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Assessing Biodeterioration in Wood Using ATP Photometry: Part II. Calculating a Conversion Factor for *Phanerochaete chrysosporium* Using ATP and Adenylate Energy Charge

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The ATP content and Energy Charge (EC) of *Phanerochaete chrysosporium* (a white rot), was measured at different phases of growth in batch culture. The nucleotide, protein and chitin contents, and the dry weight of the mycelium were measured during 4 weeks of incubation. 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 identified: class 1 included samples with EC equal to or larger than 0.65; and class 2 included samples with EC below 0.65. The average ATP content for class 1 was 4.57 nM mg^{-1} of dry weight, and for class 2, 0.99 nM mg^{-1} of dry weight. 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 12.43 $\mu g\, mg^{-1}$ of dry weight, on the third day of growth, and 87.03 $\mu g\, mg^{-1}$ of dry weight on the seventh day of growth. Similarly, the chitin content of *P. chrysosporium* varied between 4.59 $\mu g\, mg^{-1}$ of dry weight on the first day of growth and 82.25 $\mu g\, mg^{-1}$ of dry weight on the tenth day. The variability found in the protein and chitin content was not as classifiable as the ATP data. An average of all the protein and chitin measurements (protein = 48.34 $\mu g\, mg^{-1}$ of dry weight; chitin = 49.96 $\mu g\, mg^{-1}$ of dry weight), regardless of the phase of growth, was adopted as the conversion factor to estimate fungal biomass. © 1997 Elsevier Science Limited

INTRODUCTION

The use of an ATP assay to estimate biomass was developed by Holm-Hansen & Booth (1966). The estimation of biomass by measuring ATP depends upon the fact that all cells contain ATP, and also upon the assumptions that ATP is not associated with non-living particulate material, and the ratio of ATP to cell carbon is fairly constant. A factor of 250 is generally accepted for estimating the biomass of bacteria from ATP assay data (250≅C:ATP $\mu g\, l^{-1}$) (Holm-Hansen & Booth, 1966; Hamilton & Holm-Hansen, 1967; Holm-Hansen, 1969). This value is an average, based on extensive laboratory observations, and it varies somewhat with species composition and with environmental conditions. Karl *et al.* (1978) later found that multicellular organisms had ratios of less than 100. The originators of the method (Holm-Hansen & Booth, 1966) recommended that, in the absence of specific knowledge concerning the species composition of any sample, one should apply the average ATP content of representative microorganisms grown in the laboratory.

On average, the concentration of ATP of cells tested ranges from 2 to 9 nM mg^{-1} of dry weight (Karl, 1980). The concentration of ATP varies with species and with the metabolic states of cells. Environmental samples are mixtures of different species, which are often at different stages in the

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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 and 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.*, 1972). Bacterial attack of wood occurs only when the wood is saturated with water.

Three types of fungal wood decay have been recognized: white rot (e.g. *Phanerochaete chrysosporium*), brown rot (e.g. *Postia placenta*), and soft rot (e.g. *Chaetomium globosum*) (Blanchette *et al.*, 1990).

Several methods for estimating fungal biomass (e.g. ergosterol and chitin) have been used (Ride & Drysdale, 1972; Swift, 1973; Seitz *et al.*, 1979; Whipps, 1987; Gessner & Chauvet, 1993). Ergosterol and chitin are compounds found almost exclusively in the cell walls of fungi; however their detection does not give any 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.

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 ratio (Setlow & Kornberg, 1970a, b; Chapman *et al.*, 1971; Swedes *et al.*, 1975; Wiebe & Bancroft, 1975; Thomas & Dawson, 1977). The concept of Energy Charge (EC) was introduced by Atkinson and Walton (1967).

