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ORIGINAL PAPER

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Production of fungal antibiotics using polymeric solid supports in solid-state and liquid fermentation

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Abstract The use of inert absorbent polymeric supports for cellular attachment in solid-state fungal fermentation influenced growth, morphology, and production of bioactive secondary metabolites. Two filamentous fungi exemplified the utility of this approach to facilitate the discovery of new antimicrobial compounds. *Cylindrocarpon* sp. *LL*-Cyan426 produced pyrrocidines A and B and *Acremonium* sp. *LL*-Cyan416 produced acremonidins A–E when grown on agar bearing moist polyester–cellulose paper and generated distinctly different metabolite profiles than the conventional shaken or stationary liquid fermentations. Differences were also apparent when tenfold concentrated methanol extracts from these fermentations were tested against antibiotic-susceptible and antibiotic-resistant Gram-positive bacteria, and zones of inhibition were compared. Shaken broth cultures of *Acremonium* sp. or *Cylindrocarpon* sp. showed complex HPLC patterns, lower levels of target compounds, and high levels of unwanted compounds and medium components, while agar/solid support cultures showed significantly increased yields of pyrrocidines A and B and acremonidins A–E, respectively. This method, mixed-phase fermentation (fermentation with an inert solid support bearing liquid medium), exploited the increase in surface area available for fungal growth on the supports and the tendency of some microorganisms to adhere to solid surfaces, possibly mimicking their natural growth habits. The production of dimeric anthraquinones by *Penicillium* sp. *LL*-WF159 was investigated in liquid fermentation using various inert polymeric immobilization supports composed of polypropylene, polypropylene cellulose, polyester–cellulose, or polyurethane. This culture produced rugulosin, skyrin, flavomannin, and a new bisanthracene, WF159-A, after fermentation in the presence and absence of polymeric supports for mycelial attachment. The physical nature of the different support systems influenced culture morphology and relative metabolite yields, as determined by HPLC analysis and measurement of antimicrobial activity. The application of such immobilized-cell fermentation methods under solid and liquid conditions facilitated the discovery of new antibiotic compounds, and offers new approaches to fungal fermentation for natural product discovery.

Keywords Fermentation · Antibiotics · Natural products · Pyrrocidines · Acremonidins

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Introduction

New fermentation methods that influence the growth and metabolism of microorganisms enhance their value as sources of natural products and potential therapeutic compounds [20, 21]. Solid-state fermentation draws on traditional fungal fermentation technology [10–12], even ancient food fermentation methods [9], yet still offers opportunities for the development of new bioprocesses for natural products. Such alternative bioprocesses include the use of polymeric supports for mycelial attachment and growth during fermentation.

Both solid-substrate and solid-state fermentation [1, 6, 16, 30, 35, 41, 42, 46, 49–52, 55] employ a natural substrate as a carbon/energy source in the presence of little or no free water, but the broader designation, solid-state fermentation, includes the possible application of an inert substrate as a solid support in a suitable medium. Both approaches avoid free liquid and differ from liquid or submerged fermentations, which are performed in dilute solutions or slurries [4, 39, 53,

58]. Solid supports in solid-state fermentation may consist of natural materials, a variety of synthetic materials especially polymeric substances, or homogenous combinations of natural and synthetic substances, such as woven fibers, incubated in the presence of nutrients. Membrane filters used in microbiology are typically thin porous sheet structures made of cellulose esters or similar polymeric materials, and may be considered inert if they are resistant to degradation or are metabolized poorly [44]. Solid support systems applied to solid-state fermentation provide new ways to manipulate variables that influence growth and physiology [6], and, thus, to exploit fungal differentiation and developmental processes that are often linked to pathways of secondary metabolism that produce complex compounds [7, 15]. Similarly, solid supports applied to liquid fermentation permit the manipulation of these variables in new ways, as documented by an extensive literature on microbial cell immobilization by attachment, entrapment, aggregation, or containment and by a long history of its application to commercial processes [25, 26, 36, 47, 49, 54, 62, 66, 67, 73].

Solid-state fermentation has a record of successful application to the production of secondary metabolites, as the mycelial state is associated with production of many industrial compounds of this class *and* is well suited for growth on solid substrates [35]. For example, antibiotic production under such conditions is often associated with higher yields in shorter time periods compared to the alternative submerged fermentation approach, even offering processing advantages [55]. Solid-state systems, their definitions and advantages, and the physico-chemical and environmental factors that affect them have been recently reviewed by Krishna [35], as well as others [5, 33, 55, 59].

We are interested in alternate approaches to the production of bioactive microbial metabolites, and the rapid and efficient evaluation of fungi for secondary metabolite production. We have investigated variations of solid-state and liquid fermentation, as well as mixed-phase fermentation, a fermentation method with absorbent inert materials that bridges solid-state and liquid fermentation. Here we report the production of two antibiotic groups, pyrrocidines and acremonidins, by growing two fungi on an easily harvested solid support composed of polyester–cellulose fibers able to absorb a liquid nutrient medium and allow cellular attachment. Also, we report the production of four other antibiotics, two known anthraquinones, flavomannin, and a new bisanthracene, by growing another mycelial fungus in the presence of various solid supports agitated in liquid medium.

We have reported the chemical elucidation of the pyrrocidines [28] and acremonidins [29] in earlier work. Wicklow et al. [69] have reported the production of pyrrocidines by *Acremonium zeae* and have brought attention to the biocontrol potential of this organism for agricultural applications.

Materials and methods

Sources of fungal cultures

Fungal cultures *Cylindrocarpon* sp. *LL*-Cyan426 and *Acremonium* sp. *LL*-Cyan416 were obtained from the Wyeth collection. Both fungi are isolates from a mixed Douglas Fir-Hardwood forest, Crane Island Preserve, San Juan County, Washington State. *Penicillium* sp. *LL*-WF159 (subgenus *Furcatum*) was also obtained from the Wyeth Culture Collection.

Fermentation and processing of cultures

All fungal fermentations were performed at 22°C using Difco medium ingredients (Becton, Dickinson and Company, Franklin Lakes, NJ), except where indicated otherwise. D-glucose was obtained from Sigma-Aldrich, St. Louis, MO.

