Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

**Faculty of Landscape Architecture,
Horticulture and Crop Production Science**

The Effect of Different Nitrogen Sources on Mycelial Growth of Oyster Mushroom, *Pleurotus ostreatus*

– With a review concerning cultivation of the species

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– Samt information kring odling av arten

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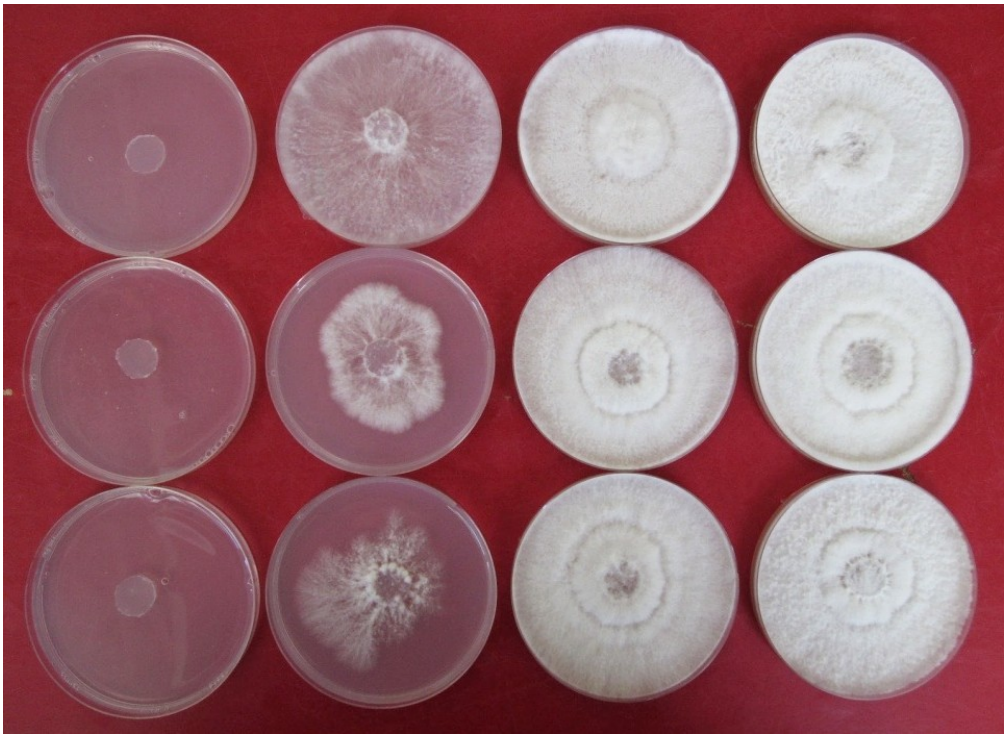

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PREFACE

During my studies at the horticultural science programme I have often stumbled upon the kingdom of Fungi. These organisms continue to amaze me, whether it is in the forms of food, diseases or the symbiotic relationships of mycorrhiza. Their extreme diversity makes them taunting and mysterious with seemingly unlimited implementations in many fields of science. In recent years my experience with home grown mushrooms, using simple hobby growth kits from Ecofungi (Ecofungi, n.d.) and Svamphuset (Svamphuset, 2016), has only strengthened my fascination with these tasty organisms. When having to decide for a subject for my project my thoughts therefore quickly went towards mushrooms. During a course in microbiology I had come to know Malin Hultberg as a most inspiring scientist and teacher. I therefore came to her for supervision and discussion of subject ideas.

An independent project is both demanding and at times trying. I have had many great lessons of how to work and how not to work. The intensive reading and writing, when trying to make sense of the subject, has been both exciting and exhausting. These experiences will be of great help when planning and conducting my future projects.

Many thanks to Malin Hultberg for the helpful supervision, clever advice, great support and discussions throughout the project!

A special thanks to Jan-Eric Englund, Department of Biosystems and Technology, Swedish University of Agricultural Sciences. I am very grateful for your advice and help concerning the statistics of this project!

I also wish to thank my family and friends for giving me love and support always.

Astrid Laursen

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ABSTRACT

The production of Oyster mushroom, *Pleurotus ostreatus,* is sustainable, both in terms of economy and environment. Cultivation can be performed successfully on a wide range of substrates, often in the form of otherwise non-productive waste from other industries, such as agriculture and forest production.

In a literary review results of studies on the fungi and its possible substrates are examined. *Pleurotus* is efficient in breaking down lignocellulosic wastes and its requirements on substrates for cultivation are therefore quite modest. Nonetheless many conditions can be regulated in order to optimise the mushroom production. Some general conclusions are made on what type of improvements the mushroom producer or scientist can make in terms of creating a better substrate.

Experiments were made on three strains of *P. ostreatus* (M 2140 and M2191 from Mycelia, Belgium, and ATCC® 44309™ from ATCC, USA). The aim is to test a chemically defined medium and a variation in nitrogen sources in it with reference to radial mycelial growth and mycelial thickness.

The results show growth on all media tested and the radial mycelial growth and thickness was observed to significantly differ depending on the available nitrogen source for three strains of *P. ostreatus*. Overall the control medium, which had a higher nitrogen content and more complex ingredients, reached the best results for both parameters. Another result is that mixtures of ammonium and the amino acid asparagine were preferred over mixtures including nitrate for all three strains.

Some strain variations were observed and from this one may conclude that the requirements of the specific strains differ. This illuminates the importance of clearly presenting the strains used in future studies. Furthermore, it is suggested to either create a strain specific substrate or "train"/adapt the specific strain for a specific substrate.

Further studies are needed for an improved understanding of *P. ostreatus* and its nutritional requirements and cultivation. In the future, additional research can improve this and the gaps in knowledge will hopefully be filled.

ABSTRAKT

Produktionen av ostronskivling/ostronmussling, *Pleurotus ostreatus*, är hållbar, både när det gäller ekonomi och miljö. Odling kan framgångsrikt ske på många olika substrat, ofta i form av icke-produktiva rester från andra branscher, såsom jordbruks- och skogsproduktionen.

I en litterär genomgång granskas resultat från studier på svampar och deras möjliga substrat. *Pleurotus* är effektiv när det gället att bryta ner lignocellulosa-haltiga restprodukter. Dess krav på odlingssubstrat är därför relativt blygsamma. Ändå finns det många förhållanden som kan regleras för att optimera svampproduktionen. Några generella slutsatser dras med hänsyn till vilka förbättringar svampproducenter eller forskare kan göra när det gäller att skapa ett bättre odlingssubstrat.

Experimenten gjordes på tre olika stammar/sorter av *P. ostreatus* (M 2140 och M 2191 från Mycelia, Belgien, och ATCC 44309 ™ från ATCC, USA). Målet var att testa radiell mycelietillväxt och myceltjocklek på ett kemiskt definierat medium som ett resultat av olika kvävekällor.

Resultaten visar tillväxt på alla testade tillväxtsmedier. Den radiella myceltillväxten och myceltjockleken observerades som signifikant olika beroende på den tillgängliga kvävekällan. Kontrollmediet, som hade högre kvävehalt och mera komplexa beståndsdelar, uppnådde de bästa resultaten för båda parametrar. Ett annat resultat är att blandningar av ammoniumnitrat och aminosyran asparagin föredrogs av alla tre stammar, framför blandningar innehållande nitrat.

Vissa skillnader observerades mellan mycelstammarna. Från detta kan man dra slutsatsen att kraven hos de specifika stammarna skiljer sig något åt. Detta belyser även vikten av att tydligt presentera vilka stammar som används i framtida studier. Det föreslås vidare att antingen skapa ett stamspecifikt substrat eller "träna"/anpassa den specifika stammen för ett specifikt substrat.

Ytterligare studier behövs för att förbättra förståelsen av *P. ostreatus* och dess näringsbehov och odlingsteknik. Fortsatt forskning kommer förhoppningsvis att fylla luckorna i vår kunskap om *P. ostreatus.*

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INTRODUCTION

The usage of mushrooms dates back many thousand years for humans and even longer for other organisms (Amuneke et al., 2011; Cotter, 2004; Melo de Carvalho et al., 2010). For hunters and gatherers the knowledge of wild animals, plants and fungi has been crucial for survival (Diamond, 1999). Luckily, development has taken us further and today the focus is no longer on recognising food but on understanding it. Through this type of knowledge we develop systems and sciences that can improve the production of our foods, including mushrooms.

However, compared to other cultivated organisms there are very limited amounts of research into mushrooms. This has led to the whole scientific field lagging behind that of plants and animals (Sonnenberg et al., 2005). This also, consequently, makes the study and cultivation of fungi a field of possibilities. Furthermore, increasing amounts of research point to the health benefits of eating mushrooms. Just to mention a few of these properties: they are anti-carcinogenic, anti-microbial, anti-viral, anti-inflammatory, good for diabetics and can lower blood pressure and strengthen your immune system (Chang, 1999; Fan et al., 2006; Gregori et al., 2007; Patel and Goyal, 2012; Rop et al., 2009). They also contain many essential minerals and vitamins (Chang, 1999; Fan et al., 2006; Kumari and Achal, 2008; Mattila et al., 2001). Only in recent years have scientists and common people started to realize the possible value of mushrooms as a healthy, sustainable and environmentally friendly food source that can maximize food production by recycling and utilizing waste products (Amuneke et al., 2011; Fan et al., 2006; Gregori et al., 2007; Khan et al., 2013; Sánchez, 2010; Thawthong et al., 2014). A cautionary note should be made, as mushrooms are known to accumulate heavy metals, sometimes to levels unfit for consumption (Baldrian, 2003; Cotter, 2004; Favero et al., 1990; Mattila et al., 2001). The amounts accumulated is correlated to the concentrations found in the substrates (Baldrian, 2003; Favero et al., 1990). Therefore, it is important to test the levels of heavy metals found in the harvested mushrooms when testing a new type of substrate.

Furthermore, mushroom production can generate equitable economic growth (Chang, 1999; Wakchaure, 2011). This impact has already been noted, both at national and regional levels, and it is expected to increase and expand in the future. This use of the conversion of agricultural and forest wastes into food in the form of edible mushrooms has been named the “non-green revolution” (Chang, 1999).

Improving the knowledge of mushroom physiology is crucial for the continued development and understanding of how to use and grow mushrooms.

BACKGROUND

First an overview of the features of *P. ostreatus* will be made. Due to the limited amount of research into fungi some references included in this review refer to close relatives of *P. ostreatus*. This is followed by an examination of cultivation techniques and possible optimisation of these.

Pleurotus is a genus in the phylum of Basidiomycota in the Fungi kingdom. It grows quickly and can be produced on many different substrates with success (Amuneke et al., 2011; Ashraf et al., 2013; Cotter, 2004; Gregori et al., 2007; Kumari and Achal, 2008; Kurt and Buyukalaca, 2010). Their fruiting bodies are gilled, often with wide flattened caps and most cultivated strains grow in clusters providing easy harvesting (Cotter, 2004). The genus can be found in many places around the world and contains a large amount of edible mushrooms of which the tree Oyster mushroom (*P. ostreatus)* is one of the most well-known. *P. ostreatus* belongs in more temperate climates where it is found growing on hardwood trees (as seen in Picture 1), branches and logs (Amuneke et al., 2011; Cotter, 2004). Depending on the specific strain it can fruit at temperatures from 7 to 27 ºC (Cotter, 2004).

Picture 1: *P. ostreatus* in natural habitat on hardwood trunk. CC0 from www.pixabay.com

The Pink oyster mushroom (*P. djamor and P. flabellatus)* should also be mentioned as it is an exceptionally fast growing mushroom, adapted to warm climates with fruiting conditions of 27-38 ºC (Cotter, 2004). Consequently, it is a great species for tropical climates. The fact that it is also known to be aggressive and able to outcompete molds, makes it a good option for earning extra money without having to make big investments on equipment for cultivation (Cotter, 2004).

All fungi have the ability to produce a varying range of enzymes. Some higher forms of mushrooms, such as *Pleurotus*, can produce a great array of sophisticated enzymes(Cotter,

2004; Knop et al., 2015; Koutrotsios et al., 2014). Another interesting fact is that many of the enzymes are extracellular, secreted into the environment; in this way the fungus start to "digest" its food before it actually reaches it (Cotter, 2004; Sánchez, 2009). As a type of white-rot fungus, *Pleurotus*, has the ability to degrade lignin. White-rot fungi are the only organisms able to do so (Baldrian, 2003; Deacon, 2006). As a result, the white-rot fungi are absolutely essential to the recycling of carbon in the biosphere (Diyarova, 2016). The important enzymes for this process are quite few: lignin peroxidase, manganese peroxidase, H_2O_2-generating enzymes and laccase (Deacon, 2006; Hatakka, 1994). These special qualities of the white-rot fungi make them highly relevant for research using waste products for both mushroom production as a food source, medicine, fodder, and as a tool in biotechnology, creating biofuel or bioremediation (mycoremediation) of contaminated land (Amuneke et al., 2011; Cotter, 2004; Kumari and Achal, 2008; Obodai et al., 2003).

Life Cycle

The natural life cycle of *P. ostreatus* consist of sporulation, spore germination grows into hyphae (primary mycelia), mycelial colonization and possible mating, primordia formation and fruiting, ending with spore release (Cotter, 2004; Deacon, 2006). In Picture 2 examples of primordia and fruiting bodies of *P. ostreatus* can be seen.

Picture 2: Author's own pictures of home-growing using Svamphuset (Svamphuset, 2016). A) Fresh primordia sprayed with water B) Subsequent fruiting bodies

Hyphal maturity in *P. pulmonarius* has been studied by Sánchez et al. (2004) They observed an increased storage of glucans, resulting in thickened cell walls. The mature hyphae were measured to be almost twice as thick as the young hyphae.

The use of enzymes varies depending on the life phase of white-rot mushrooms. For example during the mycelial growth stage of *Lentinula edodes* (Shiitake) the laccase transcripts reached their peak and then declined when going into fruiting stage (Ohga and Royse, 2001). During fruit body development, cellulase transcription peaked instead.

