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CULTIVATION OF MUSHROOM MYCELIA USING WHEY PRODUCTS AS A GROWTH SUBSTRATE

by

Boyd Sam Inglet

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

2004

ABSTRACT

Cultivation of Mushroom Mycelia Using Whey Products as a Growth Substrate

by

Boyd Inglet, Master of Science

Utah State University, 2004

Major Professor: Dr. Conly L. Hansen
Department: Nutrition and Food Science

As part of a project designed to utilize common dairy waste products profitably, reconstituted dry whey permeate and delactosed whey were tested as growth substrates for mycelia of the edible mushroom *Lentinus edodes*. This mushroom was chosen because it is possible to profitably cultivate it due to its popular culinary appeal and perceived medical benefits.

Growth experiments were performed in petri dishes containing either reconstituted dry whey permeate or delactosed whey as a growth substrate, and the measured response was the size of the growing mycelia colony. When reconstituted dry whey permeate was utilized as a growth substrate, the factors of substrate concentration, pH, and growth temperature were controlled in an effort to determine the optimal growth conditions for the mushroom mycelia. These conditions were determined by applying an analytical method known as response surface methodology (RSM). RSM is a collection of mathematical techniques that is able to determine optimal values for many variables

run simultaneously in an experiment. Mycelia were also grown on delactosed whey at different substrate concentrations in an effort to determine if this substrate would be suitable for the growth of mushroom mycelia.

Results: RSM was successfully utilized to determine the optimal growth conditions for *L. edodes* when grown on reconstituted dry whey powder. These conditions were 40 g/L substrate concentration, pH 4.97, and temperature 23.6 °C. Delactosed whey was successfully utilized as a growth substrate for *L. edodes*. However, delactosed whey concentrations above 40% v/v were lethal to the mushroom mycelia, suggesting a possible use for delactosed whey as a fungicide.

(89 pages)

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Boyd Inglet

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Whey is a by-product of the cheese manufacturing process. It is a yellowish-green liquid that remains when casein and butter fat have been separated from milk to form cheese curds. Depending on the type of cheese being manufactured, as much as 9L of whey can be generated for every kilogram of cheese produced (Lee and others 2003a, 2003b). This creates a significant amount of by-product that must be properly disposed of by the manufacturing industry. Traditionally, this by-product has been considered to be waste, and processing plants have sought the most economical means whereby it may be disposed of, including discharge into waterways and onto fields, or processing it into low value commodity powders for resale (Smithers and others 1996). More recently, however, innovative technologies have sought to recover the proteins and nutrients available in whey and a market has arisen for powdered whey. As producers attempted to fill this niche in the food processing industry, global trade in whey increased over 12% a year from the years 1995-2000 (Voorbergen and Zwanenberg 2002). In 2003 in the United States alone, 84.1 million pounds of dry whey were produced by cheese manufacturing plants and were sold for only about $0.16 per pound (USDA-NASS 2004a).

With increasing global trade in whey, there still exists a supply and demand problem: there is more supply than demand. As of 1997, only 55% of the total whey produced in the US was being further used in food applications (Anon 1997). Even

though manufacturers are now able to recover some of the value of whey by selling it in powdered form, there remains room for further exploitation of this resource.

The composition of whey is dependent upon the composition of the milk that it is derived from. Since variations in the composition of cow's milk may arise between different animals, or due to changing seasons or changing sources of feed, it is impossible to give a robust chemical composition for whey. However, it may be estimated that most whey is roughly 0.7% whey protein, 0.05% fat, 0.7% ash, 4.9% lactose, and 93.65% water on a weight by volume basis (Smithers and others 1996). Whey is rich in carbohydrate, protein, and trace mineral content, including approximately half of the original nutrients of milk (Lee and others 2003b). With so many available nutrients and a track record of being useful as a stimulant for plant growth when used as a fertilizer on fields, perhaps manufacturers could begin to take advantage of whey as a growth substrate for other economically viable products. One such crop could be the production of mushroom mycelia.

Mushrooms

Fungi comprise one of five kingdoms that make up the eukaryotic domain in the scientific classification system of living organisms (Carlile and others 2001). Mushrooms are a subset of the macro fungi that are edible by humans. Those macro fungi that have been shown to be poisonous are commonly referred to as toadstools. What consumers commonly recognize as a mushroom is actually the fruiting body of the organism, and its responsibility is to disperse spores that will propagate the species. The fruiting body itself

is a collection of slender lines of cells known as hyphae that quickly gather and rise above ground when the organism attempts to reproduce. Usually, these hyphae grow underground in a large tangled mass known as mycelia (singular mycelium). Although the fruiting body is what consumers recognize as a mushroom, it is entirely dependant for its nutrition on an extensive network of mycelium that penetrate the growth medium (Carlile and others 2001).

The mycelia network begins from a single spore. When the spore germinates, a single hypha emerges from it. As this hypha develops, it can grow branches that may in turn grow their own branches. In this fashion, a vast network of mycelia can soon grow to support the organism. The growth pattern of mycelia is unique to fungi; they exhibit apical growth. That is, they grow only at the extreme tip of the individual hypha, and it is this method of growth that allows them to penetrate and make use of virtually any substrate (Carlile and others 2001; Deacon 1980; Gow and Gadd 1995). When the mycelia begin to deplete the nutrients in the substrate, they form a fruiting body to begin the cycle anew (Gow and Gadd 1995). Figure 1 depicts the life cycle of a typical mushroom.

Mushroom growth patterns

The kinetics involved in fungal growth are not usually determined by working with mycelia growing in the natural environment. Such an environment is inherently unstable and contains too many factors that cannot be accounted for when attempting to plot the growth kinetics of a fungus (Deacon 1980). Researchers usually try to circumvent

this problem by creating an artificial environment that mimics the scenario of a fungus colonizing and then utilizing a substrate. This is accomplished by establishing a "batch" culture, where the fungi are grown in a submerged culture that contains all the growth nutrients or substrates before inoculation with mycelia (Carlile and others 2001; Deacon 1980; Deacon 1997; Gow and Gadd 1995). In this controlled environment, the fungus will be able to grow until nutrients run out in the system or until toxic by-products of metabolism accumulate. In this way, researchers can simulate what happens when a fungus invades a substrate in nature and uses it to depletion.

When grown using a batch culture, mycelia exhibit a pattern of growth that contains a lag phase, exponential growth phase, deceleration phase, stationary phase, and death or autolysis phase (Figure 2). The lag phase consists of the time required for the fungus being studied to adapt to its environment and begin to grow vigorously. This time is specific not only for each species, but also for the culture conditions of any given batch. However, if vigorously growing organisms are transferred from one medium to an identical one, no lag phase is apparent and the fungus can enter directly into the exponential growth phase (Carlile and others 2001).

The exponential growth phase is the phase where the cells of the fungus are actively dividing and multiplying. By monitoring the growth of the fungus during this period, a rate of growth, known as the specific growth rate, may be established for the species and medium combination. The specific growth rate during this exponential growth phase is considered to be the maximum growth rate attainable for the particular

growth medium, and is therefore equivalent to the maximum specific growth rate (Gow and Gadd 1995).

As nutrients become limiting in the culture, the mycelia will enter the deceleration phase of growth, where the specific growth rate will drop (Carlile and others 2001; Gow and Gadd 1995). This rate will slow even further as the substrate becomes depleted, and the fungus will enter the stationary phase, where virtually no growth is apparent. After a time in this phase, the fungus can then enter the death, or autolysis phase. In this phase the automatic machinery of the cells takes over and the cells die and become recycled into the environment, causing the fungus biomass to decrease (Gow and Gadd 1995).

This is the pattern of growth for fungi grown in a batch culture. A continuous culture refreshes the supply of media available for fungal growth and can prolong the exponential growth phase of the organism almost indefinitely (Carlile and others 2001; Deacon 1980; Deacon 1997; Gow and Gadd 1995). However, this might not always be advisable. Growing mushroom mycelia produce many products that can be beneficial for use, and many of these products are produced during the deceleration or early stationary phase of growth (Deacon 1980; Gow and Gadd 1995). Thus, while the kinetic growth patterns of fungi can be modeled using a batch culture system, this might not always prove to be the most favorable solution in every application.

The mushroom market

Currently, the largest market for mushroom production lies with harvesting the fruiting bodies of the mushrooms. These fruiting bodies are what consumers purchase in

grocery stores, and they are also the ingredients used in many food products, such as soups. In 2003, in the United States, 844 million pounds of fresh mushrooms were sold for approximately $889 million (USDA-NASS 2004b). Far and away the largest portion of these sales was of the common white button mushroom, *Agaricus bisporus*. However, specialty mushrooms also enjoyed robust sales. The value of sales for commercially grown specialty mushrooms in 2003 was $37.1 million, with the average price per pound being $2.90 (USDA-NASS 2004b).

Mushrooms enjoy an even larger market outside of the United States. China produces many times the amount of mushrooms each year as the United States. In countries such as Thailand, specialty mushroom crops such as *Lentinus edodes* are imported at the rate of about $40 million each year, and that value is expected to rise (Thepa and others 1999). With a robust worldwide market for mushrooms, producers can expect to sell their product for profit. Potential profit of specialty mushrooms is high due to the culinary aspects they possess as well as perceived medical benefits associated with them.

Functions of fungi

Fungi perform a useful function in the natural environment. Every year, global photo synthetic processes create a biomass of approximately 100×10^9 tons. The bulk of this material is cellulose (the material that makes up the rigid structure of most plants), and fungi have a major role in breaking down this material and returning carbon dioxide to the atmosphere and minerals to the soil (Carlile and others 2001; Gow and Gadd

1995). Of this 100×10^9 tons of biomass produced each year, about 20×10^9 tons are in the form of lignin (a component of woody plant tissues that serves to harden them). Most organisms are unable to metabolize lignin. White-rot fungi are an exception as they are the most effective lignin degraders in nature (Sannia and others 1991). Without their contribution to the degradation of lignin, life on earth would eventually end as the supply of carbon would gradually become converted into wood (Gow and Gadd 1995).

Fungi have become very adept at utilizing substrates not available to most organisms. Over the years, they have developed an ability to utilize virtually any carbon-containing compound as a fuel source. For example, one year the entire Indonesian air force was grounded by the growth of the fungus *Amorphotheca resinae.* The fungus was growing in the fuel tanks of the aircraft, using kerosene as its food source. The metabolism of the kerosene by *Amorphotheca resinae* resulted in corrosion along the walls of the fuel tanks (Deacon 1980). Figure 3 shows some representative carbon substrates that fungi are able to use as a growth medium.

Fungi can use this adaptability of food sources to act as parasites when they begin to use another organism as part of their food source. This can result in crop failures, such as the great potato famine in Ireland (caused by the fungus *Phytophthora infestans*) or the Great Bengal Famine (caused by the fungus *Helminthosporium oryzae*). These particular instances resulted in millions of human deaths because the host was destroyed by a parasitic fungus (Deacon 1980). Parasitism is not always a detrimental situation, however. In most cases, when a fungus acts as a parasite in the root system of a plant, it is actually beneficial to the plant. This is because the fungus is able to break down more

substrates than the plant and make the minerals and nutrients in those substrates available for the plant to use (Deacon 1980).

Fungi, including edible mushrooms, are able to utilize such a vast range of organic compounds as a food source because their mycelia excrete a variety of enzymes and chemicals that allow them to degrade these materials. These include, but are not limited to, amylases, cellulases, dextranases, galactosidases, glucanases, hemicellulases, lipases, pectinases, proteases, tannases, xylanases, organic acids, and steroid compounds (Deacon 1980; Gow and Gadd 1995). Humans can make use of the enzymes produced by these mycelia in a variety of methods ranging from food processing to waste management. Some of the chemical uses include the production of antibiotic treatments. Perhaps the most famous of these is penicillin, which is produced by *Penicillium chrysogenum*.

Fungi in the food industry

At the present time, there are a variety of functions that fungi serve in many different foods. One example for consideration is the case of rennet. Rennet is a crude extract of calf stomachs from which the enzyme chymosin may be obtained. This enzyme has traditionally been used in cheese manufacturing. However, more than half of the cheese now produced worldwide is manufactured using a fungal acid protease such as the one secreted by *Rhizomucor miehei* (Gow and Gadd 1995). Other fungi are used as fermentative agents. One example of this is *Rhizopus oligosporus*. This fungus is commonly inoculated into a soybean mixture and allowed to ferment. The end result is a product called "tempeh" that is more palatable and has a higher amount of bioavailable

protein (Deacon 1980). The uses of fungi in food processing do not end with these species. A variety of other species are also employed in making beer, wine, fruit products, cereals, tea, coffee, cocoa, and more.

Another potential use of fungi in the food processing industry is to increase safety and shelf life of foods. Fresh foods are susceptible to spoilage by a variety of bacteria and fungi (two drastic cases of fungal spoilage were discussed earlier). Extracts from certain mushroom species such as *Lentinus edodes* (commonly known as the Shiitake mushroom), have been shown to contain compounds that exhibit strong antibacterial effects toward *Staphylococcus* and *Escherichia* species (Hatvani 2001). Other mushroom extracts and their derivatives have been shown to be effective against various bacteria and fungi species. Findings such as this have allowed food processors access to natural enzymes and chemicals that can prolong the shelf life and quality of food the American public consumes. Also, some of these substances could perhaps be employed in manufacturing facilities as part of standard sanitizing procedures if they were used as surface disinfectants (Hatvani 2001).

Fungi in waste management

As we learn more about the metabolism and capabilities of mushrooms, we are able to use them in more and more diverse ways. Since it was already known that mushroom mycelia were capable of degrading lignin, researchers began testing other substrates as growth media for mycelia. It has since been discovered that a wide variety of agro-industrial wastes can be used as substrates for the growth of mushroom mycelia. For

example, the edible mushroom *Lentinus edodes* can utilize malt-containing byproducts from the brewing process, coffee husk, bracts of pineapple crown, sugarcane bagasse, sugarcane leaves, and waste from tofu manufacture as a growth medium (Hatvani and Mécs 2001). Even though these substances are considered to be waste products by the manufacturing facilities that produce them, *Lentinus edodes* is able to break them apart and use them as a substrate for growth. The end result is an organic product that has been degraded and returned to the environmental cycle, much as it would be in the natural environment.