Fungal cultures *Cylindrocarpon* sp. *LL*-Cyan-426 or *Acremonium* sp. *LL*-Cyan-416 were plated on Bennett's agar medium (10 g/l D-glucose, 1 g/l beef extract, 1 g/l yeast extract, 2 g/l N–Z amine A, 20 g/l agar) from a frozen 25% glycerol storage vial. A small agar slice bearing mycelial growth was used to inoculate 50 ml of seed culture in potato dextrose broth (PDB) in a 250-ml Erlenmeyer flask shaken at 200 rpm for 1 week. Production medium (1 l) for use with a solid support of polyester–cellulose consisted of malt extract medium (ME) with agar (25 g malt extract, 5 g peptone, 0.5 g yeast extract, 20 g agar) that had been sterilized and poured into a sterile 30 × 20 × 13 cm polypropylene tray covered with aluminum foil. The solidified agar was then overlaid with a sterile 28 × 46 cm sheet of nongauze milk-filter paper (KenAG Animal Care Group, Ashland, OH) that had been sterilized separately. The production medium was inoculated by pipeting 50 ml of seed culture fluid onto the sheet of polyester–cellulose. The inoculated tray culture was incubated stationary at 22°C. Fungal mycelia were primarily associated with the polyester–cellulose fibers and this growth pattern facilitated rapid and convenient harvest of the biomass with minimum carryover of agar and associated medium components. After 2 weeks of incubation, the milk-filter paper bearing prolific mycelial growth was easily removed from the surface of the agar, lyophilized for 5 days, and then extracted with 1 l of methanol. Liquid medium fermentations were performed in 2.8-l Fernbach flasks containing 1 l of ME medium shaken for 2 weeks at 200 rpm.

The extraction of cultures of *Cylindrocarpon* sp. *LL*-Cyan-426 and *Acremonium* sp. *LL*-Cyan-416 was performed by agitation with Diaion HP20 resin (Mitsubishi Chemical Co., Tokyo, Japan) for at least 2 h. The resin and biomass were recovered by centrifugation, and this material was lyophilized and then extracted with methanol prior to analysis. Culture broth was also lyophilized

without resin treatment, extracted with methanol, and analyzed for comparison. The resin was found to be an efficient means of concentrating natural products with excellent recovery of metabolites and antimicrobial activity. When desired, the methanol extracts were concentrated tenfold with a Thermo Electron SpeedVac Concentrator (Thermo Electron Corp., Waltham, MA) equipped with a cold-trap and vacuum.

Seed cultures of *Penicillium* sp. *LL*-WF159 were prepared as indicated above. Experiments were performed in 50-ml Erlenmeyer flasks (Pyrex No. 4442) containing 15 ml of PDB shaken at 200 rpm, conditions that favored secondary metabolite production. Nonaerated, static cultures were not productive, nor were cultures grown in Czapek-Dox Broth or Sabouraud Maltose Broth. Initial experiments with solid supports employed 50-ml shake-flasks bearing cylinders of milk-filter paper (4.5 × 10 cm, KenAG) that had been creased gently along their length and then inserted with extension into the flask stem. The vertical cylinders absorbed PDB medium added afterwards and were able to withstand both autoclaving and agitation. In subsequent experiments, thin polymeric discs 4.2 cm in diameter composed of polyurethane (HT4201 foam wipe, Wilshire Technologies, Carlsbad, CA), polypropylene (Spectrawipe 6, Baxter Healthcare, Deerfield, IL or MicroFirst wipe, Berkshire/Dupont, Great Barrington, MA/Wilmington, DE), polypropylene cellulose (Fabwipe TX3009, Texwipe Co., Kernersville, NC), or polyester–cellulose (KenAG milk-filter paper or Surex805 wipe, Berkshire, Great Barrington, MA) were employed in shake-flasks containing PDB. Cultures were either shaken at 200 rpm for a given period or were first maintained static, then shaken at 200 rpm. Both protocols generated distinctly yellow-orange cultures. After an incubation period, the cultures were lyophilized, extracted with methanol, and the methanol extracts were concentrated tenfold with a Thermo Electron SpeedVac Concentrator. Since antimicrobial activity was primarily associated with fungal biomass, immobilized mycelia could, if desired, be selectively removed from shake-flasks, and thus easily separated from medium components for harvesting and extraction—or even reinoculation into fresh media under sterile conditions. None of the polymeric supports employed in these studies contained antimicrobial activity after methanol extraction of uninoculated material, tenfold concentration, and bioassay, nor were any prominent peaks apparent after HPLC analysis of such preparations.

Analytical procedures

HPLC analysis was performed using a C18 column (YMC Co., Kyoto, Japan), YMC ODS-A, 5 μm, 120 A, 4.6 × 150 mm, and employed a linear gradient: 20–100% acetonitrile in water in 23 min and 100% acetonitrile 1 min.

Measurement of antibacterial activity

The antimicrobial activities of tenfold concentrated fungal extracts were determined by the standard agar diffusion method using selected antibiotic-sensitive and antibiotic-resistant microorganisms [17]. A volume of 20 μl of extract was dispensed into wells of an agar assay plate and the zones of inhibition were measured after 16–18 h of incubation at 37°C using a handheld digital caliper. The test microorganisms included methicillin-susceptible *Staphylococcus aureus* SA375, methicillin-resistant *S. aureus* SA310 (MR), and vancomycin-resistant *Enterococcus faecium* (VR).

Results

Production of pyrrocidines A and B by *Cylindrocarpon* sp. *LL*-Cyan426

Cylindrocarpon sp. *LL*-Cyan426 was reported earlier to produce the antibiotics pyrrocidines A and B (Fig. 1), natural products containing rare 13-membered macrocycles [28]. Solid-state fermentation with a moistened polyester-cellulosic support residing on agar, i.e., mixed-phase fermentation, was indispensable to the discovery of these compounds. The two pyrrocidines were detectable under these special growth conditions in a malt extract medium, but not in a liquid version of this medium.

