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Growth and fermentation responses of *Phanerochaete chrysosporium* to O_2 limitation

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Abstract

Phanerochaete chrysosporium BKM-F-1767 produced small amounts of ethanol from glucose, mannose, cellobiose, maltose and sucrose when grown with a limited O_2 supply in sealed bottles. Under O_2-limited growth on glucose, low levels of acetate or oxalate were produced when nitrogen was in excess or limited, respectively. Alcohol dehydrogenase activity (15 nmol/min/mg extract protein) was detected in cell extracts made from ethanol producing cells. The low levels of fermentative enzymes and products indicate that *P. chrysosporium* does not grow fermentatively, but probably survives transient oxygen limitation by fermentation. Analysis of the genome sequence has indicated multiple alcohol dehydrogenase genes that resemble ADH I and ADH II. Fermentation experiments conducted with 1-^{13}C- and 2-^{13}C-glucose produced 2-^{13}C- and 1-^{13}C-ethanol, respectively. Under conditions of 1-^{13}C-glucose incubation with nitrogen in excess 2-^{13}C-acetate was also detected. The labeling pattern is consistent with ethanol formation by glycolysis and alcohol dehydrogenase. Ethanol was detected in aspen chips aerobically colonized by *P. chrysosporium* only when the culture was purged with nitrogen gas. The data indicate that *P. chrysosporium* is able to ferment components of wood when the supply of 02 is depleted.

Keywords: Phanerochaete chrysosporium; Fermentation; Wood; Ethanol; Oxalate; White-rot fungi

1. Introduction

Phanerochaete chrysosporium is a white-rot fungus best known for lignin degradation. Wood and lignin degradation by filamentous fungi is primarily an aerobic process [1]. However, the growth of filamentous fungi in wood is probably limited by O_2 availability [2]. Lignin degradation can be enhanced with increasing CO_2 at a constant level of 02 supply [3] but is retarded by low O_2 tensions [1,4]. Growth of the fungus can take place at O_2 concentrations below that required for lignin degradation [4]. Studies of O_2 effects on wood degradation have been focused on enhancing the degradation of wood and its components [5].

P. chrysosporium has been used in many remediation studies since it produces oxidative enzymes able to degrade recalcitrant compounds [6]. Factors influencing the growth of white-rot fungi are important in these applications and others such as biopulping [7]. In biopulping, the energy-saving effect results from the growth of the organism on wood chips, with lignin modification [8,9] or carboxylic acid generation causing the effect [10]. Little is known about the substrates used for growth in the colonization of wood or of the environmental factors (local pH, O_2 supply and CO_2 concentration) encountered by these organisms while growing on wood. If we are to enhance or inhibit white-rot metabolism, we need an understanding of these influences.

Electron microscopy studies of fungal wood degradation indicate the fungal cell is often within a glycan matrix [11–15]. High resolution cryo-SEM techniques revealed *Phlebia radiata* with glycan material surrounding the fungus and even extending to the entire inside of the wood cell [16]. If the glycan in the wood cell lumen is similar to the material formed under high levels of glucose in laboratory culture [17,18], the viscosity is greater than water [19]. Gaseous exchange through the glycan material would likely be inhibited [19,20] which would retard O_2 entry and CO_2 egress. Even if the glycan slime is not formed during the invasion of wood, the fungal cells will continue to grow further into the wood and O_2 will likely become limited and CO_2 increased at some point. Even in lab cultures, pellets of *P. chrysosporium* are O_2 limited at the center of the pellets [21].

This study has focused upon the metabolic response of *P. chrysosporium* to conditions of O_2 limitation and nitrogen limitation. We have determined here that *P. chrysosporium*

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forms small amounts of ethanol upon the removal of O_2 from the culture. We have also investigated the conditions under which ethanol production takes place.

2. Materials and methods

2.1. Organism and reagents

Stock cultures of *P. chrysosporium* BKM-F-1767 (ATCC 24725) (from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, WI) were maintained on potato dextrose agar plates and were grown for 7 days then stored at 4°C until used. Chemicals were obtained from Sigma-Aldrich and were reagent grade or better.

