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MEASUREMENT OF FUNGAL BIOMASS IN WOODEN ART OBJECTS USING ATP PHOTOMETRY AND ENERGY CHARGE

by

A

Fernando Nieto

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Ann Arbor, MI 48103

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20 July 1995
Date

John J Lee
Chairman of Examining Committee
Prof. John Lee, City College

31 July 1995
Date

Richard L. Chappell
Executive Officer
Dr. Richard L. Chappell

Sharon Cosloy
Prof. Sharon Cosloy, City College

Theodore Axenrod
Prof. Theodore Axenrod, City College

Sheldon Aaronson
Prof. Sheldon Aaronson, Queens College

Robert J. Koestler
Prof. Robert Koestler, Metropolitan Museum of Art of New York

Prof. Robert Blanchette, University of Minnesota (Reader)

Supervising Committee

The City University of New York

Abstract

MEASUREMENT OF FUNGAL BIOMASS IN WOODEN ART OBJECTS USING ATP PHOTOMETRY AND ENERGY CHARGE

by

Fernando Nieto

Adviser: Professor John J. Lee

In this study ATP photometry and energy charge (EC) measurements were used to detect and quantify fungal biomass in wood. Three species of wood decaying fungi were used: *Phanerochaete chrysosporium* (white rot), *Poria placenta* (brown rot), and *Chaetomnium globosum* (soft rot). The fungi were grown in malt extract broth (MEB) for one month at 25 °C, and sampled at selected intervals to measure the ATP content and the EC of the mycelium. The mycelia were ground and extracted with cold 5% trichloroacetic acid (TCA) for 20 hours. The conversion factors calculated were based upon the EC of the mycelium. When EC was equal, or larger than 0.6, the average ATP was 4.19 nM/mg of dry weight. When EC was below 0.6 the average ATP content of the mycelium was 2.06 nM/mg of dw. Protein and chitin conversion factors were also calculated (protein/dry weight = 48.04 μg/mg of dw, and chitin/dry weight = 41.76 μg/mg of dw). These conversion factors were used to estimate the fungal biomass in wood blocks incubated in vitro with the same species of fungi. The presence of wood in the samples interfered significantly with the protein and chitin assays, but not with the ATP and Energy Charge (EC) measurements. Wood samples were ground in a Wiley mill and extracted with cold 5% trichloroacetic acid for 20 hours. After three months of incubation, birch and loblolly pine lost 16% and 14.73% of their weight when colonized by *P. chrysosporium*. Their respective biomass of *P. chrysosporium* on each of these substrates was estimated to be 40 µg and 167 µg. The growth of *C. globosum* and *P. placenta* caused negligible weight loss (1.30 and 0.7% respectively), and their respectively biomass was only 26µg and 10µg. This method was applied to assay fungal activity in three statues from the Egyptian collection of the Metropolitan Museum of Art (V Dynasty 2340 BC). The ATP measurements were not different from the background levels. It was concluded that the statues were not infected by fungi at the present time. This ATP assay technique is a rapid effective method to detect fungal biomass in wooden art objects.

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No digas en tu corazon: “Mi propia fuerza y el
poder de mi mano me han creado esta
prosperidad”, sino acuerdate de Yahveh tu
Dios, porque es el que te da la fuerza para
crear la prosperidad, cumpliendo asi la
alianza que bajo juramento prometio a tus
padres como lo hace hoy.
Deuteronomio 8:17-19

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CHAPTER 1:INTRODUCTION

General Problem

Biodeterioration may be defined as any undesirable change in the properties of a material caused by the vital activities of organisms. This research addresses a problem of interest not only for the field of biodeterioration, but for microbiology in general. The problem is how to estimate numbers of microorganisms, or biomass, in natural samples. The biodeterioration of objects of art is a potentially serious problem which is sometimes difficult to detect and/or control. The project focuses on a wooden Egyptian statue of the "Merti with Staff and Scepter" (Metropolitan Museum of Art; 26.2.4), V Dynasty 2340 BC. Studies of the ultrastructure (TEM) of samples of wood taken from this statue indicated the presence of brown rot decay which probably began soon after the closure of the tomb (Blanchette et al. 1992).

There are two general approaches to estimate the number of microbial cells or the microbial biomass present in a sample:

1. Direct methods: based on the direct count of cells or colony forming units.
2. Indirect methods: including turbidimetric methods (e.g. nephelometry), pigment absorption, and chemical methods based on the measurement of the concentration of structural molecules (e.g.: proteins, ergosterol, chitin, nucleic acids) or metabolites (e.g.: CO_2, CH_3).

Traditional assay procedures for enumerating microorganisms are often time consuming and labor intensive; a typical assay requiring one to three days to complete. The effectiveness of protocols involving growth is dependent upon microbes multiplying to levels above thresholds for detectability. However, if conditions are not favorable, or the organisms are stressed and fail to recover in time, their presence may be overlooked. Some propagules will not divide under particular conditions and are still viable (Chapelle et al., 1977). Although spores are the best known inactive propagules, dormant stages of many microorganisms in the final adjustment stage are known from collections from oligotrophic habitats. Other pitfalls of traditional growth methods are the selectivity of the media used, and the possibility of contamination. Direct methods are not useful when applied to organisms such as fungi, which are filamentous rather than unicellular. One mycelial mat could be considered one individual (e.g. zygomycetes) or a colony (e.g. ascomycetes). Indirect methods are more appropriate for fungi. Indirect methods require conversion factors to calculate the biomass from the parameter measured. Traditionally, fungal biomass is determined indirectly by measuring either the chitin or the ergosterol concentration of a sample. The approach in the present study is to use ATP measurements to estimate microbial biomass (Holm-Hansen, and Booth, 1966). ATP measurements yield different types of information (Stanley, 1986):

1.- The level of endogenous ATP and other charged nucleotides in a cell may be used as an index of energy status, a measure useful in metabolic and physiological studies.

2.- Total ATP may be used to estimate cell numbers (mass) assuming that the ATP per cell remains fairly constant (within a factor of five), and is a known value under defined conditions. This can be a rapid method to estimate microbial biomass or numbers of microorganisms.

Historical background of ATP photometry

The quantitative measurement of ATP may be done using radioisotopic methods, chromatography, or by the firefly luciferin-luciferase assay. The latter method was chosen because of its high specificity for ATP, its high sensitivity (detecting ATP concentrations as low as 10^{-10} M), and the rapidity of the laboratory procedure (results in less than one hour).

The first demonstration of firefly (*Photinus pyralis*) luminescence was done by DuBois in 1884 in Paris (McElroy 1977); but it was McElroy(1947) who discovered the role of ATP in the reaction. He observed that the luminous organs of fireflies, which no longer functioned, would respond to the addition ATP. McElroy's work was easily applied to the assay of ATP, or other enzymes or compounds that affect the ATP content value in cells. The reactions catalyzed by the firefly luciferase are:

$$E + LH_2 + ATP \xrightarrow{\quad Mg^{2+} \quad} E.LH_2\text{-AMP} + PPi \qquad (1)$$

$$\begin{tikzcd} E.LH_2\text{-AMP} + O_2 \arrow[r] & {[P^*.E\text{-AMP}] + CO_2} \arrow[d] & (2) \\ & E\text{-P} + h\mu + AMP & \end{tikzcd}$$

$$E + L + ATP \longrightarrow E.L\text{-AMP} + AMP + PPi \qquad (3)$$

E = luciferase **LH_2** = luciferin
$E.LH_2$ - AMP = enzyme bound luciferyl adenylate
P*.E - AMP = excited complex
E.L-AMP= enzyme bound dehydroluciferyl adenylate **hμ** = photon

1.- Activation of luciferin in the presence of ATP, Mg^{2+} and luciferase to form the enzyme bound luciferyl adenylate. This step is relatively specific for

ATP. In order for reaction (2) to occur D(-) LH_2 is required, L(+) LH_2 is inactive for light emission. Nevertheless the use of crude extracts of fireflies will also show luminescence when supplied with ADP or NTP, because of the presence of adenylate kinase and NDP kinase in the extract. There are several commercial sources of purified luciferin-luciferase reagent which emit light of constant intensity rather than a flash and decay.

2.- This enzyme bound luciferyl adenylate then reacts with the O_2 forming the excited complex P^*- E-AMP which then emits light resulting E-P. When substrates are present at saturating concentrations, the enzyme is strongly inhibited by the product, oxyluciferin, and normally turns over only twice.

3.- This reaction is the activation of the competitive inhibitor dehydroluciferin (L) to form enzyme bound dehydroluciferyl adenylate (E.L-AMP). This complex cannot react with O_2 to give light and the reaction is freely reversible.

When an excess of ATP is injected into a solution containing enzyme, buffer, luciferin and Mg^{2+}, a flash of light is emitted which peaks 0.3 sec later (at 25 °C) (DeLuca and McElroy, 1974). This emission decays in a biphasic manner to about 10% of the peak level within 30 sec and declines gradually over the next 30 min. The decay in light emission is due to product inhibition and slow turnover of the enzyme. At low ATP concentrations (0.2 to 10 pmoles), the light emission reaches a steady peak which is sustained for at least several minutes. At higher ATP concentrations the peak height and decay rates increase. The quantum yield of the reaction at pH 7.6 is $Q = 0.88 \pm 0.25$ photon per luciferin molecule oxidized (Seliger & McElroy, 1960). At neutral and alkaline pH, there is a single yellow-green emission band with a peak at 562 nm. At an intermediate pH 6, there is a red emission band (peak 616 nm) and at values below pH 5.5, the yellow-green emission is completely suppressed leaving only the red band. The number of light quanta emitted per luciferin molecule oxidized at acid pH is << 1(Seliger & McElroy, 1960). There are two ways to quantify the amount of ATP using the luciferase bioluminescent reaction. They are: 1) to record the peak light emission (which should occur after 25 to 30 ms after injection of the reagents) or 2) to integrate the light flux for a set time. The optimum temperature of the light reaction is 25 C and the energy of activation (∂H) is 18,500 Cal. (McElroy and Strehler, 1949).

ATP Extraction Procedures

In order to measure the ATP content, the ATP must be extracted from cells, without being degraded. A number of different media have been used for the extraction of ATP (Karl, 1980): 1) boiling aqueous buffers, 2) inorganic acids, 3) organic solvents and 4) inorganic bases. There are also physical methods for releasing ATP from cells such as abrasion, freezing/thawing, ultrasonic disruption and heating (Thore, 1981). The most important requisites for good extraction procedures include: rapid cell death and lysis, complete nucleotide release, complete and irreversible inactivation of enzyme activity, and long term stability of the extracted nucleotides (no chemical or enzymatic hydrolysis). As Karl (1980) pointed out in his major review of the subject, no single extraction method may be regarded as universally acceptable. Boiling buffers yield variable and inconsistent results when used to extract ATP from soils, sediments, and metazoans. One explanation, the "heat gradient hypothesis", suggests that inconsistent yields are due to thermal extraction efficiencies in two-phase systems (Karl et al., 1978). Nevertheless, boiling buffers have been used by many workers. Extraction with boiling Tris buffer works well when extracting from free living cells, since heat transmission is more uniform. Lundin and Thore (1975) compared the efficiency of ten different methods to extract ATP from bacteria and concluded that trichloroacetic acid (TCA) gave the highest yields and complete recovery of nucleotides added as controls. The choice of extraction method depends on the nature of the investigation. The best extraction method for a given situation is the one that gives the highest ATP and EC yields (Lundin and Thore, 1975). Lundin et al. (1986) recommended that any extractant or releasing reagent be compared with 10, 5, 2.5 and 1.25% TCA (final concentration in extract). The activity and stability of ATP converting enzymes are often dependent on divalent ions such as Mg^{2+}, therefore they also recommended that ethylenediaminetetracetic acid (EDTA) be used to chelate co-factors thereby inactivating nucleotide converting enzymes present in the extract. One drawback in the use of chaotropic acids (TCA and PCA [perchloric acid]) to extract ATP is that both are inhibitors of the luciferase reaction, thus they must be removed or diluted (at least 50 fold) from the extract before the assay can be carried out. Dilution may not be possible if the ATP content in the sample is very low, or if the device used to measure the light output has low sensitivity. TCA can be removed by the three fold liquid partition of the extract with approximately twice the volume of diethyl ether saturated with water (during the extraction, 1 min. of vigorous mixing must be used). The remaining ether is finally volatilized by bubbling with water saturated with N_2 for approximately ten minutes. PCA may also be extracted by neutralizing it with KOH and then removing the $KClO_4$ precipitate.

Since the focus of the present study is wood, the extraction procedure should be successful in extracting nucleotides from microorganisms that live inside wood cells. Wood cells, except for parenchyma cells in sapwood, and those found in the cambial zone are physiologically dead. Cells from the heart wood are filled with extractives, and those involved in conduction are hollow. To my knowledge there are no studies on ATP content in wood cells or on ATP content of microorganisms that decay wood. However there are studies done of the ATP content of plant cells. Guinn and Eidenbock(1970) tested six different extraction procedures (boiling 95% EtOH, boiling 95% EtOH +EDTA, boiling water, boiling 1 or 5 mM EDTA, 5% TCA, and 0.5N $HClO_4$) for the analysis of the ATP content of leaves, floral buds, and immature fruits of cotton. They found that the most effective method of extraction was boiling 95% EtOH, although hot water, and hot dilute EDTA also gave good recoveries. TCA and PCA were very good extractants but they had to be removed, or neutralized, after extraction, otherwise there was a strong inhibition of the light output. De Greef et al.(1978) used Tris buffer + H_2SO_4 (0.06 M, pH =7.4) and 0.7 mM EDTA to extract the ATP content of epicotylar hooks of seedlings of *Phaseolus vulgaris.* They found that the ATP content of the extract decreased rapidly within, minutes, due to ATP-ase activity. They discontinued use of PCA because it inhibited luciferase activity even when the extracts were diluted 1000-fold. At that dilution, the ATP yield with sulfuric acid extraction was about 5-10% higher than that obtained with PCA. Pamatmat and Skjoldal(1979) studied the below ground biomass apportioned by *Juncus romerianus* and *Spartina alterniflora* in estuarine environments and found that although TCA extracted, on the average, 45% more ATP than boiling $NaHCO_3$, it gave erratic values of ADP and AMP. They concluded that the extraction by boiling $NaHCO_3$ was a more satisfactory method.

Ausmus (1973) used ATP measurements to estimate total biomass in terrestrial decomposition studies. The microbial community in soils is composed of bacteria, fungi, and algae. In his study he compared seven different extraction methods, and found that sulfuric acid was the most efficient. Paul and Johnson (1977) used boiling $NaHCO_3$ and chloroform to extract the ATP from the microbial flora of soils. Ball and Atkinson (1975) used perchloric acid and boiling EtOH for extracting ATP from *Saccharomyces cerevisiae.* Both methods were equally effective in extracting the ATP from the yeast cells. Pitt and Bull (1982) used PCA for the extraction of ATP from *Trichoderma* *aureoviride.* Finally Gaunt et al.(1985) used TCA to extract ATP from *Trichoderma reesei.*

Biomass estimation using ATP

The use of an ATP assay to estimate for biomass was developed by Holm-Hansen and Booth(1966). They estimated that the ATP content of marine bacterial cells, averaged from three different species in laboratory culture, was between 0.1 and 0.2% of the dry weight, while the content in eight species of algae varied from 0.003 to 0.16%. The most serious difficulty in the estimation of biomass, using ATP, is the extrapolation of the data to units of living cells (organic carbon, dry weight, or number of bacteria of a certain dimension). Holm-Hansen and Booth (1966) estimated that the average ATP content per bacterial cell was ~1.5 X 10^{-6} ng. Extrapolating from this number they estimated the number of bacterial cells per liter at any depth in the ocean is equal to ng ATP X 0.66 X 10^{6} cells. Expanding the study to the most representative genera of marine bacteria in sea water (*Chromobacterium, Pseudomonas, Serratia, Vibrio* , and *Microccocus*) the same group (Hamilton and Holm-Hansen, 1967) found an average value of 1.5 x 10^{-9} μg of ATP per cell. This is the same value (1.5 X 10^{-6} ng) found in seawater samples.

The estimation of biomass by measuring ATP depends upon the fact that all cells, contain ATP and also upon the following assumptions (Holm-Hansen, and Booth 1966):

1.- That ATP is not associated with non-living particulate material; and

2.- The ratio of ATP to cell carbon is fairly constant.

With respect to the first assumption, Karl(1980) argued that in aquatic environments dissolved D-ATP may contribute up to 70% and 95% (fresh and saltwater respectively), of the total ATP. If this was true, Karl's results discouraged investigators from using direct injection ATP extraction techniques. His argument was quickly shown to be erroneous (Sieburth 1979). Many marine bacteria were shown to be smaller than 0.45 μm, the filter pore size he used. Using an SEM Sieburth (1979) showed that in some marine samples, most of the bacteria were between 0.25 - 0.45 μm. In the case of solid samples there may be free ATP, in which case it has to be removed enzymically, or by washing.

Holm-Hansen and Booth(1966), Hamilton and Holm-Hansen(1967) and Holm-Hansen(1969) estimated that in bacteria the ratio of C(μg/liter):ATP(μg/liter) is ~ 250. This value is an average based on laboratory observations and varies with species composition and environmental conditions. They recommended that, in the absence of specific knowledge concerning the particular species composition of any sample, one must apply their "black box number". Later they (Holm-Hansen,1970) found that 250 was a good constant for the C:ATP ratio for algae ranging in mass from <0.001 ng of C/cell to 215 ng of C/cell:

$$\text{ATP} = 0.35\% \times \text{org. C}$$

Although the average ATP content of seven algal species in exponential growth was 3.2 μg ATP/mg C, under extreme nitrogen and phosphorus deficiency the ATP content decreased to ~20% of the value. Based on the work of Skjoldal and Bamstedt (1977), Karl(1980) argued it was more logical to examine the coefficient of variation of the C/ATP ratio of a given species, or group of organisms under a defined range of environmental and physiological conditions than it was to rely on mean C/ATP ratio or its range. They (Skjoldal &

Bamstedt, 1977) compiled a table of coefficients of variation for ATP determinations of individual microbial taxa. Coefficients of variation ranged from 10% for actinomycetes to 129% for different species of bacteria. Using their data, they developed a model to predict the minimum number of species (n) in a sample to have an accuracy of ±X% in an ATP determination

$$n=(t^2{}_{0.05} \times \text{coeff. of variation})/X^2$$

X = accuracy $t^2{}_{0.05}$ = Student's T value ($\infty = 0.05$)

They showed that the greater the number of species in the sample the more accurate was the ATP measurement. In practice, however, because communities are always dominated by a few species, the biomass estimates will be most accurate when the natural microbial assemblage approaches a monoculture of a known species, and when ancillary data on the growth rate and nutrient status of a population are available. Karl(1980) estimated, that regardless of the carbon source and electron acceptor, the ATP pool in exponentially growing prokaryotes appears to be regulated around a mean of 2.0 to 6.0 nmol of ATP per mg(dry weight) of cells. During exponential growth, unicellular eukaryotes have an average ATP concentration of 2.0 to 6.0 nmol of ATP per mg(dry weight) of cells. The steady state ATP pools of metazoans are greater than those of unicellular microorganisms (Karl, 1980). There is no reasonable explanation for this. Although the accuracy of the ATP-biomass technique has been questioned, and numerous difficulties in its application have been found, it is still a rapid and reliable method for estimating total microbial biomass. The range of variation of the ATP content of the mycelium of fungi known to cause wood decay was estimated in the present study. Previous estimates of the ATP content in the mycelium of *Trichoderma aureoviride*, grown in carbon and nitrogen-limited chemostat culture, ranged between 2 and 9 µmoles/g biomass (Pitt and Bull, 1982). These values are consistent with those reported for bacteria and for other eukaryotes. They also found that the ATP content of the mycelium was correlated with the growth rate. Suberkropp et al. (1993) compared ATP to ergosterol as indicators of fungal biomass associated with decomposing leaves. They calculated a conversion factor for mycelia harvested in the stationary phase as 3.26 µmoles of ATP per gram of dry weight of *Anguillospora filiformis* and 2.96 µmoles/g dry weight for *Flagellospora curvula*.. They concluded that estimates of fungal biomass obtained using ATP concentrations were one to two times lower than those obtained using ergosterol.

In the present study the efficiency of ATP, as a biomass indicator, was compared to that of two other biomass indicators (protein, and chitin).