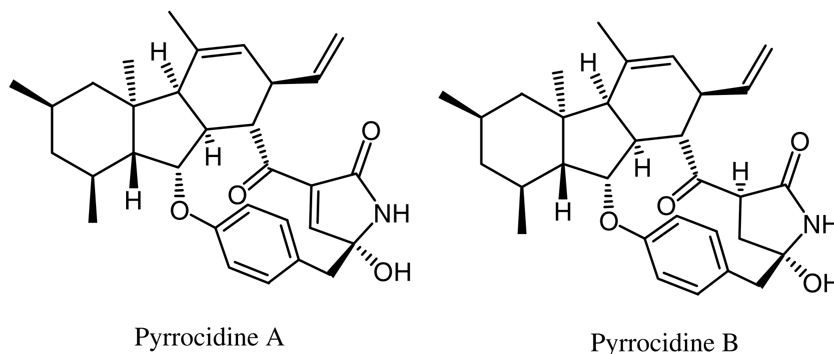

Fig. 1 Structures of pyrrocidines A and B

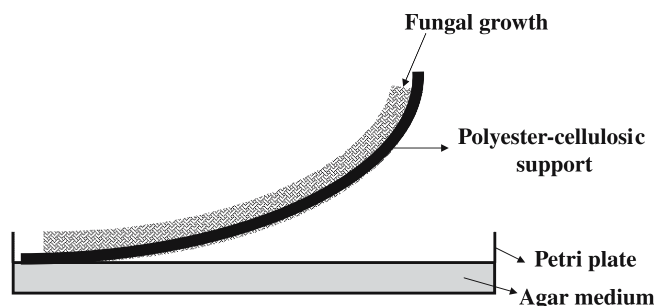

Fig. 2 Fungal growth on polyester-cellulosic support on agar surface

Cylindrocarpon sp. *LL*-Cyan426 was grown on ME agar medium bearing a polyester–cellulose support, as illustrated in Fig. 2. The fungus was also grown in liquid ME medium by conventional shake-flask fermentation. Production of pyrrocidines A and B under these two conditions was compared by HPLC analysis after extraction and concentration. In addition, antimicrobial activities associated with tenfold concentrated methanol extracts from such fermentations were monitored by the agar diffusion method using antibiotic-resistant and antibiotic-susceptible test organisms. The production of antimicrobial activity was favored by growth of strain *LL*-Cyan426 on an inert support able to absorb water and nutrients from agar medium, (Table 1). Extracts of cultures with the solid support generated larger zones of growth inhibition against strains of *S. aureus* and *E. faecium* than did comparable concentrated extracts of conventional shake-flask cultures. A concentrated (10 ×) extract of residual ME agar (post-harvest) contained only traces of antimicrobial activity. Stationary cultures in liquid ME medium also showed trace or no activity. Concentrated extracts of lyophilized whole agar cultures from petri plates bearing a lawn of growth produced small, hazy zones of antimicrobial activity.

HPLC analysis of tenfold concentrated methanol extracts revealed differences between the metabolite profiles of 2-week fungal cultures grown on agar medium under mixed-phase conditions and cultures grown by conventional liquid fermentation. Pyrrocidines A and B were produced in measurable amounts only under conditions of mixed-phase fermentation. HPLC analysis of methanol extracts of *Cylindrocarpon* sp. *LL*-Cyan426 immobilized on the solid support resolved pyrrocidine A as peak 23.9 and pyrrocidine B as a shoulder of this peak, as shown in Fig. 3a. The yield of pyrrocidine A was 21.8 mg/l, and the yield of pyrrocidine B was 3.1 mg/l. *Cylindrocarpon* sp. *LL*-Cyan426 grown in liquid culture did not produce pyrrocidine A, the more potent antibiotic of the two species, and pyrrocidine B was present at the threshold of detection, as shown in Fig. 3b. *Cylindrocarpon* sp. *LL*-Cyan426 also produced a number of illicicolins (designated “I” in Fig. 3a, b), known fungal metabolites with antibiotic activity, under both growth conditions.

The levels of polar material, consisting mainly of residual medium components, were significantly reduced in extracts of fungal growth harvested on solid supports from the agar surface, and this characteristic greatly facilitated purification of the two antibiotics. Extracts of liquid cultures contained significantly more components from the malt extract medium as represented by the early peaks in the HPLC profile of Fig. 3b (retention times of 0–15 min).

Pyrrocidines A and B have been purified and chemically characterized after fermentation by these methods and then tested against selected microorganisms [28]. Pyrrocidine A exhibited potent antibiotic activity against most Gram-positive bacteria, including drug-resistant strains, but showed only moderate activity against *S. pneumoniae*. Pyrrodicine B was less active against these test organisms.

Table 1 Effect of growth conditions on the production of antimicrobial activity by *Cylindrocarpon* sp. *LL*-Cyan426

Fermentation conditions	Antibacterial activities[a] (zone of inhibition in mm)		
	S. aureus	*S. aureus* (MR)	*E. faecium* (VR)
Solid support from ME agar	22 mm	19 mm	16 mm
Residual ME agar after removal of solid support	0	0	0
ME broth shaken	9	12	10
ME broth stationary	0	0	0

MR methicillin-resistant, *VR* vancomycin-resistant
[a]Activity determined by the standard agar diffusion method, data for 20 μl/well of the 10 × extract

Production of acremonidins A–E by *Acremonium* sp. *LL*-Cyan416

Acremonium sp. *LL*-Cyan416 was reported in earlier studies to produce the antibiotics acremonidins A–E, all natural products of polyketide origin [29]. Solid-state fermentation with a polyester-cellulosic support on ME agar was important in the discovery of these compounds since these unique growth conditions significantly elevated yields. The chemical structures of these five natural products are shown in Fig. 4.

