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CO_2 mineralization induced by fungal nitrate assimilation

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ABSTRACT

Formation of $CaCO_3$ induced by fungal physiological activities is a potential way to sequestrate atmospheric CO_2 in ecosystem. *Alternaria* sp. is a saprophytic fungus isolated from a forest soil. We examined the precipitation of $CaCO_3$ induced by the fungus in response to different levels of $Ca(NO_3)_2$ or $CaCl_2$ in agar media, and the biogenesis of $CaCO_3$ was verified by low $\delta^{13}C$ value. The formed $CaCO_3$ was identified as calcite by X-ray diffraction analysis. Square, rectangular and rhombic $CaCO_3$ crystals and amorphous calcium carbonate were observed around mycelia at higher levels of $Ca(NO_3)_2$. Acidification occurred in media at low concentrations (0 and 0.0002 M) of $Ca(NO_3)_2$, and no $CaCO_3$ formed in these media. The quantities of $CaCO_3$ formed in media increased with increasing concentrations of $Ca(NO_3)_2$ and were significantly correlated to fungal biomass, pH value and nitrite concentrations. No $CaCO_3$ was formed in media with $CaCl_2$ at all levels. These results collectively indicated that the formation of $CaCO_3$ can be induced by the fungal assimilation of nitrate. The study also revealed that biogenic crystal of $CaCO_3$ tended to grow on a silicon nucleus and the amorphous calcium carbonate (ACC) was the transient stage of $CaCO_3$ crystal.

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1. Introduction

Land and ocean uptake of carbon dioxide plays a critical role in determining atmospheric carbon dioxide levels (Reay et al., 2008). The annual global CO_2 flux from soil respiration is estimated to average (+/− S.D.) 68 +/− 4 Pg C/yr, occupying a considerable proportion in total carbon storage (Raich and Schlesinger, 1992). Soil respiration plays a critical role in the regulation of carbon cycling at the ecosystem and global scales. The microbial communities of natural ecosystems are dominated by fungi (81–95% biomass on average) (Susyan et al., 2005). So, fungi play a large role in the decomposition of litter and soil organic matter and fungal respiration may play a key role in global warming.

Sequestration or reduced emissions of CO_2 has attracted unparalleled attention due to global warming and its potential consequences on human beings. The source of marine carbonate minerals has shifted from abiotic precipitation to biogenic sources over the history of the earth (Morse et al., 2007). Except for animals that form $CaCO_3$ as an ingredient of shell skeleton, many organisms have the potential to form $CaCO_3$. Amorphous calcium carbonate (ACC) could be formed by plants and animals (Canti, 2009; Weiner et al., 2003); crystalline calcium carbonate could be formed by bacteria and algae (Bartels, 1951; Gimingham, 1911; Lee et al., 2006). Castanier et al. (1999) classified microbial Ca-carbonate precipitation into autotrophic and heterotrophic pathways. In autotrophic pathways, the Ca-carbonate precipitation was induced by CO_2 depletion in $Ca(HCO_3)_2$ solution by autotrophic bacterial uptake. In heterotrophic pathways, a pH increase was induced first during N or S cycling, then Ca-carbonate precipitation under an appropriate condition. Nitrate uptake and assimilation is a process to convert the nitrate, negative anion, into ammonium, positive ion, by organisms, and the process will lead pH increase. In the same way, nitrate uptake and denitrification by soil fungi will also induce CO_2 mineralization and reduce respired CO_2 emissions. However, there is a paucity of data on Ca-carbonate formation induced by fungi, the dominant microbes in soil. This study was carried out to test the hypothesis that nitrate assimilation will induce respiratory CO_2 sequestration by $CaCO_3$ formation in soil, and to quantify and characterize the formation of $CaCO_3$ by *Alternaria* sp., which can utilize NO_3^- as the sole N source and grows well under barren conditions, in response to different concentrations of $Ca(NO_3)_2$ in agar media.