2.2. Culture conditions

P. chrysosporium was grown in liquid cultures using B3 medium. B3 medium contained (per liter) 10 g glucose, 2 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaC1_2 \cdot 2H_2O$, 0.73 g 2,2-dimethylsuccinic acid (added as a solution adjusted to pH 4.5 using NaOH), 0.5 mg thiamine HCl, 10 ml of mineral elixer [4] and either 0.2 g (low nitrogen) or 2.0 g (high nitrogen) ammonium tartrate. Medium was inoculated by the transfer of agar plugs or by using a spore suspension [22]. Dry weight was determined by filtration through dried (60 °C, 36 h) pre-weighed filters (Whatman #42,55 mm ashless). Cultures were filtered without washing the cell material and dried for at least 24 h at 60 °C prior to weighing.

2.3. O_2-limited cultures

O_2-limited cultures were grown in sealed serum bottles (160 or 50ml total volume). Serum bottles (Fisher) were autoclaved dry, sterile medium dispensed in the volumes indicated and the bottles sealed with new sterile red rubber serum stoppers (Fisher) crimped in place. The cultures were inoculated with 0.5 ml spore suspension and incubated inverted at 37°C. Bottles were opened at specific times or samples were taken by syringe with care not to remove cell material.

2.4. Substrate trials

Sealed serum bottles (50 ml) containing 10 ml of medium (low nitrogen) and fermentative substrates (10 g/l), glucose, mannose, sucrose, galactose, cellobiose, maltose, xylose or glycerol were used to test ethanol production from these substrates. Inoculated vials were incubated at 37 °C for 12 days.

2.5. Incubation with ^{13}C enriched substrates

Cultures grown with ^{13}C glucose were first grown in 160 ml sealed bottles containing 20 ml medium with low or high nitrogen and 5 g glucose/l. The cultures were grown at 37 °C for 48 h. At that time filter sterilized 1-^{13}C-glucose, 2-^{13}C-glucose and $^{13}CO_2$ were injected into the culture vials. The vials were sampled and then incubated at 37 °C with subsequent periodic sampling. The samples were frozen until analyzed by NMR, with D_2O added to 20% (v/v).

2.6. Growth on aspen wood chips

Aspen chips (kept frozen after chipping) were processed with a Wiley mill using a 4mm screen. The milled chips were frozen until weighed into 250 ml flasks and autoclaved with 5 ml deionized water. The flasks were inoculated with 0.5ml *P. chrysosporium* spores suspended in B3 medium with no added nitrogen or carbon sources. After 9 days growth at 37 °C, one-half of the flasks were removed from the incubator, sealed and frozen. The remaining flasks were sealed with rubber stoppers and the air in the flask displaced with nitrogen gas. These were returned to the incubator for an additional 17 days after which the cultures were frozen. Flask contents were suspended with 150 ml of 20 mM phosphate buffer (pH 6.8), chilled on ice and mixed for 30s in a Waring blender. The suspension was allowed to stand on ice until all cultures were prepared and then samples were centrifuged and the acetate and ethanol determined in the supernatants.

2.7. Sample analyses

Culture samples were clarified by filtration or centrifugation. Samples were acidified (5 µl 50% w/v H_2SO_4/ml) mixed and then centrifuged at 10,000 × g for 5 min. Supernatant was filtered through 0.45 µm filters (13 mm, National Scientific) into sample vials. Ethanol and acetate were determined by gas chromatography using a Hewlet-Packard 5890A GC equipped with a flame ionization detector and a Porapac Q (Supelco) glass column (1/4 in. × 3 ft). Helium (70 ml/min) was used as the carrier gas and 1 µl samples were injected via an automated sampler. Ethanol and acetate eluted at 2.5 and 8.0 min, respectively and were quantified with calibrated standards.

Oxalic acid, glucose and lactate were determined by HPLC using a Hewlett-Packard 1050 pump and a HP UV monitor (210 nm) and Waters refractive index detector in series. The eluant was 0.005N H_2SO_4 at 0.5 ml/min at 67 °C with 80 µl samples injected into an ion 300 organic acid analysis column (Phenomenex). Lactate (17.7 min, UV), glucose (12.36 min, RI) and oxalate (9.9 min, UV) were quantified where indicated with calibrated standards.

Ammonium was determined by a modification of the method of Weatherburn [23]. Samples (or standards) in a total volume of 50 µl were placed in microtiter dish wells and 100 µl of 1% (w/v) phenol in 0.005% (w/v) sodium nitroprusside, and 100 µl of 0.84% (v/v) hypochlorite (bleach) in 0.5% (w/v) NaOH were added. The plate was incubated in a Molecular Devices Spectra Max Plus microtiter plate reader at 37 °C for 30 min and the absorbance measured at 625 nm.

Protein was determined using a bicinchoninic acid kit for protein determination (Sigma).