As was the strain of *Cylindrocarpon* sp., *Acremonium* sp. *LL*-Cyan416 was grown both on ME agar medium bearing a polyester–cellulose support (Fig. 2) and by conventional shake-flask fermentation in liquid ME medium. The production of the five acremonidins was compared by HPLC after extraction and concentration. In addition, antimicrobial activities associated with tenfold concentrated methanol extracts from such fermentations were monitored by the agar diffusion method using antibiotic-resistant and antibiotic-susceptible test organisms. The production of antimicrobial activity by strain *LL*-Cyan426 was favored by mixed-phase fermentation on moistened fibrous sheets.

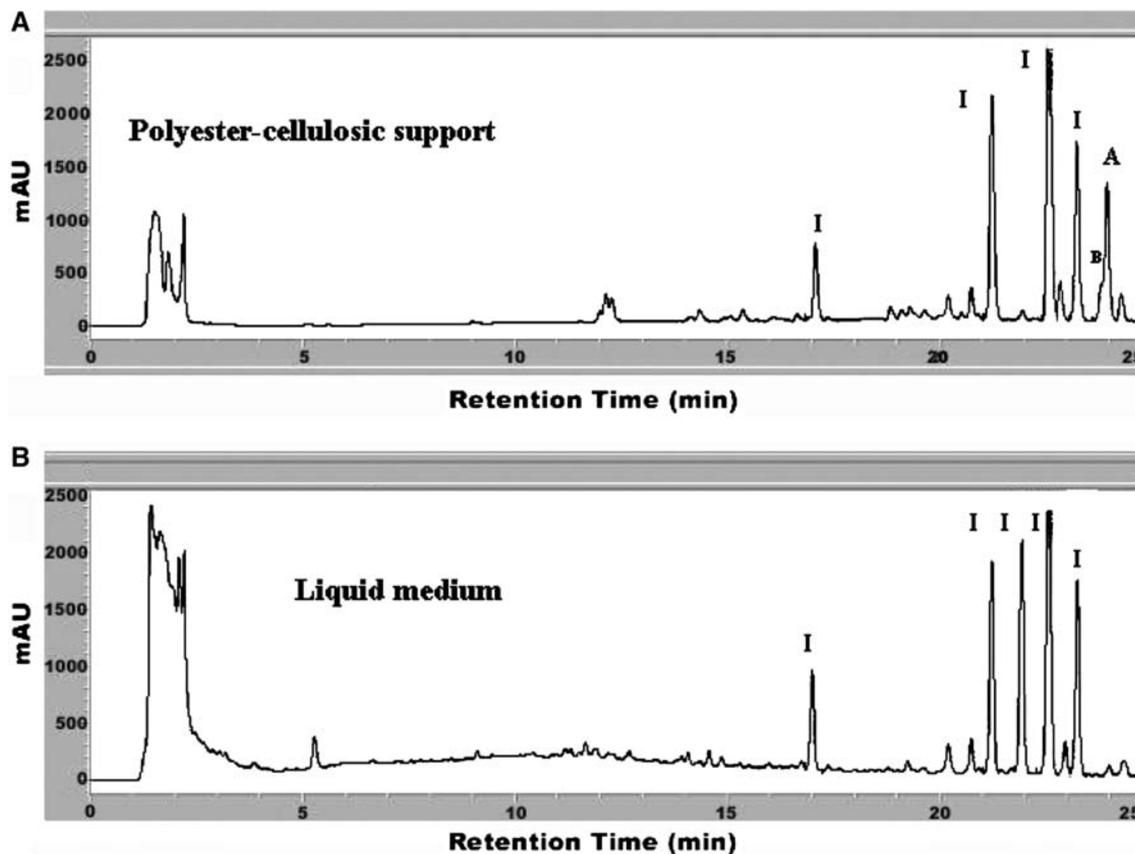

Fig. 3 **a**, **b** HPLC analysis of extracts of *Cylindrocarpon* sp. *LL*-Cyan426 showing peaks corresponding to pyrrocidines *A*, *B*, and various illicicolins (*I*)

Preparations from both types of fermentations possessed antimicrobial activity against methicillin-resistant *S. aureus* SA310, methicillin-susceptible *S. aureus* SA375, and vancomycin-resistant *E. faecium* EC379. However, extracts of fungal biomass harvested on the polyester–cellulose support were more active and generated larger zones of growth inhibition, as shown in Table 2. An extract of residual ME agar (post-harvest) showed low levels of antimicrobial activity. Concentrated extracts of lyophilized whole agar cultures from petri plates bearing a lawn of growth revealed smaller zones of antimicrobial activity, than did extracts of cultures grown on polyester–cellulose supports. Stationary cultures in liquid ME medium showed no activity.

HPLC analysis of tenfold concentrated methanol extracts revealed differences between 2-week cultures of *Acremonium* sp. *LL*-Cyan416 grown under mixed-phase conditions (Fig. 5a) and those by conventional liquid fermentation (Fig. 5b). HPLC analysis of extracts of mixed-phase fermentations revealed acremonidins A–E (peaks 19.014, 15.261, 16.378, 13.347, and 12.576, respectively). The yields of these antibiotics were: A 130 mg/l, B 4.5 mg/l, C 4.2 mg/l, D 3.1 mg/l, and E 21 mg/l. Liquid fermentation produced significantly lower levels of acremonidins A, B, D, and E and lacked acremonidin C completely, as indicated in the HPLC chromatograms shown in Fig. 5b employing a threefold expanded mAU scale for the liquid fermentation. Both types of fermentation extracts also contained known quinones (designated "Q" in Fig. 5).

Acremonidins A–E were purified from the methanol extracts of *Acremonium* sp. *LL*-Cyan416 grown by both fermentation methods. Extracts of liquid fermentations generated significantly more polar material (Fig. 5b), mostly medium components that were eluted early during HPLC (retention times of 0–10 min), whereas extracts obtained after mixed-phase fermentation had significantly lower levels of such components (Fig. 5a).