2. Methods

2.1. Fungal culture

Alternaria sp. (ITS sequence GenBank accession GU903488) was isolated from a forest litter on PDA medium with 0.2 M $Ca(NO_3)_2$ and was identified morphologically by a published guide (Larone, 2002). A test medium contains sucrose 20 g/L, K_2HPO_4 0.1 g/L, $MgSO_47H_2O$ 0.5 g/L, agar 20 g/L (the agar powder contains 0.06% nitrogen measured by elements analyzer Vario EL I, ELEMENTAR,

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Germany) and different concentrations of $Ca(NO_3)_2$. In order to examine the effect of fungal assimilation of nitrate on calcium carbonate formation, $Ca(NO_3)_2$ was replaced by $CaCl_2$ in this study (Table 1). The medium pH was adjusted to ensure pH 5.2–5.5 after autoclaving.

25 mL aliquots of the media were dispensed into 90-mm Petri dishes. A sterile dialysis membrane (4.2 × 4.2 cm) was placed on the top of solidified medium to facilitate easy removal of aerial fungal colony (Tuason and Arocena, 2009). In order to examine the interaction between calcium carbonate particles formed and fungal mycelia, a sterile microscopic glass (12 × 12 × 0.17 mm) was erected in media by the dialysis membrane. Three mycelium pellets (about 5 mm in diameter) were inoculated at the edge of Petri dishes to avoid placing the pellets on dialysis membrane. Fungal colonies were harvested after incubation at 25 °C for a month in Petri dishes sealed with Parafilm M (American National Can, Chicago, USA). Non-fungi-inoculated Petri dishes were set as a control to rule out abiotic calcium carbonate formation.

2.2. Fungal biomass

The fresh fungal mycelia on dialysis membrane were used for the test of aerial mycelia biomass, which was hypothesized to be positively related to the total fungal biomass. Aerial fungal biomass was calculated, based on the total weight of fungal colony and dialysis membrane and the weight of dialysis membrane, where $g_1 = g_0 - g_2$ (g_1, fungal biomass; g_0, total fungal colony and membrane; g_2, membrane weight). The membrane weight was measured before fungal cultivation. The aerial fungal colonies and dialysis membrane were washed for three times with distilled water gently to reduce the soluble $CaCl_2$ or $Ca(NO_3)_2$ and dried at 60 °C for 12 h. The total weight of aerial fungal colony and dialysis membrane was measured.

2.3. Nitrite and pH measurements

The media and fungal colony in the Petri dish was milled into paste and transferred into a 50-mL centrifuge tube. De-ionized water was added to reach the initial volume of 25-mL and the mixture of water and agar was homogenized after 5-min vortexing. Supernatant, which was prepared for measurement of pH and nitrite concentration, was transferred to a 10-mL centrifuge tube after centrifugation at 5, 300*g* for 15 min at room temperature. The pH values were determined with a pH meter (PHS 3C, Shanghai, China). Nitrite concentration was determined by chromatographic method according to Lowe and Evans (1964).

2.4. Extraction, quantification and identification of $CaCO_3$ crystals

The crystals were extracted by the NaClO method (Garvie, 2006). The detailed extraction steps were as follows: the fungal colonies and agar medium were transferred to a 50-mL centrifuge tube and washed six times with an identical volume of 20% alcohol in de-ionized water. Then, a duplicate volume (to the volume of agar) of 7% sodium hypochlorite solution (pH 8.5) was added to dissolve fungal mycelia and agar for two times, 12 h each time. $CaCO_3$ crystal was washed repeatedly with 20% alcohol. Then, $CaCO_3$ was dissolved in 5 mL 3 M HCl, the Ca^{2+} concentrations in appropriately diluted solution were determined by an atomic absorption spectrometer (AA800+MHS 15). The quantity of $CaCO_3$ crystal was calculated according to the Ca^{2+} concentrations measured. The $CaCO_3$ crystals were identified by powder X-ray diffraction analysis using a Rigaku D/max-2200 instrument (Rigaku Denki Co., Tokyo, Japan).

Table 1
$Ca(NO_3)_2$ and $CaCl_2$ concentrations in test medium.

