Chapter 3

Media and Growth Conditions for Induction of Secondary Metabolite Production

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Abstract

Growth media and incubation conditions have a very strong influence of secondary metabolite production. There is no consensus on which media are the optimal for metabolite production, but a series of useful and effective media and incubation conditions have been listed here. Chemically well-defined media are suited for biochemical studies, but in order to get chemical diversity expressed in filamentous fungi, sources rich in amino acids, vitamins, and trace metals have to be added, such as yeast extract and oatmeal. A battery of solid agar media is recommended for exploration of chemical diversity as agar plug samples are easily analyzed to get an optimal representation of the qualitative secondary metabolome. Standard incubation for a week at 25°C in darkness is recommended, but optimal conditions have to be modified depending on the ecology and physiology of different filamentous fungi.

Key words: Secondary metabolites, Growth media, Agar plugs, Yeast extract, HPLC

1. Introduction

Filamentous fungi can produce many different secondary metabolites. Profiles of secondary metabolites were introduced by Frisvad et al. (1–6) to show that a single strain of filamentous fungi can produce a series of secondary metabolites in a species-specific manner. The fact that many secondary metabolites can be produced by one single strain was taken up by Bode et al. (7) and called one strain many compounds (OSMAC). In recent years it has been shown that histone methylation or acetylation inhibitors, other microbes, plant extracts and other growth medium ingredients, water activity, pH, temperature, light, stress, and other factors can help express usually silent secondary metabolite gene clusters (8–26). Of the rather few tested, most secondary metabolites from

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species of fungi have been shown to have highly remarkable effects on other species. It is therefore of interest to find laboratory and industrial conditions where all these secondary metabolites can be produced in order to screen for new antibiotics, quorum sensing inhibitors, and drug lead candidates and also in general to elucidate the chemical language of organisms. It is often not known which conditions are optimal for production of any one secondary metabolite, as the ecology of the producers is usually not well known. For this reason media and growth conditions for production of secondary metabolites have been based on trial and error or tradition.

In Japan rice has been used traditionally as a substrate for secondary metabolite production and many other natural product chemists prefer this substrate (e.g., (27, 28)), while wheat has been used in Europe and corn or wheat in the USA (e.g., (29, 30)). In other studies scientists have preferred chemically well-defined media or well-defined media with added yeast extract, malt extract, corn-steep liquor, peptone, or potato extract. Secondary metabolites from marine-derived fungi are often produced on media-added seawater rather than pure water (e.g., (31)).

A major problem with chemically poorly defined media is that different brands of yeast extract (32, 33), peptone (34), malt extract (35), etc. will occasionally give different morphologies and secondary metabolite profiles. On Yeast extract sucrose (YES) agar, profiles of secondary metabolites were remarkably different depending on the brand of yeast extract (32). At least Difco yeast extract gives good results concerning secondary metabolites.

It has long been known that all filamentous fungi produce a large number of secondary metabolites, biosynthetically often produced as polyketides, terpenes, non-ribosomal peptides, and alkaloids or mixtures of those, or derived from shikimate or nucleotides. Each species produce a specific profile of secondary metabolites (2, 3), and so the concept OSMAC (7) could just as well have been an abbreviation of "one species many compounds." Some research groups have used this approach in using nutritional arrays in a small scale (9), while others have suggested a large number of media in order to increase the chance of having as many secondary metabolite families expressed as possible (36).

The medium components have a pronounced effect on secondary metabolite production. For example lactate or starch as the sole carbon source only resulted in low production of secondary metabolites in *Aspergillus niger*, but the combination of the two gave a synergistic effect, so much larger amounts of fumonisins, ochratoxins, kotanins, and naphtho-γ-pyrones were produced (23). This indicates that there are many possibilities to optimize medium composition. C/N ratios and combinations of C and N sources have not often been systematically explored regarding profiles of secondary metabolites. Experimental design is a good tool for

optimizing single secondary metabolite production in general (37), but multicriterium optimization may be needed in order to optimize the profile of secondary metabolites.