Production of anthraquinones and flavomannin by *Penicillium* sp. *LL*-WF159 in liquid fermentation

Initial experiments in liquid fermentation examined the influence of a polyester–cellulose support (KenAG milk-filter paper) on secondary metabolism with *Penicillium* sp. *LL*-WF159 according to two different shake-flask protocols (Table 3). In the first procedure, the organism was preincubated without agitation for 3 days, promoting mycelial attachment to a moistened cylinder and immobilization, and then shaken for 4 days. In the second procedure, a parallel fermentation lacking such a polyester–cellulose support was

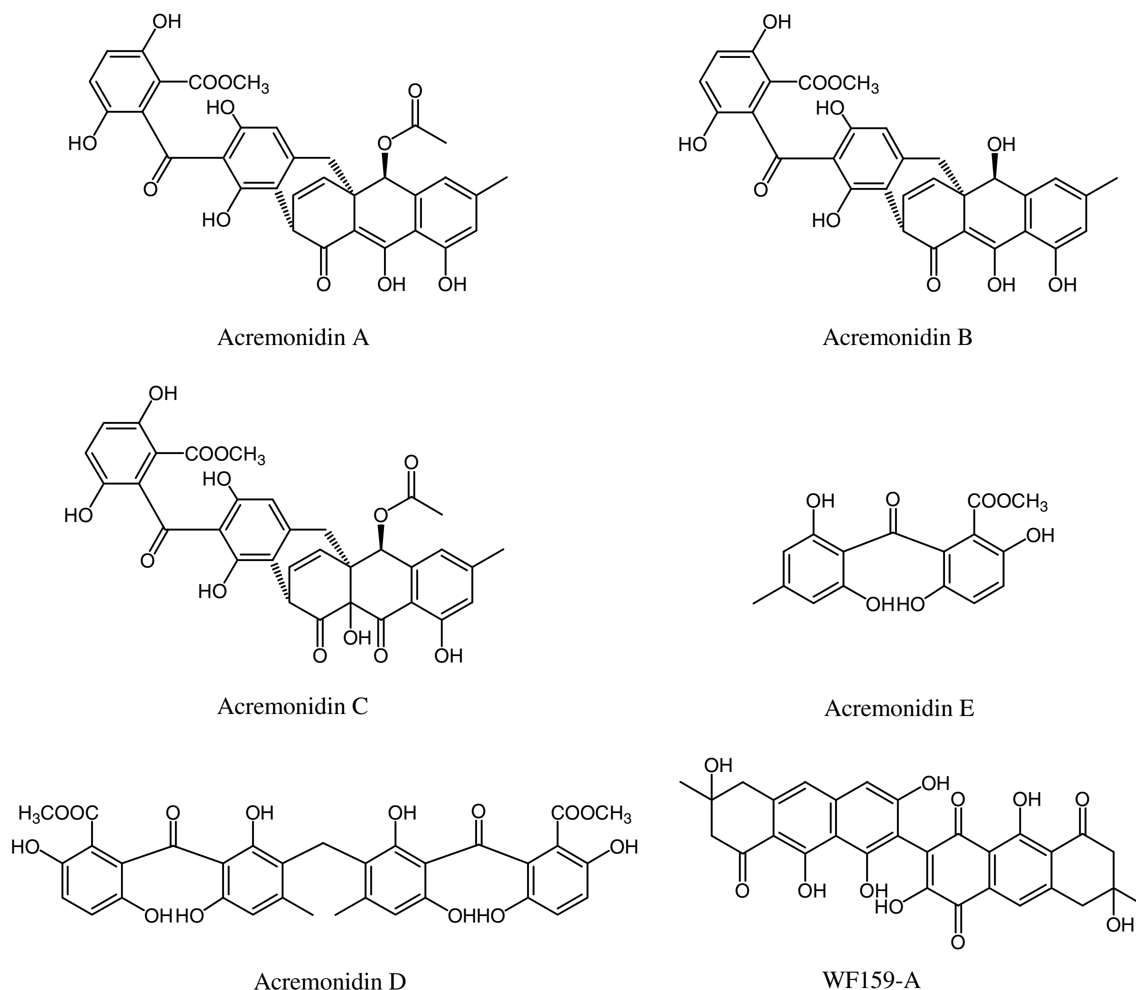

Fig. 4 Structures of acremonidins A–E and compound WF159-A

agitated without interruption for 7 days. After 7 days of fermentation, all of the mycelial growth was associated with the cellulosic cylinder and the culture fluid was clear, while conventional fermentation broth contained dispersed mycelia. All fermentation broths (including supports when present) were lyophilized, extracted with methanol, concentrated tenfold, and then analyzed for antimicrobial activity. Extracts of culture *LL*-WF159 grown in the presence of a polyester–cellulose support were more active in antimicrobial assays (Table 3). The zones of inhibition against methicillin-resistant *S. aureus* SA310, methicillin-susceptible *S. aureus* SA375, and vancomycin-resistant *E. faecium* EC379 were larger than zones obtained with standard shaken cultures (without discs). Mycelial adhesion of *Penicillium* sp. *LL*-WF159 to the solid support had an apparent effect on the production of bioactive compounds.

Shake-flask fermentations of *Penicillium* sp. *LL*-WF159 in PDB were extracted with methanol, analyzed, and subjected to bioassay-guided fractionation. All fermentation broths were typically a deep yellow. Four aromatic polyketides were isolated, and a spectroscopic analysis led to the determination of the structures of these compounds. *Penicillium* sp. *LL*-WF159 was found to produce rugulosin and skyrin [18], two anthraquinones known to possess antimicrobial activity, as well as flavomannin and new bisanthracene WF159-A (Fig. 4), in fermentations with and without a polyester–cellulose support. Purified preparations of all four compounds

Table 2 Effect of growth conditions on the production of antimicrobial activity by *Acremonium* sp. *LL*-Cyan416

Fermentation conditions	Antibacterial activities[a] (zone of inhibition in mm)		
	S. aureus	*S. aureus* (MR)	*E. faecium* (VR)
Solid support from ME agar	17 mm	17 mm	14 mm
Residual agar after removal of solid support	7	0	0
ME broth shaken	12	12	11
ME broth stationary	0	0	0

MR methicillin-resistant, *VR* vancomycin-resistant

[a]Activity determined by the standard agar diffusion method, data for 20 µl/well of the 10 × extract