Treatments	1	2 (M)	3 (M)	4 (M)	5 (M)
$Ca(NO_3)_2$ group	0	0.0002	0.002	0.02	0.2
$CaCl_2$ group	0	0.0002	0.002	0.02	0.2

2.5. Morphology of $CaCO_3$ crystals

The fungal colonies on microscopic glasses were gently washed by water containing 20% alcohol three times to remove any soluble Ca^{2+} and then freeze-dried. Crystals deposited on the microscopic glasses were examined by a KYKY-1000B scanning electron microscope (SEM, Instrumental Factory of CAS, Beijing, China) with an EDAX energy dispersive X-ray spectrometer (EDS). The microscopic glasses were stuck on Al stubs and sputter coated with Au for examination of crystal morphology and distribution.

In order to identify the carbon source of $CaCO_3$, the stable carbon isotopic compositions of $CaCO_3$, fungal mycelia, sucrose added to the media and medium mixture, were measured using a stable isotope ratio mass spectrometer (Finnigan MAT 252, Bremen, Germany). The stable carbon isotope composition is expressed as follows: $\delta^{13}C = (R_{sample} - R_{PBDstandard})/R_{PBDstandard} \times 1000‰$ (R is the mol ratio $^{13}C/^{12}C$). Secondary standards of graphite and sucrose calibrated against Pee Dee belemnite (PDB) carbonate were used for comparison. The $\delta^{13}C$ value of $CaCO_3$ was obtained by $CaCO_3$ formed in 0.2 M $Ca(NO_3)_2$level media but remaining levels were not sufficient for stable carbon isotopic composition survey.

2.6. Statistical analyses

All data were analyzed using SPSS 13. The normality of distribution and homogeneity of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. The transformed data were analyzed by two-way ANOVA with calcium and nitrate levels as the independent variables. Differences between treatments were determined by Tukey's HSD test. Correlations between variables were determined using Spearman's coefficient. Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Calcium carbonate production

After 1 month of cultivation, at all levels of 0.002, 0.02 and 0.2 M $Ca(NO_3)_2$, $CaCO_3$ were produced in media (Fig. 1) and there

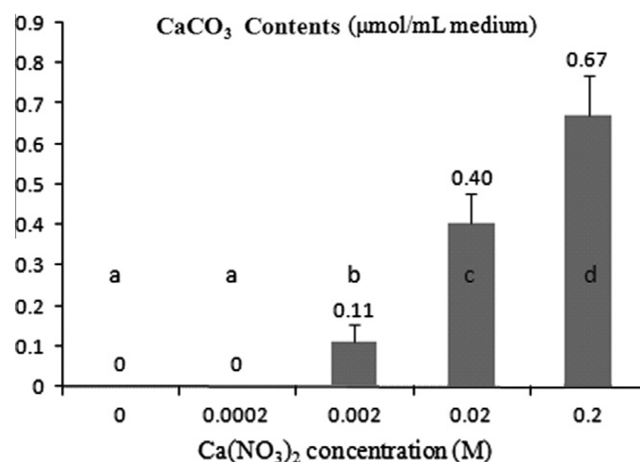

Fig. 1. Contents of $CaCO_3$ formed in media growing *Alternaria* sp. in response to different levels of $Ca(NO_3)_2$. For each analyte, bars with the same letters were not significantly different ($p > 0.05$).

was no $CaCO_3$ produced in $CaCl_2$ treatments. In the $Ca(NO_3)_2$ treatments, the differences in $CaCO_3$ contents were significant across the five levels. At 0.002, 0.02 and 0.2 M, fungal biomass of $Ca(NO_3)_2$ treatments were significantly higher than that of $CaCl_2$ treatments ($p = 0.000$). The fungal biomass (1076.6 mg on 25 mL medium) was about threefold higher in 0.2 M $Ca(NO_3)_2$ treatment than that in 0.2 M $CaCl_2$ treatment (346.4 mg). In $Ca(NO_3)_2$ treatments, fungal biomass was positively correlated with $CaCO_3$ contents produced in media (Spearman's $\rho = 0.690$, $p = 0.004$) (see Fig. 2).

After 1 month of cultivation, nitrite (NO_2^-) concentrations in media increased with initial concentrations of $Ca(NO_3)_2$ (Fig. 3). The NO_2^- concentrations in the levels below 0.02 M $Ca(NO_3)_2$ treatment were significantly different. The NO_2^- concentrations were positively correlated to fungal biomass (Spearman's $\rho = 0.715$, $p = 0.003$) and the $CaCO_3$ content (Spearman's $\rho = 0.533$, $p = 0.011$).

