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ORIGINAL PAPER

Fermentative production of self-toxic fungal secondary metabolites

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Abstract Fungi are well known for their vast diversity of secondary metabolites that include many life-saving drugs and highly toxic mycotoxins. In general, fungal cultures producing such metabolites are immune to their toxic effects. However, some are known to produce self-toxic compounds that can pose production optimization challenges if the metabolites are needed in large amounts for chemical modification. One such culture, LV-2841, was identified as the lead for one of our exploratory projects. This culture was found to be a slow grower that produced trace amounts of a known metabolite, cercosporamide, under the standard flask fermentation conditions, and extensive medium optimization studies failed to yield higher titers. Poor growth of the culture in liquid media was attributed to the self-toxicity of cercosporamide to the producing organism, and the minimum inhibitory concentration (MIC) of cercosporamide was estimated to be in the range of 8–16 μg/ml. Fermentations carried out in media containing Diaion® HP20 resin afforded significantly higher titers of the desired compound. While several examples of resin-based fermentations of soil streptomyces have been published, this approach has rarely been used for fungal fermentations. Over a 100-fold increase in the production titer of cercosporamide, a self-toxic secondary metabolite, was achieved by supplementing the production medium with a commercially available neutral adsorbent resin.

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Introduction

A vast majority of microbial secondary metabolites are produced by submerged fermentation, and many of them are self-toxic compounds affecting the growth and productivity of the producing organism [1]. Fermentations are generally performed in media containing an adsorbent resin to mitigate the self-toxicity of the product to the producer and to increase stability of the products excreted during fermentation. Many of the soil streptomyces producing highly toxic secondary metabolites have been fermented in media containing various adsorbent resins [2–13], but this approach has rarely been applied to improve secondary metabolite production in fungal fermentations [14–16]. Adsorbent resins are widely used for solid-phase extraction, isolation and purification of a wide variety of pharmaceutical products [17, 18].

Self-toxic secondary metabolites pose tremendous production optimization challenges when large amounts of natural products are needed for chemical modification. In our high-throughput screening of the natural products library samples, one such fungal culture, LV-2841, was identified as the producer of a lead compound for an exploratory project. LV-2841 was found to be a slow grower that produced trace amounts of the target metabolite, cercosporamide (Figs. 1, 2), under standard fermentation conditions. Cercosporamide was first reported as a phytotoxin produced by *Cercosporidium henningsii*, the causative agent of the brown spot disease of cassava, and it was found to have potent anticandida and antifungal activity [19, 20]. More than a decade later, cercosporamide

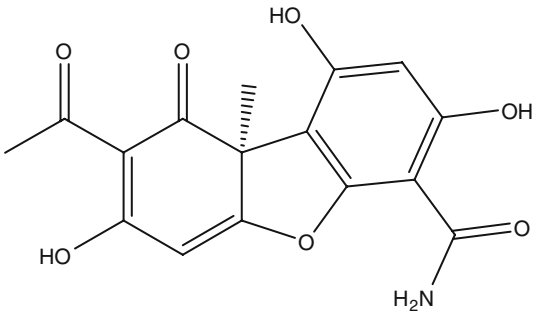


Fig. 1 Chemical structure of cercosporamide

was identified as a potent Pkc1 inhibitor through a *Candida albicans* Pkc1-based high-throughput screening assay [21]. Recently, cercosporamide has also been manufactured with other producing microorganisms such as *Lachnum fuscescens* and *Pesudaegerita websteri* [22], and cercosporamide analogs having hypoglycemic activity were prepared [23]. Cercosporamide, originally reported as a potent antifungal agent because of its selective affinity for the fungal Pkc1, appears to have additional therapeutic potentials in its new semisynthetic analogs.