The aim of this study was to calculate a conversion factor between ATP and biomass for *P. chrysosporium* to estimate fungal biomass in a wooden object. For this purpose, the changes in ATP content and EC of the mycelia of *P. chrysosporium* were estimated 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

Phanerochaete chrysosporium (Forest Products Laboratory BKMF1767) was cultured in 20 ml of Malt Extract Broth (MEB) in 25-cm^2 tissue culture flasks, at 25°C. The flasks were inoculated using 1-cm (in diameter) plugs of stock cultures maintained on Malt Extract Agar (MEA). The cultures were sampled at 1, 2, 3, 4, 7, 10, 14 and 28 days. The following parameters were measured in every sample in triplicate: adenosine nucleotide content (ATP, ADP, and AMP), dry weight (d.w.), protein, and chitin content. An uninoculated control was carried out for each measurement.

Nucleotide measurements

The nucleotides were extracted from the mycelium using cold 5% TCA for 20 h (Nieto *et al.*, in press). 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) (1000 units/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) (1000 units ml^{-1}).

The tubes were incubated for 1 h at 37°C, and then the reaction was stopped by placing the tubes in boiling water for 2 min. 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 1 s 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 centrifuged, and the supernatant was removed. The protein was extracted from the mycelium with 1 M NaOH. Extraction was aided by agitating the

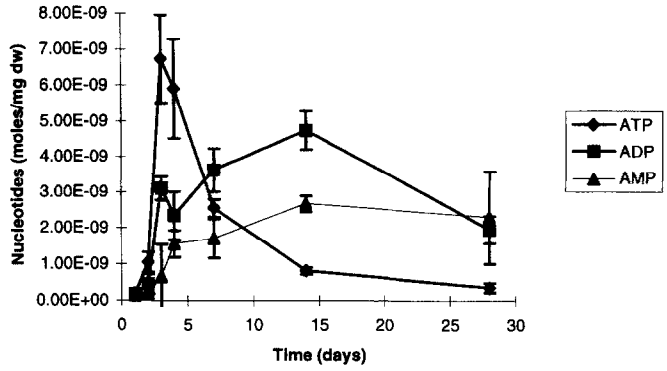

Fig. 1. Nucleotide content (moles/mg of dw) of the mycelium of *P. chrysosporium* during growth in batch culture.

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 & Drysdale (1972). 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 yielding 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

Nucleotide content and energy charge

The mean ATP content of the mycelium was 2.52×10^{-9} mol mg^{-1} of d.w. (SD = 2.65×10^{-9}). The ATP content of the mycelium increased rapidly from a value of 2.22×10^{-10} mol mg^{-1} of d.w. on the first day after transfer, to 7.28×10^{-9} mol mg^{-1} of d.w. on the third day of growth (Fig. 1). Then, the relative ATP content decreased consistently until the end of the incubation period; the ADP and AMP contents increased to a maximum value of 4.90×10^{-9} mol mg^{-1} of d.w. on the 14th day, and 2.56×10^{-9} mol mg^{-1} of d.w. after a month.

The EC values reflected this trend, 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 (Fig. 2).

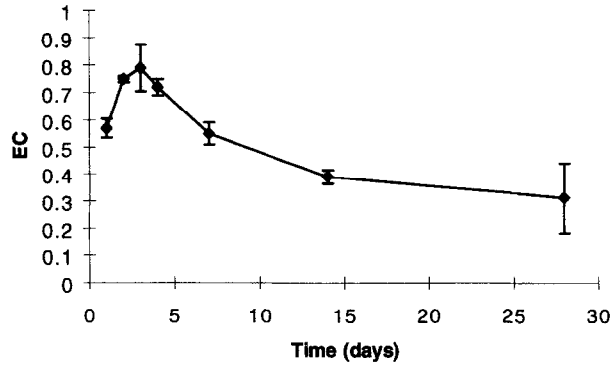

Fig. 2. Changes in Energy Charge (EC) during the growth of *P. chrysosporium* in batch culture.

The differences in ATP content and EC of the mycelium at different days of growth were tested using a one way ANOVA with pairwise comparisons (GT2 and Tukey's test). The results of the ANOVA showed that there were significant differences in both the ATP content and the EC values between sampling times ($\alpha = 0.001$).

