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Production of Coal-Solubilizing Activity by *Paecilomyces* Sp. During Submerged Growth in Defined Liquid Media

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ABSTRACT

Paecilomyces TLi, a fungus isolated from coal, had previously been shown to transform solid coal into a water-miscible liquid during surface growth on coal or on a complex, solid microbiological medium. Coal solubilization has now been demonstrated in submerged cultures grown in defined liquid minimal media. Activity under these conditions is less than that observed in surface culture, and is affected by both nitrogen and carbon nutrition. Coal solubilization occurs at low pH (≤ 4) but may be associated with pH increases during the assay period. Spectroscopic data suggest a role for alkaline catalysis in coal solubilization by this organism.

Index Entries: Coal; *Paecilomyces;* subituminous coal; coal solubilization; biosolubilization.

INTRODUCTION

The ability of microorganisms to modify and/or degrade lower-ranked coals has been the focus of considerable scientific attention in recent years. A number of fungi found associated with coal in the environment have been tested for their abilities to degrade coal in the laboratory, as have organisms (fungi and actinomycetes) known to degrade lignin (*1–7*). The solubilization of lignite and subbituminous coals by these organisms has been described. The product of this microbial activity is a water-soluble, complex mixture of oxidized organic compounds. The liquid coal product

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Table 1
Disadvantages of the Current Coal Biosolubilization Technology

Scaleup (of surface cultures) is impractical
Product contamination by organic medium components is inevitable
Product recovery is hampered by its diffusion into the solid medium
Activity measurement (as weight loss) is tedious, cumbersome, time-consuming, and subject to error

has potential as a chemical feedstock and as a substrate for other microbial processes, such as biogasification. Progress in these latter areas is limited in part by the absence of a technology with which to generate large quantities of pure liquid coal product. The current technology for microbial coal solubilization involves the cultivation of competent organisms on the surface of complex solid microbiological media. Problems inherent to this culture mode have impeded both fundamental and applied studies of this phenomenon (Table 1). A new technology should combine medium development and assay development to facilitate process optimization and critical investigations into the underlying microbial biochemistry.

Paecilomyces TLi, a fungus isolated from coal in this laboratory (*4*), was found to solubilize coal more rapidly than several other organisms tested. It has, therefore, been chosen as a model organism for both basic studies and process design. This report describes the development of defined minimal liquid media supporting growth and coal solubilization by *Paecilomyces* TLi; the development of a sensitive and rapid assay for coal solubilization in the defined system; and observations concerning the regulation and mechanism of coal solubilization by this organism.

MATERIALS AND METHODS

Organism and Culture Conditions

Paecilomyces TLi, a laboratory isolate (*4*), was maintained on potato dextrose agar slants at 30°C. This media supports profuse conidiation; conidia can be harvested by scraping and then resuspended in sterile distilled water. Surface cultures were inoculated with aqueous conidial suspensions at approximately 10^6 organisms per 100 mm petri dish. These cultures were incubated at 30°C and 80% relative humidity for 7–14 d.

Coal Solubilization

Wyodak subbituminous coal pretreated with nitric acid (*8*) was the substrate for all biosolubilization assays described in this work. Cultures were dosed with 0.5 g coal; particle size was 3–5 mm. Coal solubilization by surface cultures was measured gravimetrically (*4*).

Table 2
Defined Minimal Media for Growth of *Paecilomyces* TLi

Component	Czapek's, g/L[a]	Minimal I, g/L[a]
Carbon source[b]	1.0	1.0
Nitrogen source[c]		
$NaNO_3$	3.0	—
NH_4Cl	—	1.89
K_2HPO_4	1.0	1.0
$MgSO_4 \cdot 7H_2O$	0.5	0.5
KCl	0.5	0.5
$FeSO_4 \cdot 7H_2O$	0.01	0.01
Agar	15.	15.

[a]pH adjusted to 5.6 before autoclaving.
[b]Czapek's medium supports growth on a variety of carbon substrates; Minimal I medium supports growth on carbohydrates only.
[c]Final N concentrations: 0.035 *M*.

RESULTS

Development of Minimal Media

Coal solubilization by *Paecilomyces* TLi was previously described in surface cultures on Sabouraud maltose agar, a complex organic medium containing 10% neopeptone in addition to maltose (the primary carbon source) (*4*). Two defined minimal media which support growth of this organism in surface culture have been developed (Table 2). These media are based on inorganic salts solutions containing a single organic growth substrate (0.1% maltose in routine use). Coal solubilization has been unequivocally demonstrated on at least one of these media (Table 3).