In subsequent fermentations LV-2841 was found to have poor growth and only produced the desired metabolite in very low concentrations. Extensive medium optimization studies employing a variety of medium ingredients and other fermentation conditions (aeration and incubation temperature) failed to yield higher titers. In order to find suitable carbon and nitrogen substrates for the culture and determine their effects on secondary metabolism, we used Biolog FF MicroPlates with 95 different nutritional substrates for culture growth and production of the target secondary metabolite [24, 25]. The target compound, cercosporamide, was later found to be self-toxic to the producing organism, and in order to achieve higher titers fermentations required the presence of a suitable adsorbent resin such as Diaion® HP20 or Amberlite™ XAD-7 in the production medium. The cercosporamide titer varied from flask to flask and batch to batch, possibly due to multiple factors such as age of the culture, culture variants, media components, inoculum densities, maceration time, size of mycelia fragments, sampling error, and many other physico-chemical fermentation parameters. Each of these factors has been the subject of further investigation and will be published elsewhere. In this paper we report the effects of synthetic adsorbent resins and various carbon and nitrogen sources on the production of cercosporamide by LV-2841. A portion of this work was presented at the Annual Meeting of the Society for Industrial Microbiology held in Toronto, Canada [24, 25].

Materials and methods

Culture storage and maintenance

LV-2841, an unidentified fungal culture from our natural products collection, was stored as frozen samples in 25% glycerol at −80°C, streaked onto Difco™ potato dextrose agar (PDA) plates and incubated at 22°C for 3–4 weeks for revival and seed preparation. Difco™ dehydrated Potato Dextrose Broth medium (PDB, catalog no. 254920) was purchased from Becton–Dickinson and Company, Sparks, MD, and the medium was prepared as per the vendor's instructions.

Processing of adsorbent resin

Diaion® HP20 styrene–divinylbenzene (Supelco, catalog no. 13607) and Amberlite™ XAD7HP acrylic ester resins (Sigma–Aldrich, catalog no. XAD7) were purchased from

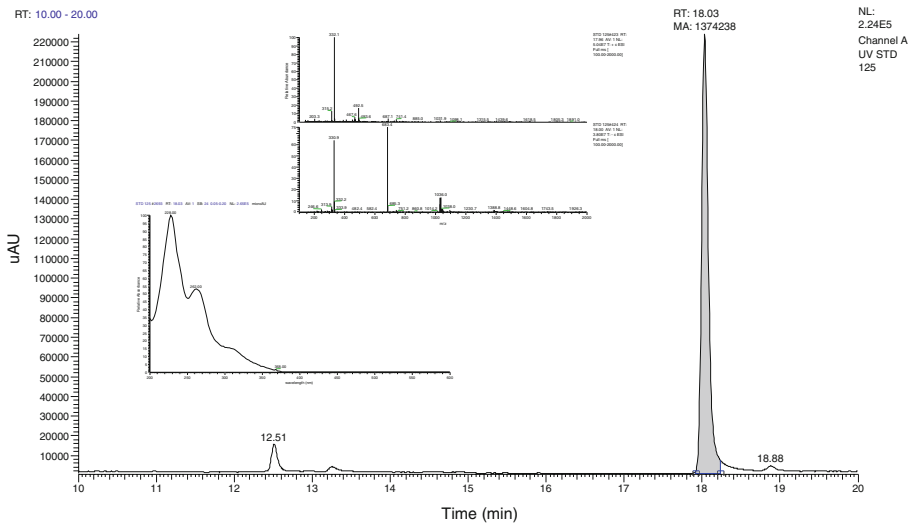


Fig. 2 LCMS and UV chromatograms of cercosporamide (125 mg/l) showing characteristic UV, mass, and retention time for the target secondary metabolite cercosporamide produced by culture LV-2841

the manufacturer. For activation of these adsorbent resins dry granules were immersed in twice the volume of methanol and stirred for an hour. Excess methanol was decanted, and the wetted resin was washed once with methanol and five to six times with DI water. Following the DI water washes, activated resin was immersed in fresh DI water and stored at 4°C until needed. Slurry of the activated resin was filtered onto a coffee filter just before use, and the weighed amounts of wet resin were added to each flask containing liquid medium.

