Chapter 5

The "FERMEX" Method for Metabolite-Enriched Fungal Extracts

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Abstract

The FERMEX (*Ferm*entation *Ex*tract) program was a highly successful source of microbial natural product molecules for pharmaceutical lead discovery. The program was based on the observation that solid fermentations of fungi generally exhibited more complex metabolite profiles than when the same strains were grown on liquid medium. To produce interference-free fermentations and improve organic product recovery, fungi colonized homogeneous media-saturated vermiculite thus promoting cellular and metabolic differentiation. Secondary metabolites in fungal cells were extracted from the substratum and medium with methyethylketone to generate metabolite-enriched screening samples. The necessary equipment, protocol, and media recipes are described along with examples of bioactive fungal metabolites produced in this system.

Key words: Fungi, Secondary metabolites, Solid-state fermentation, Drug discovery

1. Introduction

1.1. The FERMEX Method

The FERMEX (*Ferm*entation *Ex*tract) program was one of Merck Research Laboratories (MRL) most successful sources of natural product lead molecules when measured in terms of its manpower input per successful chemistry isolation project and its ability to consistently obtain and reproduce novel compounds. FERMEX operated at MRL in Rahway, New Jersey during the late 1980s and throughout the 1990s and produced fungal fermentations generated exclusively in solid media for primary screening in assays at Rahway, New Jersey and West Point, Pennsylvania. The program was based on the observation that solid fermentations of fungi generally exhibited more complex

Nancy P. Keller and Geoffrey Turner (eds.), *Fungal Secondary Metabolism: Methods and Protocols*,
Methods in Molecular Biology, vol. 944, DOI 10.1007/978-1-62703-122-6_5,

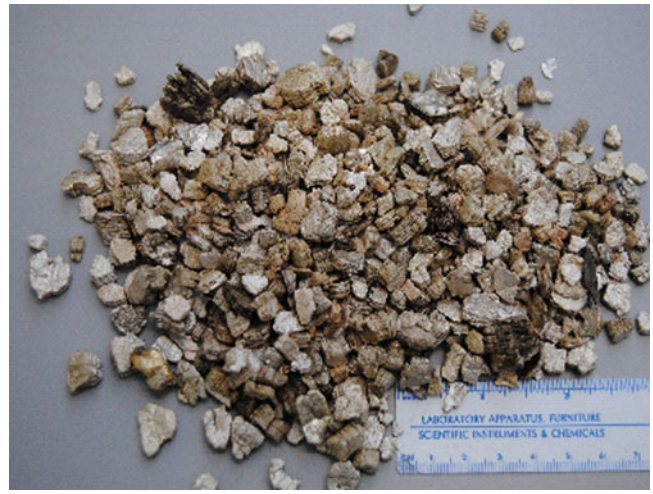

Fig. 1. The FERMEX method. Coarse particle vermiculite.

metabolite profiles than when the same strains were grown on liquid medium. Intuitively this makes sense because fungi are terrestrial organisms that penetrate, hydrolyze, and consume their substrata. However, a common problem associated with fungi grown on solid media, like agar, rice, and corn, was that often large amounts of solid material and plant metabolites would be extracted along with the fungal metabolites, diluting potency, complicating purification of fungal metabolites, and sometimes causing assay interference.

To produce "clean" solid-state fermentations (SSFs) and improve product recovery, natural matrices (rice, corn, wheat) were replaced by vermiculite (Fig. 1), an inert expanded clay mineral. The vermiculite was saturated with a defined or semidefined liquid nutrient medium to provide nutrients. To accelerate the penetration of the fungus and ensure its thorough colonization of the vermiculite, the medium–vermiculite mixture was incubated in glass tissue culture roller bottles that were rotated on cell culture roller bottle machines (Fig. 2). When the liquid to vermiculite ratio is optimized, the vermiculite forms a moist layer around the inside of the bottles. As the bottle rolls, excess liquid medium is converted to fungal biomass that penetrates and binds together the vermiculate particles (Figs. 3, 4, and 5). The fungus forms a solid matrix around a large interior surface open to the air in the bottle's central chamber stimulating sporulation (for those fungi that sporulate); a high degree of cellular differentiation is achieved corresponding to differential expression of secondary metabolite pathways within the same culture. The hyphal mass produced in roller bottle SSFs is easily broken apart and extracted to produce

Fig. 2. The FERMEX method. Roller bottles (2 l) on roller bottle machine. Note cellulose closures fastened in place with gauze and rubber bands.