Ostreolysin, a aegerolysin protein, was found to be present in *P. ostreatus* during fruiting phases, but absent during vegetative mycelial growth (Berne et al., 2007). In their study, Berne et al. found that the addition of purified Ostreolysin to a solid nutrient medium strongly stimulated primordial formation and fruiting. Mycelial growth was, however, somewhat inhibited.

Nutrients

Many nutrients are essential for fungi, at least in trace amounts. Here we will focus on a few particularly significant ones. As for most organisms the easiest way is the preferred way (Deacon, 2006). The simplest sources of carbon, such as monosaccharides, will be the first to be broken down thanks to the low energy cost of doing so. *Pleurotus* has specialised in breaking down the most complex carbon substrate: Lignin. This does not necessarily imply that it is preferred.

The only source of nitrogen that all fungi can use is amino acids (Deacon, 2006). For *P.ostreatus,* Mikeš et al. (1994) found L-glutamate and L-asparatate to be the best nitrogen sources. Supplementary, in media, ammonium is the most easily utilised and thus the preferred source of nitrogen (Deacon, 2006; Manu-Tawiah and Martin, 1988; Mikeš et al., 1994). Considering the simple molecular form, it is a good choice for a chemically defined medium. Nevertheless, it may cause pH problems in a culture medium as H^+ is released in exchange during uptake. A decreasing pH can swiftly inhibit further growth (Deacon, 2006). Ibekwe et al. (2008) found a pH of 6.4 to give optimum mycelium growth for *P. ostreatus* and a pH of 5 would inhibit growth. When lime was tested as an additive to substrates made from cotton wastes a 2 % addition, resulting in a pH of 7.8 gave the quickest and largest yields, closely followed by 0 % added lime with pH 7.2 (Khan et al., 2013). These results hint that *P. ostreatus* thrives at pH around neutral. In a comparison of nitrogen sources on peat extract media and a synthetic (defined) media the preferred sources of *P. ostreatus* were found to differ (Manu-Tawiah and Martin, 1988). On the peat extract media, yeast extract and ammonium phosphate were most successful, whereas, on the synthetic media ammonium citrate resulted in the highest biomass production. This, further implies that both pH, the source of carbon and possibly other

components in a substrate may play a role in how the uptake of nitrogen is regulated by *P. ostreatus* (Manu-Tawiah and Martin, 1988). Concerning the amounts of nitrogen available in a substrate it has been found to increase mycelial production only to a certain extent, where-after an increase in crude protein within the mycelia was observed (Manu-Tawiah and Martin, 1988; Srivastava and Bano, 1970). Nitrate is disfavoured, as a nitrogen source, due to it being harder to utilise (Deacon, 2006). Both potassium nitrate and ammonium nitrate have been found to result in the lowest yields of *P. ostreatus* mycelium compared to a range of other nitrogen sources in broth and synthetic media (Manu-Tawiah and Martin, 1988; Mikeš et al., 1994). Two enzymes, nitrate reductase and nitrite reductase, must be produced and used to transform it into the useful form of ammonium. As a control for efficiency the uptake of ammonium prevents the synthesis of membrane proteins and enzymes for utilization of other nitrogen sources (Deacon, 2006).

Specialising in wood decay through breaking down cellulose and lignin means that white-rot fungi have to be particularly efficient in their use of nitrogen (Deacon, 2006). Wood, as a source of energy, from common broad-leaved trees has exceptionally high C:N ratios of about 300:1 to 1000:1. In one study, 1:40 was been found to give optimum growth of *P. ostreatus* grown in broth media (Manu-Tawiah and Martin, 1988). Most living organisms need around 30:1 for good growth (Barron, 2003). This high efficiency is to a great extent achieved by specialised recycling and allocation of nitrogen within the mycelium for essential cell function (Deacon, 2006).

Many of the decaying fungi, including *P. ostreatus,* viewed mainly as saprophytes (living on dead organic matter) have been discovered to also have a parasitic phase in their life cycle. This makes them facultative parasites. The close relative *Hohenbuehelia* ssp. have specialized in parasitism or even predation with sticky mycelia-noose traps for nematodes, which become their only source of biological nitrogen (Barron, 2003). The discovery of this relationship led to the research of how *P. ostreatus* acquires additional nitrogen. Barron and Thorn (1987) tested five species of *Pleurotus,* and found that they also exploit nematodes as a nutrient source. They do however use a non-specific toxin and due to this their host range is not yet fully known (Barron, 2003). The vegetative hyphae produces tiny droplets of a paralyzing toxin. Once paralyzed the victims were penetrated, their interiors are digested and the nutrients are transported through the hyphae to be stored or used at other points of growth.

Phosphorous uptake is one of the fungi's triumphs. The adaptations are numerous. The thin hyphae have optimized their surface area and reach into new areas of soil (Deacon, 2006). They can release enzymes for uptake of both organic and inorganic phosphates, meanwhile accumulating any excess in vacuoles. To a great extent these are the reasons why the mycorrhizal relationship is immensely valuable to many plant species.

Manganese (Mn) is essential for the production of manganese peroxidase, one of the important ligninolytic enzymes. Additional Mn results in higher metabolic activity of the fungi (Kerem and Hadar, 1995). Kerem & Hadar (1993) observed that in general the degradation of lignin was enhanced by additions of manganese in substrates. However high concentrations could inhibit mycelial growth during the first two weeks of colonisation. With an addition of malonate the authors were able to reverse this effect.

There are signs that the ability of a fungal strain to break down complex compounds can be decreased by long term exposure to the same external factors, such as available energy sources (Cotter, 2004). This weakening effect is called strain senescence and is the result of continued production and use of an adapted set of enzymes. This could be a troubling fact for the scientists who generally keep their strains on simple media in laboratory conditions. More efficient and adaptable strains are achieved when varying the energy source slightly or through genetically crossing it with strains found in their natural habitat.

An example of using this adaptability is seen when "training" a strain of *Pleurotus ostreatus* for specific mycoremediation (Cotter, 2004). Cotter tried this by adding a strong fungicide to an agar growth medium. The mycelium stopped its growth after two days, getting dense and fluffy along its borders. After eight days the mycelium managed to adapt its enzyme production and successfully colonised the whole plate breaking down a presumably previously unencountered chemical compound.

A study by Laaksovirta and Alakuijala (1978) points toward higher accumulation of metals and minerals in decomposing fungi compared to mycorrhizal fungi. This could be due to mycorrhizal fungi getting a cleaner carbon source from the symbiotic tree. The ability of decomposers to break down and use everything from other organisms may create a type of biomagnification. This can be used as a type of mycoremediation called hyperaccumulation where the heavy metals are absorbed by the mushroom (Cotter, 2004). Here, the Fungi cannot break down the problematic molecules or compounds, which is otherwise common for

mycoremediation. Instead it filters its polluted substrate, retains the pollution and in turn becomes toxic itself.

MUSHROOM CULTIVATION

The first proof of cultivation of mushrooms was not found until around 600 CE in China (Cumo, 2015). Here, mushrooms became cultivated on tree logs. From this simple start it is now estimated that more than 2000 edible species exist (Melo de Carvalho et al., 2010) and of these 130 have been successfully cultivated by humans (Thawthong et al., 2014)

The production and consumption of edible mushrooms (and truffles) have been rising increasingly in the last two decades, as seen in Figure 1. So far, it has doubled every 10 years from 5 million tonnes 2003 to 10 million tonnes 2013 (FAOSTAT, 2016).

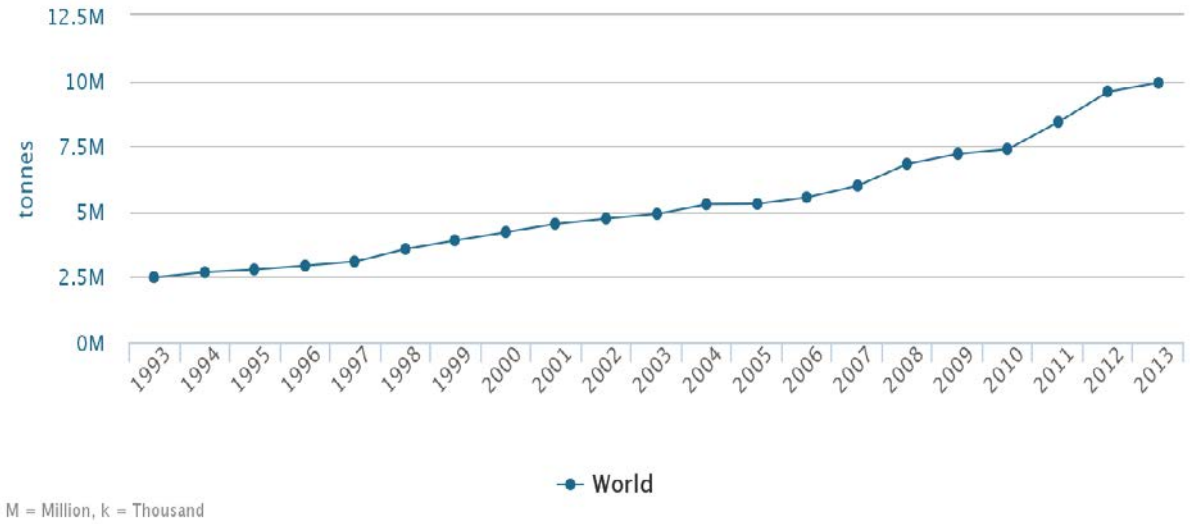


Figure 1: The increase in the production (million tonnes) of mushrooms and truffles in the world during the period from 1993-2013 (FAO, 2016).

This rise could be related to the increasing world population, which in term increases the demand for good sources of protein. This makes mushrooms an interesting alternative to meat, since it is both fast growing and can be produced on different types of waste products. *P. ostreatus* is even rated higher in protein compared to other commercial mushrooms or even other subspecies of *Pleurotus* (Ashraf et al., 2013; Cotter, 2004). It should nonetheless be noted that mushrooms consist mainly of water. Around 90 % to be more exact (Manzi et al., 1999; Morais et al., 2000; Sánchez, 2004). Ashraf et al. (2013) observed moisture contents for *P. ostreatus* to be about 86 % and about 27 % of the remaining dry matter was protein.

It is estimated that 70 % of agricultural and silvicultural materials are non-productive and go to waste (Chang, 1999). The fact that some of these can be used for food production is increasing the sustainability of mushrooms production. Additionally, the mushroom can actually make the

waste reusable after production due to its ability to break down earlier unusable lignocellulosic molecules. Several applications of Spent Mushroom Substrate (SMS), such as soil enrichment, nutritious fodder and biogas production, are being evaluated (Dashtban et al., 2009; Díaz-Godínez and Sánchez, 2002; Sánchez, 2009). An example is that *P. ostreatus* made maize straw more nutritious and digestible as a fodder after mushroom cultivation (Díaz-Godínez and Sánchez, 2002).

A weakness mentioned by several authors is that the typical mushroom producer is very devoted to growing mushrooms, but they are in general not very good at marketing their products (Cotter, 2004; Wakchaure, 2011). It is also observed by Wakchaure (2011) that a short shelf life reduces mushrooms success on the market. Nonetheless, Wakchaure has also noted a strong trend shift from canned and dried mushrooms to fresh ones. The preserved mushroom market has a continued but slow growth, whereas the market for fresh mushrooms seems to be rocketing towards the same level and beyond.

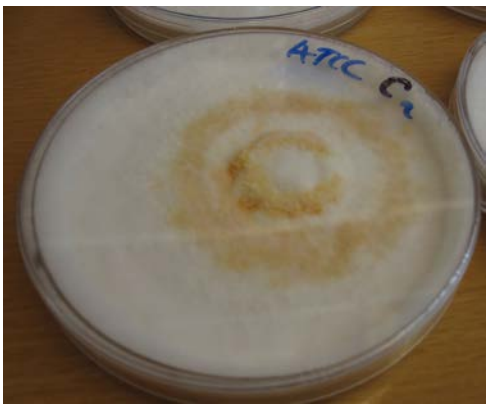
A possible limitation can be the risk of importing a foreign species. In New Zealand for example import of *P. ostreatus* is prevented since it is deemed to pose a risk to their forest industry (Hall, 2010).

SPAWN

The mycelium initially used as an original culture to inoculate a substrate or medium is called spawn. The commercially used spawn can be bought on cereal grains, supplemented sawdust or wooden dowels (Cotter, 2004). For scientific research the original spawn culture is often kept on Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA).

The creation and isolation of a spawn culture is often made from spore germination, but also cloning through tissue samples is used. The mycelium should be white and linear (Cotter, 2004) and must be uninfected and smell nice. The perception of the smell can be different therefore the imprecise adjective “nice” is used, examples could be: sweet, nutty, umami, anise-like or even the fresh humid kind of smell in a forest just after a rainfall. It is of great importance to keep good hygiene when dealing with mycelium in a production system. Bad hygiene can become time consuming and costly in terms of production loss.

If spawn is becoming over-colonized a yellow metabolite (liquid/gel) is exuded (Cotter, 2004). This same type of metabolite can be seen on fruiting substrates. Yellow metabolites were observed in the present study as seen in Picture 3.



Picture 3: Yellow droplets on strain ATCC on control medium

The amount of spawn added to a substrate should be at least 2 % of the total weight (Mycelia BVBA, n.d.). For *P. cornucopiae* close to 10 % improvements in Biological Efficiency (BE)(See formula on next page) was achieved when doubling the amount of spawn from 2,5% to 5% (Royse et al., 2004).

GROWING CONDITIONS

Several environmental factors should be controlled when growing oyster mushrooms. Most important are temperature, humidity, light, gas exchange and last but not least the substrate (Cotter, 2004; Melo de Carvalho et al., 2010; Sánchez, 2010). At different stages of the life cycle there are different needs in terms of environmental conditions (Cotter, 2004; Hall, 2010). Optimizing the conditions can almost become an art form; however, mushroom cultivation can generally succeed without following all of the technicalities.