2.8. NMR acquisition parameters

All NMR spectra were acquired using a Bruker DPX 250 spectrometer (Billerica, MA) observing ^{13}C at 62.9 MHz with a 5 mm QNP probe with a Z-gradient. Spectra were acquired at 300 K over a 15 kHz with 30° rf pulses using standard Bruker pulse program and a relaxation delay of 1.0s. All spectra were collected with 1024 scans and processed using a 2.0 Hz exponential multiplication prior to Fourier transform. The solvent was D_2O and chemical shifts were referenced by setting the a,b-1-^{13}C glucose at 97.4 and 93.6ppm and the a,b-2-^{13}C glucose at 75.9 and 73.2ppm.

2.9. Extraction of the fungi

Cells were freeze-dried overnight to remove any residual ethanol. Lyophilized cells were rehydrated in 10 mM MOPS buffer pH 7.0 and 2 mM dithiothreitol, at a suspension rate of 25 mg cells to 5 ml buffer. The cells and buffer were ground under liquid nitrogen in a mortar and pestle, thawed on ice and centrifuged 5 min at 21,000 × *g* in microcentrifuge tubes. The supernatant was used to assay alcohol dehydrogenase at 340nm using 20mM Tris (pH 8.0) buffer, 400 mM ethanol and 1 mM NAD.

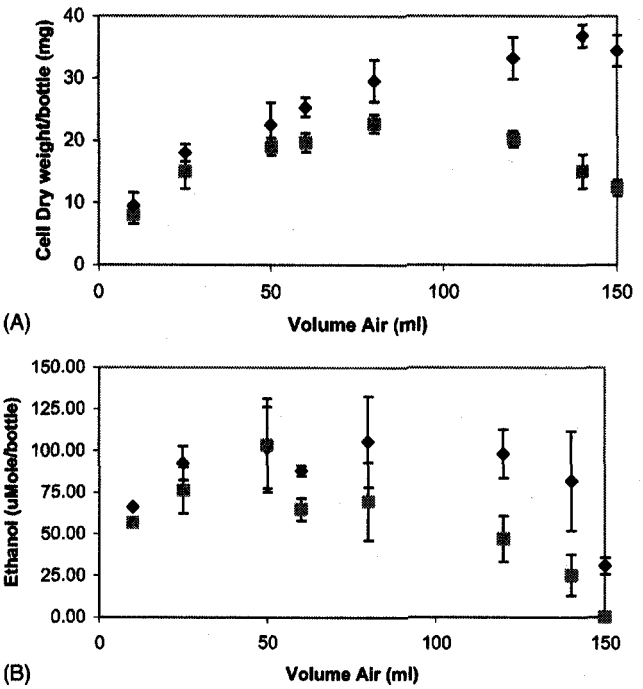

Fig. 1. Growth of *P. chrysosporium* on limited O_2 and nitrogen. *P. chrysosporium* was grown in 160ml sealed bottles with decreasing volumes of medium as described in Section 2. The data from three experiments is shown with the average ± standard deviation given for each condition. Diamonds represent the results from medium containing a high concentration of nitrogen source and the squares from medium with low concentration of nitrogen. (A) Cell dry weight/bottle and (B) ethanol in the bottle. Incubation at 37 °C for 96 h.

3. Results

3.1. O_2 limitation of growth

P. chrysosporium was grown in sealed serum bottles to determine conditions where O_2 would become depleted and nitrogen would be in excess or limited. Fig. 1A and B shows the dry weight and ethanol produced in cultures where the volume of medium was varied in sealed 160ml bottles. Growth occurred in all bottles and was well established by 48 h of incubation. After 96 h of incubation the bottles were opened and the dry weight, ethanol, acetate, oxalate and ammonium levels were determined. Glucose was detected in all samples at the end of the experiment, but the concentration was not determined.

There was a general trend of greater growth in the bottles containing higher nitrogen. Ethanol was produced in all high nitrogen-containing media, and at the end of incubation these bottles contained greater than 4 mM NH_4^+. Under conditions of 10–80ml available air the growth and ethanol production was similar for both high and low nitrogen conditions and cell growth increased with increasing available O_2.

The nitrogen supply was depleted only under two conditions. The low nitrogen medium cultures with 140 and 150 ml of air (and 20 and l0 ml of medium, respectively) exhausted all the nitrogen supply (limit of NH_4^+ detection 0.03 mM) and the bottles containing 120 ml of air and 40 ml of low nitrogen medium were almost exhausted (0.25 mM NH_4^+ remaining). All other low nitrogen conditions averaged greater than 1 mM NH_4^+ remaining.