Fig. 3. Autoclaved vermiculite bottle (1 l) before inoculation and growth of *Emericella parvathecia* (14 days, 22°C).

Fig. 4. The FERMEX method. Mycelium and vermiculite matrix formed by *Trichoderma virens* on roller bottle wall.

Fig. 5. The FERMEX method. Bottom view of roller bottles showing hollow central chamber. *Emericella navahoensis* (*left*) and *E. parvathecia* (*right*).

about 200–500 ml of organic extract from which small amounts are aliquoted into screening plates and distributed to assays; the rest is banked as a backup for initial analysis of biologically active components.

Below we describe how the FERMEX method was implemented to support MRL drug discovery programs and the necessary equipment, protocols, and media recipes. We list some examples of newly discovered fungal metabolites or known fungal metabolites that were prepared for various biology and chemistry programs at MRL.

1.2. History and Implementation

By the mid-1970s, the use of microbial fermentations in the discovery of bioactive natural products at Merck Research Laboratories and other pharmaceutical companies had focused primarily on antiparasitic, antibacterial, and antifungal targets assaying aqueous extracts or whole broth samples. These techniques were quite successful and remain perfectly valid to this day. With the advent of laboratory automation and high throughput mode-of-action assays, e.g., enzyme inhibition, receptor binding, and whole cell functional measures addressing disease states at the molecular level, traditional types of fermentation samples were explored in these more "delicate" systems. As was anticipated, and indeed borne out with experimentation, the raw microbial samples used for assay would often be too crude and resulted in false positives and uninterpretable results caused by salts, proteins, and spent media components.

Therefore, a fermentation sample preparation method was sought that circumvented these problems. It was decided to use the methods on fermentations of carefully selected microorganisms to ensure distinctive chemistry, taking into consideration that the high throughput of that period in the 1980s was on the order of 20–40 microorganisms per week rather than the screening of hundreds of microbes in the more classical programs.

Early successes were striking: mevinolin and dihydromevinolin, the first HMG CoA reductase inhibitors and which spawned the first generation of statins for the management of hypercholesterolemia, appeared literally in the first month of this new "Fermentation Products for Screening" program (1); shortly later came asperlicin, the first non-peptide antagonist to a peptide hormone discovered intentionally (2, 3).

This combined approach of preselecting distinct microorganisms and growing them to produce a cleaner organic solvent extract compatible with biochemical assays eventually evolved, as described in the following sections, into the FERMEX program, one of the most successful sources of natural product lead molecules at Merck Research Laboratories.

1.3. Biological and Chemical Basis of FERMEX Method

The advantages and benefits of using SSF for producing fungal biomass, fungal spores, enzymes, and pigments are well known. SSF processes are equally important in transforming solid materials (grains, milk products) into foodstuffs or decolorizing, bleaching, or detoxifying complex polymeric materials. SSF has been emphasized as a low technology method for converting biological and agricultural waste into high value chemical products. The design of SSF fermentors and their applications and the advantages of immobilizing cells in inert support materials for SSF have been reviewed extensively (4–6). The FERMEX method is based on a simple rotating drum bioreactor design using standard animal

cell culture roller bottles and rolling machines (Fig. 2, Bellco, Vineland, NJ, USA). Unlike SSF on natural materials, composition of production media could be carefully controlled and reproduced because all the media components were known and could be analyzed.