Industrial wastes can often mimic fungi substrates that are produced naturally. Such is the case with sawdust. Sawdust is a substance that mycelia would normally encounter in a rotting tree, but that is produced in vastly higher quantities by industrial processes. Treatment of sawdust and other wood products by the mushroom *Lentinus edodes* has proven to be successful. *L. edodes* is able to grow in these products and produce fruiting bodies (Fukushima and others 1993). This could provide an alternate use for the wood waste produced by common industrial processes.

Another process that produces a large amount of organic waste is tea manufacturing. The large amount of waste produced by this process can be used, however, as a material in which to grow and then commercially harvest the mushroom *Agaricus bisporus*, or the common white button mushroom (Gülser and Pekşen 2003).

Another mushroom whose mycelia are able to degrade agro-industrial wastes exceptionally well is *Pleurotus ostreatus*. This mushroom's mycelia have been successfully cultivated in wheat straw, flax shive, cotton stalk, hazelnut leaves, tilia

leaves, European aspen leaves, sawdust, and waste paper among others (Kerem and Hadar 1993; Yildiz and others 2002). These studies showed that mycelia can be successfully cultivated not only on naturally procured substrates such as leaves, but that they also grow well in man-made wastes such as paper. These findings are encouraging since they indicate that mushroom mycelia can be employed in order to recycle various man-made wastes.

Pleurotus ostreatus has also shown considerable process in treating olive mill wastewater. Olive mill wastewater is the liquid waste produced during the manufacture of olive oil. Most of this waste is generated in the Mediterranean region of the world, but as much as 2.5 liters of wastewater is produced for every liter of olive oil produced (Fountoulakis and others 2002). This is significant because olive mill wastewater contains a high amount of polyphenols and sugars, volatile acids, polyalcohols, and nitrogenous compounds. When these chemicals are released into the environment, they cause a considerable amount of environmental deterioration. The concentration of the phenols is an especially large concern. This is because most environmentally friendly treatments of the wastewater are not able to break down the phenols. However, since the structure of the phenolic compounds in the olive mill wastewater is similar to the structure of lignin, *P. ostreatus* has shown the ability to degrade the phenols present in the waste (Fountoulakis and others 2002). Thus, the treatment of olive mill wastewater by mycelia from *P. ostreatus* can result in an effluent that causes considerably less environmental damage.

The phenols in olive mill wastewater are not the only environmental pollutants that may be effectively degraded by *Pleurotus ostreatus*. Polychlorinated biphenyls (PCBs) are pollutants used in a variety of industrial applications such as in hydraulic and dielectric fluids. The widespread uses of PCBs, along with improper disposal practices, have made them a ubiquitous pollutant. However, *P. ostreatus* has demonstrated an ability to effectively treat soil contaminated with PCBs to lower the amount of pollution present (Ruiz-Aguilar and others 2002). In addition to PCBs, *P. ostreatus* has also been shown to effectively degrade other chemicals such as polycyclic aromatic hydrocarbons, synthetic dyes, explosives, insecticides, and neural toxins, among others (Amitai and others 1998; Baldrian and Gabriel 2003). The process of making dangerous pollutants safe for the environment using natural remedies that leave no harmful traces of themselves is commonly referred to as bioremediation. The potential that mushroom mycelia have to act in the bioremediation of various pollutants is only now beginning to be tapped.

Since it has now been discovered that fungi mycelia can be used in the breakdown of various products, both natural and man-made, efforts are also being made to discover the precise mode of action that these mycelia employ. To this end, researchers are studying the various exogenous enzymes produced by the mycelia, since it is the action of these enzymes that produces the desired bioremediation. What is being discovered is that the mycelia employ a variety of specialized and non-specific enzymes in order to function. For example, the mycelia of the mushroom Agaricus bisporus produce an *endo-1,3-ß-glucanase* in order to degrade cell wall components (Galán and others 1999).

Also, in the mycelia of vigorously growing Pleurotus ostreatus researchers have isolated veratryl alcohol oxidase, manganese peroxidase, laccase, and various glucanases (Baldrian and Gabriel 2003; Hublik and Schinner 2000; Sannia and others 1991; Sarkar and others 1997). As this species demonstrates, a variety of enzymes are present to interact with the environment and provide the mycelia with nutrients. These same enzymes may also be tapped as a potential means of treating waste products.

Fungi in the pharmaceutical industry

The uses of the various chemicals and enzymes produced by mushroom mycelia are not limited to food processing and bioremediation issues. While creating improved food products and cleaning up the environment are both laudable energy expenditures, there is another useful area where the use of fungal agents could prove very useful: pharmaceuticals.

Lentinus edodes, commonly known as the Shiitake mushroom, has been consumed for generations in Asia as a medicinal food. The perceived medical benefits associated with consuming this mushroom have combined with its appealing taste to make it the second most popular edible mushroom in the global market (Hatvani 2001). The medicinal properties of the mushroom have not been universally conceded. Many Westerners still rely on modern medicinal practices and don't concede the effectiveness of simple cures such as mushroom consumption.

Recently, though, Western and Eastern researchers alike have begun studying the Shiitake mushroom to discover if it actually contains bioactive compounds that could

promote better health. One compound that has been discovered is called Lentinan (named after the *Lentinus edodes* mushroom it was found in). Lentinan is a polysaccharide-peptide complex isolated from the mushroom that acts as a host defense potentiator (Hatvani 2001; Liu and others 1998). When this compound enters a host system, it activates the natural immuno-defensive mechanisms of the host. This has been shown to produce a significant anti-tumor effect since the host's system becomes activated to attack tumors in the body (Liu and others 1998). Of other significant interest is that the compound has also been shown to inhibit the infectivity and cytopathic effects of human immunodeficiency virus (HIV) and to block the release of herpes simplex virus type 1 cells from tissue culture cells (Hatvani 2001). Findings such as this have great potential in the pharmaceutical industry. They provide a functional product whose structure and mode of action could possibly be copied during the creation of new drug therapies. This sort of information could lead to more effective tumor and viral medications.

Another enzyme of interest that has been purified from extracts of *Lentinus edodes* is a type I DNA topoisomerase. This enzyme is normally employed in eukaryotic cells to catalyze the breakage and rejoining of DNA strands one at a time in order to remove positive and negative superhelical turns in the DNA (Kono and others 1986). With genetic research increasing rapidly at the current time, enzymes such as this that can manipulate the structure of DNA could find extensive application in industry.

Another mushroom that has been a part of traditional Asian medicine for many years is *Ganoderma lucidum*. It has been used for years in the Orient to treat a variety of afflictions including hypertension, arthritis, and bronchitis (Lee and others 2003a). Recent

studies into the activity of this mushroom have led to the discovery of polysaccharides and ganoderic acids within the mushroom that exhibit various biological activities. Notably, they have shown an ability to act as anti-tumor agents and to inhibit HIV-1 (Tang and Zhong 2003). These polysaccharides act by stimulating the immune system of their hosts, and can also have anti-inflammatory effects as well as anti-tumor effects (Lee and others 2003a). Compounds such as this could easily find application in the pharmaceutical industry, however they are in short supply. That is because *Ganoderma lucidum* grows very slowly in soil (several months to cultivate) and the yield is often poor or unreliable. Research has suggested that submerged fermentations in a liquid media could produce *Ganoderma lucidum* mycelia (and hence the bioactive compounds) much more quickly (Lee and others 2003b; Tang and Zhong 2003). Whey could possibly be a suitable liquid substrate for such fermentations.

The mushroom *Pleurotus ostreatus* has recently been discovered to contain hemolytic proteins (Berne and others 2002). These proteins are involved in the break-down of red blood cells. In humans, viral and bacterial infections frequently cause hemolysis in children since the cell membranes of their red blood cells are quite fragile (Venes and Thomas 2001). With the discovery of these new hemolytic proteins, researchers now have a new path for exploring the various mechanisms by which hemolysis can be initiated. Studies of this sort could perhaps lead to therapeutic drugs that could reduce the incidence of hemolysis in sick children.

The list of bioactive compounds contained in mushrooms will continue to grow as more mushroom species are studied. These studies can also serve to validate traditional

medicinal practices. Such was the case when *Phellinus linteus* was found to contain very high anti-tumor activity due to the various bioactive compounds it contained (Nakamura and others 2002). In some cases, we may also discover compounds that have a wide application across disciplines. Such is the case with the NAD^+-dependent glutamate dehydrogenase isolate in extracts of *Agaricus bisporus* (Kersten and others 1999). This enzyme is actually used during nitrogen fixation, so its natural application would be in an agricultural setting. However, humans use NAD^+ as a cofactor in many enzymes in the body. Understanding how the NAD^+-dependent glutamate dehydrogenase of *Agaricus bisporus* works could lead to a better understanding of how humans use NAD^+ in their own bodies. The possible applications that bioactive compounds in mushrooms could be used in are unlimited since researchers are just now beginning to tap the vast potential of the mushroom. As these compounds are discovered and explained, though, they could create a greater demand for mushrooms and their products worldwide.

Production of fungal mycelia

As researchers have become aware of the ability mushrooms have to decompose various substrates as well as of the health benefits associated with mushroom consumption, two phenomena have begun to merge. The first is that people are now realizing the potential mushrooms have for bioremediation purposes when grown on a variety of substrates (Fountoulakis and others 2002; Fukushima and others 1993; Kang and others 1993; Lee and others 2003b). The second is that researchers have discovered that the beneficial chemicals and enzymes that mushrooms produce can be extracted from

the mycelia of the species without waiting for a full fruiting body to develop (Hatvani and Mécs 2001). This second phenomenon should come as no surprise when one considers that the fruiting body of a mushroom is composed of a mass of mycelia. However, it is the combination of these two realizations that could yield the most important dividends for society. Why not utilize growing mycelia to degrade waste waters and then harvest those same mycelia for their pharmaceutical benefits?

As elementary as this proposed question sounds, there are several problems with simply attempting to grow mushroom mycelia in wastewater. The first major problem that needs to be addressed when considering such an option is that contamination could pose serious problems to the endeavor. Optimal growth temperatures for many of the species of mushrooms that researchers might wish to grow coincide with healthy growth temperatures for a variety of microorganisms. If the substrate being used can support the growth of mushroom mycelia, it can also support the growth of microorganisms. In fact, the continuous submerged culture of mushroom species has been attempted, and contamination by microorganisms has been shown to be a problem of concern (Fukushima and others 1993). This necessitates a strategy for preventing contamination by microorganisms if any large-scale production of mycelia is to be attempted. The hearty nature of mycelia offer several viable options for accomplishing this. One option is to simply add acetic acid to the reaction and maintain a low pH since this has been shown to inhibit the growth of microorganisms while still allowing growth of the mycelia (Fukushima and others 1993). Another plausible option would be to sterilize waste waters before inoculation with mycelia, much the same way as many food products are sterilized

in bulk before packaging. Care must be exercised with this option, however, since one study suggests that the method of sterilization could affect the final enzyme product obtained (Fountoulakis and others 2002).

Perhaps the next biggest concern that needs to be dealt with when the production of mycelia is attempted on a large-scale basis is the quality of the final product. If one wishes to use mycelia in not only a bioremediation role for waste waters, but also as a producer of pharmacological products, there must be some method to ascertain that the final product obtained from the mycelia is of sufficient purity or activity to serve the purpose for which it was intended. Several researchers have already discovered this problem in the course of attempting to purify a mycelial enzyme extract for further study. They have discovered that culture conditions during the growth of the mycelia (notably temperature and pH) directly affect the activity and quantity of the enzymes the mycelia produce (Kang and others 1993; Medeiros and others 1999; Morais and others 2002).

The effect that culture conditions have on the final enzyme product from mushroom mycelia will vary from species to species. Sometimes culture conditions that promote the best bioremediation effects of the mycelia will coincide with the conditions that produce the highest quality final product. Sometimes this will not be the case, and a trade-off will be necessitated. In these situations, it must be decided what is of paramount importance in the process. Is it the bioremediation of the wastewater, or the final enzyme product that might be obtained? These questions would be answered differently during different applications and so they would have to be dealt with on a case by case basis.

Some preliminary work has already been begun with the design of using cheese whey as a dual candidate for both bioremediation and growth of mycelia. This work attempted to find the optimum growth culture conditions for the *Ganoderma lucidum* mushroom species when grown on whey permeate. The primary aim of these researchers was to optimize the bioremediation effects of the mycelia, but they also wanted to see if these conditions would also give a satisfactory yield of bioactive compounds from the mycelia (Lee and others 2003a, 2003b). This work began with a sterilized whey permeate that was kept isolated from the outside environment and controlled temperature and pH in the growth medium in order to grow mycelia of *Ganoderma lucidum*. Growth of the organism was monitored as shown in Figure 5. They found that the conditions that produced the optimum growth of *Ganoderma lucidum* were pH 4.2 and 28.3 °C, which were very close to the optimal growth conditions for bioremediation purposes. Table 1 contains a summary of the bioremediation effects of the experiment. These conditions also happened to produce a high amount of bioactive polysaccharide from the mycelia (Lee and others 2003a, 2003b). From these results, the researchers were able to conclude that in the case of *Ganoderma lucidum*, whey permeate would be a suitable substrate for the cultivation of mushroom mycelia. Not only was this bonus realized, but the researchers also found that they were able to obtain mycelial product in as little as two weeks, rather than the months that production of fruiting bodies can require (Lee and others 2003a, 2003b).