Ethanol was made by all cultures except the 150ml air with low nitrogen medium indicating that this condition was not limited by the supply of O_2. This was also the only condition that had measurable oxalate (average 0.1 mM). The only growth condition where O_2 and nitrogen were both limiting in this experiment was with 20ml of low nitrogen medium in a 160ml serum bottle. Fig. 2 shows the fermentation products from a time course of incubation using these conditions. Ethanol started to accumulate between 48 and 100 h. With low nitrogen, ethanol production continued until the end of the experiment, but with high nitrogen ethanol accumulation appeared to level off after 200 h of incubation. Glucose and nitrogen were both in excess in these cultures and there did not appear to be any more evidence of growth beyond that obtained in the first 75 h. Acetate accumulated in the cultures grown with high nitrogen and was only observed in the low nitrogen cultures at the end of the experiment. Starting at approximately 100 h oxalic acid (data not shown) began to accumulate in the nitrogen limited culture, but not in the culture with excess available nitrogen. The maximum oxalate concentration (0.5 mM) was observed at the end of the experiment. Ethanol, acetate and oxalate were the only

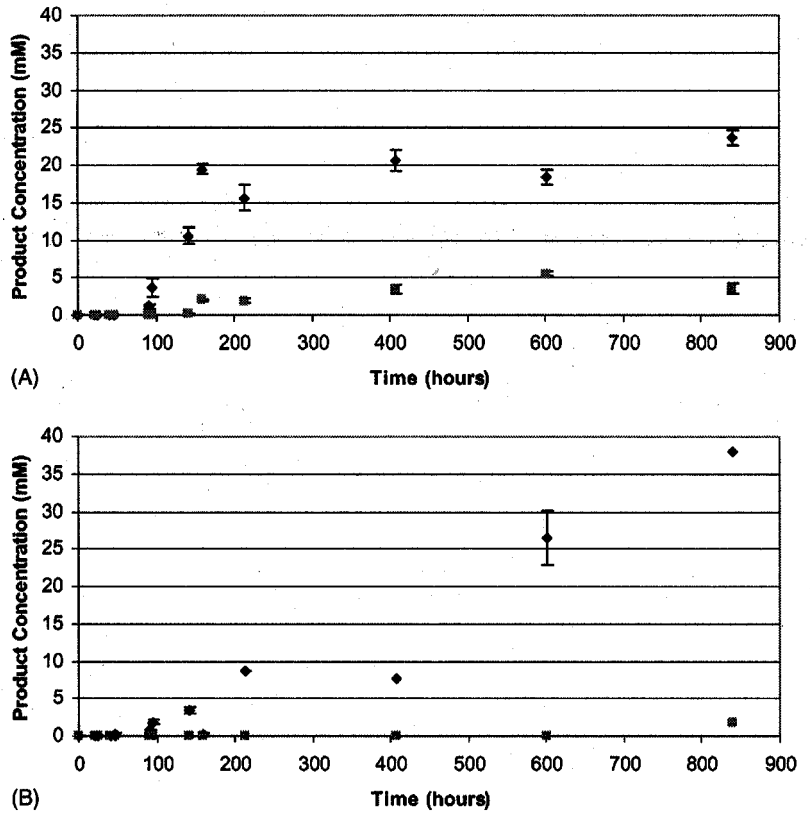

Fig. 2. Time course of *P. chrysosporium* fermentation under high (A) and low (B) nitrogen concentrations. Triplicate bottles for each condition were inoculated and samples taken at the indicated times. Bottles contained 20ml medium and 140ml of air. Data represent average with error bars for standard deviation from two combined experiments. Ethanol–diamonds, acetat —— squares.

detected metabolic products. Lactate was not observed nor were detectable amounts of formate or other organic acids.

In an attempt to separate growth and fermentation *P. chrysosporium* was grown aerobically in flasks containing high and low nitrogen. The cell contents of these flasks were transferred to new medium (with and without added nitrogen sources), sealed with rubber stoppers and purged with nitrogen gas. All of the resuspended cultures produced ethanol (Table 1). The cultures with greater cell mass (that derived from high nitrogen growth) produced more ethanol. Acetate accumulated in all of the cultures although more accumulated under conditions that had greater cell mass.