Preparation of seed inoculum

The culture was scraped from one 4-week-old large petri plate with confluent growth and placed into a small grinder (pre-sterilized); 50 ml of PDB was added, and the culture was macerated gently using 3–4 5-s pulses. The resulting culture suspension was used to inoculate two Fernbach flasks, each containing 1 l of production medium. For multiple fermentation flasks, the culture scraped from multiple plates was pooled and macerated in a larger food processor. Excessive maceration was avoided as it tended to reduce viability and productivity of the culture. Seed culture was streaked onto a PDA plate to check its purity.

Fermentative production

Difco™ potato dextrose broth with 3% w/v wet HP20 was used for fermentative production of cercosporamide. Culture LV-2841 was fermented in 100 µl, 50 ml, 100 ml or 1 l scales in microtiter plates, and 250, 500 ml or 2,800 ml Erlenmeyer flasks, respectively, at 22°C and 200 rpm for 4–5 weeks under humidified conditions. Ten-liter fermentations were conducted in a New Brunswick BioFlo 3000 fermentor with 12.2 l of PDB with 3% HP20 autoclaved at 121°C for 60 min. Seed inoculum was prepared by macerating culture growth from ten large petri plates in total volume of 1.2 l PDB. Agitation was controlled at 200 rpm using a six-blade Ruston impeller and a temperature of 22°C. Initial airflow was set at 5.0 l/min. At 185 h, airflow was lowered to 3.0 l/min to minimize foaming. During the course of fermentation pH was not controlled, and the pH ranged from 5.02 (initial) to 4.74 (final at harvest).

Extraction and titer estimation

Five milliliters of the whole broth from each flask was collected into a 15-ml polypropylene tube and centrifuged at 3,000 rpm for 10 min. The supernatant was poured off, and 5 ml of ethyl acetate was added to the tube containing the pellet (cells + resin). Extraction was performed by vortexing vigorously for 30 min followed by centrifugation, a portion (100 µl) of the extract was transferred to a small microfuge tube, chilled at −80°C for 30 min, and the solvent was evaporated under vacuum for 30 min using a Speedvac concentrator (Savant Instruments, Holbrook, NY). Dried extract was dissolved into 100 µl of DMSO and analyzed using a Hewlett-Packard model HP1100 liquid chromatograph with tandem photodiode array and mass spectral detection with either a Finnigan LCQ ion trap mass spectrometer or LCQ DECA with an ESI probe (ThermoQuest, River Oak Parkway, San Jose, CA).

HPLC conditions

Column, YMC ODS-A, 3 µm 120 Å 2 × 10 mm; solvent, 5–95% gradient mixture of water and acetonitrile containing 0.025% of formic acid in 15 min; flow rate, 0.3 ml/min; injection volume of 2–5 µl.

Results and discussion

Our natural products library containing crude and fractionated extracts of a vast diversity of fungal cultures is routinely screened against various targeted assays. Generally, only a small percentage of fungal cultures meet the strict criteria of a lead-producing culture, and some of them pose unique challenges in their cultivation/preservation, fermentation and batch-to-batch productivity.

Fungal culture LV-2841, identified as the source of a lead for one of our exploratory projects, grew very poorly in both liquid and solid fungal media, and produced only trace amounts of the targeted secondary metabolite in Difco™ potato dextrose broth (PDB). The target compound produced by the culture was identified as a known fungal secondary metabolite, cercosporamide (Fig. 1), that has been shown to have potent antifungal and fungal Pkc1 inhibitory activities [16–19]. A preliminary taxonomical investigation of LV-2841 appeared to suggest this is a new producer of cercosporamide, so we initiated additional studies to improve the production titers in various fermentation scales ranging from 100 µl to 10 l.