First, the substrate must be prepared, by creating a good substrate specific particle size and thorough pasteurization. Patel and Trivedi (2015) points out that the substrate must be able to hold the water tightly so that the air, which is needed for the mushrooms metabolism, can fill the spaces. This is only possible if the substrate is not packed too tightly. For fast colonization the water content of the substrate should be around 60-70% depending on the substrate (Amuneke et al., 2011; Ashraf et al., 2013; Kumari and Achal, 2008) and the room can be kept dark and at a temperature of 20-25 ºC (all temperatures should be strain specific). Substrate temperature should not reach above 30 ºC (Mycelia BVBA, n.d.). After complete colonization, conditions are changed in order to induce pinning (fruit body initiation). This can be done by decreasing the temperature to around 6-15 °C, increasing the humidity to 80-95% and addition of light (800-1500 lux)(Ashraf et al., 2013; Mycelia BVBA, n.d.). Due to the gas exchange of the mushroom the CO_2 concentration should be kept below 1000 ppm, otherwise deformations of fruit bodies and stem elongation can be the result. As fruiting occurs the humidity can be decreased to 85% to minimize rot. On a good substrate 2-3 flushes (induced fruiting and harvest) can be achieved.

SUBSTRATE

In many of the studies on different substrates for cultivation of *P. ostreatus* comparisons are made of different substrates and new materials for making substrates, such as various waste products (Amuneke et al., 2011; Chitamba et al., 2012; Fu et al., 1997; Gregori et al., 2007; Kerem and Hadar, 1995; Khan et al., 2013; Kumari and Achal, 2008; Kurt and Buyukalaca, 2010; Melo de Carvalho et al., 2010; Membrillo et al., 2008; Mikiashvili et al., 2006; Obodai et al., 2003; Shah et al., 2004). Surprisingly many of these studies do not specify the strain of *P. ostreatus* used and most often only one strain is examined (Amuneke et al., 2011; Ashraf et al., 2013; Chitamba et al., 2012; Khan et al., 2013; Kumari and Achal, 2008; Shah et al., 2004). Therefore, we must bear in mind that there could be differences in how well different strains grow on the same substrate. This uncertainty makes it hard to fairly compare the results of these studies. Much more research is needed to clarify the differences of growth among the strains of the *P. ostreatus* species. Presently we will examine the results, from scientific studies, with the assumption that they are as unbiased by this as possible.

In order to compare the results of many studies of substrates we must understand how they evaluate. Apart from simply valuing fast production or number of flushes they evaluate the efficiency of the substrate for mushroom production. The formula most commonly used is the BE (Melo de Carvalho et al., 2010). It is expressed as percentage and calculated accordingly:

$$BE = \frac{Fresh\ weight\ of\ biomass\ produced}{Dry\ weight\ of\ initial\ substrate} \times 100$$

The most common substrates used are wheat straw and sawdust (Gregori et al., 2007; Melo de Carvalho et al., 2010; Obodai et al., 2003). Many other types of substrates have been suggested and tested, for instance: Rice straw, sorghum, corn straw, banana waste, cotton waste, peanut sheels, weeds, palm leaves, sugarcane bargasse, paper waste, coffee husks and spent coffee grounds (Amuneke et al., 2011; Chitamba et al., 2012; Fan et al., 2003; Gregori et al., 2007; Melo de Carvalho et al., 2010; Obodai et al., 2003; Patel and Goyal, 2012; Sánchez, 2010; Thawthong et al., 2014).

In a study testing different substrates the highest mycelial growth was recorded on rice husk and fresh sawdust, the thickness, however, was described as poor on these substrates (Obodai et al., 2003). Compared to a substrate with composted sawdust mycelial growth was quicker, thinner and less dense. Additionally the composted sawdust had higher yields and supported three flushes compared to only one by the fresh sawdust. The results therefore indicate that

there is no correlation of fast mycelial growth to higher yields. The authors therefore concluded that requirements for growth probably were different from requirements for yield of fungi (Obodai et al., 2003).

In Pakistan, Ashraf et al. (2013) found that cotton waste was more successful compared to rice paddy straw and wheat straw. All of these substrates had been fermented for 4 days previous to pasteurisation and inoculation. It was also concluded that when adding nitrogen during spawn run the process becomes accelerated. In another study from Pakistan by Shah et al. (2004) the best results in yield and fruiting were recorded for sawdust as a substrate (compared to wheat straw and leaves).

Royse (2002) observed up to 2,6 times higher yields and higher BE of *P. connucopiae* on substrate supplemented with low amounts (up to 6 %) of a commercial supplement (Campbell's S-41 nutrient). The commercial supplemental nutrients were produced for *Agaricus bisporus* and therefore a nutritional supplement should still be developed for *Pleurotus* specifically. In a later study by the same author the same supplement was tested further (Royse et al., 2004). An addition from 0% to 4,5 % nutrient added to the substrate resulted in an increase in BE of almost 25%. A doubling from 4,5 % to 9 %, however ,only increased BE with just over 2 %.

Baysal et al. (2003) cultivated *P. ostreatus* on paper waste with additions of chicken manure, peat and rice husks. Increasing the proportions of peat and chicken manure had a negative effect on growth whereas rice husks accelerated overall growth.

High porosity and other features can make certain substrates dry out very fast, which make them either difficult or unsuitable for production (Obodai et al., 2003). The opposite can also happen with soft substrates that easily packs too tightly providing no space for air (Patel and Trivedi, 2015). Both of these problems can often be avoided or dealt with by mixing in other additives possessing the opposite trait (Obodai et al., 2003; Patel and Trivedi, 2015).

Furthermore, if the first trials are not successful on a new substrate it may be possible to "train" the specific strain to certain new energy sources as mentioned earlier. However it is common to mix successful substrates with a little of a less successful one to maximize the use of wastes with good BE results (Obodai et al., 2003; Patel and Trivedi, 2015). A third option is through genetics. *Pleurotus* is a heterothallic fungus, which means that sexual reproduction of spores requires two compatible individuals. An early study by Roxon & Jong (1977) found that there were differences in growth and fruiting of various haploid strains of *P. sajor-caju.* From their

results when mating different strains they concluded that the possibility of developing more efficient strains was very good. Jaswal et al. (2013) points out that more research is needed to fully understand and succeed with a breeding programme. Some desirable traits are sporeless strains, resilience to disease and climatic changes and possibly optimization of BE on specific types of substrate (Fan et al., 2006; Jaswal et al., 2013; Obodai et al., 2003).

As mentioned in the introduction, caution must be taken concerning the choice of substrates for mushrooms. Toxic compounds, such as arsenic (As), cadmium (Cd) and lead (Pb), can be accumulated in the mushrooms and are harmful even when consumed at low levels (Demirbaş, 2001; Favero et al., 1990; Laaksovirta and Alakuijala, 1978; Mattila et al., 2001). In a study performed in Turkey wild *P. ostreatus* contained on an average 1.39±0.40 mg/kg dry weight As and 3.52±1.15 mg/kg dry weight Pb (Demirbaş, 2001). In another study from Hungary the arsenic concentration levels were found to be lower than a detection limit of 0.05 mg/kg dry weight (Vetter, 2004). According to the findings of Laaksovirta and Alakuijala (1978), in Finland, the lead and cadmium concentrations in wild mushrooms were correlated with the density of traffic. As a conclusion the authors suggest that fungi growing close to roads should be advised against as a food source. Much has happened since 1978 in terms of limiting pollution, but action should, nonetheless, be taken to avoid contamination of substrates when producing mushrooms. Even though this risk of accumulation is of importance to health, it was according to Demirbas (2001) not yet known in 2001, how the accumulation physically or chemically happens. For *P. ostreatus* production Favero et al. (1990) found a correlation of levels of Cd in the substrate and levels of Cd subsequently measured in fruiting bodies produced on the substrate. The resulting concentrations of Cd reached levels above the recommended limits for weekly intake, therefore, the authors urges for alertness when using agroindustrial wastes. In a trace element comparison between commercially cultivated *P. ostreatus, L.edodes* and *A. bisporus* in Finland *P.ostreatus* had the lowest trace amounts of Pb and lower amounts of Cd compared to *L.edodes (Mattila et al., 2001).* These trace amounts were defined as harmless, since they were well below the limitations set by FAO/WHO.

OBJECTIVES

This project will aim to investigate and provide a tool for understanding how various nutrients play a role in cultivation of oyster mushroom.

A project, performed in cooperation between SLU, Ecofungi and Trelleborg Municipality, has been started with the aim to explore the possibility of cultivating mushrooms in a waste product,

biomass, from a wetland in the South of Sweden (Hultberg, 2016). A better understanding of what the oyster mushroom needs nutritionally will help further research.

The main question throughout this project is: how could Oyster mushrooms production be improved through optimization of substrates?

In the laboratory experiments the aim is to develop a chemically defined medium, focusing on testing different sources of nitrogen, for further research on oyster mushrooms. To make a chemically defined medium, all of the components must be known in their exact concentrations (Hogg, 2005). Therefore, an aim was set to create a successful growing medium without tryptone, peptone or yeast extract and similar substances that are otherwise commonly found in experiments with fungi. Yeast extract seems like the most common ingredient in growth media, also, giving good growth in other a previous study (Manu-Tawiah and Martin, 1988). Therefore, it was included as a comparison in the first experiment. It is also one of the base ingredients of the control medium (Appendix 1). Additionally, nitrate, ammonium and the amino acid asparagine were tested. With the use of these media, tests of various nitrogen sources will be made on three different strains of *P. ostreatus.* Results will be derived by evaluating the mycelial growth and thickness.

Limitations

Due to limitations in time and facilities solely mycelial growth will be examined. Further research should be continued to see if the nitrogen sources have similar effects during primordia formation and fruiting.

Materials and Methods

Literature Search

Several books on the subject were used to give basic knowledge of fungi. Using search engines, such as Google Scholar and Web of Science, a literary review of current research was made. Another tool was the “snowball effect” from using the reference lists from related research articles.

Experiments

During the experiments the growth of three different strains of *P. ostreatus* mycelia were tested on different media. The hypothesis was that there would be significant differences in growth between the media, but no significant difference between the mycelia strains. In the first

experiment three different media and a control medium were compared. These aimed to find out how well highly defined media (D1 and D2) would work compared to a more complex medium (D3). The different media were named Defined 1-3 (D1-3). The recipe to the chemically defined media were based on a chemically defined medium for liquid fungal cultures by Jim Deacon (2006). For the second experiment the focus was on the nitrogen source and mixes of different sources of nitrogen. Therefore, the media were named Nitrogen 1-10 (N1-10). The control was the same for both experiments and was a yeast and malt extract (YM) medium previously known to give successful growth for the *P. ostreatus* mycelia. It follows a recipe by ATCC (2012). Lists of ingredients for all media can be seen in Appendix 1.

SPAWN

All original mycelia was cultured and maintained on a Potato Dextrose Agar (PDA) until inoculation. Three different strains were used:

Pleurotus ostreatus strain ATCC® 44309™ from ATCC, USA. Recommended temperature for growth is 24 °C (American Type Culture Collection, 2012).

Pleurotus ostreatus strain M 2191 from Mycelia, Belgium (Mycelia BVBA, n.d.). Recommended temperature for incubation is 20-22 °C. This strain is the most common of the three in commercial production systems.

Pleurotus ostreatus strain M 2140 from Mycelia, Belgium. Recommended temperature for incubation is 20-22 °C. This strain does not produce spores.

METHOD

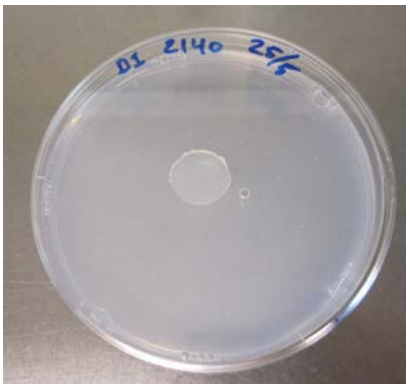
All chemicals used in the media are shown in Appendix 1. To attain the same amount of moles of nitrogen (0,024 mol of N) for all nitrogen sources as in 2 g of sodium nitrate further calculations were made (see Appendix 2).

First, stock solutions were made for the minerals and vitamins. For $FeSO_4$, $ZnSO_4$ and $CuSO_4$ 0,75g of each were mixed with 100 ml distilled water. The concentration was thus 7,5 mg/ml and 0,25 ml was needed for each medium. The Thiamine was dissolved in water to a concentration of 0,1 mg/ml. The Biotin was dissolved with 20 ml ethanol and 160 ml distilled water to a concentration of 0,05 mg/ml. Both were stored in 1ml amounts in Eppendorf tubes, which were frozen for later use.

A few adjustments in amounts were made for experiment 1 (E1) and can be seen under its subheading below. Next, 250 ml of distilled water was added to the flasks and the contents were

swirled to make the ingredients mix. Then 0,25 ml of the mineral solution was added and the contents were swirled again. Subsequently the flasks were autoclaved (CertoClav Sterilizer GmbH. A-4050 Traun/Austria) for 20 min at 120 ºC. All work with open Petri plates, ready media and mycelia was done in a laminar flow cabinet. When the media had cooled to about 60 ºC thawed biotin and thiamine was added with a pipette (250 µl and 50 µl respectively from the stock solutions). Thereafter, the media were poured onto plates and left to set overnight.

The following day the mycelia inoculum was to be added. An apple corer was used as a tool to make round 15 mm slices from the agar with mycelium. The slaps were placed, mycelium side down, at the centre of the petri plates with the different experimental media. An example is shown in Picture 4. For E1 the spawn had been started 4 weeks before inoculation and was left at room temperature for two weeks to grow and then stored the last two weeks in a refrigerator. After inoculation the plates were closed with parafilm.



Picture 4 Petri plate with D1 just inoculated with strain 2140.

For experiment 2 (E2) the inoculum from the same batch was first used, but due to infections of the inoculum for 2140 and ATCC, the experiment had to be restarted. The strain 2191 was examined and kept as an extra replicate and will be referred to as Experiment 3 (E3). New inoculum had to be prepared and the mycelia were left to grow on PA in a warm chamber (25-27 ºC) for 10 days. New media were produced and the new inoculum was added.