Adenylate Energy Charge

A number of workers have suggested that the state of growth of a population of cells can be evaluated by using the Adenylate Energy Charge (EC) ratio (Chapman et al., 1971; Wiebe and Bancroft, 1975; Setlow and Kornberg 1970 a and 1970 b; Swedes et al. 1975; Thomas and Dawson 1977). The concept of Energy Charge (EC) was introduced by Atkinson and Walton (1967). It is defined as half the average number of anhydride-bound phosphate groups per adenine moiety EC= (ATP + 1/2 ADP)/(ATP + ADP + AMP). This parameter varies between 0 and 1. The nucleotide system, in its roles of accepting, storing, and delivering energy, may be compared with an electric storage battery (Atkinson, 1969). It gives an idea of the amount of metabolically available energy in the cell. Regulatory enzymes from sequences in which ATP is regenerated are highly active at low levels of EC and decrease sharply in activity as the EC increases above a value of about 0.75. Regulatory enzymes from biosynthetic sequences, or those that consume ATP (e.g. movement) exhibit very little activity at low levels of EC, but increase as EC reaches 0.75. Curves of these two types of enzymes tend to intersect at EC values of 0.85 (Atkinson, 1969) so that intact metabolizing cells should be stabilized at energy charges between 0.8 - 0.9. Chapman et al. (1971) found that during normal growth in *Escherichia coli* the EC is regulated at a value of ~ 0.8. They also found that when the cells were glucose starved, growth ceases, the EC drops to about 0.6 to 0.7, and there is also a drop in the total adenylate pool. Stationary cultures of *E. coli* had an EC of 0.5. Death of cells coincided with a final fall in energy charge. Maintenance of viability, but not growth, seems possible at Energy Charge values between 0.5 and 0.8. Values lower than 0.5 are compatible with dormant structures such as spores or higher plants seeds, where enzymes have become dehydrated and inactive. Setlow and Kornberg(1970a) showed that dormant spores of *Bacillus megatherium* contain coenzymes and nucleotides at concentrations similar to those found in the vegetative cells, but the spores contain almost no ATP and reduced pyridine nucleotides. ATP is generated early in germination. The energy charge value in spores is about 0.1. Hutchinson and Hanson(1974), found that the ATP concentration and the EC value of *Bacillus subtilis* remained constant during the exponential growth phase (400 pmol of ATP/mg DW and EC=0.7). A drop in ATP level to about 50 pmol/mg(DW) occurred during the phase of negative acceleration. This decrease was accompanied by a reduction in EC to 0.3 - 0.5. They suggested that the decrease in EC of the cell was necessary for sporulation. Slayman (1973) obtained an average EC value for *Neurospora crassa* of 0.72, during exponential growth, a value which lies well within the range calculated by Atkinson (1969). Kahru et al.(1982) studied changes in EC and ATP during batch culture of *Thermoactinomyces vulgaris* and found that during exponential growth the EC = 0.95. During the three hours after the glucose exhaustion the EC fell steadily to a value of 0.5 and remained constant up to the end of incubation. Ball and Atkinson(1975) found that under starving conditions *Saccharomyces cerevisiae* had EC values as low as 0.14, with no loss of viability during the first 24 hours. After 7 days 66% of the cells were still viable even though the EC fell to as low as 0.03. Yeast cells seem to be able to maintain viability over a wide range of energy charge values, and when adequate growth conditions are restored, the charge rises rapidly to about 0.8. These abnormally low EC values are found not only in yeast, but also in other unicellular eukaryotes. Edwards and Lloyd (1977) obtained EC values for *Acanthamoeba castellanii* of less than 0.1 during the first 30 hours of exponential growth. This was caused by unusually large values of AMP (200 nmol/ml. It dropped to 7.5 nmol/ml after 30 h) during the first 13 h.

In summary, published EC values vary, depending on the growth phase of the population in batch cultures, from values around 0.85 to 0.9, for exponential growth, to ~ 0.5 to 0.6 for stationary cultures, and as low as 0.1-0.2 for spores or dormant stages. It is possible that the very high or very low levels of EC that have been reported may be valid, or they may be spurious, due to laboratory errors rather than natural variation. The present study attempts to determine if there is a correlation between EC and growth rate which reflects the metabolic state of the microbial populations in wooden samples. This later assessment is crucial to biodeterioration research, since it will allow the conservator to estimate the potential damage of an infestation and the effect of a particular treatment.

Wood decay

Environmental conditions, particularly relative humidity, are important in determining which agents will decompose wood. Fungi, bacteria, and insects all attack wood and use cell wall components as substrates or cause mechanical disintegration. Structural polymers in wood are gradually reduced to simpler molecules and finally to CO_2 and H_2O. This natural recycling of organic matter is an important process in terrestrial and aquatic ecosystems but it becomes a serious problem when wood of historical value, is degraded. All wood cells are composed of various ratios of cellulose (40-45 %), hemicellulose, and lignin (18-25% in hardwoods, and 25-30% in softwoods). All wood is susceptible to biological degradation. When fungi enter wood they grow from cell to cell through natural openings such as simple and bordered pits and perforation plates between vessel elements, or they may penetrate directly through the cell wall by producing a bore hole. Those cells with the most food reserves, such as ray parenchyma cells, are usually colonized first. Fungi that cause wood cell wall degradation can be separated into groups depending on the type of decay they produce (Blanchette et al., 1990): white rot; brown rot; and soft rot.

- White rot fungi are Basidiomycetes. Some species are selective and preferentially attack lignin without extensively removing cellulose and hemicellulose. Other species simultaneously remove all cell components (e.g. *Phanerochaete chrysosporium*).

- Brown rot fungi are also Basidiomycetes that degrade polysaccharides by extensive depolymerization. In advanced stages of decay, cellulose and hemicellulose have been depleted, but only limited degradation of lignin has occurred. The resulting wood which has a very high lignin content, is brown, and when dry, may break apart into cubical pieces. (e.g. the growth and affect of *Poria placenta*)

- Soft rot: is a type of decay caused by Ascomycetes and Fungi Imperfecti. It is characterized by the formation of chains of cavities with conical ends within wood cell walls. They also cause cell wall erosion (e.g. the growth of*Chaetomnium globosum* ; Greaves, 1970).

Bacteria attack wood after it is submerged in water or when it is frequently soaked by water (RH > 90%). The most common effect is the degradation of pit membranes. So far none of the bacteria capable of degrading wood cell walls have been grown axenically (Blanchette, 1990). They are all gram negative. Bacterial decay types have been separated into the following categories:

1. Bacteria that degrade pit membranes. These are best known mainly from studies of softwoods submerged in water or from softwoods sprinkled with water. The degradation of bordered pits results in pronounced increases in porosity (e.g. the growth of *Bacillus polymyxa* and *Bacillus subtilis*; Knuth, 1964).

2. Cell wall degrading bacteria. The classification of this type of attack is based on the micromorphology of the attack and three types have been described: a) erosion, b) tunneling, and c) cavitation. During erosion the bacteria grow in the wood cell lumina and attach themselves to the cell walls. The bacteria, align themselves along the microfibrils where the individual bacteria erode the cell wall in a groove-like manner. During cavitation and tunneling, the bacteria grow and degrade the fiber wall. Cavitation describes an attack which results in the formation of discrete cavities in the fiber walls. Tunneling bacteria enter the cell walls and tunnel through the entire substrate. Branching of the tunnels occurs when recently divided individuals initiate

additional tunnels in new directions. Tunneling bacteria leave a concentric arrangement of wall-like structures (Blanchette et al., 1990).

Actinomycetes colonize wood and cause a gradual degradation of wood components. Three different species of *Streptomyces* were found to colonize vessels, fibers and ray parenchyma cells and to degrade cell contents as well as vessel occlusions (*Streptomyces parvulus* , and *S. sparogenes*) in the wood of *Acer saccharium*. The investigations in which Actinomycetes have been isolated from wood suggest that these microorganisms may be more widespread in wood deterioration than is currently realized (Greaves, 1970).

The rates of bacterial degradation of wood are slower than those of fungi. Bacteria cannot compete successfully with fungi if the substrate and the environmental conditions are suitable for fungal attack. Relative humidity optima for fungi are generally lower than for bacteria. Most bacteria grow best at 100% RH. Most fungi can develop at less than 90% RH (Finstein and Morris, 1975). Waterlogging will exclude white- and brown-rot basidiomycetes. Soft-rot fungi may still be active, if oxygen levels do not limit them. Laboratory experiments have shown that hardwoods (e.g. birch and beech) containing comparatively low levels of lignin, are quickly degraded by soft rot fungi when exposed in unsterile soil. Impregnation of these timbers with an antifungal antibiotic will result in attack by wood degrading bacteria. In contrast, exposing softwoods (e.g. pine and spruce) in unsterile soil normally results in a mixture of both soft rot and bacterial attack, because the higher lignin content of the timbers inhibits soft-rot decay. It is also believed that lignin protects the plant cell wall against bacterial attack(Liese 1970; Rossell et al. 1971; Schmidt 1978).

Applications of ATP measurements to Biodeterioration

ATP photometry was used by McArthy (1983) to study microbial contamination of textiles. The purpose of his study was not to quantify the biomass but to detect the presence of contamination at its early stages. In a later study (McArthy, 1986) ATP photometry was used to compare ATP values of biocide treated and untreated samples. Kaspersson and Lindgren (1986) used ATP photometry to quantify microbial contamination in animal feeds. Littmann (1986) used ATP photometry to detect microbial contamination in drilling lubricants used to enhance the productivity of wells in oil producing zones. Microbial contamination of drilling lubricants may cause spoilage in a matter of days, so that a rapid detection method is needed in order to act as soon as possible. ATP photometry has also been used to estimate microbial numbers in stone monuments (Tiano, 1989).

ATP measurements have also been used in other applied sciences. Recently, Metge et al. (1993) used EC measurements of bacterial communities as an indicator of the level of contamination of the ecosystem due to sewage effluents. The ATP content per bacterium in the contaminant plume was twice as high as in the uncontaminated groundwater. The EC values were low (0.56 - 0.60) throughout the sampling transect and no significant differences in EC values were found between contaminated and uncontaminated groundwater. ATP measurements have also been used to study the susceptibility of Enterobacteria to ampicillin, piperacillin, and gentamicin (Wheat et al., 1988). They found that there was a good correlation between ATP bioluminescence and minimum inhibitory concentrations for most of the species tested. ATP measurements have been used in the food industry to test for the presence of microbial contamination in food (Stannard and Gibbs, 1986) and to determine the time of onset of *rigor mortis* of lamb and sardine muscles respectively (Roncales et al., 1989; Watabe et al., 1991). After rigor mortis the tissues can no longer be stored frozen.

Aims

The aims of this dissertation are:

1. to develop a reliable and reproducible procedure for the extraction of ATP from wood-decaying fungi grown in batch liquid cultures, and in wood blocks in vitro.
2. to study the correlation between ATP content and EC of the mycelium during growth in batch culture.
3. to develop a conversion factor for three representative species of wood decaying fungi (white, brown, and soft rot) which will relate ATP to biomass (measured as dry weight).
4. to apply ATP assays to "in vitro" wood decay.
5. to apply the technique developed in laboratory experiments to field case studies from the Egyptian collection of the Metropolitan Museum of Art.

CHAPTER 2: NUCLEOTIDE EXTRACTION FROM THE MYCELIUM OF THE WOOD DECAYING FUNGUS *Phanerochaete chrysosporium* GROWN IN LIQUID BATCH CULTURES AND IN WOOD BLOCKS.

ABSTRACT

The efficiency of six different extraction methods (boiling tris buffer, boiling EtOH, boiling NaHCO3, perchloric acid, trichloroacetic acid, and Extralight®) to extract ATP from the mycelium of the wood decaying fungus *Phanerochaete chrysosporium* was measured. The ATP concentration of the sample was measured using the firefly luciferin/luciferase assay. The most efficient method (ATP= 3.45×10^{-9} moles/mg dw) was extraction with cold 5% TCA for 20 hours. After the extraction, the TCA was removed with ether saturated with water. Although the amount of ATP extracted with boiling ethanol was not significantly different from that by TCA (ATP= 3.06×10^{-9} moles/mg dw), TCA was chosen over ethanol due to the potential hazards involved in boiling ethanol. The efficiency of extraction was not affected by different types of wood.

INTRODUCTION

The firefly luciferase assay of ATP (McElroy, 1947) is a rapid and highly sensitive method to detect and quantify microbial biomass (Holm-Hansen and Booth, 1966; Hamilton and Holm-Hansen, 1967; Holm-Hansen, 1970). The success of this assay depends on the ability of the investigator to extract the nucleotides from the cells in a consistent and reliable manner. No single method of extraction has been found which can be applied to every sample. The requirements for good extraction are: rapid cell death and lysis, complete nucleotide release, complete and irreversible inactivation of degradative enzyme activity, and long term stability of the extracted nucleotides. The best extraction method is the one that gives the highest ATP yield (Lundin and Thore, 1975). Extraction methods can be classified as follows: 1) boiling aqueous buffers, 2) inorganic acids, 3) organic solvents, and 4) inorganic bases (Karl, 1980). This study was undertaken to determine which method is the best for extraction of nucleotides from wood decaying fungi grown in batch liquid cultures, and in wood blocks. The growth of hyphae occurs by synthesis of new cell membrane and cell wall materials at the tip (Grove et al., 1970). The kinetics of the nucleotide extraction from an individual mycelium may be very different from that of individual cells. In this regard a mycelium could be compared to a tissue, or a multicellular organism. Therefore the diffusion time of the extractant throughout the sample becomes germane. The effect of extraction time on ATP yield also was addressed. The possible interference of wood components, such as phenols, with the measurement of the nucleotides was also studied.

MATERIALS AND METHODS

Fungi and cultures

Phanerochaete chrysosporium (FPL BKMF1767), a white rot, was used as a model for this study. 10 ml of malt extract broth (MEB) was inoculated with a plug 1 cm in diameter taken from a malt extract agar (MEA) plate. Liquid cultures were incubated at 25°C for four days (exponential growth). The whole culture was then extracted, using the six methods described below. After the extraction the samples were centrifuged and 1 ml aliquot of the supernatant was frozen at -25º C until assayed for ATP. One control and four experimentals were prepared for each extraction procedure. The uninoculated control was treated as the experimentals. The dry weight was measured for each one of the experimentals by filtering the culture, after the ATP extraction, using a cellulose acetate filter (pore size Ø=0.45 µm, Gelman cat.# 63069).

Extraction Methods

Six different methods were used and compared. They can be grouped in two different categories.

Cold extractions

1. The samples were incubated in 5% Trichloroacetic acid (TCA) for 15 min. on ice while it was being homogenized in a tissue grinder (Bagnara and Finch, 1972). TCA was removed from the extract with three changes of an equal volume of ether saturated with water. The samples were then bubbled with nitrogen to remove any dissolved ether.

or

2. The samples were incubated in 2.3 M perchloric acid (PCA) + 67 mM EDTA for 15 min. on ice while it was being homogenized in a tissue grinder. The extract was neutralized with 0.72 M KOH + 0.16 M $KHCO_3$ (Bagnara and Finch, 1972).

or

3. Ten ml of the sample were incubated with 20 ml of a proprietary agent, Extralight (ATP releasing agent manufactured by Analytical Luminescence Laboratories, ALL cat # 4025) for 15 min. at room temperature (25°C) while it was being homogenized in a tissue grinder.

Boiling extractions

1. the cultures were boiled in 20 ml of 20 mM Tris buffer + 2 mM EDTA pH 7.75 for 2 min. (Lundin and Thore, 1975).

or

2. The cultures were boiled in 20 ml of 95% ethanol for 1 min. (St. John, 1970). The samples were then bubbled with N_2 for 10 min.

or

3. The cultures were boiled in 20 ml of 0.1 M $NaHCO_3$ pH 8.5 for 5 min. (Pamatmat and Skjoldal, 1979).

For the boiling extractions the culture was transferred to a Pyrex glass test tube containing the boiling extractant. The extractant was boiled using a Bunsen burner. After boiling the mycelium, extra extractant was added to compensate for evaporation and placed on ice. They were centrifuged at 2000 rpm for 5 min. and 1 ml of supernatant was stored at -25 °C until assayed for ATP. The ethanol samples were stored at -70 °C.

Nucleotides assay

The nucleotide content of samples was measured with the aid of the following assay preparations (Pradet, 1967):

- **Tube A**: 100 µl of sample + 900 µl of 20 mM Tris (pH=7.6) buffer containing 15% (w/v) $MgSO_4$ and 12.5% K_2SO_4.

-**Tube B**: same as in tube A + 20 µl of pyruvate kinase (PK; EC 2.7.1.40) (1000units/ml) + 21 µl of 10 mM phosphoenol pyruvate(PEP).

-**Tube C**: same as in tube B + 20 µl of adenylate kinase (AK; EC 2.7.4.3) (1000units/ml).

After the tubes were incubated for 1 hour at 37°C, the reaction was stopped by placing the tubes in boiling water for 2 minutes. The tubes were cooled on ice and the ATP concentration was measured using a Monolight bioluminometer (Analytical Luminescence Laboratory cat # 2010C). The light output was measured for one second to avoid the recycling of ADP or AMP to ATP.

The ATP content of the sample was measured from tube A. The ADP content of the sample was measured indirectly by subtracting the ATP content of tube A from that of tube B. Finally the AMP content was measured by subtracting the content of tube B from that of tube C.

The volumes were pipetted using three micropipetters Socorex Micropipette Calibra 822, handling either volumes 2-10 μl, 10-100 μl, or 100-1000 μl.

A standard mixture of the three nucleotides (2.5 μM each) was assayed together with the samples, and the blank (20 mM Tris buffer), to estimate the efficiency of recovery of the three nucleotides.

ATP content was determined indirectly by measuring the light output of the extracts in the presence of a luciferin/luciferase enzymatic cocktail (Firelight, Analytical Luminescence Laboratory cat.# 2003) in the bioluminometer. This instrument is equipped with an automatic injector that delivers 100μl of a luciferin/luciferase cocktail (Firelight). When the sample is placed in the sample holder, and the reading chamber is closed the instrument will inject the luciferin/luciferase solution into the sample and start the reading at the same time. The light output peaks after the first 25 to 30 milliseconds of the beginning of the light reaction. Therefore injection of the enzyme and reading must take place at the same time.

The extracts were defrosted and diluted 100 fold with 20 mM tris (pH=7.75) to bring the concentrations within the range of sensitivity of the instrument. The ATP standards ranged in concentration from 10^{-8} M to 10^{-10} M. The instrument is linear within this range of concentrations. The luciferin/luciferase lyophilized preparation (ALL cat# 2003) was reconstituted with 5 ml of firelight buffer (ALL cat# 2550). The enzyme was allowed to stabilize for an hour before being used. Internal standards were used to determine the ATP content of the samples. The concentration of the sample was calculated using following this formula:

$$Cu = Cstd * (Lu / Lstd)$$

Lu: light output of unknown **Cu:** ATP concentration of unknown
Lstd: light output of standard **Cstd:** ATP concentration of standard

This formula calculates the concentration of the sample (**Cu**) as the product of the concentration of the ATP standard (**Cstd**), times the ratio of the light output of the sample (**Lu**) to the light output of the standard (**Lstd**).

Interference by wood

Two different types of wood, birch (hardwood), and loblolly pine (softwood), were used. The wood was ground using a Wiley mill to pass a size 60 sieve (1 mm). *P. chrysosporium* was cultured in 20 ml of MEB in 25 cm^2 tissue flasks for 6 days at 25 °C. 500 mg of ground wood was added to the cultures (approximately a 10:1 ratio of wood to mycelium). The nucleotides were then extracted overnight with 40 ml of 5% TCA. The control cultures also were extracted using 40 ml of 5% TCA, but wood was not added. Uninoculated blanks were also extracted. Three more replicates were used to measure the dry weight of the cultures.

Dry weight

Cultures were filtered through preweighed, dried, cellulose acetate membrane filters with a pore size of 0.45 µm (Geltman cat# 63069) and then dried at 60 °C for 12 hours.

RESULTS

Nucleotide extraction

The lowest ATP yields (5.77×10^{-10} moles/mg dw) (table 2.1 and figure 2.1) were extracted by Extralight. Boiling Tris buffer and boiling $NaHCO_3$ were the next least efficient extractants with ATP yields of 6.85×10^{-10} moles/mg dw and 6.65×10^{-10} moles/mg dw, respectively. The differences in ATP yield among these three extraction methods were not significant (α = 0.05). Larger ATP yields were obtained by using TCA, boiling ethanol, and PCA. PCA was significantly (α =0.05) less efficient than TCA and ethanol (1.68x10^{-9} moles of ATP/mg of dw). Ethanol and TCA were the most efficient yielding 3.06x10^{-9} moles of ATP/mg of dw (±5.23x10^{-10}) and 3.45x10^{-9} moles of ATP/mg of dw (±3.77x10^{-10}), respectively.

Table 2.1: ATP yields of the 6 extraction methods in moles/mg of dw.

	Ethanol	**TCA**	**Extralight**	**PCA**	**Tris**	**NaHCO3**
Replicate 1	3.66x10^{-9}	3.06x10^{-9}	5.37x10^{-10}	1.69x10^{-9}	6.65x10^{-10}	5.08x10^{-10}
Replicate 2	2.67x10^{-9}	3.81x10^{-9}	4.05x10^{-10}	1.79x10^{-9}	8.35x10^{-10}	7.00x10^{-10}
Replicate 3	2.87x10^{-9}	3.48x10^{-9}	7.88x10^{-10}	1.58x10^{-9}	5.55x10^{-10}	7.86x10^{-10}
Average	3.06x10^{-9}	3.45x10^{-9}	5.77x10^{-10}	1.68x10^{-9}	6.85x10^{-10}	6.64x10^{-10}
S.D.	5.23x10^{-10}	3.76E-10	1.94x10^{-10}	1.05x10^{-10}	1.41x10^{-10}	1.42x10^{-10}

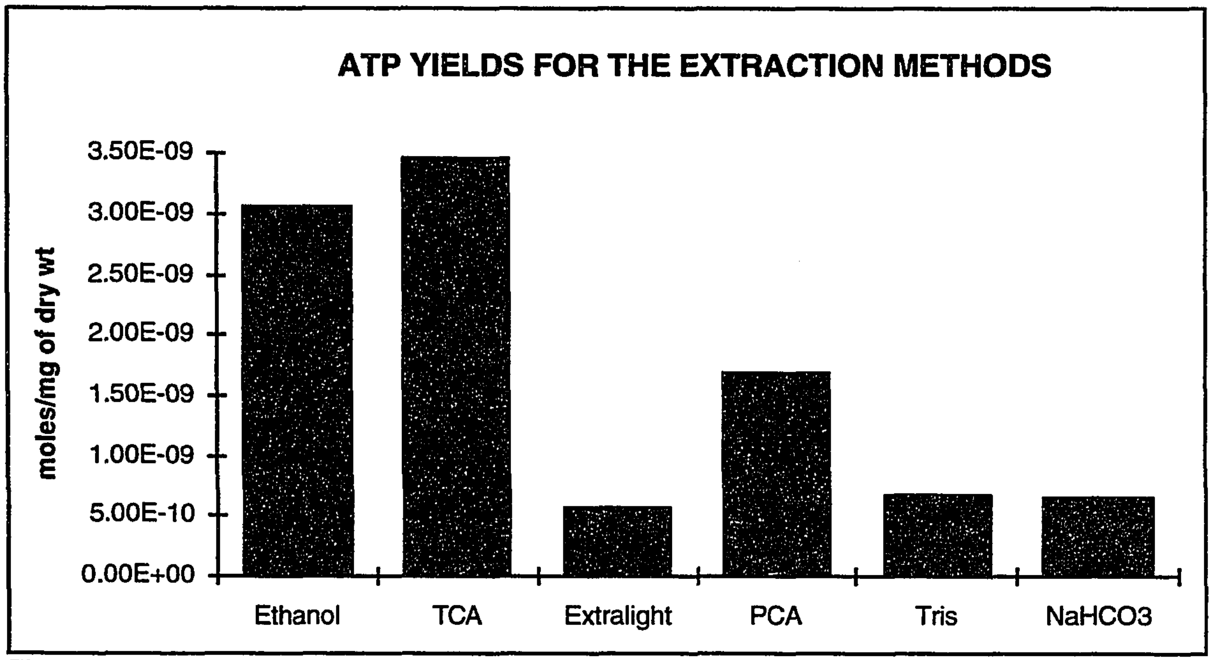

Figure 2.1: ATP yields for the six extraction methods. ATP yield is measured in moles per milligram of dry weight

The results were analyzed using a one way analysis of variance (ANOVA). This test showed that there were significant differences between the extraction efficiencies of each one of the extractants (α = 0.0001). The data was compared pairwise using the GT2 and Tukey tests. These tests showed that TCA was the reagent with the highest efficiency of extraction with a mean value of 3.45×10^{-9} moles/mg of dw ($\alpha = 0.05$). Table 2.2 shows the groupings made by Tukey's test, in which the same letter indicates no significant differences ($\alpha=0.05$) within the group.