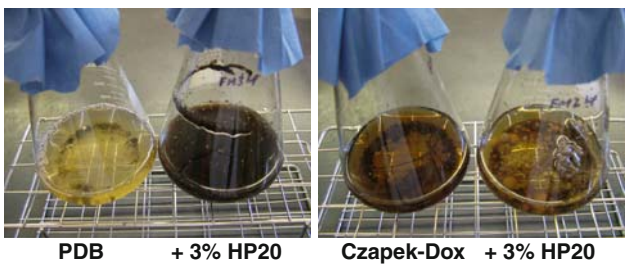


Fig. 3 Growth of culture LV-2841 producing self-toxic secondary metabolite, cercosporamide, in the DIFCO potato dextrose broth and Czapek-Dox broth unsupplemented/supplemented with 3% Diaion® HP-20 styrene–divinylbenzene adsorbent resin

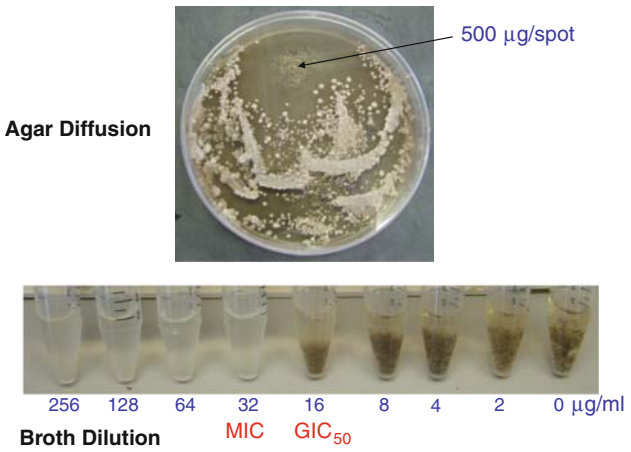


Fig. 4 Self-toxicity of cercosporamide demonstrated by the agar diffusion and broth dilution susceptibility testing methods using LV-2841 as the test organism

Culture LV-2841, plated onto potato dextrose agar (PDA), grew extremely slowly by turning into white velvety non-spreading colonies, and a dark olive color pigment was produced in 4–6 weeks. The culture was found to grow slightly better in the Czapek-Dox broth (3% saccharose, 1% dibasic potassium phosphate, 0.05% magnesium sulphate, 0.1% potassium chloride and 0.001% ferrous sulphate), but the target compound, cercosporamide, was not produced in appreciable amounts in this medium. Concentrations greater than 1–2% of ammonium sulphate or sodium nitrate completely suppressed the production of the target compound. Many other production media containing various carbon sources (20 different common sugars) and nitrogen sources were tried, but appreciable production of the target compound was seen only in the DIFCO brand PDB containing an adsorbent resin after 3–4 weeks of fermentation. Minor differences in the composition of the commercially available PDB and minor batch-to-batch variation in the same brand of PDB also affected the productivity of the culture. For example, production of cercosporamide was very poor in the Acumedia® PDB (Neogen® Corp., Lansing, Michigan) that contained potato infusion instead of the potato starch found in the DIFCO PDB. Solid media containing ground rice, ground corn, soy flour or ground Fiber-One as a base did not afford the desired product. Variations in aeration (shaking speed of 200 vs. 250 rpm) and incubation temperature of 28 and 35°C did not afford any advantage over the standard fermentation conditions conducted at 200 rpm and 22°C.

Various concentrations of adsorbent resins, ranging from 0.50 to 10% wet weight by volume of the production medium, were investigated for culture growth and cercosporamide production titer in fermentation broth. Even the smallest amount of an adsorbent resin was found to promote more growth relative to medium containing no resin (Fig. 3). Later we confirmed the self-toxicity of cercosporamide on the producing culture by testing the growth inhibitory activity of the compound against the culture using agar diffusion and broth dilution methods (Fig. 4). The minimum inhibitory concentration (MIC) of the metabolite was estimated to be in the range of 8–16 µg/ml. We concluded that in the presence of HP20 cercosporamide was efficiently sequestered by the resin, thereby mitigating its self-toxic effect during the course of the fermentation. Adsorbent resins have been in use for some