Response surface methodology

In ascertaining the optimal culture conditions for the production of *Ganoderma lucidum* mycelia, Lee and associates made use of a specialized statistical procedure known as "Response Surface Methodology," or RSM. This method involves employing a collection of mathematical and statistical techniques in situations where several independent variables influence a dependent variable or response, and the goal of the analysis is to maximize the response of the dependent variable.

The classical approach to such problems was a systematic one. The researcher would simply change one independent variable (nutrient, pH, exposure time, etc.) while holding all other variables at a fixed level, repeating the procedure for the different variables. This approach was time-consuming, and could become expensive for large numbers of variables (Adinarayan and others 2003). Utilizing RSM analysis and factorial design is one method to reduce the time and cost previously associated with such experiments.

RSM analysis assumes that the variables in question are continuous and controlled by the experimenter. The observed response of the variable, y, is assumed to be a random variable and is represented as:

$$y = f(x_1, x_2, \ldots x_k) + \epsilon$$

where:

y = observed response

x_i = variable i (i = 1, 2, ... k)

ϵ = random error

During RSM analysis, the goal is to model this function with an equation that suitably explains the data and accounts for variations present in the experiment (Hwang 1995). For responses that are well modeled with a linear function of the independent variables, the approximating function is a first-order model whereas a higher order polynomial may be necessary if the response system contains curvature (Hwang 1995). In either case, the idea is to begin with a simple modeling equation and then increase the complexity of the model until a sufficient explanation of the data is obtained (Hwang 1995; Lee and others 2003a, 2003b).

One caveat when working with RSM analysis is that it uses orthogonal first-order 2^k factorial experimental designs. These designs do not allow for an estimation of experimental error unless some of the experimental runs are repeated (Hwang 1995). Also, researchers must begin their design with some prior knowledge and understanding of the process and the process variables under investigation if they wish to obtain satisfactory results (Adinarayan and others 2003). Lee and associates dealt with these concerns by obtaining repeat values for the central point in their design and by designing the experiment so that they began with conditions already established in literature as being suitable for the growth of *Ganoderma lucidum*. Table 2 and 3 show a general model for RSM experimentation and a completed model using realistic values for the fungal growth parameters, repspectively.

Response Surface Methodology, as its name implies, produces a unique visual representation of the data for the experimenter to use during analysis. An example may be seen in Figure 4. These graphs may be used to find either maximum values, minimum

values, or saddle points that lie between two maxima (Hwang 1995). The visual nature of the graphs makes it easy to ascertain the direction in which the experiment should proceed if the correct point is not obtained at first.

Application

Mushrooms have been recognized for many years as a source of food and medicine. While their medicinal role in the Western Hemisphere has been limited, they have been a part of traditional Asian medicine for years (Lee and others 2003a; Nakamura and others 2002; Tang and Zhong 2003). This role in traditional medicine has sparked an interest in the functional mechanisms involved with mushrooms and health. Researchers have sought out bioactive compounds produced by mushrooms, and the literature is beginning to be filled with examples of the chemicals and enzymes that mushrooms produce and their possible effects on human health.

At the same time, mushrooms comprise a significant portion of the agricultural market in the United States (USDA-NASS 2004b). People consume mushrooms as part of their everyday lives. At the same time, the booming health food industry in America suggests that the general public desires to lead healthier lives. The time has come to combine this desire with a crop that is already produced and available. However, rather than simply sell more mushrooms, food processors should consider alternative methods of reaching the public with the benefits that mushrooms have to offer.

One method of marketing mushrooms and their beneficial properties could be to use mycelia as a supplement inside existing food products. This has the potential to

expose more people to the bioactive compounds in mycelia and to increase the protein quality of food at the same time (Yildiz and others 2002). Of course, simple mycelia could also be sold in an encapsulated pill form as a nutritional supplement in their own right.

Perhaps the best reason, however, that mushrooms should be seriously considered as an alternative source for nutrition and health is that their cultivation can solve a large problem in the United States. Every year the United States produces millions of tons of cheese whey (USDA-NASS 2004a). If this by-product goes untreated, it could cause great environmental damage to the country. It should be considered as a possible substrate for the cultivation of mushroom mycelia. In this fashion, not only would the bioremediation abilities of the mushrooms be realized and the pollutant effluent in America be diminished, but cheese plants could begin to recover some of the losses that they incur with by disposing of whey by selling the mycelia to pharmaceutical companies. This solution to the problem of whey production in the United States only waits upon determination of the factors that could make it an economically viable process. Then factories could begin to reduce their effluent and provide the American product with another value-added product in the same simple step.

To this end, this is a thesis designed to accomplish two objectives:

1. To utilize solid state (i.e. experimental media has been solidified through the addition of agar) fermentation experiments carried out on reconstituted dry whey permeate in order to determine the optimal growth conditions for mycelia of the

edible mushroom *Lentinus edodes* with regard to substrate concentration, temperature, and pH.

2. To determine if delactosed whey permeate could also be used as a substrate for mushroom mycelia cultivation.

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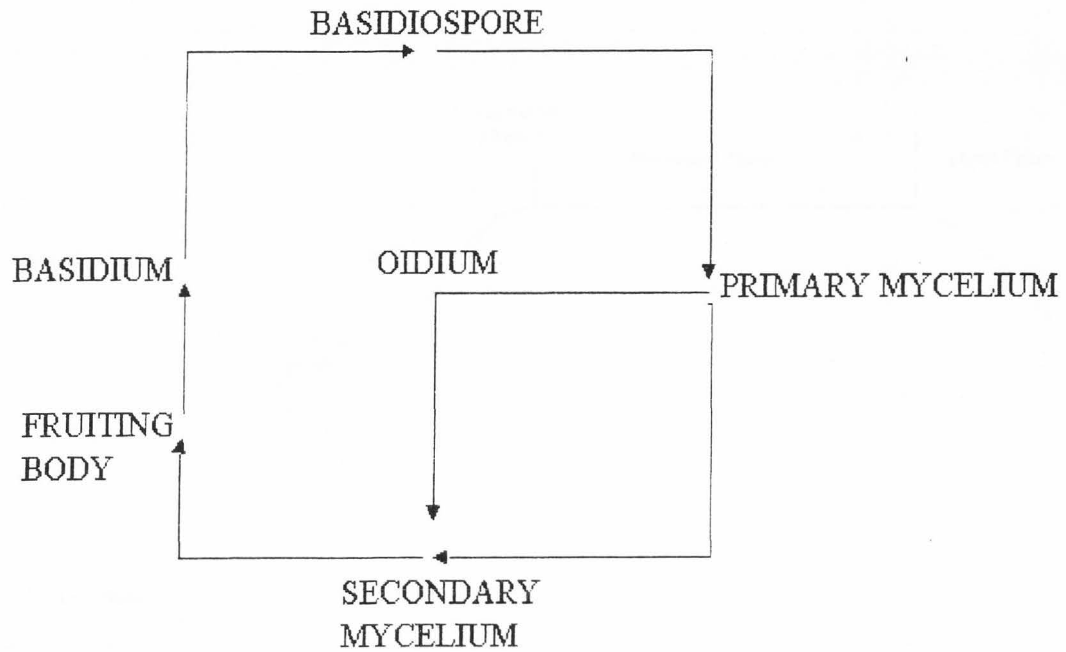


Figure 1 - Schematic representation of the life cycle of the common mushroom. As can be seen in the diagram, primary mycelia are able to divide spontaneously into secondary mycelia, or they may go through a sexual cycle to progress to the secondary mycelia stage (Carlile and others 2001).

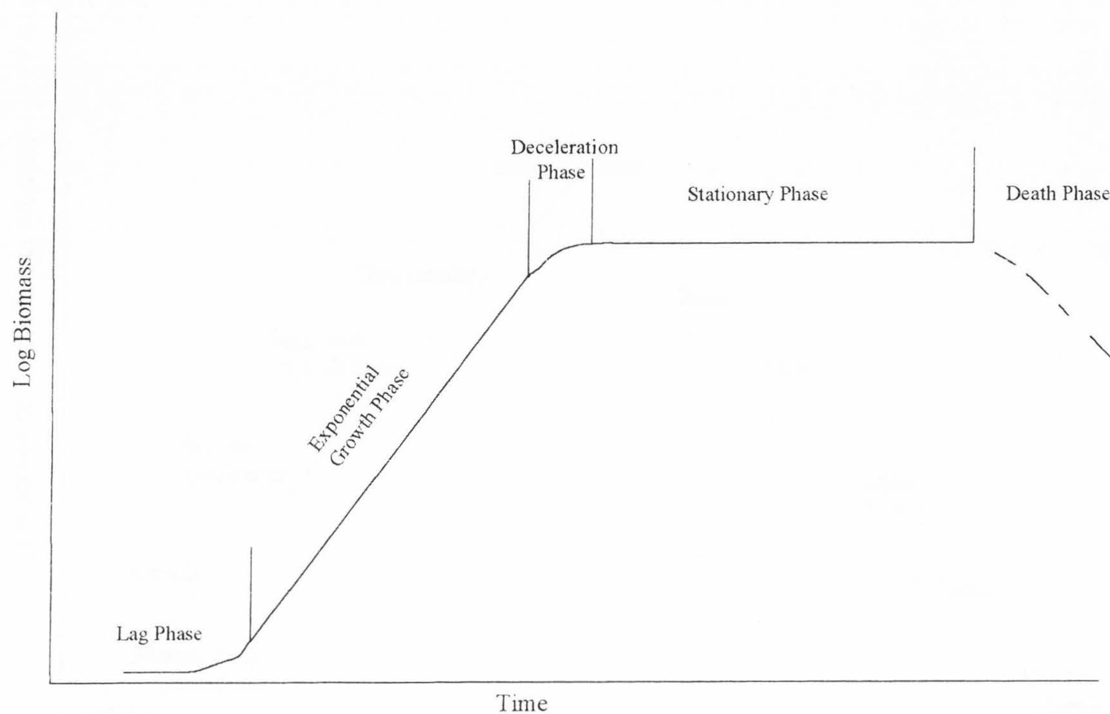


Figure 2 - Typical fungal growth curve (Deacon 1980). As illustrated by this chart, mushroom mycelia exhibit growth patterns similar to other microorganisms. An initial adjustment phase where little growth is evident is followed by rapid mycelia growth. This phase is followed by a time period where little or no growth is evident, and eventually the death of the mycelia occurs.

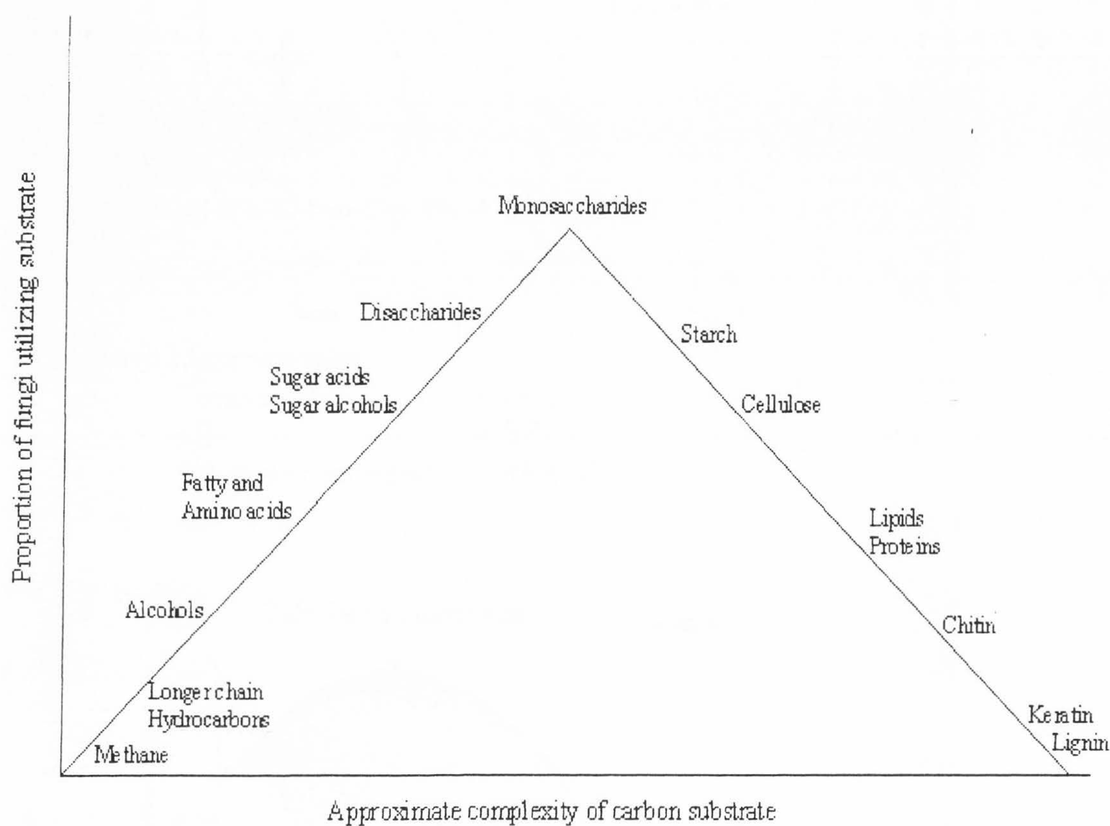
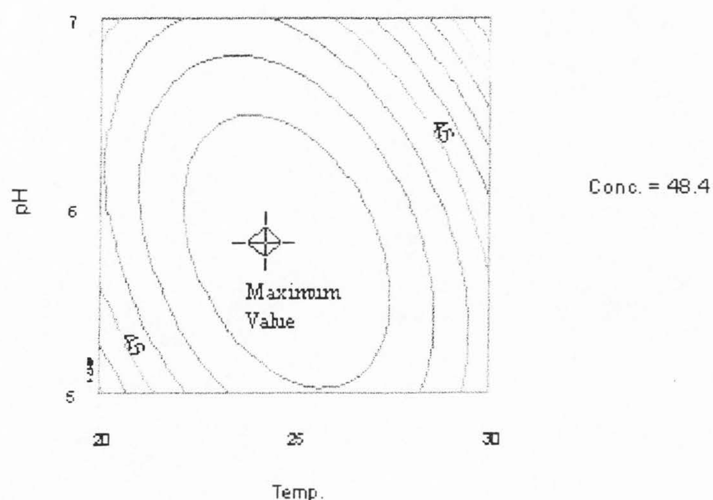


Figure 3 - Representative carbon substrates that may be utilized as a growth substrate by various fungi. Specialized mushroom species may utilize diverse compounds as simple as methane or as complex as lignin for their nutritional requirements. However, the majority of mushroom species prefer to utilize sugars and starches (Deacon 1997).