Table 2.2: Summary of results of ANOVA and pairwise comparisons.

Reagent	Mean	Tukey Groups
TCA	3.45×10^{-9}	A
EtOH	3.07×10^{-9}	A
PCA	1.69×10^{-9}	B
Tris	6.85×10^{-10}	C
$NaHCO_3$	6.65×10^{-10}	C
Extralight	5.77×10^{-10}	C

Same letters means no significant differences ($\alpha=0.05$).

Based upon these results, 5% TCA was chosen as the most efficient method for the extraction of nucleotides from the mycelium of wood decaying fungi. In order to learn more about the efficiency of TCA extraction, a time-course extraction experiment was undertaken. The same extraction protocol was followed as in the previous experiment but the length of extraction was varied. After the mycelium was ground, it was extracted in the cold for 10 minutes, 1 hour, or 20 hours. As with the previous experiment there were three replicates, and one uninoculated control. The results of this experiment are shown below (table 2.3, and fig. 2.2).

Table 2.3: Summary of the results of the time course extraction experiment in moles per mg of dry weight.

	replicate 1	replicate 2	replicate 3	Average	S.D.
10 minutes	1.53×10^{-9}	1.55×10^{-9}	2.04×10^{-9}	1.71×10^{-9}	0.29×10^{-9}
1 hour	2.43×10^{-9}	2.47×10^{-9}	1.89×10^{-9}	2.26×10^{-9}	0.32×10^{-9}
20 hours	2.54×10^{-9}	3.39×10^{-9}	3.27×10^{-9}	3.07×10^{-9}	0.46×10^{-9}

The differences in ATP yield between times of extraction were significant ($\alpha = 0.05$) (table 2.4, and figure 2.2). It was found that the extraction time was directly correlated with the ATP yield. The maximum ATP yield was obtained when the mycelium was extracted for 20 hours on ice (ATP= 3.07×10^{-9} moles / mg of dw). The results of the pairwise comparisons tests were consistent with this observation (table 2.4).

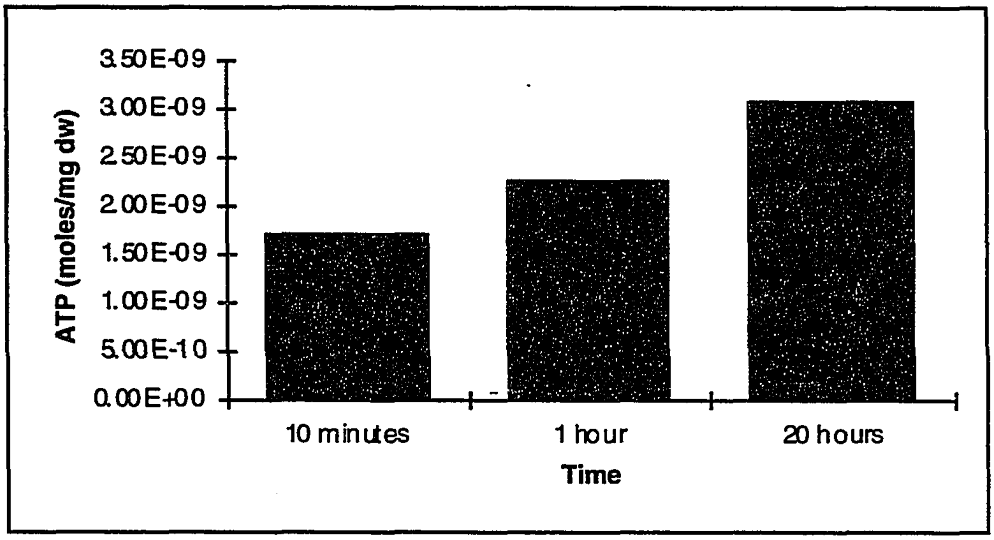

Figure 2.2: ATP yield for the time-course extraction experiment measured in moles per milligram of dry weight.

Table 2.4: Summary of the ANOVA and Tukey tests ($\alpha = 0.05$). Same letter indicates no significant differences between the samples

Extraction time	Mean	Grouping
20 hours	3.06×10^{-9}	A
1 hour	2.27×10^{-9}	A B
10 minutes	1.71×10^{-9}	B

Interference of wood

There was more interference in birch samples than those with loblolly pine (figure 2.3 and 2.4, and table 2.5). The EC values for samples with birch were also lower than loblolly pine samples (table 2.5).

Table 2.5: Nucleotide concentrations (moles/mg of dry weight), and Energy Charge values.

	ATP	ADP	AMP	EC
CONTROL	2.89E-09	5.39E-09	8.68E-10	0.61
BIRCH	1.89E-09	4.60E-09	1.58E-09	0.52
CONTROL	1.95E-09	3.67E-09	3.29E-10	0.64
LOBLOLLY	2.88E-09	3.84E-09	5.11E-10	0.65

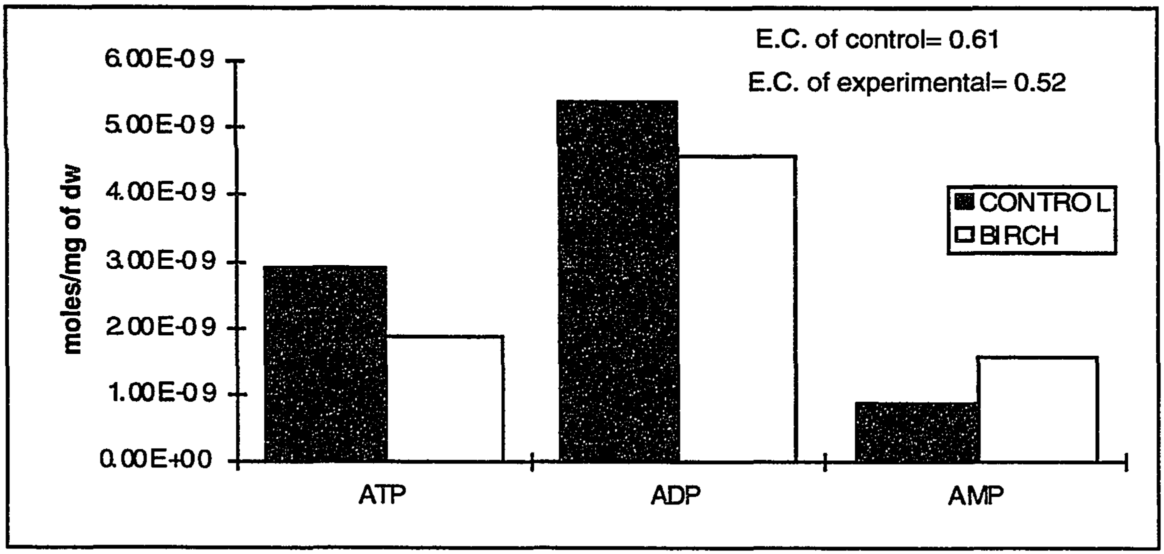

Figure 2.3: Interference of wood (birch) with the nucleotide determination (moles/mg of dw).

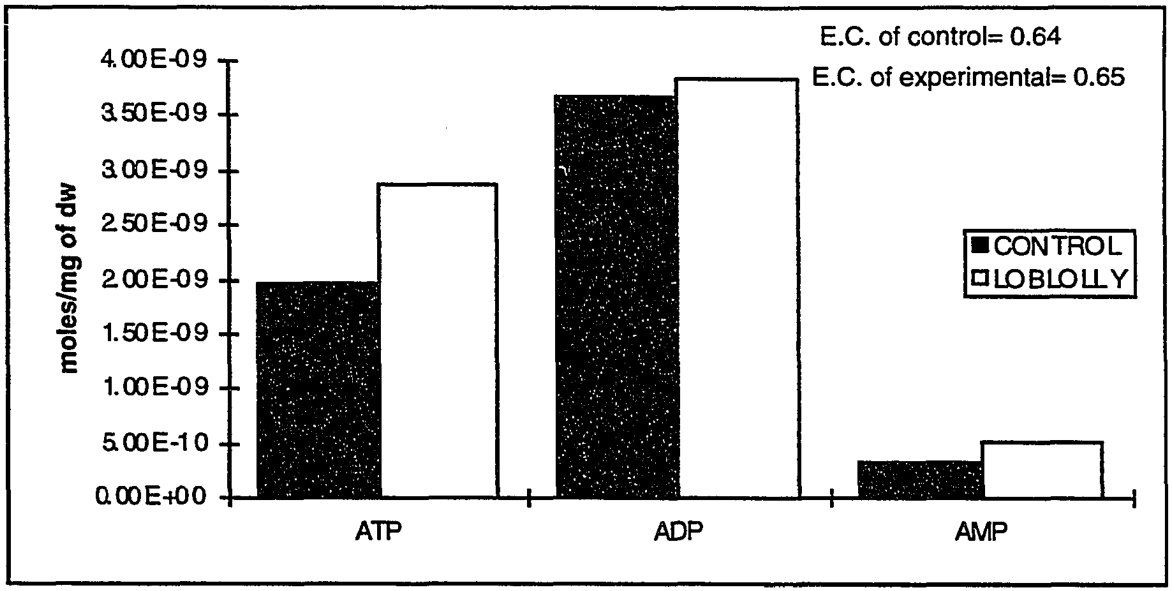

Figure 2.4: Interference of wood (loblolly) with nucleotide determination (moles/mg of dw).

The differences between the control and experimentals were tested using a one way ANOVA . No significant differences between the controls and the experimentals were found for either of the nucleotides and wood types (α=0.05).

DISCUSSION

Ethanol and TCA were the most efficient of the six different methods that were tested to extract ATP. The differences in ATP yield between these two methods were not significant. However, ethanol is potentially hazardous, because it may explode when boiling, raising the possibility of endangering the investigator and losing the sample. Therefore TCA was the method of choice. The ATP assay is very sensitive to pH and presence of salts. Although the samples extracted with PCA were neutralized using KOH and $KHCO_3$ (Bagnara and Finch, 1972), the ATP yield was significantly lower than TCA and ethanol. ATP may be lost by coprecipitation with $KClO_4$ (Guinn and Eidenbock, 1972).

Boiling buffers are known to yield inconsistent and variable results when applied to soils, sediments, and metazoans (Karl, 1980). It has been noted in the past that when extracting nucleotides from cell aggregates or tissues, the failure of diffusion of the extractant throughout the sample decreased the ATP yield (Karl et al., 1978; Karl and LaRock, 1975). Since fungi form mycelial mats during growth in batch cultures, boiling extractions may not be as efficient in rapidly disrupting the cell wall and cell membrane.

The extraction time is also a crucial factor. The best extraction time in this study was the longest time tested, 20 hours on ice. Perhaps, extraction for a longer time might produce higher yields of ATP.

TCA is considered by some researchers to be a reference method to extract ATP (Lundin and Thore, 1975). New extraction methods can be compared to TCA to determine their efficiency. In order to avoid interference with the light reaction, TCA had to be removed from the sample using ether saturated with water. The interference of TCA can be avoided by diluting the sample at least ten fold. Nevertheless, dilution of the sample compromises the sensitivity of the method. The ether was volatilized by bubbling for ten minutes with nitrogen.

Other researchers have shown that wood components interfere with nucleotide determination. Phenols bind irreversibly to proteins by means of covalent bonds or by hydrogen bonds rendering them inactive. Ground, undecayed, wood interfered with the detection and quantification of *P. placenta* using enzyme-linked immunosorbent assay (ELISA) (Jellison and Goodell, 1989). They were unsuccessful in removing phenols by using polyvinyl pyrolidone (PVP), and adsorbent polystyrene (XAD4). Nieman et al. (1978) also reported the interference of phenols with the determination of the nucleotide content of plant extracts using chromatography and UV. No interference was found when TCA was used as an extractant (Fig. 2.3 and 2.4). The differences between the controls, without wood, and the experimentals, with either type of wood, were not significant ($\alpha = 0.05$).

In summary, the most effective method to extract nucleotides from the mycelium of *P. chrysosporium* in batch cultures or in wood, is to grind the tissue and use cold 5% TCA and incubate the mixture for 20 hours in ice.

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CHAPTER 3: CHANGES IN NUCLEOTIDE CONTENT AND ENERGY CHARGE OF THE MYCELIA OF THREE SPECIES OF WOOD DECAYING FUNGI DURING GROWTH IN BATCH CULTURES.

ABSTRACT

The ATP content and Energy Charge (EC) of three different species of wood decaying fungi, *Phanerochaete chrysosporium* (a white rot), *Poria placenta* (a brown rot) and *Chaetomnium globosum* (a soft rot), were measured at different phases of growth in batch culture. The nucleotide, protein, chitin contents, and the dry weight of the mycelium were measured during four weeks of incubation. In all species, the log growth phase was associated with the highest ATP concentrations and highest EC values. The variation in the ATP content of the mycelium was classified using the EC values. Two classes were formed: class 1 included samples with EC equal or larger than 0.6; and class 2 included samples with EC below 0.6. No significant differences in ATP content were found between the three species within the two classes. The average ATP content for class 1 was 4.19 nM/mg of dw, and for class 2, 2.06 nM/mg of dw. These average values were adopted as conversion factors (ATP/biomass) to estimate fungal biomass in field samples. Protein and chitin measurements were not as consistent and varied more than the ATP values. Protein concentrations varied between 10.78 µg/mg dw, in *P. placenta* on the third day of growth, to 87.03 µg/mg dw in *P. chrysosporium* in the seventh day of growth. Similarly the chitin content of *P. chrysosporium* varied between 4.64 µg/mg dw on the first day of growth to 174.05 µg/mg in the tenth day. The variability found in the protein and chitin content of the three species was not as classifiable as the ATP data. An average of all the protein and chitin measurements (protein = 48.04 µg/mg of dw; chitin=41.76 µg/mg of dw) found for the three species, regardless of phase of growth, was adopted as the conversion factor to estimate fungal biomass.

INTRODUCTION

A factor of 250 is generally accepted for estimating the biomass of bacteria from data taken from an ATP assay (250 ≅ C:ATP µg/l) (Holm-Hansen and Booth, 1966) (Hamilton and Holm-Hansen, 1967) (Holm-Hansen, 1969). This value is an average based on extensive laboratory observations and varies somewhat with species composition and with environmental conditions. Karl et al. (1978) later found that multicellular organisms had ratios < 100. The originators of the method (Holm-Hansen and Booth, 1966) recommended that, in the absence of specific knowledge concerning the species composition of any sample, one must apply the average ATP content of representative microorganisms grown in the laboratory.

On average, the concentrantion of ATP of all living forms ranges from 2 to 9 nM/mg of dry weight (Karl, 1980). The concentration of ATP varies, not only interspecifically, but also with the metabolic states of cells. Environmental samples are mixtures of different species of microbes which are often at different stages in the cell cycle. The accuracy of the ATP assay estimates of biomass depends upon the ability of the researcher to evaluate this variation. Fungi dominate the microbial community in wood at relative humidities between 85-95%. Although bacteria are found in wood, they are usually found in the early stages of colonization (Actinomycetes), or in regions of very active wood decay. They may fix nitrogen, rather than decay the wood itself (Seidler et al., 1978). Bacterial attack of wood occurs only when the wood is saturated with water.

Three different types of wood decay caused by fungi have been recognized: white rot (e.g. *Phanerochaete chrysosporium*), brown rot (e.g. *Poria placenta*), and soft rot (e.g.*Chaetomnium globosum*) (Blanchette et al., 1990).

Several methods for estimating fungal biomass, (e.g. ergosterol and chitin), have been used (Ride and Drysdale, 1972) (Swift, 1973) (Whipps, 1987) (Gessner and Chauvet, 1993) (Seitz et al., 1979). Ergosterol and chitin are compounds found almost exclusively in the cell walls of fungi. Detection and quantification of these two molecules does not give information about the metabolic state of the biomass. Mycelial remains can be present in the sample and yet the effect upon the substrate is negligible.

The aim of this study was to estimate the changes in ATP content and EC of the mycelia of three different species of fungi, representative of the three types of wood decay (white, brown, and soft rot) at different stages of growth. The relationship between ATP and biomass was calculated. The protein and chitin contents of the mycelia also were measured, as alternative methods to estimate fungal biomass and compared to estimates made by using ATP.

MATERIALS AND METHODS

Fungal cultures

Three different species were used: *Phanerochaete chrysosporium* (Forest Products Laboratory BKMF1767), a white rot, *Poria placenta* (University of Minnesota 203A), a brown rot, and *Chaetomnium globosum* (University of Minnesota 390), a soft rot. They were cultured in 20 ml of Malt Extract Broth (MEB) in 25 cm^2 tissue cultures flasks, at 25 °C. The flasks were inoculated using 1 cm (in diameter) plugs of stock cultures of the three species maintained in Malt Extract Agar (MEA). The cultures were sampled at different times: 1, 2, 3, 4, 7,10, 14, and 28 days. The following parameters were measured in every sample: adenosine nucleotide content (ATP, ADP, and AMP), dry weight, protein, and chitin content. There were three replicates and one control (uninoculated) for each measurement.

Nucleotide measurements

The nucleotides were extracted from the mycelium using cold (on ice) 5% TCA. The samples were homogenized using a tissue grinder and then incubated overnight. The extracted cultures were centrifuged and one ml aliquot of the supernatant was stored at -20 °C. The nucleotide content of the sample was measured after preparing the following assay cocktails (Pradet, 1967):

- **Tube A**: 100 µl of extracted nucleotides + 900 µl of 20 mM Tris (pH=7.6) buffer containing 15% (w/v) $MgSO_4$ and 12.5% K_2SO_4.

-**Tube B**: same as in tube A + 20 µl of Pyruvate Kinase (PK; EC 2.7.1.40) (1000units/ml) + 21 µl of 10 mM Phosphoenol Pyruvate(PEP).

- **Tube C**: same as in tube B + 20 µl of Adenylate Kinase (AK; EC 2.7.4.3) (1000units/ml).

The tubes were incubated for 1 hour at 37°C and then the reaction was stopped by placing the tubes in boiling water for 2 minutes. The tubes were cooled on ice and the ATP concentration was measured using a bioluminometer (Monolight 2010, ALL cat # 2010C). The light output was measured for one second to avoid the recycling of ADP or AMP to ATP.

Tube A measured the ATP content of the sample. The ADP content of the sample was measured indirectly by subtracting the ATP content of tube A from that measured in tube B. Finally the AMP content was measured by subtracting the nucleotide content of tube B from that of tube C.

A standard mixture of the three nucleotides (2.5 µM of each of them) was assayed together with the samples, and a blank, to estimate the efficiency of the recovery of the three nucleotides.

Chitin and Protein

The homogenate left after the nucleotide extraction was washed and resuspended in 20 ml of distilled water. Two 5 ml aliquots of this homogenate were used to measure the chitin and protein content of the mycelium. These aliquots were centrifuged and the supernatant was removed.

Alkali (1M NaOH) was used to extract the protein from the mycelium. Extraction was aided by agitating the mixture on a shaker overnight at room temperature (25°C). The protein content was measured using Bradford's method (Bradford 1974).

The chitin content of the mycelium was measured using the colorimetric method described by Ride and Drysdale (1972). In this method, the chitin was first deacetylated using a strong hot alkali (22 M KOH at 120 °C) to yield chitosan. The chitosan was then deaminated using nitrous acid which yielded an aldehyde which was assayed colorimetrically (650 nm) after reaction with 3-methyl-2-benzothiazolone hydrazone.

Dry weight

The culture was filtered through a preweighed cellulose acetate filter membrane (0.45 μm pore size; Gelman cat.# 63069). The filters were then dried to constant weight at 60 °C overnight.

RESULTS

P. chrysosporium

The ATP content of the mycelium increased rapidly from a value of 2.22x10^{-10} moles/mg of dw on the first day after transfer, to 7.28x10^{-9} moles/mg of dw on the third day of growth (table 3.1, and figure 3.1).