Table 1 Adsorbent resin-based fermentations reported in the literature

Product	Organism	Resin used in production medium	Titer enhancement	Reference no.
Paulomycin	*Streptomyces paulus*	HP20	Fivefold increase	2
Rubradirin A	*Streptomvces achromogenes v. rubradiris* (UC®8051)	HP20	Fivefold increase	3
Esperamicin A1	*Acinomadura verrucosospora*	1% HP20	53% higher titer	4
Dynemicin	*Micromonospora chersina* ATCC 53710	HP20 or XAD8	4.7–6.9 fold increase	5
Leinamycin	*Streptomyces* sp. S-140	5% HP20	7–32 mg/l	6
Clecarmycins	*Streptomyces* sp. DO-114	5% HP20	Increased titer to 70 mg/l	7
Lymphostin	*Streptomyces* sp. KY11783	10% HP20	Increased titer to 100 mg/l	8
Lomaiviticins	*Micromonospora lomaiviriensis*	HP20	Higher titer	9
Teicoplanin	*Actiniplanes teicomyceticus*	5% HP20 or XAD-16	4.2 fold increase	10
Pristinamycin	*Streptomyces pristinaespiralis*	12% JD-1 resin	0.4–1.3 g/l	11
6-Deoxyerythronolide B	*Streptomyces coelicolor*	HP20 or XAD8	Higher titer	12
Prodigiosin-like pigment	*Serratia* sp. KH-95	7.5% HP20	13 g/l (twofold higher)	13
Trichothecene	*Myrothecium verrucaria* CL-72	HP20	75–100% increase	14
Spiroxins	*Unidentified fungus*	HP20	35 fold increase	15
Benzaldehyde	*Pycnoporus cinnabarinus*	Resin in media	100–790 mg/l	16

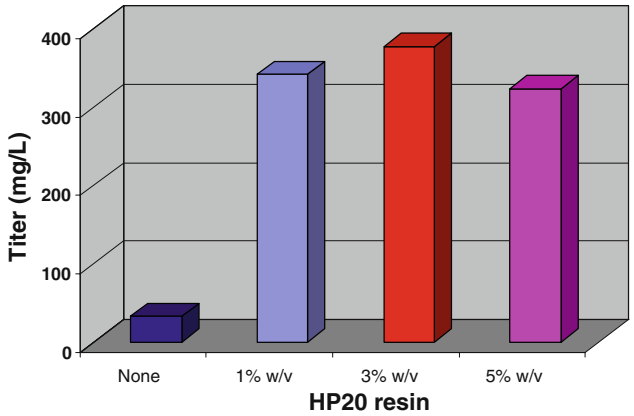


Fig. 5 Cercosporamide (mg/l) production titer in PDB and PDB containing varied amounts of activated wet HP20 resin

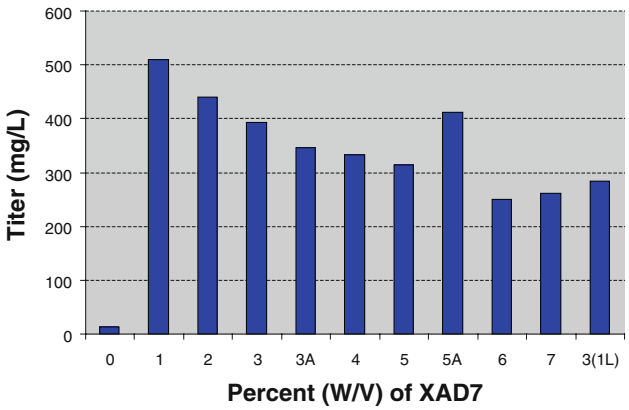


Fig. 7 Effects of various amounts (0–7 g per 100 ml PDB medium) of XAD-7 resin on the production titer of the desired secondary metabolite. Flasks 3A and 5A contained 3 and 5% w/v resin, respectively, but resin suspension in water and medium were autoclaved separately and combined just before seed inoculation. Flask 3(1L) contained 3% w/v resin in 1 l medium per Fernbach flask