More fungal biomass can be obtained in the media with higher concentrations of $Ca(NO_3)_2$. Nitrate assimilation by denitrification is a major biological process by which inorganic nitrogen is converted to ammonia and to organic nitrogen (Solomonson and Spehar, 1977). Nitrate reductase activity is significantly correlated to the amount of assimilated nitric nitrogen (Hageman and Flesher, 1960). More biomass meant higher nitrate reductase and, further, more efficient nitrogen assimilation. Our results showed that nitrite, as the first product of nitrate denitrification, was high in media with high levels of $Ca(NO_3)_2$ and was significantly correlated with fungal biomass.

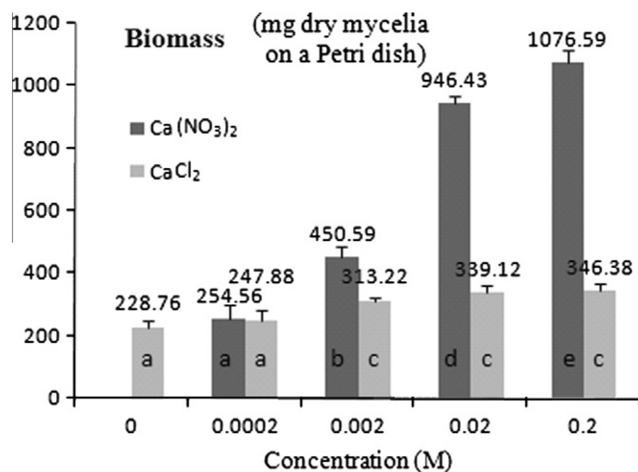

Fig. 2. Aerial fungal biomass growing on media different levels of $Ca(NO_3)_2$ and $CaCl_2$. For each analyte, bars with the same letters were not significantly different ($p > 0.05$).

Except for levels of 0 and 0.0002 M, the pH values of $Ca(NO_3)_2$ treatments were significantly higher than those of $CaCl_2$ treatments (Fig. 4). Within $Ca(NO_3)_2$ treatments, the pH values of 0.002, 0.02 and 0.2 M levels increased after 1 month of cultivation, from pH 5.5 to approximate neutral values, even to alkaline values (for 0.02 and 0.2 M $Ca(NO_3)_2$ treatments), while the pH values of all the remaining treatments decreased. Within $Ca(NO_3)_2$ treatments, pH values were positively correlated with nitrite concentrations (Spearman's $\rho = 0.452$, $p = 0.035$) and $CaCO_3$ contents (Spearman's $\rho = 0.422$, $p = 0.028$).

pH of the soil solution was found to have a significant influence on nutrient availability (Darrah, 1993). Exudation of organic acids, the main acidification agents, will increase when PO_4^{3-}, K^+ and Mg^{2+} are deficient (Paris et al., 1996). Acidification is one of adaptive mechanisms for plants or microbes toward nutrient depletion. This may be the reason why the pH of media was lower at low $Ca(NO_3)_2$ concentration in this study (Fig. 4). The decreased pH in the $CaCl_2$ treatments of 0.002 and 0.02 M levels may be related to the depletion of N source. A literature by Bago et al. (1996) also recorded decreased pH induced by extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture under nitrate depletion condition.

pH of substrate typically rises during denitrification (Fernández-Nava et al., 2008). In this study, the pH increased as nitrite accumulated in media with higher concentrations of $Ca(NO_3)_2$. Under natural conditions, nitrate of high concentration is more accessible to plants than ammonium in rhizosphere (Darrah, 1993). For the sake of denitrification, pH in nitrate-containing media was found to generally increase unless the nitrate concentration was extremely low during the corn growth (Durand and Bellon, 1994).