Submerged Culture Techniques

Bioreactor configurations proposed for microbial coal solubilization require submerged and/or agitated cultivation of the biocatalyst (*4*). These conditions may be approximated in 125-mL shake flasks containing 30 mL of liquid media. For this work, the defined minimal media described earlier were modified by the omission of agar. Submerged cultures were grown from conidial inocula (10^6/mL) and were incubated at 30°C with agitation at 50 rpm (2.5-cm stroke). Cultures grown in the two media differed with respect to final medium pH (Table 4). The extremely high buffering capacity of the pretreated coal precluded control of pH during the fermentation.

Assay Development

Results from experiments conducted in Czapek's liquid medium suggested that in comparison with surface cultures, coal solubilization is de-

Table 3
Coal Solubilization by *Paecilomyces* TLi
(Surface Cultures)[a]

Medium[b]	Coal solubilization, percent weight loss[c]
Complex	
Sabouraud maltose	12.4
Defined	
Czapek's	25.9
Minimal I	[5.3]

[a]Cultures were grown at 30°C, 80% RH for 7 d. Coal was added (0.5 g) and cultures reincubated for 7 d under the same conditions.
[b]Media contained 0.1% maltose.
[c]Controls (uninoculated medium) exhibit ≤5% weight loss. Weight loss measurements incorporate ≤20% error.

Table 4
Coal Solubilization by *Paecilomyces* TLi in Defined Media (Submerged Cultures): Final pH[a]

	Cultures without coal		Cultures with coal	
Medium	Initial	Final	Initial	Final
Czapek's	5.6	6.8	3.4–4.3	3.1–3.4
Minimal I	5.6	2.4	3.4–4.0	3.1–3.4

[a]Shake flasks (liquid medium) were inoculated with 10^6 conidia/mL ± 0.5 g coal and incubated at 30°C, 50 rpm agitation for 7 d.

pressed in submerged cultures (data not shown). However, attempts to quantitate activity in the shake flask system via the gravimetric assay used in previous work were hampered by difficulties in separating undissolved coal particles from fungal biomass. Centrifugation of the cultures at 7000 × G for 10 min permitted separation of the spent culture broth from particulate matter. Ultraviolet-visible spectroscopic scans of supernatants from experimental cultures indicated the production of a material absorbing broadly over the 420–450 nm range (Fig. 1). The chromophoric material was first detected in cultures grown in Minimal I medium to which coal was added at the time of inoculation. This material appeared in cultures during the period of active growth (Fig. 2). The material was absent in control cultures lacking coal and in uninoculated medium titrated over the pH range of the fermentation (i.e., 2.1–5.6). A similar peak was observed in cultures incubated in Czapek's medium.