Table 1
Fermentation by pre-grown aerobic cells

Growth medium	Ammonium in resuspension medium	Products average (mM) ± standard deviation		
		Ethanol	Acetate	Oxalate
Limited N	Absent	12 ± 2	1 ± 1	0.04 ± 0.02
	Present	11 ± 1	1 ± 0	0.03 ± 0.01
Excess N	Absent	20 ± 5	8 ± 1	ND
	Present	16 ± 5	7 ± 3	ND

Values represent the average ± standard deviation for triplicate determinations. ND: not detected, detection limits 0.002 mM.

Oxalate was produced under O_2 limited conditions, but was dependent on the conditions under which the inoculum was generated. Oxalate was only detected when the cells were previously grown under nitrogen-limited conditions. The cell mass from these experiments was freeze-dried for use in detection of alcohol dehydrogenase.

3.2. Ethanol from other carbon sources

P. chrysosporium produced ethanol after 12 days incubation with the following substrates: (substrate, mean (mM) ethanol ± standard deviation from duplicate cultures and replicate experiment, n = 4); glucose, 3.6 ± 1.7; sucrose, 2.5 ± 1.5; mannose, 4.2 ± 1.1; cellobiose, 6.5 ± 2.2; maltose, 3.5 ± 1.6. Glycerol supported growth but did not result in ethanol accumulation. Xylose supported growth but produced variable results in ethanol production. Galactose supported poor growth and did not result in ethanol production.

3.3. Ethanol formation from aspen

The ability of *P. chrysosporium* to produce ethanol from wood was tested with aspen. The milled aspen chips were treated and extracted as described in Section 2. Efforts

were made to provide the milled aspen substrate at close to the water content present in fresh chips. The ethanol and acetate concentrations present in these extracts are shown in Table 2. Neither ethanol nor acetate was detected in the aerobic flasks indicating that the culture had not produced either compound. The flasks that were incubated for an additional period under nitrogen contained both ethanol and acetate.

Additional controls were run separate from this experiment where ethanol and acetate were extracted and analyzed immediately upon removal from incubation and there was no difference from the values reported. Longer aerobic incubations did not produce ethanol. Since the aerobic cultures did not produce ethanol or acetate and there was no ethanol or acetate present in the wood at the start of the incubation, the products were formed as a result of continued