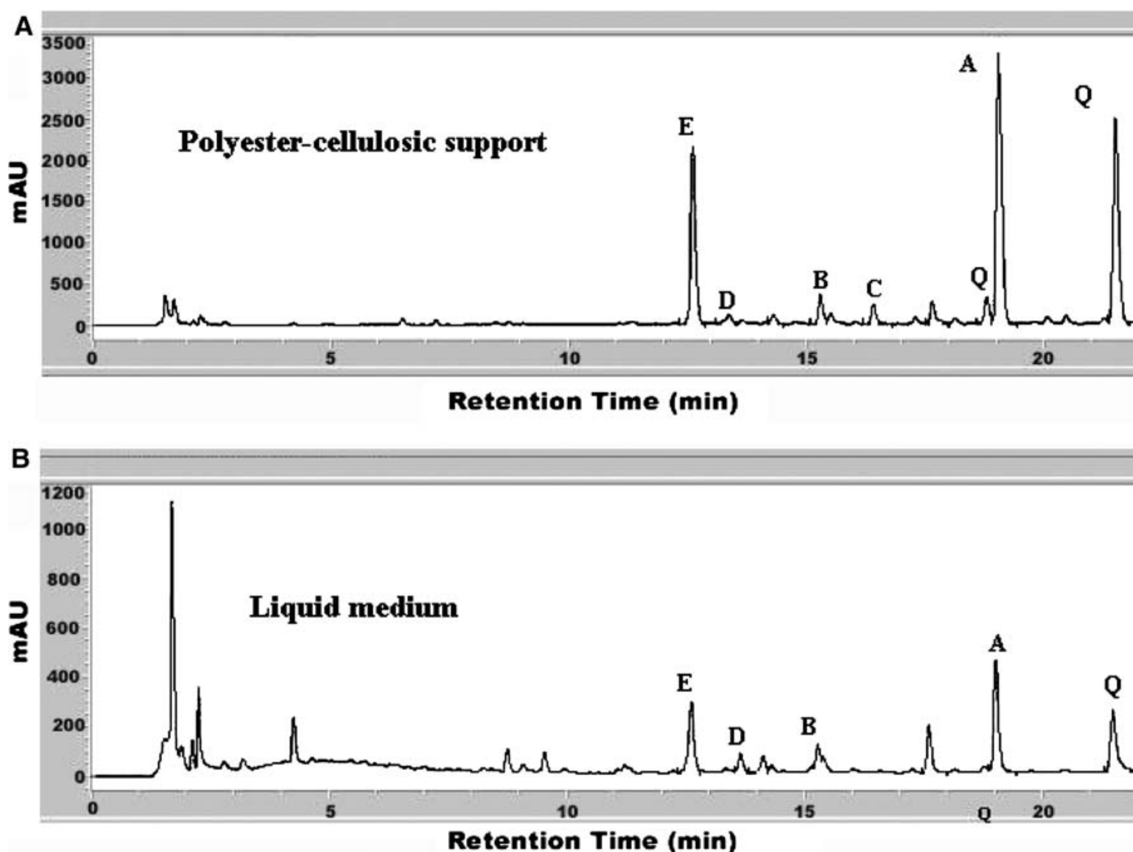

Fig. 5 **a**, **b** HPLC analysis of extracts of *Acremonium* sp. *LL*-Cyan416 showing peaks corresponding to acremonidins *A–E*, and also several quinones (*Q*)

inhibited at least one of three Mur enzymes, (UDP-*N*-acetylglucosamine enolpyruvyl transferase), MurB (UDP-*N*-acetylenolpyruvoyl glucosamine reductase), or MurC (UDP-*N*-acetylmuramate: L-alanine ligase), three cytoplasmic steps involved in the biosynthesis of peptidoglycan precursor [23].

The growth of strain *LL*-WF159 was then investigated in shake-flasks containing a polymeric disc that was free to agitate in the growth vessel. The organism was grown in PDB with and without discs composed of polyurethane, polypropylene (two types), polypropylene cellulose, or polyester–cellulose. Two different fermentation protocols were employed, and the relative yields of the four antibiotics were determined. All shake-flasks were either agitated for 2 weeks, or they were maintained static for 1 week followed by a period of shaking for 1 week. The support matrices, all of which permitted mycelial attachment and growth, not only influenced culture morphology and allowed cell immobilization (Table 4), but also elevated relative metabolite yields as determined by HPLC analysis (Table 5). Production of all four compounds in fermentations without an added support was enhanced by a static preincubation period of 1 week that allowed a mycelial mat to form on the medium surface, followed by another weeklong period of agitation. The thin white mycelial aggregate was still intact after 2 weeks, though the medium was turbid with some dispersed growth. The levels of the four antibiotics could be further elevated by the addition of a polymeric disc and the formation of an ‘‘artificial mycelial mat’’ that was free to agitate in the growth vessel (Fig. 6). The physical nature of the support system influenced the relative yields of the two bisanthraquinones and flavomannin to varying degrees (Table 5). In general, the relative yields were increased, though in a few cases, the polymeric disc lowered metabolite production owing to possible interference with mixing and aeration.

Table 3 Effect of mycelial adhesion of *Penicillium* sp. *LL*-WF159 to a polyester–cellulose support on antimicrobial zones of inhibition

Fermentation conditions	Antibacterial activities[a] (zone of inhibition in mm)		
	S. aureus	*S. aureus* (MR)	*E. faecium* (VR)
PDB shaken 7 days	12:15RC mm	9:16RC mm	8H mm
PDB with support, static 3 days, shaken 4 days	15	16	10

MR methicillin-resistant, *VR* vancomycin-resistant, *RC* zone with resistant colonies, *H* hazy zone
[a]Activity determined by the standard agar diffusion method using 10 μl/well of 10 × extract

Table 4 Culture characteristics of *Penicillium* sp. *LL*-WF159 grown in liquid medium with different polymeric discs