All plates were moved to a dark and warm growth chamber keeping the temperature between 25-27 ºC. Here, the mycelial growth was measured every day from day 2-8 (E1) or every second day from 3-9 (E2 and E3). The measurement of growth was done with a ruler showing the amount of mm that the mycelium had grown from the inoculation slab. To achieve persistency, the longest distance of the radius was always measured. This, however, means that mycelium with irregular growth could show the same results as mycelium with a regular circular growing pattern. Furthermore, very thin silk-thread-like mycelium can reach the same growth distances as fluffy (thicker) mycelium. Therefore, at least one more measurement was needed to depict how “well” the mycelium was actually growing. A classification of thickness was produced and based on rating the thickness by eye assessment. The thickness ratings assigned were: 1: bad, 2: poor, 3: good and 4: great (Figure 2).

EXPERIMENT 1

Three media were tested and compared to a control medium (ingredients can be found in Appendix 1,Table 5). Three replicates were made for each medium and strain of *P. ostreatus.* Thereby, 9 Petri plates were examined per medium, 36 plates in total. All defined media contained 0,024 moles (24 mM) of nitrogen via different sources; these are stated in Table 1.

Table 1: The three media D1-3 and their different sources of nitrogen.

Defined 1 (D1)	Defined 2 (D2)	Defined 3 (D3)
$NaNO_3$	Asparagine	Yeast extract

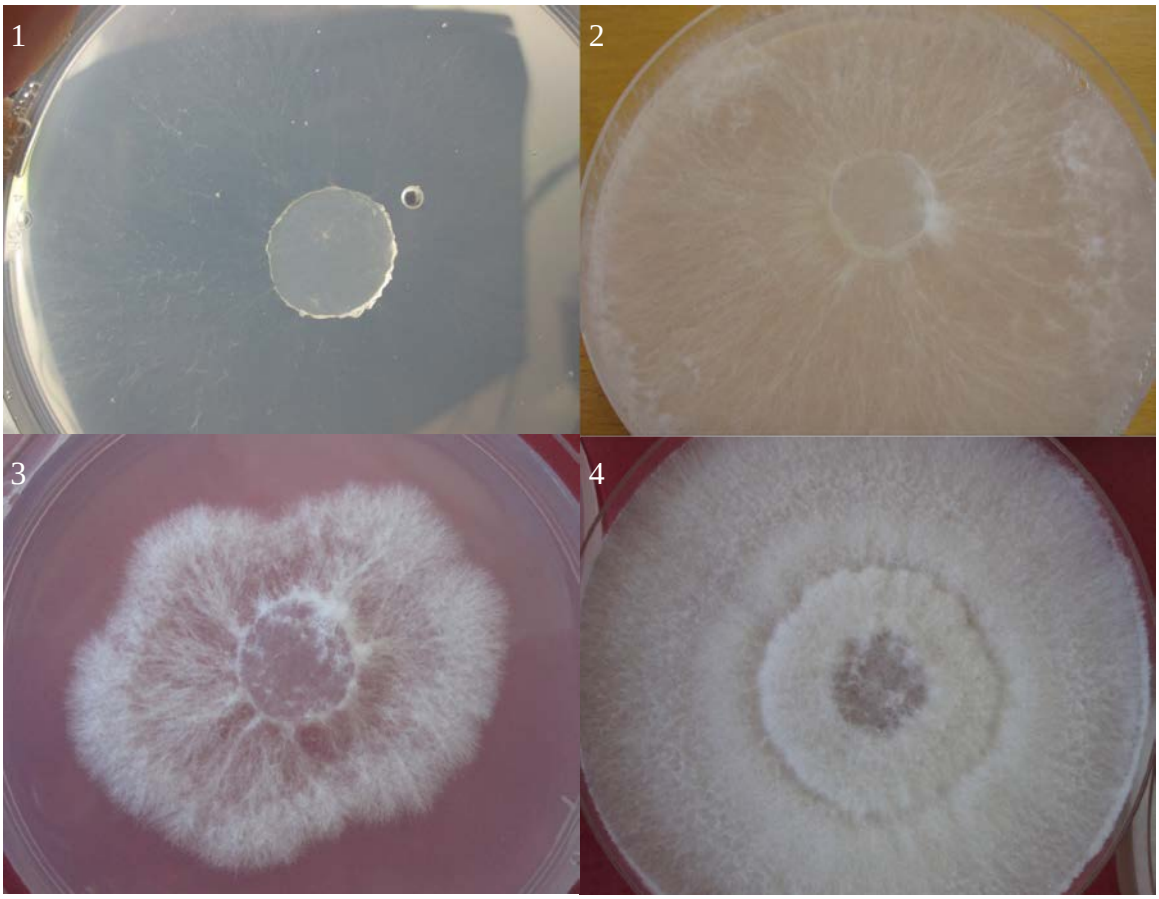


Figure 2 Pictures of mycelia with the thickness rating from 1-4.

Yeast extract contains 163,3 mg carbohydrates/g, so 3,43g contain 560 mg of carbohydrates. To adjust the amount of carbon available to the fungi only 19,5g of glucose was added to medium 3.The amount of carbon added through using asparagine in medium 2 accounts for less than 0,01g/l. Therefore no adjustments were made.

EXPERIMENT 2 AND 3

Ten defined media and a control were tested. Three replicates were made for each medium and strain of *P. ostreatus.* Thereby, a total of 99 Petri dishes were examined. The media (N1-10) were given different nitrogen sources in various mixes. An attempt was made to make a type of stairway of the mixes with proportions that would all in total provide 24 mM of nitrogen to each plate. Three types of mixes of nitrogen sources were made: 3:1, 1:1 and 1:3. Only ammonium nitrate was tested at as a single source, since it was not included previously in E1. The combinations of nitrogen sources in media N1-10 can be seen in Table 2. Calculations into amounts in grams can be seen Appendix 2 Table 6. The proportions of respective chemical source of nitrogen in the form of NH_4-N, NO_3-N and Asparagine-N can be seen in Table 3.

Table 2: Media N1-10 and the proportions of respective nitrogen sources.

N1	N2	N3	N4	N5
1 NH_4NO_3	3:1 NH_4NO_3:$NaNO_3$	1:1 NH_4NO_3:$NaNO_3$	1:3 NH_4NO_3:$NaNO_3$	1:3 NH_4NO_3/Asp
N6	**N7**	**N8**	**N9**	**N10**
1:1 NH_4NO_3:Asp	3:1 NH_4NO_3:Asp	3:1 Asp:$NaNO_3$	1:1 Asp:$NaNO_3$	1:3 Asp:$NaNO_3$

Table 3: Media N1-10 and their proportions of nitrogen from NH_4, NO_3 and Asparagine

Media	NH_4-N : NO_3-N : Asparagine-N
N1	1 : 1 : 0
N2	3 : 5 : 0
N3	1 : 3 : 0
N4	1 : 7 : 0
N5	1 : 1 : 6
N6	1 : 1 : 2
N7	3 : 3 : 1
N8	0 : 1 : 3
N9	0 : 1 : 1
N10	0 : 3 : 1

THE CONTROL

The control medium is based on a basic recipe (Appendix 1) used by the SLU laboratory. The nitrogen content was only calculated after the experiments and therefore differs from the amounts in the defined media. Bacto Malt extract contains 0,3% nitrogen, which means 3 g contain 0,009 g. Yeast extract contains 9,8% giving a total of 0,294 g in 3 g. Peptone contains 15,4 % giving a total of 0,77 g in 5 g. All in all this adds up to the fact that the control medium contains roughly calculated three times the amount of nitrogen compared to the test media.

Consequently, there are greater amounts of nitrogen and it is found in different molecular forms in the control medium.

STATISTICAL ANALYSIS

If any infections were discovered these values were marked in the tables (Appendices 3 and 4) with colours showing the type of infection (Bacteria, bacteria + mold and mold). These values were removed from further calculations. A marking using borders was used to show when the mycelium reached the edge of the petri plates. Examples of this can be seen in the raw data in Appendix 3, 4 and 5. Also means, standard deviations and graphs were produced in Excel.

In Minitab more extensive calculations were made. Analysis of Variance (ANOVA) was used to test for overall significance of data. The values were checked for outliers and none were found. The Dunnett method was used to achieve an oversight of all data from the media compared to the control and Tukey's test was employed to show significant differences between means recorded on media of one strain of *P. ostreatus* at a time. Both methods tested for significance at a level of $P<0,05$. As we have two parameters, growth and thickness, both must be taken into account. The ratings of thickness were however not as easy to use as quantified values and will therefore only be referred to as means in relation to significant differences in growth.

RESULTS

In order to simplify the inclusion of the thickness ratings we will refer to the ratings as the numbers 1, 2, 3 and 4 (as seen in Figure 2). These groups are based on the means seen in Table 3 and 4. In E1 the D1 medium is the only medium only scoring 1 as the mycelium never developed any fluffiness. A rating of 2 is seen in E2 due to very little fluffiness. D2 is rated 3 and D3 and the control both reach a 4.

Table 4: Mean values of thickness ratings for Experiment 1 on media ND1-3 and Control (C)

Mycelium	Medium	Day2	Day3	Day4	Day5	Day6	Day7	Day8
2140	D1	1,00	1,00	1,00	1,00	1,00	1,00	1,00
2191	D1	1,00	1,00	1,00	1,00	1,00	1,00	1,00
ATCC	D1	1,00	1,00	1,00	1,00	1,00	1,00	1,00
2140	D2	1,00	1,00	2,00	2,00	2,00	2,00	2,00
2191	D2	1,33	1,67	2,00	2,00	2,00	2,50	3,00
ATCC	D2	2,00	2,00	2,00	2,00	2,00	2,00	3,00
2140	D3	2,00	3,00	3,00	3,00	3,00	4,00	4,00
2191	D3	2,00	3,00	3,00	3,00	3,00	4,00	4,00

ATCC	D3	2,00	3,00	3,00	3,00	3,00	4,00	4,00
2140	C	2,00	2,67	3,00	3,00	3,00	4,00	4,00
2191	C	2,00	3,00	3,00	3,00	3,00	4,00	4,00
ATCC	C	1,67	2,33	3,00	3,00	3,00	4,00	4,00

The radial growth observed in E1 are illustrated in Figure 3. For all three strains, D3 is just below the control in radial growth and both are rated among the best (4) in thickness. This is further supported by a Tukey test (Appendix 6), created for day 7, that group together Control (mean 37,063) and D3 (mean 36,375) as significantly higher than D2 (mean 25,81) and D1 (mean 24,778).

For D1 the thickness rating was 1. Only extremely thin strings of mycelium traced over the plates, an example of this can be seen in Figure 2 as the first photo (1).

The ATCC strain shows good growth results also for D2, but only reaches a rating of 3 in thickness. The thickness rating for ATCC on D2 did reach 2 early compared to the other strains, but only reached 3 on the 8th day. There was a clear difference in growth patterns for the three strains on D2, this is illustrated in Picture 5 (from day 8). All three replicates of 2140 had irregular growth patterns with both stretching and more cloud-like formations. 2191 grew rather slowly and made a clearly fluffier and denser border, but did not seem to stretch. A completely different growth was seen for ATCC as it grew and spread quickly like a thin mycelial

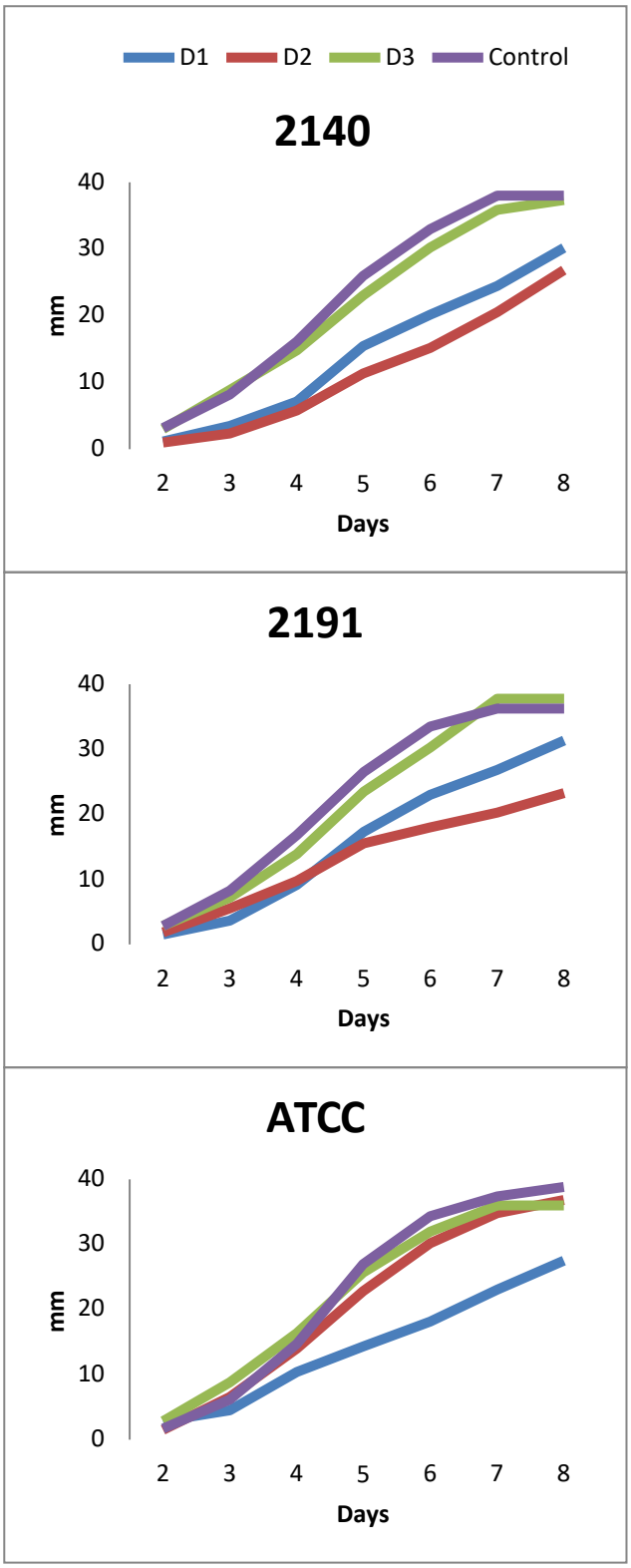


Figure 3: Radial growth of all strains separately on media D1-3 and control during the period of 2-8 days.

carpet to the edge of the petri plate. When the edge was reached it seems like ATCC started to grow more thickness.