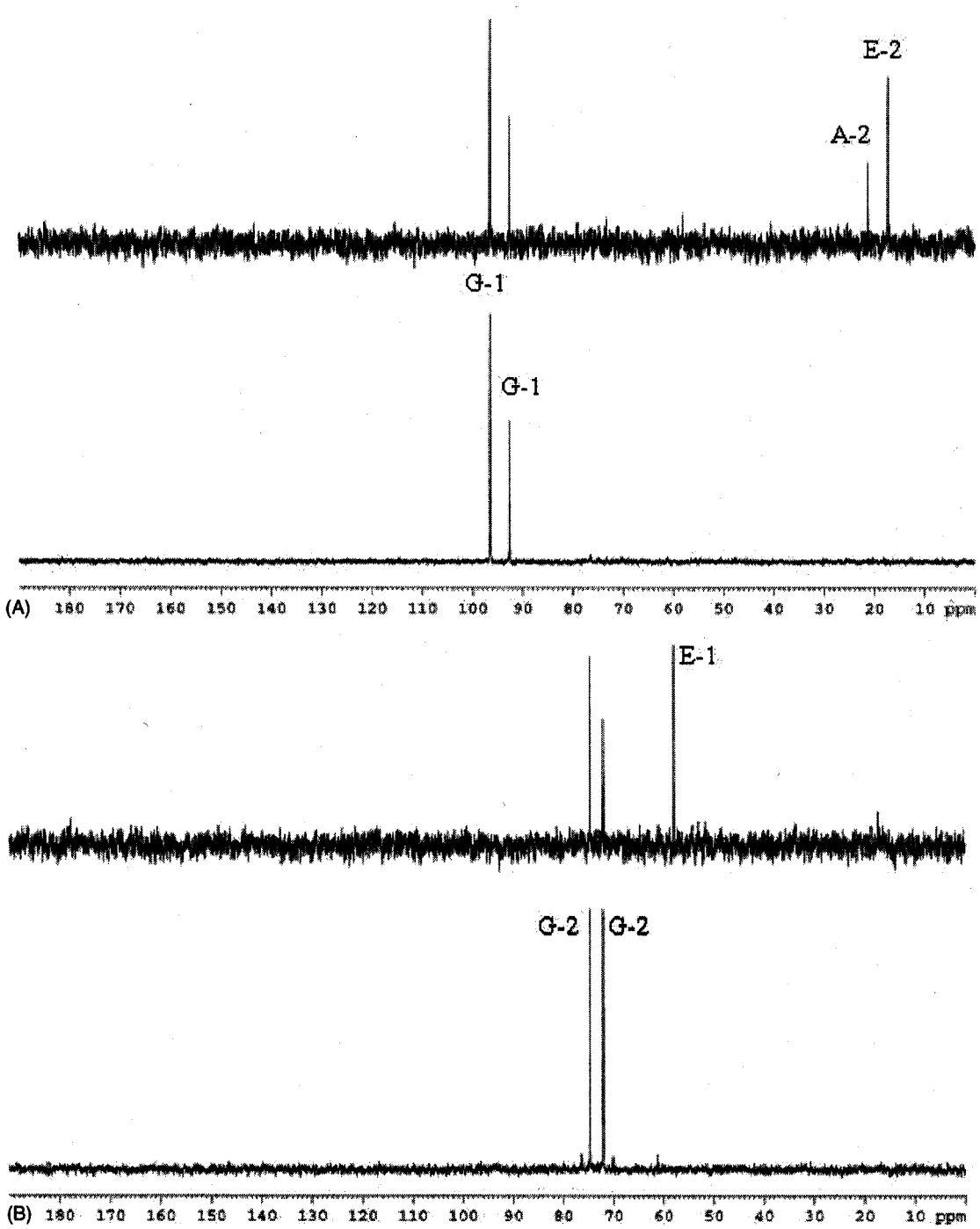

Fig. 3. ^{13}C NMR spectra of ^{13}C-glucose labeled culture filtrates. *P. chrysosporium* was exposed to 50mg 1-^{13}C-glucose (A) and 2-^{13}C-glucose (B). (A) The ^{13}C NMR spectrum for 1-^{13}C-glucose at injection (bottom spectrum) and after 7 days incubation. (B) The ^{13}C NMR spectrum for 2-^{13}C-glucose at injection (bottom spectrum) and after 7 days incubation.

Table 2
Fermentation of aspen chips

Growth condition[a]	Average ethanol (umol per flask)	Average acetate (umol per flask)	Average initial weight aspen (g)
Aerobic 9 days	ND	ND	21.2 ± 0.7
Aerobic 9 days and anaerobic 17 days	30 ± 4	96 ± 19	21.6 ± 0.2

Data from a representative experiment with the average ± standard deviation of triplicate determinations. ND: not detected, ethanol detection limit 8 umol per flask, acetate detection limit 30 umol per flask.

[a] *P. chrysosporium* grown on milled aspen chips at 37 °C.

metabolism under O^2 depletion. Oxalate or other acids were not observed in this experiment. Other experiments (data not shown) using water saturated aspen wood chips resulted in production of both ethanol and oxalate.

3.4. Alcohol dehydrogenase activity

Alcohol dehydrogenase activity (13–17nmol/min/mg protein at 25°C) was measured in extracts of the fungi producing ethanol but not in aerobically grown cells. The reduction of NAD by extracts from freeze-dried cells depended on ethanol addition and was linear with the amount of extract used. Extracts from frozen or fresh cell mass were not dependent on ethanol, Control reactions containing 0.3 mM NADH indicated there was no NADH oxidation under the same assay conditions.

3.5. Derivation of ethanol carbon

The formation of ethanol was investigated under high and low nitrogen conditions where 1-^{13}C- or 2-^{13}C-glucose was provided to the cells. The cells were first grown with unlabelled glucose, then the labeled glucose was injected into the vials after 48 h of growth. Fig. 3 shows the results of initial and final culture samples using high nitrogen medium analyzed for ^{13}C by NMR. The only products detected by GC were ethanol and acetate. The label from 1-^{13}C-glucose Fig. 3A (97.4 and 93.6ppm) appeared in 2-^{13}C-ethanol (18.3 ppm) and 2-^{13}C-acetate (22.3 ppm). The label from 2-^{13}C-glucose (75.9 and 73.2ppm) appeared in 1-^{13}C-ethanol (59.2ppm) but was not observed in 1-^{13}C-acetate, probably due the sensitivity of detecting carboxyl carbons under the conditions the spectra were acquired. Peak assignments were made for glucose by using published values for known chemical shifts. The identification of ethanol and acetate carbons were by direct addition of known compounds and observing peak coincidence. The small peaks at 18.3 ppm in Fig. 3B and 59.2 ppm in Fig. 3A could be due to natural abundance.