The advantages of SSF for fungal secondary metabolite production are intuitive. Many mycotoxins are produced in solid substrata, including aflatoxin, ochratoxin, and fumonisins. The use of SSF for antibiotic production traces its roots all the way back to some processes implemented during the development of penicillins and can be used at the production scale (4, 7), but as a discovery tool in the pharmaceutical industry, the idea never gained much traction. Likely, the reasons were needs to develop economically viable large-scale processes in stirred tank fermentors where extraction and purification of active ingredients could be carried out with liquid processes. Results from the FERMEX method started emerging in the early 1990s (Table 1). Subsequently, other research groups independently devised similar SSF methods with inert carriers, e.g., light expanded clay aggregates (8) and membrane immobilized mycelia (9), and have reaffirmed the profound effects of solid phase growth on secondary metabolite expression relative to liquid cultures. The genetic basis for differential expression is only now starting to be understood. Transcriptional analysis of *Aspergillus oryzae* demonstrated that SSF significantly upregulated the ratios of expression of genes from non-syntenic blocks relative to those from syntenic blocks (10). These non-syntenic blocks are enriched in genes for hydrolytic enzymes and secondary metabolism. Characteristics of gluconeogenesis in SSF were observed in the presence of a nutrient-rich medium, and it was proposed that lower expression of genes in the glycolytic and tricarboxylic pathway might avoid catabolite repression (11).

Early efforts explored a variety of alternatives to find the most suitable processing protocol for this new screening program. A first approach was to proceed via adsorption of whole broth onto reverse-phase resins, e.g. Amberlite XAD-2, followed by washing and solvent elution of screenable mixtures. A second period saw the rather extreme extraction with only ethyl acetate. These methods proved to be either too cumbersome for any reasonable weekly throughput or too incomplete in extractive efficiency. Further experimentation included extraction with *n*-butanol for liquid fermentations, methanol or various solvent mixtures for solid substrate fermentations. Based on its effectiveness at extracting a wide range of polarities from both liquids and solids, its convenience of use and reasonable cost, methyethylketone (MEK) eventually became the standard extraction solvent for the FERMEX program.

Table 1
Some significant fungal secondary metabolites produced by the FERMEX method

Metabolite(s)	Fungus	Application(s)	Reference(s)
Thysanone	*Thysanophora penicilloides*	Human rhinovirus 3C-protease inhibitor	(22)
Variculanol	*Emericella variecolor*	Toxicity to *Eimeria tenella*	(23)
Ophioblin K and 6-epiophiobolin K	*Aspergillus ustus*	Toxicity to *Caenorhabditis elegans*	(24)
L-696,474 (18-dehydroxy cytochalasin H)	*Hypoxylon fragiforme*	HIV-1 protease inhibitor	(18, 25–27)
3-(Dimethylaminomethyl)-1-(1,1-dimethyl-2-propenyl)indole	*Penicillium daleae*	5-HT_{1D} receptor agonists	(28, 29)
Calbistrin A	*Aspergillus versicolor*	Broad spectrum antifungal, increased human LDL receptors	(30, 31)
Maxikdiol	Unidentified coelomycete MF5717	Agonist of maxi-K (large-conductance calcium-activated potassium) channels	(32)
Apicidins	*Fusarium* spp.	Broad spectrum antiparasitic agents, histone deacetylase inhibitors	(33–35)
Ascosteroside	*Mycoleptodiscus atromaculans*	Antifungal, β-1,3-glucan synthase inhibitor	(36)
Sporandol	*Chrysosporium merdarium*	Insecticide and antiparasitic agent	(37, 38)
Equisetin	*Fusarium heterosporum*	HIV-1 integrase inhibitor	(39, 40)
Phomasetin	*Phoma* sp.	HIV-1 integrase inhibitor	(39, 40)
Cytosporic acid	*Cytospora* sp.	HIV-1 integrase inhibitor	(41)
Nodulisporic acids	*Nodulisporium* sp.	Insecticide, invertebrate-specific glutamate-gated chloride ion channels	(42–44)
Hyalodendrosides A and B	*Hyalodendron* sp.	Antifungal, β-1,3-glucan synthase inhibitor	(45)

(continued)

Table 1
(continued)

