addition at C-8; 3-acetylneosolaniol → 3-acetyl T-2 toxin.	Peplow et al. (2003)
Tri101	Acetylation at C-3; isotrichodermol → isotrichodermin.	McCormick et al. (1999)

4.1.4 Aims

As discussed, there is limited genomic information on the biodiversity of *F. venenatum*, an important resource for any biotechnological process.

1. Firstly, we aimed obtain genome sequences for each of the alternative *F. venenatum* strains gathered earlier in the project in order to complement the lack of genome sequences available.

2. The second aim of this chapter was to *identify MAT1-2* strains of *F. venenatum* and subsequently use the genomic information to elucidate the structure of the *MAT1-2* locus. *F. venenatum* thus far has been described as a heterothallic species, with the Quorn™ industrial strain recognised as a *MAT1-1* strain (King et al., 2018). As a commercial product intended for human consumption, strain improvement through directed genetic modification is not an accessible technique. Therefore, alternatives to this, such as sexual reproduction, would be of great value to future strain improvement programmes

3. Finally, previous work in the project had demonstrated that there was variation in the synthesis of the mycotoxin diacetoxyscirpenol (DAS). Thus, the final aim of this chapter was to elucidate the structure and sequence of the trichothecene biosynthetic clusters in each of the alternative *F. venenatum* strains, identifying genomic changes which might have resulted in variant phenotypes.

6.2 Methods

6.2.1 Genome Sequencing of F. venenatum

Genomic DNA (gDNA) of each *F. venenatum* strain was harvested as described previously (Chapter 2 General Methods). Genomic sequencing necessitates pure DNA without contamination by proteins or RNA, as this will disrupt the sequencing process. To ensure that DNA preparations were sufficiently pure, the DNeasy Plant kit (Qiagen) was used to manufacturer's instructions. DNA concentration was verified by Qubit analysis performed by DeepSeq (University of Nottingham).

Library construction and Illumina sequencing was performed by Novogene Co. Ltd (Figure 6.3). This raw data was then mapped to a reference *F. venenatum* genome (GenBank assembly accession: GCA_900007375; King et al., 2018). A single strain, 114-19, failed to meet the required mapping threshold (85%) and so was unable to be mapped.

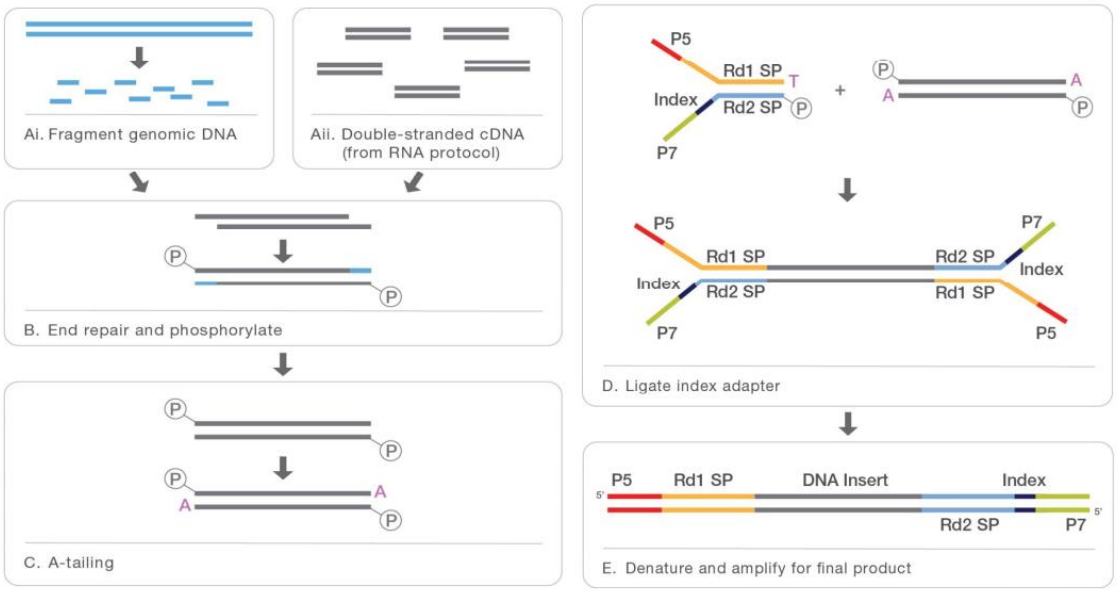


Figure 4.3. Novogene Illumina sequencing pipeline performed for each *F. venenatum* genome. Figure taken from Novogene Co. Ltd.

Once mapping was complete, a number of bioinformatic analyses were performed, focused on the differences between the query genome and the reference. These included single nucleotide polymorphism (SNP), insertion/deletion (InDel), structural variants (SVs) and copy number variation (CNVs).

For in-depth analysis of sequence variation, genome assemblies were prepared. Raw reads prepared by Illumina sequencing were assembled using SPAdes (thread value 3; Bankevich et al. 2012). The resulting contigs were then used in later analyses.

4.2.2 MAT Typing F. venenatum Strains

Sexual reproduction is an important feature for biotechnological species, as this method can be used to concentrate beneficial traits from two parental strains into a new strain. Furthermore, sexual reproduction can be a powerful non-GM methodology where regulations prevent implementation of GM techniques. In *F. venenatum*, the Quorn™ strain was described as a *MAT1-1* strain and the MAT loci of other strains have yet to be investigated (King et al., 2018).

4.2.2.1 MAT Identity Determination by PCR

In this assay, degenerate primers first developed by Kerenyi et al. (2004) for analysis of *Fusarium* species were used (Table 4.2). These primers were designed to amplify fragments of the alpha and HMG domains in the *MAT1-1-1* and *MAT1-2-1* genes respectively. As *F. venenatum* is a heterothallic species, these primer sets were expected to generate a single product per strain. In addition, *F. venenatum*-specific *MAT1-1* primers were designed using sequences from an A3/5 genome assembly provided by Andrew Armitage, East-Malling Research.

The diagnostic was performed using Phusion® High Fidelity DNA Polymerase (NEB, USA) in a final reaction volume of 20 µL (12.2 µL SDW, 4 µL 5X high-fidelity buffer, 0.4 µL 10 mM dNTP mix, 1 µL 10 mM forward primer, 1 µL 10 mM reverse primer, 50 ng DNA template and 1

U (0.4 µL) Phusion polymerase). The PCR cycling conditions were as follows: an initial denaturation step of 98 °C of 15 seconds, followed by 30 cycles of 98 °C for 10 seconds, 58 °C, 61 °C and 63 °C (fusALPHA, fusHMG and *MAT1-1* primers respectively) for 30 seconds and 72 °C for 20 seconds. A final elongation step of 72 °C for 5 minutes was performed. All reactions were performed using a Techne TC-5000 PCR Thermal Cycler. PCR products were subsequently analysed by gel electrophoresis with a 100 bp ladder used as reference for product length.

If strains generated *MAT1-2* amplicons, the PCRs were repeated as 50 µL reactions and subsequently purified using the Machery-Nagel Nucleospin Gel and PCR Clean-up kit to manufacturer's instructions. Purified products then underwent Sanger sequencing performed by DeepSeq (University of Nottingham).

Table 4.2. *Fusarium*-specific primers used in the amplification of the MAT locus alpha and HMG domains in *MAT1-1-1* and *MAT1-2-1* genes respectively.

Primer Set	Forward Primer Sequence	Reverse Primer Sequence	Approximate Product Size (bp)
fusALPHA	CGC CCT CTK AAY GSC TTC ATG	GGA RTA RAC YTT AGC AAT YAG GGC	200
fusHMG	CGA CCT CCC AAY GCY TAC AT	TGG GCG GTA CTG GTA RTC RGG	260
MAT1-1	AGC GAC TAC AAG AAC CAA CTC C	AGA GTG TTC GAA GGC ATG GG	948

6.2.2.2 Elucidation of the *MAT1-2* Locus

Once *MAT1-1* negative strains had been identified, cloning the *MAT* loci of these strains was necessitated. To do this, several primer pairs were designed to amplify the sequence between conserved genes *SLA2* and *APN1* (Table 4.3; Figure 6.4). By utilising a forward upstream primer and a downstream reverse primer, the full *MAT* locus should be retrieved. In strain A3/5 (114-1), the *in silico* prediction of amplicon length is between 8311 and 8370 bp depending on the primer combinations used.

Table 4.3. Primers used to elucidate the *MAT1-2* locus in strains which generated an amplicon in fusHMG PCRs.

Primer Set	Forward Primer Sequence	Reverse Primer Sequence
MAT12_U1	ACC AAG ATG CGA ACA TGA CG	GGT AGA GAC GCT TGT GCT CC
MAT12_U2	GTG ATG GTC CAA GTT GCT GG	CAG GAG TCT CAG CGT TCC AG
MAT12_U3	CTC CTG ACA GAG TGT TCG ACC	TCT CGT CGG CCT TCT TCT TG
MAT12_D1	CTA CCC TGA CTA CCA GTA CC	TAG GTC GAG ATC CTC CAA TTG G
MAT12_D2	GCC GTT ACT ACA AGA AGA AGG	GTC GAG ATC CTC CAA TTG G
MAT12_D3	TAA CTC TTG TGC AGC TCA GG	GAT GCG GAA GAT CTC TTA CC

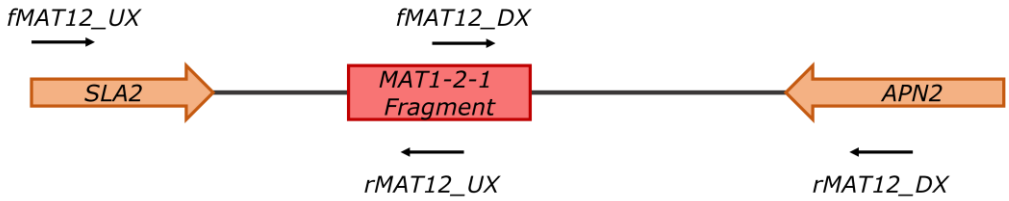


Figure 4.4. Primer position for the elucidation of the *MAT1-2* locus. The prefixes *f* and *r* refer to forward and reverse primers respectively. Arrows indicate approximate position of primers in reference to the flanking genes *SLA2* and *APN1* as well as the putative *MAT1-2-1* fragment.

The diagnostic was first performed using Phusion® High Fidelity DNA Polymerase (NEB, USA) in a final reaction volume of 50 µL (30.5 µL SDW, 10.0 µL 5X high-fidelity buffer, 1.0 µL 10 mM dNTP mix, 2.5 µL 10 mM forward primer, 2.5 µL 10 mM reverse primer, 50 ng DNA template and 1 U (0.4 µL) Phusion polymerase). Given the size of the expected amplicon, PCR cycling conditions were as follows: an initial denaturation step of 98 °C of 15 seconds, followed by 30 cycles of 98 °C for 10 seconds/kb of expected amplicon, 58 °C and 61 °C (alpha and HMG primers respectively) for 30 seconds and 72 °C for 20 seconds/kb of expected amplicon. A final elongation step of 72 °C for 5 minutes was performed. All reactions were performed using a Techne TC-5000 PCR Thermal Cycler. PCR products were subsequently analysed by gel electrophoresis with a 1 kb ladder used as reference for product length.

4.2.2.3 Confirmation by Genomic Analyses

To confirm *MAT* genotypes observed by PCR analysis, the *MAT* locus of each strain was determined from the genomic sequence. Flanking gene sequences known to be conserved in other fungi (*SLA2* and *APN1*) were obtained from a previous genome sequence provided by Andrew Armitage (East Malling Research) and used to create a frame of the *MAT* locus in each *F. venenatum* strain. Genes further upstream and downstream of these flanking regions were also included in case of structural variations which might arise (Figure 6.5).

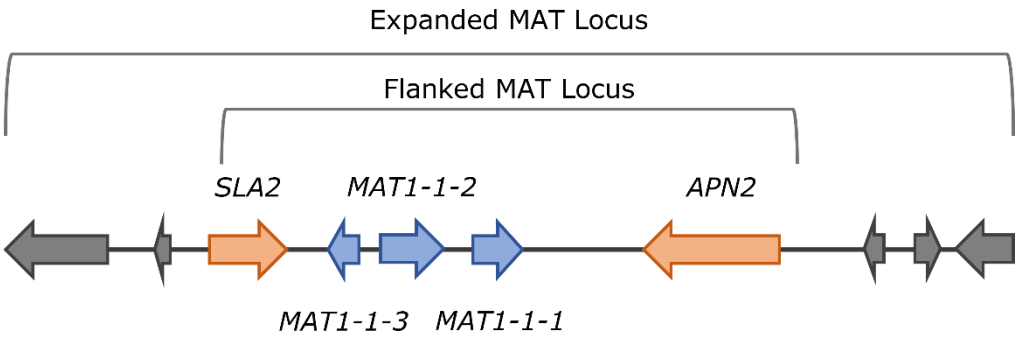


Figure 4.5. *MAT1-1* Locus Structure in *F. venenatum*. *SLA2* and *APN1* homologues are well-known flanking genes of the *MAT* locus in Sordariomycetes and can be used to frame the unknown *MAT1-2* locus. The genes further upstream and downstream were also aligned to observe structural variation of the *MAT* locus or immediate flanking regions.

The *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3* gene sequences from *F. venenatum* A3/5 were then aligned within this frame to confirm strains as *MAT1-1*. This same rationale was employed for putative *MAT1-2* strains in addition to screening with *MAT1-2-1* sequences from closely related *Fusarium* species, *F. poae* (heterothallic, GenBank Accession: AJ535631.3) and *F. graminearum* strain FG-13a (homothallic; GenBank Accession: HM474067.1). Some fusaria also contain additional *MAT1-2* genes and several of these were used to screen the putative *MAT1-2* loci for additional genes. The sequences of *F. verticillioides* MRC8560 *MAT1-2-3* (GenBank Accession: JF776853.1) and *F. xyrophilum* 62710 *MAT1-2-9* (GenBank Accession: MT876218.1) were implemented to screen putative *MAT1-2* loci for other genes.

4.2.3 Trichothecene Biosynthetic Cluster Analysis

As revealed in the previous chapter, there is a substantial difference in the *F. venenatum* strains to produce toxic metabolites, in particular the Type A trichothecene DAS. Notably, strain 114-23 appeared to lack production of toxic metabolites at all stages of upscaling. Trichothecene biosynthetic gene sequences of all *F. venenatum* strains were compared to identify sequence divergence patterns. *F. graminearum* PH-1 and *F. sporotrichioides* NRRL 3299 were also included in the comparison to observe gene divergence between these chemotype groups.

The trichothecene biosynthetic genes are separated in to three distinct loci in *F. venenatum*. Like the *MAT* loci, each strain genome was aligned to gene sequences of the A3/5 trichothecene cluster genes in addition to flanking sequences as described in King et al. (2018). Once determined, each trichothecene locus was then aligned with the remainder of the strains to map overall polymorphism patterns.

4.2.4 Sequence Data Handling and Analysis

Data-handling and sequence analysis were performed in MacVector with Assembler v.18.2.3. Alignments were performed using a ClustalW approach built within MacVector, with the following parameters: open gap penalty = 15, extend gap penalty 6.66, delay divergent = 30% and weighted transitions (Thompson et al., 1994).

4.3 Results

4.3.1 Genome Sequencing of F. venenatum

To date, 4 genome assemblies exist for the Quorn™ strain of *F. venenatum*, describing a small proportion of the available biodiversity. As for other biotechnologically important fungi, capturing genomic diversity in other strains can prove useful for further development.

An Illumina 150 bp pair-end methodology was implemented to sequence the genome. There was intraspecies variety in genome size, with two strains, 114-11 and 114-23, exhibiting genomes approximately 4 Mb shorter than the other *F. venenatum* strains (Table 4.4). There was also some variation in GC content between the isolates, with a range between 44.61 and 47.41% (114-22 and 114-1 respectively). After sequencing, each genome was compared to a reference to assess diversity in a number of features (GenBank assembly accession: GCA_900007375; King et al., 2018). One strain, 114-19, failed to meet the required mapping threshold (85%) and so was not mapped to the reference sequence. Four strains, 114-5, 114-10, 114-12 and 114-24, were unable to be sequenced at the time of writing.

Table 4.4. Basic features of *F. venenatum* genomes. Analysis by Novogene.

Strain	Genome Size (bp)	GC Content (%)	Mapping Rate (%)
114-1	38,655,166	47.41	98.56
114-2	38,631,713	46.19	98.49
114-3	38,233,701	46.98	97.09
114-4	38,324,313	46.38	98.55
114-6	38,618,867	47.36	94.53
114-7	38,630,245	46.28	99.68
114-8	38,653,004	44.63	99.54
114-9	38,392,785	45.33	97.34
114-11	34,562,753	45.17	87.61
114-13	38,398,969	47.45	95.89
114-14	38,579,490	46.63	99.67
114-15	38,401,733	46.77	98.53
114-16	38,493,692	46.97	96.90
114-17	38,255,722	46.72	98.91
114-18	38,388,280	45.91	98.41
114-19	38,217,623	46.33	82.29
114-20	38,171,667	45.00	98.69
114-21	38,263,155	45.72	99.25
114-22	38,346,470	44.61	99.22
114-23	34,584,500	46.44	87.83
114-25	38,407,880	45.95	98.60

4.3.1.1 Studying Genomic Variation in *F. venenatum*

Analysis by Novogene Co. Ltd. observed SNP, InDel, SV and CNV differences between each mapped strain and the reference genome (Table 4.5).

Genome analysis of *F. venenatum* creates 3 groups of isolates, those with low, medium, and high divergence from the reference genome (Figure 6.6). The first group contains 4 strains (114-6, 114-7, 114-8, 114-14), all of which exhibit substantial conservation with the reference genome, showing less than 300 SNPs in each case. Conversely, isolates 114-11, 114-19 and 114-23 showed greater than 1 $x10^7$ SNPs, 160,000 of which were non-synonymous mutations (Supplementary Table 4.2). Finally, the medium divergence group contains the most *F. venenatum* isolates, showing between 30 and 70,000 SNPs in each strain compared to the reference genome. These patterns of divergence are also consistent when other types of variation are studied, in this case insertion/deletions (InDels), structural variants (SVs) and copy-number variants (CNVs).

Table 4.5. Summary of genome variation across *F. venenatum* isolates. Each strain was compared against the reference genome for *F. venenatum* (GenBank assembly accession: GCA_900007375). Analysis by Novogene.

Strain	SNPs	InDels	CNVs	SVs
114-1	253	214	58	73
114-2	51,202	1,988	117	322
114-3	47,929	1,786	125	389
114-4	50,595	1,957	121	352
114-6	290	166	85	340
114-7	266	163	60	273
114-8	273	174	53	242
114-9	38,366	1,790	86	257
114-11	1,093,172	34,772	1,027	651
114-13	39,402	1,809	119	218
114-14	265	164	73	90
114-15	39,282	1,971	102	312
114-16	35,193	1,772	103	226
114-17	37,804	1,678	118	294
114-18	38,811	1,952	84	214
114-19	1,426,600	46,109	1,613	987
114-20	76,812	2,998	111	314
114-21	29,524	1,400	97	343
114-22	30,214	1,431	79	293
114-23	1,105,067	35,689	1,176	849
114-25	39,294	1953	86	222

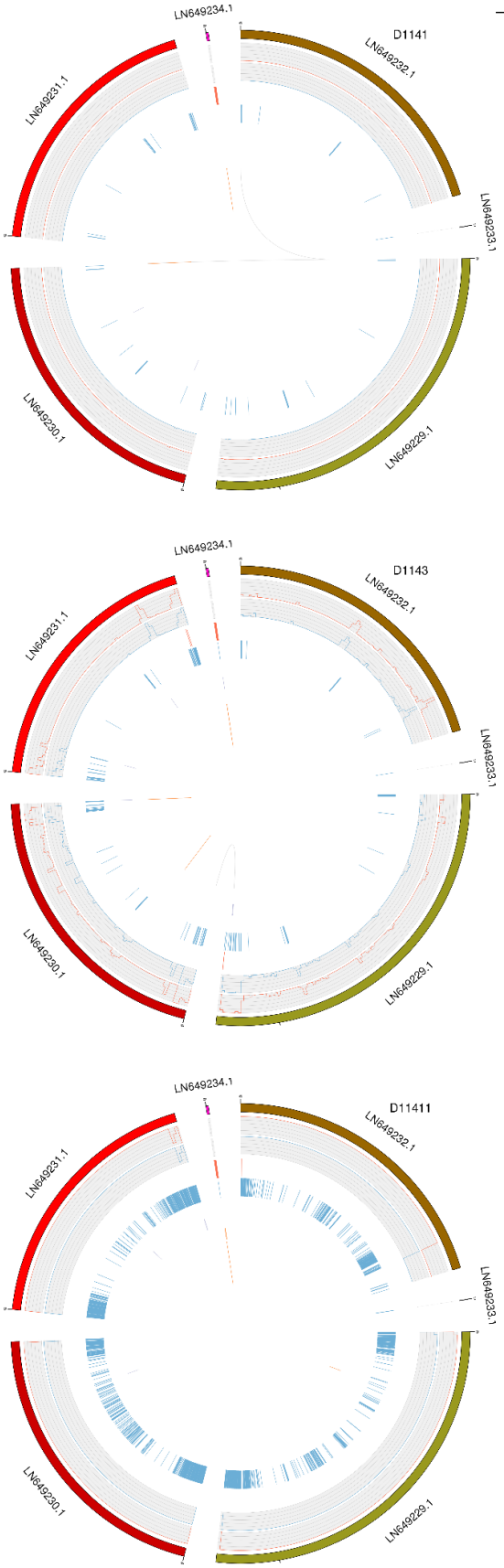


Figure 4.6. Circos plots demonstrating broad patterns of genome variation distribution in *F. venenatum*. The three examples shown here represent isolates of low, medium, and high divergence from the reference genome (114-1, 114-3 and 114-11 respectively). Rings of circos plots are arranged as follows (outer-inner); chromosome, SNPs, InDels, CNV duplication, CNV deletion, SV insertion, SV deletion, SV inversion intrachromosomal and interchromosomal translocations. For SNP/InDels, the density distribution is presented, for SV/CNV variation the location and size are shown. Constructed by Novogene.

4.3.2 MAT Typing F. venenatum Strains

4.3.2.1 MAT Identity Determination by Degenerate PCR

Previous genome sequencing projects have revealed *F. venenatum* to be heterothallic, with the Quorn™ industrial organism possessing the *MAT1-1* locus (King et al. 2018). Similar analyses have not been performed in other strains of *F. venenatum*, and thus the *MAT1-2* locus is undescribed. Using degenerate primers, we screened 25 *F. venenatum* isolates for presence of the *MAT1-1-1* alpha domain and the *MAT1-2-1* HMG domain.

19 of the 24 alternative strains produced the 200 and 946 bp amplicons indicating the strain as possessing the *MAT1-1-1* alpha domain fragment and lack amplification of the *MAT1-1-2* HMG domain. 5 strains lack the *MAT1-1-1* amplicons yet show the 260 bp amplicon for the *MAT1-1-2* HMG domain (Figure 6.7).

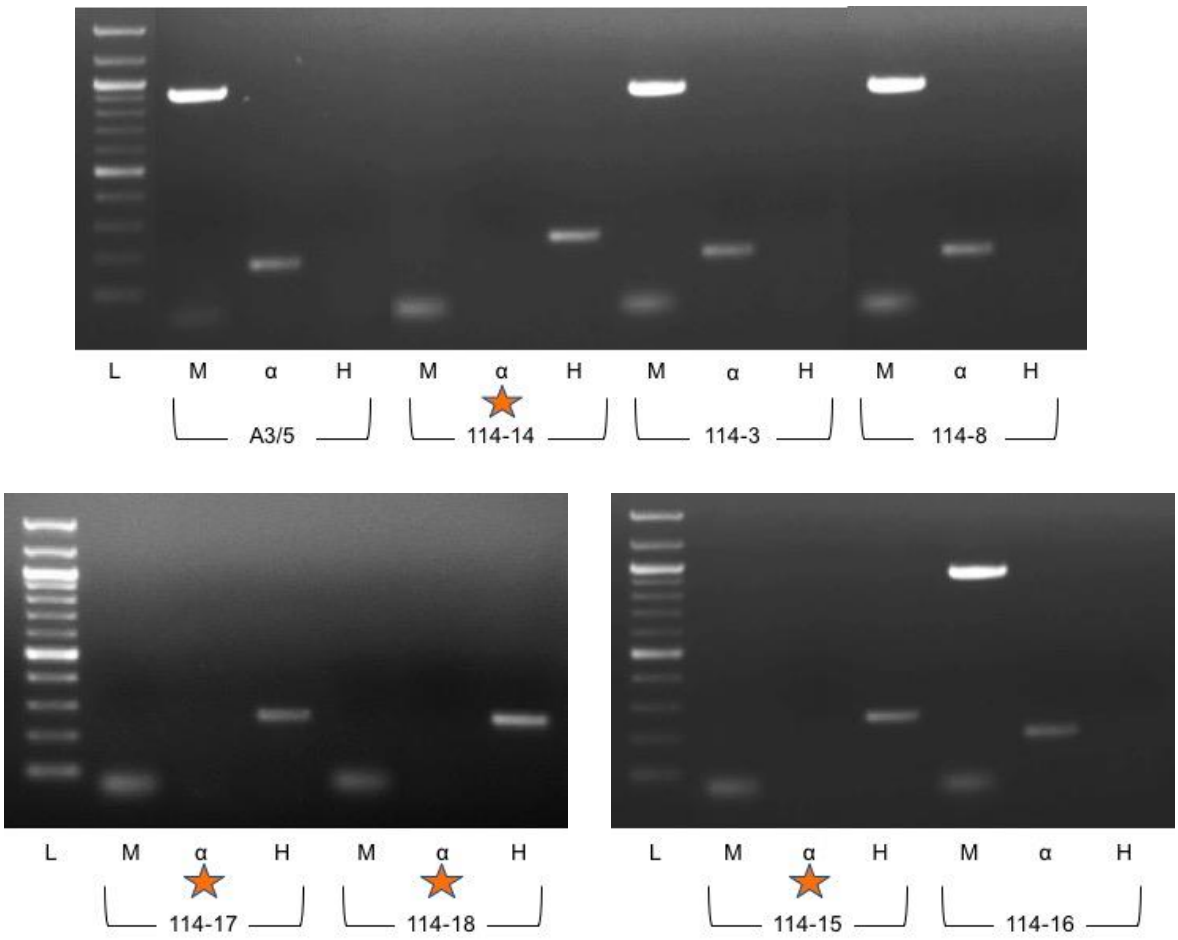


Figure 4.7. *MAT* locus "barcodes" displaying PCR products following gel electrophoresis. Orange stars indicate putative *MAT1-2* strains as determined by the degenerate HMG primers used by Kerenyi et al. (2004). Product sizes are as follows: M = 948 bp; α = 200 bp; H = 260 bp. A 100 bp ladder is loaded in wells indicated L.

These fragments were then purified and sequenced. When subjected to BLASTn analysis, the principal alignment for each putative *MAT1-2* sequence was with a putative MAT2 protein in *F. poae* (GenBank Accession: AJ535631.3). In each case, percentage identity of the fragments with this *MAT2* sequence was between 93.69 and 95.50% with a query cover of approximately 60%.

4.3.2.2 Cloning the *F. venenatum MAT1-2* Locus

Once *MAT1-2* isolates had been identified, attempts were made at "walking-out" the locus from the sequenced *MAT1-2-1* fragment. This relied on a series of primers designed to amplify the upstream and downstream regions of this fragment. PCRs aiming to amplify the whole

MAT1-2 locus were also attempted, with sequences in the *SLA2* and *APN1* homologues. The expected *MAT1-1* amplicon size based on A3/5 sequences was 8,600 bp.

Unfortunately, in all the strategies attempted no amplicons were generated with the “walking-out” primers. A number of modifications were made, including implementation of different primer combinations and *MAT1-2* isolate genomic DNA. Furthermore, the expected control amplicon from *MAT1-1* strains was unable to be retrieved, with the maximum amplicon size of ~3 kb. Both Phire II and Phusion DNA polymerases were implemented in these analyses and each failed to produce the necessary amplicon (Figure 4.8).

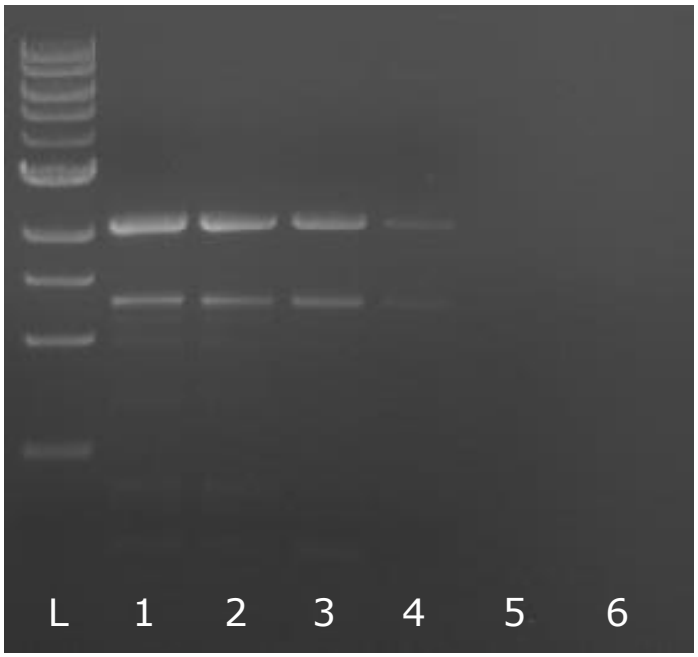


Figure 4.8. **Attempted amplification of the 114-15 *MAT1-2* locus using Phire II and Phusion DNA polymerases.** 1 and 3: 114-1 full locus amplification with Phire II and Phusion respectively. 2 and 4: 114-15 full *MAT* amplification with Phire II and Phusion. 5 and 6: Upstream and downstream region amplification of 114-15 using MAT_U1 and MAT_D1 primer sets respectively.

6.3.2.3 Validation of *MAT* Identity by Genome Analysis

Difficulty in cloning the *MAT1-2* locus necessitated a genome sequencing project to elucidate the *MAT* locus in strains which lacked *MAT1-1-1* amplicon evidence. *MAT* locus genes provided by a previous *F. venenatum* genome project were used to annotate the alternative strain genomes. In addition, several *Fusarium* spp. *MAT1-2* gene sequences were used as controls in putative *MAT1-1* strains and as screens in *MAT1-1* negative strains.

The flanking regions upstream and downstream of the *MAT* locus in each strain showed identical synteny, with each of the flanking genes exhibiting considerable (>98%) sequence conservation relative to *F. venenatum* 114-1. Each of the strains identified as *MAT1-1* in the previous PCR screen were confirmed by construction of their expanded *MAT* loci. In each case, the isolates possessed the full complement of *MAT1-1* genes demonstrated previously in A3/5; *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3*. Each of the *MAT1-1* loci show considerable sequence conservation, with many strains only showing a single SNP or none at all. The main exceptions to this are strains 114-11, 114-19 and 114-23 which show 2.79, 2.38 and 2.38% divergence respectively.

When alignment was attempted with *MAT1-2* gene sequences from closely-related species, no alignment was achieved thus confirming these strains to be heterothallic *MAT1-1* fusaria. Importantly, strain 114-14 was also found to possess the *MAT1-1* suite of genes and lacked alignment with the *MAT1-2*.

Of the remaining strains, 114-15, 114-17, 114-18 and 114-25 all showed alignment to the reference *MAT1-2-1* genes from closely related *Fusarium* species and not the *F. venenatum MAT1-1* sequences. Alignment of the expanded *MAT* locus of A3/5 (114-1) and these strains revealed sequence divergence in the region between the *SLA2* and *APN1* homologues in these alternative strains (Figure 6.9). In *MAT1-1* strains, the region between *SLA2* and *APN1* homologues was measured

to be approximately 8,400 bp. Within this region, the distance spanning the *MAT1-1-3* and *MAT1-1-1* genes is 4,544 bp in *F. venenatum* 114-1. In *MAT1-2* strains, the region between *SLA2* and *APN1* homologues was approximately 7,400 bp.

Within the suspected *MAT1-2* loci of these strains, *MAT1-2-1* genes from *F. poae* and *F. graminearum* aligned to sequences oriented on the minus strand. The *F. poae* gene sequence was used to determine the structure of the *F. venenatum MAT1-2-1* gene, including intron and exon alignment (Figure 6.9, Table 4.6). This structure is conserved in each of the 4 *MAT1-2* strains, with *MAT1-2-1* genes showing 100% sequence conservation. Similarly, the wider *MAT1-2* locus shows total sequence conservation between strains. The *MAT1-2-1* homologue was the only gene found within the locus of these strains, with other *MAT1-2* gene sequences failing to produce an alignment.

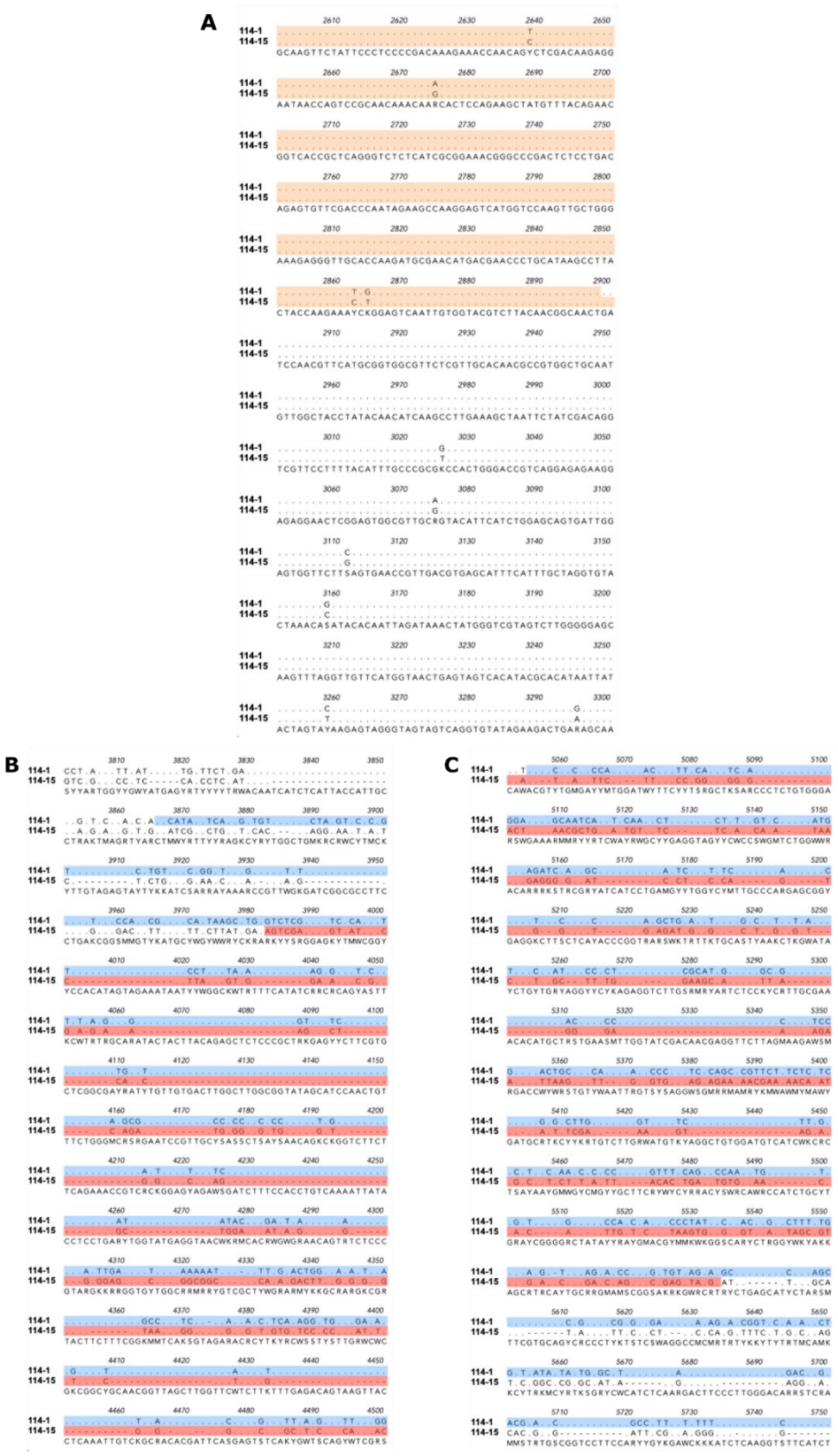


Figure 4.9. Alignment of 114-1 and 114-15 *MAT* loci. A. Upstream region of the *MAT* locus in both strains. Orange box indicates *SLA2* gene coverage in both strains, showing significant conservation between the two strains. B. The *MAT1-1-3* (blue box) and MAT1-2-1 genes (predicted from *F. poae*; red box) overlap yet sequences are expectedly divergent. C. *MAT1-2-1* continues to overlap with the region containing the *MAT1-1-2* (blue box), once again showing substantial sequence discordance.