The same pattern of labeling of ethanol with glucose was observed with cells grown with low nitrogen (data not shown). No acetate carbons were visible (and no acetate detected by GC) in the spectra for these incubations. Under both high nitrogen and low nitrogen there was no discernable ^{13}C-oxalate formation (oxalate presence detectable by HPLC in the low nitrogen growth condition) and the $^{13}CO_2$ controls also did not show any labeling.

4. Discussion

Many lines of evidence suggest that wood degrading fungi experience both O_2 and nitrogen limitation during their growth on wood. Responses to nitrogen limitation have been well described except for when combinations of nitrogen and O_2 limitation are considered. Nitrogen is growth-limiting in wood because of the large amount of available carbon and low amounts of nitrogen in the wood substrate itself [24]. Soon after growth starts on wood nitrogen is exhausted and the fungi respond by inducing secondary metabolism (oxidative enzymes and other products) that presumably will liberate further sources of nitrogen for growth [25].

The response of wood-degrading fungi to limited O_2 has never been fully described, aside from references to lower wood degradation rates and lower lignin decomposition rates [1,4]. Increasing O_2 availability accelerates wood degradation in laboratory settings, but increased O_2 never occurs in wood degrading conditions and does not mimic the natural progression of colonization and growth normally performed by these fungi. The production of glycan slime described in both wood and liquid cultures should limit the transfer of O_2 to the fungi and egress of carbon dioxide from the fungi [17–19]. These data suggest that O_2 limitation is likely to occur and that these fungi probably have mechanisms to deal with this limitation. A suggestion that anaerobic metabolism might be used in maintaining the energy charge has been made for oxygen-damaged *P. chrysosporium* [20].

We have shown here small amounts of ethanol are produced by *P. chrysosporium* from glucose and other sugars when the O_2 supply becomes depleted. Ethanol was also produced when *P. chrysosporium* was grown on aspen and the container purged with nitrogen gas. This indicates that it is possible to form ethanol using wood as a substrate. There was no ethanol detected in the aspen cultures incubated aerobically. It is possible that small regions within wood under aerobic conditions could become O_2 limited and could serve as local sites of ethanol production. Ethanol formed could then be rapidly consumed in neighboring aerobic regions of the wood.

Ethanol is probably produced by alcohol dehydrogenase. An alcohol dehydrogenase activity was detected in cell extracts from cells producing ethanol and not from those that were not producing ethanol. If the fungi were 50% (by dry weight) protein and averaged 20 mg cells from 20 ml low nitrogen medium there would be sufficient levels of alcohol dehydrogenase to form over 300 mM ethanol in 800 h. The level of alcohol dehydrogenase detected is sufficient to account for the ethanol formed in our experiments. The cessation of ethanol production shown in Fig. 2 is probably due to factors other than alcohol dehydrogenase activity.

The source of ethanol carbon was investigated using ^{13}C-glucose and $^{13}CO_2$. Ethanol could be formed from the growth substrate or from the cell mass. 1-^{13}C-Glucose label was found in 2-^{13}C-ethanol and 2-^{13}C-acetate, and 2-^{13}C-glucose label was found in 1-^{13}C-ethanol. The labeling pattern is consistent with the formation of ethanol from glycolysis and alcohol dehydrogenase.

P. chrysosporium is able to grow with low amounts of 02. Growth increased with increasing available air and was similar in high and low nitrogen media for up to 80ml of air present in sealed bottles (Fig. 1). The medium containing high nitrogen continued this trend with greater amounts of available air. Growth in the low nitrogen containing media decreased when the available air was greater than 80 ml. When the volume of medium is doubled, there is less air available but double the nitrogen available for growth. Comparing the cell mass from 10 ml of medium to that of 20 and 40ml of medium the cell material only increased 1.2-fold (20ml) and 1.6-fold (40ml) for the low nitrogen medium. There was no NH_4^+ detected in both the 10 and 20ml low nitrogen medium conditions at the end of growth. The lower than expected increase in growth could be explained by the induction of ethanol formation lowering the growth yield.