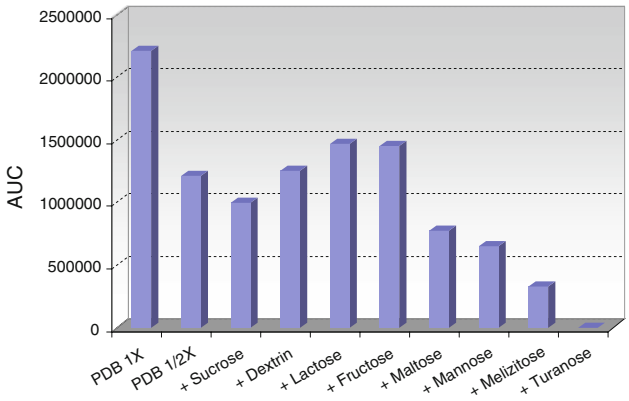
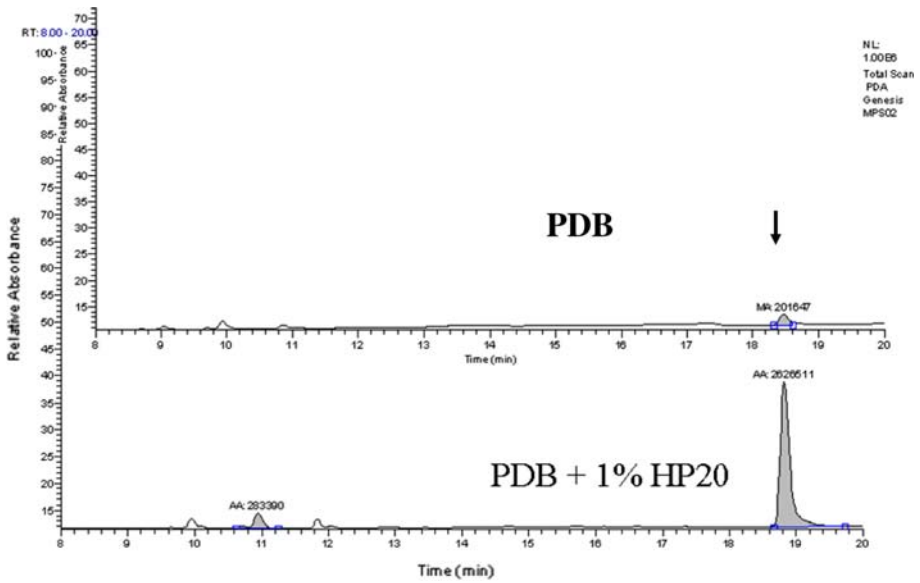


Fig. 8 Effect of various sugars (10% w/v) on cercosporamide production in 1/2X PDB with 3% HP20 (data average of duplicate flask containing 100 ml medium)

time to mitigate self-toxicity of metabolites produced by many soil bacteria (Table 1), and this technique is also known to increase stability of products during fermentations [2–13], but this approach has rarely been used for fungal fermentations [14–16].

Extensive medium optimization studies failed to identify a suitable medium for production of the desired secondary metabolite by culture LV-2841. The culture was found to produce 25–100-fold higher titers of the target compound, cercosporamide, in only DIFCO brand PDB supplemented with 1 to 5% w/v wet HP20 (Fig. 5). Of the various resins tried, Diaion® HP20 (Figs. 5, 6) and Amberlite® XAD7 (Fig. 7) appeared to give the best results when 1–3% of wet resin was used, and higher amounts of resin did not provide any advantage. We also tested a panel of 95 different substrates (C and N sources) using the FF-MicroPlates purchased from Biolog and identified some of the preferred carbon sources for LV-2841. However, these C-sources

Fig. 6 HPLC chromatogram of extracts showing target peak (indicated by an *arrow*) in fermentations done in PDB and PDB containing 1% W/V of HP20 resin



supplemented into 1/2X PDB did not afford higher titers of cercosporamide compared to the standard Difco PDB (Fig. 8). Large-scale substrate/nutritional profiling did lead to the identification of preferred substrates, but preferred substrate alone does not appear to be significant for secondary metabolism of culture LV-2841. Additional studies may be required to find suitable medium components and conditions to enhance the growth rate and the secondary metabolism pathways of the culture so that it will produce higher titers of cercosporamide. Despite the challenges encountered with the scaleup fermentations of this culture, we achieved consistent titers of 200–600 mg/l in PDB with 3% HP20, and were able to supply ample material needed for the exploratory project.

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