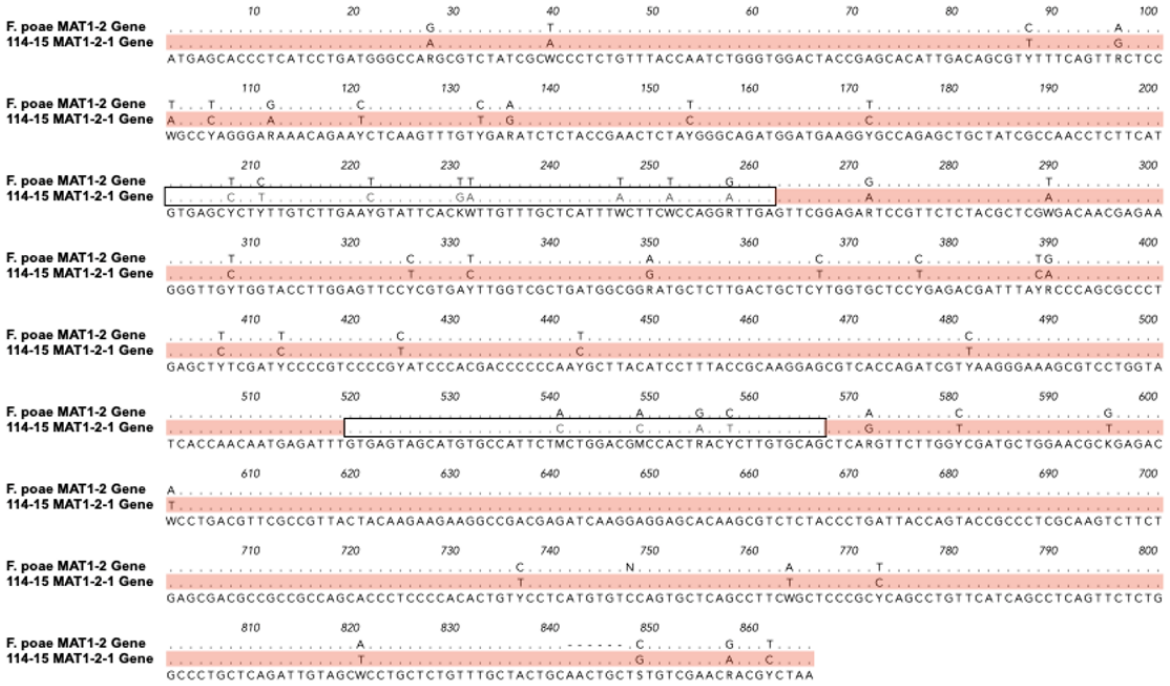


Figure 4.10. Sequence alignment of the putative 114-15 *MAT1-2-1* sequence with the *F. poae MAT1-2-1* gene (GenBank Accession: AJ535631.3). 114-15 homologue bears 93.4% sequence similarity with the *F. poae* sequence. The *F. poae* homologue is predicted to comprise of three exons, here indicated in *F. venenatum* 114-15 by the red shaded box. Predicted introns are indicated by the black outlined box. Consensus sequence is displayed below the alignment with SNPs displayed in the alignment.

Table 4.6. Physical characteristics of the *MAT1-2-1* gene in *F. venenatum*.

Characteristics	114-15	114-17	114-18	114-25
Gene Length (bp)	865	865	865	865
Introns				
Number	2	2	2	2
Range	201-262; 519-566	201-262; 519-566	201-262; 519-566	201-262; 519-566
Exons				
Number	3	3	3	3
Location	1-200; 263-518; 567-865	1-200; 263-518; 567-865	1-200; 263-518; 567-865	1-200; 263-518; 567-865

To summarise, of the 25 *F. venenatum* isolates studied here, there is a distinct *MAT1-1* bias, with a *MAT1-1*:*MAT1-2* identity distribution of 21:4. The identities of each strain are summarised below in Table 4.7.

Table 4.7. *MAT* Identity of *F. venenatum* strains following PCR and genomic analyses.

Strain	Source of Isolation	MAT Type
114-1	Compost, Marlow	*1-1*
114-2	*Zea mays*, Germany	*1-1*
114-3	Unknown, Finland	*1-1*
114-4	Quorn Deposit	*1-1*
114-5	Quorn Deposit	*1-1*
114-6	Quorn Deposit	*1-1*
114-7	Quorn Deposit	*1-1*
114-8	Illinois, USA	*1-1*
114-9	*Triticum sativum*, Austria	*1-1*
114-10	*Cucurbita maxima*, New Zealand	*1-1*
114-11	*Spinacia oleracea*, Sweden	*1-1*
114-12	*Solanum tuberosum*, Finland	*1-1*
114-13	*Triticum sativum*, Austria	*1-1*
114-14	Unknown	*1-1*
114-15	Unknown	*1-2*
114-16	Unknown	*1-1*
114-17	Unknown	*1-2*
114-18	Unknown	*1-2*
114-19	*Zea mays*, Australia	*1-1*
114-20	Soil, Turkey	*1-1*
114-21	Soil, Australia	*1-1*
114-22	Soil, Australia	*1-1*
114-23	*Triticum sativum*, Austria	*1-1*
114-24	Unknown	*1-1*
114-25	Unknown	*1-2*

4.2.3 Trichothecene Biosynthetic Cluster Analysis

For each strain of *F. venenatum*, the *tri* cluster genes were each aligned to the genome and the sequences extracted. Once gathered, all 25 sequences were then aligned to illustrate common polymorphism patterns.

For the largest biosynthetic cluster, all 24 alternative strains contained the full complement of 11 genes, each displaying the expected synteny for this region. Most of the strains displayed 100% alignment with each other. However, 3 strains (114-11, 114-19 and 114-23) demonstrated notable divergence (95.4, 94.8 and 95.4% respectively) from the main group of strains. Pairwise alignment of 114-11 and 114-23 revealed that these sequences shared 99.9% sequence similarity, whereas 114-19 showed 94.2% similarity.

Comparative genomics between 114-1 and 114-23 revealed that the latter possessed a full suite of *tri* genes with the exception of *tri1* where no alignment was produced. The sequence divergence for each gene is displayed in Table 6.8. Sequence divergence in this region varies between genes up to a maximum of 9.2% (*tri15R*). This level of divergence is matched in the flanking genes either side of the *tri5* locus (g3123 and g3135), showing between 92.0 and 95.0% similarity to the 114-1 sequence. The genes encoding regulatory elements (*tri5* and *tri10*) show the greatest conservation with less than 2.0% divergence in each case.

Table 4.8. Sequence divergence of the *tri* genes in isolate 114-23, a strain demonstrated to lack DAS biosynthesis. Numbers indicate percentage alignment relative to isolate 114-1.

Tri Gene	114-23
Tri1	N/A
Tri3	96.0
Tri4	97.0
Tri5	98.2
Tri6	97.6
Tri7	97.2
Tri8	95.8
Tri9	97.5
Tri10	98.3
Tri11	97.0
Tri12	96.7
Tri13	97.2
Tri14	96.4
Tri15	93.9
Tri15R	91.8
Tri16	96.1
Tri101	96.6
g3123	97.1
g3135	94.8

6.2.4 Reassessment of PCR-Based Diagnostic

One focus of the previous chapter in this thesis saw the development of a PCR-based diagnostic for the discovery of *F. venenatum* strains from the environment. The primers designed in this diagnostic were retested using the 22 genomic sequences described in the current chapter, with the amplification product predicted *in silico*.

In each isolate, there was a 100% nucleotide residue match for both primer binding sites. 19 of the strains were predicted to show the expected 831 bp amplicon (Table 4.9). There was some variation in the genomic position, with the amplicon occurring within an 8 kb range. The amplicon for strain 114-17 was 4 bp shorter and appeared approximately 60 kb ahead of the reference strain. The amplicons of strains 114-11 and 114-23 were both predicted to have a significantly shorter size (788 bp). Similarly, the genomic position showed greater removal from the other strains, occurring approximately 130 kb ahead in the genome relative to the reference strain. This truncated amplicon was identical between strains 114-11 and 114-23 but showed 740 conserved residues with the expected 831 bp amplicon. Aligning the sequences also demonstrated that there were two deletions within this region, the most significant was 41 bp in length, starting at residue 110 in the full-length amplicon. The second deletion was 2 bp in length and occurred at residues 575 and 576 in the full amplicon.

Table 4.9. Predicted amplicon size from application of PCR-based diagnostic primers (ven1).

Strain	Product Size (bp)	Amplicon Genomic Position
114-1	831	631822:632652
114-2	831	628735:630565
114-3	831	623843:624673
114-4	831	629313:630143
114-6	831	626994:627824
114-7	831	629202:630032
114-8	831	631738:632568
114-9	831	628101:628931
114-11	788	497484:498270
114-13	831	628846:629676
114-14	831	625127:625957
114-15	831	628120:628950
114-16	831	627827:628657
114-17	827	568116:568942
114-18	831	627082:627912
114-19	831	623947:624777
114-20	831	623258:624088
114-21	831	623999-624829
114-22	831	630762:631592
114-23	788	496225:497012
114-25	831	628197:629027

4.4 Discussion

Sequencing of fungal genomes has proven to be a powerful tool in biotechnology, enhancing historic industrial processes and unlocking cryptic metabolites for further investigation. In 2017, de Vries and colleagues released 10 genome sequences from varied members of the *Aspergillus* genus. Here, they demonstrated the power of comparative genomics methods, revealing a wealth of information concerning diverse biological processes including asexual and sexual reproduction, plant biomass degradation and secondary metabolism (de Vries et al. 2017). Primarily, comparative genomic studies in *Fusarium* spp. have yielded important information linking genotypes to pathogenic features (Ma et al., 2010). For *F. venenatum*, comparison with *F. graminearum* showed that the former lacked two genes involved in pathogen-host interactions (King et al., 2018). This might explain why this same study showed *F. venenatum* to be a superficial pathogen, failing to transmit infection between inoculated wheat kernels and the subcutaneous layer of tomatoes.

While interspecies comparisons are often implemented for these studies, intraspecies comparative genomics are particularly useful in understanding the nuances of cultivar-phytopathogen interactions. Many plant species and their phytopathogens engage in a genetic arms race, with plant cultivars exhibiting resistance to specific phytopathogen strains. For instance, understanding the evolution of new pathogenic *Magnaporthe* oryzae races relies on the identification of novel effector genes by comparing fungal isolates (Singh et al. 2019). Similarly, we employed comparative genomics to investigate important features in the 25 *F. venenatum* isolates thus far obtained. The work presented in this chapter is incomplete, as the genomic data was received late into this thesis. While relatively limited, the insights gleaned from this first review have formed a solid foundation for further study of *Fusarium venenatum* genomic variation.

4.4.1 Comparative Genomics of F. venenatum

Each of the *F. venenatum* isolates obtained earlier in this thesis had been obtained from culture collections or as gifts from researchers under the *F. venenatum* classification. Cloning of the *TEF1α* fragment and subsequent sequencing also confirmed these classifications (see Chapter 3). Analysis of broad genomic properties reveals that the consensus genome size is approximately 38 Mb. Notably two isolates, 114-11 and 114-23, show a reduced genome size of approximately 34 Mb. Closely-related *Aspergillus* species in section *Nigri* show similar divergence in genome size to that seen here in *F. venenatum*, ranging between 34 and 37 Mb (*A. niger* CBS 513.88 and *A. luchuensis* CBS 106.47 respectively; de Vries et al., 2017)). Furthermore, *F. venenatum* 114-11, 114-19 and 114-23 showed substantial lack of mapping to the reference genome compared to the other isolates (87.61, 82.29, 87.83% respectively). The divergence between these isolates and the reference genome is also reflected in the number of polymorphisms. For instance, 114-11 and 114-23 show approximately 4,400-fold more SNPs relative to 114-1. These parameters suggest that these isolates, particularly 114-11 and 114-23, might constitute a novel species or subspecies. This would be unsurprising for the fusaria, a genus comprised of several species complexes which continue to increase in size and complexity (Dongzhen et al. 2020). Elucidation of this finding is beyond the scope of this study yet would make an interesting pursuit for projects in the future.

There are limitations to the genomic data presented in this chapter, most notably when comparing percentage identity of a strain with the supposed SNPs it possesses. For instance, strain 114-8 showed 273 SNPs compared to the reference genome, yet there was a reported difference of over 3% in GC content (114-8: 44.63% vs. A3/5: 47.50%). The reason for this difference is unclear, and further investigation into the disparity is required.

4.4.2 MAT *Typing* F. venenatum *Strains*

Until this study, only the *MAT1-1* locus of *F. venenatum* had been described, establishing the fungus as a heterothallic species but without the complementary partner (King et al., 2018). Here we describe the *MAT1-2* locus of *F. venenatum* as containing a single gene, *MAT1-2-1*, homologues of which are found in closely related heterothallic species such as *F. poae* (Kerényi et al. 2004). Heterothallic *Fusarium* species can show a number of genes in the *MAT1-2* loci, for example *MAT1-2-3* and *MAT1-2-9* genes (Montoya-Martinez et al. 2019). Alignments of example genes from *Fusarium MAT1-2* isolates was attempted in the now recognised *MAT1-2 F. venenatum* isolates, yet no significant alignments were found. We support the hypothesis that the *MAT1-2-1* gene is conserved both across *Fusarium* spp. and more widely in the Ascomycota. The appearance of this gene in both heterothallic and homothallic species suggests that this gene is essential for mating, while other genes in the *MAT1-2* locus provide species-specific auxiliary functions (Dyer et al., 2016).

Description of the *MAT1-2* locus from the genome provides insight into early work attempting to clone the region. Once the *MAT1-2* locus had been determined, it was revealed that the *MAT1-2-1* gene was oriented on the minus strand. This orientation suggests that the lack of amplification when cloning the *MAT1-2* was a consequence of incorrect orientation of the primers placed in the cloned *MAT1-2-1* fragment. Reverse complementation of these primers and subsequent *in silico* calculation of the amplicon product confirms this.

4.4.2 Trichothecene Cluster Analysis

Reclassification of *F. venenatum* from *F. graminearum* was made on the basis of the former producing the mycotoxin DAS, a Type A trichothecene rather than DON, a Type B trichothecene (O'Donnell et al. 1998). Synthesis of DAS was also shown in 4 other *F. venenatum* isolates, all showing a lack of Type A trichothecenes such as the T-2 and

HT-2 toxins (Laraba et al. 2021). In the previous chapter, we showed there was variation among *F. venenatum* strains to produce DAS, with isolate 114-23 lacking biosynthesis across upscaled fermentations (see Chapter 6). Intraspecies comparative genomics in *F. venenatum* show considerable sequence conservation in the trichothecene loci, with 18/21 strains showing 100% similarity.

The three remaining isolates showed sequence divergence of between 13.0 and 20.0%. Of these, 114-23 failed to show DAS biosynthesis at any stage of upscaling. Mycotoxin contamination is yet to occur in Quorn™ mycoprotein, yet discovery of a strain lacking its production is a significant finding. At the level of the genotype, isolate 114-23 contains the full suite of trichothecene biosynthetic cluster genes, with the exception of *tri1* which failed to produce an alignment. The *tri1* gene product hydroxylates the C-7 and C-8 positions in the trichothecene structure. This step is required for the production of Type B trichothecenes (conversion of calonectrin to 7,8-dihydroxycalonectrin) and is also involved in the synthesis of 3-acetylneosolaniol, the precursor of the T-2 toxin (McCormick et al., 2006A). Based on the currently understood DAS biosynthetic pathway, lack of *tri1* would not prevent DAS biosynthesis. This suggests that either *tri1* has a thus-far undiscovered role in DAS synthesis or there is another feature of this isolate which might halt the trichothecene pathway. 114-23 shows considerable divergence from 114-1 and the negating feature or features may not be obvious from a direct comparison of non-synonymous SNPs.

4.4.3 Reassessment of PCR-based Diagnostic Primers

A PCR-based diagnostic was designed in the previous chapter with the goal of identifying soil samples which contained *F. venenatum. F. venenatum* was not isolated from the environment, but 24 alternative strains were obtained during the project.

The genomic data from these alternative strains was used to validate the ven1 primers in silico. To that end, the diagnostic proved a success, with each of the 21 strains tested in this chapter generating an amplicon using the ven1 primers. Of these, 18 strains produced an amplicon of the expected size (831 bp). The predicted amplicon for strains 114-11 and 114-23 was 787 bp, a truncation of 44 bp. Strain 114-19 shows a similar number of SNPs to 114-11 and 114-23, yet the amplicon generated by this strain is identical to the expected 831bp fragment. It is difficult to conclude from these findings whether they support the sister species hypothesis presented in this chapter however it should be noted that 114-19 showed considerable divergence in SNP identity, yet still showed a predicted amplicon resembling the expected.

4.4.4 Conclusions and Future Work

With the *F. venenatum MAT1-2* locus and *MAT1-2-1* gene now described, a number of important studies could be carried out. *F. venenatum* is a heterothallic species and thus requires two partners of opposing mating types to undergo sexual reproduction, yet this life cycle is yet to be discovered in *F. venenatum*. In recent years, discoveries of cryptic sexual cycles have occurred in several fungal species of great interest to human society (Dyer and O'Gorman, 2011). This can be investigated in *F. venenatum* with the confirmation of *MAT1-2* strains detailed in this study. Part of this work would have entailed transcription analysis of mating type genes and additional genes implicated in sexual reproduction. This screening would have observed transcription across a number of media which have been shown to induce sexual reproduction in other fungal species, for instance carrot and V-8 media for *Fusarium* spp. (Gomes et al. 2020). Primers had been designed for this purpose in *MAT1-1* strains yet funding limitations prevented the undertaking of this work. From this work, sexual crosses could then be performed and indeed was planned for this project, yet greater focus was placed on obtaining additional strains to implement in the mating trials.

Here we compare the trichothecene biosynthetic clusters of 21 *F. venenatum* strains to glean insight into the variation seen in the previous chapter. The main finding from this search was that the non-DAS producer, 114-23, lacked the *tri1* gene. The role of this gene can be elucidated in a number of ways. As described in the previous chapter, transcription analyses of the trichothecene biosynthetic cluster in this isolate would provide insights into the progression of the biosynthesis pathway and the stage at which synthesis is halted. In addition, incorporating the *tri1* gene sequence from *F. venenatum* A3/5 via transformation and subsequent screening would reveal lack of this gene is the bottleneck to producing DAS.

This study was primarily concerned with comparisons of mating type identity and trichothecene cluster analysis, yet these genomes can be applied to a number of research avenues. As discussed in the introduction to this chapter, genomes hold a wealth of information for discovering unique metabolites (Brakhage, 2013). Application of *in silico* biomining approaches, such as antiSMASH or SMURF, might reveal metabolites unique to *F. venenatum* which may later be upscaled. Further to this, there may be elements of *F. venenatum* biology which may warrant investigation by Quorn™ and thus provision of 20 unique genome sequences will prove to be useful in future research projects.

5

A Change is Gonna Come: Investigating Replacements for the Quorn™ Mycoprotein Carbon Source

5.1 Introduction

5.1.1 Fungal Nutrition in the Environment

In nature, filamentous fungi present one of three heterotrophic lifestyles to obtain their supply of carbon from the environment: saprotrophy, biotrophy or necrotrophy. Of these, most filamentous fungi will behave as free-living saprotrophs, consuming dead organic matter available in the environment, for example leaf-litter or decaying wood. By contrast, biotrophs gain nutrients from a living host, either to mutual benefit (in the case of mycorrhizal fungi or lichens) or by covertly siphoning nutrients in a parasitic manner. Meanwhile, necrotrophs are pathogenic - invading the host organism (primarily plants) and engaging in direct cellular warfare causing host cytolysis and subsequent release of nutrients (Kabbage et al. 2015).

In their role as free-living saprotrophs, fungi are the principal decomposers of organic material and key to nutritional cycling within an ecosystem. When a fungus is colonising a new environment, the mycelium network will exhibit rapid growth, characterised by sparsely branched hyphae with each tip behaving like a dowsing rod, searching for nutrient rich patches in the environment (Brand and Gow 2009). When a hypha detects a suitably nutrient-rich zone, the rate of extension slows down while branching rate increases, thereby allowing the

mycelial surface area to maximise capture of the nutrient source. Such nutritious zones can take the form of carbon-dense matter, for example plant litter, or digested litter rich in nitrogen and micronutrients.

Environmental carbon substrates encountered by a saprotrophic fungus are heterogenous, rarely comprising of a single sugar or carbohydrate type. In addition, simple sugars are unlikely to be readily available due to the presence of competing microorganisms. For instance, a typical agricultural residue such as wheat straw or sugar cane bagasse is likely to comprise of 30-40% cellulose, 20-30% hemicellulose and 15-35% lignin (Moore et al. 2011). Therefore, to successfully survive and proliferate a fungus must have efficient mechanisms for prioritising the acquisition of energetically favourable carbon substrates. There are two key processes to consider: first, the secretion of hydrolytic enzymes by the fungus into the environment and second, uptake of the carbon substrate. Upon the detection of insoluble, polymeric plant-derived substrates or their derivatives, filamentous fungi upregulate the transcription of genes such as cellulases, hemicellulases and ligninases (Aro et al. 2004). These enzymes can be secreted to form a film surrounding the hyphae, while some are fixed to the hyphal wall, extracellular matrix or to the substrate itself (Evans et al. 1991). Immobilisation of the enzyme allows the fungus to exert some control over substrate acquisition, as localising the substrate and the subsequent hydrolysis products to the immediate proximity of the mycelium allows the exclusion of competing organisms.

Once extracellular hydrolysis has released fermentable sugars from target polymers, permease enzymes catalyse the transport of mono- and disaccharides into cytoplasm from the periplasmic space for the ultimate purpose of generating ATP. In this capacity, the genomes of filamentous fungi have shown substantial gene enrichment for sugar transporters compared to yeast species, allowing rapid uptake of various sugars from the environment. Two of the most prolific cellulase producing fungi, *Trichoderma reesei* and *A. niger* harbour 256 and 113 sugar transporter proteins in their predicted proteomes respectively, compared to just 43

in *S. cerevisiae* (Sloothak et al. 2016). Such metabolic flexibility of filamentous fungi allows them to utilise a variety of hexoses and pentoses released as a result of complex substrate hydrolysis, even when the sugars are at low concentrations in the environment. This treasury of hydrolytic and permease enzymes might explain the ubiquitous appearance of filamentous fungi in most environmental niches across the globe.

5.1.2 Regulation of Carbon Substrate Acquisition

Possessing a wealth of enzymes is problematic if those enzymes are improperly regulated. For metabolic efficiency, the fungus needs to detect a carbon substrate, upregulate the appropriate enzymes for its degradation and/or uptake while suppressing the expression of gene products which are excessive to utilise that substrate. The critical mechanism which allows a fungus to prioritise the utilisation of a favoured environmental carbon substrate is termed carbon catabolite repression (CCR; Adnan et al. 2017). CCR mechanisms are not exclusive to fungi, having been studied extensively in other microorganisms such as the Enterobacteriaceae and bacilli (Deutscher 2008). CCR allows a microorganism to assimilate glucose and other energetically favourable carbon substrates before switching to less profitable sources.

Much of the work to understand CCR in filamentous fungi has been performed in *A. nidulans*, mutants of which have facilitated the discovery of a variety of *Cre* genes integral to CCR function; *CreA, CreB, CreC* and *CreD* (Adnan et al. 2017). Homologues of these genes and equivalent systems of CCR have been identified in several filamentous fungi including *Neurospora crassa*, *Botrytis cinerea* and *Fusarium graminearum* (Ebbole 1998; Tudzynski et al. 2000). The activity of the CCR is briefly summarised in Figure 4.1. The key regulator in this system is the gene *CreA*, which encodes a C_2H_2 zinc finger transcription factor protein (Ries et al. 2016). When glucose is detected by *A. nidulans*, CreA binds to 5'-SYGGRG-3' consensus sequences in the promoter of genes

encoding enzymes for secondary carbon substrate utilisation such as lignocellulose and represses their transcription (Cubero and Scazzocchio 1994; de Assis et al. 2021). For example, upon detection of glucose the *CreA* homologue *Cre1* in *T. reesei* acts to repress the xylanolytic system by directly repressing transcription of the xylan-inducible xylanase *xyn1* (Mach et al. 1996). Similarly, in *A. nidulans* CreA represses expression of xylanases *xlnA*, *xlnB* and *xlnD* as well as xylose transporters *XtrA, XtrB, XtrC* and *XtrD*, prioritising the uptake and assimilation of glucose instead (Ruitjer et al. 1997; de Assis et al. 2021). This has important implications when considering commercial systems aiming to utilise heterogeneous carbon derivatives from waste streams where glucose is likely to repress utilisation of other sugars like xylose.

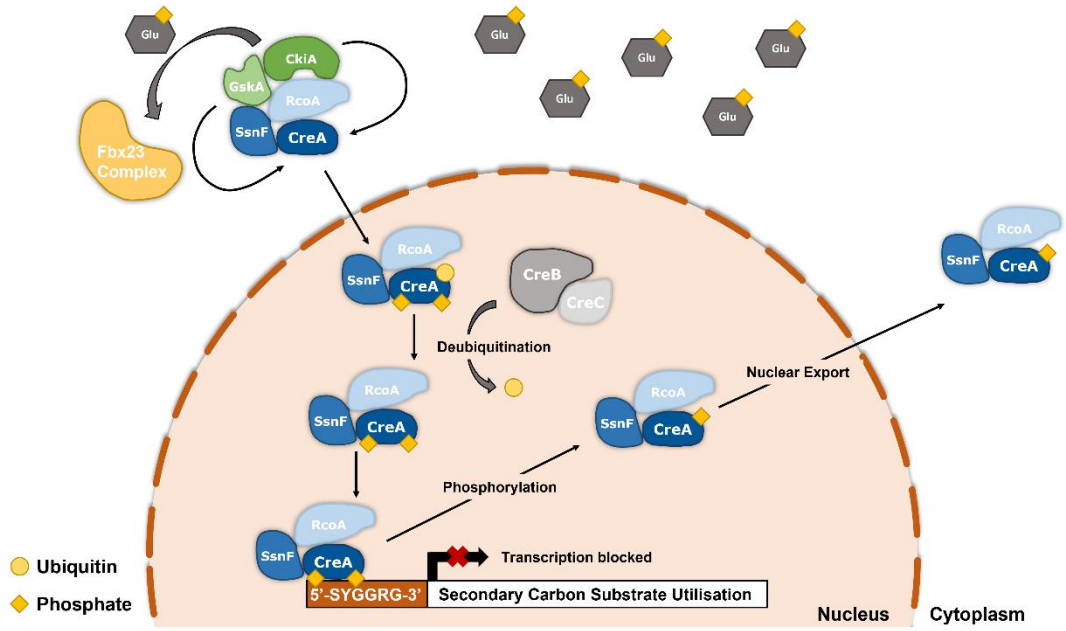


Figure 4.1. Proposed model for CreA activity in CCR regulation in the model fungus *Aspergillus nidulans*. CreA exists in the cytoplasm complexed with corepressors RcoA and SsnF in addition to the Fbx23 complex and the protein kinases GskA and CkiA (de Assis et al. 2018). During derepressing conditions, the cytoplasmic CreA complex is bridged to the Fbx23 ubiquitin ligase complex by GskA, targeting the corepressor complex for degradation (de Assis et al. 2018). Conversion of glucose to glucose-6-phosphate during glycolysis is a crucial signal in CCR, causing the dissociation of Fbx23 from the complex and allowing GskA and CkiA to phosphorylate CreA at several residues, facilitating translocation of the corepressor complex across the nuclear membrane (de Assis et al. 2021). Once the complex has been transported into the nucleus, CreA is ubiquitinated by a heterotrimeric complex of CreD/HulA/ApyA, leading to eventual degradation by the proteosome (not pictured). When glucose is abundant, the CreB-CreC deubiquitylation complex removes ubiquitin moieties from CreA preventing proteasomal degradation (Lockington and Kelly, 2002). The now-active CreA is able to bind to consensus sequences in the promoters of target genes, repressing their transcription.

5.1.3 Carbon Substrate in Commercial Processes

The metabolic plasticity of filamentous fungi is coupled with the diverse array of compounds they are able to synthesise, making these organisms promising candidates for a variety of biotechnological applications (Meyer et al. 2020). Several compounds produced by filamentous fungi are now synthesised in commercial processes, significant examples including β-lactam antibiotics and the statins (Elander 2003; McLean et al. 2015). The carbon substrate choice for these industrial processes is crucial as it will have effects on the growth rate of the organism. To that end, glucose is commonly used as a commercial substrate as it is often the most energetically favourable carbon substrate in addition to many microorganisms encoding machinery for glycolysis. Key exceptions include bacteria such as *Clostridium* spp. which utilise syngas (carbon monoxide and hydrogen) as a substrate to synthesis acetate, butanol, ethanol in processes termed ABE fermentations (Dhakal and Acharya, 2021).

However, growth is not the only parameter to consider as carbon substrate choice is likely to have implications for synthesis of the desired product. For instance, while lactose is non-fermentable by many filamentous fungi and yeasts, the heterodisaccharide is crucial in triggering the production of hemicellulolytic enzymes in industrial *T. reesei* fermentations (Fekete et al. 2007). This fungus can produce cellulases in quantities up to 80 g/L, yet optimal synthesis requires induction by cellulose-derived disaccharides or other stimulatory carbohydrates (Fonseca et al. 2020). To date, lactose is the only inducing sugar demonstrating cost-effectiveness when liquid culture is necessitated, even despite reduced biomass accumulation relative to the use of cellulose (Seiboth et al. 2007). As a further example, gibberellic acid is an important hormone used in the brewing and agriculture industries, demonstrating a multitude of beneficial effects on plant development (Rojas et al. 2001). While some gibberellic acid is produced by plant extraction and chemical synthesis, the vast majority is produced by fermentation of *Fusarium fujikuroi* (Rios-Iribe et al. 2011).

Glucose has historically been employed as the commercial substrate, yet plant oils have been used in combination with glucose to further optimise gibberellic acid production (Rios-Iribe et al. 2011). *F. fujikuroi* also synthesises the polyketide bikaverin, a red pigment which has exhibited antitumour and antioomycete activities (Zhan et al, 2007; Son et al. 2008). Rodríguez-Ortiz et al. (2010) fed *F. fujikuroi* several carbon substrates and discovered that sucrose was able to induce greater production of bikaverin relative to other sugars including glucose, fructose and xylose. Thus, moving away from glucose as a carbon substrate can offer great optimisation in the production of natural products in industrial settings.

For any industrial process there is commercial pressure to reduce the costs of manufacturing a product to maximise potential profit. Furthermore, there is mounting public pressure for industrial processes to seek out methods with reduced environmental impact. Both factors are true of industrial microbial fermentation processes, where there is an increasing trend towards using agro-industrial wastes to provide carbon, nitrogen and micronutrients such as iron, magnesium etc. (Ritala et al. 2017). The potential for successful use of such substrates has been shown by the commercial production of citric acid by *A. niger* utilising a range of different waste substrates such as apple and grape pomace, molasses, and rape seed oil (Ramesh and Kalaiselvam, 2011). The main concern with the use of waste substrates over refined sugar is that batch composition variability will be much greater in the former, which may lead to undesirable or unexpected results in the final product.

5.1.4 Carbon Substrate in SCP Production

Unlike the processes described above, the production of single-cell protein (SCP) aims to optimise the production of proteinaceous biomass rather than the synthesis of a particular chemical compound. As such, the most important aspect of a carbon substrate is the effect on cell biomass. Many production systems have been used to manufacture SCP, using both eukaryotic and prokaryotic organisms (Ritala et al.

2017). Most of these organisms have been heterotrophs and have demonstrated the ability to utilise a broad range of carbon substrates, many from waste streams including cheese whey, prawn shells and waste from agricultural and brewing industries (Yadav et al. 2016; Rhishipal and Phillip 1998; Ahmadi et al. 2010). Importantly, many of these processes focus on SCP production for feed where the quality of ingredients is not as strict as in human food (Whittaker et al. 2020). The best-known commercial process generating SCP suitable for human consumption is Quorn™ mycoprotein by Marlow Foods. As described in Chapter 1 (Section 1.5.3) the industrial organism *F. venenatum* A3/5 is grown continuously in a culture medium consisting of glucose, nitrogen, vitamins, and mineral salts for a period of approximately 30 days. Glucose has been used as the carbon substrate for several decades, with the current high-purity glucose syrup produced by digestion of either wheat or maize starch using the enzyme glucoamylase. There has only been very limited investigation into the growth of the Quorn™ strain on feedstocks other than glucose, with the most comprehensive report published by Anderson et al. in 1975, who examined the use of a limited range of simple sugars as alternatives to glucose. Waste substrates which have proved successful in other microbial fermentations are not as easily implemented in the Quorn™ industrial process. Instead, greater focus should be placed on low-cost food-grade wastes such as high-fructose syrups (HFSs), which are more readily available thanks to a decline in use in the soft drink manufacturing (Lima et al. 2011). Discovering a carbon substrate with lower cost but maintaining the same level of product quantity and quality would be of great benefit to the Quorn™ business.

5.1.5 Chapter Aims

The high-cost glucose substrate currently used as a feedstock by the Quorn™ industrial process presents a significant overhead in the production of mycoprotein. Consequently, the aims of this chapter were twofold:

1. First, to investigate the effect of using alternative carbon substrates on the production of mycoprotein, with the goal of seeing whether a more cost-effect feedstock might be found. The carbon substrates tested were those considered as likely to be suitable for an industrial process and included simple sugars and substrates available from other commercial/industrial processes. Growth tests were performed first at a laboratory bench scale before upscaling to the bioreactor scale.
2. Second, to screen the alternative strains of *F. venenatum* obtained in Chapter 3 to assess whether any of the these exhibited better growth than the current A3/5 production strain. This included examination of aspects such as faster and more efficient growth and also lowered levels of mycotoxin production.

Results of these investigations could conceivably lead to alternatives to the current production paradigm implemented by Quorn™.

5.2 Materials and Methods

All chemicals listed were supplied by Sigma-Aldrich (Poole, Dorset, UK) or Fischer Scientific, unless stated otherwise. Most chemicals were of analytical or molecular biology grade, the only exceptions food-grade high fructose syrups and the ionic fluid-derived substrates. Sterilisation was achieved by autoclaving at 121 °C for 20 minutes unless specified otherwise.

5.2.1 Preliminary Growth Screening

Prior to screening of alternatives, initial growth flask studies were undertaken to characterise the Quorn™ production paradigm – A3/5 on glucose. For this, 250 mL triple-baffled Erlenmeyer flasks were filled with 50 mL of RHM broth medium supplemented with D-glucose for a final concentration of 1.00% (Section 2.2.1). This investigation was comprised of two time course studies, the first of which observed growth over 7 days with mycelia from sample flasks harvested every 24 hours. The second trial focused on elucidating the exponential phase, observing 60 hours of growth with sample flasks extracted evert 6 hours from 18 hpi. In each case, triplicate flasks were setup for each time point, incubated at 28 °C in the dark at 150 rpm.

Following the appropriate incubation time, each mycelium sample was harvested under vacuum filtration using a Buchner flask setup, resulting in deposition of the culture mycelium on a pre-weighed Whatman No. 1 filter paper. Following harvest, each sample was placed in a 50 mL centrifuge tube which was flash-frozen in liquid nitrogen. Samples were then lyophilised overnight at -40 °C. Once completed, the samples were weighed, and the final mass recorded.

5.2.2 Characterising Fusarium venenatum *Growth on New Substrates*

5.2.2.1 Microfermentation Assay

A high-throughput screening method was devised to process the large number of assay conditions. 96-well flat-bottomed plates (Cellstar®; Greiner Bio-one, Austria) were used in combination with an Epoch microplate spectrophotometer (BioTek Instruments, USA) to assess *F. venenatum* growth, using optical density (OD) accumulation as a proxy.

Direct comparison of culture growth in microfermentations required the selection of descriptive metrics, a range of which have been used for other microorganisms grown in 96-well microtitre plates (Kurokawa and Ying, 2017). Of these, the most commonly used are calculations of doubling time (DT) and growth rate (μ). These metrics are calculated using values obtained during exponential growth of the cultures. In *F. venenatum*, exponential growth in microtitre plates appeared to have two distinct phases occurring between 10-30 and 30-50 hpi. Both exponential phases were described using doubling times, termed the early and late doubling times (DT_E and DT_L respectively; Equation 5.1). DT_E was calculated between 15 and 21 hours, while DT_L was calculated between 36 and 42 hours.

Equation 5.1. *F. venenatum* doubling time calculation. 0.301 = log(2), h = time period studied in hours (=6), ODi = initial optical density value, ODf = final optical density value.

$$DT(hours) = \frac{(-0.301 \times h)}{(logODi - logODf)}$$

Validation of Method

Initially, the suitability of this assay was tested by comparing the OD of these microwell cultures with dry cell weights (DCWs) over 96 hours. These DCW values were then correlated with the equivalent values obtained from shake flask cultures. The primary concern was a potential

disparity in mass transfer of oxygen, the importance of which has been discussed (see Section 1.5.3).

In each trial, 150 µL of RHM (Section 2.2.2) containing glucose at a final concentration of 3.00% (w/v) was dispensed into each well of the microtitre plate. 15 µL of a *Fusarium venenatum* A3/5 macroconidial suspension was used to inoculate each well to a final concentration of 1 x 10^5 spores/mL. Plates were then sealed with a single layer of parafilm and placed into the Epoch microplate spectrophotometer. Incubation conditions were 28 °C with heated lid option set at 35 ºC, with optical density readings (600 nm) taken for each well every 30 minutes for 120 hours. Cultures were static, as shaking was found to drive pellet formation (see Supplementary Figure 5.1). OD_{600} readings were first taken in a microwell plate reader and mycelial samples were then taken for each time point (12, 24, 36, 42 and 48 hpi), filtered through a pre-weighed Whatman No. 1 filter paper and freeze-dried altogether to obtain a DCW value. These values were then plotted to establish a correlation between the two values. For each time point, twelve replicate wells per growth sample were assessed in this manner.

Screening of Alternative Carbon Sources

Once suitability of the microwell growth assay system had been established, these microfermentation screens were used to assess the effect of different carbon substrates on growth in *F. venenatum* A3/5. Several sugars were investigated as an alternative to glucose (Table 5.1). These substrates were in D-conformation with ≥98% purity. Each sugar was dissolved in SDW before sterilisation with a pre-sterilised vacuum-driven filter system (0.22 µm pore size; Millipore Corporation, Billerica, USA). Final concentration in culture was 3.00% (w/v), chosen as this mimics the glucose concentration used in the steady state Quorn industrial process. The key exception to this was in the case of starch, which is typically insoluble in H_2O. For this substrate, soluble starch was implemented which can achieve concentrations of 2% (w/v), prepared in 1.5x RHM broth and filter-sterilised as described above.