Ethanol formation by itself does not account for the observed growth differences (bottles with 120ml air, Fig. 1) and the trend for higher growth in all of the high nitrogen media. There was still some NH_4^+ present (0.25 mM) in the condition where 40ml of low nitrogen medium was present indicating that more nitrogen could still be obtained by these cultures. The high nitrogen medium had 1.6-fold higher growth at the same volume of available air. Under these conditions there was clearly more nitrogen available for growth and more growth had been achieved in the high nitrogen containing medium using the same volume of air. These data suggest there might be more efficient growth at higher nitrogen levels.

There is no evidence of strictly fermentative growth with the fungus, The relationship of cell mass to air available was not linear but followed a trend of increasing cell mass with increasing air. The data presented in Fig. 1 for the high nitrogen conditions show that ethanol was produced in every case. There was sufficient O_2 present to allow growth, which decreased the O_2 to a level where ethanol production was induced. Ethanol production in the time course experiments (Fig. 2) appeared after most of the growth had taken place (first 72 h). Likewise the transfer of fungi from aerobic to O_2-limited conditions results in fermentation, but no increase in biomass (data not shown). In nature, fermentative metabolism may allow the maintenance of active cells which could exploit new sources of O_2 as they become available.

The synthesis of oxalate by *P. chrysosporium* is interesting since it was observed only after the nitrogen supply was exhausted. This is in contrast to other reports where oxalate was produced in stationary phase or under conditions of high nitrogen content [26,27]. Under conditions of long-term O_2 limitation (Fig. 2) oxalate accumulated during the incubation only when NH_4^+ was limited. Oxalate was produced when the cultures were grown aerobically under nitrogen limitation and transferred to O_2-limited conditions with and without NH_4^+. However, when aerobic cells from nitrogen sufficient conditions were transferred to NH_4^+ excess or absent media under O2-limited conditions there was no synthesis of oxalate, but there was fermentation. Exhaustion of the nitrogen supply while the culture is growing appears to be required for oxalate production.

The production of oxalate does not appear to be regulated by the availability of O_2. Less O_2 is required in the formation of oxalate from glucose than the conversion to CO_2. Brown-rot fungi and some white-rot fungi have been proposed to make oxalate as a result of a deficiency of 2-oxoglutarate dehydrogenase [28,29]. In these fungi the production of oxalate may be obligate and less O_2 is required for glucose metabolism to oxalate than to CO_2. The data indicate that with *P. chrysosporium* O_2-limited cells can ferment regardless of the nitrogen content, but oxalate production is not turned on unless nitrogen is limiting.

The *P. chrysosporium* genome has been sequenced and is available to the public (http://www.jgi.doe.gov/JG_microbial/html/index.html). Using the reported alcohol dehydrogenase (ADH) I and II sequences of *Aspergillus nidulans* [30,31] and ADH I of *A. flavus* [32] the genome of *P. chrysosporium* was searched using the TblastN program. The ADH I sequences from both *Aspergillus* species identified six scaffolds of the *P. chrysosporium* genome with bit scores higher than 50 and 8 with values less than 1E−04. The ADH II sequence identified six different scaffolds of the *P. chrysosporium* genome with bit scores higher than 50 and 9 with values less than 1E−04.Investigation into the induction of these potential ADH genes will indicate their involvement in the production or use of ethanol.

Fermentative metabolism of the filamentous fungi might not be sufficient to produce recoverable amounts of products, but is likely to be more widespread than generally believed. Aerobic filamentous fungi are more known for organic acid production than for ethanol formation [33]. *Fusarium, Aspergillus, Mucor, Polyporus, Neurospora, Monilia, Paecilomyces,* and *Rhizopus* are able to produce ethanol [34–36]. *Fusarium* is able to grow anaerobically [37]. *Rhizopus* is able to produce up to 31 g/l ethanol [38]. A *Trichoderma harzianum* isolate has recently been reported to ferment a variety of substrates, including cellulose, to ethanol in a minimal medium [39]. These filamentous fungi indicate that the Ascomycetes, Zygomycetes and Deuteromycetes all have representatives that are able to produce ethanol. Recently an unidentified Basidiomycete (H2) was reported to produce ethanol from sawdust [38], Now with the addition of *P. chrysosporium* there are identified ethanol producing species in all classes of the Amastigomycota.

P. chrysosporium and other white-rot fungi can use ethanol as a substrate for growth [40]. This work indicates that *P. chrysosporium* is also able to produce ethanol under

O_2-limited conditions. Ethanol is able to support both the growth of *P. chrysosporium* and lignin degradation [25]. While the production of ethanol may improve fungal viability it also can serve as a future substrate to be used when O_2 is no longer in short supply.

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