Picture 5: All three strains on medium D2. From left to right 2140, 2191 and ATCC.

In E2 the thickness ratings will be referred to as follows: N3 was rated as 1. N1, N2, N4, N9 and N10 was rated 2. N5, N6, N7, and N8 reached a rating of 3. The control was the only media to have mycelium reach the rating of 4.

Table 5: Mean values of thickness ratings for Experiment 2 on media N1-10 (1-10) and Control (C)

Mycelium	Medium (N)	Day3	Day5	Day7	Day9
2140	1	2,00	2,00	2,00	3,00
2191	1	2,00	2,00	2,67	2,67
ATCC	1	2,00	2,00	2,67	2,67
2140	2	1,67	2,00	2,00	2,33
2191	2	2,00	2,00	2,67	2,67
ATCC	2	2,00	2,00	2,00	2,67
2140	3	1,00	1,00	1,00	1,00
2191	3	1,00	1,00	1,00	1,00
ATCC	3	1,00	1,00	1,00	1,00
2140	4	2,00	2,00	2,00	2,50
2191	4	2,00	2,00	2,33	2,00
ATCC	4	1,67	2,00	2,67	2,67
2140	5	2,00	3,00	3,00	3,00
2191	5	2,33	2,67	3,00	3,00
ATCC	5	2,00	3,00	3,00	3,00
2140	6	2,00	2,00	2,67	2,67
2191	6	2,00	2,00	3,00	3,00
ATCC	6	2,00	2,00	2,67	3,00
2140	7	2,00	2,00	3,00	3,00
2191	7	1,67	2,00	3,00	3,00
ATCC	7	2,00	2,00	2,67	3,00
2140	8	1,67	2,00	2,33	3,00
2191	8	2,00	2,00	3,00	3,00
ATCC	8	2,00	2,00	2,00	2,67
2140	9	1,67	2,00	2,67	2,67
2191	9	1,33	2,00	3,00	3,00
ATCC	9	1,67	2,00	2,33	2,67

2140	10	1,67	2,00	2,33	2,67
2191	10	1,00	2,00	3,00	3,00
ATCC	10	1,67	2,00	2,00	2,67
2140	C	3,00	4,00	4,00	4,00
2191	C	3,00	4,00	4,00	4,00
ATCC	C	3,00	4,00	4,00	4,00

To get an overview of the development of each separate strain on all media in E2, Figures 4 (strain 2140), 5 (strain 2191) and 6 (strain ATCC) were made. Here the means of growth are plotted for each day of measurement. As in E1 the control is most successful in both growth and thickness. N6 (50/50 NH_4NO_3/Asp) places second on growth for all strains and mostly reaches a rating of 3 in thickness. Example from day 9 is shown in Picture 6.

Picture 6: N6 on day 9. In the order 2140, 2191 and ATCC from above

For all graphs we can see that the growth decreases strongly for day 9. This is accounted for by the fact that the mycelium were reaching the border of the petri plates. Day 7 was chosen as a time for comparison as the mycelia were still growing radially or had only just reached the border. It should be noted that the control media shows a slight decrease in radial growth already on Day 7 and this is probably due to the mycelium reaching the border. Day 5 was too early as differences in whether each

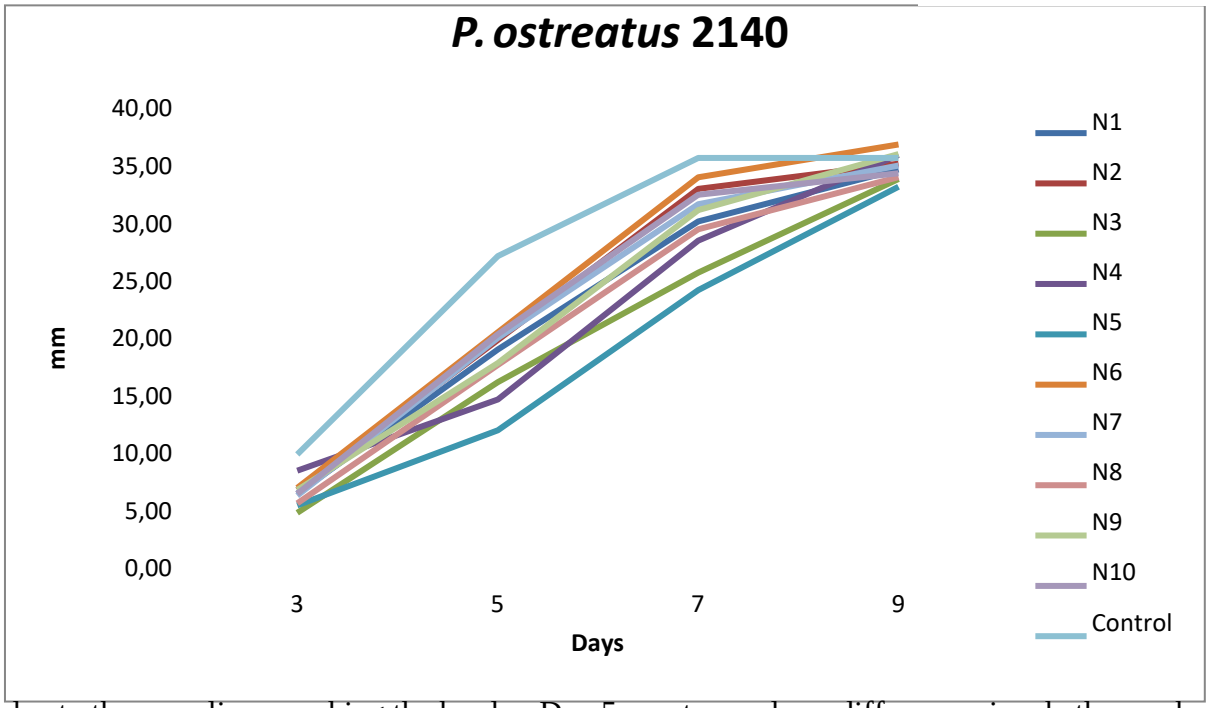


Figure 4: Mean growth (in mm) by the strain 2140 during the period from day 3-9 on media N1-10.

individual had a strong or weak start was still detectable. These differences cannot be explained since they varied for all strains and all media, however it could be due to levels of success when applying the inoculum, such as achieved level of contact with the medium, or the stage of development of the specific part of mycelium from the inoculum plates.

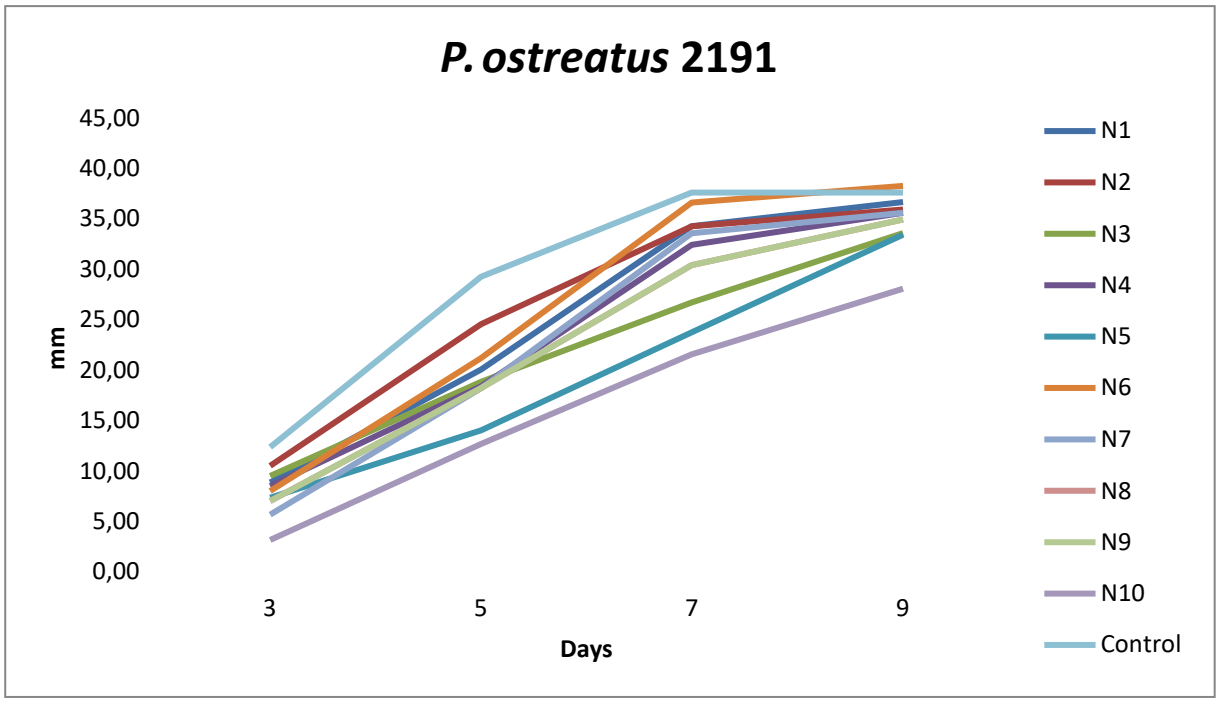


Figure 5: Mean growth (in mm) by the strain 2191 during the period from day 3-9 on media N1-10.

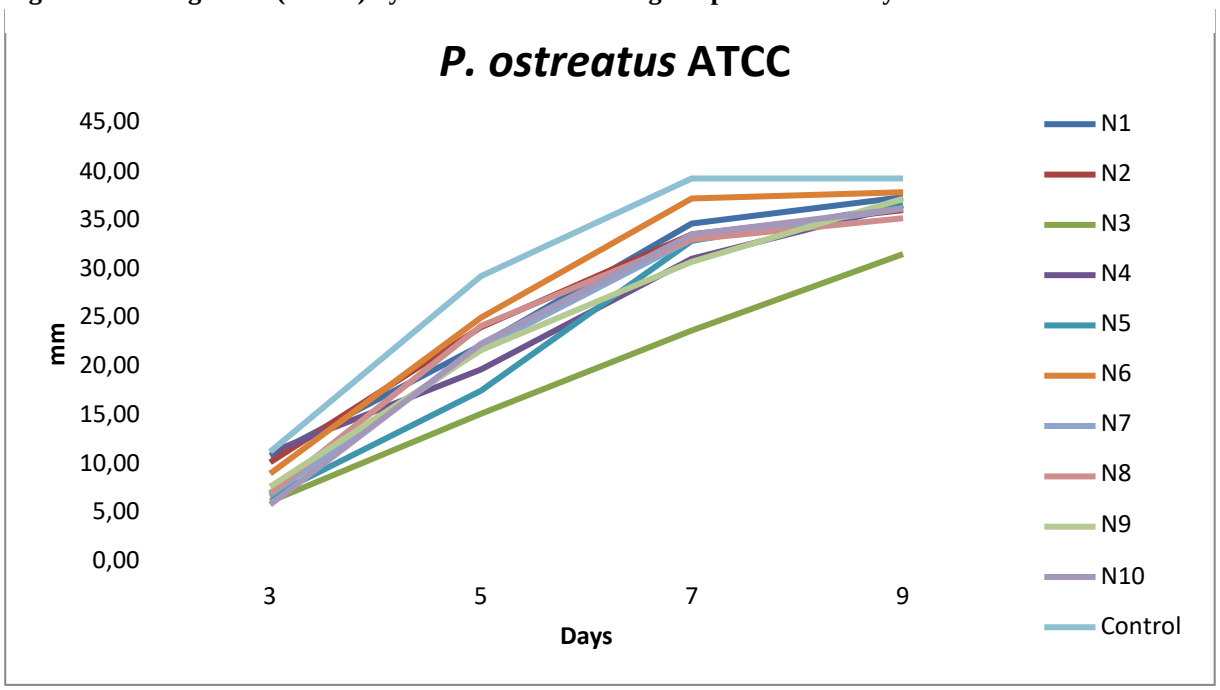


Figure 6: Mean growth (in mm) by the strain ATCC during the period from day 3-9 on media N1-10.

A test of variance (ANOVA) shows highly significant results for growth media ($p<0,0001$), a significant interaction of medium*mycelium ($p<0,0003$) and mycelium ($p<0,0036$). The details can be seen in Appendix 7. Secondly a Dunnett test was made to compare N1-10 to the control for the means of all strains per medium on day 7. The N6, N2, N7 and N1 were grouped with the control whereas the other media were significantly different from the control. This indicates that the four media grouped with the control were the ones that performed better for the parameter of growth. Of these four N1 and N2 performed poorly on the thickness rating whereas N6 and N7 are among the good (See Table 4).

We generally see a similar pattern on the graphs (Figure 4-6) as to that of the Dunnett test. However, significance was found in the ANOVA test and some differences clearly exist in the graphs considering the best versus the worst results for each strain. Therefore separate Tukey's tests were made for each strain. Details can be seen in Appendix 7.

Tukey's test for strain 2140 only shows medium N3 and N5 as significantly lower than the rest. In the results for strain 2191, N3, N9, N5 and N10 are significantly lower. For strain ATCC only N3 is significantly lower. A few interesting similarities and differences are worth being mentioned. The means of N6 rates second after the control for all strains. The strains grew significantly different on medium N10. By using the Tukey method the strains 2140 (mean 33,33) and ATCC (mean 33,50) were grouped together and the 2191 strain (mean 21,67) was significantly lower. For strain 2140 and ATCC results for N10 were above average, whereas for strain 2191 N10 gave the worst result. Interestingly, in table 4, we can see that the mean thickness rating for 2191 was higher.