Table 5.1 List of sugars used to assess *F. venenatum* carbon substrate flexibility in microwell growth assay system. In most cases, each sugar was used at a final concentration of 3% (w/v). In the case of starch, a more concentrated broth was prepared to account for the reduced solubility in H_2O.

C Substrate	Structure	Reducing Sugar?	Supplier
Glucose	Hexose monosaccharide	Yes	Quorn Foods, UK
Galactose	Hexose monosaccharide	Yes	Honeywell Fluka™, USA
Fructose	Pentose monosaccharide	Yes	Sigma-Aldrich, USA
Lactose	Disaccharide, galactose-β-1→4-glucose.	Yes	Honeywell Fluka™, USA
Sucrose	Disaccharide, glucose-β-1→2-fructose.	No	Thermo Fisher Scientific, USA
Maltose	Disaccharide, glucose-α-1→4-glucose.	Yes	Applichem, Germany
Cellobiose	Disaccharide, glucose-β-1→4-glucose.	Yes	Sigma-Aldrich, USA
Xylose	Pentose monosaccharide	Yes	Thermo Fisher Scientific, USA
Starch	Glucose homopolymer, α-1→4-linkage (>C100)	No	Thermo Fisher Scientific, USA

In some cases, *F. venenatum* cultures were fed a mix of glucose and an alternative sugar substrate such as sucrose, xylose or fructose. The aim was to understand whether *F. venenatum* cultures could be fed a combination of sugars in the mycoprotein production process without total substitution of the currently used glucose syrup.

In addition to the pure sugar compounds, a number of alternative carbon sources were trialled (Table 5.2). These were either relatively cheap commercially available sugar syrups that were readily available for use in an industrial process (as recommended by the Marlow Foods industrial sponsor), or a lignocellulosic breakdown product provided by Tsinghua University (China). This latter substrate was a thick, brown liquid and was composed of xylose (33.8%) and glucose (11.2%) (Wei-Chien Tu, Pers. Comm.). When used as a carbon substrate, these samples were adjusted to 3% w/v of the final culture composition.

Table 5.2. Commercial syrups considered for use as a carbon substrate for A3/5 fermentations. The composition of each syrup is listed as % w/v: DX = Dextrose; DP2 = Dextrose disaccharide; DP3 = Dextrose trisaccharide; DE = Dextrose equivalent; ISC = Invert sugar conversion.

Commercial Syrup	Xylose	Fructose	DX	DP2	DP3	DE	ISC
C*Sweet M01521	-	-	-	12.5	7.5	-	-
C*Sweet M10170	-	-	~1.1	~16.7	~2.2	-	-
C*Sweet 1112	-	-	5	2.5	2.5	10	-
C*TruSweet 1750	-	~8.9	~11.1	-	-	-	-
Tereos SI	-	-	-	-	-	-	20
Tsinghua Sample	11.2	-	33.8	-	-	-	-

Screening of Alternative Strains

Not only was the carbon substrate under scrutiny, but so too was the production organism. Quorn™ mycoprotein has been produced by a single strain of *F. venenatum* since its discovery in the 1960s, leaving a wealth of unexplored biodiversity. Thus, each *F. venenatum* strain was screened for growth with glucose or sucrose as the carbon substrate in the microwell assay as described above. A list of the strains used in this study can be found in Table 3.3.

5.2.2.2 Shake Flask Fermentations

Following preliminary screening in microwell plates, it was necessary to upscale to shake flasks to determine whether observed patterns were maintained under growth conditions bearing greater similarity to the Quorn™ fermenter model.

To that end, 50 mL of RHM broth (Section 2.2.2**)** supplemented with the necessary carbon source to reach a final sugar concentration of 3% (w/v) was dispensed into 250 mL triple-baffled Erlenmeyer flasks (Kimble, USA). Baffled flasks were used to replicate the procedure at the mycoprotein production facility in Billingham. Cultures were grown at 28 ºC in the dark at 150 rpm on an orbital shaker platform. The growth profile for each carbon substrate was assessed every 24 hours for 7 days, with an additional sample at 36 hours to further define the exponential phase. For each time point, 4 replicate flasks were prepared.

Later experiments used a reduced timescale, taking samples within a 72-hour time period. Harvesting of mycelium was described as in Section 5.2.1.

5.2.2.3 Five L Batch Fermentation

Promising candidate alternative carbon (sugar) sources identified from the growth flask trials (Section 5.2.3.2) were trialled further by upscaling to 5 L batch fermentations. Fermentations were carried out in a 5 L jacketed stirred-tank bioreactor (Infors Labfors 5; 7.5 L capacity, 5L working capacity, Figure 5.2). Fermentation experiments were designed and monitored using the eve® bioprocessing software.

Seed cultures of F. venenatum A3/5 were prepared by inoculating 24.75 mL of RHM broth (Section 2.2.2) contained in a 100 mL shake flask with 250 µL of macroconidial suspension (1 x 10^7 spores per mL) from cryostocks. These seed cultures were then incubated for 48 hours until sufficient mycelium was generated (28 °C, 150 rpm).

5 L of RHM medium comprising a carbon source (glucose, sucrose or an equimolar combination of the two) was used as the culture broth. The medium was then pH adjusted to a value of 6 before decanting into the bioreactor for autoclaving. Once preparation of the autoclaved bioreactor was complete, the vessel was inoculated with the prepared seed cultures which were then incubated under the following conditions: temperature = 28 °C, pH = 5.80, impeller rotation speed = 750 rpm, air flow = 5 L/min (1.0 vvm). Offline fermentation broth samples were taken at 24, 36-, 48-, 72- and 96-hours post-inoculation (hpi). Three 30 mL samples of the fermentation broth were drawn from the vessel using a sterile pipette and dispensed in to 50 mL centrifuge tubes. These samples were frozen at -20 °C until required. As before, separation of the mycelium and supernatant was achieved using miracloth (Section 4.2.1). The mycelium was lyophilised, and the sample weight recorded.

Microscopy analysis of the samples from each fermentation broth were undertaken. 10 µL of each broth was dispensed onto a glass slide with

a coverslip and observed by phase-contrast light microscopy under X100-X400 magnification. For each sample, checks were made to ensure lack of any contamination and the mycelial morphology was observed by eye and noted.



Figure 5.2. Laboratory set up for batch fermentation growth in a 5 L stirred-tank reactor (STR). The headplate was fitted with pH (Hamilton), temperature, dO_2 (Mettler Toledo) and capacitance (Aber Instruments) probes for automatic fermentation homeostasis. pH was maintained using 1 M phosphoric acid and 1 M NaOH controlled by peristaltic pumps. Temperature was maintained by a heated jacket encapsulating the inner fermentation vessel. Mass transfer of oxygen was achieved through the sparging hose, triple baffle setup and two double angle-bladed impellers.

5.2.3.4 Ten L Batch and Steady State Fermentation

As a final scale-up trial, *F. venenatum* A3/5 and a putative non-DAS producing isolate (114-23; see Chapter 6) were screened for growth on both glucose and sucrose in 10 L scale batch and steady-state fermentations using facilities at the Marlow Foods industrial sponsor.

Seed cultures for the fermentations were generated by inoculating 36 mL of broth with 4 mL of a glycerol stock of either *F. venenatum* A3/5 or 114-23. The broth comprised of mineral salts, ammonia salts as a source of nitrogen and glucose (exact media details are proprietary; personal communication, M Taylor). The baffled flask was then incubated (28 °C, 110 rpm) for 48 hours.

Batch Fermentation

Batch growth was performed in a 15 L bioreactor (Applikon,10 L working volume) and controlled via EzControl software with live data logged via Applikon Lucullus software. Batch growth medium comprised sterile additions of 3.00% (w/v) glucose or sucrose (w/v) and a separate set of mineral salts (specific medium composition is excluded as a confidential trade secret; pers. comms., Mark Taylor). After all components were added, medium pH was adjusted to a value of 6 using 1 M ammonium hydroxide. The vessel was equilibrated to 28°C, set at an agitation of 600 rpm (two Rushton impellors at 10 cm distance) and 10 L/min air (1.0 vvm) and when equilibrated, the dO_2 was set to 100% under these conditions. Off gas analysis was calibrated against air for both CO_2 and O_2 content and measured continuously throughout. A setpoint of 30% dO_2 was mandated as a minimum, against a cascade control of 600-1000 RPM and 10-20 L/min air. A pH of 6 was maintained with an on-demand feed ammonium hydroxide.

Batch fermentations were maintained until carbon sources were exhausted, with broth samples taken at 24, 26, 28, 30 and 48 hpi. In

steady state fermentations, cultures were grown until exponential phase was reached and then broth samples taken after 24 hours. DCW was typically measured for a 5 mL sample although larger volumes were used for early, low biomass samples. These samples were taken via sterile syringe with filter attached to a vacuum filtration device. Most of the water was removed, the cell pellet washed, and the remaining pellet was dried to constant weight in a 60 °C oven. Dried mycelium samples were then weighed, and DCW mg/mL of broth determined directly. Measurements were made in triplicate and a mean and SEM determined.

5.2.3 Impact of Carbon Sources on F. venenatum Morphological Traits

5.2.3.1 Changes to Macroconidial Germination Patterns

When considering a new carbon source, it was necessary to assess whether this gave rise to variations in germination rate or changes in patterns of mycelial growth. This was important given that significant changes in either from existing growth behaviour of *F. venenatum* may have implications both for seed culture preparation and growth in the industrial production system. This was particularly considered a potential risk because macroconidia are more complex than many fungal asexual spores, with each unit comprising of several individual compartments (cells) divided by septation. Germ tube formation can occur from any one of these cells but is most likely to originate from one of the polarised cells (Harris 2005). The Quorn™ commercial product requires long, sparsely branched hyphal networks, and therefore substrates which promote germination from the interstitial cells may be undesirable.

Promising candidate strains of *F. venenatum* (114-8 and 114-18) were studied to observe any differences in macroconidial germination rate and growth pattern when each was cultured in the presence of glucose, sucrose or an equimolar mix of the two sugars. 25 mL of RHM broth was

prepared in 250 mL baffled Erlenmeyer flasks in triple replicate. Each set of replicate flasks was supplemented with either glucose, sucrose or an equimolar mix to a final concentration of 3.00% (w/v). Macroconidial suspensions were prepared from PDA plates as described previously (Section 2.2.1) and inoculated at a final concentration of 1 x 10^5 macroconidia/mL. Liquid cultures were incubated on a rotary shaker (28 °C; 150 rpm) for a total period of 8 hours, with triplicate 100 µL samples taken from the flasks every 2 hours.

From each aliquot, 10 µL was pipetted onto a microscope slide, with a cover slip placed over the solution and viewed by phase-contrast light microscopy using a 40x objective lens (HBO 50 microscope, Carl Zeiss, Welwyn Garden City, Hertfordshire, UK). Per aliquot, 100 spores were counted and photographed using the Motic Images Plus software (v. 3.0, Motic). The spores were then grouped by their observed morphotype (dormant, swollen or germinating). If a spore was observed to be germinating, the number of germ tubes were counted.

5.2.4 Assessing Utilisation of Carbon Sources

Efficient utilisation of carbon substrates during exponential phase may vary depending on the strain and/or carbon substrate in question. This in turn will have repercussions for the industrial process, should an alternative strain/carbon substrate be found. If a strain appears to have an improved growth rate yet requires a significantly greater amount of carbon, the process economics may not be suitable for upscaling.

F. venenatum cultures were setup as previously described (Section 5.2.3.2), with glucose and sucrose used as the carbon sources at 3.00% (w/v). Triplicate flasks were prepared for a number of time points; 0, 24, 36, 48 and 72 hours. Once incubation had terminated, supernatant and mycelium were separated using miracloth, the mycelium freeze-dried and the weight recorded, with the supernatant saved for analysis, with measurements made as soon as possible to avoid any change in chemical composition.

The supernatant was processed using a Sucrose/D-glucose test kit according to manufacturer's instructions (Megazyme, Ireland). This colorimetric assay relies upon the activity of β-fructosidase, glucose oxidase and peroxidase to measure the levels of sucrose and glucose through generation of quinoneimine (Figure 5.3).

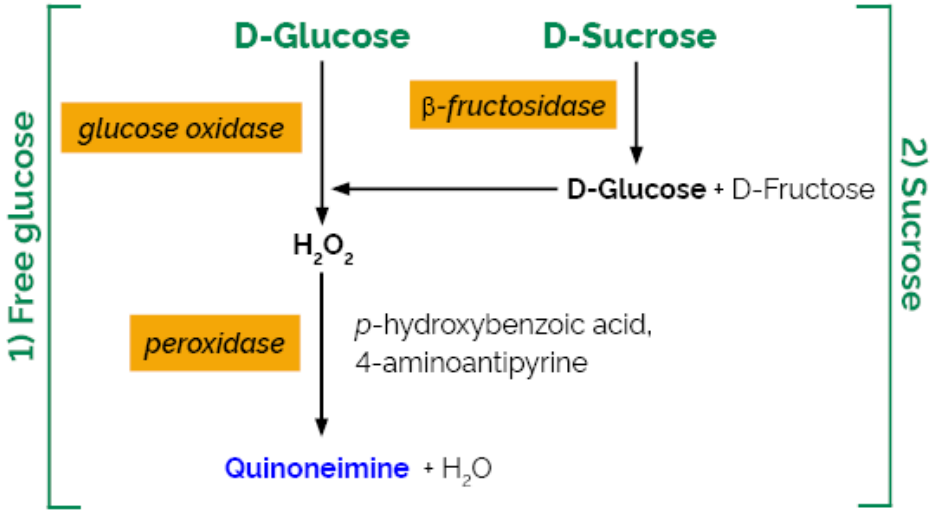


Figure 5.3. Sucrose/D-glucose assay procedure. Sucrose is hydrolysed by beta-fructosidase to D-glucose and D-fructose. D-glucose (either as media substrate or sucrose breakdown product) is then oxidised to H_2O_2. H_2O_2 then acts as a substrate for peroxidase, which in combination with *p*-hydroxybenzoic acid and 4-aminoantipyrine, yields quinoneimine. This compound generates the colour change allowing the measurement of the original carbon substrates. The absorbance at OD_{510} is directly proportional to the amount of glucose or sucrose remaining in the medium. Figure is reproduced from product page (www.megazyme.com).

5.3 Results

5.3.1 Characterising Quorn™ Strain Growth on New Substrates

5.3.1.1 Initial *F. venenatum* A3/5 Growth Studies

In initial experiments, growth of the Quorn™ A3/5 production strain was studied using baffled shake flask cultures. In the first trial, *F. venenatum* cultures had reached stationary phase by 48 hpi, with 4.3 ± 0.06 mg/mL generated (Figure 5.4A). Biomass decreases in each of the time points following, suggesting that stationary phase at around 48 hours. Subsequent analysis of the exponential phase studied biomass accumulation was determined every 6 hours between 18 and 60 hours in cultures incubated with RHM media and 1.00% glucose (w/v) (Figure 5.4B). This revealed that stationary phase occurred at 36 hpi (4.9 mg/mL ± 0.2) in these conditions.

Repeating this experiment at 3.00% glucose (w/v) to better replicate the conditions at Quorn™ showed that at 48 hours, cultures were now in exponential phase. Cessation of exponential phase occurred at approximately 48 hours whereby the cultures entered a stationary phase with no appreciable further increases in DCW accumulation (Figure 5.4C; 48 hpi - 10.69 ± 0.22 mg/mL; 72 hpi – 11.20 ± 0.21 mg/mL; 96 hpi – 10.05 ± 0.26mg/mL).

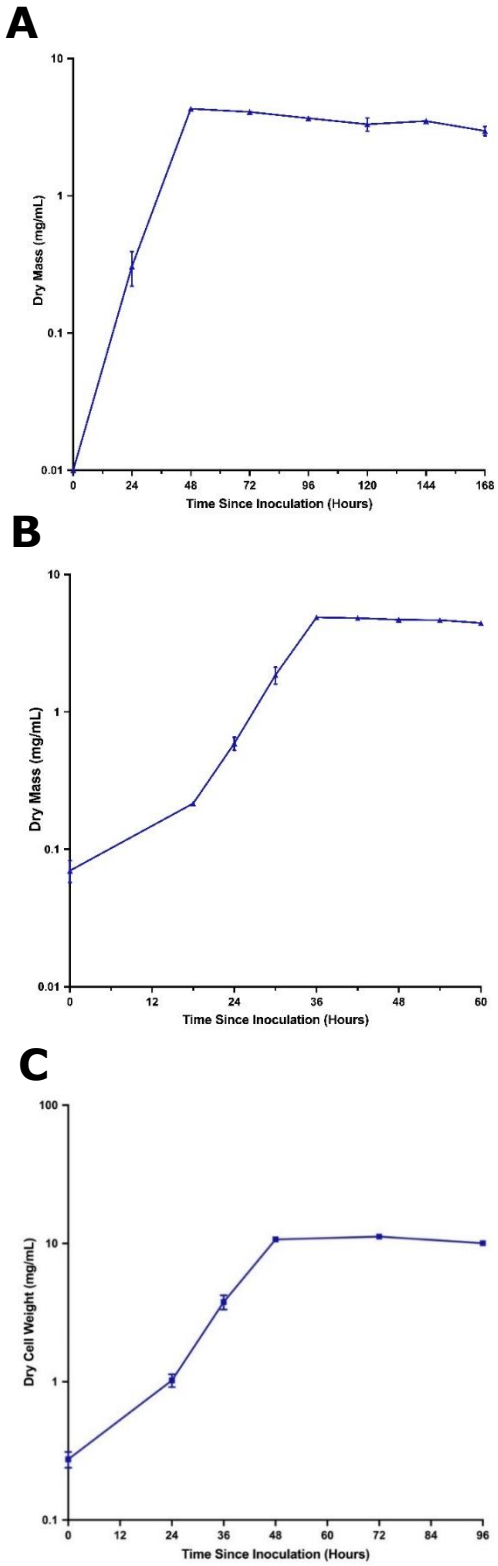


Figure 5.4. Semilogarithmic growth curves of *F. venenatum* to elucidate growth patterns as determined by dry cell weight in baffled shake flask culture. A – Original time course observing growth on 1.00% glucose over 168 hours, showing 4.3 ± 0.06 mg/mL at 48 hpi. B – Elucidation of exponential phase growth on 1.00% glucose, observed every 6 hours between 18 and 60 hpi, with cultures showing 4.9 mg/mL ± 0.2 at 36 hpi. C – Growth over 96-hour period on 3.00% glucose. Average dry mass accumulated at mid-exponential phase (36 hpi) = 3.77 ± 0.45 mg/mL). Data points indicate mean average, ± SEM, n = 4.

5.3.1.2 Validation of Microfermentation Method

Shake flask fermentations were soon found to be inadequate for the rapid screening of the large number of conditions demanded by the project. As a result, an alternative microfermentation system was evaluated, with the goal of more rapidly screening growth resulting from different carbon substrates and *F. venenatum* strains. Optical density (OD) is often used to measure the culture density of bacteria and yeast. Unlike the propagation of such single-celled organisms, a colony forming unit of filamentous fungi, likely a spore, will expand into a multicellular network which may not be adequately represented by an OD value. Therefore, any correlation first had to be assessed between biomass accumulation and OD reading within microfermentation cultures. OD readings were taken at a wavelength of 600 nm to avoid confounding values from media colouration.

A linear relationship was found, with a positive correlation (R_2 = 0.98) between *F. venenatum* culture optical density and biomass accumulation observed at OD_{600} (Figure 5.5; Table 5.3). Both biomass and optical density increased as cultures proceeded through exponential growth, with the greatest value for both responses occurring at 48 hours pi. In these conditions, two distinct growth phases were apparent in the exponential growth phase, steeper initial phase (DT_E) and a shallower later phase (DT_L) (Figure 5.6).

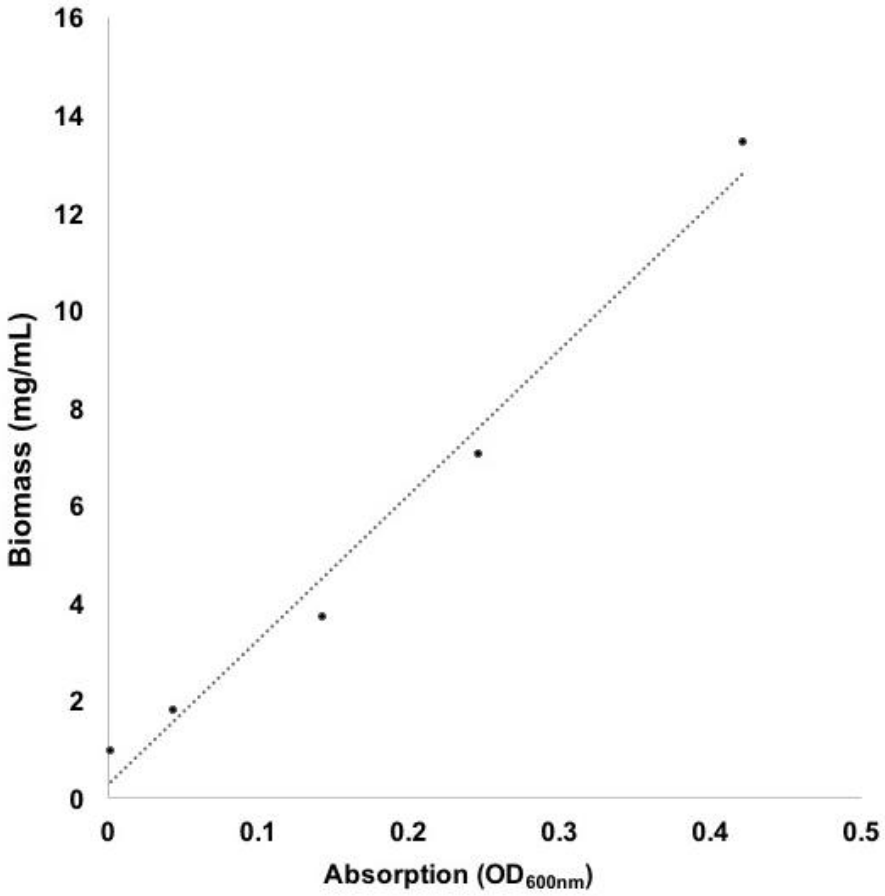


Figure 5.5. Biomass accumulation during growth of *F. venenatum* in microfermentation trial. Points represent mean average values. R^2 value = 0.98, n = 12.

Table 5.3. Comparison of growth metrics during *F. venenatum* microfermentation growth. Values represent mean average.

Time Since Inoculation (Hours)	Biomass (mg/mL)	Absorption (OD_{600})
12	0.95	0.0020
24	1.78	0.0445
36	3,70	0.1430
42	7.03	0.2468
48	13.41	0.4217

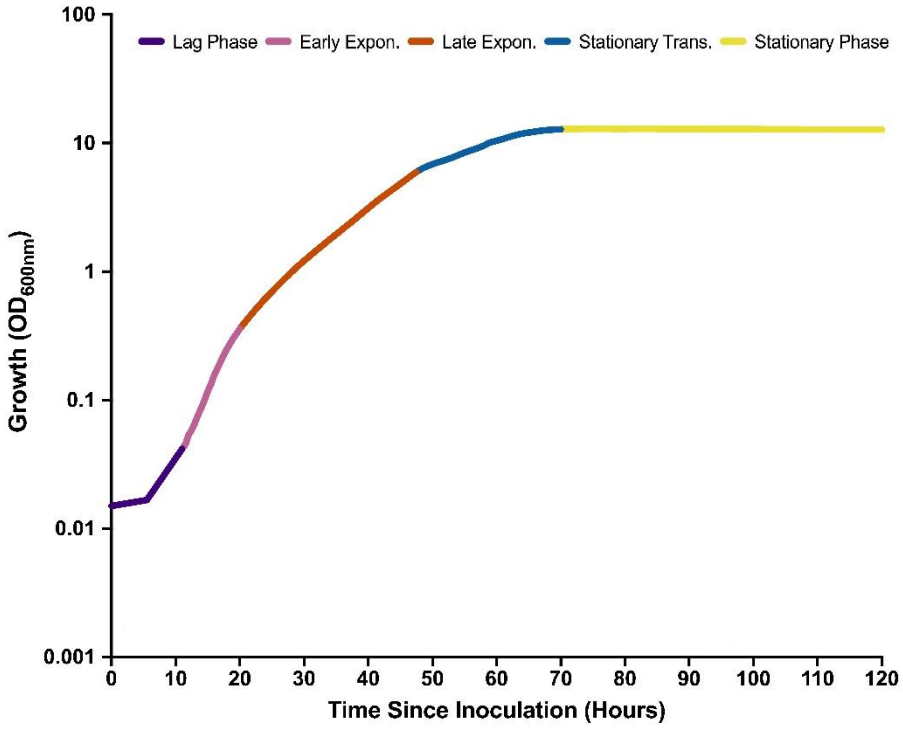


Figure 5.6. Demonstration of growth pattern in microfermentation plates, with each of the stages colour coded. Of interest are the early and late exponential (characterised by DT_E and DT_L respectively). After approximately 20 hours post-inoculation, the steep exponential growth becomes shallower and consequently the time taken to double optical density is greater.

5.3.1.2 Characterisation of *F. venenatum* Carbon Substrate Flexibility by Microfermenation Assays

The newly developed microtitre plate OD-based method was used to screen growth of *F. venenatum* A3/5 on various alternative carbon sources. As discussed earlier in the chapter, cultures screened in this manner demonstrated two exponential phases which necessitated the use of two distinct doubling times (DT_E and DT_L) as growth metrics to describe culture growth profiles. DTE was calculated between 15 and 21 hours, while DTL was calculated between 36 and 42 hours (Section 5.2.1.1).

The flexibility of *F. venenatum* substrate utilisation was first explored with three monosaccharides (glucose, galactose and xylose) and two

disaccharides (lactose and sucrose). Both sucrose and xylose appeared to be promising substrates with similar doubling times to glucose-fed cultures and no significant difference between DTE and DTL when compared to glucose (Figure 5.5, Supplementary Table 5.1). However, the maximum growth period of xylose-fed cultures occurred later into incubation, suggesting an increase in lag phase and/or slower assimilation into the cell. By contrast, galactose and lactose-fed cultures showed significantly higher DTL doubling times (Two Way ANOVA – $F(4,107) = 56.33$, $df = 4$, $p < 0.0001$; Dunnett's Multiple Comparisons – galactose $p < 0.0001$; lactose $p < 0.0001$) and an increased lag phase duration, although intriguingly galactose exhibited an earlier exponential growth phase with DTE values with no significant difference to glucose (4.07 ± 0.44 and 2.87 ± 0.46 hours, respectively; $p = 0.84$).

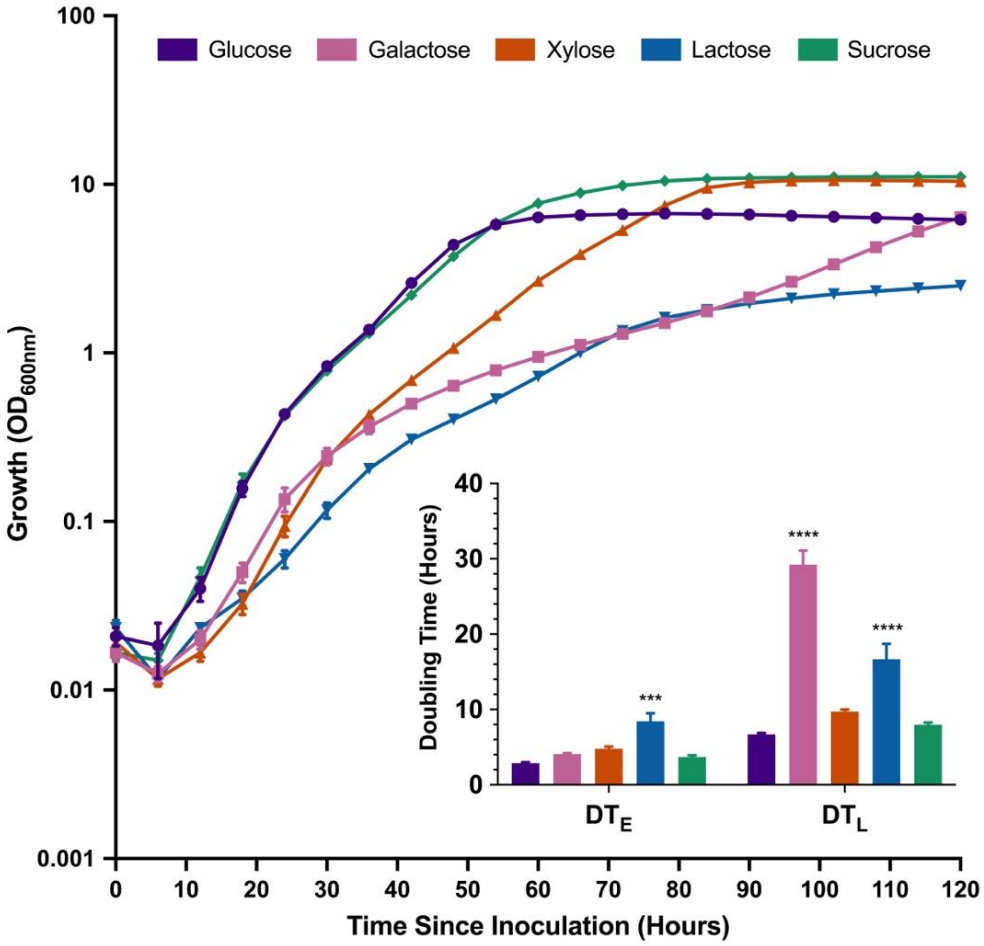


Figure 5.5. Growth of *F. venenatum* A3/5 on different sugar substrates as assessed by microfermentation growth assay. Sugars typically associated with plant tissues (xylose and sucrose) show similar doubling times to that of glucose, while galactose and lactose sugars demonstrate considerably reduced growth rate. DT_E = Early doubling time; DT_L = Late doubling time. Error bars indicate ± SEM, n = 12. Asterisks indicate significant difference (Two Way ANOVA/Dunnett's Multiple Comparisons) when compared to the glucose control: * = 0.05, ** = 0.01, *** = 0.001, **** = <0.001.

Investigating Sugar Feedstock Blends

While the current Quorn™ industrial process uses a pure glucose syrup as the carbon feedstock, complete substitution of the carbon source may be unnecessary. Should there be reluctance for complete substitution of glucose, implementing a low-cost carbon substrate without having to compromise on growth rate or other production parameters would be of great benefit. As such, feedstock blends comprising glucose with either sucrose or xylose were screened for effects on *F. venenatum* A3/5 growth.

Feedstock blends were created by first mixing various ratios of glucose and sucrose to a final concentration of 3% (w/v) total sugar. The growth profiles in each blend were almost identical, with no significant effect on either DT_E or DT_L doubling time metrics when compared to the glucose control (Figure 5.6A; Two Way Anova – $F(4,50) = 0.7861$, $df = 4$, $p = 0.5397$). Similarly, there was no significant difference observed between DT_E values when mixing various ratios of glucose and xylose to a final concentration of 3% (w/v) total sugar (Figure 5.6B). However, when xylose became the predominant sugar in the blend, DT_L values become significantly higher than that of the glucose control (Two Way Anova – $F(10, 154) = 26.29$, $df = 10$, $p < 0.0001$) (Dunnett's multiple comparisons; G3 – 5.56 ± 0.52; G1.5:X1.5 – 6.78 ± 0.45, $p = 0.02$; G1:X2 = 8.63 ± 0.85, $p < 0.0001$).

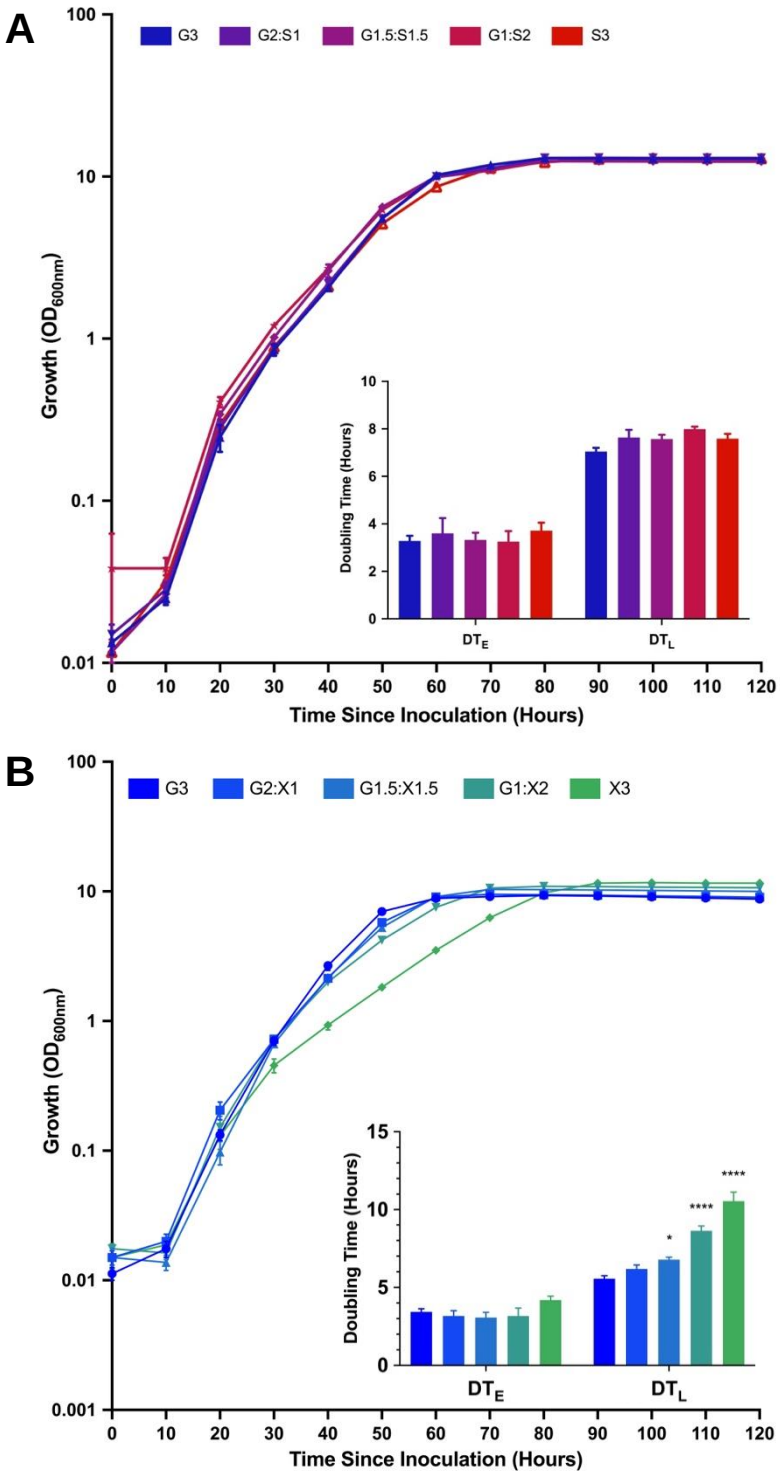


Figure 5.6. Growth of *F. venenatum* A3/5 on different sugar blends as assessed by microfermentation growth assay. A. Growth on glucose (G) and sucrose (S) blends, with relative % indicated by key at top of figure. B. Growth on glucose (G) and xylose (X) blends, with relative % indicated by key at top of figure. DT_E = Early doubling time; DT_L = Late doubling time. Error bars indicate ± SEM, n = 12. Asterisks indicate significant difference (Two Way ANOVA followed by Dunnett's Multiple Comparisons) when compared to the glucose control.

Glucose Homopolymers

Several glucose homopolymers are available as low-cost carbon substrates, and thus were promising targets for screening. The disaccharides maltose and cellobiose were studied in addition to starch, a polymer comprising several hundred glucose monomers in length. Furthermore, 50:50 blends of each polymer with glucose were investigated as a potential path to feedstock cost reduction.

There were no significant differences in DT_E values between glucose and the alternative substrates (Figure 5.7). However, as incubation progressed, the cultures fed glucose polymers showed a significant increase in doubling time when compared to the glucose control (Two Way ANOVA – $F(9, 119) = 7.79$, $df = 9$, $p < 0.0001$; Dunnett's test – Maltose = $p < 0.0001$; Cellobiose = $p < 0.0001$; Starch $p = 0.0006$). Notably, maltose induced the slowest growth of the homopolymers with DT_L values significantly greater than both cellobiose and starch ($p = 0.0001$ and <0.0001 respectively). Blending homopolymers with glucose appeared to relieve this reduction in growth (Figure 5.7). Glucose-fed cultures reached stationary phase entry around 50 hpi, approximately 20 hours prior to the cultures fed a glucose homopolymer.