Calculated Maximum value:

Temperature	= 24.23
pH	= 5.81
Substrate concentration	= 48.4 g/l

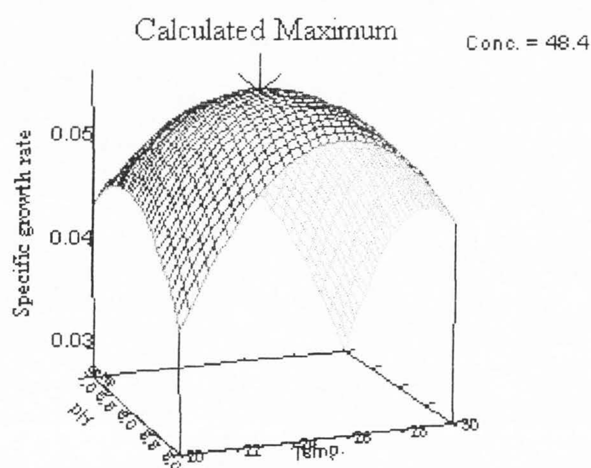


Figure 4 - Typical graphical outputs of an RSM analysis. The top graph is a two-dimensional representation of the listed values for the calculated maximum. It is read in the same way one would read a topographical map. The bottom graph is a three-dimensional representation of the same values.

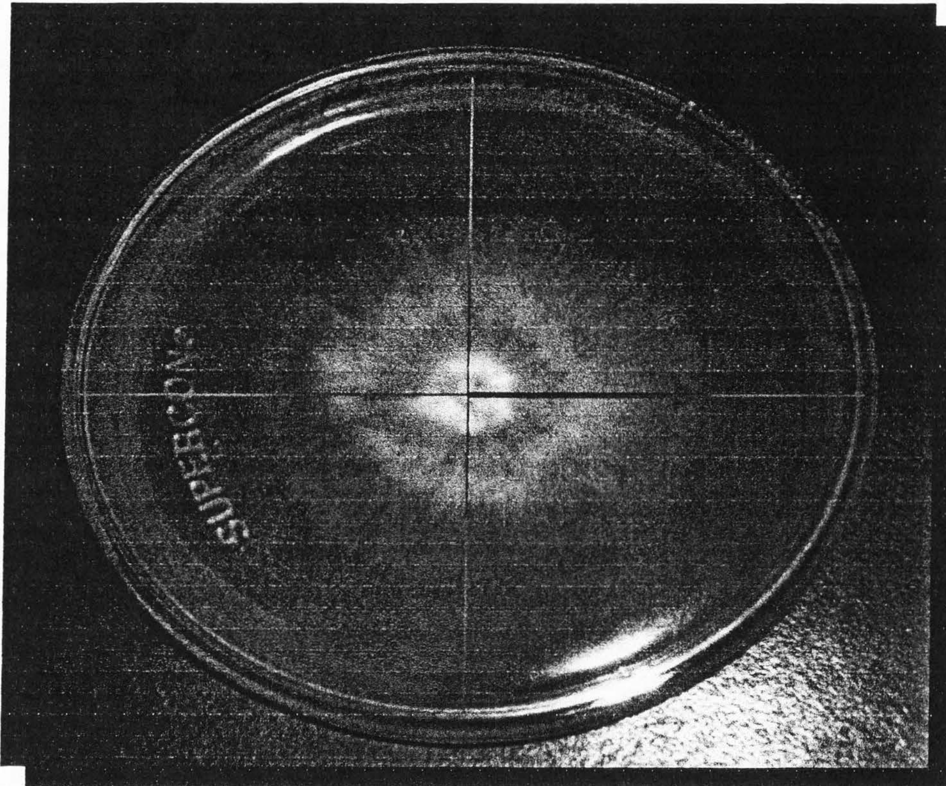


Figure 5 - Growing mycelia exhibit radial growth patterns. This makes it possible to determine the size of a growing colony by measuring from the center of the colony to any point on the outer edge. Multiplying this value by 2 yields the diameter of the colony.

Table 1: Growth conditions for *Ganoderma lucidum* and the residual soluble chemical oxygen demand (SCOD) of the spent media. The media originally contained 52993 mg SCOD. (Lee and others 2003a)

pH	Temperature °C	Residual SCOD (mg)
3.5	25	6751
4.5	25	4049
3.5	35	10254
4.5	35	7758
4.0	30	4454
4.0	37.1	8452
4.0	22.9	6227
4.7	30	4632
3.3	30	8871

Table 2: A general model for solid state fermentation experiments utilized in response surface methodology. All table values, such as 1, -1, 0, a, and "-a," are coded variables. X1, X2, and X3 represent substrate concentration, pH, and temperature.

General Model

Trial #	X1	X2	X3
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	"-a"	0	0
10	a	0	0
11	0	"-a"	0
12	0	a	0
13	0	0	"-a"
14	0	0	a
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0

Table 3: A model used for the analysis of the growth of *Agaricus bisporus*. The coded values of the general model in Table 2 have been replaced by the experimental values utilized. For each trial number, three individual petri dishes were prepared according to the values specified in the table. The dishes were then innoculated with mycelia, and the growth of the mycelia was observed and recorded.

Agaricus bisporus

	-1	0	1	a	"-a"
Whey permeate concentration (X1)	50	60	70	70	50
pH (X2)	3	4	5	5	3
temperature°C (X3)	20	25	30	30	20

Factor level settings			
Trial #	X1	X2	X3
1	50	3	20
2	70	3	20
3	50	5	20
4	70	5	20
5	50	3	30
6	70	3	30
7	50	5	30
8	70	5	30
9	50	4	25
10	70	4	25
11	60	3	25
12	60	5	25
13	60	4	20
14	60	4	30
15	60	4	25
16	60	4	25
17	60	4	25
18	60	4	25
19	60	4	25

CHAPTER II

CULTIVATION OF *LENTINUS EDODES* MYCELIA USING DRY WHEY PERMEATE AS AN ALTERNATIVE GROWTH SUBSTRATE: OPTIMIZATION OF GROWTH PARAMETERS UTILIZING RESPONSE SURFACE METHODOLOGY[1]

Abstract

A novel approach to utilizing dry whey permeate, the cultivation of mycelia of the edible mushroom *Lentinus edodes*, is introduced. Response surface methodology (RSM) was successfully applied in order to determine the combination of substrate concentration, temperature, and pH that would result in a maximal mycelial growth rate. The settings predicted to maximize growth were determined to be 40 g/L substrate concentration, pH 4.97, and temp 23.6 °C. Subsequent verification of these levels agreed with model predictions. The results of these experiments suggest that whey permeate could be utilized as a growth substrate for the cultivation of mycelia from the edible mushroom *L. edodes*, helping to utilize this by-product of the cheese manufacturing industry.

Introduction

Whey is the serum portion of milk, containing 4-5% lactose, that remains after most milk proteins have been removed during the cheese making process. Depending on

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[1]Coauthored with Conly Hansen, Professor, Utah State University

the type of cheese being manufactured, as much as 9L of whey can be generated for every kilogram of cheese produced (Lee and others 2003a, 2003b). This creates a significant amount of by-product that must be properly disposed of by the manufacturing industry. Traditionally, this by-product has been considered to be waste, and processing plants have sought the most economical means to dispose of it (Smithers and others 1996). More recently, however, innovative technologies have been developed to recover the valuable nutrients of whey, notably proteins (Cifuentes and others 1993; Xiong 1992) . As producers attempted to fill this niche in the food processing industry, global trade in whey increased over 12% a year from the years 1995-2000 (Voorbergen and Zwanenberg 2002). In 2003 in the United States alone, 84.1 million pounds of dry whey were produced by cheese manufacturing plants and were sold for only about $0.16 per pound (USDA-NASS 2004a).

With increasing global trade in whey, however, there still exists a supply and demand problem: there is more supply than demand. As of 1997, only 55% of the total whey produced in the US was being further used in food applications (Anon 1997). Whey is rich in carbohydrate, protein, and trace mineral content, including approximately half of the original nutrients of milk (Lee and others 2003b). At the present time, a common treatment for whey is to send it through a filtration process designed to retain specific nutrients (notably proteins) for further use. These retained nutrients are known as the "retentate," and the remainder of the whey becomes whey permeate. Table 4 shows a compositional comparison of some nutrients present in whey and whey permeate. This table shows that the main compositional difference between whey and whey permeate is

that whey permeate typically contains less protein and carbohydrate than whey while containing higher mineral levels. With so many available nutrients, whey and whey products should be considered as growth media for economically valuable products.

In 2003, in the United States, 844 million pounds of fresh mushrooms were sold for approximately $889 million, with the value of sales for specialty mushrooms being $37.1 million, at an average price per pound of $2.90 (USDA-NASS 2004b). Specialty mushrooms enjoy particularly large markets in countries such as Thailand, where the mushroom *Lentinus edodes* is imported at the rate of about $40 million each year (Thepa and others 1999).

Aside from their culinary appeal, mushrooms enjoy robust sales due in part to their inclusion in traditional Asian medicine (Lee and others 2003a; Tang and Zhong 2003). Recently, however, researchers have begun isolating specific bioactive compounds found in mushrooms so that their functional mechanisms can be more fully elucidated (Galán and others 1999; Sannia and others 1991; Sarkar and others 1997). Another feature of note is that mushrooms are able to utilize various chemicals and enzymes to penetrate and make use of almost any substrate as a growth medium (Carlile and others 2001). This ability has led to research where various pollutants and environmental toxins as well as agro-industrial wastes have been used as a growth substrate by various mushroom species (Amitai and others 1998; Fountoulakis and others 2002; Hatvani and Mécs 2001; Ruiz-Aguilar and others 2002).

Of specific interest is the mushroom *Lentinus edodes*, commonly known as the Shiitake mushroom. The perceived medical benefits associated with consuming this

mushroom have combined with its appealing taste to make it the second most popular edible mushroom in the global market (Hatvani 2001). Recent research into this mushroom, however, has revealed the presence of many bioactive compounds that function as host defense potentiators, antibacterials, anti-tumor agents, anti-HIV agents, and DNA topoisomerases (Hatvani 2001; Kono and others 1986; Liu and others 1998).

Research has shown that many of the beneficial bioactive compounds contained in mushrooms can be extracted from the mycelia of the species without waiting for a full fruiting body to develop (Hatvani and Mécs 2001). This is useful since mycelia can be produced much more quickly than fruiting bodies (Lee and others 2003a, 2003b). However, the efficacy of these compounds has also been shown to be directly affected by the culture conditions in which the mycelia were grown (Medeiros and others 1999; Morais and others 2002).

Since whey is an abundant resource waiting to be tapped, and since mushroom species are able to use virtually any organic substance as a growth substrate, the question is simply how best to combine the two in order to utilize an abundant resource to produce a valuable product. With these facts in mind, the purpose of this research was to use dry whey permeate as a growth medium for mycelia of the mushroom species *Lentinus edodes* and to find the combination of temperature, pH, and substrate concentration that resulted in optimal mycelial growth as determined by growth rate.

Materials and Methods

Cheese whey and microbial strain (preliminary experimentation)

Dried whey permeate powder (see composition information in Table 4) was purchased from Samik Co., Korea, and dissolved in distilled water in order to obtain differing concentrations (10, 20, 30, 40, 50, 60, 70, and 80 g dry powder per liter) of whey. Since the purpose of the research was to provide information about the treatment of raw cheese whey with mushroom mycelia, no additional nutrients were added. Commercial agar (Becton Dickinson and Co, Sparks, Md., U.S.A.) used for bacterial plate counts was added to the different concentrations at the rate of 1.5% w/v and the solutions were then mixed and autoclaved at 120°C for 20 min in order to ensure sterility. The solution was then poured into petri dishes and allowed to solidify. These dishes became the growth media for the mycelia used.

L. edodes (KCTC 6735) was obtained from the Korean Collection for Type Cultures (KCTC) and maintained in a potato dextrose agar (PDA) slant at 4°C. The seed culture of *L. edodes* was transferred to petri dishes containing PDA media and incubated at 25°C for 4 days. Mycelial agar discs (5mm) were then cut using a round cutter and used as inocula for subsequent experiments. After inoculation, the whey-containing petri dishes were placed into an incubator at 25 °C.

Data collection
(preliminary experimentation)

Petri dishes inoculated with growing mycelia were removed from the incubator every 24 hours for 10 days in order to collect growth data. Since the colonies grow in a circular fashion, the data was collected by using standard laboratory calipers to measure the diameter of each mycelial colony in mm as it was growing on the petri dish. The diameter was measured in two different places and the average of the two values was used as the size of the colony.

Analysis (preliminary experimentation)

The collected data were entered into Sigma Plot (version 6.0; Systat Software Inc, Richmond, Calif., U.S.A.) software and linear analysis produced an equation representing the slope of the growth curve generated by the data. The slope of this curve represented the growth rate of the mycelia and was assumed to be the maximum growth rate of the mycelia under the given conditions.