Figure 7 illustrates the results of the means from day 7. The means of each strain are shown with standard deviation for each medium. We again see differences among the strains. Especially on N5, N9 and N10. In general the standard deviations for N4, N9 and N10 are greater. This makes these results more uncertain.

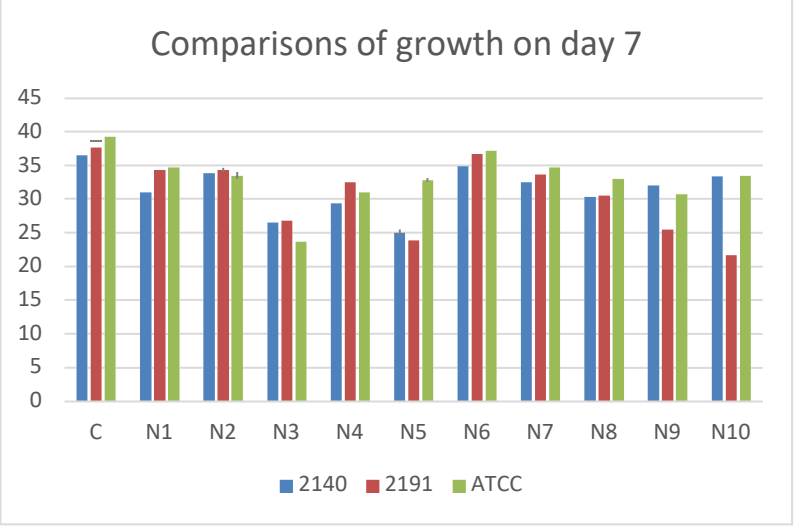
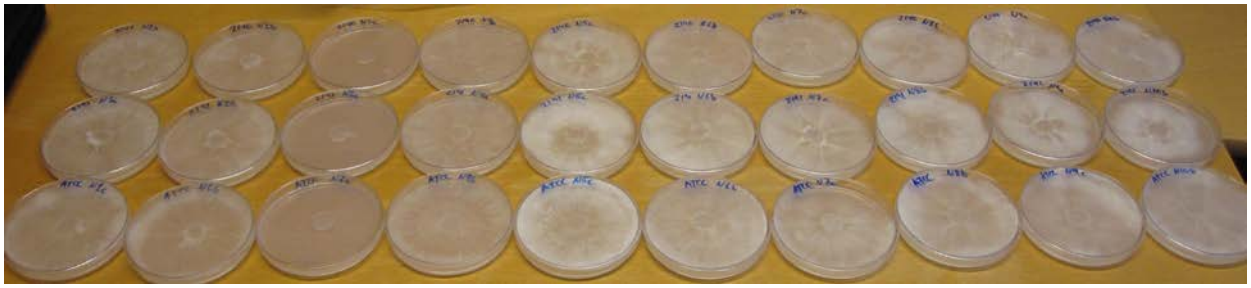


Figure 7 The mean growth reached (mm) on day 7 for all three strains of mycelia on the Control (C) and N1-10 media. Error bars are included illustrating the standard deviation.

As for E1, somewhat different patterns of growth were noted among the strains. It seems that strain 2191 often got slightly denser, at least on N5-10. This and other differences can be seen in Picture 7. ATCC generally seemed to be slightly faster, but mostly stayed as a thin carpet, rating 2 and 3 in thickness. The 2140 seemed to have a more stable growth, but slightly slower. It only just reached around and above 20 mm on day 5 for all test media. The two other strains had reached mostly above and on some media even closer to 25 mm.



Picture 7: Strains in order 2140, 2191 and ATCC from above. Media from left to right in the order N1-10.

The E3 became a replicate of the results of the 2191 strain in E2. A comparison is seen in Figure 8. Mostly the results are similar, even though the age of the inoculating mycelium was different (only 10 days for E2 over a month for E3). One important difference is seen in N6 where the E3 results are among the worst and are significantly lower than the results in E2 where it was the best.

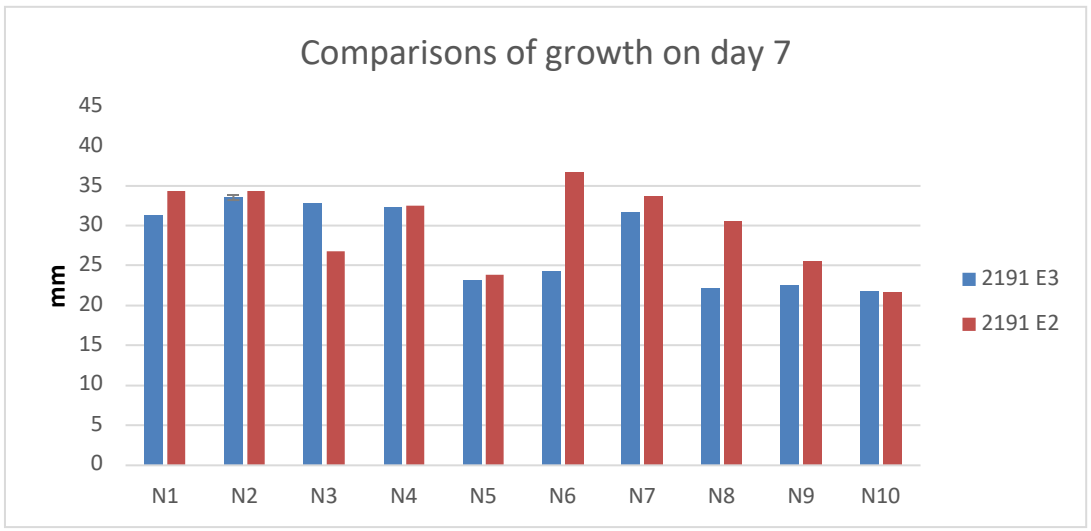


Figure 8: The mean growth reached (in cm) on day 7 for the 2191 strain in experiments 1 and 2. Error bars are included illustrating the standard deviation.

DISCUSSION

The present literature review of previous scientific studies has established that many types of substrate can be used for production of *P. ostreatus.* In most cases the best substrates are waste products from other types of production, be it agricultural or silvicultural. This makes the production of *P. ostreatus* a potential sustainable crop, both in terms of economy and environment.

The requirements of *P. ostreatus* are generally modest. However the right conditions of growth and fruiting are of course important for optimisation. Apart from this it is hard to pinpoint exactly what works and why. Generally, an optimized substrate should have the right consistency, neither too dense nor too porous (Obodai et al., 2003; Patel and Trivedi, 2015). Additions of certain nutrients will enhance the growth, but in high amounts this effect is reversed. Manganese in a combination with malonate would be interesting to add in future studies.

The experimental part of this study shows that defined media can be used for mycelial growth. Another result is that radial mycelial growth and thickness differ largely due to the available nitrogen source. Further, Manu-Tawiah and Martin (1988) argues that the preference and use of nitrogen source may to some extent be depend on what sources of carbon are available. This would have been another interesting aspect to test. Also, in hindsight, it would have been relevant to measure the pH of the media, as the uptake of nitrogen could have caused a drop in pH, resulting in stunted growth. Both high and low pH can inhibit growth (Ibekwe et al., 2008; Khan et al., 2013). A correlation between radial growth and mycelial thickness was observed. Differences between the used strains were generally low, but some interesting exceptions were noted. Differences in strains can indicate that they have slightly different requirements. This suggests that optimal substrates could be developed for each specific strain.

Strain variation is not uncommon and occurs in other studies as well (Sastre-Ahuatzi et al., 2007; Téllez-Téllez et al., 2005). An example is when five strains of *P. ostreatus* were tested for sensitivity to 2-deoxyglucose, a toxic compound similar to glucose (Sastre-Ahuatzi et al., 2007). Here, Sastre-Ahuatzi *et al* (2007) found significant differences between some of the strains concerning growth and laccase production respectively. Téllez-Téllez et al. (2005) concluded that growth patterns were similar between strains of the same species, but found significant differences between all strains in their study. This calls for a requirement of specifying which strain, not just species, is being used in all studies.

If a fungal strain does not perform well on a new substrate one can look at the properties of the substrate and attempt to improve them as seen in studies by Obodai et al. (2003) and Patel & Trivedi (2015). A second approach would be to "train" the mycelia, as done by Cotter (2004), in order to adapt it and thereby achieve better results. A third approach can be to look into the genetics of the specific strain and try to improve it as suggested by Jaswal et al. (2013) and Roxon & Jong (1977).

Overall, in this study, the control was observed to give the best growth in E1 and E2. Maybe this could have been predicted, as the control media contained 3 times more nitrogen than the treatments, and in varied forms. However, the results were far from 3 times better, even though, as mentioned earlier, the last days of growth may have been limited by the border of the plates. Also, it correlates with earlier results where the nitrogen concentration only increases growth to a certain level, after which it causes an increase in crude protein within the mycelia (Manu-Tawiah and Martin, 1988; Srivastava and Bano, 1970). In E1 nitrate, the amino acid asparagine and yeast extract was compared. Yeast extract was observed to be favourable among these three. Asparagine was more favourable compared to nitrate. This suggests that *P. ostreatus* thrive on complex food sources and organic nitrogen may be preferred over mineral nitrogen. In E2, ammonium was also included as a nitrogen source, moreover, nitrate and asparagine were used again. This experiment showed that a mixture of asparagine and ammonium was generally preferred over the mixtures with nitrate. This correlates with previous studies where amino acids, L-glutamate and L-aspartate, yeast extract and ammonium resulted in the best growth and nitrates resulted in the lowest growth (Manu-Tawiah and Martin, 1988; Mikeš et al., 1994).

The interesting difference observed in E1 on medium D2 is puzzling. It seems that ATCC in general grows faster and has no problem adapting to any of the media. The growth pattern observed for strain 2191 reminds of the growth pattern explained by Cotter (2004) as he trained a strain of *P. ostreatus* for mycoremediation. The pattern is that the growth stops and the mycelia thicken along the outer border. It would have been interesting to follow these plates for longer to see if the strain would start to grow quickly again.

The contradicting differences observed for N6 in E2 and E3 are very hard to explain. It could be due to the age difference of the inoculating mycelia, since mycelia kept on the same medium for longer periods adapt to this and therefore may have a slower response on a new medium (Cotter, 2004). However, this would be more likely if bigger differences were observed and more replicates had been made to strengthen the results.

To get a better understanding of the effect of these different nitrogen sources a further study following the *P. ostreatus* through all its life phases, from mycelia to fruiting bodies, should be made. This could also lead to a further conclusion on the effect of thickness versus radial growth. The connection of the thickness to quality of yield was made in a previous study (Obodai et al., 2003). Also the additional thickness of mature hyphae observed in *P. pulmonarius* (Sánchez et al., 2004) strengthens the argument that the thickness is as important as the radial growth for a successful total growth of the fungi.

Additional research could look into the addition of Ostreolysin at the end of colonisation of a substrate. Maybe this could maximise the fruiting and yield (Berne et al., 2007). Could this be added as a spray to initiate fruiting and avoid the inhibition of growth if added at an earlier stage?

Due to the ability of mushrooms to accumulate potentially toxic substances (Cotter, 2004; Demirbaş, 2001; Laaksovirta and Alakuijala, 1978; Vetter, 2004) more studies and thorough trials should be done on possible new substrates, such as the wetland biomass, in order to ensure that no toxins will be found it the final product.

DIFFICULTIES AND SUGGESTED IMPROVEMENTS

At times it was difficult to find literature dealing with exactly what was searched for. Often either the focus or the species was slightly different than what was wished for. In the future, additional research can improve this and slowly the gaps of knowledge will hopefully be filled.

Deacon advises to use a chemically defined liquid medium in order to avoid possible impurities of the agar when attempting to determine the minimum nutrient requirements of a fungus (2006). We were, however, not trying to find a minimum but rather to optimise. It is still noteworthy since creating a low baseline of nutrients would make it easier to explore at which amounts the fungi truly thrives by using a stairway model. That is “making steps” with continuously increasing amounts of a specific nutrient. In this study we only explored different sources of nitrogen, it would have been interesting to further explore at which amounts of nitrogen the growth would be more efficient. When is the amount too small for the fungus to succeed and at what levels will the amounts become toxic?

The measurements of radial growth of this study were done by hand using a ruler and the thickness ratings were done on what was visually noticeable. This makes both measurements quite crude. Using a smart image-processing programme could be possible. An example of this

is seen in a study where staining was also used (Sánchez et al., 2004). It is, however, important that the calibration of both size and sensitivity is made correctly. An open source programme such as ImageJ should be able to handle this (ImageJ, 2012). Calibrating and using the ImageJ program for leaf area measurements and cell counts with area measurements seem to be two good techniques (Reinking, L., 2007). They could possibly work for mycelial growth area measurements as well. In this programme it is possible to calibrate by using a known length on the image, such as the diameter of a petri plate, then use a segmentation tool to select the mycelia on the image where after the program calculates the area. To simplify the work we recommend using a dark background and a camera with high resolution to take pictures directly from above.

Several of the replicates were removed due to infections. More replicates of each strain on each medium would make the results more certain and thereby also strengthen the significances of the results. Additional runs/replicates of the experiments should be done to improve the certainties of the results and further avoid uncertainties such as the case of differences for N6 in E2 and E3.

CONCLUSIONS

In conclusion, it is shown that a chemically defined medium can be used for studies of *P. ostreatus*. The radial mycelial growth and thickness was observed to significantly differ depending on the available nitrogen source for three strains of *P. ostreatus*.

The control, containing yeast extract, gave the overall best results for both radial growth and mycelial thickness. It had faster radial growth, producing a fluffier mycelial mat, than the test media. For the test media a general pattern was observed where mixtures of ammonium and the amino acid asparagine were preferred over mixtures including nitrate for all three strains.

Some strain differences were observed and from this, one may conclude that the requirements of the specific strains differ. This illuminates the importance of clearly presenting the strains that are used in future studies. Furthermore, it indicates that it is possible to create the substrate according to strain specific needs. An alternative solution could be to “train”/adapt the specific strain for a specific substrate.