The $\delta^{13}C$ values of the formed $CaCO_3$ in medium, fungal mycelia, sucrose and medium mixture were $-10.62 \pm 0.54‰$, $-14.71 \pm 0.32‰$, $-12.12 \pm 0.02‰$ and $-17.82 \pm 0.12‰$, respectively. ^{13}C species was abundant in $CaCO_3$ compared to fungal mycelia, sucrose and medium mixture. ^{12}C species was enriched in fungal mycelia compared with sucrose but was depleted compared to medium mixture. Previous studies indicate that fungal biomasses were enriched in ^{13}C relative to the growth medium (Henn and Chapela, 2000; Henn et al., 2002). The higher $\delta^{13}C$ values of $CaCO_3$, compared with those of fungal mycelia, revealed that the respired CO_2 by fungi was rich in ^{13}C. A previous study also indicated that

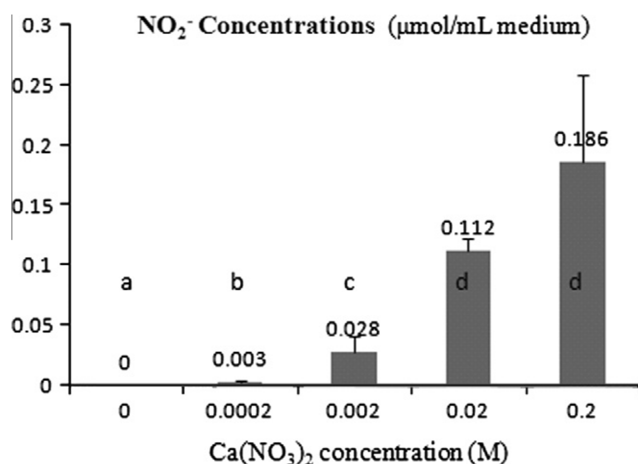

Fig. 3. Concentrations of nitrite (NO_2^-) produced in media growing *Alternaria* sp. in response to different levels of $Ca(NO_3)_2$. For each analyte, bars with the same letters are not significantly different ($p > 0.05$).

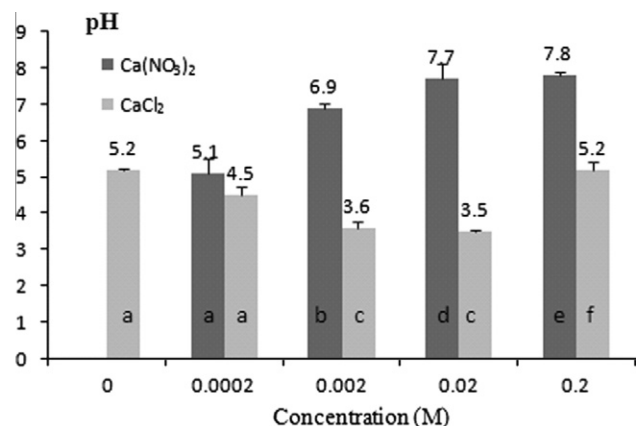

Fig. 4. pH values of media with different levels of $Ca(NO_3)_2$ and $CaCl_2$ after one month of cultivation of *Alternaria* sp. For each analyte, bars with the same letters are not significantly different ($p > 0.05$).

the respired CO_2 is not enriched in ^{12}C relative to the fungal biomass (Henn and Chapela, 2000). Many studies showed that fungal biomass was enriched in ^{13}C relative to the growth medium (Henn et al., 2002). However, our results showed that the biomass of *Alternaria* sp. was enriched in ^{12}C relative to sucrose but depleted in ^{12}C relative to medium mixture. This may indicate that the fungus not only assimilated sucrose, but also decomposed agar as C and energy source.

