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Assessing Biodeterioration in Wood Using ATP Photometry: Part I. Nucleotide Extraction and Wood Interference

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The efficiency of six different extraction methods (boiling tris buffer, boiling EtOH, boiling $NaHCO_3$, 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 $= 3.45 \times 10^{-9}$ moles/mg dw) was extraction with cold 5% TCA for 20 h. After the extraction, the TCA was removed with ether-saturated water. Although the amount of ATP extracted with boiling ethanol was not significantly different from that by TCA (ATP $= 3.06 \times 10^{-9}$ moles/mg dw), TCA was chosen over ethanol due to the potential hazards involved in boiling ethanol. The efficiency of extraction was the same for both birch and loblolly pine. © 1997 Published by Elsevier Science Limited

INTRODUCTION

The firefly luciferase assay of ATP (McElroy, 1947) is a rapid and highly sensitive method to detect and quantify microbial biomass (Holm-Hansen, 1970; Hamilton & Holm-Hansen, 1967; Holm-Hansen & Booth, 1966). 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 & 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 was 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 (Grooves & Bracker, 1970; Grooves *et al.*, 1975). 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 was addressed as well as the possible interference of wood components, such as phenols, with the measurement of the ATP.

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MATERIALS AND METHODS

Fungi and cultures

Phanerochaete chrysosporium (FPL BKMF1767), a white rot fungus, was used as a model organism for this study. Ten millilitres 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. The whole culture was then extracted, using one of the six methods described below. After the extraction the samples were centrifuged and a 1 ml aliquot of the supernatant was frozen at −25°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 ∅ = 0.45 μm, Gelman cat.# 63069).

Extraction methods

Six different methods were used and compared. Three cold extractions methods were performed as follows: (1) the samples were incubated in 5% trichloroacetic acid (TCA) for 15 min on ice and homogenized in a Potter-Elvejhem tissue grinder (Bagnara & Finch, 1972). TCA was removed from the extract with three changes of an equal volume of ether-saturated water. The samples were then bubbled with nitrogen to remove any dissolved ether; (2) the samples were incubated in 2.3 M perchloric acid (PCA) +67 mM EDTA for 15 min on ice and homogenized in a tissue grinder. The extract was neutralized with 0.72 M KOH +0.16 M $KHCO_3$ (Bagnara & Finch, 1972); (3) 10 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) and homogenized in a tissue grinder.

Three boiling extraction protocols were followed: (1) the cultures were boiled in 20 ml of 20 mM tris buffer +2 mM EDTA pH 7.75 for 2 min (Lundin & Thore, 1975); (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; (3) the cultures were boiled in 20 ml of 0.1 M $NaHCO_3$ pH 8.5 for 5 min (Pamatmat & Skjoldal, 1979).

For the boiling extractions the culture was transferred to a Pyrex glass test tube containing the boiling extractant. After boiling the mycelia, extra extractant was added to compensate for evaporation, placed on ice, and cooled samples were then centrifuged at 2000 rpm for 5 min. One ml of the resultant supernatant was stored at −25°C until assayed for ATP. The ethanol samples were stored at −70°C.

ATP assay

The ATP concentration of the samples was measured using a Monolight bioluminometer (Analytical Luminescence Laboratory cat. # 2010C) equipped with an automatic injector. The 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.

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} 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 the following equation:

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

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*).

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

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. Five hundred mg of ground wood were 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 h.

RESULTS

ATP extraction

The lowest ATP yields were obtained after extraction by Extralight (5.77×10^{-10} moles/mg dw), boiling tris buffer (6.85×10^{-10} moles/mg dw), and boiling $NaHCO_3$ (6.65×10^{-10} moles/mg dw) (Fig. 1). The differences in ATP yield among these three extraction methods were not significant ($\alpha = 0.05$). Larger ATP yields were obtained by using TCA, boiling ethanol, and PCA. PCA was significantly ($\alpha = 0.05$) less efficient than TCA and ethanol (1.68×10^{-9} moles of ATP/mg of dw). Ethanol and TCA were the most efficient yielding 3.06×10^{-9} moles of ATP/mg of dw ($\pm 5.23 \times 10^{-10}$) and 3.45×10^{-9} moles of ATP/mg of dw ($\pm 3.77 \times 10^{-10}$), respectively.

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 ($\alpha = 0.0001$). 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 ($\alpha = 0.05$).

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.

Time-course experiment

In order to learn more about the efficiency of TCA extraction, a time-course extraction experiment was undertaken. The same extraction protocol for TCA was followed as in the previous experiment except the length of time for extraction was varied. After the mycelium was ground, it was extracted in the cold for 10 min, 1 h, or 20 h. As with the previous experiment there were three replicates, and one uninoculated control. The results of this experiment are presented in Fig. 2.