Further studies are needed for an improved understanding of the species and its needs.

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APPENDIX 1

Table 6. A table of the ingredients of the control and test media Defined (D) 1-3 and Nitrogen (N) 1-10.

Control	Test media - D1-3 and N1-10
Yeast extract 3.0 g	Nitrogen source 0,024 moles of N equal to amount in $NaNO_3$ 2 g
Malt extract 3.0 g	KH_2PO_4 1 g
Dextrose 10.0 g	$MgSO_4$ 0,5 g
Peptone 5.0 g	KCL 0,5 g
Agar 20.0 g	$CaCl_2$ 0,5 g
DI water 1000 ml	$FeSO_4$, $ZnSO_4$, $CuSO_4$ 0,0075g
	Glucose 20,0 g
	Agar 20,0 g
	DI water 1000 ml
	Biotin 10 µg
	Thiamine 100 µg

APPENDIX 2

NITROGEN CALCULATIONS

Nitrogen weighs approximately 14 g/mole

Weight in moles:

$NaNO_3$ = 85 g/mole

Asparagine ($C_4H_8N_2O_3$)= 132,1 g/mole

NH_4NO_3 = 80 g/mole

In 2 g of $NaNO_3$ there is therefore 2/85 ≈ 0,024 moles of N. The weight of the N is then: 14 g/mol × 0,024 mol = 0,336 g

In both Asparagine and NH_4NO_3 there are 2 N in each molecule and therefore half the amount of moles are needed to achieve the same amount of N. 0,024/2 = 0,012 moles.

Amount of Asparagine needed: 0,012 mol × 132,1 g/mol = 1,6 g

Amount of NH_4NO_3 needed: 0,012 mol × 80 g/mol = 0,96 g

The Yeast extract (Ducheta, Batch 040032) contains approximately 9,8% Nitrogen. Therefore we can calculate: x × 0,098 = 0,336 g

x = 3,43 g of yeast extract.

Table 7 Shows the media D1-3 and their different sources of nitrogen.

Defined 1 (D1)	Defined 2 (D2)	Defined 3 (D3)
$NaNO_3$ 2 g	Asparagine 1,6 g	Yeast extract 3,43 g

Table 8 Shows the media N1-10 and their Nitrogen sources with proportions and the equivalent weight in grams of each.

N1	N2	N3	N4	N5
NH_4NO_3 0,96 g	75/25 NH_4NO_3/$NaNO_3$ 0,72 g/0,51 g	50/50 NH_4NO_3/$NaNO_3$ 0,48 g/1,02 g	25/75 NH_4NO_3/$NaNO_3$ 0,24 g/1,53 g	25/75 NH_4NO_3/Asp 0,24 g/1,189 g
N6	**N7**	**N8**	**N9**	**N10**
50/50 NH_4NO_3/Asp 0,48 g/0,793 g	75/25 NH_4NO_3/Asp 0,72 g/0,396 g	75/25 Asp/$NaNO_3$ 1,184 g/0,51 g	50/50 Asp/$NaNO_3$ 0,739 g/1,02 g	25/75 Asp/$NaNO_3$ 0,396 g/1,53 g

APPENDIX 3

Table 9 Raw data of growth in mm, with mean values and standard deviation, from Experiment 1.

								36,5
								37
								37
2140	a	2	7,5	12	19,5	25,5	34,5	39
	b	4	10	16,5	25,5	32	36	36
	c	3	9	16	24	33	37	37
	mean	3,00	8,83	14,83	23,00	30,17	35,83	37,33
	St. deviation	1,00	1,26	2,47	3,12	4,07	1,26	1,53
2191	a	2,5	7	12,5	21,5	30	36	36

	b	3	7,5	14	23,5	28,5	37	37
	c	3	7	15	25,5	32	38,5	38,5
	mean	2,83	7,17	13,83	23,50	30,25	37,75	37,75
	St. deviation	0,29	0,29	1,26	2,00	2,47	1,06	1,06
ATCC	a	1,5	7	14	22,5	29,5	35	35
	b	3	8,5	16	24	32	36	36
	c	4	11	19	30,5	34,5	37	37
	mean	2,83	8,83	16,33	25,67	32,00	36,00	36,00
	St. deviation	1,26	2,02	2,52	4,25	2,50	1,00	1,00
Mycelia Control	Day	2	3	4	5	6	7	8
2140	a	2,5	6,5	14	24,5	31,5	36	36
	b	3,5	9	17,5	25,5	34	37	37
	c	3,5	9	17	26,5	32	39	39
	mean	3,17	8,17	16,17	26,00	33,00	38,00	38,00
	St. deviation	0,58	1,44	1,89	0,71	1,41	1,41	1,41
2191	a	2,5	8	17,5	27	34,5	37	37
	b	4	8,5	16	26	32,5	35,5	35,5
	c	2	7,5	13,5	23	25	25	26
	mean	2,83	8,25	16,75	26,50	33,50	36,25	36,25
	St. deviation	1,04	0,35	1,06	0,71	1,41	1,06	1,06
ATCC	a	2	7	16	29,5	38	38	38
	b	1,5	6,5	15	28	35,5	39	39
	c	1,5	5	13	23,5	29,5	35	39,5
	mean	1,66666 6667	6,16666 6667	14,6666 6667	27	34,3333 3333	37,3333 3333	38,8333 3333
	St. deviation	0,28867 5135	1,04083 3	1,52752 5232	3,12249 8999	4,36844 7474	2,08166 5999	0,76376 2616

Table 10 Raw data of thickness with mean values from Experiment 1.

Thickness on D1	Day	2	3	4	5	6	7	8
2140	a	1	1	1	1	1	1	1
	b	1	1	1	1	1	1	1
	c	1	1	1	1	1	1	1
	mean	1,00	1,00	1,00	1,00	1,00	1,00	1,00
2191	a	1	1	1	1	1	1	1
	b	1	1	1	1	1	1	1
	c	1	1	1	1	1	1	1
	mean	1,00	1,00	1,00	1,00	1,00	1,00	1,00
ATCC	a	1	1	1	1	1	1	1
	b	1	1	1	1	1	1	1
	c	1	1	1	1	1	1	1
	mean	1,00	1,00	1,00	1,00	1,00	1,00	1,00
Thickness on D2	Day	2	3	4	5	6	7	8
2140	a	1	1	2	2	2	2	2
	b	1	1	2	2	2	2	2

	c	1	1	2	2	2	2	2
	mean	1,00	1,00	2,00	2,00	2,00	2,00	2,00
2191	a	1	1	2	2	2	2	3
	b	2	2	2	2	2	2	3
	c	1	2	2	2	2	2	3
	mean	1,33	1,67	2,00	2,00	2,00	2,00	3,00
ATCC	a	2	2	2	2	2	2	3
	b	2	2	2	2	2	2	3
	c	2	2	2	2	2	2	3
	mean	2,00	2,00	2,00	2,00	2,00	2,00	3,00
Thickness on D3	Day	2	3	4	5	6	7	8
2140	a	2	3	3	3	3	4	4
	b	2	3	3	3	3	4	4
	c	2	3	3	3	3	4	4
	mean	2,00	3,00	3,00	3,00	3,00	4,00	4,00
2191	a	2	3	3	3	3	4	4
	b	2	3	3	3	3	4	4
	c	2	3	3	3	3	4	4
	mean	2,00	3,00	3,00	3,00	3,00	4,00	4,00
ATCC	a	2	3	3	3	3	4	4
	b	2	3	3	3	3	4	4
	c	2	3	3	3	3	4	4
	mean	2,00	3,00	3,00	3,00	3,00	4,00	4,00
Thickness on Control	Day	2	3	4	5	6	7	8
2140	a	2	2	3	3	3	4	4
	b	2	3	3	3	3	4	4
	c	2	3	3	3	3	4	4
	mean	2,00	2,67	3,00	3,00	3,00	4,00	4,00
2191	a	2	3	3	3	3	4	4
	b	2	3	3	3	3	4	4
	c	2	3	3	3	3	3	3
	mean	2,00	3,00	3,00	3,00	3,00	3,67	3,67
ATCC	a	2	3	3	3	3	4	4
	b	2	2	3	3	3	4	4
	c	1	2	3	3	3	4	4
	mean	1,67	2,33	3,00	3,00	3,00	4,00	4,00

APPENDIX 4

Table 11 Raw data of growth in mm, with mean values and standard deviation, from Experiment 2.

Mycel E2 start 20/6		Bacteria	Mold	Bac + mold	Edge reached
Mycelia on N1	Day	3	5	7	9
2140	a	11	21,5	32,5	35
	b	8	17	29,5	35
	c	11,5	21	31	36,5
	mean	7,33	19,83	31,00	35,50
	St. deviation	1,61	2,47	1,50	0,87
2191	a	7	14	31,5	37,7
	b	9,5	22	35,5	37
	c	10,5	24,5	36	35,5
	mean	9,00	20,17	34,33	36,73
	St. deviation	1,80	5,48	2,47	1,12
ATCC	a	9,5	21	33	36
	b	11,5	22	36	39
	c	11,5	23,5	35	37
	mean	10,83	22,17	34,67	37,33
	St. deviation	1,15	1,26	1,53	1,53
Mycelia on N2	Day	3	5	7	9
2140	a	8,5	21,5	35	37
	b	8	21	33	35,5
	c	6,5	19,5	33,5	35,5
	mean	7,67	20,67	33,83	36,00
	St. deviation	1,04	1,04	1,04	0,87
2191	a	8	20	34	36,5
	b	10,5	25,5	34,5	36
	c	13,5	28,5	34,5	35,5
	mean	10,67	24,67	34,33	36,00
	St. deviation	2,75	4,31	0,29	0,50
ATCC	a	8	21,5	33	36
	b	10	25,5	33,5	36
	c	12,5	25	34	36
	mean	10,17	24,00	33,50	36,00
	St. deviation	2,25	2,18	0,50	0,00
Mycelia on N3	Day	3	5	7	9
2140	a	5,5	16,5	26	33
	b	6,5	18	29,5	37
	c	5	16,5	24	34
	mean	5,67	17,00	26,50	34,67

	St. deviation	0,76	0,87	2,78	2,08
2191	a	8	16,5	25	32,5
	b	13	21	28	34,5
	c	8	19,5	27,5	34
	mean	9,67	19,00	26,83	33,67
	St. deviation	2,89	2,29	1,61	1,04
ATCC	a	5,5	12,5	21	30,5
	b	6	16	25	33
	c	7	17	25	31
	mean	6,17	15,17	23,67	31,50
	St. deviation	0,76	2,36	2,31	1,32
Mycelia on N4	Day	3	5	7	9
2140	a	6,5	13	28	34,5
	b	8	15	28,5	35,5
	c	13,5	18,5	31,5	38
	mean	9,33	15,50	29,33	36,75
	St. deviation	3,69	2,78	1,89	1,77
2191	a	6	15	29	35
	b	11,5	22,5	34,5	37
	c	8,5	18	34	35
	mean	8,67	18,50	32,50	35,67
	St. deviation	2,75	3,77	3,04	1,15
ATCC	a	8,5	13,5	22	34
	b	11,5	21,5	35	
	c	13,5	24	36	37
		11,166666	19,666666		38,5
	mean	67	67	31	36,5
		2,5166114	5,4848275	7,810249	2,291287
	St. deviation	78	57	676	847
Mycelia on N5	Day	3	5	7	9
2140	a	6	11	25	34
	b	6,5	15	25,5	35,5
	c	6,5	12,5	24,5	32,5
		6,3333333	12,833333		
	mean	33	33	25	34
		0,2886751	2,0207259		
	St. deviation	35	42	0,5	1,5
2191	a	6	10,5	23,5	34
	b	8	15,5	22,5	32
	c	8,5	16,5	25,5	34,5
	mean	7,50	14,17	23,83	33,50
	St. deviation	1,32	3,21	1,53	1,32
ATCC	a	6	17	32,5	
	b	7,5	19	33	37
					36,5

	c	6,5	16,5	33	36
	mean	6,6666667	17,5	32,83333	36,5
	St. deviation	0,7637626	1,3228756	0,288675	0,5
Mycelia on N6	Day	3	5	7	9
2140	a	5,5	16,5	32	37
	b	9,5	24	36,5	37
	c	8,5	23,5	36	39
	mean	7,83	21,33	34,83	37,67
	St. deviation	2,08	4,19	2,47	1,15
2191	a	6	18	35	36
	b	8,5	23,5	34	36
	c	10	22,5	41	43
	mean	8,17	21,33	36,67	38,33
	St. deviation	2,02	2,93	3,79	4,04
ATCC	a	6,5	23	36,5	37
	b	10	25,5	38	38,5
	c	10,5	26,5	37	38
	mean	9,00	25,00	37,17	37,83
	St. deviation	2,18	1,80	0,76	0,76
Mycelia on N7	Day	3	5	7	9
2140	a	5,5	16	29	35,5
	b	8,5	24,5	33,5	35
	c	7,5	22	35	37
	mean	7,17	20,83	32,50	35,83
	St. deviation	1,53	4,37	3,12	1,04
2191	a	5	18	35	36,5
	b	8	20	34	35,5
	c	4,5	17	32	35
	mean	5,83	18,33	33,67	35,67
	St. deviation	1,89	1,53	1,53	0,76
ATCC	a	6	21,5	35	36
	b	8	23,5	35,5	36
	c	6,5	20,5	33,5	36,5
	mean	6,83	21,83	33,00	36,17
	St. deviation	1,04	1,53	1,80	0,29
Mycelia on N8	Day	3	5	7	9
2140	a	5	15	28,5	34
	b	7	19,5	29,5	35
	c	7,5	21	33	35,5
	mean	6,50	18,50	30,33	34,83
	St. deviation	1,32	3,12	2,36	0,76