Table 3.1:
Changes in nucleotide concentrations (moles/mg of dry weight) and Energy Charge(EC) values of *Phanerochaete chrysosporium.* as a function of time (μ and ∂ are the average and standard deviation of three data points).

Time	ATP		ADP		AMP		EC	
days	μ	∂	μ	∂	μ	∂	μ	∂
1	2.22E-10	0.41E-10	2.05E-10	0.92E-10	1.33E-10	0.37E-10	0.58	0.02
2	1.21E-9	3.53E-11	6.02E-10	1.81E-10	1.96E-10	0.11E-10	0.75	0.00
3	7.28E-9	1.11E-9	3.25E-9	0.30E-9	1.66E-10	2.35E-10	0.83	0.01
4	6.70E-9	1.20E-10	2.70E-9	3.32E-10	1.59E-9	0.52E-9	0.73	0.03
7	2.65E-9	2.47E-10	3.98E-9	0.71E-10	1.44E-9	0.40E-9	0.57	0.02
14	8.80E-10	5.60E-12	4.90E-9	0.67E-9	2.56E-9	0.15E-9	0.40	0.01
28	2.97E-10	1.06E-10	1.92E-9	0.53E-9	3.04E-9	0.92E-10	0.23	0.05

μ: average; ∂: standard deviation.

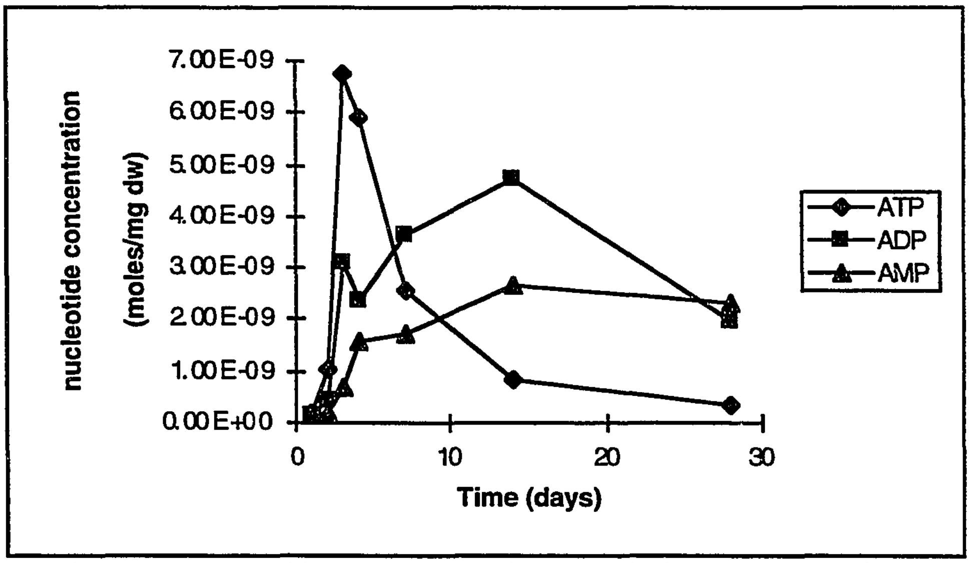

Figure 3.1: Nucleotide content (moles/ mg of dw) of the mycelium of *P. chrysosporium* during growth in batch culture.

Afterward the relative ATP content decreased consistently until the end of the incubation period, whereas, the ADP, and AMP contents increased to a maximum value of 4.90x10^{-9} moles/mg of dw on the fourteenth day, and 2.56x10^{-9} moles/mg of dw after a month.

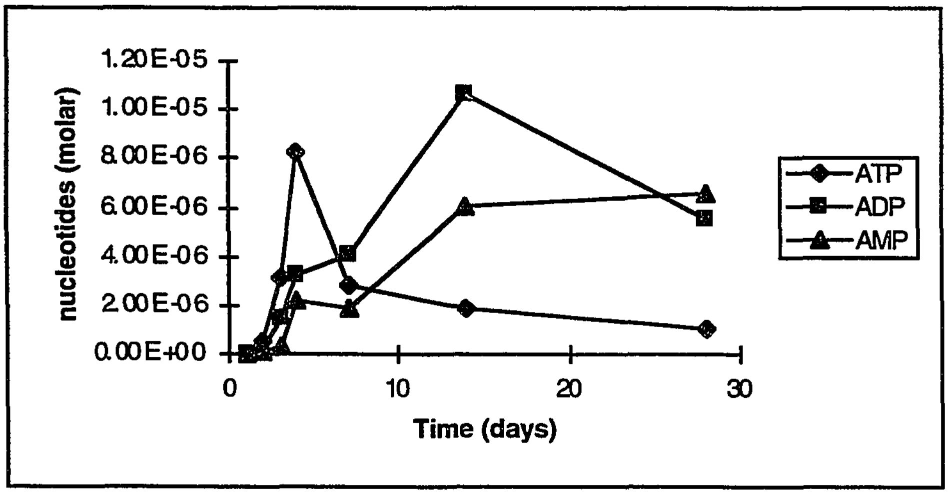

Figure 3.2: Nucleotide content of the cultures of *P. chrysosporium* (µg/ml)

The total nucleotide concentrations of the culture (figure 3.2) follow those of the nucleotide content per unit of dry weight (figure 3.1).

The EC values reflected these trends, increasing from 0.58, on the first day of incubation, to 0.83, on the third day, and then decreased to a low value of 0.23 after a month (figure 3.3).

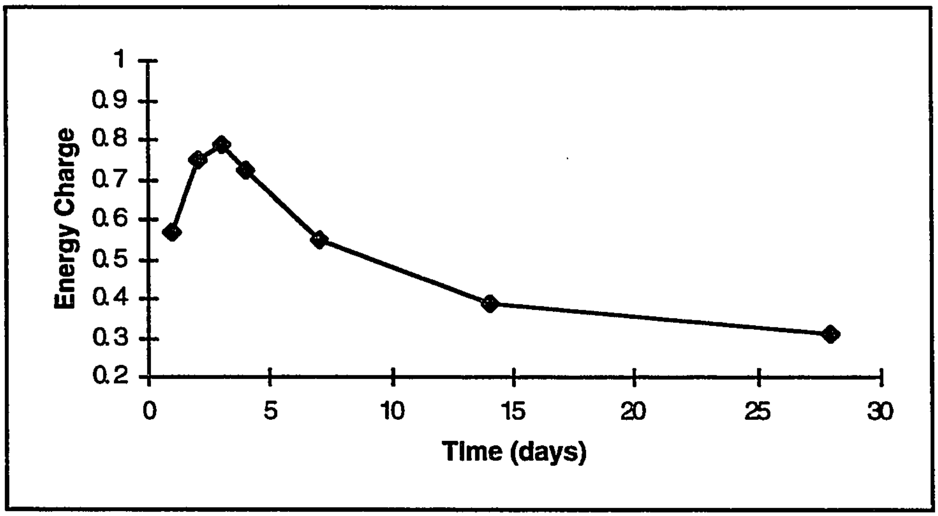

Figure 3.3: Changes in Energy Charge (EC) during the growth of *P. chrysosporium* in batch culture

P. chrysosporium grew rapidly during the first four days of exponential growth phase, a characteristic which was consistent with increasing values of EC (0.58 to 0.83).

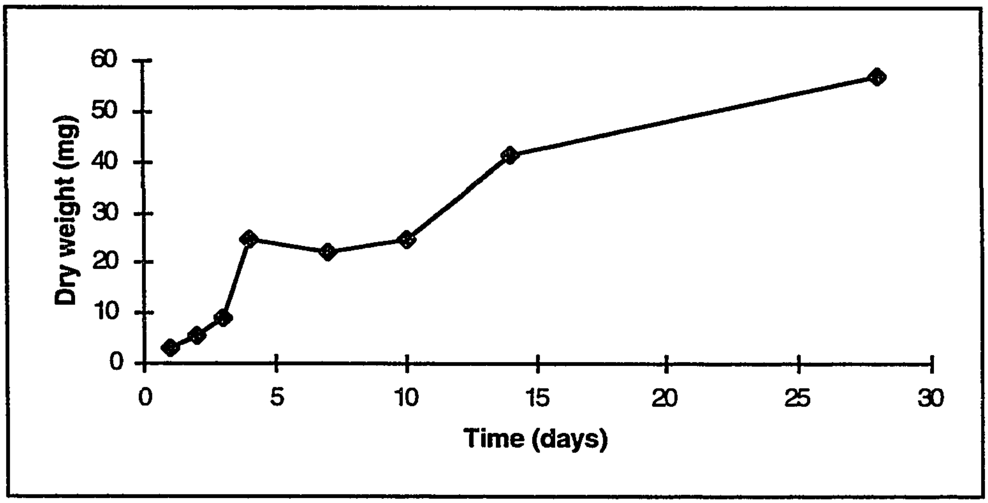

Figure 3.4: Changes in dry weight during the growth of *P. chrysosporium* in bacth culture

The differences in ATP content and EC of the mycelium at different days of growth were tested using a one way ANOVA and Tukey's test for pairwise comparisons. The results of this test showed that there were significant differences in both the ATP content and the EC values between sampling times ($\alpha = 0.0001$).

Tukey's test classified the data into three different groups based on ATP contents of the mycelium (table 3.2). The first group (labeled A in table 3.2) included the third, and fourth days. The ATP concentrations of the mycelium on these days were 7.28×10^{-9} and 6.70×10^{-9} moles/mg of dw respectively. The EC values were 0.83 and 0.73 respectively. The second group of data (labeled B in table 3.2) included the second and seventh days, with ATP concentrations being 1.21×10^{-9} and 2.65×10^{-9} moles/mg of dw respectively. The EC values were 0.75 on the second day, and 0.57 on the seventh day. The latter value of EC indicated a change in growth of the fungi from the exponential phase to the phase of negative acceleration. It is important to note that the ATP content for the seventh day was larger than for the second day but the EC value was lower. Therefore higher ATP content did not necessarily correlate with faster growth. The third group (labeled C in table 3.2) of values included the first, fourteenth, and twenty-eighth days with the lowest range of ATP values (2.22×10^{-10} to 8.80×10^{-10} moles/mg of dw) and EC values (0.23 to 0.55). The differences between these groups were significant ($\alpha = 0.05$), although there was some overlap between groups B and C.

Table 3.2: Results of the one way ANOVA and pairwise comparisons of ATP contents of the mycelia of *P. chrysosporium* as a function of time.

Tukey Groupings	Average	Time (days)	EC
A	7.28×10^{-9}	3	0.83
A	6.70×10^{-9}	4	0.73
B	2.65×10^{-9}	7	0.57
C B	1.21×10^{-9}	2	0.75
C	8.80×10^{-10}	14	0.40
C	2.97×10^{-10}	28	0.23
C	2.22×10^{-10}	1	0.55

(Tukey's test, α =0.05). Same letters indicates no significant differences. Units are moles/mg of dry weight.

The chitin and protein content of the mycelium showed a different pattern of changes than that for ATP. Although the total protein and chitin content of the mycelium increased with time, they decreased in relation to the dry weight. Increases of biomass and faster growth rates are coupled with decreases in the protein and chitin content of the mycelium. There was a time lag between the changes in biomass and the changes in protein and chitin content of the mycelium. The protein content of the mycelium varied from a minimum value of 17.14 μg/mg of dry weight on day two to a maximum value of 87.03 μg/mg of dw on day seven (table 3.3).

Table 3.3: Protein content of *P. chrysosporium*, *P. placenta*, and *C. globosum* during growth (μg/mg of dry weight).

Time	P. chrysosporium		P. placenta		C. globosum	
(days)	μ	∂	μ	∂	μ	∂
1	34.45	0.18	82.23	0.32	64.44	3.79
2	17.14	1.80	71.57	0.00	43.59	1.94
3	69.20	8.23	10.78	0.15	35.52	1.86
4	64.28	8.75	45.43	0.47	50.40	0.08
7	87.03	0.00	65.63	1.93	40.36	1.54
10	66.29	13.76	56.19	2.81	28.14	0.09
14	69.87	4.35	43.69	0.38	48.25	3.86
28	29.16	0.25	29.27	6.17	23.96	1.02

μ: average; ∂: standard deviation.

Table 3.4: Chitin content of the mycelium of *P. chrysosporium*, *P. placenta* and *C. globosum* during growth (μg/mg of dw).

Time	**P. chrysosporium**		**P. placenta**		**C. globosum**	
(days)	μ	∂	μ	∂	μ	∂
1	4.64	0.01	26.51	0.37	18.69	0.18
2	6.14	0.73	23.17	0.23	17.13	1.40
3	54.34	2.88	6.48	0.08	17.25	0.44
4	38.60	0.46	51.46	0.66	63.05	0.84
7	68.77	0.20	39.44	1.49	40.10	0.15
10	174.05	7.88	40.46	18.02	37.42	0.70
14	29.85	1.49	72.87	11.33	102.17	16.98
28	64.35	7.20	67.16	3.40	54.03	13.43

The changes in biomass of the mycelium of *P. chrysosporium* followed an exponential increase during the first three days of the growth (figure 3.3). Nevertheless the protein content of the mycelium did not reach its maximum value until the seventh day (87.03 μg/mg of dw) (figure 3.5).

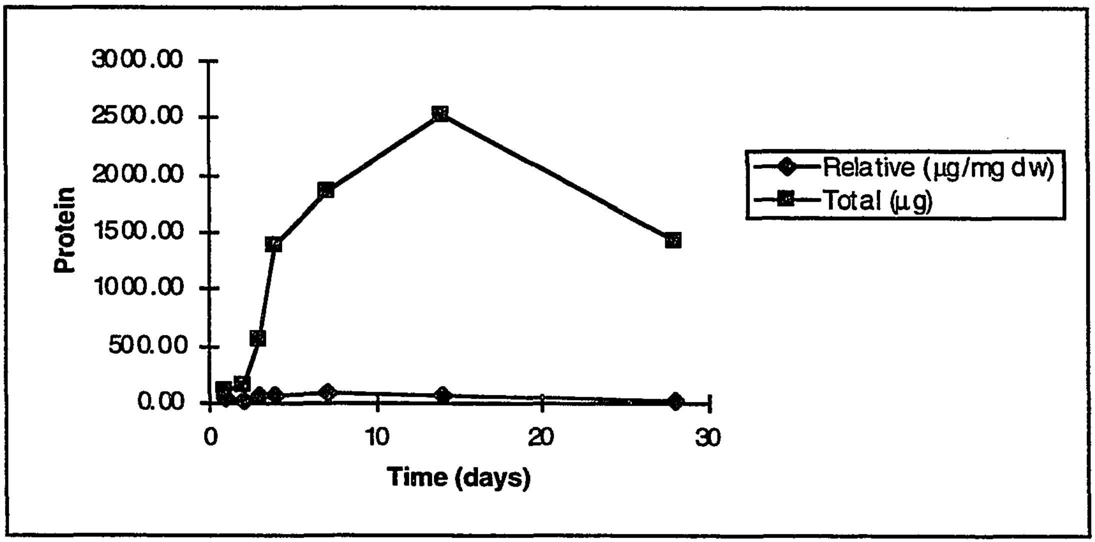

Figure 3.5: Changes in protein content of the mycelium of *P. chrysosporium* during growth in batch culture.

The data for protein content were also analyzed using a one way ANOVA with Tukey's test for pairwise comparisons, and significant differences were found (α =0.0001) between the protein content in different phases of the growth cycle. Statistical analysis divided the data into two groups which were significantly different from each other (α =0.05). The first group, labeled A, included protein values between 64.28 and 87.03 µg/mg of dry wt. corresponding to days 3, 4, 7, 10, and 14. The second group, labeled B, included protein contents ranging between 17.14 and 34.45 µg/mg of dw for days one, two, and twenty eight (table 3.5).

Table 3.5: Results of one way ANOVA for the protein/mg of dw content of *P.chrysosporium.*

Tukey Groupings	Average	Time (days)
A	87.03	7
A	69.87	14
A	69.20	3
A	66.29	10
A	64.28	4
B	34.45	1
B	29.16	28
B	17.14	2

The chitin content of the mycelium of *P. chrysosporium* increased with increasing biomass, from a minimum of 4.64 µg/mg of dw, on the first day, up to a maximum value of 137.18 µg/mg of dry wt. on the tenth day of incubation.

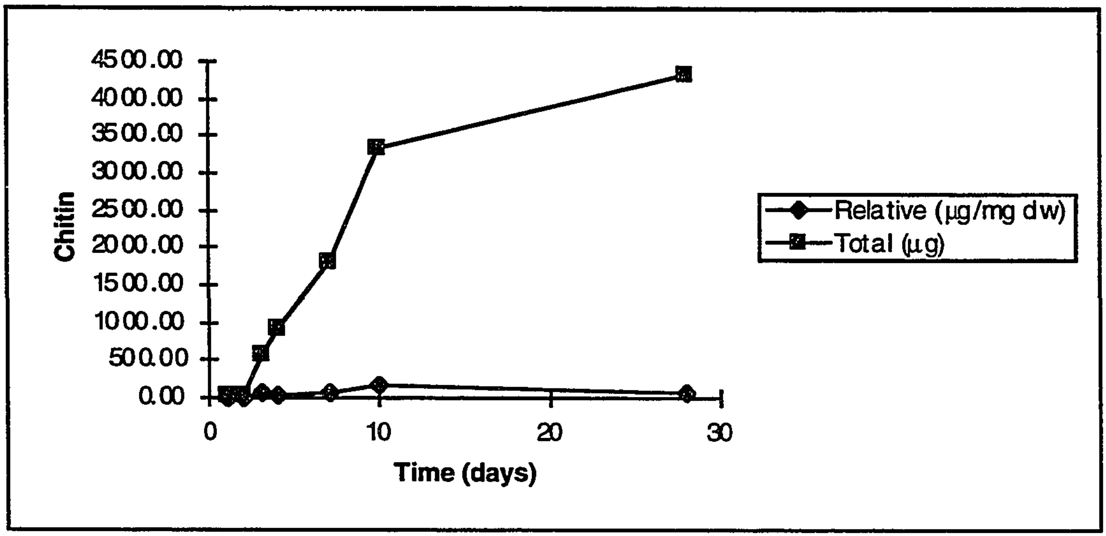

Figure 3.6: Changes in chitin concentration of the mycelium of *P. chrysosporium* (µg/mg of dw) during growth in batch culture.

After the tenth day, the chitin content of the mycelium decreased to a final value of 64.35 µg/mg of dw. For instance, between the third and fourth days the chitin content decreased from 54.34 μg/mg of dw to 38.60 μg/mg of dw. Similar changes were observed in the protein content of the mycelium, particularly during periods of rapid increase of biomass (table 3.4, and figure 3.6). The data for the chitin content were also analyzed using an ANOVA with pairwise comparisons. As the mycelium grew significant differences in the concentration of chitin, were found. Tukey's test classified the data into four groups (table 3.6). The maximum chitin content (174.05 μg/mg dw) was recorded on the tenth day of incubation (Group A). Group B included the seventh, third, and twenty-eighth days, with chitin values ranging from 54.34 to 68.77 μg/mg of dw. Group C included the fourth and fourteenth days, with chitin values of 38.60 and 29.85 μg/mg of dw respectively. The values of chitin were low during the first few days of incubation (group D; 4.64 and 6.14 μg/mg of dw).

Table 3.6: Results of ANOVA for the chitin content of *P. chrysosporium*. (μg/mg dw).

Tukey Groupings	Average	Time
A	174.05	10
B	68.77	7
B	64.35	28
B	54.34	3
C	38.60	4
C	29.85	14
D	6.14	2
D	4.64	1

Overall, the chitin and protein content increased steadily with the biomass of the mycelium. Changes in protein, and chitin of the mycelium lagged behind those of ATP and EC. Changes in ATP and particularly EC were coupled with changes in the rate of increase of the biomass of the mycelium . Growth took place even when the EC was low.

P. placenta

The growth of *P. placenta* was not as rapid as that of *P. chrysosporium*. It had a two day lag phase followed by steady linear increase in biomass until the fourteenth day, after which there was a slight decrease in biomass (figure 3.7).

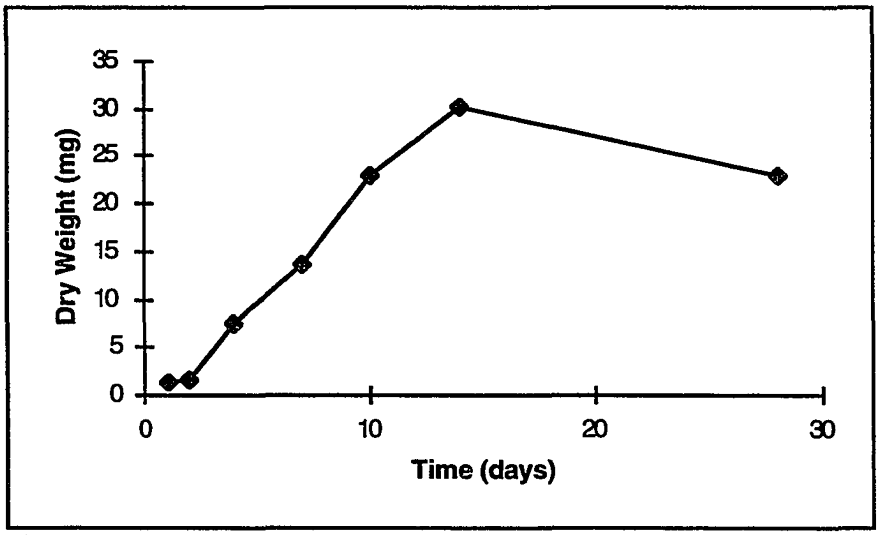

Figure 3.7: Changes in the dry weight changes of the mycelium of *P. placenta* in batch culture (mg).