Metabolite(s)	Fungus	Application(s)	Reference(s)
Nalanthalide	*Chaunopycnis pustulata*	Blockage of voltage-gated potassium (Kv1.3) channels and L-type Ca^{2+} channels	(46)
Candelalides	*Sesquicillium candelabrum*	Blockage of voltage-gated potassium (Kv1.3) channels	(47)
Dihydrotubingensin A, B	*Aspergillus tubingensis*	No activity	(48)
Sordarins	*Rosellinia subiculata*	Antifungal, elongation factor 2 inhibitor	(19)
Integramides A and B	*Dendrodochium* sp.	HIV-1 integrase inhibitors	(49)
Xylarenal A and B	*Xylaria liquidambar*	Selective binding to neuropeptide Y receptor Y5	(20)
Xanthonol	Non-sporulating fungus MF6460	Insecticidal activities against larvae of *Lucilia sericata*, *Aedes aegypti*, and anthelmintic against *Haemonchus contortus*	(50)
Coccidiostatin A	*Penicillium rugulosum*	Antiparasitic activity against *Besnoitia jellisoni*, *Eimeria tenella*, and *E. mitis*	(51)
Mellamide	*Aspergillus melleus*	Insecticidal activity in bioassays against larvae of *Lucilia sericata* and *Aedes aegypti*	(52)

2. Materials

2.1. Source of Strains

To obtain strains for screening, fungi were isolated at Rahway, New Jersey from living plants (12, 13), plant litter (14, 15), herbivore dung (16), soils (17), spores from field-collected fungi (18–20), and other materials (21). These sets of fungi were supplemented with strains deposited in the Merck Culture Collection and strains supplied from collaborating laboratories. Strains spanned most of the major groups of cultivable filamentous Ascomycota and Basidiomycota.

2.2. Infrastructure and Equipment

1. Autoclave with capacity to autoclave large tissue culture bottles or large media bottles.
2. Climatic chamber or controlled temperature room for maintaining stable fermentation conditions. Mesophilic fungi are normally grown at between 20 and 30°C. If possible, relative humidity should be maintained at 60% or higher, especially in arid climates.
3. Freezers (–80°C or liquid N_2) for cryopreservation of fungal strains.
4. Stereo- and bright field microscopes for checking purity and identity of strains.
5. Tissue culture roller machine (e.g., Fig. 2, Bellco bottom drive roller apparatus, Bellco Glass, Vineland, NJ, USA). The number of positions of the machine depends on the intended throughput. Our machines have 45 positions.
6. Rotary shaker with clamps for 250-ml Erlenmeyer flasks (e.g., New Brunswick Scientific, USA, Kühner AG, Switzerland or other manufacturer) for aerated culturing of inoculum.
7. Level P2 biological safety cabinet for culture and media manipulations.
8. Ventilated chemical safety cabinet for handling of volatile organic solvents, extraction of fermentations, and solvent evaporation.
9. Regulatory approved disposal system for biohazards and used chemicals.
10. Liquid handling station (e.g., Tecan Genesis, Biomex FX) for distribution of extracts in 96-well assay plates.

2.3. Glassware, Ingredients and Reagents

1. Petri dishes and agar media appropriate for fungi, e.g. malt extract agar, potato-dextrose agar.
2. Cryovials with sterile 20% glycerol and storage boxes for culture preservation.
3. Automatic and disposable pipettes, Transfertubes (Spectrum Laboratories, Rancho Dominguez, CA).