The ATP means were compared pairwise using Tukey and GT2 tests. These tests classified the data into two different groups. The first group included data from the third and fourth days with ATP concentrations of 6.74×10^{-9} and 5.91×10^{-9} mol mg^{-1} of d.w., respectively. The second group included the rest of the data (the ATP concentration ranged between 2.08×10^{-10} and 2.56×10^{-9} mol mg^{-1} of d.w.). The differences between these two groups were significant ($\alpha = 0.05$).

The EC data yielded three groups. The first group included EC data from the second, third and fourth days (EC = 0.72–0.79). The second group included the first and seventh days (EC = 0.57 and 0.55). The third group included the 14th, and 28th days (EC 0.31 and 0.38). The data were grouped into two classes. The first class included data points with EC greater than or equal to 0.65, and a

mean ATP content of 4.57×10^{-9} mol mg^{-1} of d.w., corresponding to the second, third and fourth days of growth. The second class included data with EC lower than 0.65, and a mean ATP content of 0.99×10^{-9} mol mg^{-1} of d.w., corresponding to the first, seventh, 14th, and 28th days. The ATP means for these classes were compared (GT2 and Tukey's test) and found to be significantly different ($\alpha = 0.05$).

Chitin and protein content

The total protein and chitin content of the mycelium varied greatly with time (Figs 3 and 4). 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 per unit of weight of the mycelium.

The mean protein content of the mycelium was 48.34 μg mg^{-1} of d.w. with a minimum value of 12.43 μg mg^{-1} of d.w. on the second day, and a maximum value of 87.03 μg mg^{-1} of d.w. on the seventh day.

The average chitin content of the mycelium of *P. chrysosporium* was 49.96 μg mg^{-1} of d.w. The chitin content of the mycelium increased with increasing biomass, from a minimum of 4.59 μg mg^{-1} of d.w., on the first day, up to a maximum value of 82.25 μg mg^{-1} of dry wt on the tenth day of incubation.

After the tenth day, the chitin content of the mycelium decreased to a final value of 64.35 μg mg^{-1} of d.w. For instance, between the third and fourth days, the chitin content decreased from 54.34 μg mg^{-1} of d.w. to 38.60 μg mg^{-1} of d.w. Similar changes were observed in the protein content of the mycelium, particularly during periods of rapid increase of biomass (Figs 3 and 4).

Overall, the total chitin and total 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.

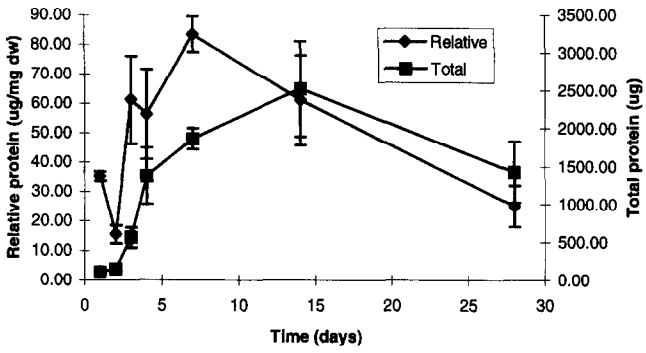

Fig. 3. Changes in protein content of the mycelium of *P. chrysosporium* during growth in batch culture.

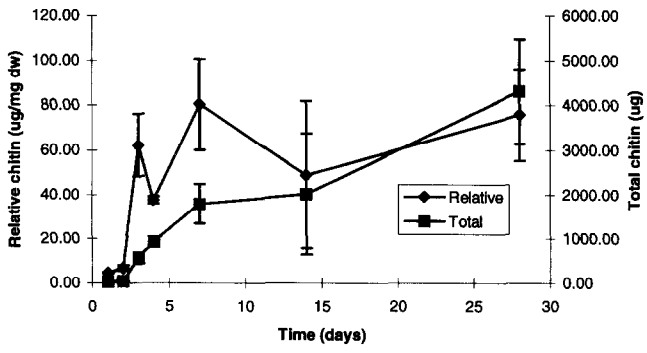

Fig. 4. Changes in chitin content of the mycelium of *P. chrysosporium* during growth in batch culture.