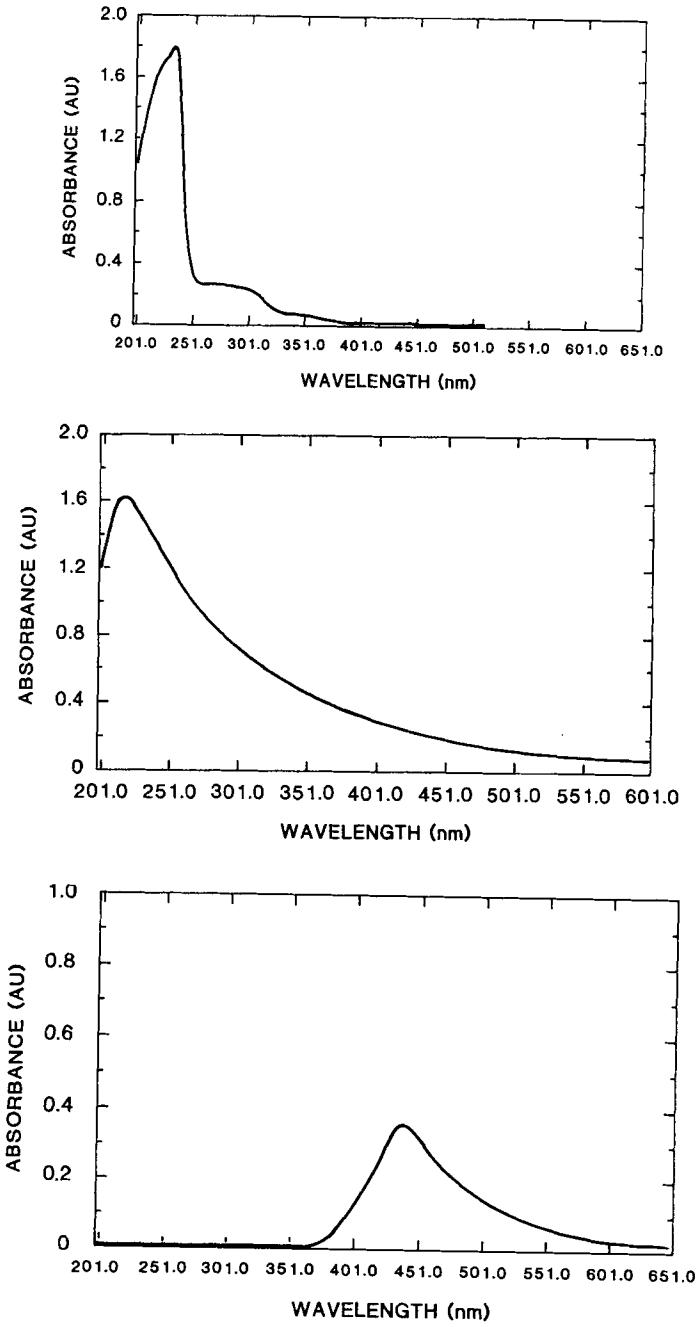

Fig. 1. Spectroscopic scans of spent broth from *Paecilomyces* TLi incubated with coal (submerged cultures). Minimal I medium in shake flasks was inoculated with 10^6 conidia/mL; 0.5 g coal was added to experimental cultures at the time of inoculation. Controls contained fungus only or coal only in uninoculated medium. Experimental cultures and controls were incubated at 30°C for 3 d. A) Cells-only control vs uninoculated medium; B) Coal-only control vs uninoculated medium (1:10 dilution); C) Experimental cultures (cells + coal) vs coal-only control.