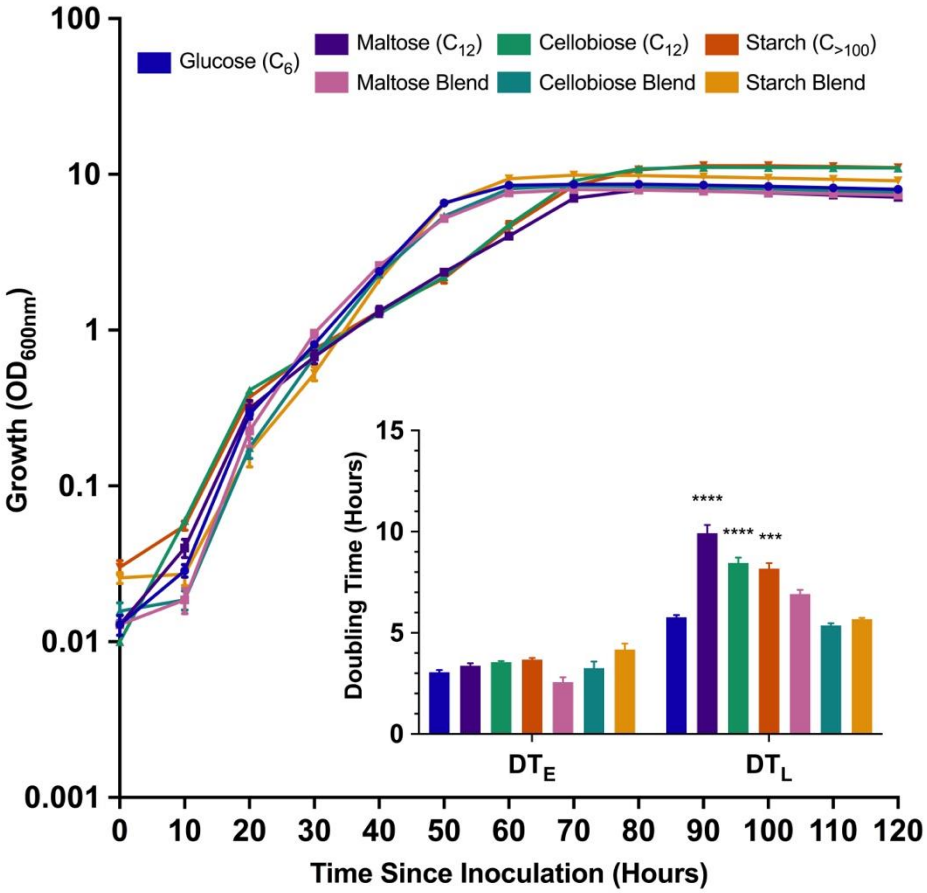


Figure 5.7. Growth of *F. venenatum* A3/5 on different glucose homopolymers as assessed by microfermentation growth assay. Different polymers are indicated by key at top of figure. DT_E = Early doubling time; DT_L = Late doubling time. Error bars indicate ± SEM, n = 7. Asterisks indicate significant difference (Two-Way ANOVA/Dunnett's Multiple Comparisons) when compared to the glucose control: * = 0.05, ** = 0.01, *** = 0.001, **** = <0.001.

Commercial Syrups

Commercial sugar syrups provide a promising alternative to using pure sugars, becoming increasingly available following the reduction in usage in food and drink industries. The current Quorn™ industrial system implements a pure glucose syrup, yet many low-cost alternatives exist, comprising large sugar polymers or blends of glucose with other sugars, such as fructose. *F. venenatum* A3/5 growth was assessed using a selection of commercially available sugar syrups, each varying in its composition.

None of the sugar syrups assessed showed any significant difference in either DT_E or DT_L doubling times when compared to the glucose control

(Figure 5.8). Particularly noteworthy is the result with the syrup C*Sweet M01521 as this carbon substrate contained only polymeric sugars with no glucose. However, it is cautioned that growth of *F. venenatum* A3/5 in this particular set of microfermentations was stalled compared to other growth studies presented here. This is apparent in both doubling time metrics, particularly the DT_E values for each culture as well as in the stationary phase entry. In other examples, stationary phase is reached between 50 and 60 hours, yet in this case stationary phase does not occur until at least 70 hpi. This pattern was maintained following a repeat experiment with fresh spore suspensions and media preparation, so the cause was not identified.

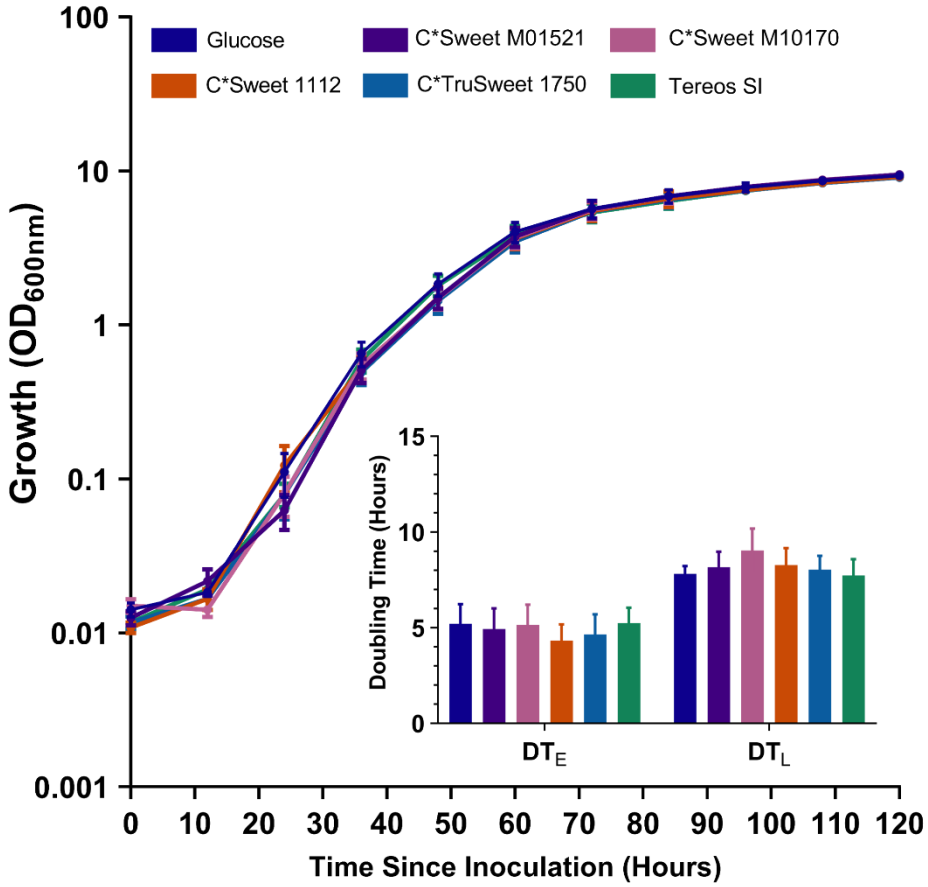


Figure 5.8. Growth of *F. venenatum* A3/5 on different commercial sugar syrups as assessed by microfermentation growth assay. Different syrups are indicated by key at top of figure. DT_E = Early doubling time; DT_L = Late doubling time. Error bars indicate ± SEM, n = 12. Statistical test compared DT values of each substrate to the glucose control (Two Way Anova/Dunnett's Multiple Comparisons).

F. venenatum A3/5 was also fed a syrup produced by breakdown of lignocellulosic substrates, comprising mostly glucose (33.8%) but also containing xylose (11.2%) and other unknown sugars (Figure 5.9). The cultures fed the lignocellulosic syrup exhibited an increased lag phase, and significantly slower DT_E value (Two Way ANOVA – $F(1,92) = 17.70$, $df = 1$, $p < 0.0001$). However, the lignocellulosic syrup-fed cultures showed significantly lower doubling times than the glucose-fed cultures in the later DT_L, exponential growth phase ($p = 0.0253$).

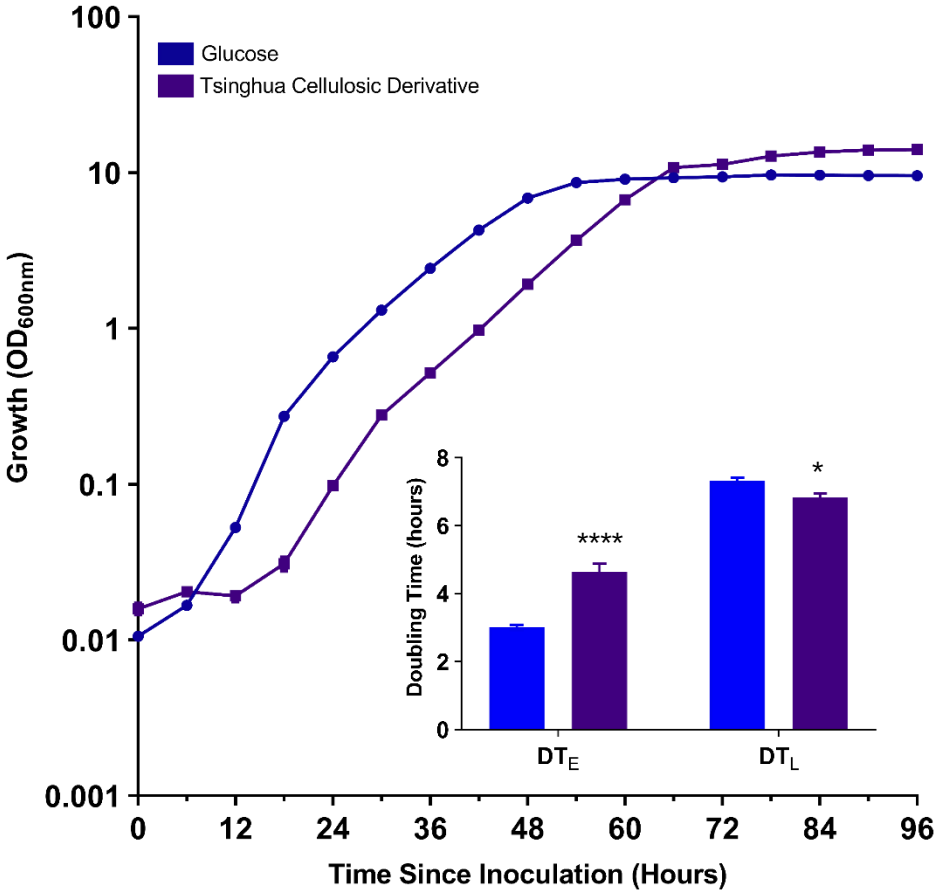


Figure 5.9. Growth of *F. venenatum* A3/5 on feedstocks produced from breakdown of lignocellulosic material as assessed by microfermentation growth assay. DT_E = Early doubling time; DT_L = Late doubling time. Error bars indicate ± SEM, n = 24. Statistical test compared DT values of each substrate to the glucose control (Two Way Anova/Dunnett's Multiple Comparisons); * = 0.05, ** = 0.01, *** = 0.001, **** = <0.001.

5.3.2 Characterisation of Alternative Fusarium venenatum Strains

5.3.2.1 Microfermentations of Alternative Strains with Sucrose

Each of the 25 strains of *F. venenatum* in the culture collection (Table 3.3) were screened for growth in the microfermentation system using both 3.00 % (w/v) glucose and 3.00 % (w/v) sucrose as feedstocks. The latter sugar was included as results above had identified sucrose as a potential alternative carbon source for the mycoprotein fermentation process.

It was found that almost all of the strains exhibited no differences in growth on both glucose and sucrose when compared to A3/5 (Figure 5.10). There was no significant difference between A3/5 and any of the alternative strains when fed glucose and DT_E and DT_L were used as metrics. However, when sucrose was used as the carbon substrate, one strain (114-3) did show a significant increase in DT_L value (Two Way ANOVA – $F(31, 125) = 3.95$, $df = 31$, $p < 0.0001$; Dunnett's multiple comparisons – $p = 0.026$). This strain also showed a significantly lower final absorption than all other strains (Figure 5.10). Nevertheless, some strains exhibited interesting growth features. For each sugar, strain 114-15 shows very rapid early phase growth, achieving optical density values significantly greater than the other strains at 10 and 20 hpi. Strains 114-3 and 114-6 show unusual growth patterns, exhibiting slower entry to the stationary phase compared to strains forming the consensus, noting that 114-6 only demonstrated this change when incubated with sucrose (Figure 5.10; Supplementary Table 5.1).

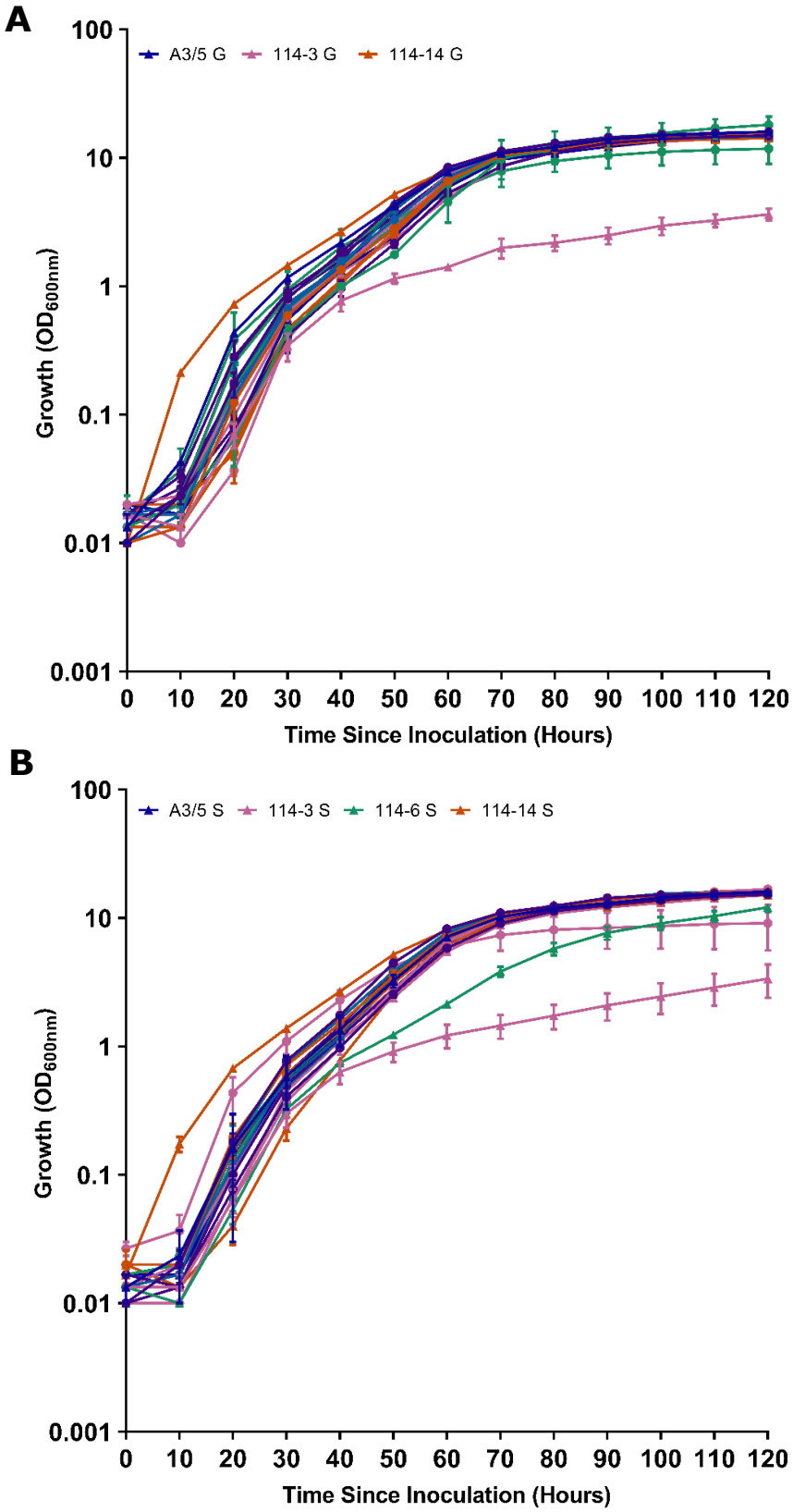


Figure 5.10. Growth of twenty-five different strains of *F. venenatum* A3/5 on glucose and sucrose as assessed by microfermentation growth assay. A. Glucose B. Sucrose. Strains exhibiting interesting features are highlighted in the key at the top of the figure. Values indicate mean average of three replicates ± SEM.

5.3.3 Scale-up Studies of Candidate Strains with Sucrose

5.3.3.1 Shake Flask Culture

While there appeared to be little difference between doubling times of *F. venenatum* strains based on the initial microfermentation screens, several strains had other features of interest which made them prime candidates for upscaling to trials with shake flask culture. These strains included 114-15 (a MAT1-2 strain) and 114-23 (exhibiting reduced mycotoxin production, see Chapter 6). Initially, each strain of *F. venenatum* was fed either glucose, sucrose or a blend of the two sugars at 3.00% (w/v) total sugar given that earlier screens had identified sucrose as a candidate for replacing glucose as the carbon feedstock used by Quorn™.

For A3/5, the dry cell weight from shake flask growth showed no significant difference in biomass accumulation between the three substrates (Figure 5.11). As seen in the microfermentation assays, the transition between exponential and stationary occurred at around 48 hpi for each of the three sugar substrates. Glucose-fed cultures showed significantly greater biomass accumulation than those provided with the sugar blend at 48 hours (Two-way ANOVA – F (2, 30) = 2.79, df – 2, p =0.78; Dunnett's multiple comparisons – p = 0.023), but this difference was no longer evident as cultures entered stationary phase (Figure 5.11A).

Strain 114-15 also showed a lack of significant difference in dry cell weight accumulation between the carbon feedstocks. The only significant difference detected was at 48 hpi, where glucose-fed cultures had significantly greater (Two-way ANOVA – F (2, 30) = 2.79, df – 2, p =0.62; Dunnett's multiple comparisons – p = 0.045) DCW than those fed sucrose. DCW in glucose-fed cultures at 36 hours was significantly less in this strain compared to A3/5 and 114-23 (Two-way ANOVA – F (2, 30) = 0.07, df – 2, p =0.78; Dunnett's multiple comparisons – p = 0.0003 and

0.0006 respectively), yet this difference was no longer evident at 48 hours where the strain appeared to transition to stationary phase.

Strain 114-23 showed no significant difference in biomass accumulation between carbon feedstocks at each time point studied (Two-way ANOVA – $F(2,30) = 2.10$, $df = 2$, $p = 0.1370$). Furthermore, there was no significant difference between DCW accumulation in this strain when compared to A3/5.

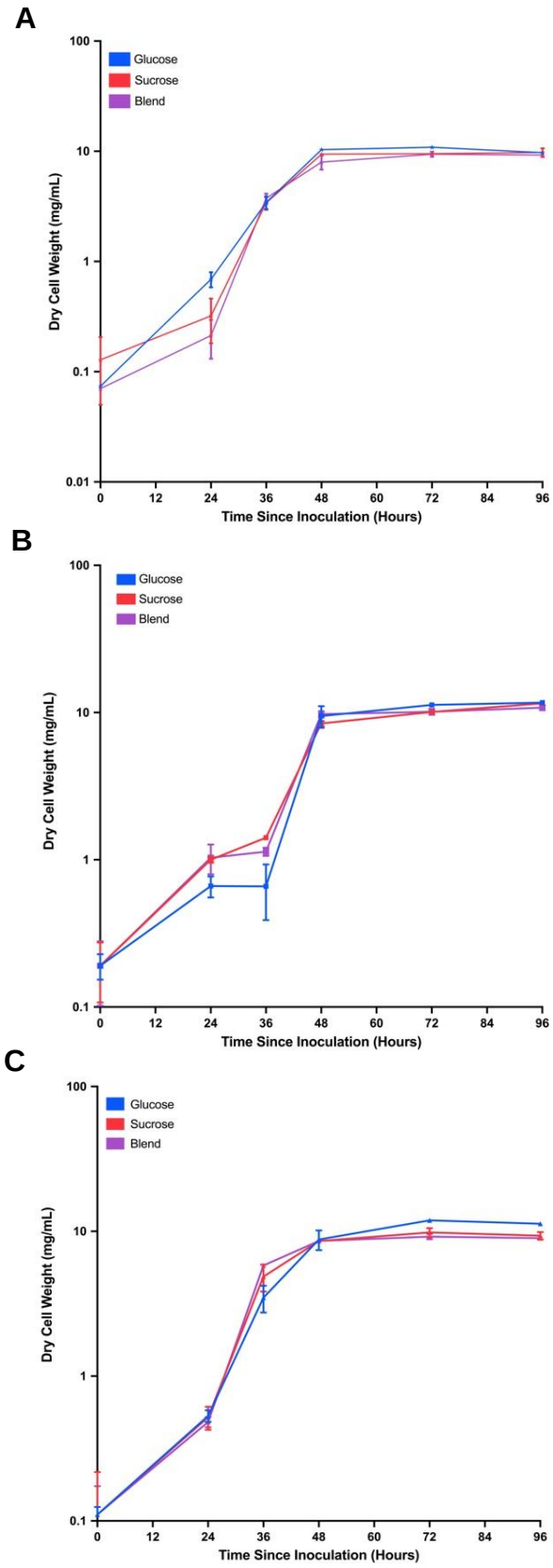


Figure 5.11. Growth of *F. venenatum* in shake flask culture using different sugar sources. Key on graphs indicates the particular sugar substrate. A. Quorn™ industrial strain, A3/5. B. Strain 114-15. C. Strain 114-23. Of these strains, 114-15 appears to show a reduction in growth at 36 hpi, while at 48 hours growth differences show no significant difference (Two-Way ANOVA and Dunnett's Multiple Comparisons test). Growth profiles show mean average values ± SEM, n = 3.

5.3.3.2 A3/5 Five L Bioreactor Fermentations

The Quorn™ A3/5 production strain was incubated in 5 L batch fermentations and fed either glucose, sucrose, or a blend of the two sugars. Growth profiles were obtained for all three substrates whether using offline biomass samples to assess growth (Figure 5.12; Table 5.4). The patterns of growth witnessed between the two measurement types were similar. Exponential phase occurred between 36 and 48 hours, with cultures entering stationary phase by 72 hours. Biomass samples showed no significant difference between feedstocks across any timepoint suggesting that utilisation and assimilation of both carbon substrates is similar in *F. venenatum* A3/5 (Two-way ANOVA – $F(2,31) = 1.49$, $df = 2$, $p = 0.24$; Figure 5.12).

Overall growth patterns were similar to those observed in microfermentations as well as in shake flasks. However, samples taken at 36 hours showed markedly lower biomass in these bioreactor fermentations than the other growth methods. Capacitance readings confirm that these samples were taken during the exponential phase however.

Table 5.4 Accumulated Biomass in *F. venenatum* A3/5 5L STR fermentations.

Time Since Inoculation (Hours)	Accumulated Biomass (mg/mL)			Capacitance (pF/cm)		
	Glucose	Sucrose	Blend	Glucose	Sucrose	Blend
24	1.09 ± 0.10	0.77 ± 0.15	0.65 ± 0.15	0.47	0.052	0.068
36	1.94 ± 0.26	0.84 ± 0.16	1.79 ± 0.09	1.31	1.22	1.12
48	10.30 ± 1.98	8.98 ± 0.10	9.63 ± 1.10	8.87	8.91	6.30
72	15.42 ± 1.38	13.99 ± 0.15	13.77 ± 0.44	15.92	13.70	9.38
96	13.88 ± 0.95	13.87 ± 0.13	12.63 ± 0.12	21.38	17.69	11.03

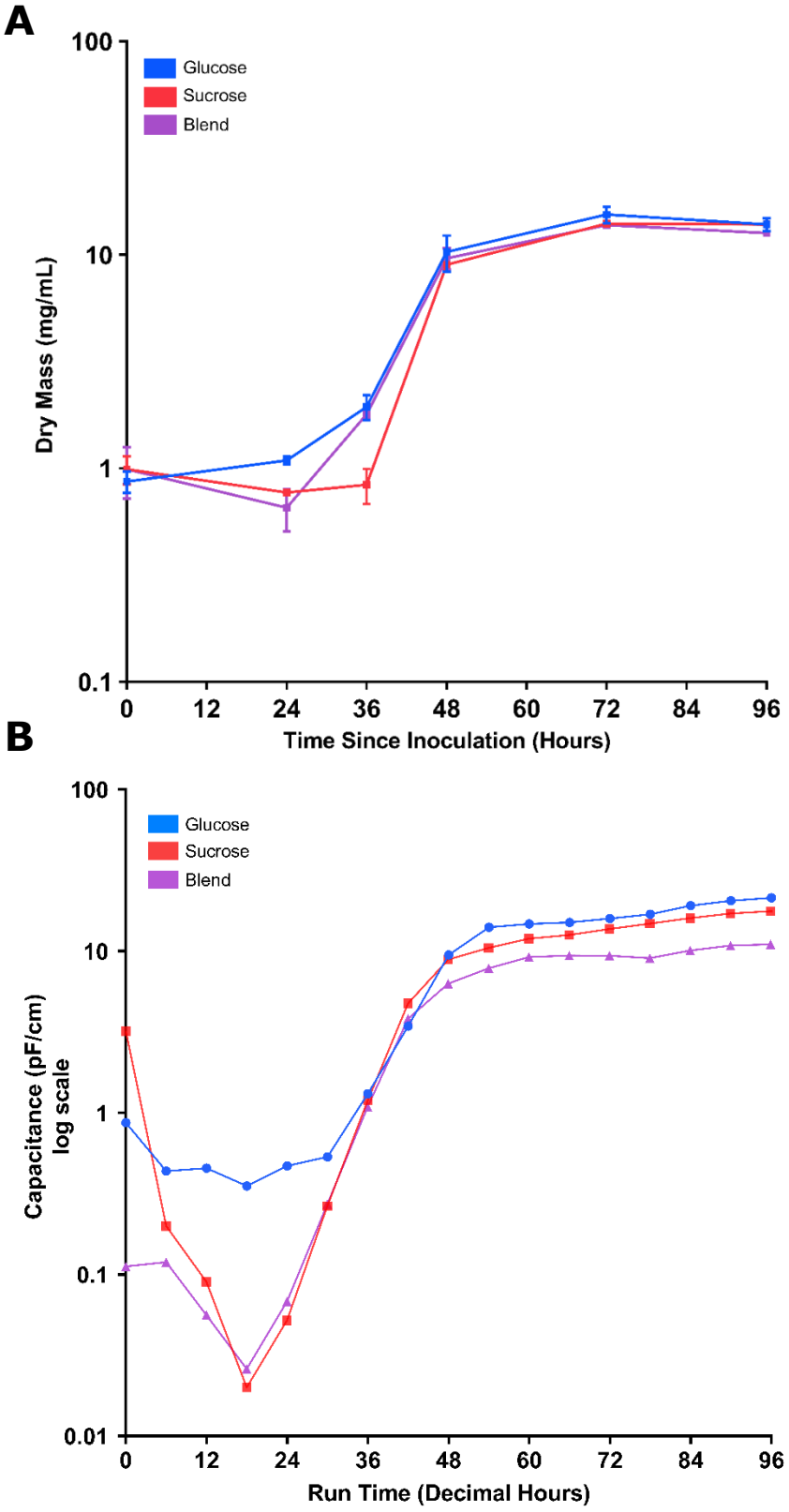


Figure 5.12. Growth of *F. venenatum* A3/5 in 5 L bioreactor batch culture using different sugar sources. Key on graphs indicates the particular sugar substrate. A: Dry biomass accumulation. Mean average values are plotted ± SEM, n = 3 pseudoreplicates. B: Online capacitance readings taken throughout the fermentations, observed using the Aber Instruments SCADA probe.

5.3.3.3 Ten L Batch and Steady State Bioreactor

The final stage in assessing growth of *F. venenatum* with glucose or sucrose involved upscaling to 10 L reactions, implementing both batch and steady-state conditions for *F. venenatum* strains A3/5 and 114-23 (a putative non-mycotoxin producing strain, see Chapter 6).

In batch conditions, biomass accumulation showed different patterns according to sugar substrate and strain (Figure 5.13). Sucrose-fed cultures of A3/5 showed significantly greater biomass accumulation at 28 and 30 hpi than glucose-fed cultures (Two-way ANOVA – $F = (3,14) = 22.26$, df = 3; Dunnett's multiple comparisons, $p = <0.0001$ and 0.012, respectively), although no difference in final biomass. By contrast, 114-23 showed no difference in biomass accumulation between glucose- and sucrose-fed cultures and showed significantly lower biomass than A3/5 cultures during exponential phase ($p = 0.0023$). For all test conditions, exponential growth begun to plateau between 30 and 48 hpi. Notably, biomass accumulation in these fermentations was approximately 10-fold lower than those established in previous shake flask and 5 L fermentations.

Continuous fermentations of A3/5 and 114-23 were also performed at a modest dilution rate of 0.125 h^{-1}. For both strains there was significantly greater biomass accumulation in sucrose-fed cultures than those incubated with glucose once exponential phase had been established (One-way ANOVA – $F = 54.09$, $p < 0.0001$; Holm-Šídák's multiple comparisons – A3/5, $p = 0.009$; 114-23, $p = 0.011$; Figure 4.13B). There was a clear distinction between the two strains assessed, with A3.5 showing 137 and 135% greater biomass accumulation than 114-23 in glucose and sucrose-fed cultures, respectively. This was found to be significantly different (glucose, $p = 0.0002$; sucrose, $p = 0.0001$).

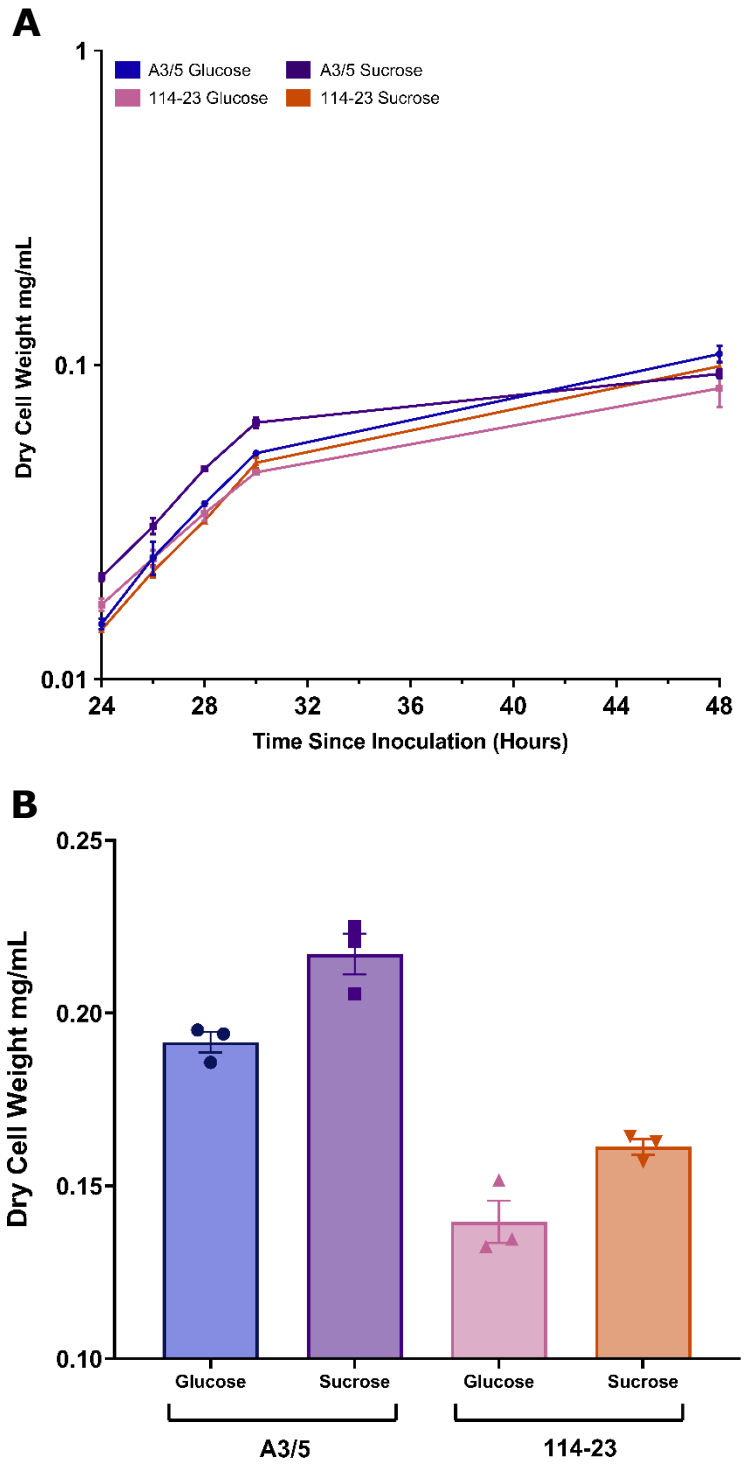


Figure 5.13. Growth of strains *F. venenatum* under pilot plant conditions as assessed by dry cell weight. A: A comparison of batch culture exponential phase biomass accumulation in strains A3/5 and 114-23 fed with either glucose or sucrose. B: Steady-state growth systems of A3/5 and 114-23 maintained at a dilution rate of 0.125 h^{-1}. Values indicate mean average of three replicates, error bars = ± SEM.

5.3.4 Impact of Carbon Sources on F. venenatum *Morphological Traits*

5.3.4.1 Effect of Sucrose on Germination Pattern

Substituting glucose for an alternative carbon source may drive changes in germination rate. The impacts on *F. venenatum* A3/5 growth were explored in 4 media compositions; the proprietary RHM medium composition supplemented with either glucose, sucrose or 50:50 blend of the two sugars, alongside potato dextrose broth (PDB) as a nutrient-rich comparison. In each case, the appearance of cell morphotypes (dormant, swollen or germinating) was monitored and where cells demonstrated germination, the number of germination events per spore enumerated.

There was no significant difference between the morphotype distribution in cultures fed glucose, sucrose or a blend of the two (Figure 4.12A). By comparison, macroconidia inoculated in PDB showed rapid macroconidial swelling, with a significantly greater number of spores having swollen two hours post-inoculation compared to glucose-fed cultures. This did not promote germling emergence, as 6 hours' post-inoculation there was no significant difference in the number of germinating spores compared to glucose-fed cultures.

Due to the multicellularity of *Fusarium* macroconidia, the germ tube morphotypes could be further categorised. The initial germination events in macroconidia showed a bias at the polar cells, thought to allow the most efficient exploration of the environment (Harris 2005). The number of germ tubes originating from each macroconidium were counted and photographed (Figure 5.12B). 4 hours post-inoculation, only single germ tubes were observed from each germinating macroconidia on all media types. Cultures incubated with sucrose demonstrated a significantly greater number of germinating spores than those incubated with glucose or the sugar blend (32.78% ± 4.34 versus 15.11% ± 7.11 and 15.82 ±

5.89) (Two-way ANOVA – F (3,19) = 0.00; DF = 3, p > 0.99; Dunnett's multiple comparisons – p = 0.037) (Table 5.3). By comparison, macroconidia incubated in PDB showed a reduced frequency of germination (62.8% ± 3.7) by 6 hours' post-inoculation compared to all the other media. Interestingly, further incubation for 2 hours in PDB resulted in a significant number of interstitial cell germination events (triple germlings = 13.5 % ± 3.5; quadruple germlings = 47.5 ± 5.5) occurring in the macroconidia, which were not observed in growth on the other media types, with no observable difference between the germination patterns of the three sugar sources at either 6 or 8 hours post inoculation.

An identical experiment was carried out for strain 114-15, although the effect of PDB was not assessed (Figure 5.13). Generally, *F. venenatum* 114-15 exhibited similar germination patterns to A3/5 but the carbon substrate has more pronounced effects on germination patterns. First, glucose was quicker to induce germination than sucrose, having a significantly greater number of single germlings at 4 hours post inoculation (Two-Way ANOVA – F (2,18) = 0.00, DF = 2, p > 0.999; Dunnett's multiple comparisons – p = 0.0034) (Table 5.4). Whereas in A3/5, the impact of the sugar blend closely resembled the patterns presented by glucose, in 114-15 the sugar blend instead exhibited greater similarity to sucrose. Thus, at 6 hpi sucrose and the sugar blend show greater numbers of double germlings than glucose (p = 0.0002 and <0.0001 respectively). This pattern continues, with sucrose-fed cultures showing more total germlings than glucose-fed cultures at 8 hpi. Unlike A3/5, some macroconidia of this strain show three germ tube emergence events, although this occurred at low prevalence (Glucose 8 hours; 1.80% ± 1.03).