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 mycelium of *P. chrysosporium*. Nevertheless, 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 & 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. Swift (1973) also used chitin content to measure mycelial biomass in decaying wood tissue. His measurements were based on the white rot fungus, *Triametes versicolor*, grown in media with different C:N ratios. The conversion factor calculated by Swift varied between 12.4 μg mg^{-1} in medium with a C:N ratio of 45:1 to 16.5 μg mg^{-1} 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 $\mu g\,mg^{-1}$ d.w. in the former to 38–66 $\mu g\,mg^{-1}$ d.w. in the latter. Some of the variability that he observed might reflect differences in the 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 (Swift, 1973; Seitz *et al.*, 1979), losses in dry weight and redistribution of carbohydrates and nitrogenous compounds supported continued growth of the mycelium under nitrogen-limited conditions (Cowling & 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, increases in total mycelial biomass were not always associated with increases in protein and chitin content. This may be caused by changes in the thickness of the cell walls of the hyphae during growth, as a result of changing extension rates of the tips (Marchant, 1978). The chitin values of the cell walls of vegetative mycelium are different from sporophore walls (Elliot & 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 as a result of irregular branching and abnormally thick cell walls, when grown in batch cultures (Lysek & Esser, 1971).

Another factor that 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 & 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 & Chauvet, 1993). The values of ergosterol to mycelial biomass in 12 species of aquatic hyphomycetes varied from 2.3 to 11.5 $mg\,g^{-1}$ of d.w. (average 5.5 $mg\,g^{-1}$) (Gessner & Chauvet, 1993). The differences between species were significant ($\alpha = 0.0001$) in 75% of the comparisons (ANOVA). Salt-marsh fungi have similar ergosterol values (2–16 $mg\,g^{-1}$) (Newell *et al.*, 1987). Ergosterol values of three other fungi, *Alternaria alternata*, *Aspergillus flavus* and *Aspergillus amstelodami* fell into 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 48.34 $\mu g\,mg^{-1}$ of d.w. (SD = 24.32) and chitin was 49.96 $\mu g\,mg^{-1}$ of d.w. (SD = 37.33).

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 $nM\,mg^{-1}$ of d.w. 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^{-1} d.w. (Suberkropp *et al.*, 1993).

The ATP concentrations of the mycelium of *P. chrysosporium* varied greatly. This variance was correlated with the EC of the mycelium. 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 & Bancroft, 1975). In this study, EC values above 0.65 were associated with phases of rapid growth. *P. chrysosporium* showed a clear exponential phase during the first 4 days of growth. The ATP and EC values found during this phase (on the third day) were the highest in the whole study (6.74×10^{-9} mol of ATP mg^{-1} of d.w. and EC = 0.83). This rise in ATP and EC preceded the actual increase in biomass. This anticipation of growth ws also observed by Wiebe & Bancroft (1975) in bacterial communities from the Western North Atlantic Ocean. Growth and maintenance of viability took place at EC values below 0.65.

In summary ATP and Energy Charge were found to be reliable estimates of fungal biomass and activity. When using ATP estimates, it is recommended that two different conversion factors be used, depending on the EC value of the mycelium. If the EC of the sample is equal to or larger than 0.65, the ratio of ATP/dry weight equaled 4.57×10^{-9} mol mg^{-1} of d.w. When the EC in a sample is below 0.65, the ratio of ATP/dry weight equaled 0.99 nm mg^{-1} of d.w. These results have also been substantiated by testing two other fungi

(i.e. *Chaetomium globosum* and *Postia placenta*) in a similar manner. In both of these cases, two different conversion factors for ATP are recommended, again based upon the EC values.

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