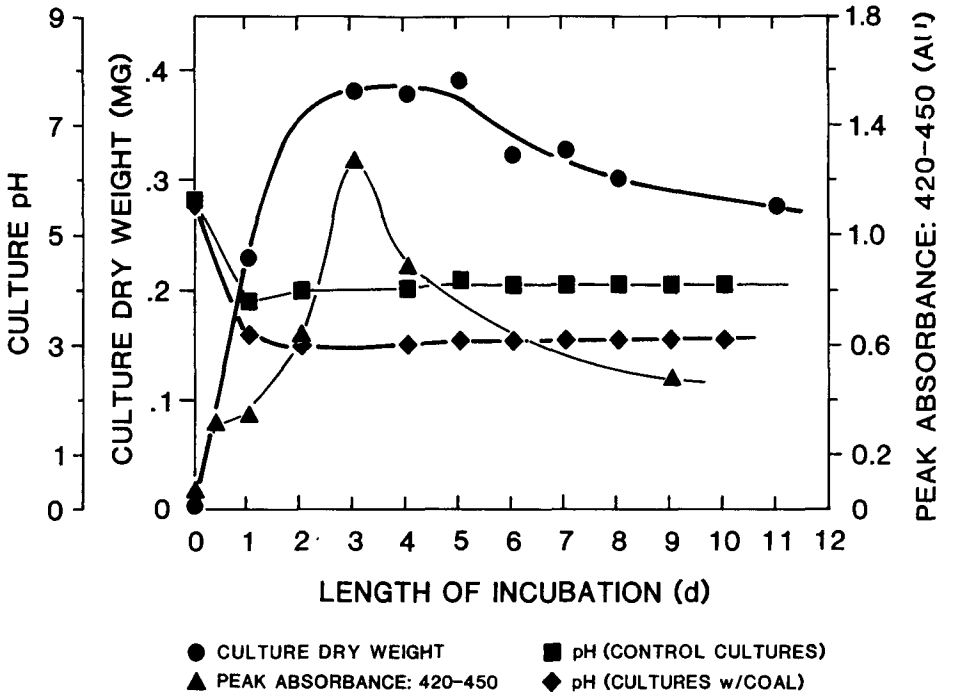

Fig. 2. Kinetics of growth, pH changes, and production of chromophoric material by *Paecilomyces* (submerged cultures). Minimal I medium (shake flasks) was inoculated with 10^6 conidia/mL; 0.5 g coal was added to experimental cultures at the time of inoculation. Controls contained fungus only, or coal only in uninoculated medium. Experimental cultures and controls were incubated at 30°C and 50 rpm. Replicates were harvested by centrifugation. Growth (dry weight) was measured in control cultures. Absorbance data reflect difference spectra over the 420–450 nm range for experimental cultures vs coal-only controls.

Genesis of the Chromophoric Material

Coal is solubilized by dilute alkali at pH values of 6 and greater. It has, therefore, been suggested that biological coal solubilization occurs as a fortuitous result of pH increases associated with growth (*6*). In the present work, material with spectroscopic characteristics identical to those of the biogenic chromophore was produced by incubation of coal with dilute alkali at pH 6.55 and higher (data not shown).

Effect of Growth Substrate

The organism used in this work had acclimated to growth on complex carbohydrate-based media. However, it was able to utilize a number of other carbon sources as well when grown on Czapek's medium (Table 5). Coal solubilization was evident on two of the noncarbohydrate substrates tested, i.e., benzoate and vanillin (Table 6).

Table 5
Growth of *Paecilomyces* TLi on Various Carbon Substrates (Submerged Cultures)[a]

Carbon substrate	Growth	Final pH
Carbohydrates		
Maltose (control)	+ + + +	6.8
Glucose	+ + + +	6.8
Starch	+ + +	7.2
Noncarbohydrates		
Acetate	+ + +	8.7
Benzoate	+ +	7.2
Butyrate	+	7.2
Methanol	+	ND[b]
Phenol	±	6.9
Phthalate	+	7.1
Vanillin	±	6.7

[a]Czapek's medium containing the carbon source at 1% final concentration (shake flasks) was inoculated with 10^6 conidia/mL and incubated at 30°C and 50 rpm for 5 d. Growth is expressed on a relative basis, from + + + + (excellent) to ± (poor).
[b]ND = not determined.

Table 6
Noncarbohydrate Carbon Substrates Supporting Coal Solubilization by *Paecilomyces* TLi (Submerged Cultures)

		Final pH[c]	
Carbon substrate[a]	Peak absorbance[b]	Control	Experimental
Maltose (control)	0.35	7.1	3.2
Benzoate	0.80	5.8	3.7
Vanillin	0.12	5.6	3.1

[a]Czapek's medium containing the carbon source at 0.1% final concentration (shake flasks) was inoculated with 10^6 conidia/mL and spiked with 0.5 g of coal at the time of inoculation. Cultures were incubated at 30°C, 50 rpm, and harvested by centrifugation.
[b]Absorbance data reflect difference spectra over the 420–450 nm range for experimental cultures vs coal-only controls.
[c]pH was measured in control cultures containing cells only and in experimental cultures containing coal.

DISCUSSION

Development of defined liquid media for coal solubilization by *Paecilomyces* is a first step in the creation of a viable technology for coal biosolubilization. Benefits yielded by this effort include easier product recovery, decreased product contamination, and increased potential for scaleup (Fig. 3). Refinement and further validation of a rapid spectrophotometric assay for coal solubilization will aid in future technology development.