Cheese whey and microbial strain
(optimization experiment)

Dried whey permeate powder (see composition information in Table 4) was purchased from Samik Co., Korea, and dissolved in distilled water in order to obtain differing concentrations (40, 50, and 60 g dry powder per liter) of whey. Since the purpose of the research was to provide information about the treatment of raw cheese whey with mushroom mycelia, no additional nutrients were added. Commercial agar (Becton Dickinson and Co, Sparks, Md., U.S.A.) used for bacterial plate counts was

added to the different concentrations at the rate of 1.5% w/v and the solutions were then mixed and autoclaved at 120°C for 20 min in order to ensure sterility. Before pouring the solution into petri dishes, the pH was adjusted by addition of 0.5 M NaCl or 0.5 M NaOH as needed to meet the experimental parameters in Table 5. The solution was then poured into petri dishes and allowed to solidify. These dishes then became the growth media for the mycelia used.

L. edodes (KCTC 6735) was obtained from the Korean Collection for Type Cultures (KCTC) and maintained in a potato dextrose agar (PDA) slant at 4°C. The seed culture of *L. edodes* was transferred to petri dishes containing PDA media and incubated at 25°C for 4 days. Mycelial agar discs (5mm) were then cut using a round cutter and used as inocula for subsequent experiments. After inoculation, the whey-containing petri dishes were placed in incubators at different temperatures according to the design listed in Table 5.

Data collection (optimization experiment)

Petri dishes inoculated with growing mycelia were removed from the incubator every 24 hours for 10 days in order to collect growth data. Since the colonies grow in a circular fashion, the data was collected by using standard laboratory calipers to measure the diameter of each mycelial colony in mm as it was growing on the petri dish. The diameter was measured in two different places and the average of the two values was used as the size of the colony.

Analysis (optimization experiment)

The collected data were entered into Sigma Plot (version 6.0; Systat Software Inc, Richmond, Calif., U.S.A.) software and linear analysis produced an equation representing the slope of the growth curve generated by the data. The slope of this curve represented the growth rate of the mycelia and was assumed to be the maximum growth rate of the mycelia under the given conditions. These rates were then entered into EChip (version 7.01; ECHIP Inc, Hockessin Dela., U.S.A.) software along with their corresponding environmental conditions in order to perform analysis according to response surface methodologies (RSM) and obtain the set of conditions that would maximize the mycelial growth rate.

RSM analysis involves employing a collection of mathematical and statistical techniques in situations where several independent variables influence a dependent variable or response, and the goal of the analysis is to maximize the response of the dependent variable. The mushroom mycelia experiment was designed after the model of a 2 X 3 orthogonal design (pH, temperature, and substrate concentration at two levels each) with all points being done in triplicate, and the center points of the design being replicated 5 times as previously described (Adinarayana and others 2003; Hwang 1995; Lee and others 2003a, 2003b). This type of design was used to minimize the number of trials needed to obtain statistically relevant results. See Table 5 for the values used in this experiment.

Results and Discussion

RSM analysis requires that the researcher must first obtain estimate values for the variables in question. These initial estimates are then used in the model and experimentation as a starting point for analysis. It is of great importance to have good estimates because good estimates result in more rapid determination of peak criteria. For this experiment, estimates of temperature and pH for growth of *L. edodes* were based on values that have been used for the cultivation of this mushroom in the past (Hatvani 2001; Kono and others 1986; Ruiz-Aguilar and others 2002). An estimate for concentration of substrate (whey permeate) was not available since this substrate has not previously been used for the cultivation of mushroom mycelia.

In order to obtain an estimate for the substrate concentration to use in the experimental design, we performed some preliminary experiments testing the growth rate of *L. edodes* in various concentrations of whey permeate. This was accomplished by preparing petri dishes with various concentrations of the reconstituted dry whey permeate substrate as described. The growth of *L. edodes* was then observed and recorded to provide a measure of its ability to utilize the substrate.

It was expected that the mycelia would exhibit some form of substrate inhibition. Specifically, it was suspected that increasing substrate concentrations would yield higher growth rates up to some maximum substrate concentration, at which point higher concentrations of substrate would actually begin to inhibit the growth of *L. edodes*. This type of substrate inhibition is commonly modeled with the Haldane equation (Shi and

others 1999). However, modeling the response with this equation produced unsatisfactory results (Figure 6). Therefore, it was necessary to use a different equation to model the effect of substrate inhibition on the mushroom mycelia. We employed a new equation suggested by Shi and others (1999) with very satisfactory results (Figure 7). The r-squared value for this modified equation was 0.95 and the standard error of estimate was 0.11. Analysis of model terms was significant for all terms at $p=0.05$. Thus, we concluded that this model adequately described the substrate inhibition effect of whey permeate on mycelia of the mushroom *L. edodes*. Based on this equation, we assumed that a concentration of 50 g/L (reconstituted as described above) was the substrate concentration that would maximize mycelial growth rate. This value was used as the initial estimate for RSM model building and analysis.

A total of 15 trials were run to approximate the response surface for the mycelial production of *L. edodes*. In order to find a maximum response (highest mycelial growth rate), increasingly complex equations from linear to partial cubic were sequentially tested to model the data obtained from the trials in Table 5. When using RSM analysis, the maximum response is determined by setting the partial derivatives of the modeling equation to zero with respect to the independent variables. This determines the point where responses are no longer increasing or decreasing: they have leveled off at either a maximum or minimum value. This type of modeling must be done with a higher order equation that can model the curvature created when the response levels off at maximum or minimum value.

When the data were analyzed using a partial cubic model, the p-value of regression was significant at the 1% α level and lack of fit was not significant at the 5% α level. The regression coefficient and residual standard deviation of the partial cubic model were 0.99 and 0.16, respectively. This indicated that curvature existed within the defined response surface and that the partial cubic model was an accurate representation of the data. Therefore, this equation was used to create the response surface and determine a set of conditions that would maximize mycelial growth. The conditions determined as providing maximal mycelial growth rate were 40 g/L substrate concentration, pH 4.97, and temp 23.6 °C. Maximum and minimum growth rates under these conditions were estimated to be 6.88 and 5.94 mm/d, respectively.

Two- and three-dimensional representations of the response surface generated by analysis with Echip are shown in Figure 8a and 8b. These surfaces show a clear peak and constant contour lines, suggesting that the independent variables are not interdependent. Statistical analysis of the variables involved in the model show that the individual variables are highly statistically significant ($p<0.0001$). This was expected and reflected by the fact that the research was designed to identify optimal values for each of the variables simultaneously. The three-way interaction of the variables was not significant at the 5% α level, suggesting that all three variables were not simultaneously interdependent. Of the three possible two-way interactions among the variables, two of the interactions (whey concentration×pH and pH×temperature) were not significant at the 5% α level. The third interaction, whey concentration×temperature, was significant at the 5% α level (p value 0.013). This suggests an interaction where the values obtained for

concentration and temperature will vary based on the values of one another. This relationship can be seen in Figure 9. The relationship of the non-significant interactions may be seen in Figure 14 and 15 (Appendix). In Figure 9 the interaction between the variables manifests itself in the form of the saddle shape the graph displays. As implied by the shape, there is more than one region where a local maximum may be achieved. However, one of these points could be more desirable than another based on factors such as ease of maintaining the given conditions. Researchers should be mindful of this interaction whenever *L. edodes* is grown on reconstituted dry whey permeate.

In order to verify the accuracy of the model predictions, an additional 5 trials of the experiment were run under the optimal growth conditions predicted by the model (temperature 23.6 °C, pH 4.97, and 40g/L substrate concentration). These trials resulted in an average growth rate of 6.39 mm/d with a standard deviation of 0.22. This average growth rate was larger than any rate observed in the initial experimental trials. Since the growth rate fell in the expected region, and the standard deviation in the results was small, it was concluded that the model was able to accurately predict optimal growth conditions for *L. edodes* mycelia when grown using reconstituted dry whey permeate as a growth substrate.

Conclusions

It is readily apparent that growing mycelia of the mushroom *L. edodes* exhibit a growth pattern that is affected by substrate inhibition. However, this inhibition cannot be satisfactorily modeled by conventionally used equations. A slightly modified substrate

inhibition equation gives satisfactory modeling of the behavior of the growing mycelia and allows for accurate predictions to be made regarding the effect of substrate concentrations on the mycelia.

RSM analysis designed to optimize substrate concentration, pH, and temperature during the cultivation of *L. edodes* mycelia is effective in providing statistically sound conclusions. For the growth of *L. edodes* on reconstituted dry whey permeate, these conditions were determined to be 40 g/L substrate concentration, pH 4.97, and temp 23.6 °C. However, researchers should be aware that the variables of substrate concentration and temperature exhibit interdependence and should therefore not be analyzed independently of one another.

The optimal conditions determined by this experiment are valid for mycelia of the edible mushroom *L. edodes* when grown on reconstituted dry whey powder in petri dishes. The results of this experiment suggest that whey permeate could provide a viable growth substrate for the commercial cultivation of *L. edodes* mycelia. Before these results may be applied in a full scale industry, however, they should be tested in small reactors in order to determine that the optimal conditions are the same if the mycelia are grown in a liquid substrate. It could then be possible to scale up the fermentors and make use of liquid whey permeate on a large scale.

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Table 4: Representative values of the average composition of rehydrated whey and whey permeate powders. The values are based on a reconstitution ratio of 40 g day powder per liter.

pH = 6.5 (whey powder)
pH = 5.8 (whey permeate powder)

	Whey	Whey Permeate		Whey	Whey Permeate
Parameter	mg/L	mg/L	Parameter	mg/L	mg/L
Total Solids	44,120	46,320	Ammonium	47.5	0
Total Suspended Solids	3,160	2,053	Potassium	905.3	16,852
Total Volatile Solids	40,280	39,812	Magnesium	129.7	572
Volatile Suspended Solids	2,840	1,652	Calcium	43.6	2.240
Total Chemical Oxygen Demand	47,844	38,286	Nitrite	1.6	21
Carbohydrate	39,577	35,087	Nitrate	23.2	26
Protein	4,181	3,209	Total Nitrogen	698	514
Sodium	269.5	4,711	Phosphate	370	202
Chloride	657.5	266	Sulphate	50.8	40

Table 5: Values used in the design of the response surface methodology experiment to determine optimal growth conditions for *Lentinus edodes* when grown on reconstituted dry whey permeate. The linear design section includes values for trials used to fit a linear model. The quadratic design section includes values for trials used in combination with the linear trials to fit the data to a modified partial cubic model. The final column lists the calculated maximum growth rate attained for each trial. These values were used for the RSM analysis of the experiment.

Experimental design & radial growth rate					
	Trial	Independent Variables			Growth Rate mm/day (Collected Data)
		Dry Whey Permeate Concentration (g dry powder/l)	Media pH	Incubation Temp °C	
Linear Design	1	40	4	20	2.6
	2	60	4	20	1.2
	3	40	6	20	1.5
	4	60	6	20	0.8
	5	40	4	30	0.5
	6	60	4	30	0.5
	7	40	6	30	0.3
	8	60	6	30	0.1
	9	50	5	25	3.8
	9	50	5	25	3.7
	9	50	5	25	4.1
	9	50	5	25	4.0
	9	50	5	25	3.9
Quadratic Design	10	40	5	25	6.3
	11	60	5	25	4.1
	12	50	4	25	6.1
	13	50	6	25	2.5
	14	50	5	20	3.7
	15	50	5	30	1.1

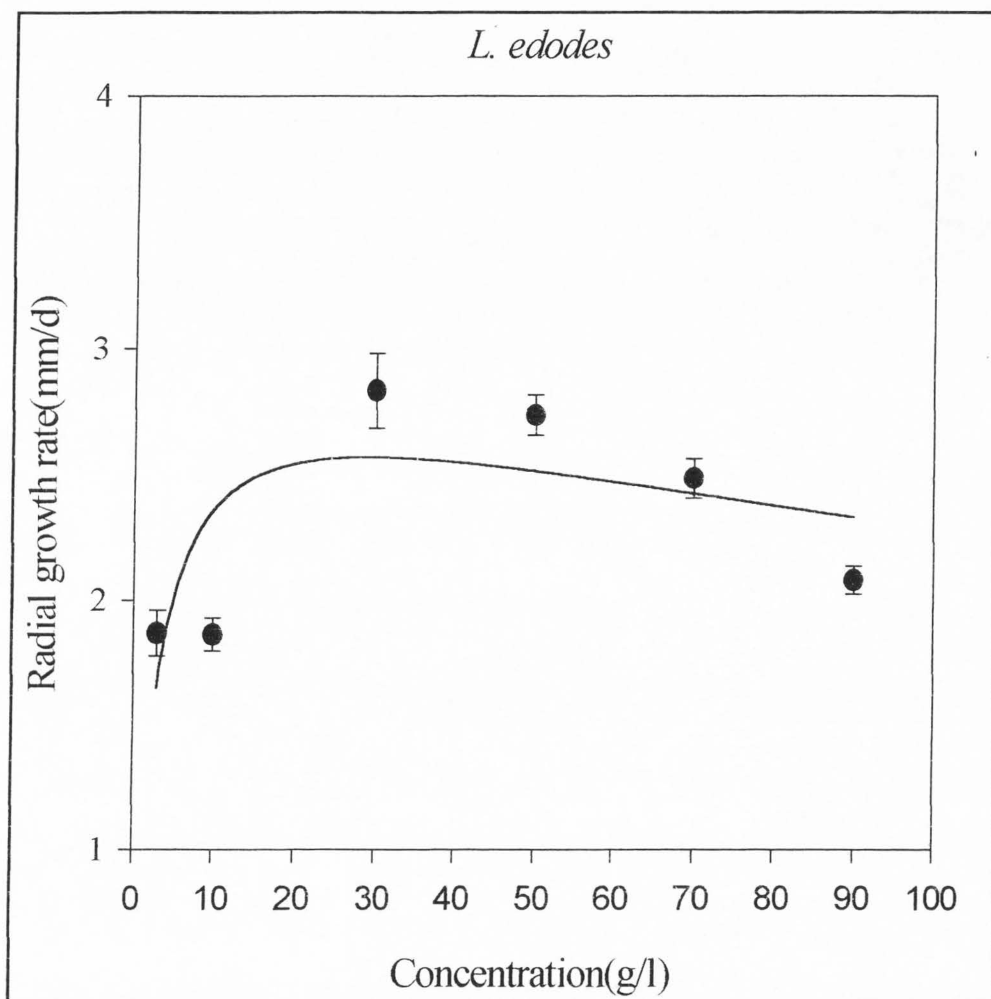

Figure 6 - The maximal radial growth rate of *Lentinus edodes* grown on reconstituted dry whey permeate decreases at high concentrations of the growth substrate. The nature of this inhibition is modeled here using the Haldane equation (Shi and others 1999).