Disc composition[a]	Disc source	Culture appearance[b]
No added disc	None	Turbid culture fluid
Polyurethane	Wilshire	Fungal biomass attached to support
Polypropylene 1	Baxter	Fungal biomass attached to support
Polypropylene 2	Berkshire/Dupont	Fungal biomass attached to support
Polypropylene cellulose	Texwipe	Fungal biomass attached to support
Polyester–cellulose	Berkshire	Fungal biomass attached to support

[a]Discs were 4.2 cm in diameter and resided on the bottom of 50-ml Erlenmeyer shake-flasks containing 15-ml PDB
[b]All cultures were either shaken at 200 rpm at 22°C for 2 weeks or were maintained static for 1 week then shaken for 1 week at 200 rpm at 22°C. All cultures were shades of yellow-orange after 2 weeks

Table 5 Relative yields of four metabolites in shaken and stationary/shaken fermentations with *Penicillium* sp. *LL*-WF159

Disc added to PDB	Flavomannin		Compound WF159-A		Rugulosin		Skyrin	
	Shake[a]	Stat/shake[b]	Shake[a]	Stat/shake[b]	Shake[a]	Stat/shake[b]	Shake[a]	Stat/shake[b]
PDB only	418	4,205	184	460	1,955	4,586	245	1,068
Polyurethane	3,784	9,384	779	758	8,784	6,778	984	1,246
Polypropylene 1	140	9,049	1,167	1,048	5,918	7,578	942	1,759
Polypropylene 2	89	9,683	606	889	6,287	7,563	1,448	1,352
Polypropylene cellulose	316	6,197	1,003	758	5,407	7,852	829	1,573
Polyester–cellulose	157	4,585	778	533	4,020	6,329	397	1,037

All productive cultures were dark yellow to yellow-orange revealing colors characteristic of anthraquinones. Static 2-week cultures contained pale white, nonpigmented mycelia
[a]Shaken 14 days at 200 rpm
[b]Static 7 days, then shaken 7 days at 200 rpm

Considering the two different fermentation protocols (Table 5), flavomannin production was significantly enhanced by stationary preincubation that permitted mycelial mat formation on the surface of the medium or a comparable period of growth during which the mold infiltrated the supplemented disc. For example, the production of flavomannin was enhanced tenfold by static preincubation of the mycelial mat before agitation, and even more when a disc was employed for mycelial immobilization. The extent of the enhancement was related to the makeup of the disc. A polyurethane disc employed in a 14-day static/shaken fermentation was especially effective in elevating flavomannin production, and the level of this compound was 22-fold greater than that attained after a standard 14-day fermentation lacking any supplement. The 14-day shaken fermentation with disc elevated flavomannin levels ninefold. The two fermentation protocols incorporating a polymeric support typically resulted in similar relative yields of rugulosin, skyrin, and the new bisanthracene compound. However, both types of fermentations (shaken or static/shaken) that incorporated a disc support consistently outperformed standard shake-flask fermentations that lacked any polymeric disc, resulting in higher relative yields of these three compounds (Table 5).

Morphological and physiological changes have been observed with a number of other mycelial fungi grown by shake-flask fermentation with a PUF support in our laboratory. Mycelial attachment to the support is common in such agitated cultures, and changes in developmental patterns and secondary metabolism, pigment production, for example, are sometimes evident (R. Bigelis and H. He, unpublished observations).

Discussion

Fungi are a remarkably diverse group of microorganisms that include an estimated 1.5 million species [27, 34]. The manipulation of fungal growth conditions in new ways magnifies their chemical diversity and increases their potential as sources of useful secondary metabolites, and possibly new therapeutic compounds [20–22]. Alternative approaches to fungal growth and secondary metabolism in the fermentation laboratory complement bioprospecting for natural products with unique microbial isolates from unexplored or extreme environments [14]. While the developmental or ecological role fungal secondary metabolites may be complex and poorly understood [15, 22], the regulatory networks that modulate natural product production can be manipulated during fermentation to generate new antimicrobial compounds. As reported here, alternative approaches using solid supports in solid-state or liquid fermentation facilitated the discovery of two new classes of natural products, pyrrocidines and acremonidins [28, 29], as well as a new bisanthracene, by revealing new compounds, elevating their yields, and simplifying harvesting/processing steps. Thus, fermentation under solid conditions with a support bearing liquid medium, and

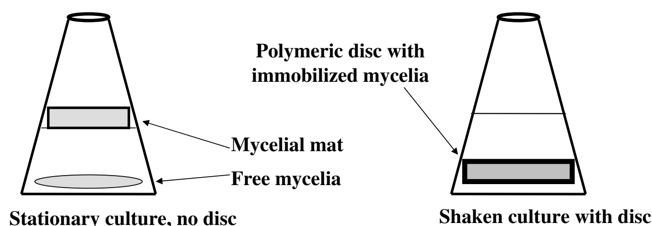

Fig. 6 Growth of *Penicillium* sp. *LL*-WF159 as a mycelial aggregate in stationary and shaken flasks

fermentation in liquid medium bearing solid supports with immobilized mycelia enhanced the discovery process for new natural products. The solid fermentation approach reflected growth conditions more commonly encountered in nature, typically environments populated by terrestrial fungi. These growth conditions are discussed below together with applications to natural products discovery.

The culturing of fungi on static supports in abundant liquid medium, i.e., mixed-phase fermentation with solid and liquid environments, exploits various environmental stimuli that influence metabolism, morphogenesis, and differentiation—and consequently natural product profiles. The physicochemical characteristics of the support system and its interaction with available water, oxygen, and medium components are primary variables that can influence growth and metabolism under these conditions. Various other factors, some subtle, may also play a role in mediating the response of the colonizing organisms to the support system. These factors include water activity, water absorbency and retention, availability of the gaseous phase in interstitial spaces and aeration, porosity, accessible surface area, surface topographies and architecture, mediators of adhesion and anchorage (such as adhesins as agents in biofilm formation), and air-interface-induced development and aerial stimulation, as well as nutrient and waste product absorbance and diffusion [26, 52, 60, 61, 65, 70]. Some characteristics of the support system may change significantly during growth stages of the culture and may even generate gradients of environmental conditions that produce nutrient limitations simulating the variability found in nature. The support environment itself may also reflect subtle characteristics of natural fungal habitats, ranging from inorganic native locales to living hosts. Some supports such as cellulosic or synthetic papers, cellulosic pulps, fabrics, membranes, plastic foams, sponges, porous inorganic particulates, and especially agricultural products may closely mimic natural substrates. Such conditions may elicit growth habits related to proliferation, cellular attachment, and differentiation.