3.2. Morphology and identification of crystals

XRD analysis confirmed that the presence of monocrystal calcite (purified with NaClO method) formed in the medium. The amount of formed $CaCO_3$ was closely related to the Ca^{2+} concentration, CO_2 or HCO_3^- concentrations and ambient pH value. The rise of pH caused by denitrification, together with high concentrations of calcium and respired CO_2 or HCO_3^-, provided appropriate conditions for $CaCO_3$ formation. $CaCO_3$ crystals of different shapes were formed around the mycelia on microscopic glasses with higher $Ca(NO_3)_2$ levels. The sizes of crystals ranged from a maximum of 10 μm to less than 1 μm at minimum. At 0.2 M $Ca(NO_3)_2$ level, $CaCO_3$ particles of the most quantity were formed. At 0.02 and 0.002 M level, certain quantities of $CaCO_3$ particles were formed. At the levels of 0.0002 and 0 M, there were no particles produced around mycelia. At 0.02 and 0.002 M $Ca(NO_3)_2$ levels, the $CaCO_3$ crystals always kept a distance from fungal mycelia. At 0.2 M $Ca(NO_3)_2$ levels, the crystals were much closer to mycelia than those of the above two levels. This may be due to higher respired CO_2 concentration (Castanier et al., 1999) or carbonic anhydrase concentration (Dong et al., 1993) near mycelia, which made the $CaCO_3$ tend to dissolve, and at the higher level of $Ca(NO_3)_2$, the concentration of calcium pH were high enough to capture the respired CO_2 near fungal mycelia immediately. The SEM image of $CaCO_3$ particles and mycelia growing on microscopic glasses in response to different levels of $Ca(NO_3)_2$ were shown in Supplementary materials as in Fig. S1.

A loose texture was observed at the center of many $CaCO_3$ crystals. The SEM–EDS analysis indicated that silicon was abundant in the loose texture and calcium was abundant in the peripheral area, which tended to be compacted (see Fig. S2 in Supplementary materials). The crystal shapes of biogenic calcite were different from the abiotically formed calcite (Lian et al., 2006). The crystal shape of calcite was not only affected by the microbial species (Castanier et al., 1999), but also by the microbial excretions, such as exopolysaccharides, amino acid, taurocholate, lecithin, chitosan and cholesterol (Braissant et al., 2003; Payne et al., 2007), solution composition, and pH (Ziegler, 2008). In this study, the formation of biogenic $CaCO_3$ crystals may be affected by many factors, such as ambient pH, ionic activity, and fungal excretions. Kitano et al. (1979) found that dissolved silica would favor the precipitation of $CaCO_3$ in solution. Our results that loose structures rich in silicon existed in some $CaCO_3$ crystals supported their finding strongly. The loose structure here also indicated that the crystals probably grew on a silicon nucleus initially.

Amorphous calcium carbonate (ACC) was also found on some microscopic glasses. These ACC included vague film along the mycelia, which appeared to be at an early stage of development and thin film of calcium around mycelia appeared to be at a later stage (see Fig. S3 in Supplementary material). The formation of ACC film may indicate the respired CO_2 or HCO_3^- was captured immediately by high concentration calcium at neutral pH value before the crystal was formed. Many scientists considered that ACC was the precursor phase of calcite or other crystal types (Pecher et al., 2009; Politi et al., 2004; Raz et al., 2003). About 10 μm long threads, another form of ACC, extended from poorly crystalline $CaCO_3$, were observed on microscope glasses. Growing threads from the poorly crystalline confirmed the precursor phase theory, i.e., the threads were at ACC stage and the poorly crystalline was at calcite-becoming stage (see Fig.S4 in Supplementary material).

It is predictable that nitrogen deposition will continuously elevate in the future on regional and global scales (Dentener et al., 2006; Reay et al., 2008). Nitrate (NO_3^-) is the fate of atmospheric NO_x (Brown et al., 2004). Nitrate can be assimilated by microorganisms and plants. The nitrate assimilation processes include nitrate uptake and transport into cell by nitrate transporter, with the sequential reduction to nitrite and ammonium, catalyzed by the enzymes nitrate reductase and nitrite reductase, respectively (Crawford and Arst Jr., 1993). The reduction of nitrate will lead pH increase. As a result, these available nitrogen inputs will not only increase the uptake of carbon dioxide (Reay et al., 2008), but also increase the mineralization of carbon dioxide induced by NO_x reduction in terrestrial ecosystem. Uptake and mineralization of carbon dioxide can slow the process of global warming. Apart from nitrogen deposition, appropriate nitrogen fertilization management may be exerted in nitrogen-limited area to improve biomass and CO_2 will be mineralized subsequently in some extent.

4. Conclusions

Provided with higher concentrations of calcium and nitrate in substrate, fungal metabolism induced the formation of calcium carbonate by denitrification. The results showed a potential pathway to sequestrate CO_2 or reduce CO_2 emissions from calcicolous soil by nitrate fertilization. Further studies on the nitrate fertilization management are needed.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.08.080.

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