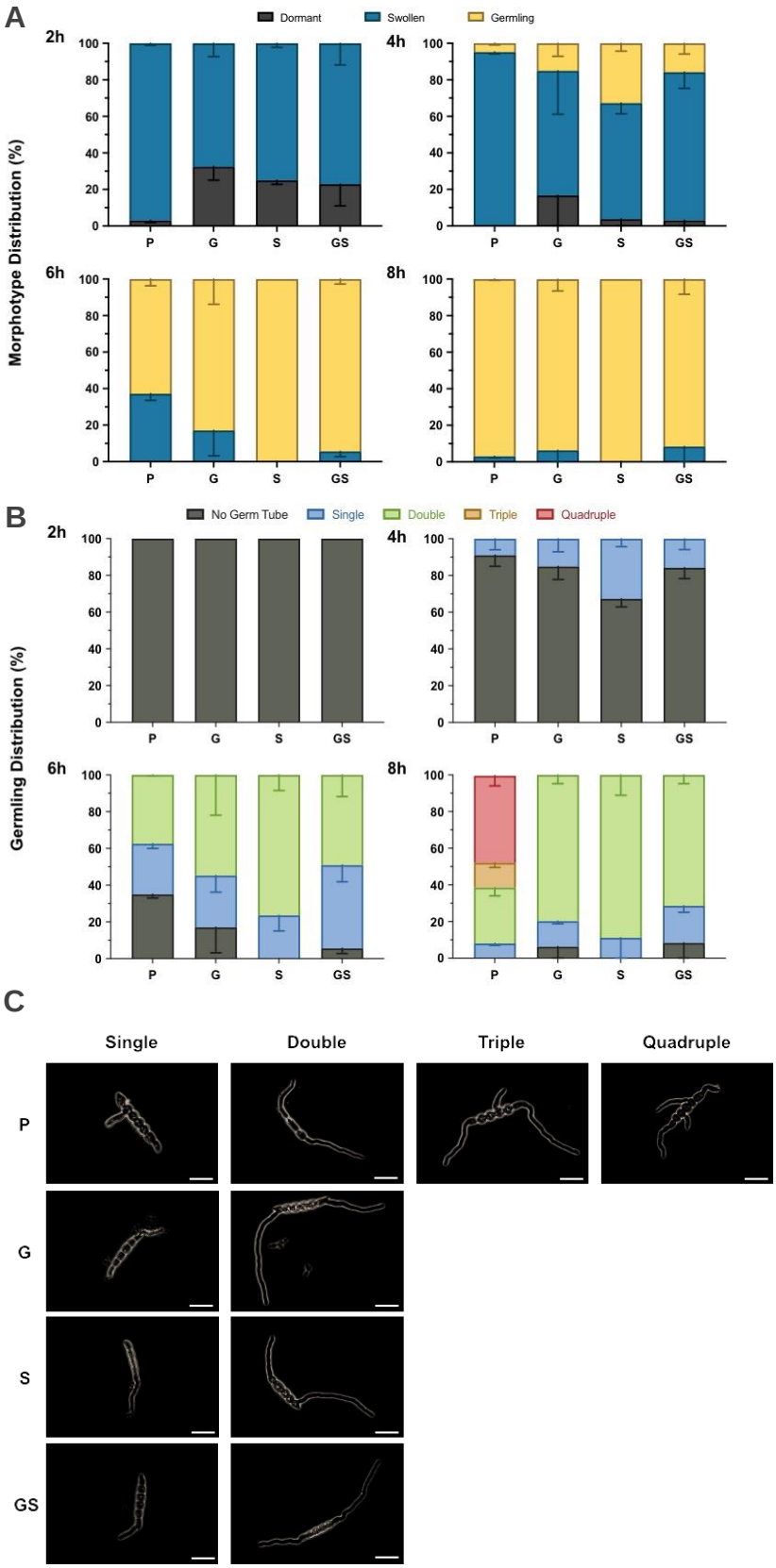


Figure 5.13. Effect of sugar source (glucose, sucrose or blend of both) on germination patterns of *F. venenatum* A3/5 macroconidia. A. Morphotype distribution across media types observed over the initial 8 hours of growth. B. Germling types distributed in each media type. C. Micrographs representing germination stages in each media type. P = PDB, G = RHM broth + glucose (3% w/v), S = RHM broth + sucrose (3% w/v). GS = RHM broth + glucose and sucrose (1.5% w/v each). Data represented as mean average values ± SEM. n = 3, scale = 50 µm.

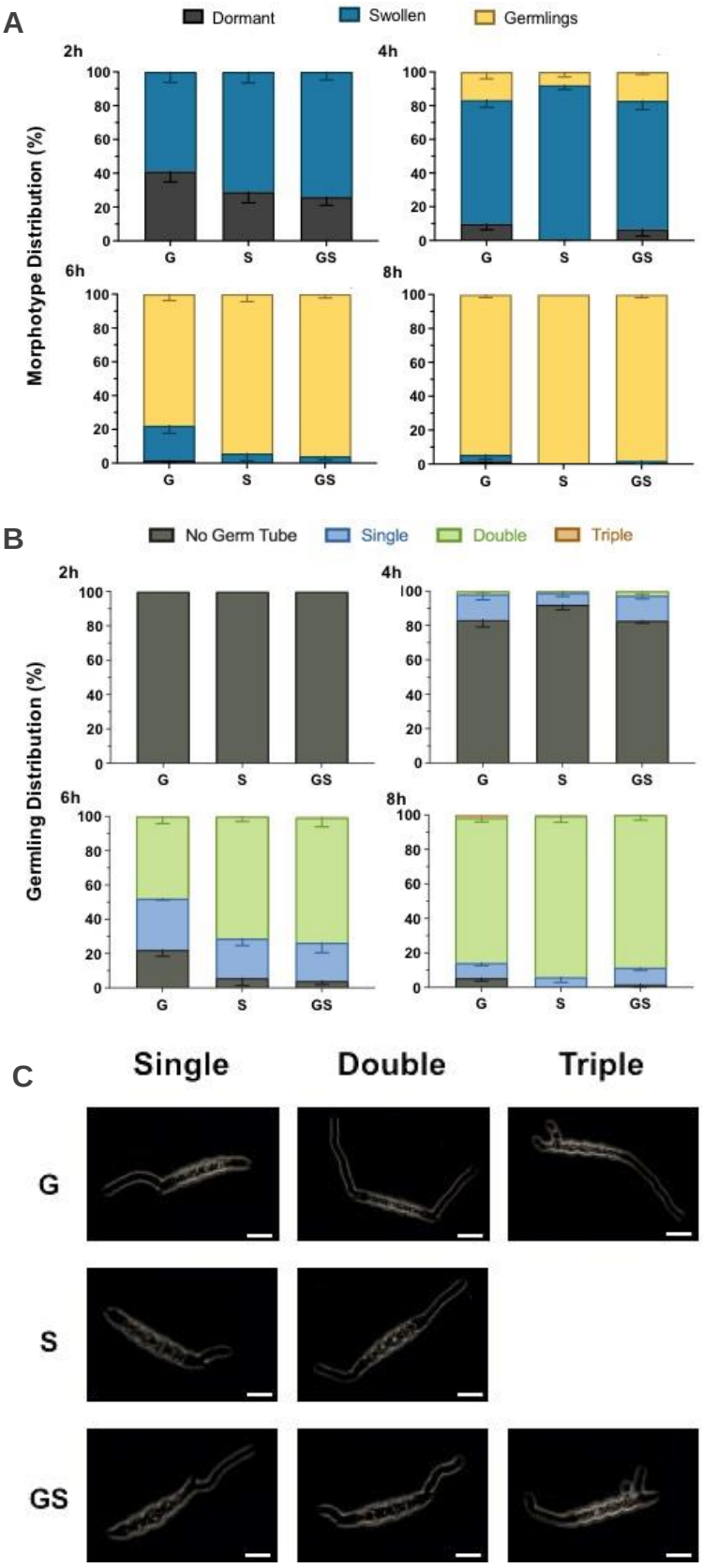


Figure 5.14. Effect of sugar source (glucose, sucrose or blend of both) on germination patterns of *F. venenatum* strain 114-15 macroconidia. A. Morphotype distribution across media types observed over the initial 8 hours of growth. B. Germling types distributed in each media type. C. Micrographs representing germination stages in each media type. G = RHM broth + glucose (3% w/v), S = RHM broth + sucrose (3% w/v). GS = RHM broth + glucose and sucrose (1.5% w/v each). Data represented as mean average values ± SEM. n = 3, scale = 50 µm.

Table 5.3. Distribution of morphotypes and germlings in strains A3/5 in response to carbon substrate changes. Values indicate mean average of 3 replicates, +/- SEM. "-" indicate categories in which no spores of that type were counted.

Time Since Inoculation (Hours)	Morphotype Distribution (%)			Germling Distribution (%)				
	Dormant	Swollen	Germling	No Germ Tube	Single	Double	Triple	Quadruple
Glucose								
2	32.42 ± 7.35	67.58 ± 7.25	-	-	-	-	-	-
4	16.67 ± 16.67	68.22 ± 23.78	15.11 ± 7.11	84.89 ± 7.11	15.11 ± 7.11	-	-	-
6	-	17.04 ± 13.84	82.96 ± 13.84	17.04 ± 13.84	28.15 ± 9.01	54.81 ± 21.94	-	-
8	-	6.25 ± 6.25	83.75 ± 6.25	6.25 ± 6.25	13.94 ± 1.44	79.81 ± 4.81	-	-
Sucrose								
2	25.00 ± 2.27	75.00 ± 2.27	-	-	-	-	-	-
4	3.70 ± 3.70	63.52 ± 5.88	32.78 ± 4.34	67.22 ± 4.34	32.78 ± 4.34	-	-	-
6	-	-	100.00 ± 0.00	-	23.54 ± 8.48	76.46 ± 8.48	-	-
8	-	-	100.00 ± 0.00	-	11.11 ± 11.11	88.89 ± 11.11	-	-
Blend								
2	22.86 ± 11.90	77.14 ± 11.90	-	-	-	-	-	-
4	3.03 ± 3.03	81.15 ± 5.89	15.82 ± 5.89	84.18 ± 5.89	15.82 ± 5.89	-	-	-
6	-	5.55 ± 2.27	94.44 ± 2.78	5.55 ± 2.78	45.37 ± 9.12	49.07 ± 11.82	-	-
8	-	8.34 ± 8,34	91.67 ± 8.34	8.34 ± 8.34	58.34 ± 41.67	33.34 ± 33.34	-	-
PDB								
2	3.05 ± 1.17	96.95 ± 1.17	-	-	-	-	-	-
4	-	95.15 ± 0.98	4.85 ± 0.98	95.15 ± 0.98	4.85 ± 0.98	-	-	-
6	-	37.17 ± 3.71	62.83 ± 3.71	37.17 ± 3.71	27.50 ± 2.50	39.67 ± 0.50	1.67 ± 1.67	-
8	-	2.96 ± 0.71	97.04 ± 0.71	2.96 ± 0.71	8.00 ± 1.00	27.46 ± 4.50	9.00 ± 4.73	31.67 ± 16.15

Table 5.4. Distribution of morphotypes and germlings in strain 114-15 in response to carbon substrate changes. Values indicate mean average of 3 replicates. "-" indicate categories in which no spores of that type were counted.

Time Since Inoculation (Hours)	Morphotype Distribution (%)			Germling Distribution (%)				
	Dormant	Swollen	Germling	No Germ Tube	Single	Double	Triple	Quadruple
Glucose								
2	41.10 ± 6.27	58.90 ± 6.27	-	-	-	-	-	-
4	9.75 ± 3.49	73.50 ± 4.25	16.74 ± 4.13	83.26 ± 4.13	14.95 ± 3.27	1.80 ± 1.03	-	-
6	1.58 ± 0.86	20.60 ± 4.54	77.83 ± 3.77	22.18 ± 3.77	29.90 ± 0.94	47.92 ± 4.23	-	-
8	1.75 ± 0.90	3.74 ± 2.66	94.50 ± 1.77	5.47 ± 1.75	8.80 ± 1.66	83.98 ± 2.38	1.74 ± 0.89	-
Sucrose								
2	28.97 ± 6.29	71.03 ± 6.49	-	-	-	-	-	-
4	0.56 ± 0.56	91.56 ± 2.59	7.89 ± 2.99	92.11 ± 2.99	6.99 ± 2.16	0.90 ± 0.90	-	-
6	-	5.72 ± 4.37	94.28 ± 4.37	5.71 ± 4.36	23.04 ± 4.15	71.25 ± 2.96	-	-
8	-	-	100.00 ± 0.00	-	6.03 ± 3.09	93.29 ± 3.58	0.68 ± 0.68	-
Blend								
2	25.89 ± 6.49	74.11 ± 4.86	-	-	-	-	-	-
4	6.63 ± 4.06	76.25 ± 5.25	17.13 ± 1.54	82.87 ± 1.54	14.61 ± 1.99	2.52 ± 1.63	-	-
6	-	4.09 ± 2.19	95.52 ± 2.19	4.09 ± 2.19	22.30 ± 5.95	72.78 ± 5.28	0.83 ± 0.83	-
8	-	1.85 ± 1.85	98.15 ± 1.85	1.85 ± 1.85	9.64 ± 1.63	88.51 ± 2.97	-	-

5.3.5 Elucidating Carbon Substrate Consumption

It was necessary to evaluate the consumption of glucose and sucrose by each strain to confirm that differences in biomass accumulation were not simply a consequence of greater substrate uptake. To this end, a Novozyme™ glucose/sucrose colorimetric assay kit was employed. This analysis was performed for A3/5, 114-8 and 114-23 and was used to compare glucose and sucrose consumption in triplicate shake flask cultures.

For each strain, there was limited consumption within the first 24 hours of growth. It was noted that results at this point showed unexpected sugar values of over 30 g/L (an error rate of approximately +10%), but such data were consistent for almost conditions (Figure 5.15). For A3/5, there was no significant difference in the consumption of glucose or sucrose (Two-Way ANOVA – $F(1, 4) = 0.577$, $df = 1$, $p = 0.49$), with approximately 3.5 and 25.5 g/L of both sugars consumed by 36 h and 48 h respectively. By 72 h, both sugars had been totally consumed, producing 0.37 and 0.32 g of biomass of per gram of glucose and sucrose, respectively.

Strain 114-8 demonstrated a similar pattern of consumption, but with a greater consumption of both sugars in the initial 36 h growth period (glucose = 10.6 g/L, sucrose = 10.0 g/L). At the onset of late exponential/early stationary phase (48 hpi) sugar consumption was similar to A3/5, with 25.0 and 27.8 g/L of glucose and sucrose consumed, respectively. This strain also demonstrated no significant final difference in biomass conversion between carbon feedstocks with 0.39 g of biomass per gram of glucose as opposed to 0.37 g/g in sucrose-fed cultures at 72h (Two-Way ANOVA – $F(1,4) = 0.40$, $df = 1$, $p = 0.56$). As with A3/5, both carbon substrates show almost total depletion by 72 hpi with only 0.30 glucose and 1.6 g/L sucrose remaining.

Finally, strain 114-23 showed the greatest difference in utilisation of the carbon substrates. Glucose was much more readily taken up by this strain within the first 48 hours showing significant lower remaining analyte as compared to sucrose with over half of the sucrose remaining in the medium at 48 hpi (16.14 g/L ± 1.74; Two-Way ANOVA – $F(1, 16) = 35.31$, df – 1, $p < 0.0001$; Šídák's multiple comparisons – $p = 0.0001$). However, there was no significant difference in final biomass accumulation, with glucose- and sucrose-fed cultures showing 0.39 and 0.37 g of biomass to g of sugar respectively.

There was no significant difference between final biomass accumulation and therefore carbon utilisation between A3/5 and 114-8 (One-Way ANOVA – $F(5,8) = 10.66$, $p = 0.0359$; Šídák's multiple comparisons – $p = 0.98$ and 0.99). Conversely, strain 114-23 demonstrated significantly lower biomass accumulation when grown on sucrose relative to A3/5, but this was not witnessed in glucose-fed cultures (Šídák's multiple comparisons – $p = 0.008$ and 0.1947 respectively).

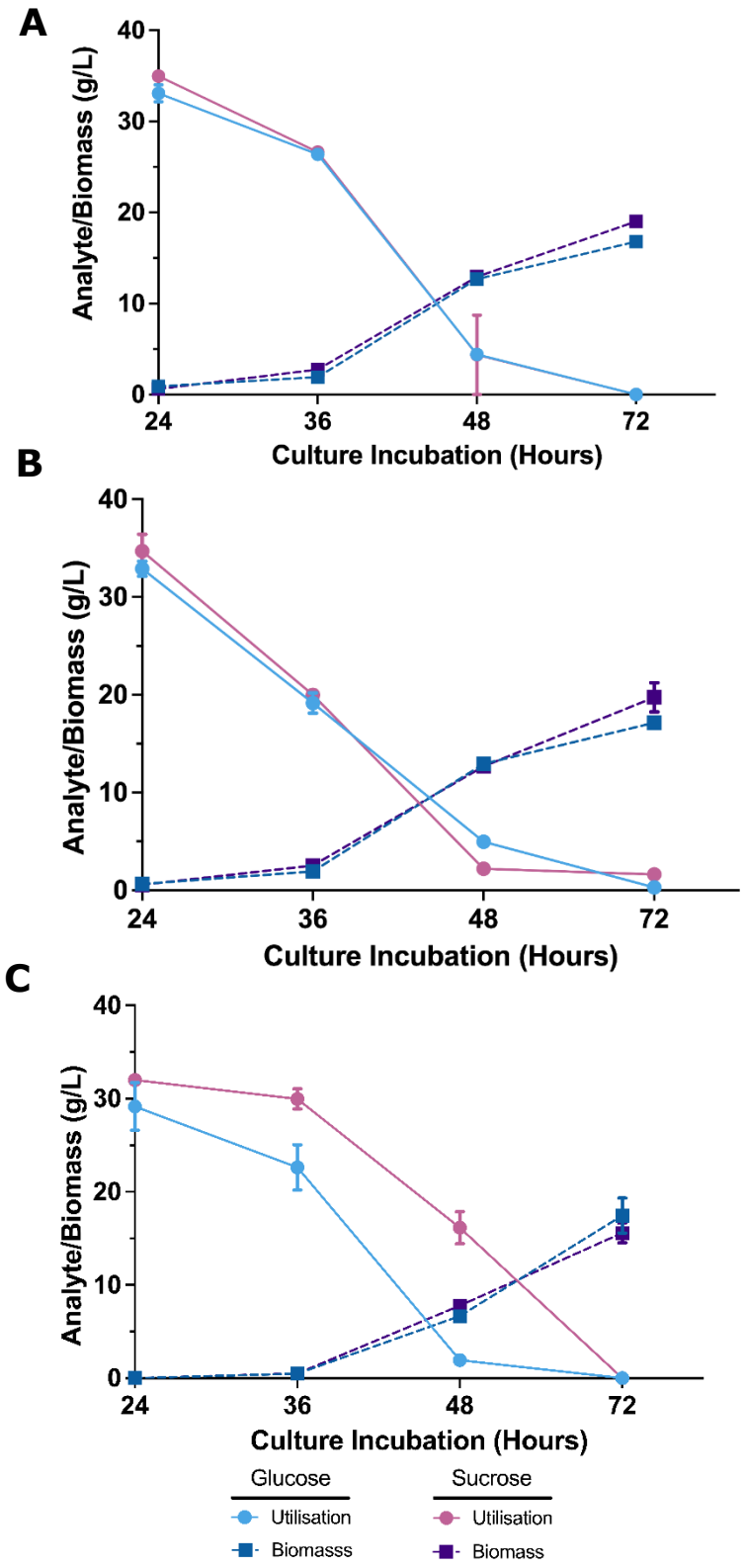


Figure 5.15. Utilisation of glucose and sucrose by strains of *F. venenatum* according to a glucose/sucrose colorimetric assay, plotted alongside recorded biomass. A. The Quorn™ strain A3/5 B. Strain 114-8 C. Strain 114-23. Values show mean average of three replicates ± SEM.

5.4 Discussion

All industrial processes which result in a commercial product must account for the cost of raw materials required. In commercial microbial processes, culture medium contributes a significant cost while great efforts are undertaken to optimise culture medium for output product quantity and quality (Singh et al., 2017). This is no less true in the Quorn™ mycoprotein industrial process where the carbon feedstock used is a highly-purified yet costly glucose syrup, which requires several shipments to the production site each day (RI Johnson, Pers. Comm.). As demand for Quorn™ products continues to grow, mycoprotein production capacity will be required to expand to meet that demand which will in turn necessitate an increased number of shipments of the glucose syrup. The current approach will likely become unsustainable and thus alternative approaches to providing a feedstock need to be explored. Work presented in this chapter assessed several carbon feedstock alternatives to the glucose syrup currently implemented in the mycoprotein industrial process. If productivity of the industrial process was to be preserved, it was essential that the growth rate remained equal if not faster than that of the current industrial feedstock, and that efficiency of biomass conversion remained constant or showed improvement. In addition, a range of strains of *Fusarium venenatum*, from a culture collection (established in earlier thesis work) were screened for possible differences in growth rates on sugar substrates. Importantly, these studies were performed at a fixed source and concentration of nitrogen, with only the carbon substrate changed. As discussed elsewhere, optimising the carbon:nitrogen ratio in a microbial fermentation is crucial to optimising growth rate and may modulate biosynthesis of important metabolites (Moreira et al., 2020).

5.4.1 Establishment of Microfermentation Assay

In the present study, we chose to determine success of alternative conditions through assessment of doubling time and later biomass

accumulation. Conditions presenting a loss of productivity would not be considered as a potential replacement for the Quorn™ industrial process. Turbidimetric analyses of growth have traditionally been implemented to study bacterial growth yet has been effectively used for studying filamentous fungus growth (Medina et al. 2012).

By establishing and then validating a microwell high-throughout screening system, alternative conditions were able to be screened rapidly before potential upscaling. This system does present drawbacks in that the fermentation system was likely too far removed from the Quorn™ industrial conditions and was used instead to assess substrate flexibility and utilisation of *F. venenatum* A3/5 and alternative strains. This is most evident following the appearance of two exponential phases (DT_E and DT_L), which complicates elucidation of growth patterns. Microwell plates were not shaken, a strategy to avoid pellet formation, yet the trade-off was reduced oxygenation. Perhaps appearance of the slower DT_L is a consequence of reduced oxygenation once the supply in medium was no longer sufficient for the growing mycelium.

This is remedied in shake flask fermentations, where strategies were implemented to increase oxygen transfer such as increasing agitation through using triple-baffled flasks. This model allowed confirmation of patterns observed at the level of the microplate, for instance the similarity in growth between glucose- and sucrose-fed cultures. However, shake flask cultures lack several important bioprocess controls such as pH, oxygenation and temperature, all of which are crucial for a commercial process. As growth proceeds in these batch cultures, production of organic acids by the fungus would acidify the medium which could limit growth (Hassan et al. 2012).

Five L Bioreactor studies observing growth by biomass accumulation and capacitance were undertaken with all the necessary bioprocess controls. The number of these trials was ultimately limited due to instrument component breakage and subsequent extended lead times with the replacement. Nevertheless, these provided the most accurate

model of the mycoprotein fermentation process available at Nottingham. Capacitance probe readings (facilitated by Aber Instruments) were particularly useful, providing online measurements of growth and thus greater resolution of growth patterns. Similarly, the nature of capacitance means that these readings were true of the live biomass in the fermentation, particularly important for detection of stationary phase entry. There are several items to note with regards to the capacitance probe readings. First, the initial decline in capacitance values witnessed in the first 18 hours of growth were an artifact of the capacitance probe setup. Once mycelial networks begin to expand, these differences are lost. Second, late-stage capacitance values showed substantial variation between the carbon substrates, yet these differences are not reflected in the dry biomass. In each carbon substrate, sporulation occurred following entry into stationary phase. We hypothesise that sporulation would have a greater effect on capacitance than biomass accumulation. Thus, the differences in stationary phase capacitance values might reflect differing rates of sporulation, with the glucose-fed cultures producing the most, followed by sucrose and finally the blend. This cannot be confirmed however, as spore counts were not quantified.

However, the Quorn™ mycoprotein production process is a continuous system, and thus it was required that proposed conditions be tested in this way. Fortunately, promising studies undertaken using steady-state systems available at the Quorn™ Fermentation Development Centre, to provide crucial information on the behaviour of sucrose in particular. In both A3/5 and 114-23, sucrose was found to give significantly greater dry cell weight values relative to glucose. This might suggest that growth on glucose is limited in some way, while provision of both glucose and fructose (in the form of sucrose) facilitates simultaneous utilisation and thus allowing greater biomass accumulation.

5.4.2 Utilisation of Alternative Sugar Feedstocks

The Quorn™ industrial organism, *Fusarium venenatum* A3/5 was first screened in culture medium supplemented with sugar substrates

available in crystalline form, namely galactose, xylose, lactose and sucrose. Results demonstrated that, as with many filamentous fungi, *F. venenatum* exhibits metabolic plasticity in the carbon substrates it can utilise, with growth observed with all of the sugar feedstocks. However, certain differences were evident. *F. venenatum* showed more limited growth on sugars rarely found in the environment. For example, lactose is the principal sugar found in milk and is regarded as a waste product from cheese-manufacture or whey-processing industries and is increasingly viewed as a potential carbon source in commercial processes (Pasotti et al., 2017). Several filamentous fungi are capable of utilising lactose, including *T. reesei*, but with relatively low growth rates (Fekete et al. 2007; Seiboth et al. 2007), and this also appeared to be true for *F. venenatum.* Thus, lactose was considered to be the weakest candidate of the sugar feedstocks examined.

By contrast, the combination of xylose and sucrose were shown to support good biomass accumulation. Both of these sugars are important components of lignocellulosic waste streams, and utilisation of such feedstocks could provide substantial cost savings. Indeed, the ability to metabolise these sugars is often a first aim in genomic engineering of industrial organisms such as the bacterial workhorses *Escherichia coli* and *Psuedomonas putida* (Löwe et al. 2020). Unlike yeast and bacteria, filamentous fungi are naturally equipped with a suite of enzymes to facilitate utilisation of plant storage sugars such as sucrose and xylose (Sloothak et al. 2016). This is particularly true in genera such as *Fusarium* which demonstrate a primarily phytopathogenic lifestyle and thus it follows that *F. venenatum* would be expected to show the ability to metabolise these sugars.

While many fungi can use D-xylose as a sole carbon source, this sugar was unable to drive growth to the same level as glucose in *F. venenatum* A3/5. This is likely explained by the expanded biochemical pathway required for D-xylose to be metabolised, where D-xylose is eventually converted to D-xylulose-5-phosphate before entry into the pentose

phosphate pathway (Li et al. 2014). This conversion requires the activity of three enzymes: D-xylose reductase, xylitol dehydrogenase and xylulokinase, the expression of which will have to be induced and is not usually constitutive in filamentous fungi, thus potentially accounting for the delay in growth (Terenbieniec et al. 2021). In the present study it was shown that when glucose became the majority carbon substrate in blends containing both glucose and xylose, doubling times were restored to similar rates as when glucose was used as a pure substrate. It is possible that these sugars were not used simultaneously but instead this effect was a consequence of CCR by glucose on xylose utilisation These results suggested that xylose, and indeed lignocellulosic derivatives containing xylose, could be used to supplement the current glucose syrup but would be unable to replace it. It is also noted that the use of high purity xylose is unfeasible as a commercial carbon feedstock because it is prohibitively expensive. The more likely scenario is therefore that xylose might be used as a component in lignocellulosic breakdown products which are increasingly available following global interest in second generation biofuels (DEFRA, 2019).

The possibility of using cellulosic-derived substrates as a potential feedstock was also explored, given that these are increasingly desirable in industrial biotechnology due to perceived environmental and cost benefits (Govil et al., 2020). In the present study, we trialled one such feedstock produced from ionic liquid treatment of lignocellulosic material. When A3/5 was incubated in the presence of this cellulosic derivative (comprising 33.8% glucose and 11.2% xylose), there was an increased lag phase which had not occurred in previous trials using high-purity glucose and xylose in the blends. This might have been a consequence of carry-over of growth inhibitors following hydrolysis of the lignocellulose, a well-reported issue that has impeded progress in the use of these alternative substrates (Sakai et al. 2007). Nevertheless, there was a significant decrease in the late exponential doubling time when this substrate was used relative to glucose, suggesting that cellulosic derivatives containing blends of glucose and other sugars

show promise, despite the treatment processes required to produce them not yet upscaled sufficiently to an industrial scale. Elsewhere, there has been very limited published data regarding the growth of the Quorn™ strain on substrates other than glucose, with the most comprehensive report published in 1975 (Anderson et al. 1975). This report examined the use of a limited range of simple sugars, with general statements made regarding their usage by the fungus such as the limited utilisation of lactose, as seen in the present study.

Studies were also undertaken to assess whether commercially available syrups, consisting primarily of glucose polymer blends, might provide an alternative to the high-cost glucose syrup currently used for Quorn™ production. All of the sugar syrups assessed showed similar DT_E and DT_L doubling times to the glucose control, indicating that these all might provide viable alternative feedstocks, an exciting outcome. Interestingly, the syrup C*Sweet M01521 demonstrated similar rates of growth of *F. venenatum* A3/5 on glucose, even though this sugar syrup consisted only of dimeric and trimeric glucose polymers. This was in apparent contradiction to results of earlier work demonstrating that growth of *F. venenatum* A3/5 was much reduced when either maltose, cellobiose or starch were used as the only carbon substrate (Anderson et al., 1975), although growth was recovered when these polymers were blended with glucose. This reason for this discrepancy was unclear but may relate to the presence of single or multiple substrates.

Sucrose eventually became the main focus for the present study, demonstrating three key advantages for use as a carbon feedstock in industrial microbial fermentation: low purchase price, abundance and ease of storage. It was shown that sucrose-fed *F. venenatum* A3/5 cultures reliably produced growth rates with no significant difference to those of cultures fed with glucose. This was maintained throughout each level of batch upscaling, from microfermentation to 250 ml flask culture to 5 L and eventually 10 L STR fermentation. While the results from batch fermentations were promising, it was necessary to evaluate

growth under steady-state continuous fermentation to have greater similarity to the Quorn™ mycoprotein production process. Sucrose-fed cultures also showed no significant difference in dry cell weight accumulation in this continuous system when compared to glucose-fed cultures. Thus, results indicate the possibility of using sucrose as a main feedstock, an exciting possibility given the potential commercial cost and logistic benefits.

One potential complication might relate to the nature of sucrose hydrolysis and uptake by *F. venenatum* in liquid culture. Some examples of sucrose transporter genes have been reported, for example in the plant pathogen *Ustilago maydis* and *A. niger* (Whal et al., 2010; Peng et al. 2018). A comprehensive comparative genome study by de Vries and colleagues in 2017 demonstrated that *A. niger* encoded four transporters for sucrose, with two exhibiting high affinity. In addition to direct uptake, filamentous fungi and yeasts hydrolyse sucrose to D-glucose and D-fructose by the action of invertases (EC 3.2.1.26), enzymes belonging to the glycoside hydrolase GH32 family. Filamentous fungi are well known for their secretory capacity, and both intracellular and extracellular invertases have been shown to exist in filamentous fungi such as *A. oryzae* and *Paecilomyces variotii* (Kurakake et al. 2008; Giraldo et al. 2012). If the principal invertase activity of *F. venenatum* was extracellular, it would be expected that the enzyme concentration in the fermentation medium would be minimal at the beginning of the industrial process, and continuous feeding of new material would likely maintain the low enzymatic concentration. As a consequence, there is the risk that less sucrose would be hydrolysed, and growth would be poorer than on glucose (which does not require breakdown by an extracellular enzyme). That said, results from the continuous fermentation system suggested that reduced invertase activity, whether intracellular or extracellular, was not observed in continuous versus batch culture. This data supports the concept of substitution of glucose with sucrose as the industrial feedstock, with no loss of productivity whilst achieving a significant reduction in cost.

F. venenatum A3/5 was also incubated with glucose:sucrose blends of varying composition, which resulted in no significant changes in growth rate nor in DCW accumulation. This highlighted the possibility that sucrose could be used to supplement fermentations using the current glucose syrup, rather than as a complete substitute. This would enable sucrose to be utilised as an emergency carbon substrate, stored on site in case of failure to receive shipments of glucose syrup. Unfortunately, a key experiment involving the use of both glucose and sucrose in a continuous system was unable to be performed due to time constraints. This would have provided insights into the nature of carbon catabolite repression when both glucose and sucrose were used as carbon substrates.

Furthermore, the similarity of the carbon feedstock blends to the pure substrates suggests that these sugars are metabolised simultaneously, with a lack of diauxic growth which might otherwise be expected when feeding cultures with combined carbon feedstocks.

5.4.3 Utilisation of Different Strains

Each of the twenty-four additional *F. venenatum* isolates studied showed similar growth rates to strain A3/5 when incubated with either D-glucose or sucrose, with no strain showing statistically higher growth rates than A3/5 under the test parameters. This was disappointing as it had been hoped to identify a faster growing strain. Nevertheless, the fact that all strains maintained the same levels of productivity supported further testing of these alternative strains should they demonstrate other beneficial characteristics, such as lowered mycotoxin production relative to the A3/5 production strain (explored in Chapter 6). In addition, the observation of similar growth by all strains on both D-Glucose and sucrose confirmed earlier findings with A3/5 regarding the possible use of sucrose as a feedstock. This remained true when certain strains were upscaled, in the case of 114-23, to the point of continuous fermentation. Furthermore, in pilot substrate utilisation experiments with three test

strains, glucose and sucrose were consumed at similar rates during exponential growth.

The impact of sugar substrate on germination pattern was also studied. The importance of carbon substrate has been explored in other filamentous fungi such as *A. niger* (Hayer et al., 2013). This study demonstrated that different pyranose sugars could support germination, subsequent outgrowth, or both. D-glucose both initiated germination as well as subsequent outgrowth. A recent study in *A. nidulans* and *Botrytis cinerea* showed that fructose and sucrose were similar to glucose in stimulating early-stage germination (Chung and Kim, 2018). Quorn™ mycoprotein is produced in a continuous fermentation and so lagging germination rates caused by alternative substrates would delay fermentation thus reducing productivity. Confirmation that there is no change in macroconidia germination demonstrates that sucrose or implementation of blends will not have negative effects on productivity.

Finally, pilot studies were undertaken to study sugar substrate uptake and efficiency of biomass conversion. Biomass conversion efficiency is a more powerful metric for studying the effects of carbon substrate. While not seen in this study, this approach could be more widely implemented to identify strains with more efficient consumption of the key sugars glucose and sucrose. Unfortunately, isolate 114-23 demonstrated decreased efficiency relative to the other two strains examined. As this pattern was common to both substrates, this is less likely to be a consequence of hydrolysis machinery but perhaps the uptake of monosaccharides or subsequent processing for the generation of energy.

5.4.4 Critique and Future Work

The majority of the research presented in this chapter was performed using reagent grade sucrose of >99.00% purity. Should the investigation of sucrose as an industrial substrate be taken further, use of lower purity sucrose supplies available to industry, such as refined cane sugar

varieties, would need to be assessed to ensure there is no inhibitory effects on growth. Such feedstocks often contain trace metals such as calcium, magnesium, manganese, iron and zinc. Each of these metal ions can show inhibitory effects in filamentous fungi, both on growth and synthesis of target metabolites as demonstrated by *Aspergillus* species in citric acid biosynthesis (Kristiansen et al., 1999).

Measurements of carbon consumption were not performed routinely during this study. Some limited observations were made using an enzymatic assay for strains A3/5, 114-8 and 114-23 but these were not expanded upon. In future, work of this nature will require analysis of carbon consumption in each study. There are well-reported HPLC analyses for detecting and quantifying sugar compounds from culture broth (Honda et al., 1989; Gonzalez et al., 2020). This analysis is simple to undertake and can process a large number of samples as in this study.

In addition, Quorn™ mycoprotein is a well-known example of a successful SCP product and is one of the few permitted for human consumption. As the name suggests, an important parameter of microbial fermentations for SCP production is the quantity and quality of the protein composition within the biomass. It has previously been demonstrated that a change in carbon substrate can have alter both the quantity and composition of amino acids present in the mycelium of *F. venenatum*. Anderson and Solomons (1984) replaced glucose as the carbon substrate with the pentose sugar ribose and observed a corresponding reduction of amino acid content in fermentations of *F. venenatum* A3/5 (394 and 494 mg/g respectively), although results were not statistically validated. Investigations of this nature had been planned for this study, with keen interest to be paid to the effects on amino acid composition following substitution of glucose with sucrose. While preliminary investigations were made, this area of the work is now pursued in other PhD projects (K Szepe, Pers Comm.).

A final consideration was the risk that the use of sucrose as a putative replacement for glucose might result in increased mycotoxigenesis, as

seen elsewhere in filamentous fungi (Jiao et al. 2008). This topic is addressed in Chapter 5 of the present study.

5.4.5 Conclusions

The aim of this chapter was to discover a challenger to the current Quorn™ industrial paradigm, either an alternative carbon substrate and/or a fungal strain which demonstrated growth advantages over the current strain. Several alternatives were investigated. It was concluded that sucrose or commercial syrups hold particular promise for use in the mycoprotein production system. Meanwhile, almost all strains showed similar growth rates on both glucose or sucrose as compared to *F. venenatum* A3/5, meaning that any strain with a particularly desirable characteristic(s), such as lowered mycotoxin profile, could present a valuable alternative to the A3/5 production strain. These areas were investigated in work described in the next chapter of this thesis.

6

Is Your Sweetness My Weakness?: The Impact of Carbon Source on Mycotoxigenesis in *Fusarium venenatum*

6.1 Introduction

Secondary metabolites are often defined as low molecular weight compounds synthesised in the later 'idiophase' stages of microbial growth rather than early and exponential growth phases. These compounds are not required for growth, yet many confer some competitive advantage to the producer organism, be it offensive (e.g. toxic metabolite production) or defensive (e.g. pigment protection against UV irradiation; Keller, 2015). Fungal secondary metabolism is well-known for the vast diversity of natural products often belonging to one of three chemical classes: polyketides, non-ribosomal peptides (NRPs) or terpenes (Hoffmeister and Keller, 2007). Many of these compounds demonstrate bioactive features that are useful in human society; antibiotic, antiviral, enzyme inhibitory and so on, and thus understanding the mechanisms of their production is key to unlocking their potential.

Restriction of secondary metabolism to the later stages of growth suggests a degree of temporal regulation. Such control is most likely the result of co-ordinated of genetic and environmental inputs. Coordinating these multitudinous signals is necessary if a microorganism is going to survive and proliferate in the environment, facilitating flexibility in responses to changes in the environmental conditions it aims to

colonise. Adaptation to a wide variety of environmental conditions not only relies on an internal homeostatic system but also ensuring that the surrounding environment is suitable for compounds which the fungus might excrete, allowing them to operate in suitable conditions. As a consequence, environmental conditions are key modulators of secondary metabolism, implicated in the creation of a complex hierarchical structure of influences on the secondary metabolite profile presented by a microorganism (Brakhage 2013). A number of environmental parameters, their genetic regulation and how they relate to the production of secondary metabolites, will now be described.

6.1.1 pH

Recognition of ambient pH by a fungus is an important signal with implications for growth, development, secondary metabolism, and host infection. By modulating extracellular pH, phytopathogenic fungi are able to coordinate later features of infection (e.g. mycotoxin production) at the appropriate stage of host colonisation. Pathogenicity and ochratoxin A biosynthesis were found to be controlled by ambient pH in *Aspergillus carbonarius*, where excretion of gluconic acid was crucial to establishing infection and subsequent toxin production (Maor et al., 2017). Likewise, *Penicillium expansum* utilises gluconic acid to achieve host tissue acidification, paving the way to biosynthesis of the toxic metabolite patulin which requires lower pH for optimal production (Barad et al., 2014). Similarly, low extra-cellular pH is required for Type B trichothecene production in *Fusarium graminearum*, as demonstrated by increased *TRI* gene expression (Gardiner et al., 2009; Merhej et al., 2010).