The ATP content of the mycelium reflected this trend, increasing until the seventh day of incubation (ATP=5.32×10^{-9} moles/mg and EC= 0.72) (table 3.7 and figure 3.8). The concentrations of the other nucleotide followed this same trend (figure 3.9). The EC value of the mycelium stayed above 0.7 except for the first day of growth and the last two (table 3.7 and figure 3.10).

Table 3.7

Nucleotide concentrations (moles/mg of dry weight) and Energy Charge(EC) values for the mycelium of *Poria placenta* during growth in batch culture.

Time	ATP		ADP		AMP		EC	
days	μ*	∂*	μ	∂	μ	∂	μ	∂
1	$2.31X10^{-10}$	1.77E-10	8.69E-10	6.62E-10	$2.84X10^{-10}$	$1.64X10^{-10}$	0.47	0.02
2	$2.37X10^{-9}$	$0.51X10^{-9}$	$1.05X10^{-9}$	$0.23X10^{-9}$	$4.97X10^{-10}$	$0.54X10^{-10}$	0.75	0.01
4	$4.23X10^{-9}$	$0.22X10^{-9}$	$3.34X10^{-9}$	$1.22X10^{-9}$	$4.14X10^{-10}$	$5.86X10^{-10}$	0.76	0.02
7	$5.32X10^{-9}$	$0.35X10^{-9}$	$4.56X10^{-9}$	$0.63X10^{-9}$	$1.66X10^{-9}$	$0.35X10^{-9}$	0.72	0.06
10	$4.70X10^{-9}$	$0.32X10^{-9}$	$3.42X10^{-9}$	$0.15X10^{-9}$	$1.07X10^{-9}$	$0.55X10^{-9}$	0.70	0.06
14	$1.21X10^{-9}$	$0.08X10^{-9}$	$3.26X10^{-9}$	$0.40X10^{-9}$	$1.75X10^{-9}$	$0.02X10^{-9}$	0.45	0.01
28	$1.31X10^{-9}$	$0.30X10^{-9}$	$1.46X10^{-9}$	$0.13X10^{-9}$	$1.46X10^{-9}$	$0.13X10^{-9}$	0.49	0.03

* μ and ∂ are the average of three data points.

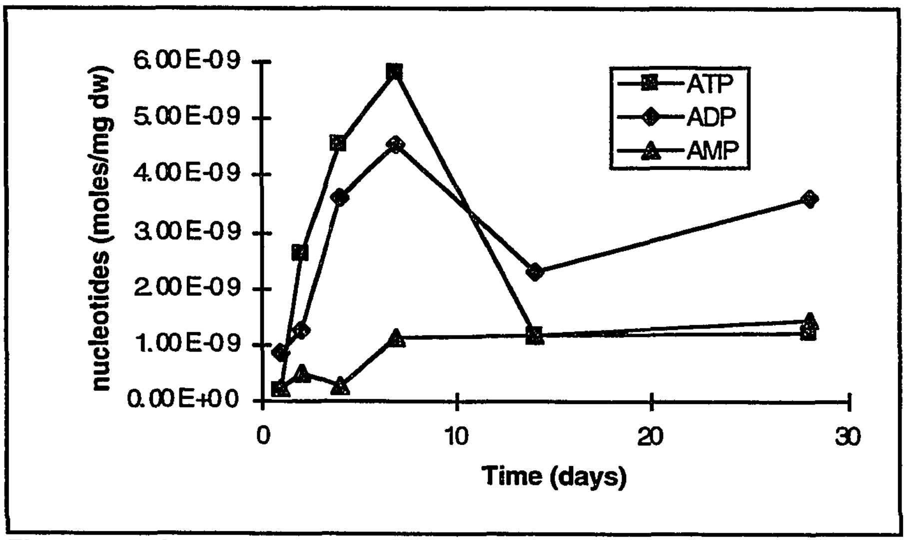

Figure 3.8: Changes in nucleotide concentrations of *P. placenta* (moles/mg of dw) during growth in batch culture.

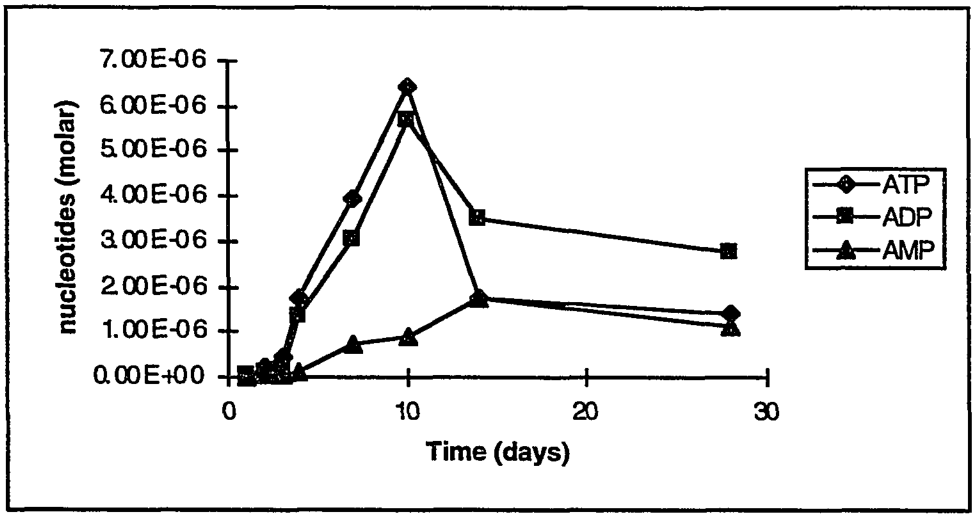

Figure 3.9: Changes in nucleotide concentrations of *P. placenta* (molar) during growth in batch culture.

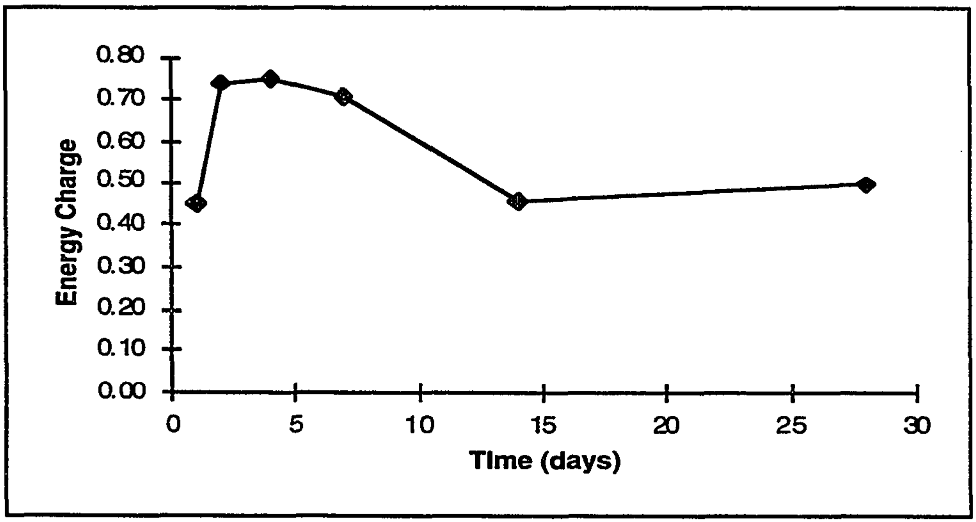

Figure 3.10: Changes in EC of *P. placenta* during growth in batch culture.

The data for ATP and EC were analyzed using a one way ANOVA with pairwise comparisons. There were significant differences in both the ATP content and energy charge as the mycelia grew. Tukey's test classified the data into three groups (table 3.8). Group A included samples from the fourth, seventh, and tenth days of growth with EC values above 0.7, and ATP contents ranged from 4.23×10^{-9} to 5.32×10^{-9} moles/mg dw. Group B included samples from the second, twenty-eighth, and fourteenth days. The EC for the second day was 0.75, but the ATP content was significantly lower than those in group A

(ATP=2.37×10^{-9} moles/mg of dw). The phase of positive acceleration occurred during the second day of incubation (between the lag phase of growth and beginning of exponential growth of the mycelium). This was reflected in a higher EC value but lower ATP content. The ATP content for the first day was 2.31×10^{-10} moles/mg; ten times lower than in the second day. The other days included in group B had EC values below 0.7 and lower ATP contents (ATP=1.21×10^{-9} moles/mg of dw EC=0.45 after two weeks, and 1.39×10^{-9} moles/mg of dw EC=0.49 after one month) (table 3.8). Except for the first day (lowest ATP content 2.31×10^{-10} moles/mg of dw), the third group, labeled C, overlapped with group B in all its range.

Table 3.8 : Results of the pairwise comparisons of the ATP content (Tukey and GT2 tests) of the mycelia of *P. placenta* during growth in batch culture.

Tukey Groupings	Average	Time	EC
A	5.32×10^{-9}	7	0.72
A	4.70×10^{-9}	10	0.70
A	4.23×10^{-9}	4	0.76
B	2.37×10^{-9}	2	0.75
C B	1.39×10^{-9}	28	0.49
C B	1.21×10^{-9}	14	0.45
C	2.31×10^{-10}	1	0.47

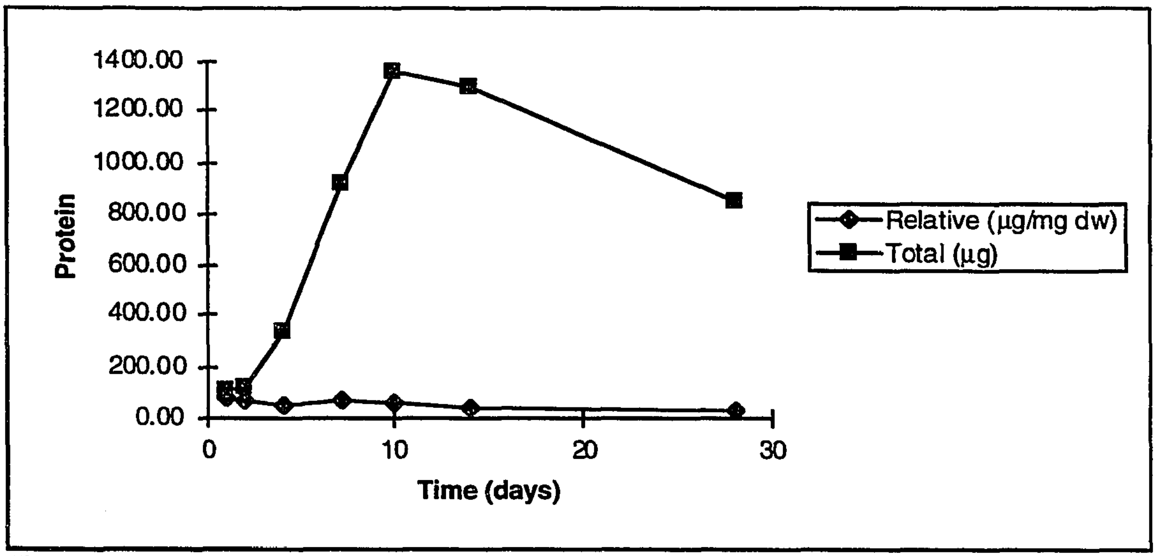

Figure 3.11: Changes in the protein concentration of the mycelium of *P. placenta* during growth in batch culture.

Protein concentrations of the mycelium ranged, on average, from values of 10.45 µg/mg to 83 µg/mg dry weight (table 3.3 and figure 3.11) . Overall protein concentration of the mycelium (per unit weight) decreased as the mass of the mycelium increased. Nevertheless, the total protein content of the culture increased with time (figure 3.11). The protein data was analyzed using one way ANOVA with pairwise comparisons (table 3.9). Tukey's test classified the data in four different groups (α=0.05). The first one included only the first day of incubation with a protein content of 82.23 µg/mg of dry weight. The second group encompassed the log phase of growth (from the second to the tenth day) with protein content ranging from 56.19 to 71.57 µg/mg of dw.

Table 3.9: Results of one way ANOVA for the protein content of the mycelium of *P. placenta* during growth in batch culture.

Tukey Groupings	Average	Time
A	82.23	1
B	71.57	2
C B	65.63	7
C	56.19	10
D	45.43	4
D	43.69	14
E	29.27	28
E	10.77	3

The third group included samples from the fourth and fourteenth days, with protein values of 45.43, and 43.69 µg/mg respectively. Finally the fourth group included samples from the third and twenty-eighth days with the lowest protein values (29.27 µg/mg of dw and 10.77 µg/mg of dw respectively). Overall, the protein concentration per milligram of dry weight of the mycelium of *P. placenta* decreased throughout the growth period. Statistical analysis of the data suggested that there were significant differences in the protein content of the mycelium at different phases of the growth cycle.

After an initial decline during the first days of growth (26.5 to 6.5 µg/mg of dry weight), the chitin content of the mycelium of *P. placenta*, increased steadily until the fourteenth day when it reached a maximum concentration of 72.87 µg/mg of dry weight (table 3.4 and figure 3.12). The total chitin content per ml of culture increased with time until the fourteenth day, and then decreased until the end of the incubation period.

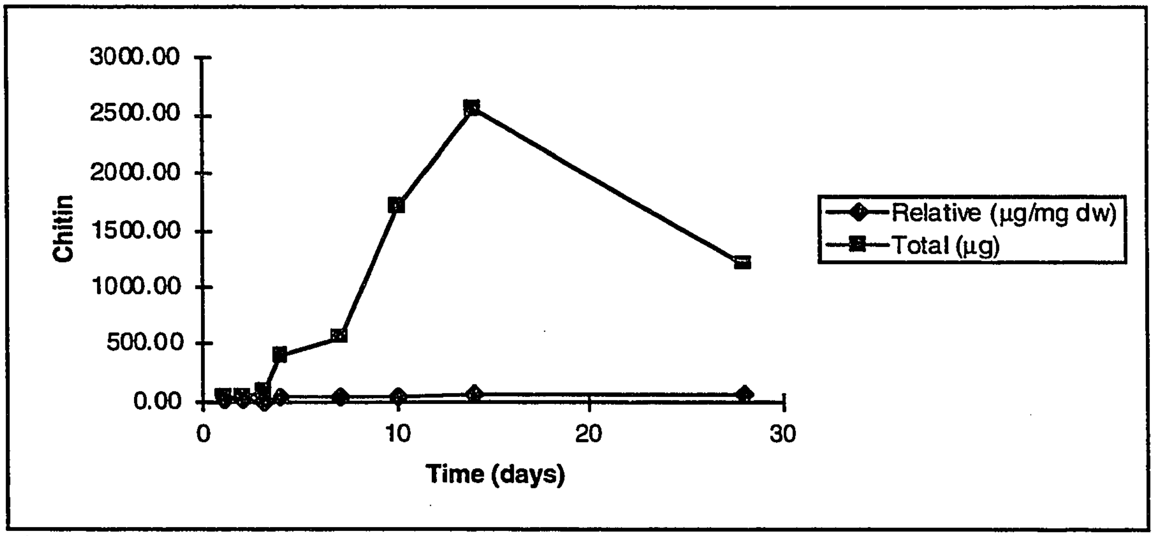

Figure 3.12: Changes in the chitin content of the mycelium of *P. placenta* during growth in batch culture .

The data was analyzed using an ANOVA with pairwise comparisons (table 3.10).

Table 3.10: Results of the ANOVA on the chitin content of the mycelium of *P. placenta* during batch culture.

Tukey Groupings	Average	Time (days)
A	72.87	14
A	67.16	28
B A C	51.46	4
B C	40.46	10
B C	39.44	7
D C	26.51	1
D C	23.17	2
D	6.48	3

Overall, the chitin content of the mycelium varied from a low of 6.48 to a peak of 72.87 µg/mg of dry weight. When the data for chitin was analyzed using a one way ANOVA with pairwise comparisons, significant differences were found in the chitin concentration as the mycelia grew. The pairwise comparisons divided the data into four different groups (table 3.10). The ranges of the groups overlapped. There was no overlap in the data groups A (including samples from the fourth , fourteenth, and twenty-eighth days with chitin contents between 51.46 - 72.87 µg/mg of dw) and B (including samples from the first three days with chitin values between 6.48 and 26.51µg/mg of dw). Groups B, and C (chitin contents between 23.17 and 51.46 µg/mg of dw) overlapped at their upper and lower ranges with A, and D respectively. Overlapping indicates large variance within groups.

C. globosum

C. globosum was the slowest growing of the three fungi tested. It grew continuously, at a linear rate, throughout the period of observation (figure 3.13).

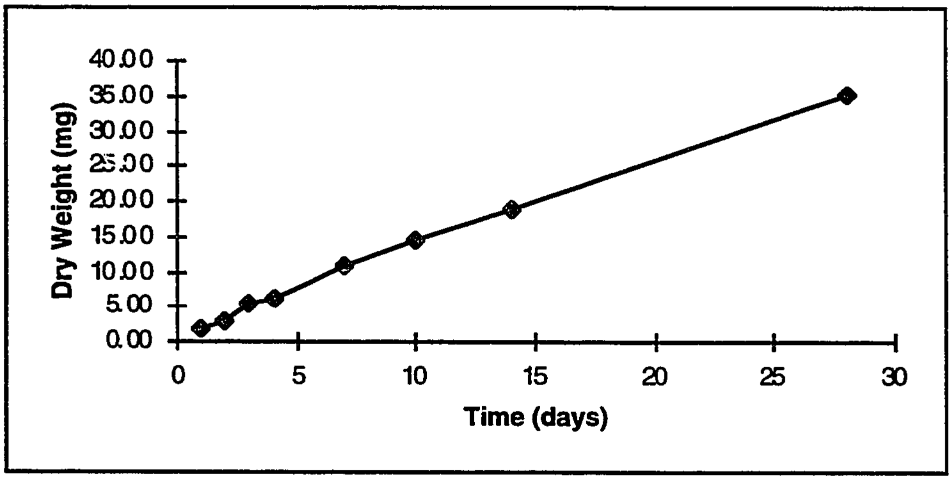

Figure 3.13: Changes in dry weight (mg) of the mycelium of *C. globosum* during growth in batch culture.

This growth pattern was associated with low EC values during the whole period of incubation (0.43 ≤ EC ≤0.60) (figure 3.16 and table 3.11). The lowest ATP concentrations were found during the first three days and the last day of incubation (ATP= 1.46×10^{-9} to 1.71×10^{-9} moles/mg of dw). During the intervening days of incubation the ATP content stayed between 3.13 - 3.63×10^{-9} moles/mg of dw (figure 3.14 and table 3.11). The total nucleotide concentrations of the cultures increased until the seventh day and then remained at that level until the end of the growth period (figure 3.15). The EC values were between 0.4 - 0.6 during the whole period of growth.

Table 3.11: Nucleotide concentrations (moles/mg of dry weight) and Energy Charge(EC) values of the mycelium of *Chaetomnium globosum* during growth in batch culture.

Time	ATP		ADP		AMP		EC	
days	µ*	∂*	µ	∂	µ	∂	µ	∂
1	1.65X10^{-9}	0.07X10^{-9}	3.40X10^{-9}	0.95X10^{-9}	1.10X10^{-9}	0.07X10^{-9}	0.51	0.01
2	1.46X10^{-9}	0.09X10^{-9}	2.02X10^{-9}	0.50X10^{-9}	1.00X10^{-9}	0.15X10^{-9}	0.48	0.03
3	1.71X10^{-9}	0.00X10^{-9}	1.95X10^{-9}	0.64X10^{-9}	0.96X10^{-9}	0.43X10^{-9}	0.60	0.00
4	3.63X10^{-9}	0.25X10^{-9}	4.90X10^{-9}	1.38X10^{-9}	1.69X10^{-9}	0.16X10^{-9}	0.57	0.01
7	3.32X10^{-9}	0.06X10^{-9}	4.04X10^{-9}	1.11X10^{-9}	3.06X10^{-9}	0.57X10^{-9}	0.55	0.01
10	3.43X10^{-9}	0.11X10^{-9}	4.14X10^{-9}	0.22X10^{-9}	1.72X10^{-9}	0.78X10^{-9}	0.53	0.06
14	3.13X10^{-9}	0.15X10^{-9}	3.31X10^{-9}	1.43X10^{-9}	3.25X10^{-9}	1.50X10^{-9}	0.43	0.06
28	1.57X10^{-9}	0.00X10^{-9}	1.48X10^{-9}	0.35X10^{-9}	1.38X10^{-9}	0.20X10^{-9}	0.51	0.01

* µ and ∂ are the average and standard deviations of three data points.

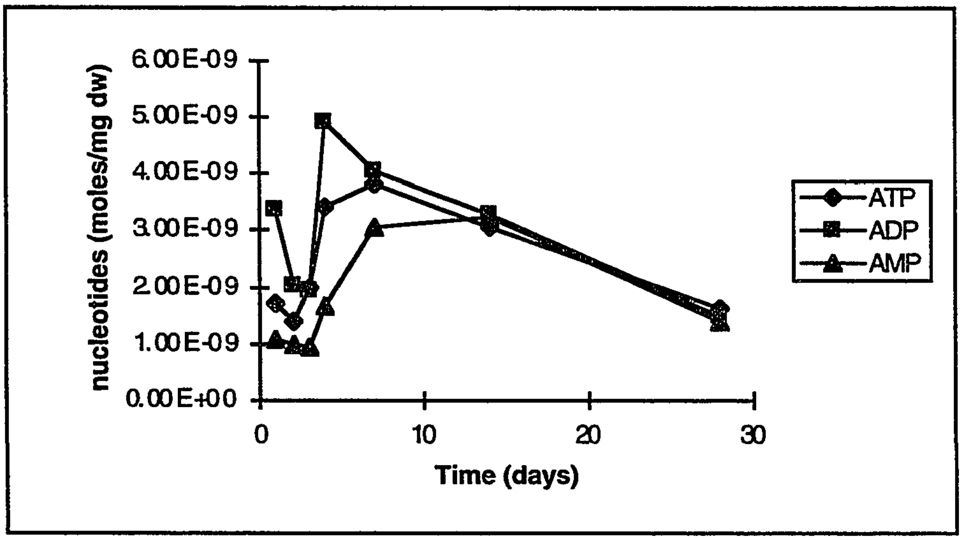

Figure 3.14: Changes in nucleotide concentrations (moles/mg of dw) of the mycelium of *C. globosum* during growth in batch culture .