Fungal interactions with native solid substrates are known to influence growth and physiology. These interactions may initiate a cascade of events that influence differentiation. Artificial membranes and etched surfaces have been used as tools to mimic leaf topography and induce the development of fungal infection structures in vitro [24, 31, 32, 40, 71]. Obligate plant pathogens possess a unique and precise capability for topographical perception and thigmotropic response to plant microarchitecture [31, 64, 72]. For example, the differentiation program of the rust fungus *Uromyces appendiculatus* is responsive to leaf surface topography of the bean *Phaseolus vulgaris*. The ability of *U. appendiculatus* to infect the host plant and mobilize a developmental sequence involving gene expression, mitosis, and specific morphological adaptation is triggered by such surface signals [31, 72]. The topographical signal can orient growth, promote infection structure formation, and induce cell differentiation along with nuclear division [2, 3, 31]. The first infection structure forms directly over the stomata, through which it penetrates and forms other structures. *U. appendiculatus* is even responsive to the microfabricated ridges on silicon wafers or plastic membrane replicas of the leaf surface that simulate stomatal guard cells [3, 31]. This organism's exquisite sensitivity to substrate elevation is remarkable: the induction of appressorium formation occurs at ridge heights of 0.5 μm, while those less than 0.25 μm or greater than 1.0 μm are significantly less inductive.

Though the mechanisms of surface recognition that can trigger fungal development are poorly understood, the hydrophobicity of the substratum is known to be an important variable in the differentiation processes of some fungi [24, 45]. Some pathogenic fungi undergo specific developmental processes that are influenced by such interactions with surfaces. The firm attachment of plant pathogenic fungi to plant surfaces is believed to be essential for prepenetration development and successful infection, followed by processes of differentiation. The formation of the appressorium by the rice blast fungus *Magnaporthe grisea* is primarily determined by the hydrophobicity of the contact surface [38]. Similarly, *U. appendiculatus* spore and germling adhesion and induction of appressoria are closely related to the degree of hydrophobicity of substrata [63]. A role for hydrophobins, hydrophobic proteins located on the cell surface, has been proposed as mediators of these types of responses to surfaces [60, 61, 68]. Other fungi, however, can attach to a variety of surfaces, including artificial surfaces, and their behavior does not reveal a linkage between adhesion and surface hydrophobicity. Surface properties such as charge, texture, and hardness may play a role in these cases. The corn leaf pathogen *Cochliobolus heterostrophus* is an example of a plant pathogenic fungus that is insensitive to surface hydrophobicity and differs from *M. grisea*, *U. appendiculatus*, *Botrytis cinerea*, *Colletotrichum* sp., *Candida albicans*, *Nomuraea rileyi*, *Metarhizium anisopliae* and a number of other fungi that have been shown to adhere with greater tenacity to more hydrophobic surfaces [13, 63]. Moreover, fungal surface hydrophobicity can vary among species, as well as within one species, depending on the age of the fungus and the composition of the growth medium [57].

Environmental stimuli associated with solid-state fermentation using polymeric materials may create advantages over conventional liquid fermentation for production of certain fungal metabolites, especially when such stimuli favor cellular differentiation that occurs in the natural state. Investigations with lichen fungi by Culberson and Armaleo [19] parallel findings reported here with *Cylindrocarpon* sp. *LL*-Cyan426 and *Acremonium* sp. *LL*-Cyan416. Their experiments with the mycosymbiont *Cladonia grayi* suggest that the characteristic of secondary metabolism of natural lichen is linked to their aerial habit of growth. Quantitative analysis of polyketide metabolite yield coupled with culturing on nylon microfilters [48] placed on agar medium revealed that secondary metabolism could be induced under restrictive conditions. Disaggregated fungal mycelia were seeded by suction on nylon filters forming a mat before placement on solid medium. Filters bearing the attached fungal mat were removed at set time points, harvested, and analyzed for the production of a depside and two depsidones found in the natural lichen. The three natural products appeared a few days after transfer of mycelia from liquid to solid medium. Induction of the pathway was enhanced on drier substrates, and was correlated with growth of aerial mycelia. Decreased water activity was critical for this lichen mycobiont pathway, along with exposure to air. Specialized growth conditions are important for the study of this group of organisms, and their requirements provide insights into factors that may mediate secondary metabolism in other classes of fungi.

The relationship between morphological differentiation, such as sporulation, and secondary metabolism suggests independent processes regulated by a common mechanism [8, 22]. Most solid-state fermentation protocols are associated with environmental conditions suitable for sporulation or conidiation [49], cellular development programs that can influence secondary metabolism and thus natural product profiles [4]. And, solid-state fermentations involving a polymeric support system are suitable for the induction of such fungal differentiation patterns. The sporulation exhibited by diverse filamentous fungi, especially species of *Penicillium* and *Aspergillus*, is stimulated primarily by of exposure of hyphae to air. Additional stimuli associated with solid substrates and added supports, such as surface effects, dessication, osmotic stress, and nutrient limitation, also influence developmental processes able to alter growth and physiology, as well as the production of natural products [6, 24, 37, 43, 56, 73].

We have described the production of antimicrobial metabolites by three fungi in media containing different inert solid supports composed of polymeric materials. These approaches not only improved yields of some polyketide natural products, but also facilitated the experimental process from fermentation to extraction to analysis, raising the question of other applications. It is conceivable that such immobilized-cell fermentation methods could be extended to miniaturized formats with small, absorbent fibrous discs amenable to rapid automated screening. Alternative methods of this nature could simplify and even condense some of the multiple steps necessary for microbial natural product discovery.

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