Response to shifts in extracellular pH is governed by the transcription factor PacC, originally described in *A. nidulans*, and its homologues, which become active during alkaline conditions (Peñalva et al., 2008). Generally, PacC acts as a positive regulator of mycotoxin biosynthesis. Gene knockout studies in a variety of fungi have demonstrated that the

activity of PacC and its homologues varies across the kingdom. Decreased virulence and secondary metabolism have been witnessed in *P. expansum* and *A. ochraceus* following deletion of PacC homologues (Chen et al., 2018; Wang et al., 2018). In *P. expansum*, nine of the patulin biosynthetic cluster genes contain at least one binding site for the homologue of PacC (PePacC), suggesting direct interaction of the transcription factor (Li et al., 2021). Conversely, upregulated trichothecene biosynthesis was observed in knockout mutants of the *F. graminearum* homologue *Pac1*, suggesting that the transcription factor acts as a negative regulator of mycotoxigenesis, thus demonstrating potential divergence in function (Merhej et al., 2011).

6.1.2 Light

Of the environmental factors reported to impact secondary metabolism, the effect of light and the fungal molecular response it elicits is the best understood. The presence or absence of light modulates fungal development and secondary metabolism through the broadly conserved velvet family of regulatory proteins (Bayram and Braus, 2012). First characterised in *Aspergillus nidulans*, the founding protein of this complex, the velvet protein VeA, responds directly to the presence of light. Illumination of a growing fungus prevents VeA from crossing the nuclear envelope, restricting the regulator to the cytoplasm (Bayram et al., 2008). In the absence of light, VeA enters the nucleus where it is able to form a trimeric complex with two other proteins, the velvet complex protein VelB and the chromatin-modifying methyltransferase LaeA (Stinnett et al., 2007; Bayram et al., 2008). In the dark, cytoplasmic VeA forms a heterodimer with the nuclear import protein KapA, which facilitates localisation of VeA as well VelB into the nucleus. The VeA/VelB heterodimer then combines with LaeA in the nucleus, forming the complex responsible for the initiation of secondary metabolism and entry into the sexual cycle (Bayram et al. 2008; Lind et al. 2015).

The role of this complex in coordinating light detection and secondary metabolism has been revealed through gene deletion mutants across the fungal kingdom. *veAΔ* mutants in *A. nidulans* were incapable of aflatoxin and penicillin production (Kato et al., 2003), a pattern seen in VeA homologues both within the aspergilli (e.g. *A. flavus*, Calvo et al., 2004; Duran et al., 2007) and wider afield (e.g. *FvVE1* - *F. verticillioides*, Li et al., 2006) with knockout mutants unable to generate toxic metabolites. In *A. nidulans*, *laeA* loss-of-function mutants lack expression of the AlfR transcription factor, which activates production of sterigmatocystin (Bok and Keller, 2004). As with VeA, LaeA homologues have since been shown to positively regulate mycotoxin biosynthesis across the Ascomycota (e.g. *Cochliobolus heterostrophus* – Wu et al., 2012; *F. oxysporum* – Lopez-Berges et al., 2013; *Trichoderma reesei* – Seiboth et al., 2012). Kim et al. (2013) created transgenic strains of *F. graminearum* which placed control of a luciferase reporter system under the control of the Tri6 promoter, the transcription factor responsible for controlling transcription trichothecene biosynthetic cluster. Deletion of the LaeA homologue in *F. graminearum* (*FgLaeA*) led to a significant reduction of luminescence, implicating *FgLaeA* as a regulator of these transcription factors.

6.1.3 Nutrition

Nutritional availability, variety and source of culture medium components can also have drastic effects on secondary metabolite biosynthesis in filamentous fungi. Once detected, changes in nutritional availability will stimulate a fungus to respond accordingly. For instance, plant pathogenic fungi will rely upon the detection of storage sugars within the infected plant organ to stimulate mycotoxigenesis (Uppala et al., 2013).

Fatty acids, particularly oleic acid, have been associated with stimulating aflatoxin and sterigmatocystin synthesis in *Aspergillus flavus, A. parasiticus* and *A. nidulans* (Maggio-Hall. et al., 2005). Octanoic acid can promote the biosynthesis of pigment formation in *Monascus ruber* by 30-

50%, while also reducing the level of citrinin produced (Hajjaj et al., 2000). Similarly, plants with high-lipid content (notably soybean and peanut) were found to have significantly greater aflatoxin B_1 accumulation than those with reduced lipid profiles (Ma et al., 2015). *Aspergillus* spp. colonise lipid-rich crops, like peanut and soy but also target lipid bodies in some plants (e.g. wheat kernels) rather than the starch granules favoured by other fungi (Keller et al., 1994). As we shall see, metabolic coupling of nutritional availability and mycotoxigenesis is speculated to relate to the mode of infection in the phytopathogen in question.

In addition, the availability and type of nitrogen feedstock is also an important consideration for fungal secondary metabolism. Genome-wide microarray experiments in *F. fujikuroi* under nitrogen-sufficient and nitrogen-limiting conditions demonstrated that expression of 30 out of 45 putative secondary metabolite gene clusters depended on the quantity and quality of the nitrogen source used in culture (Wiemann et al., 2013) Availability of amino acids is crucial, particularly for those secondary metabolites whose construction rely upon NRP-synthase enzymes (e.g. penicillin and cyclosporine A). However, supplementation of culture media with different amino acids can have disparate effects on toxic metabolite synthesis. The addition of 50 mM tryptophan had polarising effects on mycotoxigenesis in *A. flavus* and *A. parasiticus*. The former showed significantly reduced aflatoxin B_1 and B_2 biosynthesis, whereas the latter had significantly increased B_1 and G_2 biosynthesis (Wilkinson et al., 2007). The disparate response of these closely-related fungi might highlight differences in lifestyle, with *A. flavus* a dedicated plant pathogen but *A. parasiticus* primarily existing as a saprotroph.

Nitrogen limitation leads to the induction of amino acid biosynthetic gene pathways in addition to aminoacyl tRNA synthases (Pusztahelyi et al., 2015). Nitrogen starvation in *F. graminearum* activated the trichothecene biosynthetic pathway with a subsequent increase in deoxynivalenol (DON) production (Audenaert et al., 2014). Similar effects have also been witnessed in *Cochliobolus heterostrophus* (Turgeon and Baker,

2007) and *Alternaria alternata* (Markham and Hille, 2001). The nature of the nitrogen source is important too. Inorganic sources can have opposing effects on secondary metabolism – ammonium activated the expression of aflatoxin genes in *A. parasiticus* while the use of nitrate proved to be inhibitory (Feng and Leonard 1998; Bagheri-Gavkosh et al., 2009). Variability in the source of a particular nutrient type can also impact the type and quantity of secondary metabolites produced by a filamentous fungus. Yeast extract sucrose (YES) medium is often cited as a reliable screening medium for mycotoxin production. However, several studies have reported lack of reliability between brands of yeast extract when used for this purpose (Filtenborg et al., 1990; Sørensen and Sondergaard, 2014). Indeed, it was found that yeast extract components could have inhibitory effects on trichothecene biosynthesis, and toxin production was restored when these components were extracted from the medium using ethyl acetate (Tanaka et al., 2019).

Nitrogen metabolism is mediated by a complex genetic regulatory system which prioritises the use of energetically favourable nitrogen sources, particularly ammonium and glutamine. When primary nitrogen sources in the environment are limited, upregulation of structural genes such as degradative enzymes and permeases facilitates the ability of a fungus to switch to less easily utilised nitrogen sources including nitrate, urea, and amino acids among others (Tudzynski 2014). In the Ascomycota, this is facilitated by GATA family transcription factors, the primary gene known as areA in *A. nidulans* (Chant et al., 2003). AreA and its orthologues have been shown to mediate de-repression of genes involved in secondary nitrogen source utilisation in the absence of primary sources, a system resembling creA and carbon catabolite repression (Wiemann and Tudzynski, 2013). Following this, utilisation of a secondary substrate requires substrate-specific gene activation the best studied example of which is nitrate utilisation in *A. nidulans*, involving the synergistic activity of areA and the pathway-specific regulator NirA (Berger et al., 2008).

Further research indicated that AreA had additional roles in regulating the production of secondary metabolites. A key breakthrough was made in *F. fujikuroi*, where it was the decades-long industry understanding that nitrogen starvation was required to achieve maximal levels of gibberellic acid (Bu'lock et al., 1974). It was shown that deletion of AreA in *F. fujikuroi* completely abolished gibberellic acid production (Mihlan et al., 2003). Similar areA dependency of secondary metabolism was also witnessed in *F. verticillioides*, where areA deletion mutants were incapable of synthesising fumonisin toxins on mature maize kernels and *in planta* growth is resumed only following supplementation with ammonia (Kim and Woloshuk, 2008). This same study also demonstrated that transcription of the fumonisin biosynthetic cluster genes *fum1*, *fum8* and *fum12* was abolished under inducing *in vitro* conditions. Conversely, constitutive expression mutants were able to produce fumonisins under ordinarily repressive conditions, thereby demonstrating the importance of areA activity in coupling extracellular nitrogen detection and secondary metabolism.

The carbon substrate is also an important consideration for secondary metabolism regulation in filamentous fungi. Carbon substrates are well-documented in their ability to affect mycotoxin biosynthesis as seen in patulin (Zong et al., 2015), ochratoxin A (Medina et al., 2008), fumonisin (Sørensen et al. 2009) and trichothecene production (Jiao et al., 2008). When used as the sole carbon source, glucose promoted the biosynthesis of citrinin, a toxic polyketidic metabolite, in *Penicillium citrinum* (Li et al., 2017). This was suspected to be a result of increased acetyl-CoA and malonyl-CoA flux through the glycolytic pathway, providing more precursor molecules for polyketide synthesis. Synthesis of tenuzonic acid, a notable inhibitor of eukaryotic protein synthesis, showed induction in A*lternaria alternata* when cultured with glucose, sucrose and maltose (Brzonkalik et al., 2011). However, general patterns of induction are difficult to establish given the complexity of mycotoxin biosynthetic pathways and the potential for genetic variability within them. For instance, increasing sucrose concentration produced a

corresponding decrease in patulin production in *P. expansum*, yet this was modulated by changes in culture pH and medium supplementation with apple phenolic compounds (Kumar et al., 2016). Despite this, sucrose is often cited as a universal mycotoxin producer, thought to be a consequence of the sugar comprising a large proportion of soluble sugars in plants and thus an important signalling molecule in plant pathogens (Takahashi et al., 2001).

As in the case of nitrogen metabolism, carbon substrate choice is also tightly controlled by genetic regulatory networks by a system termed carbon catabolite repression (CCR). The mechanism of CCR is discussed in greater detail in Chapter 4, but here we will examine the direct influence of the system on secondary metabolism. Once again, such a system allows the fungus to optimise growth in relation to the extracellular conditions it finds itself.

Like in nitrogen metabolism regulation, implementation of CCR revolves around the activity a global regulator, in this case creA. Unlike areA, this regulator is primarily known for repressing the transcription of genes required for alternative carbon substrate utilisation in the presence of glucose or other energetically favoured sugars (explained further in Chapter 4.1). Given the importance of carbon substrate in secondary metabolism, it would be expected that creA is involved in this system, yet there is there is limited literature on its direct impact. Aflatoxin biosynthesis was reduced 16-fold in *A. flavus ΔcreA* mutants compared to the wildtype, with a parallel downregulation in aflatoxin biosynthetic gene transcription (Fayin et al., 2018). However, this study did not explore creA binding site enrichment (5'-SYGGRG-3') in the promoter regions of these biosynthetic genes, which might have provided insight into the nature of creA involvement. Interestingly, Kumar et al. (2016) demonstrated that increasing sucrose concentration *in vitro* with *P. expansum* had a negative impact on *laeA* transcription and thus patulin biosynthesis. creA binding sites were found in the promoter of *laeA*, suggesting that when glucose is detected by the fungus, *laeA* is downregulated, coordinating CCR and light responses.

The impact of carbon substrate on secondary metabolism has been well-described within the genus *Fusarium*. The production of several metabolites in *F. avenaceum*, including some mycotoxins (moniliformin, fusarin C etc.) was modulated by the carbon source used in its culture (Sørensen and Giese 2013). *F. fujikuroi* produced significantly greater amounts of the reddish-pigment bikaverin in cultures fed sucrose relative to glucose-fed cultures (Rodríguez-Ortiz et al., 2010). Fumonisin B_1 biosynthesis was upregulated when amylopectin was used as the major carbon substrate in *F. verticillioides* cultures (Bluhm & Woloshuk, 2005). When sucrose replaced glucose as the primary carbon source, *F. proliferatum* demonstrated increased *FUM* gene transcription and production of fumonisins, supporting other evidence of sucrose as a universal mycotoxin inducer (Achibón et al., 2021). Thus, how a carbon source impacts synthesis of metabolites is dependent on the species and often the strain of the fungus in question and may relate to the method of infection in plant-pathogenic fungi. Jiao et al. (2008) studied the effect of 12 carbon sources on the production of Type B trichothecenes (DON and 3-acetylDON; 3ADON) in 9 strains of *F. graminearum*. When glucose was replaced with sucrose or one of two fructo-oligosaccharides (1-kestose and nystose), each of the strains showed significant increases in trichothecene yield. Conversely, a number of other sugars, namely amylose, cellobiose, fructose and maltose drove mycotoxigenesis in a strain-specific manner. Such subtlety in mycotoxigenesis induction is likely a consequence of cultivar infection capacity, with some strains more adapted to the infection of certain host types with particular compositions of storage sugars.

Similar to other members of the *Fusarium sambucinum* species complex (FSSC), the Quorn™ fungus, *F. venenatum* has shown the capacity to produce Type A trichothecenes (Laraba et al., 2021). Indeed, the production of type A trichothecenes was a key feature facilitating the reclassification from *F. graminearum* which relies on Type B trichothecene synthesis (O'Donnell et al., 1998). The primary mycotoxin produced by *F. venenatum* is diacetoxyscirpenol (DAS) and is similar in

structure to the more well-known T-2 toxin (Figure 6.1). The presence of an isovalerate group at the C-8 position in T-2 toxin is the key difference between the two, enabling an additional linkage to be formed between the toxin and the A site of the 60S ribosomal subunit in eukaryotes (Wang et al., 2021). Despite the supposed additional toxicity expected by the addition of an isovalerate, there is some disparity in toxicity, as observed in swine. Intravenous administrations of DAS and T-2 toxin gave lethal dose (LD_{50}) values of 0.3 and 1.3 mg/kg respectively, suggesting that DAS showed greater toxic capability than T-2 toxin (Haschek et al., 2002).

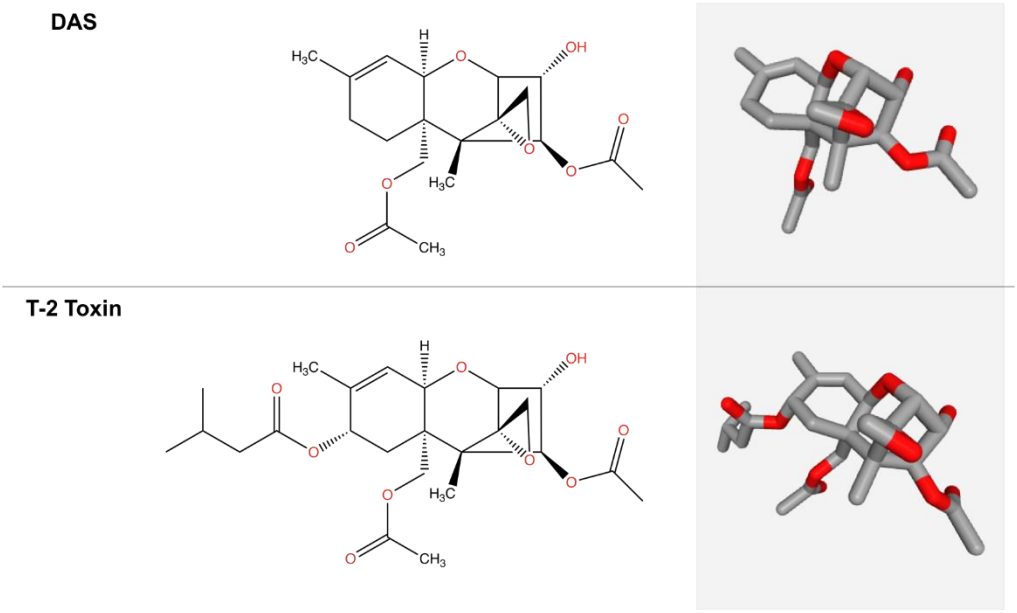


Figure 6.1. Comparison of the molecular structure of DAS (diacetoxyscirpenol) and T-2 toxin. DAS lacks a functional group at the C-8 position, which is filled by an isovalerate group in T-2 toxin. DAS can form several linkages with the 60S ribosomal subunit, utilising the C-3-hydroxyl group, the double bond between C-9 and C-10, and also the epoxide ring formed at C-12 and -13. The isovalerate substituent at C-8 confers an additional bonding site in T-2 toxin. Shaded box displays the 3D structure of the corresponding toxin, acquired from PubChem. Toxin chemical structures drawn using ChemDraw.

To date, analysis of *F. venenatum* trichothecene production has only been studied in the Quorn™ production strain given its biotechnological importance. However, this is likely to give a biased perspective of the

species' capacity for toxicity and thus a broader study of mycotoxigenicity in the species is warranted. Similarly, a study investigating the impact of different carbon substrates (i.e. other than glucose) on DAS production has yet to be performed, an important consideration given the proposed changes to the Quorn™ industrial process in this thesis.

6.1.4 Chapter Aims

Earlier work in the present project had identified sucrose as a promising alternative to the glucose feedstock currently used as the carbon substrate for production of Quorn™. However, many countries have strict limits on the quantity of mycotoxins that can be present in any given foodstuff, be it intended for human or animal consumption [Commission Regulation (EC) No 1881/2006, UK]. Throughout decades of production, the current Quorn™ fermentation system has failed to show any detectable mycotoxin presence (R Johnson, pers. comm.). Thus the aims were as follows.

1. While sucrose is a commercially appealing prospect, it was necessary to observe the effect of sucrose implementation in *F. venenatum* mycotoxigenesis given the body of evidence indicating that using sucrose as the primary carbon substrate can lead to increased toxin synthesis in filamentous fungi (Achibón et al., 2021).

2. Furthermore, carbon substrate-driven mycotoxigenesis has been shown to vary both within genera and within species (Jiao et al., 2008). The second aim of this chapter was to assess toxin biosynthesis in the newly established culture collection of 24 strains of *F. venenatum*, on both glucose and sucrose.

3. Finally, it was aimed to identify promising strain and substrate combinations which would then be upscaled to 10 L batch and steady state fermentations to assess possible mycotoxin

production of any new strains/sucrose feedstock in a model more similar to that used in the Quorn™ industrial process.

6.2 Materials & Methods

All chemicals were of analytical or molecular biology grade. Most were supplied by Fisher or Merck with exceptions outlined where necessary.

Unless stated otherwise, sterilisation was achieved by autoclaving at 121 °C for 20 minutes.

6.2.1 Growth Media & Solutions

6.2.1.1 Yeast Extract Peptone Dextrose (YEPD)

900 mL of this medium was prepared as follows: Add 20.0 g peptone and 10.0 g yeast extract to SDW and make up to 900 mL. Post-autoclave, 100 mL of sterilised 20% (w/v) glucose solution was added to the broth to yield a final sugar concentration of 2% (w/v).

When required, this broth was supplemented with filter-sterilised polymyxin B sulphate at a final concentration of 15 µg/mL. This acts as a permeabilisation agent during the *Kluyveromyces marxianus* bioassay.

When solid media was necessary, 15 g/L of bacteriological agar was added to the broth.

6.2.1.2 Diacetoxyscirpenol Mycotoxin Standard

Stock solutions of the type A trichothecene diacetoxyscripenol (DAS) were prepared to a final concentration of 1 mg/mL in acetonitrile (ACN; Apollo Scientific, Cheshire, UK) These were refrigerated at 4 °C until required.

Chromatographic standards were prepared from this stock, with each dissolved in ACN; 100 ng/µL, 50 ng/µL, 25 ng/µL, 10 ng/µL, and 5 ng/µL. These were stored in amber vials at 4 °C to ensure stability of the chemical.

6.2.3 Solvent Extraction of Trichothecenes

Trichothecenes were extracted from both the mycelium and supernatant of fungal cultures using ethyl acetate (EtAc) to gauge the relative trichothecene production following replacement of carbon substrates, *F. venenatum* strain or both. After the intended incubation time, cultures were harvested using miracloth gauze inserted into a plastic funnel, allowing the separation of the two culture components with the supernatant collected in one Duran bottle per sample. Mycelium from the miracloth filter was dried, broken up and weighed before placingin individual Duran bottles.

30 mL of EtAc was added to each of the sample Duran bottles. Mycelial samples were sonicated for 10 minutes in a sonicating water bath at room temperature before the addition of anhydrous sodium sulphate. The organic layer was then filtered through Whatman No. 1 filter paper placed in a glass funnel before collection in a round bottom flask. For supernatant samples, the organic phase of the extract was poured over a Whatman No. 1 filter filled with anhydrous sodium sulphate, with the solvent once again collected in a round bottom flask.

Once collected, samples were evaporated under reduced pressure and elevated temperature using a rotary evaporator. Following evaporation, 350 µL of acetonitrile (ACN) was added to each dried extract for the collection of approximately 300 µL of extract. Once each extract was sufficiently dissolved, each sample was then dispensed into an amber vial and stored at 4 °C.

6.2.4 Chromatographic Analyses of Extracts

High-Performance Liquid Chromatography (HPLC) and GC-MS (Gas Chromatography Mass Spectrometry) analyses were undertaken to assess production of DAS by *Fusarium venenatum* cultures.

6.2.4.1 HPLC

High-performance liquid chromatography (HPLC) analyses of the culture extracts were performed on a Dionex UltiMate 3000 (Thermo Scientific) fitted with an Accucore C18 column (Thermo Scientific; 150 mm x 2.1 mm, 2.6 µm) maintained at 30 °C. A bipolar solvent system consisting of dH_2O + 0.1% formic acid (A) and 100% ACN (B) was utilised. The following gradient was applied at a flow rate of 0.5 mL/min: 0.0-5.0 min 5% B, 5.0-8.0 min 55% B, 8.0-9.0 min 70% B, 9.0-11.0 min 100% B, 11.0-12.0 min 100% B and 11.0-12.0 5% B. 20 µL of a sample was loaded in each case. DAS retention time was established using DAS standards (100, 75, 50, 25, 10 and 5 ng/µL). DAS production in *F. venenatum* culture extracts was based on retention time and mass spectra comparisons with DAS standards.

6.2.4.2 GC-MS

Gas chromatography-mass spectrometry (GC-MS) analyses of the ethyl acetate extractions were performed on a Trace 1310 gas chromatograph fitted with a TraceGold-5MS column (Thermo Scientific; 30 m, 0.25 mm, 1.0 µm). Mass spectrometry of the samples was facilitated by a Trace ISQ Single Quadrupole Mass Spectrometer in electron ionisation (EI) mode. Helium was used as the carrier gas at 20 mL/min in splitless flow. The column was held at 150 °C for 1 minute following injection, before heating to 280 °C at 30 °C/min after which it was held for 7.7 minutes. 10 µL of sample was loaded in each case. DAS retention time was established using DAS standards (75 and 50 ng/µL). DAS production in *F. venenatum* culture extracts was based on retention time and mass spectra comparisons with DAS standards and NIST (National Institute of Standards and Technology; www.nist.gov/srd) reference library.

6.2.5 Quantification of DAS Production

To gauge the retrieval of trichothecenes from fungal cultures, solvent extractions were made from RHM media spiked with DAS. A stock solution was prepared by dissolving 1 mg of DAS in 1 mL of ACN. From this stock solution, two dilutions (100 ng/µL, 10 ng/µL) were made. Each solution was added to individual 50 mL samples of RHM set up in triplicate. These spiked media samples were then processed using the same supernatant extraction process as detailed above, with the exception that EtAc was added to the supernatant samples as two volumes of 15 mL to increase extraction efficiency. Once extracted, these samples were then injected on to the GC-MS and analysed as outlined previously.

DAS quantification was calculated by comparing the average area-under-the-curve (AUC) values of each standard of known concentration with the average AUC value for each spiked extract.

6.2.6 Investigating DAS Production in A3/5 and other strains of F. venenatum

A variety of culture conditions were used to characterise the production of DAS in *F. venenatum*. These involved either substituting different carbon substrates in the medium and/or use of alternative *F. venenatum* strains for the inoculum.

In each case, 50 mL of RHM culture medium (Section 2.2.2) in a triple-baffled 250 mL flask was inoculated to a final concentration of 10,000 macroconidia/mL. Flasks were incubated at 28 °C in the dark on a rotary shaker at 150 rpm for the required period. RHM medium was supplemented with the appropriate carbon substrate as described below.

6.2.6.1 DAS Production Profile in A3/5

Choice of carbon substrate can have a profound effect on a number of metabolic characteristics of a fungus, including mycotoxigenesis. It was therefore necessary to understand the pattern of DAS production in A3/5 across the full growth period. 50 mL RHM cultures in triple-baffled flasks were prepared with one of five sugar compositions (Table 6.1).

Table 6.1. Final sugar composition of various RHM broths used to assess possible mycotoxigenesis in A3/5 cultures. G, F and S refer to glucose, fructose and sucrose, respectively.

Broth	% Glucose (w/v)	% Fructose (w/v)	% Sucrose (w/v)
RHMG	3.00	-	-
RHMF	-	3.00	-
RHMS	-	-	3.00
RHMGF	1.50	1.50	-
RHMGS	1.50	-	1.50

Each culture was incubated in the dark at 28 °C on a rotary shaker at 150 rpm, for a duration of 24, 36, 48, 72, 96, 120 or 144 hours. Following incubation, the mycelium and supernatant of each culture were subjected to EtAc extractions as described previously. Production of DAS was assessed by GC-MS and quantified by area-under-the-curve analysis.

6.2.6.2 Evaluating Carbon Substrate Effect on Mycotoxigenesis in A3/5 Cultures

F. venenatum A3/5 was grown in the presence of several alternative carbon sources (Table 6.2) assessed previously in the project. These cultures were incubated for a period of 36 or 120 hours to evaluate mycotoxigenesis during exponential and stationary phases of growth, respectively.

Table 6.2. Various sugars used as carbon substrates to assess possible mycotoxigenesis during A3/5 fermentations. The composition of each sugar is listed: DX = Dextrose; DP2 = Dextrose disaccharide; DP3 = Dextrose trisaccharide; DE = Dextrose equivalent; ISC = Invert sugar conversion.

Sugar	% Fructose (w/v)	% DX (w/v)	% DP2 (w/v)	% DP3 (w/v)	% DE	ISC
Maltose	-	-	20	-	-	-
Cellobiose	-	-	20	-	-	-
C* Sweet M01521	-	-	12.5	7.5	-	-
C*Sweet M10170	-	~1.1	~16.7	~2.2	-	-
C* Sweet 1112	-	5	2.5	2.5	10	-
C* TruSweet 1750	~8.9	~11.1	-	-	-	-
Tereos SI	-	-	-	-	-	20

6.2.7 Characterising Species-wide DAS Biosynthetic Behaviour

6.2.7.1 Fermentation of *F. venenatum* Strains

Each of the 24 alternative strains were characterised for DAS production, although to a lesser degree than for A3/5. DAS biosynthesis was observed in RHMG and RHMS media under identical shake flask conditions used to characterise A3/5 (see section 5.2.6). Each culture was incubated for a period of 36 or 120 hours to capture exponential and stationary phase profiles respectively. As before, EtAc extracts were performed on each of these cultures followed by analysis on GC-MS and subsequent quantification.

6.2.7.2 Evaluating Extract Toxicity by Yeast Bioassay

Kluyveromyces marxianus GK1005 exhibits vulnerability to trichothecene activity whilst demonstrating considerable insensitivity to other mycotoxins. Therefore, this yeast can be used effectively in a colorimetic bioassay to assess presence of trichothecenes in culture

extracts (Engler et al., 1999). For this study, the *K. marxianus* bioassay was used to observe non-DAS-related toxic activity in extracts from strains which did not show DAS presence.

K. marxianus pre-cultures were prepared by the addition of a single yeast colony to 10 mL YEPD in a 100 mL Erlenmeyer flask. Pre-cultures were incubated at 35 °C on a rotary shaker at 150 rpm for 24 hours. Following incubation, the culture was diluted to a value of 0.018 OD_{450} in YEPD medium supplemented with polymyxin bisulphate (PMBS). The subsequent bioassay was prepared in a 96-well microtitre plate (Greiner Cellstar®, Greiner Bio-One, Austria). Each well contained 150 µL of diluted yeast culture and 10 µL of filtered sample.

These microtitre plates were then wrapped with a single layer of semi-transparent, flexible film (Parafilm, Bemis Company Inc., Wisconsin, USA) and placed into a microplate reader (Epoch 2, BioTek Instruments). Plates were incubated for 72 hours at 35 °C with reciprocal shaking at 567 cpm. OD_{540} readings were taken every 30 minutes for the incubation duration.

Assessment of growth inhibition was made by comparing the average doubling time of the yeast cultures. Doubling time was measured in exponential phase between 15h and 18h and calculated using the following formula:

Equation 6.1. *Kluyveromyces marxianus* doubling time calculation. 0.301 = log(2), h = time period studied in hours (=3), ODi = initial optical density value, ODf = final optical density value.

$$\frac{(-0.301 \times h)}{(logODi - logODf)}$$

In addition, corrections were made to reduce interference due to individual well differences on each culture. The minimum OD_{540} value was determined for each culture, as this would describe the contribution of the well and initial medium to the optical density value. A value of 0.01 was added to each value in the dataset to avoid complications with logarithmic plots of 0 values.

Two experiments were performed using this methodology, validation of DAS toxicity in addition to screening DAS presence in *F. venenatum* culture extracts, as follows.

Validating DAS Toxic Effects in K. marxianus

To investigate the extent of DAS toxicity in *K. marxianus*, nine replicate cultures were treated with one of eight concentrations of DAS or supplemented with H_2O. The final concentrations of DAS were as follows: 0.70, 0.56, 0.42, 0.28, 0.14, 0.12, 0.08, 0.04 and 0.02 nM.

Once incubation was complete, an assessment was undertaken to determine whether concentrations were fungistatic or fungicidal. 100 µL was taken from three wells of each culture condition and used to inoculate individual 9 cm YEPD agar plates. These plates were then incubated at 35 °C for 24 hours. Cultures demonstrating a lack of growth in microwell conditions but which produced colonies on solid media would imply fungistatic concentrations, whereas lack of growth in both would indicate fungicidal activity.

Assaying F. venenatum *Extract Toxicity*

This bioassay was also used to determine the bioactivity of DAS produced by *F. venenatum* strains. Mycelial extracts generated from *F. venenatum* strains with apparent reduced mycotoxigenesis were implemented in this analysis. Due to the nature of the Quorn commercial product, only mycelial extracts were screened for toxic effects. Of each extract tested, serial dilutions (1:4; 1:16) were also assessed.

Each extract was added to triplicate cultures. A series of controls were implemented for each assay; a toxin negative control (SDW) and two positive toxic controls; a DAS standard (100 ng/µL), and a known sample of DAS produced by *F. venenatum* A3/5 (sucrose-fed, 120 hpi) as determined by GC-MS analysis.

6.2.8 Ten L Batch and Steady State Fermentation at Quorn™ Pilot Plant

Given the promise of strain 114-23 as a non-DAS synthesising isolate, the mycotoxigenic capacity of this strain was compared with A3/5 using facilities available at the Quorn Foods Fermentation Development Centre in Billingham. Fermentation work using 10 L bioreactors and growth-related analyses were performed by Dr. Mark Taylor (Quorn Foods, Billingham) the results of which have already been discussed in Chapter 4.

Each strain was cultured with either glucose or sucrose (330 g/L) under conditions described previously (Chapter 5.2.3.5). Each batch fermentation was conducted through to stationary phase, exceeding the point at which the carbon source had been exhausted. Steady state fermentations were first grown in glucose until 26 hpi. At this point, the carbon substrate was either maintained as glucose or switched to sucrose. Samples for mycotoxin analysis were taken 37 hpi.

Mycotoxin analysis of the mycelial paste was subsequently carried out by Premier Analytical Services (High Wycombe, Buckinghamshire). Mycelial paste samples were screened for the presence of several mycotoxins known to be produced by *Fusarium* spp. in addition to DAS, namely: deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15-ADON), fusarenone X, nivalenol, neosolaniol, T2 triol, T2 toxin, HT2 toxin and fusarins. Detection limits for each mycotoxin was 10 µg/kg with the fusarins as the only exception, as these were detectable to a limit of 5 µg/kg.

6.3 Results

6.3.1 Establishing Chromatographic Analyses of DAS

6.3.1.1 HPLC

It was necessary to establish a reliable method for trichothecene detection wa before the mycotoxigenic capacity of *F. venenatum* could be judged. Initially, analysis by HPLC were attempted as described by Thimm et al. (2002) and Omurtag et al. (2007). DAS standards of a known concentration were injected onto the HPLC (Figure 6.3). DAS was detected in all standards at 5.20 minutes post-injection. However, the limited detection of DAS relative to the amount injected on to the system indicated that HPLC was insufficiently sensitive for the desired purpose.

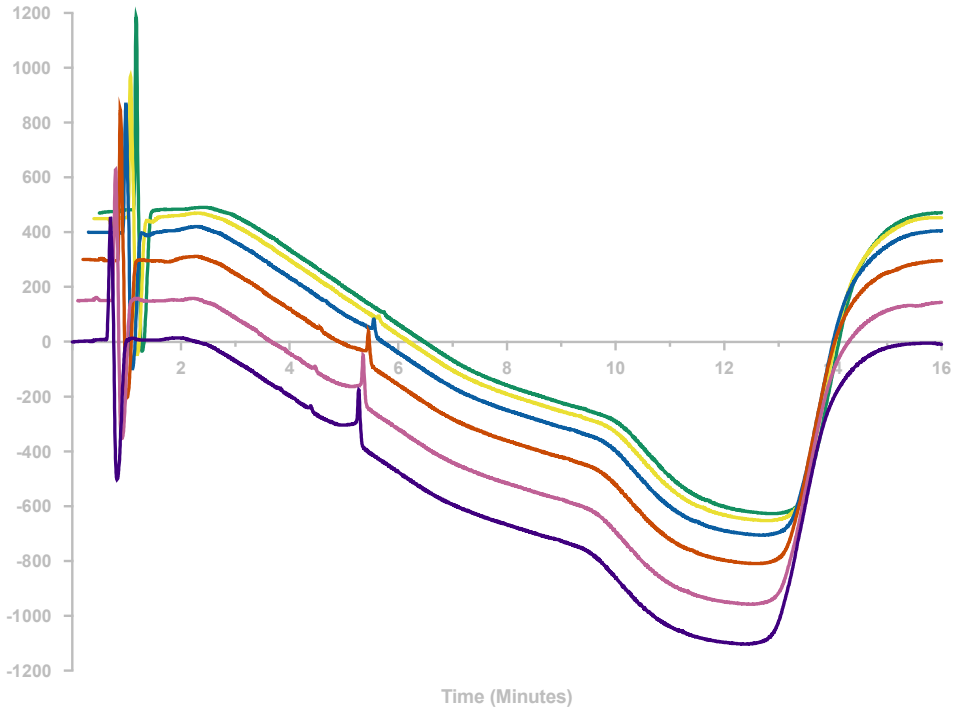


Figure 6.3. HPLC analyses of diacetoxyscirpenol (DAS) standards of known concentration. DAS detection occurs at 5.20 minutes post-injection. Purple = 2000 ng; Pink = 1500 ng; Orange = 1000 ng; Blue = 500 ng; Yellow = 200 ng; Green = 100 ng. Absorption at 205 nm.

6.3.1.2 GC-MS

Following limited success with HPLC, GC-MS analyses were then attempted, as methodologies had been established for the detection of DAS from liquid culture (Müller et al., 1997; Ismail et al., 2011; Laraba et al., 2021). DAS standards were easily detected, producing a strong peak at approximately 14.70 minutes post-injection (Figure 6.4A). Confirmation of compound identity was facilitated using the NIST library (Figure 6.4B). This method enabled considerably more sensitive detection of DAS than the previous HPLC method, and thus was used preferentially.