2191	a	7	23	31,5	36
	b	8	16	29,5	34
	c	6,5	16	29	33,5
	mean	7,17	18,33	30,50	35,00
	St. deviation	0,76	4,04	1,41	1,41
ATCC	a	6	24,5	32,5	34
	b	8,5	25,5	35	35,5
	c	6,5	22,5	31,5	36
	mean	7,00	24,17	33,00	35,17
	St. deviation	1,32	1,53	1,80	1,04
Mycelia on N9	Day	3	5	7	9
2140	a	5,5	14	28,5	36
	b	8	20,5	33,5	36,5
	c	9,5	21,5	34	38
	mean	7,6666667	18,666667	32	36,83333
	St. deviation	2,0207259	4,0722639	3,041381	1,040833
2191	a	4	13,5	22	32
	b	6,5	15,5	26,5	33,5
	c	5	14,5	28	34
	mean	5,17	14,50	25,50	33,17
	St. deviation	1,26	1,00	3,12	1,04
ATCC	a	4	20	30,5	34
	b	7,5	23	37	40
	c	6	22	24,5	37,5
	mean	7,6666667	21,666667	30,66667	37,16667
	St. deviation	2,0207259	1,5275252	6,251666	3,013856
Mycelia on N10	Day	3	5	7	9
2140	a	5,5	17	30,5	
	b	8,5	24	35	35
	c	8	22,5	34,5	35,5
	mean	7,33	21,17	33,33	35,17
	St. deviation	1,61	3,69	2,47	0,29
2191	a	3	11	16	24,5
	b	3,5	12	22,5	26,5
	c	3,5	15,5	26,5	33,5
	mean	3,33	12,83	21,67	28,17
	St. deviation	0,29	2,36	5,30	4,73
ATCC	a	6	23	34,5	
	b	6,5	23	35	36
	c	5	21	31	37
					35,5

	mean	5,83	22,33	33,50	36,17
	St. deviation	0,76	1,15	2,18	0,76
Mycelia on C	Day	3	5	7	9
2140	a	10,5	26,5	36,5	36,5
	b	11	29,5	36,5	36,5
	c	15	26,5	37	
	mean	10,75	28,00	36,50	36,50
	St. deviation	0,35	2,12	0,00	0,00
2191	a	12,5	29	38	38
	b	11,5	28	38	38
	c	13,5	31	37	37
	mean	12,50	29,33	37,67	37,67
	St. deviation	1,00	1,53	0,58	0,58
ATCC	a	9	27	40	40
	b	13,5	31,5	38,5	38,5
	c	14	23	23	
	mean	11,25	29,25	39,25	39,25
	St. deviation	3,18	3,18	1,06	1,060660172

Table 12 Raw data of thickness with mean values from Experiment 2.

Thickness on N1	Day	3	5	7	9
2140	a	2	2	2	3
	b	2	2	2	3
	c	2	2	2	3
	mean	2	2	2	3
2191	a	2	2	3	3
	b	2	2	2	2
	c	2	2	3	3
	mean	2	2	2,666666667	2,666666667
ATCC	a	2	2	3	3
	b	2	2	3	3
	c	2	2	2	2
	mean	2	2	2,666666667	2,666666667
Thickness on N2	Day	3	5	7	9
2140	a	1	2	2	3
	b	2	2	2	2
	c	2	2	2	2
	mean	1,666666667	2	2	2,333333333
2191	a	2	2	3	3
	b	2	2	3	3
	c	2	2	2	2
	mean	2	2	2,666666667	2,666666667
ATCC	a	2	2	2	2
	b	2	2	2	3

	c	2	2	2	3
	mean	2	2	2	2,666666667
Thickness on N3	Day	3	5	7	9
2140	a	1	1	1	1
	b	1	1	1	1
	c	1	1	1	1
	mean	1	1	1	1
2191	a	1	1	1	1
	b	1	1	1	1
	c	1	1	1	1
	mean	1	1	1	1
ATCC	a	1	1	1	1
	b	1	1	1	1
	c	1	1	1	1
	mean	1	1	1	1
Thickness on N4	Day	3	5	7	9
2140	a	2	2	2	2
	b	2	2	2	2
	c	2	2	2	3
	mean	2	2	2	2,50
2191	a	2	2	2	2
	b	2	2	3	2
	c	2	2	2	2
	mean	2	2	2,333333333	2
ATCC	a	1	2	2	2
	b	2	2	3	3
	c	2	2	3	3
	mean	1,666666667	2	2,666666667	2,666666667
Thickness on N5	Day	3	5	7	9
2140	a	2	3	3	3
	b	2	3	3	3
	c	2	3	3	3
	mean	2	3	3	3
2191	a	2	3	3	3
	b	2	2	3	3
	c	3	3	3	3
	mean	2,333333333	2,666666667	3	3
ATCC	a	2	3	3	3
	b	2	3	3	3
	c	2	3	3	3
	mean	2	3	3	3
Thickness on N6	Day	3	5	7	9
2140	a	2	2	3	3
	b	2	2	2	2
	c	2	2	3	3
	mean	2	2	2,666666667	2,666666667

2191	a	2	2	3	3
	b	2	2	3	3
	c	2	2	3	3
	mean	2	2	3	3
ATCC	a	2	2	3	3
	b	2	2	2	3
	c	2	2	3	3
	mean	2	2	2,666666667	3
Thickness on N7	Day	3	5	7	9
2140	a	2	2	3	3
	b	2	2	3	3
	c	2	2	3	3
	mean	2	2	3	3
2191	a	1	2	3	3
	b	2	2	3	3
	c	2	2	3	3
	mean	1,666666667	2	3	3
ATCC	a	2	2	3	3
	b	2	2	2	3
	c	2	2	3	3
	mean	2	2	2,666666667	3
Thickness on N8	Day	3	5	7	9
2140	a	1	2	3	3
	b	2	2	2	3
	c	2	2	2	3
	mean	1,666666667	2	2,333333333	3
2191	a	2	2	3	3
	b	2	2	3	3
	c	2	2	3	3
	mean	2	2	3,00	3,00
ATCC	a	2	2	2	2
	b	2	2	2	3
	c	2	2	2	3
	mean	2	2	2	2,666666667
Thickness on N9	Day	3	5	7	9
2140	a	1	2	3	3
	b	2	2	3	3
	c	2	2	2	2
	mean	1,666666667	2	2,666666667	2,666666667
2191	a	1	2	3	3
	b	2	2	3	3
	c	1	2	3	3
	mean	1,333333333	2	3	3
ATCC	a	1	2	2	2
	b	2	2	2	3
	c	2	2	3	3

	mean	1,666666667	2	2,333333333	2,666666667
Thickness on N10	Day	3	5	7	9
2140	a	1	2	3	3
	b	2	2	2	2
	c	2	2	2	3
	mean	1,666666667	2	2,333333333	2,666666667
2191	a	1	2	3	3
	b	1	2	3	3
	c	1	2	3	3
	mean	1	2	3	3
ATCC	a	2	2	2	3
	b	2	2	2	3
	c	1	2	2	2
	mean	1,666666667	2	2	2,666666667
Thickness on C	Day	3	5	7	9
2140	a	3	4	4	4
	b	3	4	4	4
	c	3	4	4	4
	mean	3,00	4,00	4,00	4,00
2191	a	3	4	4	4
	b	3	4	4	4
	c	3	4	4	4
	mean	3	4	4	4
ATCC	a	3	4	4	4
	b	3	4	4	4
	c	3	3	4	4
	mean	3,00	4,00	4,00	4,00

APPENDIX 5

Only 2191, E3, start 4/6	Day	3	5	7	9	11
N1	a	9,5	19,5	31	36,5	36,5
	b	12	21	32	37	37
	c	8,5	16	31	36	36
	mean	10,00	18,83	31,33	36,50	36,50
	St. deviation	1,80	2,57	0,58	0,50	0,50
N2	a	5,5	19	33	36	36
	b	7	22	34	37	37
	c	9,5	22,5	33,5	36	36
	mean	7,33	21,17	33,50	36,33	36,33
	St. deviation	2,02	1,89	0,50	0,58	0,58
N3	a	9	20	32	37	37
	b	10	21,5	34	37,5	37,5
	c	8	20,5	32,5	37	37
	mean	9,00	20,67	32,83	37,17	37,17
	St. deviation	1,00	0,76	1,04	0,29	0,29
N4	a	8	19	32	36	36
	b	7	19	33,5	37	37
	c	8,5	19	31,5	39	39
	mean	7,83	19,00	32,33	37,33	37,33
	St. deviation	0,76	0,00	1,04	1,53	1,53
N5	a	2	11,5	25	34,5	37
	b	3,5	12	23	36	38
	c	2,5	9,5	21,5	35	36,5
	mean	2,67	11,00	23,17	35,17	37,17
	St. deviation	0,76	1,32	1,76	0,76	0,76
N6	a	3,5	11	23	34	36
	b	4	14,5	23	32,5	36
	c	3	12,5	26	36	37,5
	mean	3,50	12,67	24,00	34,17	36,50
	St. deviation	0,50	1,76	1,73	1,76	0,87
N7	a	10,5	23	34,5	38	38
	b	7,5	18	30,5	35	36
	c	7	18,5	30	35,5	36
	mean	8,33	19,83	31,67	36,17	36,67
	St. deviation	1,89	2,75	2,47	1,61	1,15
N8	a	1,5	11	20,5	35	35
	b	3	11,5	22	33,5	39
	c	3,5	12	24	37	38
	mean	2,67	11,50	22,17	35,17	37,33
	St. deviation	1,04	0,50	1,76	1,76	2,08
N9	a	3	12,5	20,5	35,5	36,5

	b	3	13,5	23	36,5	36,5
	c	2	11	24	32	35
	mean	2,67	12,33	22,50	34,67	36,00
	St. deviation	0,58	1,26	1,80	2,36	0,87
N10	a	2,5	10	22,5	26,5	35,5
	b	3	11,5	24	32	36
	c	3	10	19	20	34
	mean	2,83	10,50	21,83	26,17	35,17
	St. deviation	0,29	0,87	2,57	6,01	1,04

Thickness E3	Day	3	5	7	9	11
N1	a	1	2	2	2	2
	b	2	2	2	2	2
	c	2	2	2	2	2
	mean	1,666666667	2	2	2	2
N2	a	2	2	2	2	2
	b	2	2	2	2	2
	c	2	2	2	2	2
	mean	2	2	2	2	2
N3	a	2	2	2	2	2
	b	2	2	2	2	2
	c	2	2	2	2	2
	mean	2	2	2	2	2
N4	a	2	2	2	2	2
	b	2	2	2	2	2
	c	2	2	2	2	2
	mean	2	2	2	2	2
N5	a	1	2	2	2	3
	b	1	2	2	2	3
	c	2	2	2	2	3
	mean	1,333333333	2	2	2	3
N6	a	1	2	2	2	3
	b	1	2	2	2	3
	c	2	2	2	2	3
	mean	1,333333333	2	2	2	3
N7	a	2	2	2	2	3
	b	2	2	2	2	3
	c	2	3	2	2	3
	mean	2	2,333333333	2	2	2
N8	a	1	2	2	2	3
	b	1	2	2	2	3
	c	1	2	2	2	3
	mean	1	2	2	2	3
N9	a	1	2	2	2	3
	b	1	2	2	2	3

	c	1	2	2	2	2
	mean	1	2	2	2	2,666666667
N10	a	1	2	2	2	3
	b	1	2	2	2	3
	c	1	2	2	2	3
	mean	1	2	2	2	3

APPENDIX 6

Statistical tests from Minitab belonging to Experiment 2.

Tukey's test for all strains on all test media and control (0) on day 7:

```
Grouping Information Using the Tukey Method and 95% Confidence

Medium  N    Mean  Grouping
0       8  37,063  A
3       8  36,375  A
2       8   25,81    B
1       9  24,778    B

Means that do not share a letter are significantly different
```

APPENDIX 7

Statistical tests from Minitab belonging to Experiment 2

Two-way ANOVA of day 7 using mycelia (2140, 2191, ATCC) and medium (N1-10 and control = 0) as factors.

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Medium	10	1140,45	114,045	14,59	<0,0001
Mycelia	2	96,36	48,182	6,17	0,0036
Medium*Mycelia	20	488,58	24,429	3,13	0,0003
Error	63	492,29	7,814		
Total	95	2226,31			

Dunnett test for all strains on all media on day 7:

Dunnett Multiple Comparisons with a Control (0)

```
Grouping Information Using the Dunnett Method and 95% Confidence

Medium       N    Mean  Grouping
0 (control)  7  37,786  A
6            9  36,222  A
2            9  33,889  A
7            9  33,611  A
1            9  33,333  A
8            8  31,375
4            9   30,94
10           9   29,50
9            9   29,39
5            9   27,22
3            9  25,667

Means not labeled with the letter A are significantly different from the control level
mean.
```

Tukey's test for 2140 on all test media and control (0) on day 7

```
Grouping Information Using the Tukey Method and 95% Confidence

Medium  N    Mean  Grouping
0       2   36,50  A
6       3   34,83  A
2       3  33,833  A
10      3   33,33  A
7       3   32,50  A B
9       3   32,00  A B
1       3  31,000  A B C
8       3   30,33  A B C
```

```
4       3   29,33  A B C
3       3   26,50    B C
5       3  25,000      C

Means that do not share a letter are significantly different.
```

Tukey’s test for 2191 on all test media and control (0) on day 7

```
Grouping Information Using the Tukey Method and 95% Confidence

Medium  N    Mean  Grouping
0       3  37,667  A
6       3   36,67  A
2       3  34,333  A B
1       3   34,33  A B
7       3  33,667  A B
4       3   32,50  A B C
8       2   30,50  A B C D
3       3  26,833    B C D E
9       3   25,50      C D E
5       3  23,833        D E
10      3   21,67          E

Means that do not share a letter are significantly different.
```

Tukey’s test for ATCC on all test media and control (0) on day 7

```
Grouping Information Using the Tukey Method and 95% Confidence

Medium  N    Mean  Grouping
0       2  39,250  A
6       3  37,167  A
7       3  34,667  A
1       3  34,667  A
10      3   33,50  A B
2       3  33,500  A B
8       3   33,00  A B
5       3  32,833  A B
4       3   31,00  A B
9       3   30,67  A B
3       3   23,67    B

Means that do not share a letter are significantly different.
```