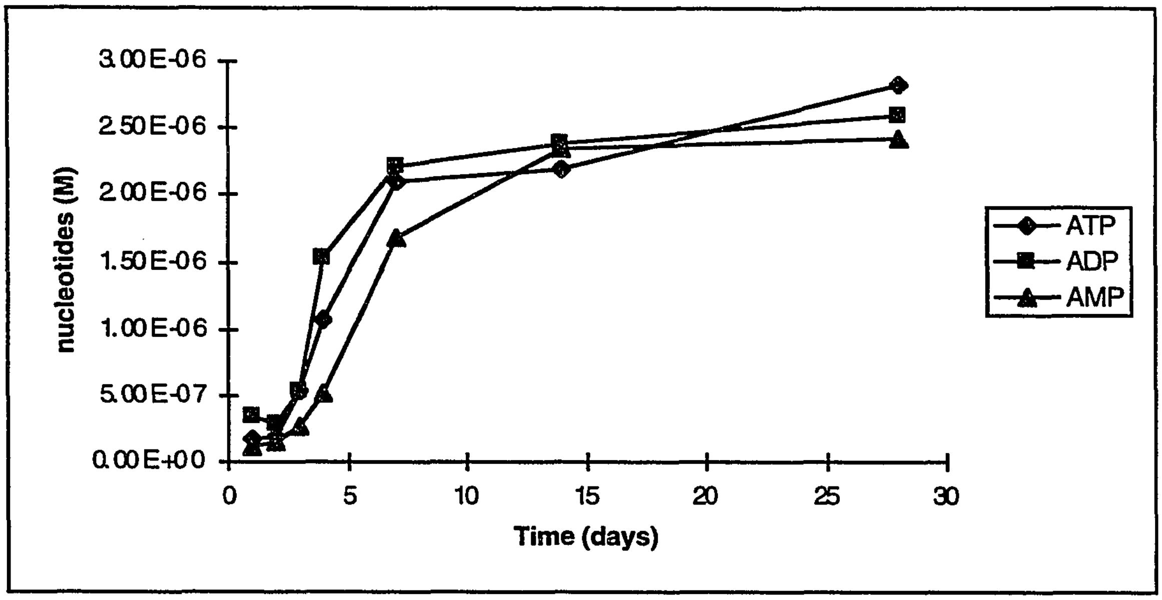

Figure 3.15: Changes in nucleotide concentrations (M) of the mycelium of *C. globosum* during growth in batch culture .

The ANOVA test for the ATP content of the mycelium found significant differences at different stages of the growth cycle (α =0.001). Pairwise comparisons classified the data in two groups with significant differences (α=0.05). The first group included the highest values of ATP (2.63 - 3.63x10^{-9} moles/mg of dw) for the fourth, seventh, tenth, and fourteenth days (table 3.12). The second group included the lowest ATP values (1.46 - 1.71x10^{-9} moles/mg of dw) corresponding to samples from the first three and twentyeigtht days (table 3.12).

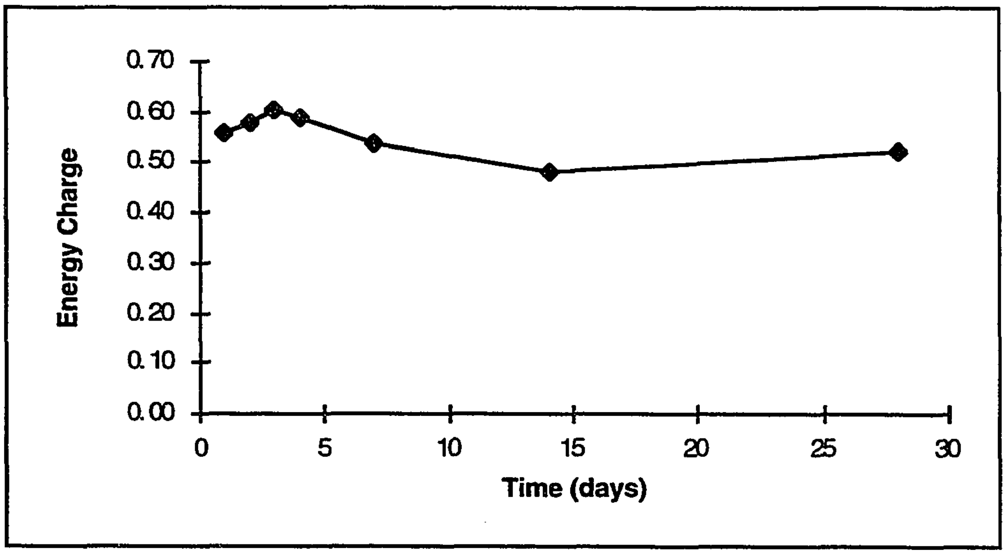

Figure 3.16: Changes in EC during growth of *C. globosum* in batch culture.

Table 3.12: Results of the one way ANOVA of pairwise comparisons of the mycelium of *C. globosum* for the ATP content, during various phases of batch culture

Tukey Groupings	Average	Time	EC
A	3.63x10^{-9}	4	0.48
A	3.43x10^{-9}	10	0.53
A	3.32x10^{-9}	7	0.55
B A	2.63x10^{-9}	14	0.43
B	1.71x10^{-9}	3	0.60
B	1.65x10^{-9}	1	0.51
B	1.57x10^{-9}	28	0.51
B	1.46x10^{-9}	2	0.48

The EC values for the mycelium of *C. globosum* (0.4 and 0.6), were lower than those found for the other two fungi. The growth rate of *C. globosum* was also slower (figure 3.13). However the average ATP concentrations of the mycelium, considered in proportion to dry weight, were larger than those found in the mycelia of the other two fungi. Protein concentrations in *C. globosum* were similar to those in *P. placenta*, with a small decline at the beginning of the growth, followed by a peak and subsequent decline (figure 3.17). Overall the protein concentration of the mycelium ranged between 64.44 µg/mg of dry weight in a sample from the first day to 23.96 µg/mg of dry weight in a sample from the twenty-eighth day (table 3.3, figure 3.17). The total protein concentrations of the cultures increased with time until the fourteenth day. This pattern is similar to that found for the other two species.

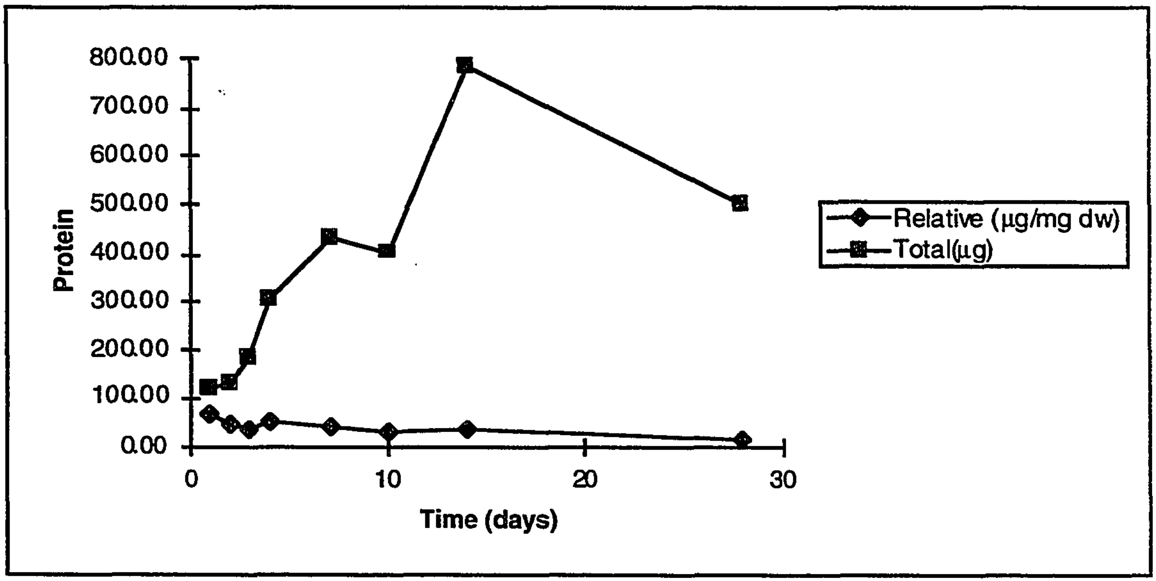

Figure 3.17: Changes in protein concentration during growth for *C. globosum*.

Statistical analysis of the protein data showed significant differences between the measurements taken on different days of incubation, but Tukey's test could not separate the data into groups because there was too much overlap (table 3.13).

Table 3.13: Results of one way ANOVA for the protein content of the mycelium of *C. globosum* during growth in batch culture.

Tukey Groupings	Average	Time
A	64.44	1
B	50.40	4
C B	48.25	14
C B D	43.59	2
C D	40.36	7
E D	35.52	3
F E	28.14	10
F	23.96	28

As the culture grew there were smaller changes in protein values than there were in the other two species. Changes in the amount of chitin in the mycelium of *C. globosum* were similar to those of the other two species, increasing with the biomass of the mycelium (figure 3.18). They ranged from 17.13 μg/mg on the second day, to 102.17 μg/mg of dw on the fourteenth day. The data for chitin concentration were analyzed using a one way ANOVA with pairwise comparisons. Significant differences were found dividing the data into two groups. The highest value of chitin (102.17 μg/mg of dw) was obtained in a sample taken on the fourteenth day of growth; it was distinct from all other measurements (table 3.14).

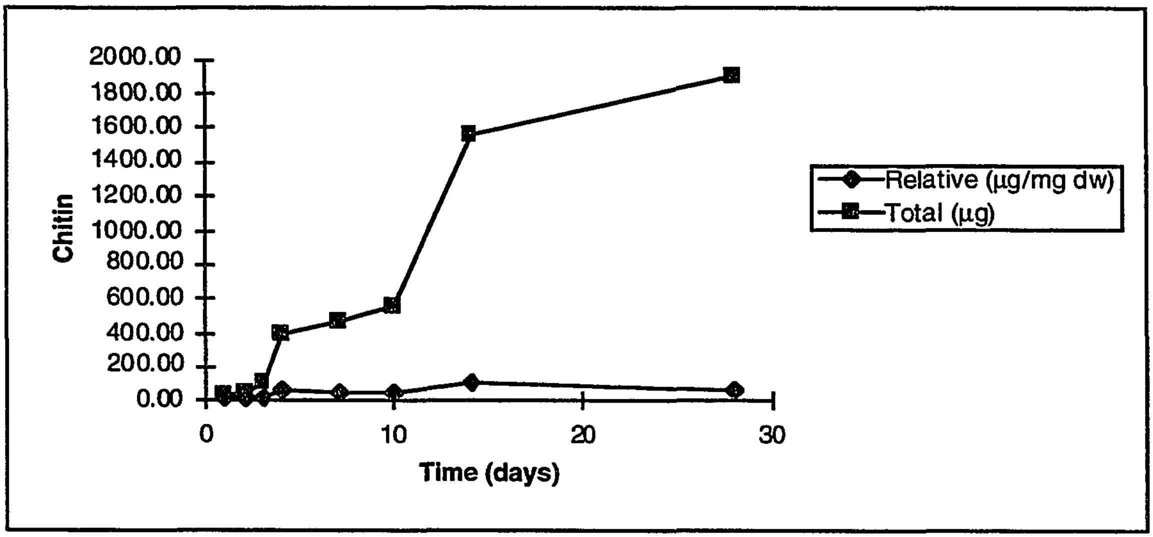

Figure 3.18: Changes in the chitin content of the mycelium of*C. globosum* as it grew in batch culture.

The second group included samples from the fourth, seventh, tenth, and twenty eighth days. They had chitin concentrations ranging from 37.42 to 63.05 μg/mg of dw (table 3.14). The third group had the lowest chitin values (17.13 and 18.69 μg/mg of dw).

Table 3.14: Results of ANOVA of chitin in the mycelium of *C. globosum* during growth in batch culture.

Tukey Groupings	Average	Time
A	102.17	14
B	63.05	4
B	54.03	28
C B	40.10	7
C B	37.42	10
C	18.69	1
C	17.25	3
C	17.13	2

DISCUSSION

Inherent in the application of indirect chemical methods to estimate biomass, is the assumption that the content of the chemicals in the mycelium per unit of dry weight is relatively constant. None of the parameters used in this study remained constant during growth of the mycelia of any of the three fungi studied. A conversion factor can be calculated as an average of all the values found for each parameter. Such a conversion factor could be used to estimate the biomass of a sample with an error equal to the standard deviation of the data used to calculate the conversion factor. For example, Ride and Drysdale(1972) used chitin content as a way to estimate fungal biomass in plant tissue. They used a conversion factor of 80 μg of glucosamine per mg of dry weight for *Fusarium oxysporum f. lycopersici* . They found, however, that other species used in their study, and grown in the same type of medium, had much lower chitin values (average value of 38 μg of glucosamine per mg of dry weight). Swift (1973) also used chitin content to measure mycelial biomass in decaying wood tissue. His measurements were based on the white rot fungus, *Coriolus versicolor*, grown in media with different C:N ratios. The conversion factor he calculated varied between 12.4 μg/mg in medium with a C:N ratio of 45:1 to 16.5 μg/mg in medium with ten times more carbon. Whipps (1987) studied the changes in chitin content in different species of ectomycorrhizal fungi. The chitin content of the mycelia of two of the fungal species, *Laccaria laccata* and *Suillus bovinus*, grown in the same medium, ranged from 16 - 23 µg/mg dry weight in the former to 38 - 66 µg/mg dry weight in the latter. Some of the variability he observed might reflect differences in age of the mycelium, or changes in the composition of the medium. Since protein and chitin contents are affected by the C:N ratio of the medium (Seitz et al., 1979, and Swift, 1973), losses in dry weight and redistribution of carbohydrates and nitrogenous compounds supported continued growth of the mycelium under nitrogen limited conditions (Cowling and Merrill, 1966). These changes took place both in batch culture and inside wood.

In this study the protein and chitin content of the mycelium varied throughout the incubation period. The total content of protein and chitin in the mycelium increased, following the increases in biomass. Yet, per unit of dry weight, the protein and chitin content of the mycelium decreased as the mycelium matured. This may be caused by changes in the thickness of the cell walls of the hyphae during growth, due to changing extension rates of the tips (Marchant, 1978). It is also known that the chitin values of the cell walls of vegetative mycelium are different from that of sporophore walls (Elliott and Wood, 1978). The chemical composition of the cell wall is also different between monokaryons and dikaryons (Marchant, 1978). Mutant strains of some fungi (e.g. *Podospora anserina)* show zonated growth due to irregular branching and abnormally thick cell walls, when they are grown batch cultures (Lysek and Esser, 1971).

Another factor which affects the chemical composition of cell walls (per unit of weight) is that there are differences between the actively growing peripheral zone and the rest of the mycelial mass. The ratio between the growing zone and the cell mass decreases with time. It is mainly in the growing portion of the mycelium where nutrient accumulation occurs (Bornefeld and Lysek, 1972). If new synthesis does not occur, the main pools of cell wall constituents (e.g. chitin, ergosterol, protein, and other carbohydrates) will be reduced in relation to the increasing diameter of the colonies.

Other components of fungal cell walls, such as ergosterol, show the same variation (Gessner and Chauvet, 1993). The values of ergosterol to mycelial biomass in twelve species of aquatic hyphomycetes varied from 2.3 to 11.5 mg/g of dry weight (average 5.5 mg/g) (Gessner and Chauvet, 1993). The differences between species were significant (α=0.0001) in 75% of the comparisons (ANOVA). Salt-marsh fungi have similar ergosterol values (2 - 16 mg/g) (Newell et al., 1987). Ergosterol values of three other fungi, *Alternaria alternata*, *Aspergillus flavus* and *Aspergillus amstelodami* fell in the same range (Seitz et al., 1979).

Taking the variability of chitin and protein observations into consideration, it seems most reasonable to estimate the fungal biomass of an unknown fungus by using an average conversion factor of all the concentrations found in this study. The average protein concentration of the mycelium was 48.04 µg/mg of dry weight (s.d.=20.56) and chitin was 41.76 µg/mg of dry weight (s.d.= 17.55).

There are only a few published data which use ATP measurements to estimate fungal biomass. Pitt and Bull (1982) reported that the ATP content in the mycelium of *Trichoderma aureoviride* was between 2 and 9 nM/mg of dry weight. The ATP levels of fungi found in decomposing leaves, *Anguillospora filiformis* and *Flagellospora curvula*, were respectively 3.26 and 2.96 nM of ATP/mg dw (Suberkropp, 1993) .

The ATP concentrations of the mycelia of the three fungi used in this study varied greatly. Nevertheless this variance was correlated with the EC of the mycelium. In all three species the highest ATP contents were associated with high EC values, and high growth rates. EC is known to correlate with growth rates of natural communities (Chapman et al, 1971, Wiebe and Bancroft 1975). In this study EC values above 0.7 were associated with phases of rapid growth. Only *P. chrysosporium* showed a clear exponential phase during the first four days of growth. The ATP and EC values found during this phase (on the third day) were the highest in the whole study (7.28×10^{-9} moles/mg of dw and 0.83). This rise in ATP and EC preceded the actual increase in biomass. This anticipation of growth also was observed by Wiebe and Bancroft (1975) in bacterial communities from the Western North Atlantic Ocean. This suggests that ATP/EC estimations are better indicators of growth than changes in protein or chitin. Another important observation noted in this study is that growth took place even at values of EC below 0.6.

In order to classify the variance found in ATP content of the mycelium between and within species of fungi the data for ATP content were divided into three different groups based on the EC values. Class one included EC values equal or larger than 0.6. Class two included EC values between 0.6 and 0.4, and the values of class three were below 0.4. These classes were based upon the results from the ANOVA done for each species. This classification was tested using a one way ANOVA with pairwise comparisons. It was found that there were significant differences between classes one and two, and one and three (α=0.05), but not between classes two and three. Therefore class three was subsumed into class two. The average ATP content for class one was 4.19 nM/mg of dw, and for class two 2.06 nm/mg of dw. The next step was to test the differences between species within each of these two classes. A second

ANOVA with pairwise comparisons was performed. The comparison of the results of energy charge mesurements showed that there were no significant differences between the species tested. It was concluded that ATP and Energy Charge were reliable indicators of fungal biomass. When the EC of the sample is equal or larger than 0.6, the ratio of ATP/dry weight equals 4.19 nm/mg of dw. When the EC in a sample is below 0.6, the ratio of ATP/dry weight equals 2.06 nm/ mg of dry weight.

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CHAPTER 4: ESTIMATION OF THE FUNGAL BIOMASS IN DECAYED WOOD USING MEASUREMENTS OF ATP AND ENERGY CHARGE.

ABSTRACT

Measurements of ATP and Energy Charge (EC) were used to estimate the changes in biomass of three species of wood decaying fungi (*P. chrysosporium,* a white rot, *P. placenta,* a brown rot, and *C. globosum* , a soft rot), grown in wood blocks. The wood blocks were inoculated with the three species and incubated for three months at 25 °C and 95% RH. The blocks were sampled every month for concentrations of ATP, protein, chitin, EC, and weight loss. Protein and chitin data were used as alternative methods to estimate fungal biomass. The possible interference of wood chemicals with the protein and chitin assays was studied. As shown in a previous study the chemicals in wood did not interfere with the measurement of nucleotides. EC levels for the three fungi remained below 0.52. The ADP and AMP concentrations were at least ten fold larger than the ATP. The ATP data was converted into biomass using a conversion factor (ATP/Biomass = 2.06 nM/mg of dry weight for EC values below 0.6). After three months of incubation the biomass of *P. chrysosporium* was larger than the other two species. This correlated with faster fungal degradation of the substrate (weight loss 5% per month) compared to less than 2% for *P. placenta* and *C. globosum. P. chrysosporium* produced a larger biomass when grown on loblolly (167 µg/g of wood) than on birch (40 µg/g of wood) but the weight loss of the wood was larger in the birch blocks (16% versus 14.73 %). Protein measurements were below the level of sensitivity of the assay. Wood completely interfered with the chitin assay. As a field test, three statues from the Egyptian collection were sampled and their nucleotide content was measured. The ATP measurements were below the background level. It was concluded that there was no fungal growth in the statues at this time.

INTRODUCTION

Indirect chemical methods to estimate microbial biomass are based upon the measurement of a structural or functional chemical component, present in microbial cells. Fungal biomass can be estimated by using measurements of ergosterol (Newell et al., 1987) or chitin (Ride and Drysdale, 1972) as well as with methods used for bacteria (e.g. protein and total N). ATP measurements, together with the Energy Charge (EC) values of the mycelia can be used to estimate the biomass of fungi at different phases of the growth cycle of batch cultures (Atkinson, 1969) (Chapman et al., 1971).

There are three different types of wood decay caused by fungi. White rot is caused by the degradation of lignin by Basidiomycete fungi. Some species of white rot decay both lignin and cellulose (Blanchette et al. 1990). Brown rot occurs when the cellulose and the hemicellulose of the cell wall is removed by other Basidiomycete fungi (Blanchette et al. 1990). Finally, soft rot is the result of the degradation of cellulose from the wood, by Ascomycete or Fungi Imperfecti (Blanchette et al. 1990).