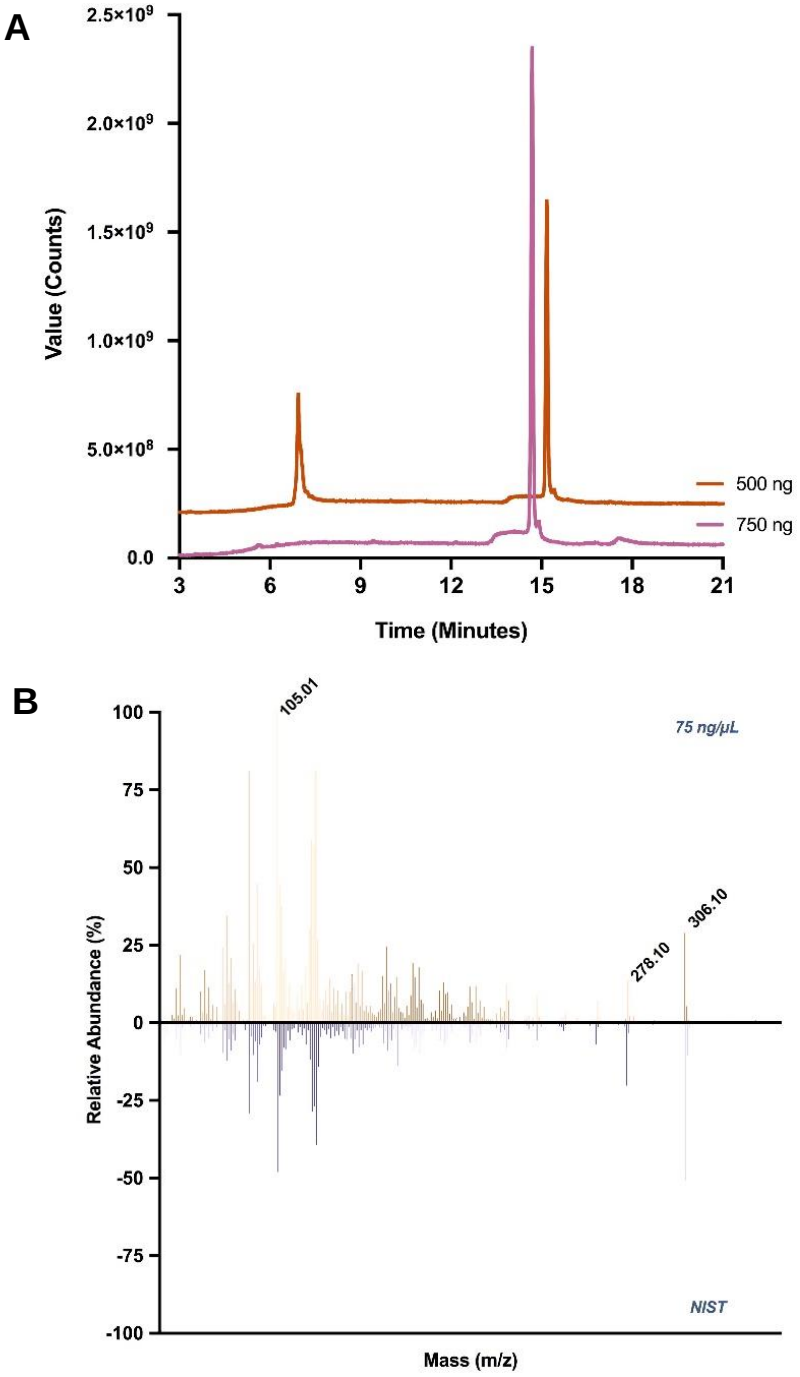


Figure 6.4. Representative gas chromatography (GC) results of diacetoxyscirpenol (DAS) standards. A. GC-MS profiles of two DAS standards, 50 and 75 ng/µL (500 and 750 ng total respectively). DAS peak occurs approximately 14.70 minutes post injection. 500 ng trace shifted +0.5 minutes in x-axis. B. Mass spectra of peak #279 of the 75 ng/µL sample (orange) and DAS as reported by the NIST library (blue). NIST library predicted peak #279 to be DAS with 92% likelihood.

6.3.2 Quantification of DAS Extraction

An understanding of the efficiency of extraction of metabolites was required to assess the comparative mycotoxigenic capacity of *F. venenatum* in different growth conditions. RHM medium was therefore spiked with different concentrations of DAS and these samples then underwent EtAc extraction to determine the approximate extraction efficiency of the methods used. From this data, the established limit of detection for this method was 10 ng/µL. Results are shown in Table 6.3. Given these results, any reported mycotoxin concentrations should be assumed to be between 20 and 25% greater than the values stated due to loss by extraction.

Table 6.3. Evaluation of efficiency of methods used for secondary metabolite extraction Note that between 20 and 25% of total DAS is lost due to the extraction method. Therefore, the true values of DAS production will be between 20 and 25% greater than those reported in this chapter.

Standard Concentration (ng/µL)	Standard AUC (± SEM)	Mean Spiked Sample AUC (± SEM)	Extraction of Total DAS (%; ± SEM)
100	2,422,336 (± 6228)	1,874,697 (± 110,235)	77.39 (± 0.50)
10	33,935 (± 2606)	25,403 (± 1194)	75.56 (± 3.52)

6.3.3 Evaluating Carbon Substrate Effect on Mycotoxigenesis in A3/5 Cultures

Various sugars were assessed for their ability to induce DAS production in A3/5 cultures. These carbon sources included promising alternatives to the glucose feedstock currently used by Quorn Foods, as well as highly pure glucose as a control. DAS biosynthesis in A3/5 was assessed across the full growth period (0 – 144 hours post-inoculation; hpi) in batch conditions. Glucose, fructose and sucrose were assessed in this manner in addition to equimolar blends of these substrates. Note that exponential phase growth was predicted between 24 and 48 hours.

DAS was not detected in glucose-fed mycelium extracts nor in the equivalent supernatant samples even at the final stationary phase of growth (Figure 6.5; Table 6.4). By contrast, sucrose-fed extracts showed significant DAS enrichment in the late exponential/early stationary phase (48 hours) in both mycelial and supernatant extracts, although DAS production was not observed before this point (Figure 6.5; Table 6.4).

Extraction of DAS peaked at 72 hours (equivalent to 18.91 mg/g of mycelium), declining in later stationary phase cultures until no longer detectable at 144 hours. DAS was also detected in mycelial extracts from cultures fed an equimolar mix of glucose and sucrose, although here the first detection did not occur until 72 hours post inoculation. These samples produced less DAS than the equivalent samples from sucrose-fed cultures (GS3 = 15.91 mg/g). In both cases, the peaks assumed to be DAS showed >90% likelihood to be correct when the mass spectra were compared with DAS from the NIST library (Figure 6.5). DAS was more readily detected in culture supernatant extracts than the equivalent mycelial extract. Sucrose-fed supernatant extracts showed substantial enrichment for DAS, both when used as the sole carbon substrate but also when used in combination with glucose (Figure 6.5).

Extracts from fructose-fed cultures did not show detectable levels of DAS. When glucose and fructose are used as a combined feedstock,

imitating the hydrolysis products of sucrose, extracts failed to demonstrate detectable DAS levels (

Table 6.4 6.4).

Table 6.4. Comparison of glucose, fructose, and sucrose in synthesising DAS across 144 hours. Values indicate ng/µL of GCMS sample.

Time (Hours Since Inoculation)	Substrate				
	Glucose	Fructose	Sucrose	Glucose:Fructose	Glucose:Sucrose
36					
Mycelium	N/A	N/A	N/A	N/A	N/A
Supernatant	N/A	N/A	N/A	N/A	N/A
48					
Mycelium	N/A	N/A	>1000	N/A	N/A
Supernatant	N/A	N/A	>1000	N/A	N/A
72					
Mycelium	N/A	N/A	>1000	N/A	>1000
Supernatant	N/A	N/A	>1000	N/A	>1000
96					
Mycelium	N/A	N/A	>1000	N/A	>1000
Supernatant	N/A	N/A	>1000	N/A	>1000
120					
Mycelium	N/A	N/A	>1000	N/A	>1000
Supernatant	N/A	N/A	>1000	N/A	>1000
144					
Mycelium	N/A	N/A	N/A	N/A	N/A
Supernatant	N/A	N/A	N/A	N/A	N/A

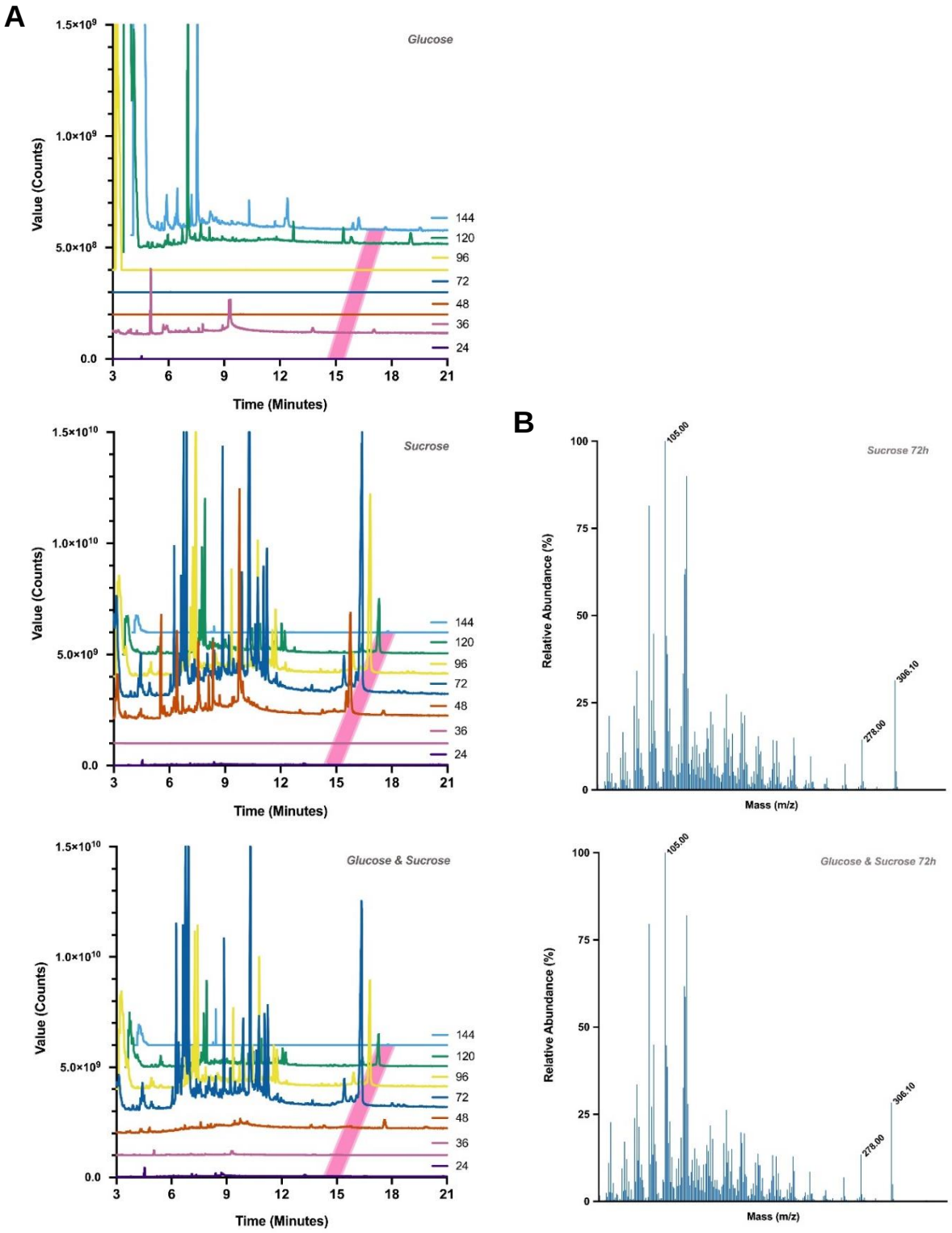


Figure 6.5. GC-MS results showing metabolite production in A3/5 extracts from cultures grown in RHMG and RHMS. A. DAS biosynthesis in either glucose, sucrose, or glucose/sucrose -fed cultures, as measured from culture mycelium extracts. Each spectrum is shifted by +0.5 minutes on the x-axis relative to the time point before. Different coloured lines correspond to different time points (in hours) of extraction as shown on right hand side of graph. Shaded box indicates expected GC column passage time for DAS detection. B. Mass spectra for the DAS peaks produced by cultures incubated for 72h. For each suspected DAS peak in sucrose- or glucose/sucrose-fed 72h extracts, NIST library predicted 94 and 92% likelihood respectively.

Two other disaccharides, the glucose homodimers, maltose (α-(1→4) glucosyl-glucosyl) and cellobiose (β-(1→4) glucosyl-glucosyl bonds) were also investigated for their impact of mycotoxin production in *F. venenatum* A3/5. Both failed to induce mycotoxin production, with both mycelial and supernatant extracts failing to produce demonstrable DAS following culture incubation for 120 hours (Figure 6.6; Table 6.5). Meanwhile, two commercial sugars produced detectable, albeit trace, amounts of DAS. These sugars were C*TruSweet 1750 (0.51 µg/g; 629.78 µg/L) and the invert sugar convert Tereos (0.43 µg/g; 627.22 µg/L) samples. These feedstocks were the only commercial feedstocks which contained approximately equimolar amounts of glucose and fructose. Other commercial sugar sources assayed failed to induce any DAS production (Figure 6.6).

Table 5.5. Detection of DAS in alternative dimeric sugars and high fructose syrups. Values indicate ng of DAS in 10 µL sample.

Substrate	Mycelium	Supernatant
Maltose	N/A	N/A
Cellobiose	N/A	N/A
C*Sweet M01521	N/A	N/A
C*Sweet M10170	N/A	N/A
C*Sweet 1112	N/A	N/A
C*TruSweet 1750	14.06	1049.63
Tereos SI	10.51	1045.36

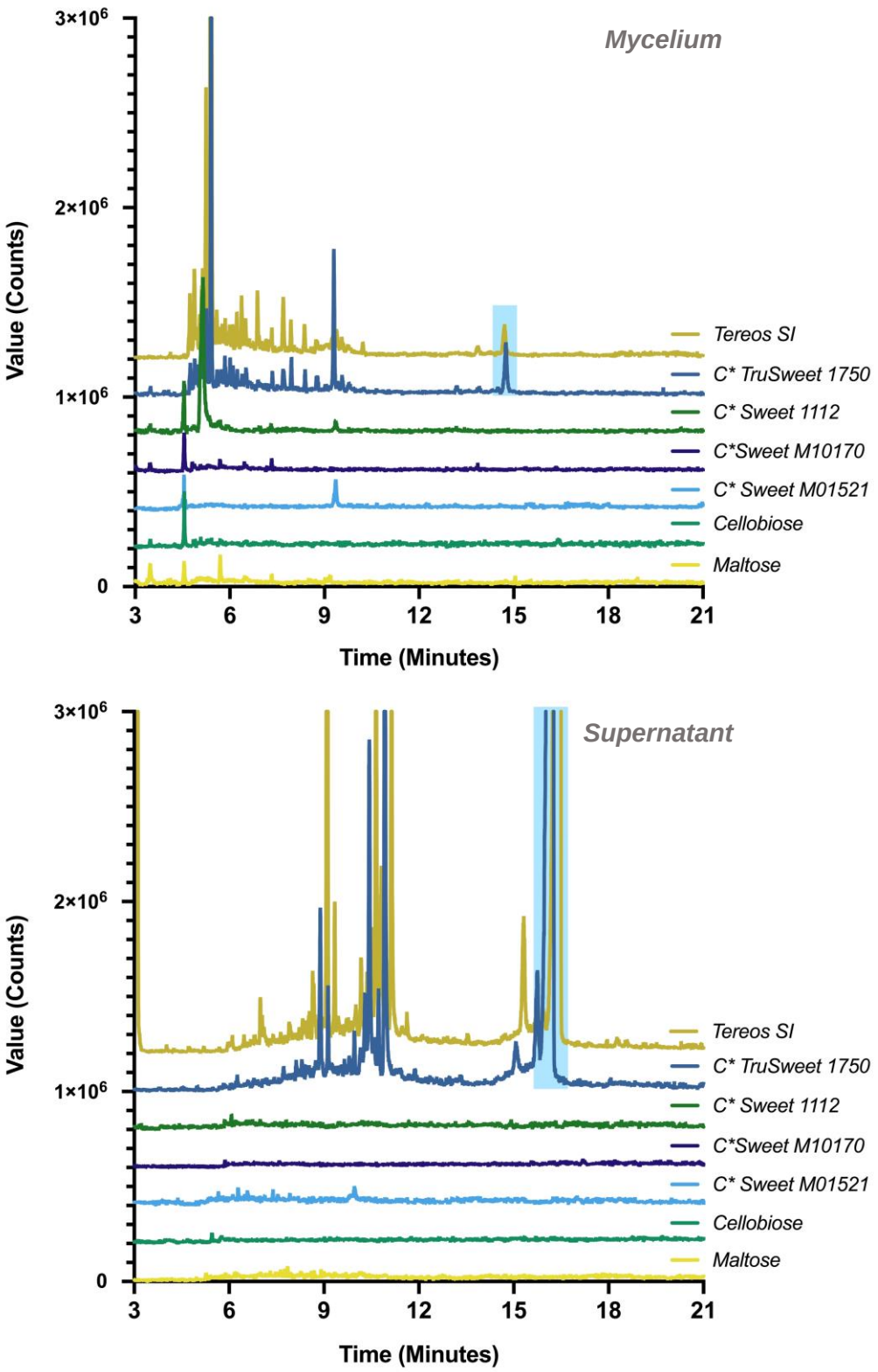


Figure 6.6. GC-MS results showing metabolite production in A3/5 extracts following incubation with alternative carbon substrates. Different coloured lines correspond to different sugar substrates as shown on right hand side of graph. Shaded box indicates presence of DAS in each case.

6.3.3 Characterising Species-wide DAS Biosynthetic Behaviour

6.3.3.1 Qualitative Screening

It was necessary to screen each *F. venenatum* strain previously acquired during the project for their capacity to produce DAS. Strains presenting a reduced mycotoxigenic profile might then provide an alternative to the current A3/5 production strain. Of particular interest were strains 114-8 and 114-15, as both of these strains had been found to be possible candidates to replace A3/5 when previously assessed by growth (Section 5.3.2). All 24 strains were cultured in the presence of glucose or sucrose for an incubation period of 36 or 120 hours, representing exponential and stationary phases, respectively. 114-10 was not studied due to insufficient formation of macroconidia to produce inoculum.

DAS was only detected in extracts which were derived from cultures in the 120-hr stationary phase (Figure 6.7). The toxin was not detected in 36 hr exponential phase culture extracts from any of the strains when using either glucose or sucrose as the feedstock (data not shown). The different carbon substrates demonstrated substantial differences in their ability to induce mycotoxigenesis in *F. venenatum*. DAS was identified in the supernatant extracts from four strains at stationary phase incubated with glucose; 114-11, 114-12, 114-14 and 114-24. Strains 114-11 and 114-12 exhibited substantial quantities of DAS in the supernatant sample with limited enrichment in mycelial extracts. DAS was detected to a lesser degree in the 114-24 supernatant extract and undetected in the mycelial sample. Unusually, DAS was detected in mycelial extracts of 114-14 but was not found in the supernatant (Table 6.7, Table 6.6).

Conversely, sucrose had a more profound, yet varied, effect on DAS production. DAS was detected in the late-stage supernatant of 21 of the 24 sucrose-fed *F. venenatum* strains, including A3/5 (114-1), while occurring in the mycelial extracts of 12 strains (Figure 6.7; Table 6.6).

The greatest producer appeared to be strain 114-12, which showed substantial toxicity while two more strains, 114-5 and 114-11 also showing substantial DAS biosynthetic potential. Importantly, while sucrose appears to be a species-wide inducer, some strains appeared to lack notable DAS production in the 120-hour sucrose-fed samples. The aforementioned strains of interest, 114-8 and 114-15 produced low levels of DAS when compared to A3/5. Furthermore, strains 114-9, 114-19, and 114-23 showed a distinct lack of mycotoxigenesis when fed either glucose or sucrose (Table 6.6).

Table 6.6. DAS enrichment in *F. venenatum* strains when extracted from stationary phase (120 hpi) cultures. Values indicate ng of DAS in 10 µL sample. N/A indicates samples in which DAS was not detected.

Strain	Glucose		Sucrose	
	Mycelium	Supernatant	Mycelium	Supernatant
A3/5	N/A	N/A	>1000	>1000
114-2	N/A	N/A	N/A	>1000
114-3	N/A	N/A	N/A	>1000
114-4	N/A	N/A	N/A	N/A
114-5	N/A	N/A	N/A	>1000
114-6	N/A	24.59	315.86	>1000
114-7	N/A	N/A	N/A	>1000
114-8	N/A	N/A	12.67	46.98
114-9	N/A	N/A	N/A	N/A
114-11	640.44	>1000	>1000	>1000
114-12	797.35	>1000	>1000	>1000
114-13	N/A	N/A	280.80	>1000
114-14	589.13	N/A	N/A	>1000
114-15	N/A	18.45	308.48	>1000
114-16	N/A	N/A	N/A	25.70
114-17	N/A	14.64	143.66	302.42
114-18	N/A	25.21	37.68	>1000
114-19	N/A	N/A	N/A	N/A
114-20	N/A	N/A	>1000	>1000
114-21	N/A	84.86	93.53	>1000
114-22	N/A	N/A	N/A	34.18
114-23	N/A	N/A	N/A	N/A
114-24	15.08	>1000	234.56	>1000
114-25	N/A	N/A	N/A	N/A

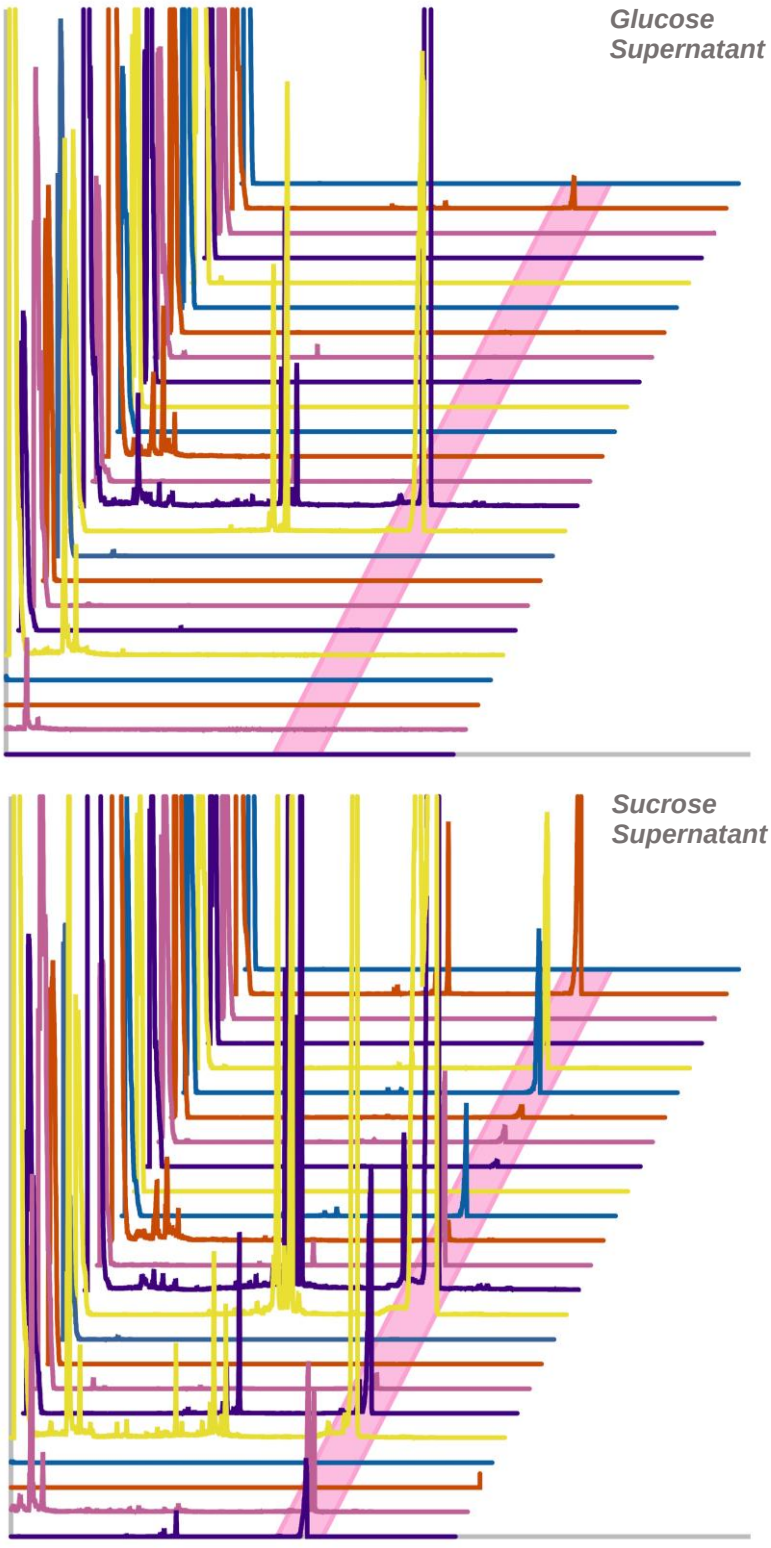


Figure 6.7. GC-MS profile results showing metabolite production in supernatant extracts of various strains of *F. venenatum* after 120 hours growth when fed either glucose or sucrose. Different coloured lines correspond to different strains of *F. venenatum,* strains ascend from A3/5 (114-1) at the bottom of the graph to 114-25 at the top. The spectrum for each strain is shifted +0.5 minutes on the x-axis relative to the spectrum below it. 114-10 is not included. Shaded box indicates expected time of DAS detection. substrates as shown on right hand side of graph.

6.3.3.2 Validation of DAS Toxicity by *K. marxianus* Bioassay

It was necessary to assess *K. marxianus* sensitivity to trichothecenes before use with *F. venenatum* extracts. This study revealed that DAS was effective at limiting growth and proliferation of *K. marxianus* at concentrations greater than 5.13 x 10^{-3} ng/10 µL (Figure 6.8A). Lower concentrations exhibited some growth inhibition, with significantly increased lag time duration in concentrations between 3.30 x 10^{-3} and 5.13 x 10^{-3} ng/10 µL . Only cultures treated with SDW or 1.10 x 10^{-3} and 2.20 x 10^{-3} ng/10 µL concentrations demonstrated reportable doubling times between 15 and 18 hpi (2.55 ± 0.17, 3.33 ± 0.50 and 2.77 ± 0.15 respectively). Doubling times of cultures treated with 1.10 x 10^{-3} and 2.20 x 10^{-3} ng/10 µL DAS were insignificantly different from the SDW control.

There appeared to be nuances of growth inhibition when concentrations which completely restricted *K. marxianus* growth in microfermentations conditions were then plated on agar. Cultures treated with 1.03 x 10^{-2} ng/10 µL DAS demonstrated abolition of growth in microwell plates, yet colony formation was observed when cultures were plated (Figure 5.8B, 4.3 ± 2.6 colonies). The number of colonies formed were approximately 100-fold lower than cultures treated with 5.13 x 10^{-3} ng/10 µL DAS (415 ± 136 colonies). Cultures treated with DAS concentrations greater than 1.03 x 10^{-2} ng/10 µL failed to form colonies on agar. This suggests that DAS has concentration-dependent toxic activity, fungistatic at concentrations lower than 1.03 x 10^{-2} ng/10 µL or fungicidal at concentrations greater than 1.54 x 10^{-2} ng/10 µL.

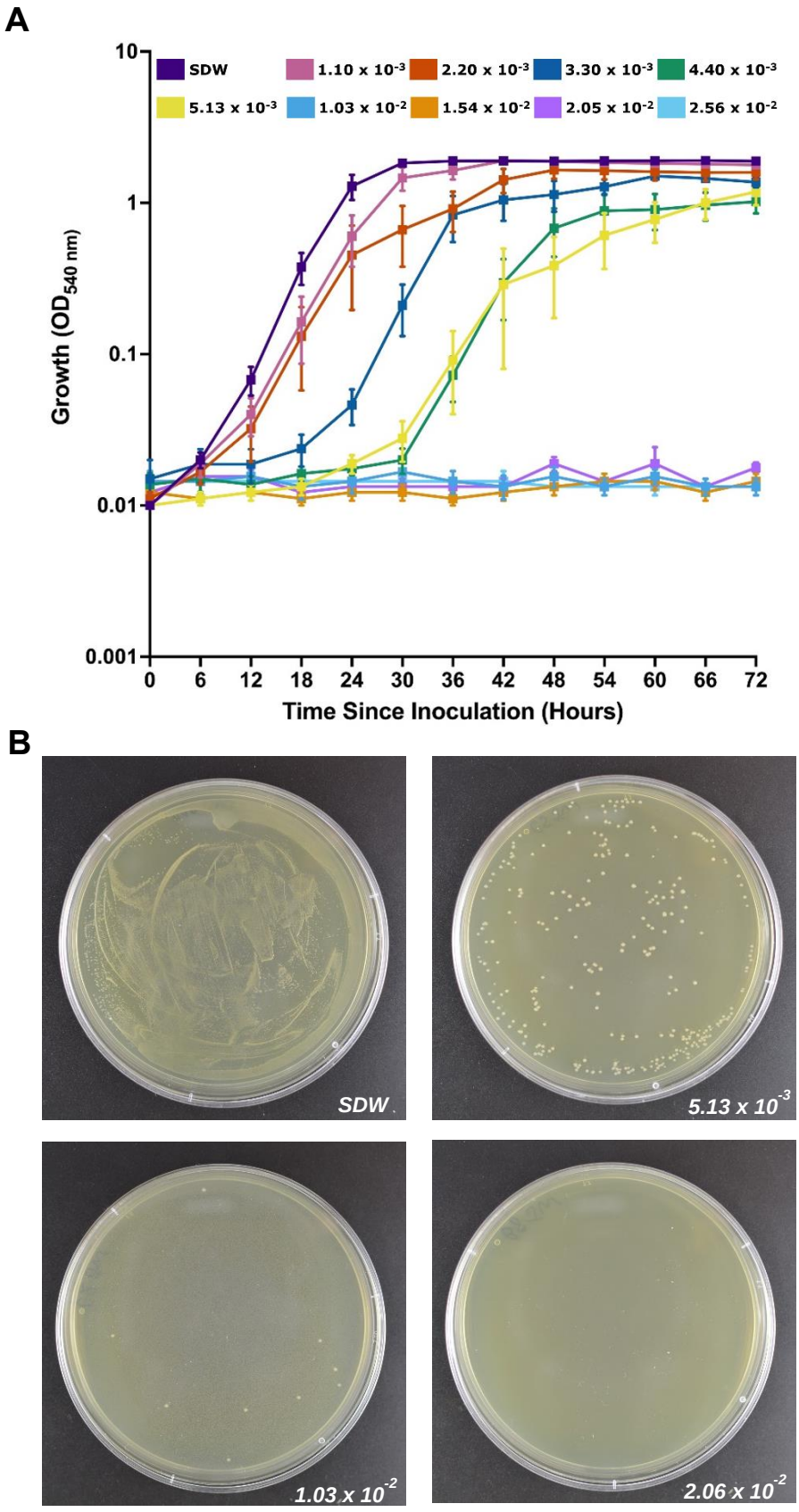


***Figure* 6.8. Growth of *K. marxianus* in response to diacetoxyscirpenol (DAS) treatment.** Values given as ng/10 µL. A. Growth profiles of *K. marxianus* cultures treated with DAS. n = 8, Error bars ± SEM. B. *K. marxianus* colony formation on YEPD following 72 hours' incubation with DAS. n = 3.

Assaying Toxicity of F. venenatum *Culture Extracts*

Extracts from strains 114-9, 114-19 and 114-23 were found to have reduced amounts of DAS compared to the other strains tested. Extracts from these strains were added to *K. marxianus* cultures to confirm reduced mycotoxigenesis rather than lack of DAS biosynthesis.

Extracts from glucose-fed 114-9 cultures showed no significant toxic effect compared to the water supplement control. By contrast, the undiluted sample from the sucrose-fed culture increased the lag phase duration, increasing the doubling time at the 15-18 hr mark more than 12-fold relative to the glucose-fed culture extract (49.30 ± 11.85 and 3.85 ± 0.68 hours respectively). Meanwhile, 114-19 extracts were markedly toxic, causing complete inhibition of growth in both extract groups across all three dilutions. Conversely, *K. marxianus* growth was not inhibited when treated with mycelial extracts from 114-23 fed either glucose or sucrose (Figure 6.9). Each undiluted extract from this strain increased doubling time relative to the negative toxic control (SDW = 2.26 ± 0.12 hours; glucose = 3.33 ± 0.30 hours; sucrose = 3.55 ± 0.26 hours), yet these changes were found to be insignificant when queried by pairwise comparisons test (glucose, p-value = 0.99; sucrose, p-value = 0.99; Dunn's test). However, there was some suggestion of toxicity in sucrose samples, as subsequent dilutions have a lesser inhibitory effect on doubling time (S3 1:1 = 3.55 hours ± 0.26; 1:4 = 2.94 hours ± 0.08; 1:16 = 2.88 hours ± 0.22). Again, statistical analysis suggested the doubling time reductions in each dilution was insignificantly different from the undiluted extract (1:4, p-value = 0.99; 1:16, p-value = 0.99; Dunn's multiple comparisons).

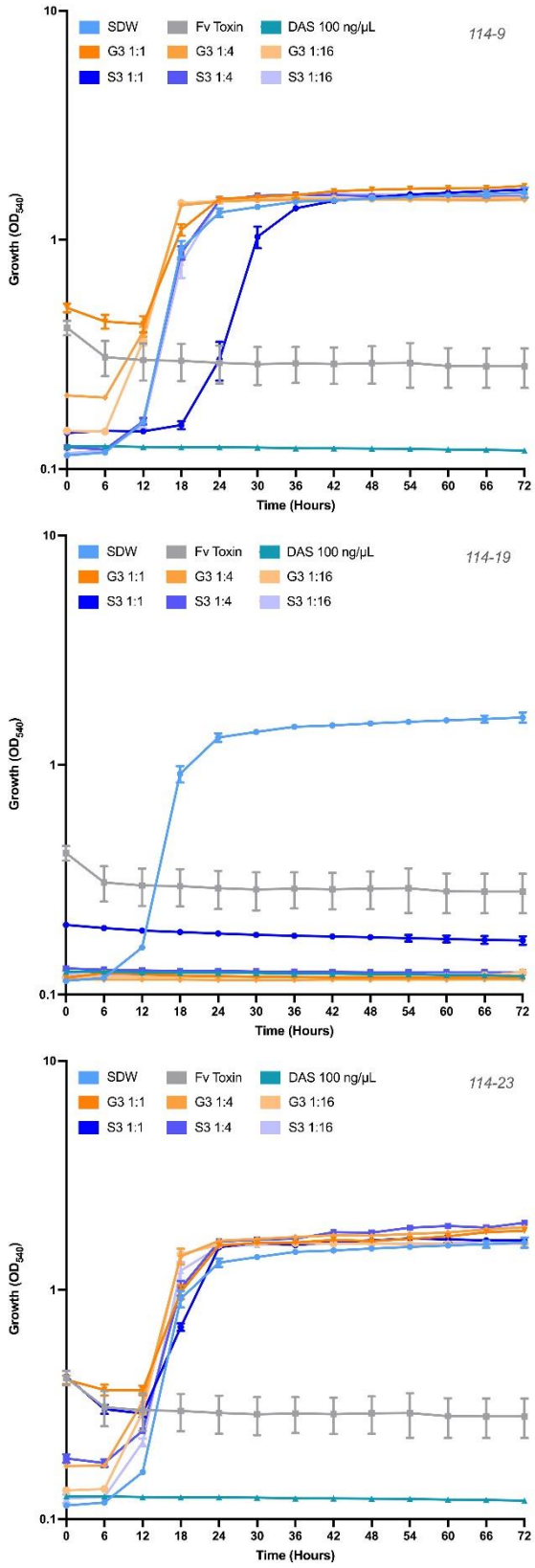


Figure 6.9. Growth of *K. marxianus* in response to *F. venenatum* extracts from isolates 114-9, 114-19 and 114-23. Each isolate is indicated on to right hand of graph. Different colour lines indicate growth resulting from use of different substrates as shown by the key on the graph. Deviations from control culture growth suggest that other toxic metabolites are produced by strains. n = 3, Error bars ± SEM.

6.3.3.3 Reassessing Toxicity in Candidate Strains

Due to the potential importance of these results, DAS biosynthesis when using sucrose as a growth substrate were repeated in triplicate for strains A3/5, 114-8, 114-15 and 114-23. Extractions were made from stationary phase (120 hpi) cultures.

Strains 114-8 and 114-15 had been identified as strains of interest earlier in the project, 114-8 as a potential closely-related strain to A3/5 and 114-15 due to suspicion of possessing a MAT1-2 genotype (see Chapter 4). As per the original strain assessment, none of the strains showed detectable DAS enrichment in mycelial extracts, despite growth on sucrose. Both strains were found to have reduced mycotoxigenesis in supernatant extracts compared to A3/5, although this was not found to be significant (Figure 6.10). 114-8 and 114-15 produced similar quantities of DAS to those each strain produced in the qualitative strain screen. Furthermore, DAS was not detected in either the mycelium or the supernatant of 114-23 culture extracts. Other peaks were produced in these extracts yet were unable to be identified confidently due to lack of appropriate standards.

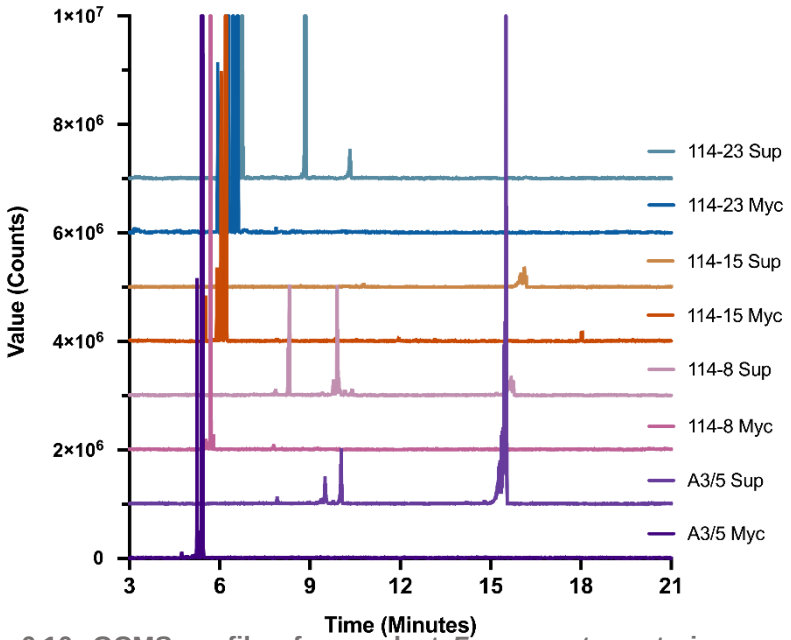


Figure 6.10. GCMS profiles from select *F. venenatum* strains grown in shake flask culture. 114-8 and 114-15 showed reduced DAS production, whereas the toxin was not detected in 114-23 extracts. Chromatogram profiles display the mean average of triplicate extraction values.