The differences in ATP yield between times of extraction were significant ($\alpha = 0.05$) (Fig. 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 h on ice ($ATP = 3.07 \times 10^{-9}$ moles/mg of dw). The results of the pairwise comparisons tests (Tukey and GT2) were consistent with this observation.

Interference of wood

There was more interference in birch samples than those with loblolly pine (Fig. 3) as seen by lower ATP values for birch than loblolly.

No significant differences between the controls and the experimentals were found for either of the wood types using a one-way ANOVA.

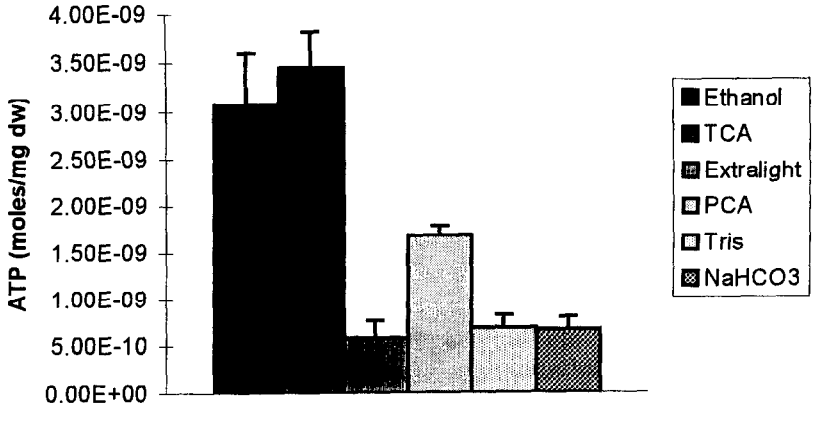

Fig. 1. ATP yields for the six extraction methods. ATP yield is measured in moles per milligram of dry weight.

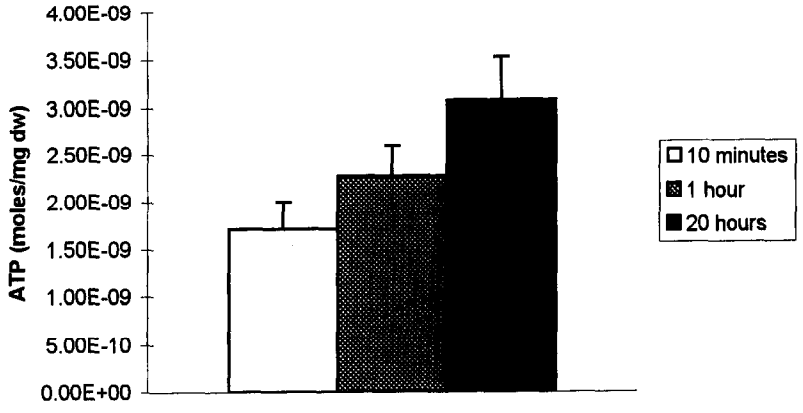

Fig. 2. ATP yield for the time-course extraction experiment measured in moles per milligram of dry weight.

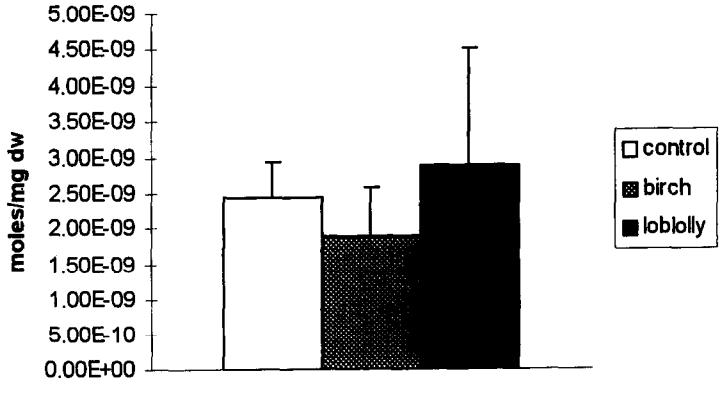

Fig. 3. Interference of wood (birch and loblolly) with the ATP assay (moles/mg of dw).

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. 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 & Finch, 1972), the ATP yield was significantly lower than TCA and ethanol. ATP may be lost by coprecipitation with $KClO_4$ (Guinn & 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 & LaRock, 1975; Karl *et al.*, 1978). 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 h on ice.

TCA is considered by some researchers to be a reference method to extract ATP (Lundin & 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 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.

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 & 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. 3). 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 tested 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 h in ice.

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