4. Adhesive labels or marking pens.
5. Autoclavable glass roller bottles. Several sizes are available ranging from 1 to 4 l. In FERMEX, 2 l tissue culture bottles (Wheaton, Bellco) were used for fermenting strains for the extract collection (Fig. 1), and 4 l tissue bottles were used for scale-up of fermentation for chemistry purification projects. However, recently to conserve resources, we have scaled down the primary extract fermentations using 1 l media bottles (e.g. Fisher, VWR) which are large enough to roll on the roller apparatus (Fig. 2). Glass bottles are reusable and are inert to the organic solvent extract.
6. Closures for roller bottles. Roller bottles are closed with a breathable lid during fungal growth. We have used reusable silicone closures (Silicone Sponge Closure for Flasks with 38 mm OD Neck), cellulose stoppers (Figs. 2 and 3), or cotton. The latter two need to be taped to the bottles so they don't fall out while rolling. During the solvent extraction, the breathable closure is replaced with the bottle's normal screw-cap closure.
7. Vermiculite. Vermiculite is readily available in the USA and parts of Europe. Extra coarse particle size is necessary, e.g., as Grade V4 (Fig. 1). The fine particle sizes used in horticultural is unsatisfactory because the particles clog and do not form proper pore spaces. Before use, the vermiculite must be sieved to remove dust and fine particles. This is best done outdoors, but if done inside, then use adequate ventilation and respiratory protection against the dust.
8. 250-ml Erlenmeyer flasks.
9. Ingredients for media. Media selection will depend on the kinds of fungi grown and the investigator's experience and preferences. Because one of the goals of the technique is to produce clean interference-free extracts, generally media with defined or soluble complex ingredients are used. See references in Table 1 for examples and formulations of different media.
10. Solvents for extraction. Historically MEK or acetone has been used, but other solvents could be used. DMSO for suspension of dried extracts.

3. Methods

1. Storage of frozen strains. An agar slant or Petri plate of each strain is used to prepare frozen vegetative mycelia, by aseptically transferring a portion of the growth into seed medium and incubating on a gyratory shaker (220 rpm) at 22–25°C and until sufficient amount of biomass is obtained. The length

of time required to obtain sufficient biomass for each culture is noted, and cultures are designated fast (1–4 days seed growth) or slow (5–10 days seed growth) for subsequent experimentation. If pellets or clumps form, the culture flask can be shaken at 150 rpm with small sterile ceramic balls and cylinders for 30–60 min to macerate the mycelial mass and facilitate its pipetting. Several vials (2 or 4 ml each) of each culture are stored in 10–20% glycerol at −80°C until used. Cultures are routinely stored at least 6 months or longer before being used in screening. After assay and chemistry analysis, repetition and scale up of fermentations may not be expected for a year or more after the culture was initially grown. The 6-month storage period prior to screening eliminates those few organisms unable to survive long-term ultracold freezing.

2. Organization and scheduling. The FERMEX program operated on a continuous weekly schedule and accumulated a large collection of screening extracts over time. Each strain is usually grown on three different production media that vary significantly in their composition and levels of primary and trace nutrients in order to express a large range of secondary metabolism. Over the course of several weeks, the schedule is rotated among 10–12 different production media (see examples in references). Approximately 240–300 extracts are generated per month.

   The strains were inoculated from frozen vials into seed media and then transferred to production media when sufficient biomass had grown. Strains were selected and segregated into weekly sets according to whether they grow slowly or quickly. A typical weekly schedule during a 6 week period is illustrated in Table 2.

3. Inoculum media. To minimize mycelium from pelleting and therefore accelerating growth, polymeric agents, e.g., agar, carboxymethyl cellulose or polyacrylic acid, are added to the inocula (53, 54). Three media typically useful are Sabouraud’s maltose broth emended with yeast extract and agar (SMYA; neopeptone 10 g, maltose 40 g, yeast extract 10 g, agar 4 g, distilled H_2O 1,000 ml), yeast malt extract with Junlon (polyacrylic acid) (YMEJ; yeast extract 4 g, malt extract 8 g, glucose 4 g, Junlon 1.5 g, distilled H_2O 1,000 ml), and KFA (corn steep powder 2.5 g, tomato paste 40 g, glucose 10 g, oat flour 10 g, agar 2 g, trace element solution 10 ml, distilled H_2O 990 ml, with the trace element solution containing $FeSO_4 \cdot 7H_2O$ 1.0 g, $MnSO_4 \cdot H_2O$ 1.0 g, $CuCl_2 \cdot 2H_2O$ 0.025 g, $CaCl_2$ 0.1 g, H_3BO_3 0.056 g, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.019 g, $ZnSO_4 \cdot 7H_2O$ 0.2 g in 1,000 ml distilled H_2O).