6.3.4 DAS Production in Upscaled Fermentations

6.3.4.1 Ten L Batch Fermentations

114-23 was identified as a putative non-synthesiser of DAS after incubation with sucrose, a trait which would be desirable in the Quorn production system, either directly or as a target for future strain improvement. To this end, 114-23 was compared to A3/5 in pilot fermentation conditions at the Fermentation Development Centre by Dr Mark Taylor. Both batch and continuous fermentations were investigated for each strain using both 3.00 % glucose and 3.00% sucrose as feedstocks. Mycotoxin screening was performed by Premier Analytical Services (High Wycombe, Buckinghamshire).

As demonstrated in preliminary shake flask trials earlier in the study, *F. venenatum* A3/5 does not produce DAS when glucose is used as the sole carbon substrate nor was it found to produce any of the screened mycotoxins. Consistent with sucrose shake flask studies, each of the replicate sucrose-fed A3/5 batch fermentations produced DAS (12 and 732 µg/kg of paste). In these conditions, DAS is the only mycotoxin produced above the detection limit of 10 µg/kg. By contrast, mycelial paste from strain 114-23 did not demonstrate detectable levels of the screened mycotoxins when cultures were fed either glucose or sucrose. This finding was consistent with shake flask cultures presented earlier in the study.

6.3.4.3 Ten L Steady State Fermentation

Steady state fermentations performed at the Fermentation Development Centre (Quorn Foods, Billingham) provided the closest model to the Quorn production process available. This fermentation strategy was used to investigate both the effect of glucose substitution with sucrose in A3/5 but also the putative non-mycotoxigenic strain 114-23.

Initial incubations were performed with 1.00% glucose before switching to a regime where each strain was fed either 3.00% glucose or sucrose as the sole carbon source. The results from Premier Analytical Services (High Wycombe, Buckinghamshire) showed that none of the fermentations screened synthesised any mycotoxins at a detection limit of 10 µg/kg. These included Type A trichothecenes (DAS, T-2 triol, T2 toxin, HT2 toxin), Type B trichothecenes (DON, 3AcDON, 15AcDON) and other mycotoxins known to be synthesised by the fusaria (Nivelanol, Fusarenone X, Fusarins).

6.4 Discussion

Quorn™ mycoprotein underwent considerable safety testing before demonstrating it was safe for sale as a commercial product. A key element of this evaluation was the risk to human consumption due to toxin production by the fungus (Whittaker et al., 2020). Researching alternatives to the established A3/5 and glucose production paradigm, be it industrial strain or carbon substrate, necessitated screening for toxic metabolites before consideration as a replacement.

Results from the present chapter demonstrated that there is substantial variation in the mycotoxigenic profile amongst *F. venenatum* strains. In addition, it was shown that DAS production in *F. venenatum* is directly influenced by the carbon source used in liquid culture as discussed below.

6.4.1 Influence of Carbon Substrate on Mycotoxigenesis in A3/5 Batch Culture

The influence of carbon substrate on DAS biosynthesis had not been extensively elucidated in *F. venenatum*. Studies in phytopathogenic fungi have demonstrated that carbon substrates, particularly those considered to be major storage molecules in plant tissues (e.g. sucrose, mannose and raffinose) can have significant impacts on mycotoxigenesis. While several carbon substrates had been considered in this project, the disaccharide sucrose was considered a promising alternative, enabling similar rates of growth but with greater economic advantages when compared to the use of glucose syrup by Quorn Foods.

Biosynthesis of DAS was examined in batch fermentation of *F. venenatum* A3/5 using GC-MS. The impact of five different carbon feedstocks on DAS production in the A3/5 Quorn™ industrial organism was examined: glucose, fructose and sucrose as sole carbon sources in

addition to equimolar combinations of glucose with the other sugars. Glucose failed to induce DAS production in batch fermentations, a finding that is consistent with mycotoxin testing performed on mycoprotein paste by Quorn foods (Rob Johnson, pers. comms.). Glucose has previously been reported to display limited induction of mycotoxigenesis in other *Fusarium* species including those well-known for their pathogenicity such as *F. graminearum* (Jiao et al., 2008), *F. proliferatum* (Li et al., 2017) and *F. asiaticum* (Kawakami et al., 2014).

Conversely, stimulation of mycotoxin biosynthesis by sucrose has been reported for many filamentous fungal species (Brzonkalik et al., 2012; Jiao et al., 2008). In the present study it was found that production of DAS by *F. venenatum* A3/5 increased markedly in sucrose-fed cultures when compared to those incubated in the presence of glucose. When quantified from shake flask culture, the estimates of mycotoxin production were much higher than expected. This was likely a consequence of the peaks containing DAS possibly also due to the presence of other metabolites of a similar molecular weight which contributed to the overall peak area. This could be remedied by refining the GCMS protocol for the specific detection of DAS, elucidating any confounding peaks. However, the inductive effect of sucrose on mycotoxin biosynthesis is not limited to the trichothecene family of mycotoxins. *Fusarium* species in the Liseola section including *F. proliferatum* and *F. verticillioides* utilise fumonisin toxins as the primary toxic metabolite during host invasion. When sucrose is used as a carbon feedstock in liquid culture, these species have demonstrated increased fumonisin production and transcription of *FUM* biosynthetic clusters (*F. proliferatum*, Li et al., 2017; *F. verticillioides*, Achimón et al. 2021).

This universal pattern of mycotoxigenesis induction by sucrose suggests a conserved mechanism of action. Jiao et al. (2008) speculated that structural elements of the fructo-oligosaccharides 1-kestose and nystose were critical to driving production of the Type B trichothecene 3-acetyldeoxynivelonol (3ADON), specifically citing the β-(2→1) fructosyl-

fructosyl bonds as inductive structural elements. Nakajima et al. (2016) explored this structural hypothesis of trichothecene induction further, concluding that non-reducing sugars possessing α-(1→2) (glucosyl/xylosyl)-fructosyl linkages were sufficient to induce trichothecene production in *F. graminearum*. This may explain why sucrose, formed by an α-(1→2) glucosyl-fructosyl linkage, is capable of stimulating 3ADON biosynthesis in *F. graminearum,* but its component monosaccharides (glucose and fructose) demonstrate restricted mycotoxin biosynthesis as they lack the α-glycoside structural element (Jiao et al., 2008). This pattern was also observed in the research presented in this chapter, particularly exemplified by the lack of DAS production in cultures fed equimolar mixes of glucose and fructose. Furthermore, sucrose induced Type B trichothecene biosynthesis in *F. asiaticum*, but three structural isomers of sucrose with different α-glycoside linkages (turanose α-(1→3), maltulose α-(1→4) and palatinose α-(1→6)) failed to do so (Kawakami et al. 2014). This further supports the structural hypothesis, suggesting that the α-(1→2) glucosyl-fructosyl bond in sucrose has a specific role in regulating mycotoxigenesis and perhaps other elements of secondary metabolism.

The ambiguous ecological niche of *F. venenatum* in the environment has been discussed previously in Chapter 3. The induction of mycotoxigenesis by sucrose lends weight to theories of ancestral or latent phytopathogenicity. Sucrose is loaded into the phloem of plants before distribution to sink organs, such as wheat kernels, which are often sites of infection for phytopathogens (Bihmidine et al., 2013). Thus, sucrose might act as an environmental signal indicating to an invading fungus that plant resources are available for plunder. Jiao et al. (2008) demonstrated that sucrose was able to substantially increase DON biosynthesis in all 16 strains of *F. graminearum* tested. In *F. graminearum*, production of DON is required for symptomless infection and avoidance of plant defence initiation (Brown et al., 2017). Thus, it is possible that coupling sucrose detection and toxin biosynthesis is an adaptation to optimise infection of the plant host. *F. venenatum* is

incapable of causing significant infection of wheat or tomato plants, yielding only superficial infection wounds (King et al., 2018). However, *F. venenatum* still possesses a full suite of *tri* genes and as this work has shown, is still capable of producing DAS. One hypothesis considered in chapter 3 is that *F. venenatum* cannot act as a pathogen alone, instead relying as a commensal on invasion by other plant pathogens. As part of the invasion, these partner organisms will likely produce mycotoxins to achieve pathogenicity. As the trichothecene biosynthesis pathway progresses, the compound undergoes alternating rounds of hydroxylation and acetylation, activating and inactivating the toxin molecule respectively (Kimura et al., 1998; McCormick et al., 2011). *F. venenatum* may retain the full suite of *tri* genes not for use in offense but as a defensive mechanism, allowing the fungus to neutralise trichothecenes which may have been produced by the primary pathogen it is parasitising.

F. venenatum A3/5 displayed a lack of DAS production during liquid culture with fructose. Similarly, the limited capacity of fructose to induce mycotoxigenesis in filamentous fungi has been reported in other literature (Jiao et al., 2008; Jian et al., 2019). In the cell, fructose is phosphorylated by hexokinase to fructose-6-phosphate which then enters the glycolytic pathway (Khosravi et al., 2018). Unlike glucose catabolism, fructose processing avoids the pentose phosphate pathway (PPP), which is key to generating a pool of NADPH, the main donor of electrons in anabolic reactions producing fatty acids, nucleotides and reduced glutathione (Masi et al., 2021). Many steps in trichothecene biosynthesis are facilitated by cytochrome P450 activity, which in turn require the association of NADPH-cytochrome P450 reductases (CPRs; Malonek et al., 2004, Etzerodt et al., 2017). Thus, if the NADPH pool is depleted in fructose-fed cultures due to reduced flux through the PPP, the activity of cytochrome P450 enzymes is diminished, which would have implications for trichothecene biosynthesis. It is possible that there is conversion between fructose-6-phosphate and glucose-6-phosphate facilitated by phosphoglucoisomerase (PGI) to equilibrate the

compounds, allowing some NADPH to be produced through the PPP, but this is perhaps not sufficient for considerable trichothecene biosynthesis.

The impact of carbon substrate on growth assessed in this project demonstrated that some feedstocks were less effective at driving growth in *F. venenatum*. Importantly for the work in this chapter, when fructose was used as the sole carbon source there was a significant increase in doubling time compared to glucose-fed cultures. Consequently, directly comparing the temporal DAS production profile may be inaccurate as snapshots of different growth stages might have been captured for each culture condition. If fructose was a potent inducer of mycotoxigenesis, it might be expected that this latent inductive ability would be seen in later culture extracts, as it would reach stationary phase later than the other cultures. This was not the case, DAS failing to be detected throughout the batch growth cycle.

In addition to pure sugar substrates, a number of commercial substrates were investigated for use as alternatives to the currently used glucose syrup. Several of these, particularly the C*Sweet range, did not appear to upregulate DAS biosynthesis when used to feed A3/5 cultures. These present an interesting opportunity, as they demonstrate greater economic feasibility than the current glucose syrup. Interestingly, the only syrups to present detectable DAS levels were the invert sugar syrup and C*TruSweet 1750, both of which contained fractions of fructose. Contrastingly, when equimolar blends of glucose and fructose were assessed in this study, they did not seem to induce DAS biosynthesis. It might be suspected that these substrates, particularly the invert sugar, contain low levels of sucrose which are sufficient to induce the trace levels of DAS observed.

Much of the work presented in this chapter was performed in batch culture, a system necessitated by laboratory limitations with undertaking continuous culture studies. These batch conditions showed that sucrose did not induce DAS biosynthesis until late exponential phase/early

stationary phase. When sucrose-fed A3/5 steady state fermentations were performed, analysis revealed that none of the major *Fusarium* mycotoxins were synthesised and detected in the mycelium. However, time constraints meant that for the most part this study lacked screening of culture supernatants, which were found to be a major location for trichothecenes where tested in the present work. However, the main methodology used of screening mycelium is identical to the quality control performed by Quorn Foods on mycoprotein paste for sale. As such, lack of supernatant analysis may not be important for the industrial process. Such a finding implies that sucrose could substitute glucose as the industrial carbon substrate, conferring lower costs and greater stability of substrate acquisition without risk of increased toxicity in the mycelium.

6.4.2 Species-wide DAS Biosynthetic Behaviour

Previous studies of mycotoxin production in *F. venenatum* have been limited and have focussed mainly on the A3/5 Quorn production strain, a consequence of its biotechnological relevance. Results from this chapter provides insights into DAS biosynthesis across the species biodiversity, building on recently published studies by Laraba et al. (2021) using 7 strains of *F. venenatum.*

Most of the *F. venenatum* strains studied showed limited DAS production when glucose constituted 3% of the total medium composition. While glucose had a limited effect on A3/5 mycotoxigenesis, glucose-fed cultures of 114-11, 114-12 and 114-24 showed much greater DAS production. DAS was undetectable in the remainder of the strains, suggesting that mycotoxin induction by glucose is strain specific. If *F. venenatum* has some limited potential for phytopathogenicity, strain-specific effects might be interpreted as subtleties in host preference of these strains.

By contrast, sucrose showed much greater potency as an inducer of mycotoxin production. Extracted DAS was between 3- and 1,600-fold greater in those strains which showed restricted capacity for DAS production in glucose-fed conditions when compared to the equivalent sucrose-fed culture extracts. Species-wide induction of DAS biosynthesis by sucrose was perhaps expected given previous reports of the inductive capacity of sucrose both across *Fusarium* genus and more widely in filamentous fungi (Jiao et al., 2008; Brzonkalik et al., 2011). In the case of *F. venenatum*, many of the strains which failed to produce detectable DAS under glucose-fed conditions produced substantial quantities of the mycotoxin when sucrose was used as the sole carbon source. However, of promise was that extracts from three strains; 114-9, 114-19 and 114-23 did not show detectable DAS in either condition, and other strains (114-7, 114-8, 114-14 and 114-18) showed much lower levels of DAS production that A3/5 when grown on sucrose.

To confirm the lack of DAS production by these putative non-DAS producers, the impact of culture extracts on the growth of the yeast *K. marxianus* GK1005 (which is sensitive to DAS) was evaluated. Importantly, supernatant extracts generated from 114-23 failed to show significant growth inhibition of *K. marxianus* cultures. Failing to inhibit *K. marxianus* growth suggests the extract lacks toxic metabolites, DAS or any other. The same bioassay revealed that extracts derived from 114-19 were toxic to *K. marxianus*, attenuating growth for the whole growth course of 72h. It could be hypothesised that this strain is able to synthesise a toxic metabolite other than DAS, perhaps a precursor in that same pathway as a consequence of an interrupted trichothecene biosynthesis process.

The study performed by Larabe et al. (2021) is the only other study to observe mycotoxin production across *F. venenatum* strains. It was shown that 114-19 (R-2273) was able to produce DAS, and thus is likely to account for the toxicity witnessed in the *K. marxianus* bioassay. If this is the case, it is possible that there is a gap in sensitivity between the

current GC-MS analysis and the *K. marxianus* bioassay. Furthermore, the work undertaken in this chapter was unable to demonstrate DAS biosynthesis from strains 114-3, 114-4 and 114-22. Laraba et al. (2021) showed that these strains were capable of producing DAS, and the toxic metabolites butenolide (BUT; 114-3 and 114-22), calonectrin (CAL; 114-3) and isotrichodermol (ITD; 114-22). Recovery of BUT, CAL and ITD by ethyl acetate extraction has been shown in previous work and so might have been extracted during this project (Laraba et al., 2021). ITD and CAL are trichothecene precursors to DAS which have shown toxic effects in eukaryotic cells and so may be responsible for the toxicity demonstrated by strain 114-19 (Bondy et al., 1991; Tanaka et al., 2019). Butenolide is a mycotoxin which poses potential health risks for humans and animals, albeit with low pharmacological toxicity, through the consumption of infected grain, for example in the case of Kashin-Beck disease (Guo et al., 2011). The GC-MS profiles generated by 114-9 and 114-19 extracts were screened using diagnostic m/z values (BUT = 99 m/z; CAL = 290 m/z; ITD = m/z), but this analysis failed to suggest that these toxic metabolites had been produced. Discrepancy of results with Laraba et al. (2021) could be a consequence of experimental error or differences in medium composition used for mycotoxin production. Specific strategies were undertaken by Laraba et al. (2021) to induce mycotoxigenesis such as the use of agmatine broth, in addition to rice- and cornmeal-based broths, whereas the current study was designed to assess mycotoxin production only in the liquid media used in the industrial process at Quorn Foods.

Importantly, strain 114-23 showed restricted ability to generate DAS as detected by GC-MS and the extract harboured no significant toxic effects on *K. marxianus*. Lack of toxin production on a cheap, mycotoxigenic carbon substrate like sucrose makes these strains a significant alternative to the Quorn production strain, as the current glucose syrup is one of the greatest overheads of production (Whittaker et al., 2020). Promisingly, results from process upscaling showed that strain 114-23 lacked DAS production when fed sucrose in batch and steady-state

conditions. The mycotoxin analysis of these cultures demonstrated that this strain failed to synthesise other toxins including Type A trichothecenes later in the pathway (T-2 and HT-2). This analysis did not reveal whether the trichothecene pathway was interrupted earlier and that a toxic metabolite such as culmorin was produced instead. While this remains a possibility, analysis of the toxic extract in yeast bioassays suggests that there was no toxic metabolite present which could limit the growth of *K. marxianus*.

6.4.3 Future Prospects

While the work performed in this chapter has provided promising prospects for future industrial exploitation of *F. venenatum*, various future work could provide important further insights into mycotoxin production in *F. venenatum*.

First, of industrial importance would be transcriptional analysis of the trichothecene biosynthetic cluster. qRT-PCR methodologies could be implemented to assess transcription of specific genes when cultures were incubated in either glucose, sucrose or a 50:50 blend and observed over batch culture progression. In particular, the genes *tri5*, *tri6* and *tri10* would be important targets for analysis, as the products of these genes represent the first enzyme in the biosynthetic pathway (trichodiene synthase), a regulator of biosynthesis and export pump respectively (Hohn et al., 1986; Seong et al., 2009). This experiment had been intended for this study, with primers suitable for RT-PCR investigations designed for all trichothecene biosynthetic cluster genes. However, financial and time constraints on the project meant that this analysis was not undertaken.

Second, mycoprotein contamination with trichothecenes could be abolished entirely using genetic modification. *Tri5* encodes the trichodiene synthase enzyme, converting farnesyl pyrophosphate to trichodiene and initiating the trichothecene biosynthetic pathway (see

Chapter 1.; McCormick et al., 2011). Deletion of this gene in *F. venenatum* abolishes DAS production in addition to other trichothecenes (Miller and MacKenzie, 2000). This study observed cultures incubated for 7 days grown in MYRO medium, a sucrose-containing medium with consistent ability to induce mycotoxigenesis in trichothecene-synthesising fungi (Miller et al., 1991). Strain improvement strategies involving traditional mutagenesis such as UV or gamma radiation might allow *tri5* to become inactivated, but such non-specific strategies are much less efficient at achieving specific goals and also introduce potential unwanted mutations (Ohnishi et al., 2002). Production of a GM strain using targeted mutagenesis strategies such as CRISPR-Cas9 or error-prone PCR is a viable research strategy, yet opinion to GM food remains a controversial topic in Europe (Ichim 2020). Investigating DAS production in such a mutant under the Quorn fermentation model described in the study would be of great interest, as it is expected that DAS production would be abolished and thus sucrose could be used as the carbon substrate in mycoprotein production without risk.

Finally, as discussed in Chapter 5, changes in response to sucrose implantation or alternative strains would need to be investigated to prevent changes to the favourable Quorn™ amino acid make-up. A number of strains appeared to have promisingly low DAS biosynthesis other than 114-23, such as isolates 114-4 and 114-8. There was limited time to investigate these in more detail during this study, yet such strains could present an attractive option.

6.4.4 Conclusion

The aim of this study was to assess the pattern of DAS biosynthesis following changes in carbon substrate in addition to analysis across the *F. venenatum* species with the aim of finding a potential alternative to the Quorn industrial process. The work in this chapter has shown that changes in carbon substrate have important repercussions for mycotoxin biosynthesis, as seen in other filamentous fungi. A3/5, while showing substantial DAS biosynthesis in late-stage cultures, does not

produce DAS when incubated with sucrose in a steady state culture. This, combined with the findings in Chapter 4 that there is no difference in growth in steady state culture, suggest that sucrose could be used to replace glucose syrup. Furthermore, 114-23 is a promising alternative to the Quorn paradigm, appearing to lack the capacity to produce mycotoxins on either glucose or sucrose, both in batch and steady state conditions. While A3/5 still carries the threat of producing DAS on sucrose, 114-23 has no such danger. Based on these results, 114-23 in combination with sucrose could replace the current Quorn production paradigm, both eliminating the threat of mycotoxigenesis and significantly reducing the production overheads involved in using glucose syrup.

7

Summary and Future Outlook

The work in this thesis aimed to broaden the research horizons of the Quorn™ mycoprotein process beyond that of the established strain and carbon substrate paradigm. The principal findings of the work are laid out below, laying a strong foundation for future research.

7.1 Expanding Known Biodiversity

7.1.1 Key Accomplishments

- Established largest known collection of *Fusarium venenatum* strains to-date (25).
- Confirmed scarcity of *F. venenatum* in the environment, in agreement with other studies studying *Fusarium* communities worldwide.
- Developed PCR-based diagnostic for screening soil for *F. venenatum* incidence. Confirmed as compatible with alternative *F. venenatum* strains by *in silico* analysis.
- Generated 21 unique *F. venenatum* genomes, laying the foundation for deeper analysis of *F. venenatum* biology and genetics.
- First report of the *F. venenatum* MAT1-2 locus structure and *MAT1-2-1* gene.
- Identified four MAT1-2 strains, paving the way for elucidation of the *F. venenatum* sexual cycle.

7.1.2 Proposed Future Work

- Use successful gene targets identified in PCR-based diagnostic and use to develop LAMP assays as in King et al. 2019 or similar.
- Perform transcriptional analysis of *MAT* genes in both *MAT1-1* and *MAT1-2* strains on various crossing media to observe if changes in gene regulation occur.
- Undertake investigations to attempt to induce the sexual cycle in *F. venenatum*, by implementing several mating techniques and using crossing media known to be successful for triggering sex in other filamentous fungi.
- Genomic manipulation studies to facilitate functional characterisation of the *MAT* genes in *F. venenatum* through creation of *MAT*-null strains as well as transferral of *MAT* loci between strains.

7.2 Challenging the Quorn™ Paradigm

7.2.1 Key Accomplishments

- Demonstrated that *F. venenatum* shows considerable carbon substrate flexibility, with growth supported by a range of sugar compounds.
- Blends of glucose and alternative carbon substrates support growth and provide potential low-cost avenues for the Quorn™ industrial process.
- Alternatives to pure sugar feedstocks were considered, including high fructose syrups and a lignocellulosic breakdown product provided by Tsinghua University. Both results showed that the Quorn™ industrial process was able to move towards implementing waste carbon substrates.
- A3/5 exhibited similar growth rates on both glucose and sucrose, with no apparent changes in utilisation of substrate or germination pattern.

- As observed in other literature, sucrose was demonstrated to induce mycotoxigenesis in *F. venenatum*, similar to other species in the *Fusarium* genus. However, when A3/5 was grown in continuous fermentation with sucrose, DAS and other non-trichothecene toxins, common to *Fusarium* species, such as the fusarins, were not detected.
- A species-wide analysis of DAS production was undertaken, observing the effect of carbon substrate on the synthesis of the mycotoxin. This work revealed that some strains of *F. venenatum* potentially lacked mycotoxigenesis on sucrose, with one strain confirmed to lack mycotoxigenesis during stationary phase.
- Genomic data was used to analyse the trichothecene biosynthetic cluster arrangement of 21 *Fusarium venenatum* strains but did not provide insight into the variation of trichothecene biosynthesis between strains.

7.2.2 Proposed Future Work

- Further work could explore the intergenic regions, assessing promoter and/or transcription factor binding site sequence variability.
- Carbon substrate can have an impact on the amino acid profile presented by a fungus, as exemplified by ribose in *F. venenatum* (Anderson and Solomons, 1984). Given the heterodimeric structure of sucrose, changes in amino acid content may be attributed to the utilisation of fructose, a ribose sugar. Research into this topic will be undertaken by a PhD project focusing on the modulation of amino acid content in *F. venenatum*.
- Extended continuous fermentation studies will be necessary to determine if the change in carbon substrate will have implications for the occurrence of morphological mutants such as the undesirable c-variant.

7.3 Concluding Remarks

This thesis set out to explore how changes to carbon substrate and *F. venenatum* strain might lead to an improved commercial production system. In doing so, we have built upon and maintained the continuum of Quorn™ development beginning with Lord Rank's dream. In exploring this aim, not only have potential alternatives been uncovered, but in addition this work has established a robust foundation for further work to drive development into the future. While my chapter in this story has come to a close, it has been a privilege to be part of the research continuum for such a revolutionary player in biotechnology.

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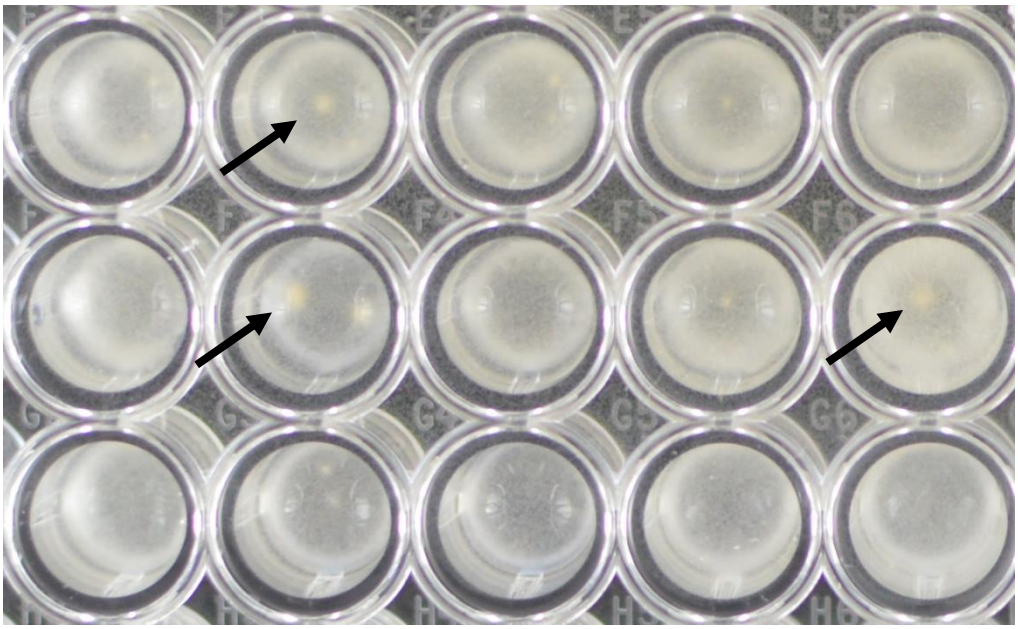
Supplementary Material

Supplementary table 3.1. Details of soil samples taken during the course of the present study.

Code	Sample Name	Site of Sample
SL_001	Blackcurrant plot	East Malling Research
SL_002	Apple orchard	East Malling Research
SL_003	Grape plot	East Malling Research
SL_004	Grass	East Malling Research
SL_005	Strawberry plot	East Malling Research
SL_006	Cereal plot	East Malling Research
SL_007	Soil by Trough	Billingham
SL_008	Ragwort	Billingham
SL_009	Front Lawn	Billingham
SL_010	Under Compost	Nottingham Garden
SL_011	Vegetable patch	Nottingham Garden
SL_012	Strawberries	Nottingham Garden
SL_013	Back Garden	Sheffield
SL_014	2nd Wheat F2	Bunny
SL_015	2nd Wheat F4	Bunny
SL_016	Sugar Beet Washed Soil	Sutton Bonnington
SL_017	Field 5 Near Bales	Quorn Village
SL_018	Maize	Quorn Village
SL_019	Field 3	Quorn Village
SL_020	Field 4	Quorn Village
SL_021	Field 5	Quorn Village
SL_022	Field 6	Quorn Village
SL_023	Field 9	Quorn Village
SL_024	“Gent's Corner”	Marlow Allotments
SL_025	P22 Clay	Marlow Allotments
SL_026	P36	Marlow Allotments
SL_027	P48A Topsoil (Compost)	Marlow Allotments
SL_028	P48A Compost Bin	Marlow Allotments
SL_029	P70 Compost	Marlow Allotments
SL_030	P70 Under Compost Bin	Marlow Allotments
SL_031	P104 Under Poppies	Marlow Allotments
SL_032	P79	Marlow Allotments
SL_033	P73 Compost	Marlow Allotments
SL_034	P73 Under Compost	Marlow Allotments
SL_035	P42 Clay	Marlow Allotments
SL_036	P71	Marlow Allotments
SL_037	P89	Marlow Allotments
SL_038	Leisure Park Grass	Marlow Town
SL_039	Edge Soil	Marlow Town
SL_040	River Mole Hill	Marlow Town
SL_041	Below Wood Compost	Marlow Town
SL_042	Forest	North Kesteven
SL_043	Field 1	North Kesteven
SL_044	Field 2	North Kesteven
SL_045	Field 3	North Kesteven
SL_046	Field 4	North Kesteven
SL_047	Field 5	North Kesteven
SL_048	Field 6	North Kesteven

Supplementary table 3.*2*. Identity summary of the putative Fusarium colonies isolated from the environment. In each case, the top three BLASTn results for the TEF-1α sequence are given.

Isolate	Origin Soil Sample	Primary Hit Species	Secondary Hit Species	Tertiary Hit Species
SI_006	SL_001	*Alternaria tenuissima* KUC1573	*Alternaria alternata* LA3	*Alternaria alternata* LA2
SI_046	SL_002	*F. oxysporum* Fo47	*F. oxysporum* FRC O-1890	*F. oxysporum* 170
SI_060	SL_002	*F. oxysporum* Fo47	*F. oxysporum* FRC O-1890	*F. oxysporum* 170
SI_064	SL_003	*F. graminearum* ITEM 6530	*F. graminearum* ITEM 6510	*F. graminearum* ITEM 6509
SI_083	SL_003	*F. graminearum* ITEM 6530	*F. graminearum* ITEM 6510	*F. graminearum* ITEM 6509
SI_188	SL_006	*F. graminearum* ITEM 6530	*F. graminearum* ITEM 6510	*F. graminearum* ITEM 6509
SI_451	SL_016	*F. culmorum* ITEM 7161	*F. culmorum* ITEM 7160	*F. graminearum* ITEM 6530
SI_468	SL_016	*F. culmorum* ITEM 7161	*F. culmorum* ITEM 7160	*F. graminearum* ITEM 6530
SI_480	SL_016	*F. culmorum* ITEM 7161	*F. culmorum* ITEM 7160	*F. graminearum* ITEM 6530
SI_497	SL_020	*Neocosmospora rubicola* CBS 101018	*Neocosmospora rubicola* CBS 320.73	*Neocosmospora rubicola* CBS ICMP: 17329.
SI_572	SL_020	*Neocosmospora rubicola* CBS 101018	*Neocosmospora rubicola* CBS 320.73	*Neocosmospora rubicola* CBS ICMP: 17329.
SI_586	SL_020	*F. vanettenii* CBS 123669	*F. solani-melongenae* CBS 101573.	*F. solani* H06A_1F_1a
SI_597	SL_020	*F. culmorum* ITEM 7161	*F. culmorum* ITEM 7160	*F. graminearum* ITEM 6530
SI_692	SL_023	*F. oxysporum* Fo5176	*F. oxysporum* f. sp. *koae* 44.	*F. oxysporum* 170
SI_719	SL_023	*F. solani* CBS 119600	*F. phaseoli* CBS 265.50	*F. solani-melongenae* CBS 101573.
SI_763	SL_025	F. graminearum ITEM 6530	F. graminearum ITEM 6510	F. graminearum ITEM 6509
SI_791	SL_026	*F. hostae* NRRL 29642	*F. incarnatum* JSDTF.i..1901	*F. nygamai* FRC M-7492.
SI_809	SL_026	*F. hostae* NRRL 29642	*F. incarnatum* JSDTF.i..1901	*F. nygamai* FRC M-7492.
SI_888	SL_029	*F. culmorum* ITEM 7161	*F. culmorum* ITEM 7160	*F. graminearum* ITEM 6530
SI_898	SL_029	*F. oxysporum* Fo5176	*F. oxysporum* f. sp. *koae* 44.	*F. oxysporum* 170
SI_889	SL_029	*F. culmorum* ITEM 7161	*F. culmorum* ITEM 7160	*F. culmorum* Class2-1B
SI_1026	SL_034	*F. oxysporum* Fo47	*F. oxysporum* FRC O-1890	*F. oxysporum* 170
SI_1036	SL_034	*F. hostae* NRRL 29642	*F. incarnatum* JSDTF.i..1901	*F. nygamai* FRC M-7492.
SI_1045	SL_034	*F. oxysporum 170*	*F. oxysporum* Fo5176	*F. oxysporum* f. sp. *koae* 44
SI_1073	SL_035	*Chaetominum globosum*	*Diaporthe foeniculina* CAA955	*Diaporthe foenicula* EFA 465
SI_1123	SL_037	*F. oxysporum* Fo47	*F. oxysporum* FRC O-1890	*F. oxysporum* 170
SI_1239	SL_041	F. graminearum ITEM 6530	F. graminearum ITEM 6510	F. graminearum ITEM 6509
SI_1254	SL_041	F. graminearum ITEM 6530	F. graminearum ITEM 6510	F. graminearum ITEM 6509
SI_1359	SL_045	*F. oxysporum* Fo47	*F. oxysporum* FRC O-1890	*F. oxysporum* 170
SI_1364	SL_045	*F. oxysporum* Fo47	*F. oxysporum* FRC O-1890	*F. oxysporum* 170
SI_1374	SL_045	*F. oxysporum* Fo47	*F. oxysporum* FRC O-1890	*F. oxysporum* 170
SI_1378	SL_045	*F. oxysporum* Fo47	*F. oxysporum* FRC O-1890	*F. oxysporum* 170



Supplementary Figure 4.1. Photograph of *F. venenatum* cultures in microwell plates following shaking incubation. Arrows indicate mycelial pellets, an undesirable growth morphology.

Supplementary Table 4.1. DTE and DTL values calculated for each of the *F. venenatum* isolates used in this study. Values represent hours ± SEM, n = 3.

Strain	Glucose		Sucrose	
	DT_E	DT_L	DT_E	DT_L
A3/5	3.83 ± 0.24	10.16 ± 0.75	6.81 ± 1.80	7.14 ± 1.33
114-2	2.76 ± 0.05	7.86 ± 0.26	2.99 ± 0.17	7.95 ± 0.12
114-3	4.31 ± 0.86	4.58 ± 0.93	3.69 ± 0.61	5.05 ± 0.37
114-4	2.81 ± 0.12	7.70 ± 0.91	2.77 ± 0.18	7.68 ± 0.73
114-5	2.99 ± 0.13	10.43 ± 0.47	3.09 ± 0.18	9.24 ± 0.90
114-6	3.03 ± 0.20	8.95 ± 1.95	3.23 ± 0.24	11.58 ± 1.08
114-7	6.65 ± 3.90	6.87 ± 0.51	3.25 ± 0.30	6.44 ± 0.13
114-8	3.43 ± 0.56	5.40 ± 0.42	3.26 ± 0.26	4.75 ± 0.24
114-9	2.66 ±0.28	6.66 ± 0.45	2.98 ± 0.20	7.71 ± 0.21
114-10	3.48 ± 0.79	9.60 ± 0.71	3.48 ± 0.03	8.51 ± 0.47
114-11	2.95 ± 0.08	9.94 ± 0.35	3.52 ± 0.42	9.02 ± 0.63
114-12	3.60 ± 0.80	11.35 ± 1.22	5.32 ± 3.49	9.79 ± 1.58
114-13	2.61 ± 0.14	6.21 ± 0.08	3.00 ± 0.31	7.53 ± 0.37
114-14	7.26 ± 0.36	10.86 ± 0.50	6.29 ± 0.34	11.52 ± 0.02
114-15	2.67 ± 0.13	6.06 ± 0.36	2.69 ± 0.13	6.34 ± 0.45
114-16	3.91 ± 0.53	9.82 ± 1.64	3.63 ± 0.44	7.86 ± 0.46
114-17	2.97 ± 0.24	7.32 ± 0.52	2.81 ± 0.19	7.64 ± 0.44
114-18	2.72 ± 0.04	6.53 ± 0.15	3.16 ± 0.04	6.15 ± 0.02
114-19	3.13 ± 0.19	8.92 ± 0.90	3.09 ± 0.09	7.94 ± 0.64
114-20	2.60 ± 0.17	6.23 ± 0.25	3.64 ± 0.08	5.60 ± 0.31
114-21	3.30 ± 0.02	7.30 ± 0.47	3.25 ± 0.27	7.34 ± 0.91
114-22	4.30 ± 0.62	10.74 ± 2.45	2.96 ± 0.41	9.39 ± 0.36
114-23	3.51 ± 0.73	10.04 ± 0.86	3.01 ± 0.21	7.92 ± 0.61
114-24	4.07 ± 1.37	9.42 ± 0.81	3.80 ± 0.83	9.08 ± 0.56
114-25	2.95 ± 0.25	9.52 ± 2.03	3.11 ± 0.10	8.64 ± 0.27