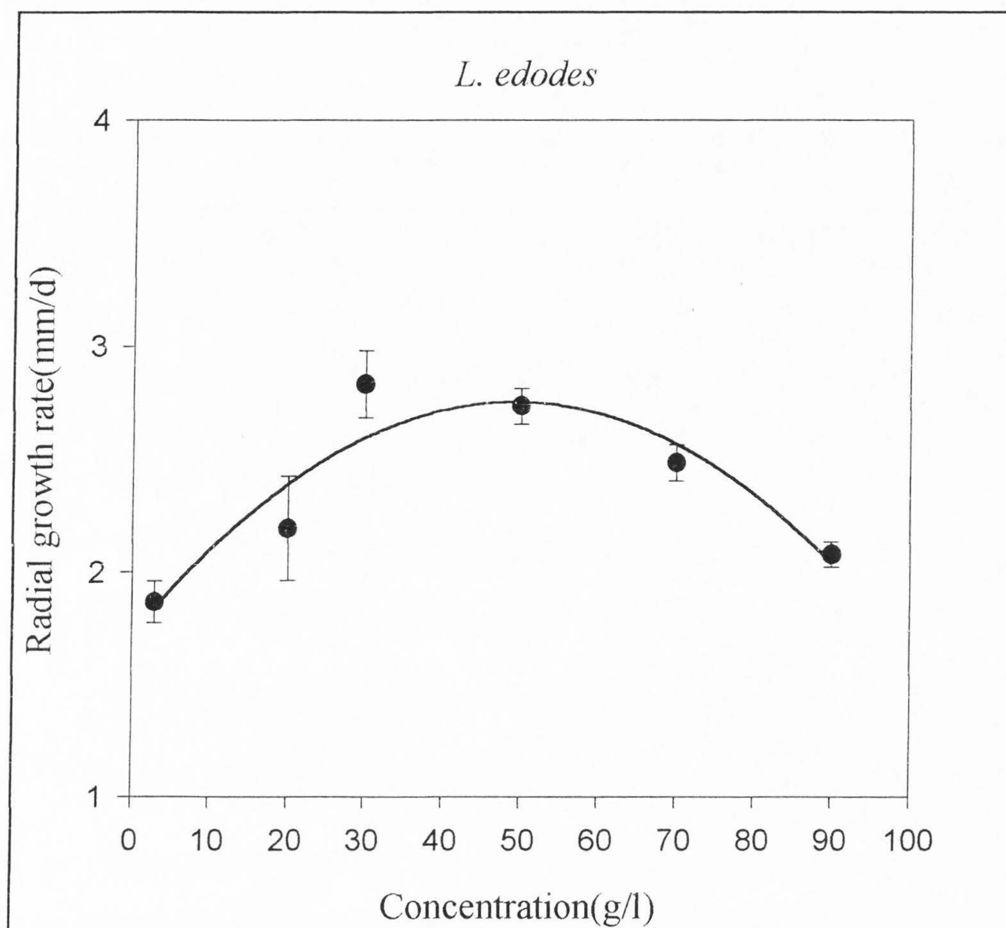

Figure 7 - Accurate modeling of the nature of the substrate inhibition exhibited by *Lentinus edodes* grown on reconstituted dry whey permeate requires the use of a different modeling equation. The nature of this inhibition is modeled here using the revised equation proposed by Shi and others (1999).

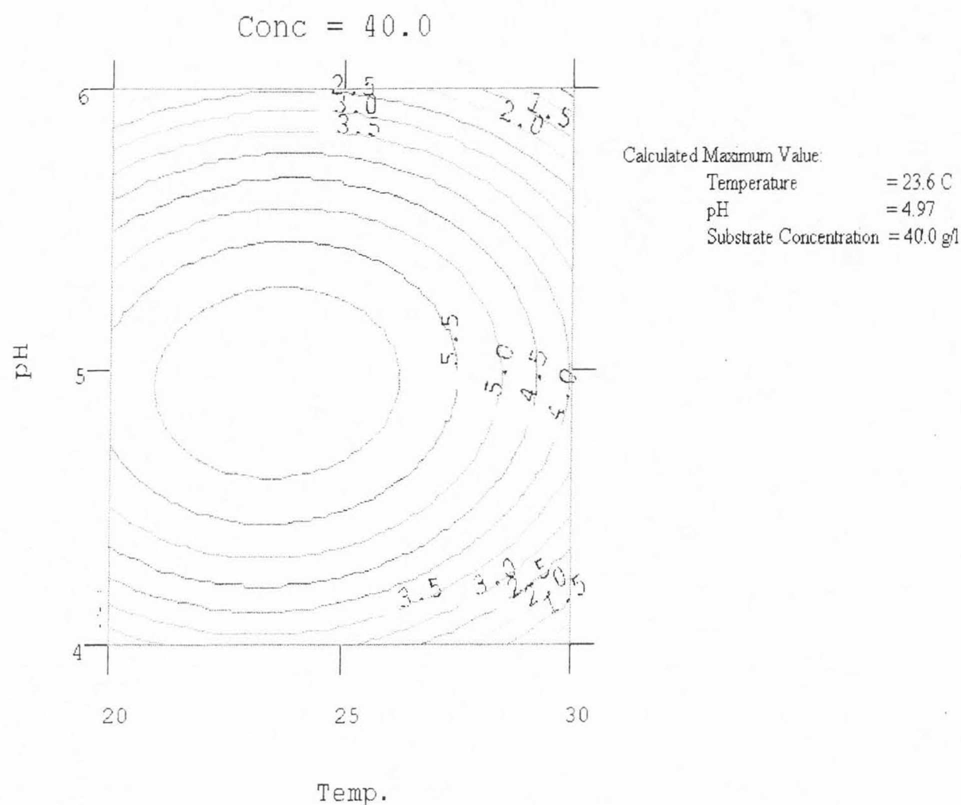

Figure 8a - Two-dimensional representation of the response surface generated to describe the conditions resulting in the maximal mycelial growth rate for *Lentinus edodes*. Values at the growth maximum are given. The graph is read like a topographical map, with the concentric circles representing increasing values for the maximal growth rate. At the maximal growth conditions given, the value for growth rate becomes 6.39 mm/day.

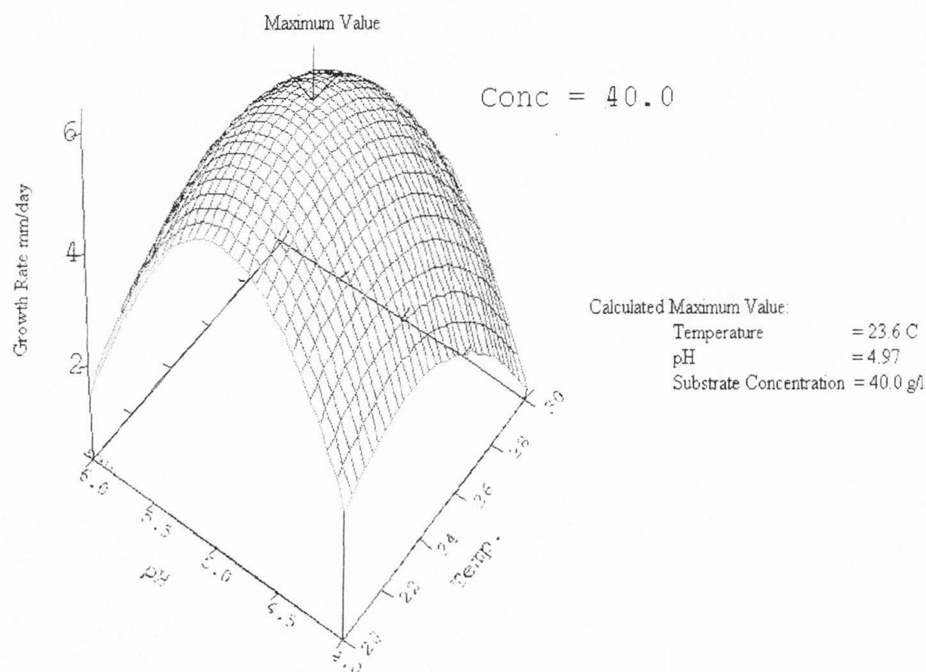

Figure 8b - Three-dimensional representation of the response surface generated to describe the conditions resulting in the maximal mycelial growth rate for *Lentinus edodes*. Values at the growth maximum are given. The three variables of substrate concentration, temperature, and pH form the base of the graph as it peaks toward a maximal value of 6.39 mm/day.

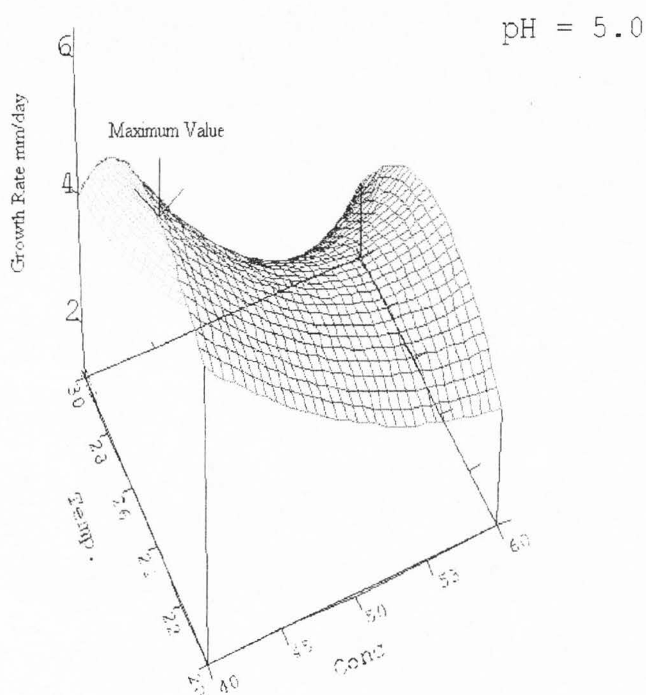

Figure 9 - Three-dimensional representation of the response surface showing the interaction between the variables of temperature and substrate concentration. The interaction of these variables creates a "saddle point" in the response surface. These points result in multiple local maxima. Careful analysis of the design and application of the research will designate which maximum is more acceptible to the researcher.

CHAPTER III

CULTIVATION OF *LENTINUS EDODES* MYCELIA USING DELACTOSED CHEESE WHEY PERMEATE AS AN ALTERNATIVE GROWTH SUBSTRATE[2]

Abstract

A novel approach to utilize delactosed cheese whey permeate was introduced by cultivating mycelia of the edible mushroom *Lentinus edodes* using delactosed cheese whey permeate as a growth substrate. The mycelium were grown in petri dishes containing solidified substrate with various concentrations of delactosed cheese whey permeate, and their growth patterns were observed. The delactosed whey permeate proved to be a viable substrate for the cultivation of the mycelium. Relatively low concentrations of the substrate yielded the most satisfactory results, while high concentrations of the substrate proved lethal to the growing mycelia. Further studies could focus on optimization techniques for higher yield and on exploring the fungicidal properties of delactosed whey permeate for possible future application in the food industry.

Introduction

Whey is the serum portion of milk, containing 4-5% lactose, that remains after most milk proteins have been removed during the cheese making process. Depending on the type of cheese being manufactured, as much as 9L of whey can be generated for every

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[2]Coauthored with Conly Hansen, Professor, Utah State University

kilogram of cheese produced (Lee and others 2003a, 2003b). Traditionally, this by-product has been considered to be waste, and processing plants have sought the most economical means to dispose of it (Smithers and others 1996). More recently, however, innovative technologies have been developed to recover the valuable nutrients of whey, notably proteins and lactose (Cifuentes and others 1993; Xiong 1992). A popular treatment of whey now is to recover the salable proteins it contains by means of a filtration process. The whey remaining from this process is referred to as whey permeate. In an attempt to further utilize whey permeate, producers often recover the salable lactose it contains. This is often done by the addition of salts to the whey, causing the lactose to precipitate out of solution. The product left after this treatment is called "delactosed" whey (it still contains significant portions of lactose). As producers attempted to introduce whey and whey products into the food processing industry, global trade in whey increased over 12% a year from the years 1995-2000 (Voorbergen and Zwanenberg 2002). In 2003 in the United States alone, 84.1 million pounds of dry whey were produced by cheese manufacturing plants and were sold for about $0.16 per pound (USDA-NASS 2004a).

With increasing global trade in whey, however, there still exists a supply and demand problem: there is more supply than demand. As of 1997, only 55% of the total whey produced in the US was being further used in food applications (Anon 1997). Whey is rich in carbohydrate, protein, and trace mineral content, including approximately half of the original nutrients of milk (Lee and others 2003b). With so many available

nutrients, whey and whey products should be considered as growth media for economically valuable products.

In 2003, in the United States, 844 million pounds of fresh mushrooms were sold for approximately $889 million, with the value of sales for specialty mushrooms being $37.1 million, at an average price per pound of $2.90 (USDA-NASS 2004b). Mushrooms enjoy an even larger market outside of the United States. China produces many times the amount of mushrooms each year as the United States. Specialty mushrooms enjoy particularly large markets in countries such as Thailand, where the mushroom *Lentinus edodes* is imported at the rate of about $40 million each year (Thepa and others 1999).