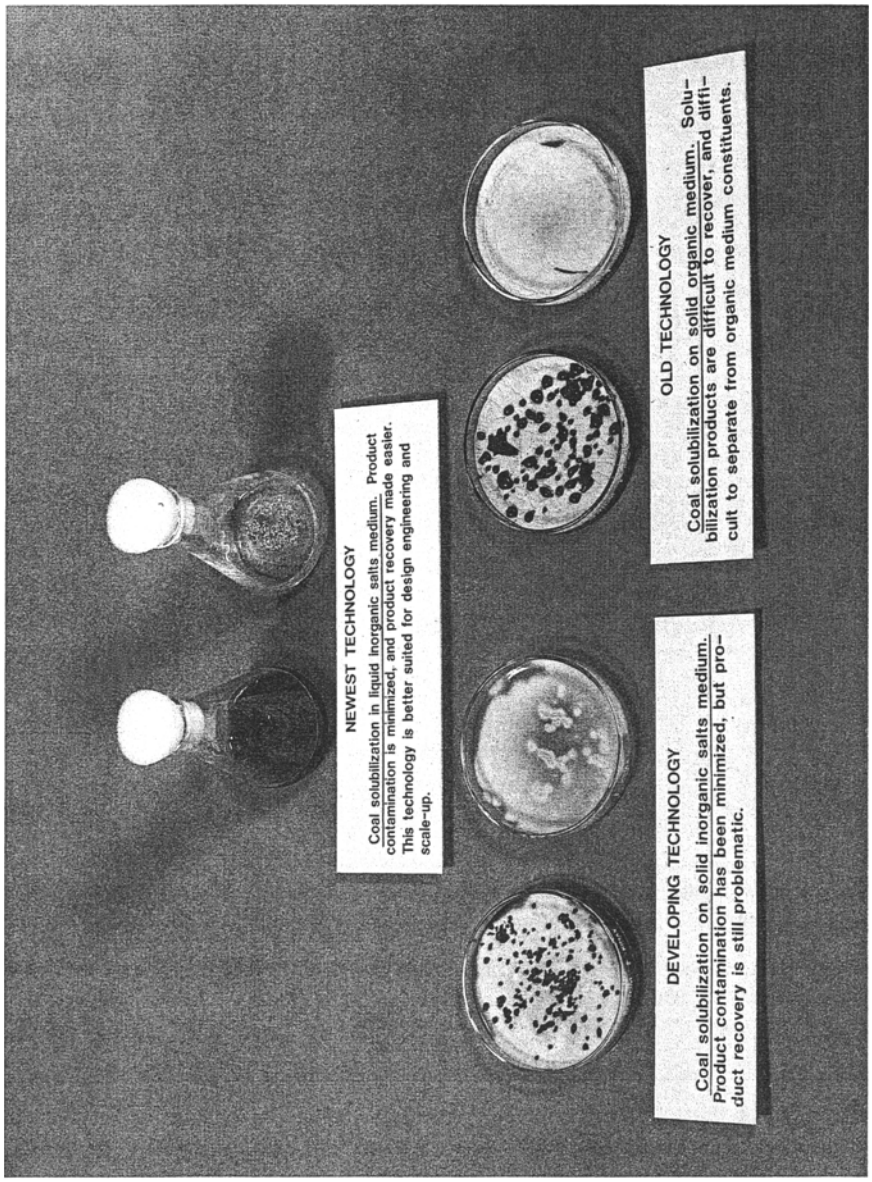

Fig. 3. Summary of technology development described in text.

Certain differences between surface and submerged cultures were evident. Submerged cultures were apparently less active toward coal than were surface cultures. Also coal solubilization in surface cultures was characteristic of nongrowing cells (*4*); in contrast, solubilization by submerged cultures coincided with the growth phase. The basis for differences between submerged and surface culture is unknown, and requires further study.

The noncarbohydrate growth substrates used in this work were chosen for their structural similarity to coal's presumed monomeric units, and, hence, its putative degradation products. Preliminary analysis of the liquid coal product indicated the presence of various oxidized, polar compounds, including organic acids and aldehydes (*4*). Coal's polyaromatic structure is hypothesized to yield aromatic acids and aldehydes, possibly including benzoate and vanillin, upon microbial degradation. These presumptive coal degradation products may be inducers of coal-solubilizing activity, much as some aromatic alcohols that are lignin degradation products induce ligninase (*9*). In any event, the ability to solubilize coal while utilizing key degradation products would make coal a suitable growth substrate for this organism.

The role of alkali in the coal biosolubilization is not entirely clear. Much of the activity reported previously was measured in alkaline or alkaligenic media (including Sabouraud maltose), and thus may reflect nonspecific nonbiological alkaline catalysis. Even in this work, the highest rates were measured in a defined alkaligenic (Czapek's) medium. The detection of activity in an acidogenic system (Minimal I) indicates that biological systems need not be alkaline *per se* in order to support coal biosolubilization. However, the chromophoric material produced within the acidic system had spectral qualities identical to those of the material produced by the action of alkali on coal. This finding suggests that coal solubilization by this organism is catalyzed by a specific alkaline catalyst of microbial origin. The detection and isolation of such an agent will be facilitated in defined liquid culture media.

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