In this study ATP concentrations and EC were used to estimate the fungal biomass in wood decayed in vitro. This estimate was compared to those based on measurements of chitin and protein concentrations. Extracts from plant matter are rich in phenolic compounds which combine with proteins reversibly, by hydrogen bonding and irreversibly by oxidation followed by covalent condensations. This may result in inactivation of assay enzymes (e.g. luciferase). The interference of chemical compounds found in wood, with the three methods of biomass estimation was studied.

MATERIALS AND METHODS

Interference by wood constituents

Two different types of wood, birch (hardwood), and loblolly pine (softwood), were used. The wood was ground in a Wiley mill to pass through a size 60 sieve (2 mm). *P. chrysosporium* was cultured in 20 ml of malt extract broth (MEB) in 25 cm^2 tissue flasks for 6 days at 25 °C. The flasks were inoculated using plugs of stock cultures (1 cm in diameter) of the three species maintained in malt extract agar (MEA). The interference of wood components with the nucleotide assay was studied previously (see materials and methods, and results of chapter 2). In order to study the interference of wood with the protein and chitin measurements, the cultures were homogenized for ten minutes using a tissue grinder (Potter-Elshelm), and equal aliquots of the homogenate were extracted in the presence of wood (10:1 ratio of wood to mycelium) and in its absence. Protein extraction was accomplished by hydrolyzing the tissue with using 1 M NaOH overnight on a shaker. The protein content was measured using Bradford's method (Bradford, 1976). Chitin extraction was done by hydrolyzing it with concentrated KOH (22 M) at 120 °C for one hour. The residues were measured using the methods described in Ride and Drysdale (1972).

Wood block test

Two types of wood (birch and loblolly) were used. The wood blocks were 2 cm x 2 cm x 1cm. The wood blocks were dried in the oven at 60 °C until the dry weight was constant. The dry weights were recorded, and the blocks were then rehydrated in distilled water overnight. Then they were dry-blotted with a paper towel. The blocks were placed in screw-capped jars containing 15 cm^3 of vermiculite and 10 ml of distilled water. The jars were then autoclaved at 120 °C for 45 minutes, and placed in a humidified chamber at 98% RH until inoculated. The water content of the wood was 50 %.

Three species of fungi were used to inoculate the wood blocks: *Phanerochaete chrysosporium* (Forest Products Laboratory BKMF 1767) (white rot), *Poria placenta* (University of Minnesota 203A) (brown rot), and *Chaetomnium globosum* (University of Minnesota 390) (soft rot). They were grown for four days in Malt Extract Broth (MEB) at 25°C on a shaker. The liquid cultures were inoculated using a 1 cm plug, from malt extract agar (MEA) plates of each fungus. The supernatant was removed and the mycelium was used to inoculate the wood blocks. Four blocks (three replicates and one control, uninoculated) were sampled after one, two, and three months, for the concentrations of nucleotide, protein and chitin, and also for substrate degradation (loss of weight).

Wood decay

The wood decay tests were disassembled. The blocks were dried in a convection oven at 60 °C to a constant dry weight. The weight loss was calculated by subtracting the dry weights before and after incubation.

Nucleotide measurement

The block was cleaned of superficial fungal growth using a heat-sterilized razor blade and then it was ground in a Wiley Mill to pass through a size 60 sieve (2 mm). The nucleotides were extracted from the ground wood using 20 ml of 5% TCA; extraction was overnight at 0°C. A one ml aliquot was removed and stored in the freezer until assayed. The assay procedure for the measurement of the ATP, ADP, and AMP concentrations has been already described in a previous section (see materials and methods of chapter 1).

Chitin and protein measurement

After the extraction of the nucleotides, the samples were centrifuged and the supernatant was discarded. In order to extract the proteins, 10 - 15 ml of 1M NaOH were added to the samples and left in a shaker overnight. The samples were then centrifuged and the supernatant used to measure the protein concentration (Bradford, 1974). The chitin was extracted using 10-15 ml of concentrated KOH (22 M) at 120 °C. The chitin content of the samples was measured following the methods of Ride and Drysdale (1972).

Field Test

Three different objects from the Egyptian collection of the Metropolitan Museum of Art were sampled:

1. **"Merti with Staff and Scepter"** Dynasty 5 (2350 B.C.) MMA# 26.2.4 (this statue was analyzed using TEM at the beginning of this study) (Blanchette et al. 1992)

2. "**Merti's wife**" Dynasty 5(2350 B.C.) MMA# 26.2.5, and
3. "**A Courtier**" Dynasty 6 (2345-2181 B.C.) MMA# 59.50.2

Small fragments of wood (~ 2 mg) were retrieved from areas of apparent decay. The fragments were flamed briefly to eliminate surface contamination, and then they were extracted using cold 5% TCA for 20 hours. The nucleotide content of the sample was measured following the same procedure already described in a previous section (see materials and methods of chapter 2).

RESULTS

Synergism by wood constituents

Protein assay

Higher concentrations of protein were measured in samples that were extracted in the presence of wood (birch or loblolly) than in the controls (table 4.1, and Figure 4.1).

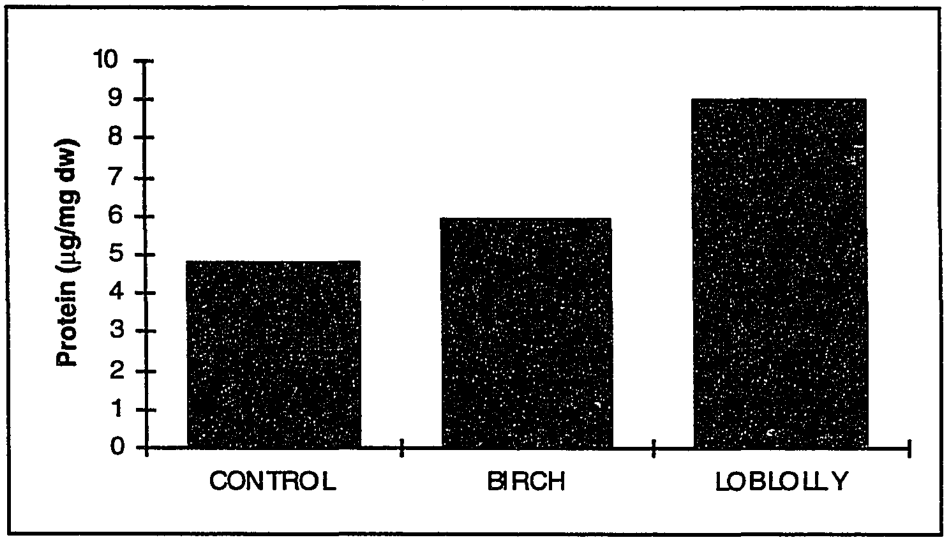

Figure 4.1: Interference of wood with protein assay.

Table 4.1: Results of analysis of interference of wood with protein assay (μg of protein/ml).

	Average	**St. dev.**
CONTROL	4.82	0.35
BIRCH	5.92	0.23
LOBLOLLY	9.03	0.50

Those samples with birch had protein concentrations one order of magnitude larger than the control, whereas, those with loblolly had protein concentrations twice the control. These data was also analyzed using a one way ANOVA and significant differences between the controls and the experimentals were found, for both types of wood (α =0.02 for birch and α =0.01 for loblolly).

Chitin assay

The presence of wood completely interfered with the color reaction required for the determination of the chitin content. After the extraction of the samples with concentrated KOH at 120 °C the color of the sample was dark brown. When the samples were treated to determine the glucosamine content (Ride and Drysdale, 1972) using 3-methyl-2-benzothiazolone hydrazone (MBTH), there was no color reaction. The absorbance of the samples at 650 nm was zero.

In order to avoid the interference with the chitin assays, the wood was removed before the extraction, by resuspending the ground wood in distilled water. The sample was then vortexed and the wood allowed to settle. An aliquot of the supernatant containing the mycelium was then removed and used to measure the protein and chitin concentrations.

Wood block tests

Nucleotide measurements

The total ATP of the mycelia of the three fungal species increased with time which was correlated with increases in biomass of the mycelium (table 4.2, and figures 4.2, 4,3, 4.4, and 4.5). The largest ATP concentrations were found in *P. chrysosporium* grown in loblolly pine (ATP ranged from 1.53×10^{-12} to 3.22×10^{-10} m/gram of wood). The concentrations of ADP and AMP were at least ten fold larger than the ATP of the three species. The EC values found for all 3 species were less or equal to 0.52, which indicates low activity of the mycelium (table 4.2 and figure 4.6).

Table 4.2: Nucleotide concentrations (moles/gram of wood), and EC values in the wood block tests.

		1 MONTH	2 MONTHS	3 MONTHS
P. chrysosporium (Birch)	**ATP**	$1.67 \pm 0.28 \times 10^{-11}$	$1.06 \pm 0.65 \times 10^{-10}$	$8.67 \pm 4.18 \times 10^{-11}$
	ADP	$4.39 \pm 1.08 \times 10^{-9}$	$5.16 \pm 0.83 \times 10^{-9}$	$4.40 \pm 1.24 \times 10^{-9}$
	AMP	$1.43 \pm 0.53 \times 10^{-9}$	$2.09 \pm 0.71 \times 10^{-9}$	$8.89 \pm 1.33 \times 10^{-10}$
	EC	0.37 ± 0.06	0.36 ± 0.04	0.43 ± 0.01
P. chrysosporium (Loblolly)	**ATP**	$4.83 \pm 1.13 \times 10^{-11}$	$1.18 \pm 0.17 \times 10^{-10}$	$3.22 \pm 0.87 \times 10^{-10}$
	ADP	$4.86 \pm 0.62 \times 10^{-9}$	$3.09 \pm 0.06 \times 10^{-9}$	$4.14 \pm 0.60 \times 10^{-9}$
	AMP	$2.85 \pm 1.06 \times 10^{-9}$	$2.05 \pm 1.23 \times 10^{-9}$	$6.86 \pm 7.07 \times 10^{-10}$
	EC	0.32 ± 0.04	0.39 ± 0.08	0.47 ± 0.05
P. placenta (Birch)	**ATP**	$1.53 \pm 0.98 \times 10^{-12}$	$1.83 \pm 0.18 \times 10^{-11}$	$5.41 \pm 2.70 \times 10^{-11}$
	ADP	$9.59 \pm 0.52 \times 10^{-10}$	$1.40 \pm 0.68 \times 10^{-9}$	$9.58 \pm 1.05 \times 10^{-10}$
	AMP	$3.75 \pm 0.72 \times 10^{-11}$	$1.35 \pm 0.84 \times 10^{-10}$	$2.38 \pm 1.33 \times 10^{-10}$
	EC	0.48 ± 0.00	0.45 ± 0.05	0.43 ± 0.03
C. globosum (Birch)	**ATP**	$1.70 \pm 0.95 \times 10^{-11}$	$8.91 \pm 2.70 \times 10^{-12}$	$2.46 \pm 0.32 \times 10^{-11}$
	ADP	$7.92 \pm 2.36 \times 10^{-10}$	$3.64 \pm 0.26 \times 10^{-10}$	$3.22 \pm 0.42 \times 10^{-10}$
	AMP	$2.34 \pm 1.14 \times 10^{-10}$	$1.90 \pm 0.00 \times 10^{-9}$	$3.64 \pm 3.66 \times 10^{-9}$
	EC	0.43 ± 0.04	0.52 ± 0.06	0.50 ± 0.03

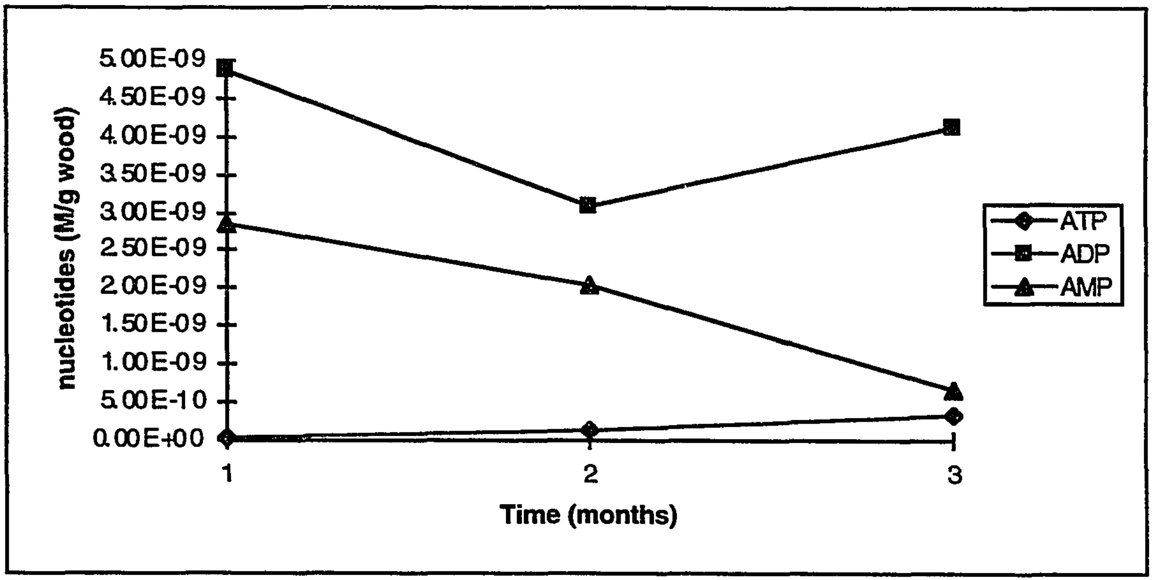

Figure 4.2: Changes in nucleotide concentrations in *P. chrysosporium* grown on loblolly pine.

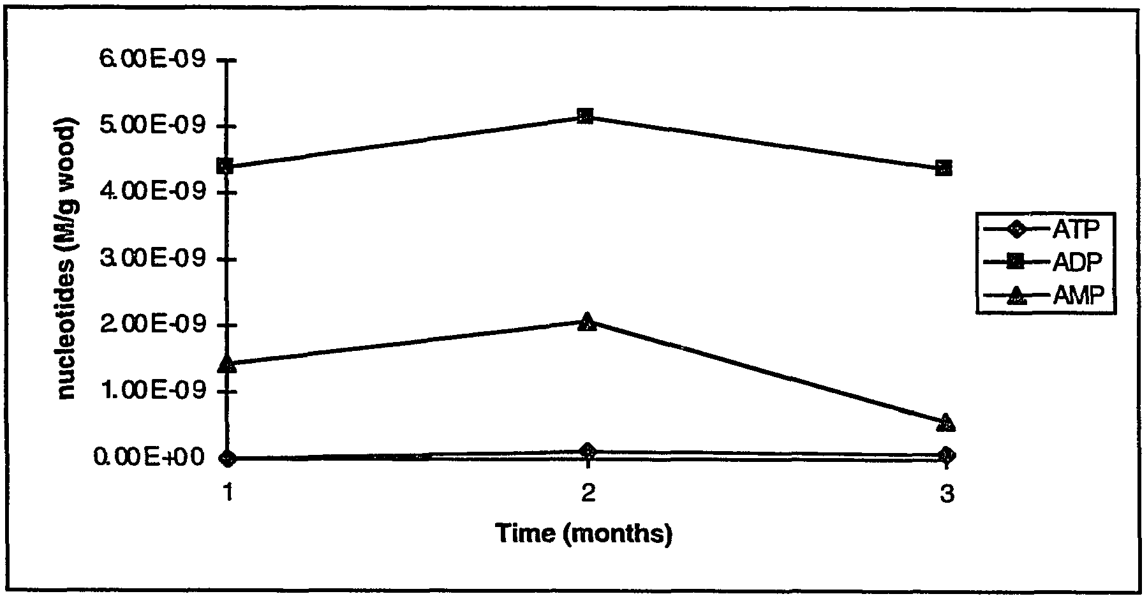

Figure 4.3: Changes in nucleotide concentrations in *P. chrysosporium* grown on birch.

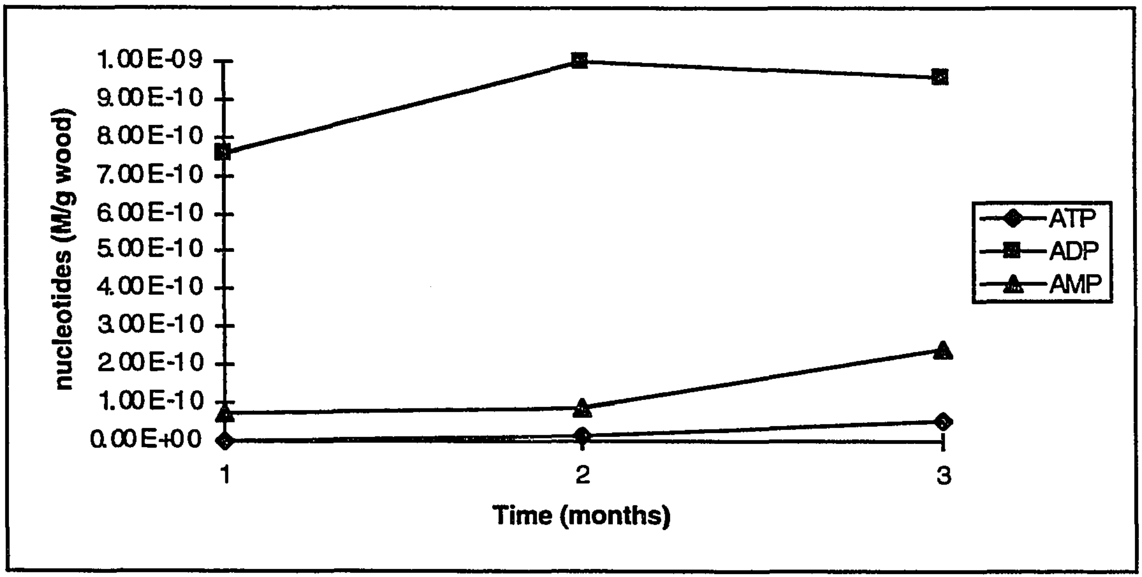

Figure 4.4: Changes in nucleotide concentrations for *P. placenta* grown on birch.

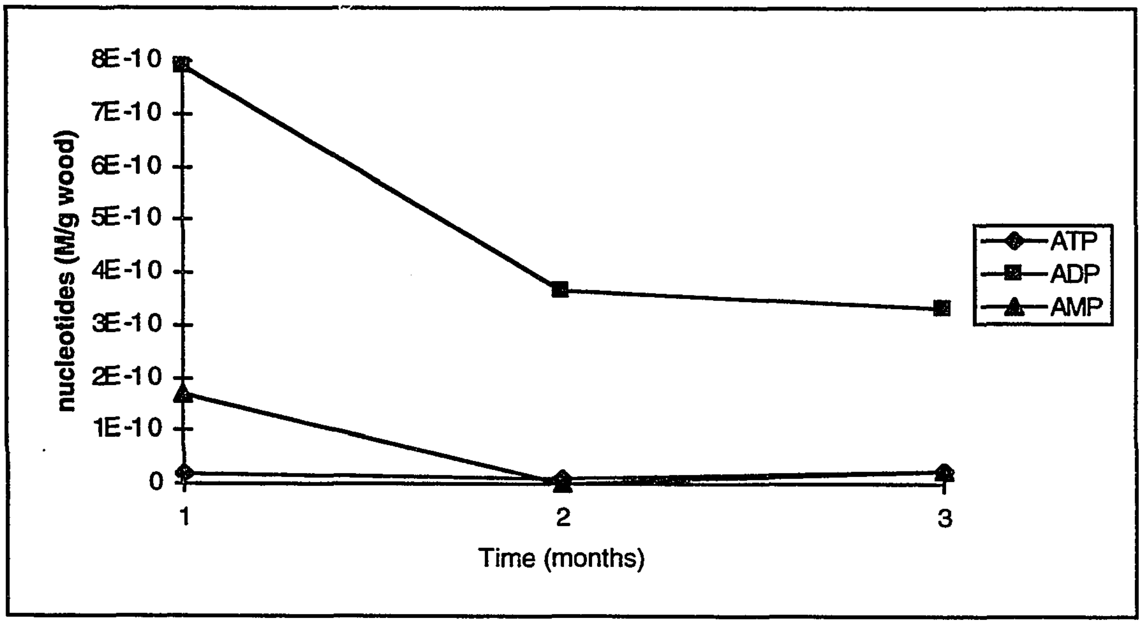

Figure 4.5: Changes in nucleotide concentrations in *C. globosum* grown on birch.