Aside from their culinary appeal, mushrooms enjoy robust sales due in part to their inclusion in traditional Asian medicine (Lee and others 2003a; Nakamura and others 2002; Tang and Zhong 2003). Recently, however, researchers have begun isolating specific bioactive compounds found in mushrooms so that their functional mechanisms can be more fully elucidated (Baldrian and Gabriel 2003; Sannia and others 1991; Sarkar and others 1997). Another feature of note is that mushrooms are able to utilize various chemicals and enzymes to penetrate and make use of almost any substrate as a growth medium (Carlile and others 2001; Deacon 1980). This ability has led to research where various pollutants and environmental toxins as well as agro-industrial wastes have been used as a growth substrate by various mushroom species (Fukushima and others 1993; Hatvani and Mécs 2001; Kang and others 1993; Yildiz and others 2002).

Of specific interest is the mushroom *Lentinus edodes*, commonly known as the Shiitake mushroom. The perceived medical benefits associated with consuming this

mushroom have combined with its appealing taste to make it the second most popular edible mushroom in the global market (Hatvani 2001). Recent research into this mushroom, however, has revealed the presence of many bioactive compounds that function as host defense potentiators, antibacterials, anti-tumor agents, anti-HIV agents, and DNA topoisomerases (Hatvani 2001; Kono and others 1986; Liu and others 1998).

Research has shown that many of the beneficial bioactive compounds contained in mushrooms can be extracted from the mycelia of the species without waiting for a full fruiting body to develop (Hatvani and Mécs 2001). This is useful since mycelia can be produced much more quickly than fruiting bodies (Lee and others 2003a, 2003b). However, the efficacy of these compounds has also been shown to be directly affected by the culture conditions that the mycelia were grown in (Kang and others 1993; Medeiros and others 1999; Morais and others 2002).

Since whey is an abundant resource waiting to be tapped, and since mushroom species are able to use virtually any organic substance as a growth substrate, the question is simply how best to combine the two in order to utilize an abundant resource to produce a valuable product. With these facts in mind, the purpose of this research was to explore the possibility of using delactosed whey permeate as a growth substrate for the mycelium of the edible mushroom *Lentinus edodes*.

Materials and Methods

Cheese whey and microbial strain

Liquid delactosed whey permeate was obtained frozen from Glanbia Foods, Inc., in Gooding, Idaho. It was stored frozen and then allowed to thaw completely in a refrigerator (40 °F) for 3 days prior to use. *L. edodes* (ATCC 20635) was obtained from the American Type Culture Collection (ATCC) and maintained in a potato dextrose agar (PDA) slant at 4 °C. The seed culture of *L. edodes* was transferred to petri dishes containing PDA media and incubated at 25 °C for 4 days. Mycelial agar discs (5mm) were then cut using a round cutter and used as inocula for subsequent experiments.

Preparation of the petri dishes was as follows: delactosed whey permeate was mixed with distilled water to create six different concentrations of substrate on which to test mycelial growth (100% v/v, 80% v/v, 60% v/v, 40% v/v, 20% v/v, 10% v/v). Standard bacterial plate count agar (Becton Dickinson and Co, Sparks, Mary., U.S.A.) was added at 1.5% w/v to create a solid media. The solutions were then autoclaved at 120 °C for 20 min to ensure sterility. The solutions were then poured into sterile petri dishes and allowed to solidify, after which time they were inoculated with an agar disc cut from the seed culture grown on PDA media. Six separate petri dishes were prepared and utilized for each substrate concentration. After inoculation, the whey-containing petri dishes were placed in an incubator at 30 °C.

Data collection

Petri dishes inoculated with growing mycelia were removed from the incubator every 24 hours for 8 days in order to observe the colonies and collect growth data. Since the colonies grow in a circular fashion, the data was collected by using a standard ruler delineated in millimeters to measure the diameter of each mycelial colony as it was growing on the petri dish. The diameter was measured in two different places and the average of the two measurements was used as the size of the colony in mm. Comparison of day to day averages provided the data necessary to estimate the growth of the colonies in mm per day.

Analysis

Each different concentration of substrate was observed for growth of *L. edodes* mycelia. The collected data were entered into Sigma Plot (version 7.0; Systat Software Inc, Richmond, Calif., U.S.A.) software and linear analysis produced an equation representing the slope of the growth curve generated by the data. The slope of this curve represented the growth rate of the mycelia and was assumed to be the maximum growth rate of the mycelia when grown under the given conditions.

Results and Discussion

Previous experience with mycelia in this laboratory have shown that it takes about 3 days to be able to tell if a growth substrate will support the growth of a particular strain of mushroom mycelia. Before that time, the mycelia can actually live using the nutrients

contained in the agar disc they were transplanted on. In the case of some substrates, after the mycelia have exhausted the resources in the disc, they are not able to utilize the substrate they were planted on and cease growing. They enter a dormant stage and wait for a more favorable growth environment.

In the case of delactosed whey permeate, within 24 hours after innoculation of the petri dishes, the pellets planted in the three highest concentrations (100%, 80%, and 60%) had died. The mycelia had not entered a dormant waiting stage. They had completely and quickly been killed (Figure 10). This was evidenced by the fact that the live mycelia visible on the innoculation plug had disappeared. However, the mycelia planted in the low concentrations of delactosed whey permeate (10%, 20%, and 40%) were showing typical signs of slow growth as the mycelia reached toward the substrate.

Thinking at this time that perhaps delactosed whey permeate could be too acidic for the growth of mushroom mycelia, a pH reading was taken of the original full strength delactosed whey permeate. The sample had a pH of 4.98, which is within the pH growth range of *L. edodes* that has been observed previously in this lab (data not published), indicating that the pH of the substrate was not too low to support growth of the mycelia.

After 8 days of observation, it was clear that the mycelia of *L. edodes* could grow on the lower concentrations of delactosed whey permeate (Figure 11). Having established that delactosed whey permeate could be used as a growth substrate, the data collected during the experiment (see Table 6 and Figure 13, Appendix) were then analyzed using Sigma Plot in order to ascertain the growth rates of the mycelia (Figure 12). These data plainly showed that *L. edodes* could grow quite rapidly at low concentrations of

delactosed whey permeate, but that at high concentrations the substrate actually became lethal to the mycelia.

Conclusions

This pilot study suggests that it would be possible to utilize delactosed whey permeate as a growth substrate for the cultivation of mycelia from the edible mushroom *Lentinus edodes*. These mycelia could then find application in many food products or health treatments. In order to make the process useful on a large scale, further optimization tests, such as those performed by Lee and others (2003a), should be performed in order to discover ideal growth conditions for the mycelia. The fact that high concentrations of delactosed whey permeate proved to be lethal to the mycelia suggests that perhaps there are compounds contained in delactosed whey permeate that could be extracted and used commercially as a fungicide. This avenue of study could prove to be useful in many aspects of the food manufacturing process since whey and whey products have not been shown to be harmful to human health. Also, since mycelia from the mushroom *L. edodes* were able to utilize delactosed whey permeate as a growth substrate, further study could reveal that it is possible to cultivate other mushrooms using delactosed whey permeate as a growth medium.

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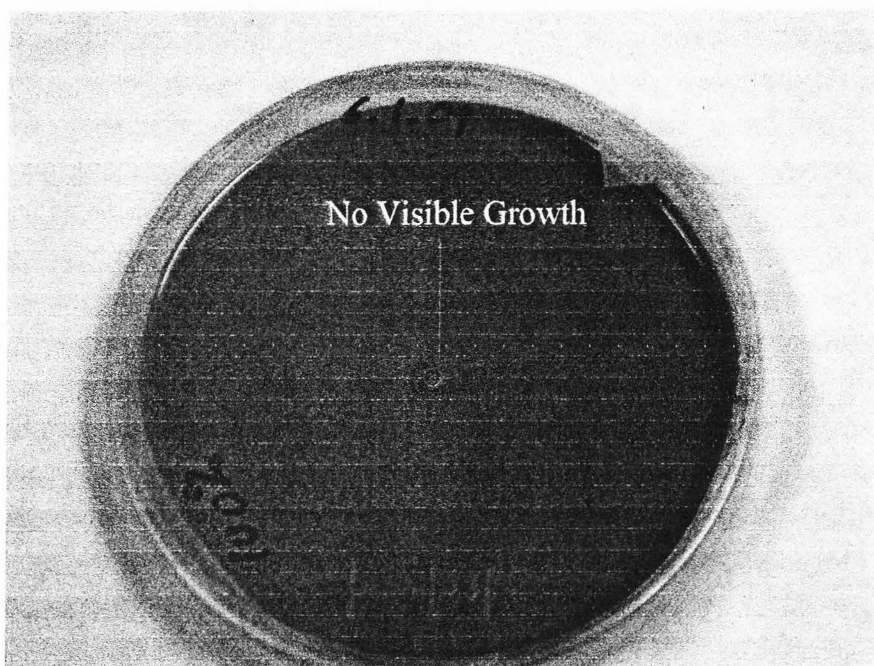


Figure 10 - The result of incubating mycelia from the mushroom *Lentinus edodes* in 100% v/v delactosed whey permeate for eight days. No new growth is visible, and the original growth present on the innoculation plug has disappeared. This suggests the delactosed whey permeate has exhibited a fungicidal effect at this concentration.

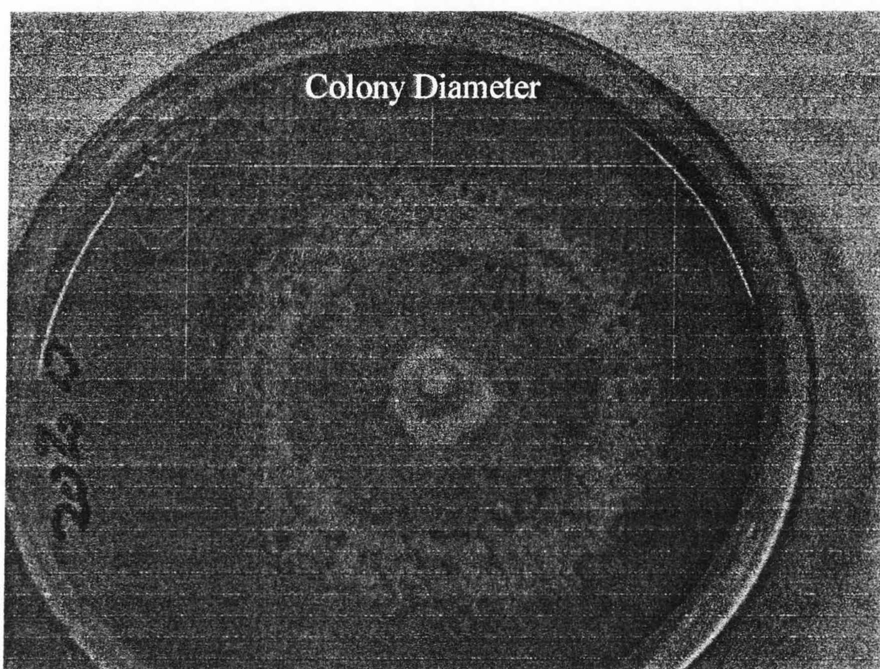


Figure 11 - The result of incubating mycelia from the mushroom *Lentinus edodes* in 20% v/v delactosed whey permeate for eight days. New growth is visible and abundant. This suggests that delactosed whey permeate could be an effective growth substrate for the growth of *L. edodes* at this concentration.

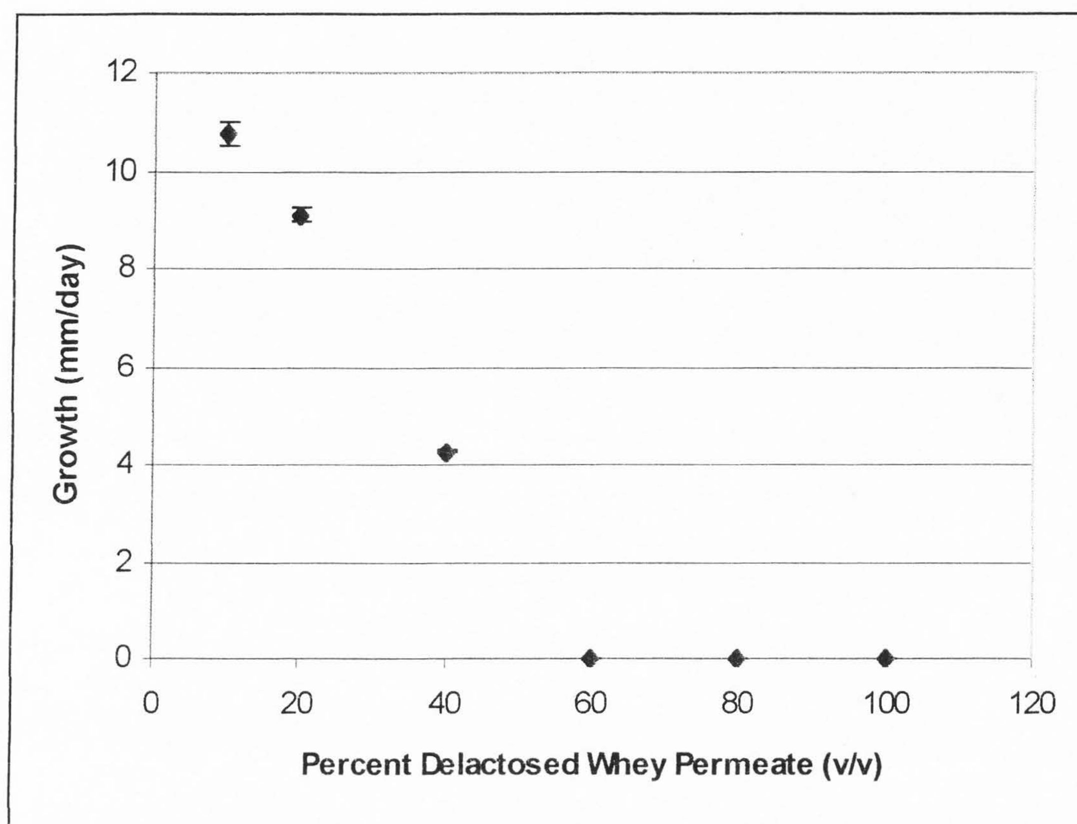


Figure 12 - Maximum mycelial growth rate for *Lentinus edodes* grown in six different concentrations of delactosed whey permeate. Values at each concentration were obtained by averaging the results of six replicates. These data suggest that low concentrations of delactosed whey permeate promote the growth of *Lentinus edodes* whereas high concentrations prevent growth.