4. Inoculum production. To revive frozen strains, vials are thawed at room temperature and a portion of the mycelial discs or

Table 2
Weekly schedule for inoculation, growth, and extraction of three sets of fungi using the FERMEX method

Week	Day 1	Day 2	Day 3	Day 4	Day 5
1	Set A-Start inocula (1–4 days fast growers)	A inocula to production (1 day)	A inocula to production (2 days)	A inocula to production (3 days)	A inocula to production (4 days) Set B-Start inocula (5–7 days slow growers)
2			B inocula to production (5 days)	B inocula to production (6 days)	B inocula to production (7 days)
3	Set C-Start inocula (1–4 days fast growers)	C inocula to production (1 day)	C inocula to production (2 days)	C inocula to production (3 days)	C inocula to production (4 days)
4	Set A Extraction (17–20 days)				
5	Set B Extraction (17–19 days)				
6	Set C Extraction (17–20 days)				

mycelial suspension is aseptically transferred to a 250-ml flask with 50 ml of SMYA, KFA, or YMEJ medium. The rest of the contents of the cryovial are transferred to a Petri plate with malt extract agar or potato-dextrose agar. Flask are incubated on a gyratory shaker (200–220 rpm, 22–25°C) 3–10 days or until abundant cells are produced.

5. Culture quality control. Mycelium cultured from cryovials should be compared to inoculum plated onto the same medium. The plates can be saved and compared to growth on vermiculite to check for possible contamination.
6. Preparation of vermiculite and roller bottles. Pre-sifted vermiculite is added to roller bottles and sealed with breathable closures (see Table 3 for relative proportions). The bottles are autoclaved with the autoclave's dry cycle twice, with at least one full day between each autoclaving (Fig. 3). A large supply of vermiculite bottles can be prepared in advance and stored for subsequent use.
7. Media preparation. The liquid media are weighed and mixed in large batches and then aliquoted and autoclaved in single

Table 3
Relative proportions of inoculum, medium, vermiculite, and solvent extract for different roller bottle dimensions

Container and dimensions	Volume inoculum (ml)	Volume liquid medium (ml)	Volume vermiculite (cm^3)	Solvent extraction with methyethyl ketone (ml)
Media bottle (1 l capacity)	7	120	340	150–165 to yield 125 of 1 WBE
Small roller bottles (105 × 210 mm, 2 l capacity)	15	225	675	300–325 to yield 250 of 1 WBE
Large roller bottles (105 × 420 mm, 4 l capacity)	25	550	1,250	600–650 to yield 500 of 1 WBE

Whole broth equivalent (WBE) is the extract concentration relative to the original fermentation volume

bottles or flasks, one for each roller bottle according to the proportions in Table 2.

8. Inoculation of roller bottles. Clean and turn on the biosafety cabinet. On a nearby table or laboratory cart, arrange each inoculum flask with its corresponding roller bottle and liquid media aliquot. Label each container with the strain number and medium type, using either preprinted adhesive labels or a lab marker. Labels should go on the bottles' inclined neck so they are not rubbed off by the roller machine cylinders.

   Inside the biosafety cabinet, aseptically pipette an aliquot of inoculum into its corresponding liquid medium. Close the liquid medium container and shake it vigorously to disperse the cells. Quickly pour (or pipette) the inoculated liquid into the corresponding vermiculite roller bottle and close it. Make sure the closures are securely fastened so they will not loosen on the roller machine (Fig. 2).