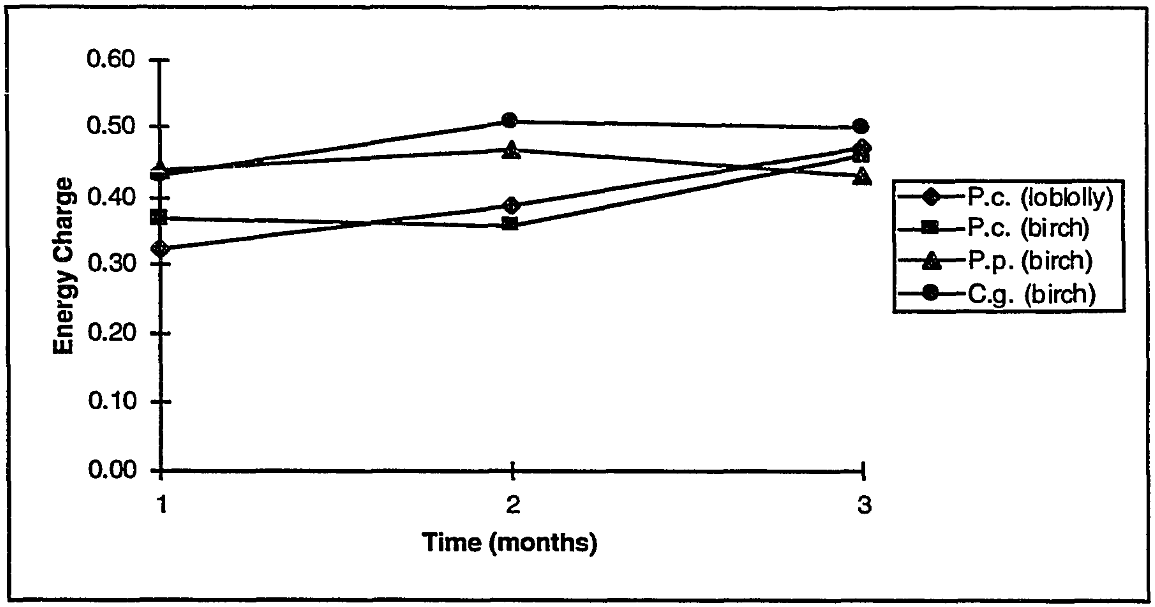

Figure 4.6: Changes in EC of *P. chrysosporium (P.c.) P. placenta (P.p.), and C. globosum (C.g.).*

The ATP concentrations are converted into biomass using the conversion factor calculated in the previous chapter for this particular range of EC (ATP / dry weight = 2.06 nM/mg of dry weight) (table 4.3). *P. chrysosporium* had the largest ATP values in both birch and loblolly pine, and therefore the largest biomass (table 4.3 and figure 4.9). The biomass produced by *P. chrysosporium* was larger when grown in loblolly than in birch (167 and 40 μg of dw/g of wood respectively). *P. placenta* had a biomass of 26 μg/g of wood, after three months. The least productive of the three fungi was *C. globosum* (10 μg/g of wood) (table 4.3 and figure 4.7)

Table 4.3: Calculation of fungal biomass (μg of dw/g of wood) on the basis of ATP values

Species/wood	1 MONTH	2 MONTHS	3 MONTHS
P. chrysosporium **(Birch)**	8	51	40
P. chrysosporium **(loblolly)**	23	57	167
P. placenta **(birch)**	0.7	6	26
C. globosum **(birch)**	8	4	10

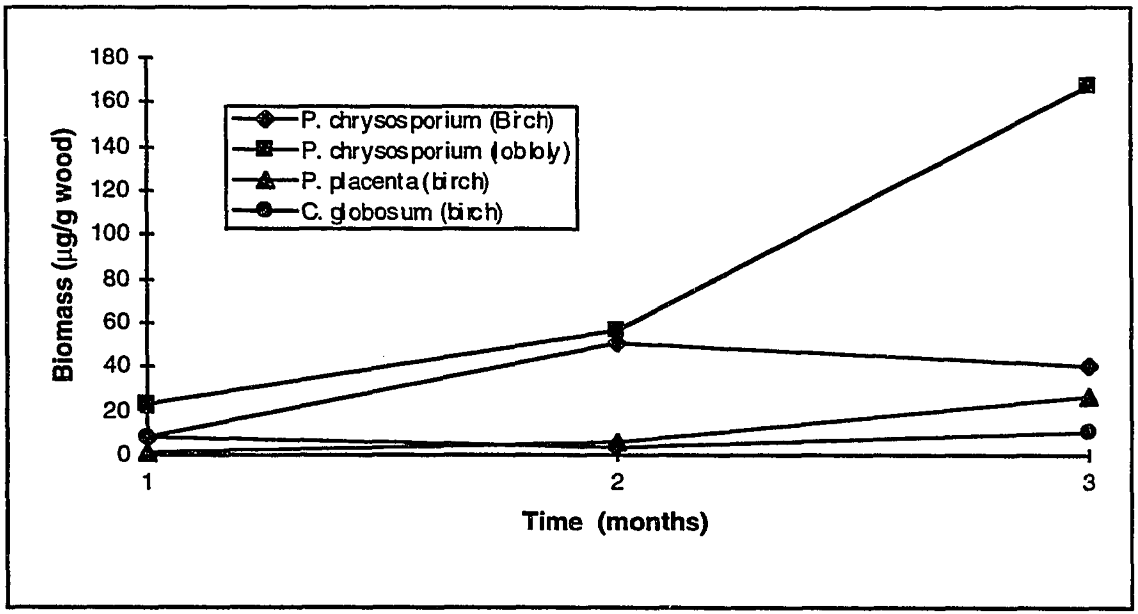

Figure 4.7: Changes in biomass of the fungi based on the ATP measurements.

Protein measurements

The optical density (O.D. at 595 nm) of protein extracted from the wood block samples were below the threshold of sensitivity of the system (~ 4.3 µg/ml). O.D. values for protein concentrations below 4.3 µg/ml were indistinguishable from the blanks. Values around this threshold are not linear and although the protein concentration for the sample can be extrapolated from the regression line, it will be an overestimation. Therefore when these protein data were converted into biomass values using the factor calculated in the previous chapter (48.04 µg of protein/mg of dry weight) the apparent biomass of mycelia were much higher than those calculated using ATP. The Bradford protein (Micromethod) assay is not valid for this application.

Chitin measurements

The chitin content of the blocks was low. The largest concentrations of chitin were found in the first sample of *C. globosum* (185.33 µg/g of wood). This was probably due to the large concentration of spores found on the surface of the block (table 4.4). Spores are known to have larger chitin content than the rest of the mycelium (Marchant, 1978) (Elliott and Wood, 1978). The next highest chitin contents were found in the mycelium of *P. chrysosporium* growing on birch (23.49 µg/g of wood) (table 4.4).

Table 4.4: Chitin content of the wood blocks (µg/g of wood).

	1 MONTH	2 MONTHS	3 MONTHS
***P. chrysosporium* (Birch)**	*	8.02 ± 4.62	23.49 ± 17.97
***P. chrysosporium* (loblolly)**	3.19 ± 0.04	6.41 ± 2.01	3.17 ± 0.08
***P. placenta* (birch)**	*	5.44 ± 2.91	2.52 ± 0.03
***C. globosum* (birch)**	185.33 ±45.15	44.31 ± 49.93	45.72 ± 17.38

* indicates sample loss

The content of chitin was used to calculate biomass using the conversion factor calculated in the previous chapter (41.76 µg/mg of dw) (table 4.5).

Table 4.5: Biomass estimates based upon chitin content values (mg of dry weight/g of wood)

	1 MONTH	2 MONTHS	3 MONTHS
***P. chrysosporium* (Birch)**	-	0.19	0.56
***P. chrysosporium* (loblolly)**	0.08	0.15	0.08
***P. placenta* (birch)**	-	0.13	0.06
***C. globosum* (birch)**	4.44	1.06	1.09

Biomass estimates calculated from measurements of chitin were much lower than those obtained by calculating values from ATP.

The wood colonized by *P.placenta* and *C. globosum* lost only 0.17% and 1.30 % weight per month, whereas that colonized by *P. chrysosporium* lost 5 % (table 4.6, and figure 4.8). *P. chrysosporium* degraded more birch than it did loblolly pine (16% versus 14.73 %), even though its biomass was larger in loblolly (167 µg/g wood) than it was on birch blocks (40 µg/g wood).

Table 4.6: Percent Weight Losses

	1 MONTH	2 MONTHS	3 MONTHS
***P. chrysosporium*.**(birch)	5.22	11.33	16.00
P. chrysosporium (loblolly)	3.94	9.23	14.73
P.placenta (birch)	0.00	0.13	0.17
C. globosum (birch)	1.23	0.90	1.30

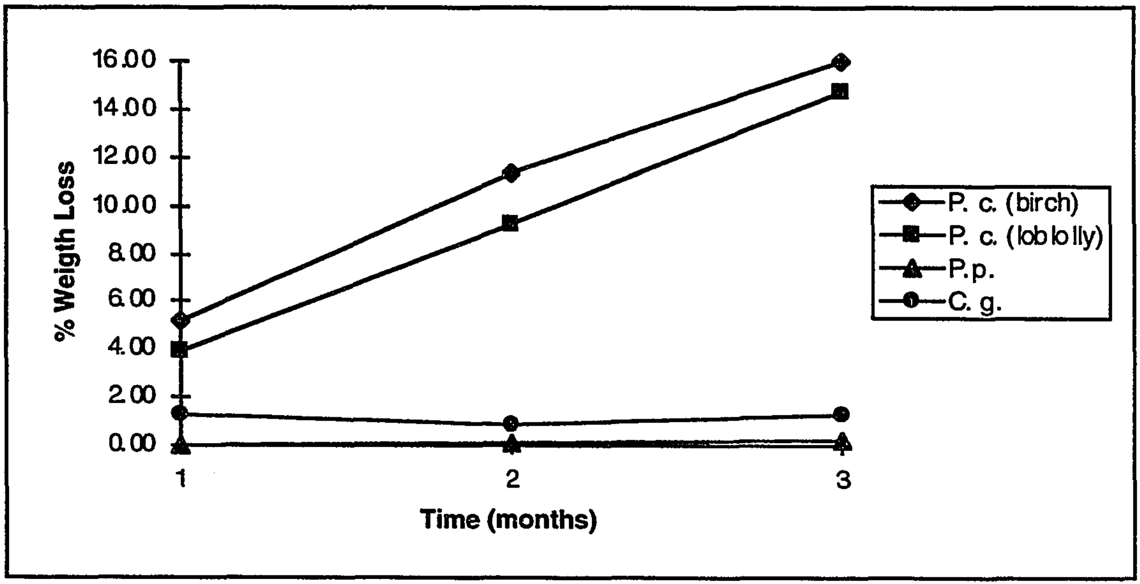

Figure 4.8: Loss of weight (%) of wood infested with *P. chrysosporium*, *P. placenta*, or *C. globosum*.

DISCUSSION

Fungal biomass estimates calculated using ATP values were in consonance with the data on weight loss. Larger weight losses were associated with larger biomass estimates. The amount of wood decayed by *P. chrysosporium* was significant (5% loss per month). Although there was greater biomass of *P. chrysosporium* when it grew in loblolly pine than when it grew in birch, the weight loss was smaller. The biomass estimates obtained from the protein measurements were not reliable because the protein present was below the level of sensitivity of the assay and because wood components interfered with the assay. Loblolly interfered more with the measurements than did birch. Plant tissues are known to present special difficulties for the isolation of enzymes and organelles, because of the presence of large quantities of secondary products such as phenols (Loomis, 1974). Phenols can bind irreversibly to proteins by means of covalent bonds, or by hydrogen bonding rendering them inactive. Phenols may also interfere with traditional methods of protein determination. For instance the biuret method, depends on the blue color formed when Cu^{2+} complexes with peptide bonds under alkaline conditions. However, Cu^{2+} forms colored complexes with phenols as well. Bradford's method for quantitative analysis of protein (Bradford, 1976) makes use of the fact that Coomasie Brilliant G250 perchloric acid changes color when the dye binds to protein (Reisner et al., 1975). Phenols may bind the dye causing a color change similar to that caused by the binding of proteins. Therefore the presence of wood might inflate the protein values.

The presence of wood also interfered with the chitin assay. When the same assay was applied measuring the fungal biomass in tomato, broad bean, and pea leaflets infected with five different fungi no interference was found (Ride and Drysdale, 1972). Ride and Drysdale (1972) extracted the chitin from acetone homogenates of the plant leaflets. In the present study the wood was not treated prior to the measurement of the nucleotide, protein and chitin concentrations. Swift (1973) also studied the applicability of chitin measurements as a method for estimating fungal biomass in decayed wood tissue. He used an acid hydrolysis of the chitin instead of hot alkali. He concluded that unless the samples were purified using an ion exchange separative procedure, components other than hexosamines of the acid hydrolysate of wood tissue may produce colorimetric reactions.

Methods of biomass estimation based upon cell wall components can be misleading, since inactive fungal hyphae are measured as well as active hyphae. Conversion factors based upon cell wall constituents have larger variance. Many factors affect the chemical composition of the cell walls. Different stages in the life cycle of the fungus may differ in chitin or protein content (Marchant, 1978; Elliot and Wood, 1978). Because hyphal tips are growing fast they have thinner cell walls, and therefore lower chitin and protein contents (Marchant, 1978). The advantage of using chitin or ergosterol is that they are specific for fungi. In this study, *C. globosum* produced large numbers of conidia over the surface of the blocks after one week of incubation. This was reflected in large measurements of chitin and protein. Yet the weight loss of wood colonized by *C. globosum* was insignificant.

Neither of the types of wood interfered with the nucleotide determinations (see results in Chapter 2). This is not always the case, and in a previous study it was found that the presence of wood interfered with a chromatographic assay of nucleotides (Nieman et al., 1978). ATP assay and EC estimation together provide a rapid an accurate estimate of the biomass and its phase of growth. Fungi with EC values below 0.7 are still active. In spite of EC as low as 0.36 *P. chrysosporium* was capable of decaying the wood block at a rate of 5% per month. EC values are important in understanding the estimates of fungal biomass. The weight loss for loblolly pine wood blocks incubated with *P. chrysosporium* was smaller than that of the birch blocks. Softwoods have a higher lignin content , and it is thought to be a defense against decay. Brown rot fungi are known to inflict considerable structural damage to the wood while causing small weight losses (1 to 2 %, Wilcox 1978). Brown rot fungi cause a sharp reduction in the degree of polymerization (DP) of cellulose without much weight loss and removal of lignin (Cowling 1961). Richards (1954) found 50% loss in toughness by the time there was 1% weight loss. It is also known that *C. globosum* grows very slowly in wood without nutrient supplements (Savory, 1954, Worral and Wang, 1991). Those timbers that are embedded in soil from which mineral nutrients can diffuse are most severely attacked by soft rot (Savory, 1954).

As an application of this study three statues form the Egyptian collection of the Metropolitan Museum of Art were sampled and their nucleotide content was measured. None of the samples tested had values above background. Based upon these results it is fair to conclude that these statues are not infected, at the present time. These results have been corroborated by TEM studies of replicate samples of the same statues (Blanchette pers. com.). The samples were free of fungal growth. It was found that in some of these samples the secondary layer of the cell wall was completely absent due to chemical attack. When archeological objects were removed from their burial sites, and preserved to prevent their deterioration, they were covered with bees' wax or paraffin. This wax has to be removed using solvents. This treatment might have damaged the cell walls of the wood. ATP measurements were quite useful in assessing the state of deterioration of these archaeological artifacts.

There have been other attempts to estimate fungal biomass in natural samples. Jellison and Goodell (1989) used ELISA to detect and quantify fungal biomass in wood. Their procedure involves the preparation of monoclonal antibodies against every species of fungi involved. Although they were able to detect the fungus (*Poria placenta*) they could not get rid of cross reactions with other species. The presence of ground undecayed wood interfered with the assay (Jellison and Goodell, 1989). Although they were able to detect *P. placenta* even when the sample was diluted over 1:10,000, they were unsuccessful in removing the phenols using polymers, such as polyvinylpirrolydone (PVP), and XAD-2 resins. More recently Johnston and Aust (1994) have used PCR and restriction analysis to detect *P. chrysosporium* in soil samples. They also experienced difficulties in differentiating between species within the genus *Phanerochaete.*

Concluding, it has been found that nucleotide measurements are a rapid and accurate method to detect and quantify fungal biomass in wood. Although chitin and protein have been often used to estimate fungal biomass, in this study they were not sensitive enough to measure small amounts of fungal biomass.

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CHAPTER 5: SUMMARY

This project started out as an attempt to answer two questions frequently asked in the field of microbiology: how many microbes are in a sample?, and how active are they? The context of the project was art conservation and biodeterioration. From the point of view of art conservation, the answers for the questions above, will determine the course of treatment for an object. Presence or absence of microbial growth on, or in an object is important, but what is more important is knowing if the microbes are active (and therefore likely to damage the art object). Museum collections are usually maintained under controlled environments (T=18-20 °C and RH=50-55 %) so that fungal or bacterial growth is, presumably, prevented. Problems, however, may arise if the relative humidity fluctuates in the case, or if the internal moisture content of an object is actually much higher than the standard museum environment.

This project was conceived after some apparent deterioration problems, microbial in nature, were discovered on a statue in the Egyptian collection of the Metropolitan Museum of Art of New York (**"Merti with staff and scepter"** MMA 26.2.4 V dynasty 2340 B.C). The wood from the base of the statue was badly deteriorated. The exact cause was unknown. A first assessment of the state of decay of the wood was done using TEM (Blanchette et al., 1992). The micrographs from this study showed the presence of fungal hyphae that resembled a brown rot within decayed cell walls. Soft rot decay was also found in another statue of the same collection: **"The wife of Merti"** (MMA 26.2.5) V dynasty from Saqqara. These hyphae were not active and it is thought that the decay occurred soon after the closing of the tomb. The analysis carried out by Blanchette et al.(1992) were from external fragments. Since the statue seemed to still be deteriorating, a technique for detecting low levels of fungal biomass was desirable to ascertain if microbes were active inside the statue.

It was decided that the method of choice should be accurate, rapid, and most important, it should be able to detect small amounts of biomass. There are many methods to estimate the microbial biomass in a sample. Direct methods are those that estimate numbers of cells using microscopy or plate counts. Indirect methods estimate the biomass or the number of cells in a sample by measuring a chemical compound present in the cells (i.e. protein, DNA, chlorophyll, ergosterol).

For this study ATP was evaluated as a method to estimate fungal biomass in works of art. The quantitative measurement of ATP may be done using radioisotopic methods, chromatography, or the firefly luciferine-luciferase system, also known as ATP photometry. The easiest and most rapid method seemed to be to measure ATP concentrations in a sample using the bioluminescent reaction of the firefly system. This method can detect ATP concentrations as low as 10^{-10} M. In addition to ATP, ADP and AMP were measured by converting them into ATP using substrate specific enzymes (Pradet, 1967). The relative concentrations of the three adenosine nucleotides were expressed as a ratio known as the Adenylate Energy Charge (EC).

In order to measure the ATP concentration of the cell, the sample has to be extracted using a chemical reagent. The first part of this study compared the efficiency of six different extractants to release ATP from cells. *Phanerochaete chrysosporium* was used to test the efficiency of six different extraction reagents and procedures. They were: boiling Tris buffer, boiling EtOH, boiling NaHCO3, Trichloroacetic acid, Perchloric acid, and a proprietary releasing agent called Extralight, manufactured by Analytical Luminescence Laboratory (ALL). The results showed that the most efficient method was 5% TCA on ice for 20 hours. In addition, it was found that using a homogenizer during extraction enhanced the ATP yield and reduced the variance among replicates.

The possible interference of the assay caused by wood constituents was also examined. No interference was found with the ATP assay. Samples containing wood could be assayed without having to treat the sample in advance.

Protein and chitin assays were also performed. In these experiments it was found that significant interference occurred in both assays in the presence of wood.

The second set of experiments studied the changes in ATP, protein, and chitin content, EC, and dry weight of the mycelium during the growth of three species of wood decaying fungi. *Phanerochaete chrysosporium* (white rot); *Poria placenta* (brown rot); and *Chaetomnium globosum* (soft rot). The ATP content of the mycelium of the three species ranged between 2.22 nM/ mg of dw and 6.40 nM/mg of dry weight. The EC values followed a similar pattern starting at low values of 0.4 - 0.5 at the beginning of the incubation period, increasingly rapidly during the first days of incubation to values around 0.8, and finally decreasing to low values around 0.3 - 0.4 towards the end of the experiment. There was a positive correlation between ATP content and EC values. Two conversion factors were calculated to estimate biomass from ATP measurements. The average ATP content of the mycelium for values of EC equal or larger than 0.6 was 4.19 nM/mg of dw, and for EC values below 0.6, the average ATP content was 2.06 nM/mg of dw. These values were adopted as the conversion factors to be used in the future for estimation of fungal biomass. The differences in ATP content between the two classes were significant, but there were not significant differences among species within the two classes.

Overall the protein concentration of the mycelium of the three fungi ranged from 17.14 µg/mg of dw and 87.03 µg/mg of dw. The average value for the protein content of the mycelium was 48.04 µg/mg of dw (s.d.=20.56). Similarly the chitin content ranged from 4.64 µg/mg of dw and 174.05 µg/mg of dw. The average content for the three species was 41.76 µg/mg of dw (s.d.=17.55). These averages were adopted as the conversion factors to estimate biomass based upon protein and chitin measurements.

The conversion factors listed above were used to estimate the fungal biomass in wood blocks incubated with the same three species. The ATP measurements detected *Poria placenta* biomass concentrations as low as 0.7 µg/g of wood. The protein and the chitin measurements were not sensitive enough at this low concentration of fungus. The measurements for ATP were well within the levels of sensitivity of the assay. *P. chrysosporium* was the species that produced the largest biomass and greatest decomposition of the wood. After three months of incubation *P. chrysosporium* formed a mycelium wieghing 40 µg and 160 µg/g wood in birch and loblolly respectively. Even though it produced more biomass when grown on loblolly (16 % and 14.73% respectively) the mineralization was greater in the latter substrate. The EC values for all the species were below 0.54.

As an application of these results, three statues of the Egyptian collection were sampled and their ATP and EC were measured. The ATP measured was not significantly different from the background. It was concluded that these statues were not infected. These statues were part of the permanent exhibition at the museum. The environmental conditions inside the cases are closely controlled and maintained at about 18-20°C and %RH between 50 and 55 %. These results have been corroborated by an ultrastructural study of the same statues (Blanchette per. com.)

In this study it has been shown that ATP measurements have the potential for use to detect and quantify fungal biomass in wooden objects of art. Among the advantages of the method are its sensitivity, rapidity, and accuracy. When ATP measurements are accompanied by EC values, this information can be used to predict the state of activity of the biomass. In the case of the fungi used in this study, values as low as 0.4 were compatible with wood decay. The variance found in this study for the ATP content of the mycelium was attributed to differences in physiological state of the mycelium, rather than interspecific differences.

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