CHAPTER IV

GENERAL SUMMARY AND CONCLUSIONS

This research was concerned with investigating these two objectives:

1. To utilize solid state (i.e. experimental media has been solidified through the addition of agar) fermentation experiments carried out on reconstituted dry whey permeate in order to determine the optimal growth conditions for mycelia of the edible mushroom *Lentinus edodes* with regard to substrate concentration, temperature, and pH.
2. To determine if delactosed whey permeate could also be used as a substrate for mushroom mycelia cultivation.

In regard to the first objective, this research revealed two points that are of importance to this research in particular, and the study of mushroom mycelia cultivation in general:

a. It was discovered that growing mushroom mycelia exhibit signs of substrate inhibition that are commonly seen during the growth of microorganisms or while studying enzyme activity. Normally, the effects of substrate inhibition can be accurately modeled by a commonly applied equation known as the Haldane equation. However, the inhibition pattern exhibited by the mycelia was not modeled accurately by this equation. For this reason, it was necessary to use a modified equation that better described the effects of substrate inhibition on growing mycelia. While

these results only apply specifically to mycelia of the species *L. edodes* when they are grown in reconstituted dry whey permeate, they still serve as a signal to all scientists working with mushroom mycelia that it could be necessary to use an alternative model in order to obtain accurate representations of mycelial growth patterns.

b. Response surface methodology (RSM) was successfully applied to analyze the growth of the mycelia grown in reconstituted dry whey. This analytical method was able to coordinate values of three different independent variables in order to maximize a growth response with a very small number of experimental trials (15). Analysis by RSM revealed that the optimal growth conditions for *L. edodes* when grown on reconstituted dry whey permeate were 40 g/L substrate concentration, pH 4.97, and temperature 23.6 °C.

In regard to the second objective, it was discovered that mycelia of the mushroom *L. edodes* could effectively be grown using delactosed whey permeate as a growth substrate. This information could be of special importance to the dairy industry as it could provide a new avenue for the disposal of a prevalent waste product. The mycelia grew best at low concentrations of the substrate, so plans to utilize delactosed whey permeate on a large scale would have to take into account that the substrate could not be utilized at full strength. However, delactosed whey permeate also exhibited a surprise effect on the mushroom mycelia. It was expected that high concentrations of the substrate would cause the mycelia to grow extremely slowly. During experimentation, however, it was observed

that high concentrations of substrate were actually lethal to the growing mycelia. This result suggests that delactosed whey permeate contains chemicals and properties that could allow it to function as a natural fungicide. Further research into this area could reveal areas where the natural fungicidal properties of delactosed whey permeate would be of use to the food processing industry.

APPENDIX

Table 6: Data collected during the growth of *Lentinus edodes* on delactosed whey permeate. Six plates (A to F) were prepared containing the indicated amount of substrate (10%, 20%, or 40% delactosed whey permeate v/v) and were innoculated with *Lentinus edodes*. The diameter of the colonies in each plate was measured twice daily, and these measurements were used to create the average colony size in mm. Differences in average size between days was used to calculate the growth rate.

10 % Delactosed whey permeate (v/v)

Day	Plate A		Plate B		Plate C		Plate D		Plate E		Plate F		Average
	1	2	1	2	1	2	1	2	1	2	1	2	(mm)
1	5	5	5	5	5	5	5	5	5	5	5	5	5.0
2	8	8.5	7	7	8	7.5	8.5	8	7	8	8	7	7.7
3	8	9	8	7.5	7.5	8	9	7.5	7.5	9.5	8	9	8.2
4	39	37	36	34.5	40	39	38	38	38.5	38	41	41	38.3
5	49.5	50	45	47	50	51	50.5	48	49	47	51	49	48.9
6	61	62	56	57	59	62	58	56	59	60	59	60	59.1
7	70	72	68	68	74	74	74	72	72	73	68	65	70.8

20 % Delactosed whey permeate (v/v)

Day	Plate A		Plate B		Plate C		Plate D		Plate E		Plate F		Average
	1	2	1	2	1	2	1	2	1	2	1	2	(mm)
1	5	5	5	5	5	5	5	5	5	5	5	5	5.0
2	8	7.5	9	9	8	9	9.5	9.5	9	9	8	8	8.6
3	10.5	9.5	10	9	11.5	10.5	10	10	9	10	10.5	11	10.1
4	35	34	30	29	28	28	30	31	32	33	25	24.5	30.0
5	42	43	39.5	39	36	38	41	40	41	42	34	35	39.2
6	51	51.5	47	46	48	46	48	47.5	49	49.5	42	41	47.2
7	62	60	56	56	57	57	59	60	60	60	51	51	57.4
8	68	68.5	66	66	65	66	68	68	70	71	61	59	66.4

40 % Delactosed whey permeate (v/v)

Day	Plate A		Plate B		Plate C		Plate D		Plate E		Plate F		Average
	1	2	1	2	1	2	1	2	1	2	1	2	(mm)
1	5	5	5	5	5	5	5	5	5	5	5	5	5.0
2	6.5	6	7	6	6	7	7	7	7	7	7.5	7.5	6.8
3	10	10	10	10	9.5	9.5	9.5	10	10	10	10.5	10	9.9
4	11	10	10.5	10	10	10	10	10	10	9	10	9.5	10.0
5	14	15	17	16	14	14.5	16.5	18	16	15	17	16	15.8
6	19	19	21	22	19	19.5	21	20.5	21	19	21	20	20.2
7	23	23	25	25	24	24	26	25	23	24	25	25	24.3
8	27.5	27	29	28	27	28	30	30	28	29	29.5	30	28.6

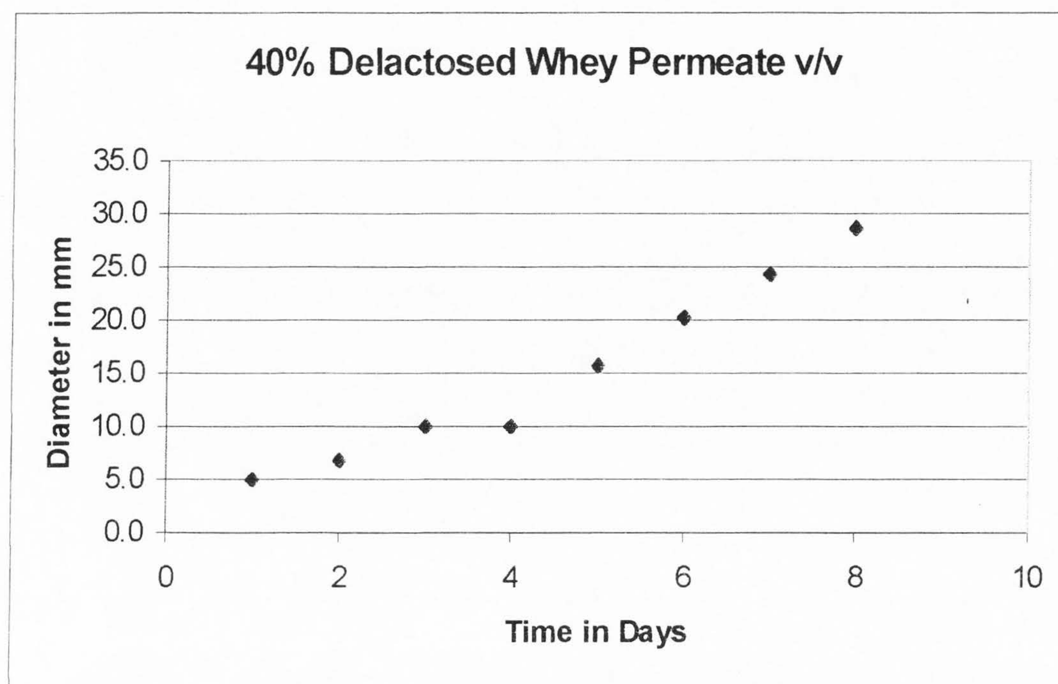
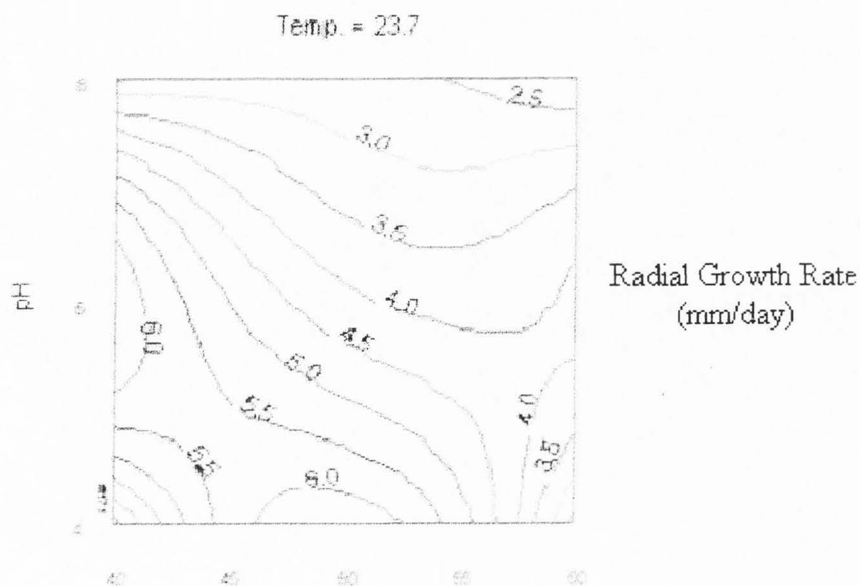


Figure 13 - Graphical representation of the data collected during the growth of *Lentinus edodes* on 40% delactosed whey permeate v/v. The mycelial colonies exhibit a "lag" growth phase where erratic growth is apparent while they adjust to the new growth substrate. This is indicated by the portion of the graph where the diameter does not increase at a steady rate. After the mycelia became adjusted to the growth substrate, they exhibited a constant increase in size. This constant increase represents the maximal myclial growth rate and is shown by the portion of the graph that forms a straight line with a steadily increasing slope.



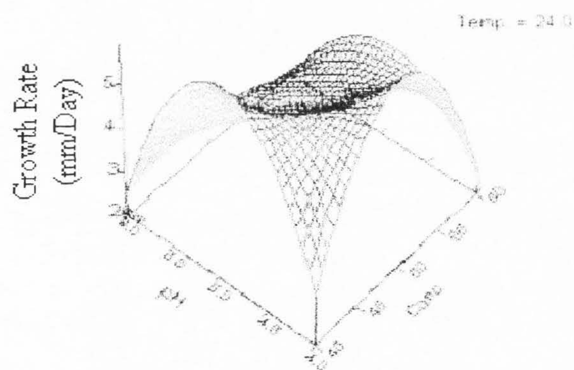
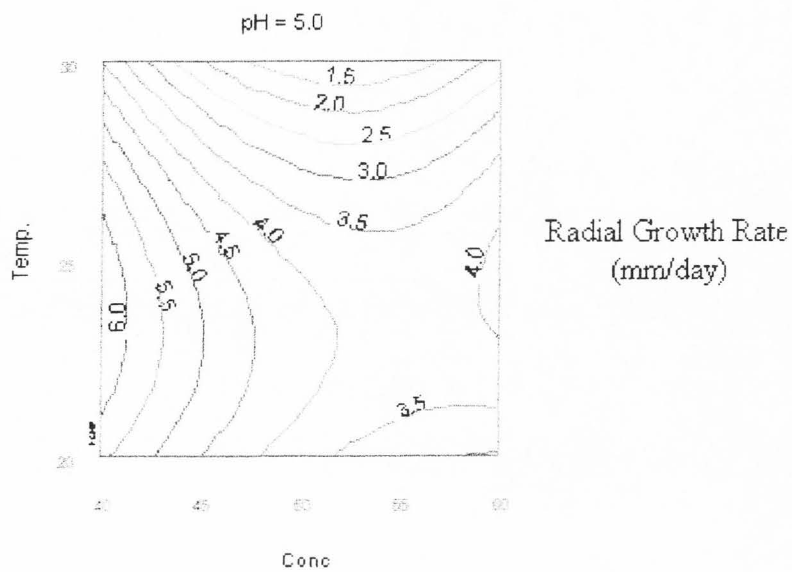


Figure 14 - Two and three-dimensional RSM displays for the growth of *Lentinus edodes* on whey permeate when temperature remains fixed. The pH and substrate concentration are allowed to vary.



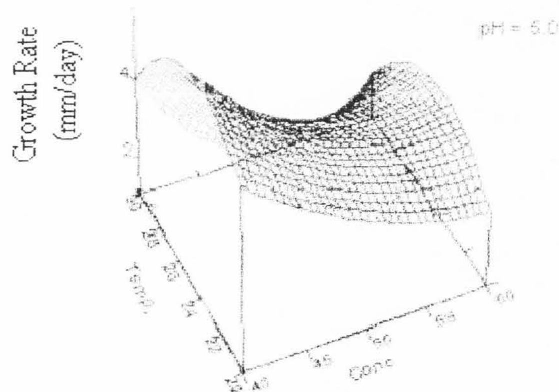


Figure 15 - Two and three-dimensional RSM displays for the growth of *Lentinus edodes* on whey permeate when the pH remains fixed. The temperature and substrate concentration are allowed to vary.