9. Incubation of roller bottles. The roller machine should be located in a humidified controlled temperature room or climatic chamber. Inoculated roller bottles are placed on the machine and the machine set at about 4–6 rpm. Excess speed is unnecessary and only wears out the drive mechanisms. As the bottles roll, observe that the vermiculite starts to coat the bottles. Some excess liquid may roll in the bottom of the bottle, but as each fungus grows, the liquid will be absorbed. Depending on the kind of fungus, the vermiculite is usually immobilized by a mycelial matrix in about 2–5 days (Figs. 3, 4, and 5). Once, the matrix is solidified, the bottles can be incubated on

a shelf, or the roller machine can be turned off and used as incubator shelf. The incubation time can vary depending on the kinds of fungi. For robustly growing ascomycetes, like *Aspergillus*, *Hypoxylon*, or *Botryosphaeria* species, 14 days may be adequate, but slower growing ascomycetes and basidiomycetes may require to 3–4 weeks or longer to fully colonize the bottles and exhaust the nutrients.

10. Extraction. In a chemical fume hood, add MEK (ca. 1.2–1.4 times the nominal fermentation volume) to the roller bottles. Close the bottles with the solvent-resistant caps and shake vigorously for 2–3 min and roll them on the roller apparatus for 3–4 h. Mycelium matted vermiculite clumps in the occasional recalcitrant sample are broken up with a long sturdy glass rod prior to rolling. After extraction, the extracts are filtered on a Buchner funnel, and the vermiculite mass is pressed down by hand with a plastic Erlenmeyer flask. The exhausted vermiculite is thoroughly dried in a fume hood and subsequently disposed in a biohazard container for incineration.

    The resulting extract, nominally at a 1× concentration, i.e. 1 ml broth equivalent (WBE) per ml, is sufficiently concentrated for screening, confirmation of hits, dereplication and at least preliminary chromatographic fractionation.

11. Preparation of screening samples. A sufficient aliquot is dried down under N_2 flow or vacuum, weighed, and redissolved in DMSO to give at least 6 ml of a 10 mg/ml solution, typically corresponding to a three- to sixfold concentrate over the original fermentation. This concentration is used for two main reasons: firstly, it yields fluid solutions for pipetting stations; secondly, assuming a 50- to 100-fold dilution into the assay medium because of limitations in DMSO concentration in most biochemical assays, it would result in crude extracts assayed at 100–200 μg/ml, and therefore, ultimately yielding low micromolar or more potent compounds, based on the presumption that the active metabolites represented at most a few percent of the crude extract. Approximately one-half of this DMSO solution is aliquoted into multiple 96-well conical-bottom assay plates, with 80 samples in the plate's center ten columns. Both 10 μl/well copies are prepared for single assays and 100 μl/well copies are prepared for laboratories running multiple screens over time. The remainder of the DMSO sample is retained as a backup in 2-ml mother plates. Assay and mother plates are heat-sealed and stored at −20°C until use. The bulk MEK extract is kept in reserve in glass bottles in a 4°C cold room.

12. Screening and follow-up of active extracts. Confirmation and prioritization of screening hits, which might include a dose–response, a counterscreen, and/or secondary assays, is carried

out on “fresh” DMSO aliquots recovered from the mother plates and a “new” sample is prepared from the bulk reserve MEK extract. Confirmed samples of interest are then analyzed by HPLC or LC/MS for purposes of dereplication and semi-quantitative analyses of known compounds.

Chromatographic fractionation of samples passing all tests then proceeds by using one-third of the remaining original MEK extract. This strategy is usually successful in either, full resolution of the active in ca. 20% of the cases; or in securing a chromatographic standard of the target compound to guide eventual refermentation, fermentation optimization, and scale-up in nearly all other cases. Enough MEK reserve remains for an occasional accident, or in anticipation of subsequent actives, for which one-half of the remainder would be used each time. In no instance should a sample be completely depleted; a few milliliter voucher of the original MEK should always be kept in permanent reserve for use in confirmation assays and for side-by-side with a replenishment fermentation and extraction.

Furthermore, by proceeding in this fashion, one not only has the benefits of duplicate samples but also samples with which to run comparative analyses in the rare cases of mismatch between DMSO and MEK reserves.

For chromatographic follow-up and identification of actives, the nature and composition of the vast majority of the FERMEX extracts is such that a first crude separation by gel filtration on Sephadex LH-20 (Pharmacia) in methanol followed by reverse-phase HPLC is a rapid and largely successful way of proceeding.

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