Addition of suboptimal concentrations of fungicides, preservatives, and histone effectors to the media may also enhance secondary metabolite production (10, 18, 21, 22, 38). Plant secondary metabolites may trigger secondary metabolite production by epigenetic remodeling (13, 26) and thus the addition of plant extracts to many media often boost secondary metabolite production. Here a general procedure for exploring the secondary metabolome using a simple agar plug extraction procedure with different kinds of media combined with (UPLC)-DAD-MS is presented.

2. Materials

2.1. Fungal Inoculation

1. Media for production of conidia:
   (a) CYA (Czapek yeast autolysate agar) for *Penicillium*, *Aspergillus*, and *Paecilomyces*: $NaNO_3$ (3 g/L), yeast extract (5 g/L), sucrose (30 g/L), $K_2HPO_4{\cdot}3H_2O$ (1.3 g/L), Czapek concentrate (10 mL/L), agar (15 g/L) in 1 L ddH_2O. Autoclave for 15 min, at 121°C on liquid cycle.
   (b) Czapek concentrate: KCl (5 g/100 mL), $MgSO_4{\cdot}7H_2O$ (5 g/100 mL), $FeSO_4{\cdot}5H_2O$ (0.1 g/100 mL), $ZnSO_4{\cdot}7H_2O$ (0.1 g/100 mL), $CuSO_4{\cdot}5H_2O$ (0.05 g/100 mL) in 100 mL ddH_2O.
   (c) Malt extract agar (MEA) for most filamentous fungi: Malt extract (20 g/L), glucose (20 g/L), peptone (1 g/L), $ZnSO_4{\cdot}7H_2O$ (0.01 g/L), $CuSO_4{\cdot}5H_2O$ (0.005 g/L), agar (25 g/L) in 1 L ddH_2O, pH approximately 4.7, so it is advisable to autoclave 500 mL water with the malt extract and 500 mL with the remaining ingredients, then mix after autoclaving. Autoclave for 15 min, at 121°C on liquid cycle (see Note 1).
2. Spore Suspension Medium (SSM): Tween 80: 0.5 g/L, agar 2 g/L, ddH_2O to 1 L, distributed in 4 mL vials, autoclaved, and stored in a refrigerator (39).

2.2. Media for Secondary Metabolite Production

1. YES agar: Sucrose (150 g/L), yeast extract (20 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $ZnSO_4{\cdot}7H_2O$ (0.01 g/L), $CuSO_4{\cdot}5H_2O$ (0.005 g/L), agar (15 g/L) in 1 L ddH_2O. Autoclave for 15 min, at 121°C on liquid cycle (see Note 2) (1, 2, 40, 41).
2. CYA agar (see above) (1, 2, 41, 42) (see Note 3).

3. Potato dextrose agar (PDA): 200 g sliced potatoes are boiled in 1 L of ddH_2O and sieved, add glucose (20 g/L), agar (20 g/L), $ZnSO_4{\cdot}7H_2O$ (0.01 g/L), $CuSO_4{\cdot}5H_2O$ (0.005 g/L). pH is approximately 5.6. Autoclave for 15 min, at 121°C on liquid cycle (1, 2, 41).
4. Mercks Malt Extract (MME) agar: Malt extract (30 g/L), soy peptone (3 g/L), $ZnSO_4{\cdot}7H_2O$ (0.01 g/L), $CuSO_4{\cdot}5H_2O$ (0.005 g/L), agar (20 g/L) in 1 L ddH_2O. Autoclave for 15 min, at 121°C on liquid cycle (43).
5. Wickerhams Antibiotic Test Medium (WATM): $NaNO_3$ (2 g/L), glucose (2 g/L), saccharose (30 g/L), yeast extract (Difco) (2 g/L), peptone (3 g/L), corn steep solids (5 g/L), $KH_2PO_4{\cdot}3H_2O$ (1 g/L), KCl (0.2 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $FeSO_4{\cdot}7H_2O$ (0.01 g/L), $ZnSO_4{\cdot}7H_2O$ (0.01 g/L), $CuSO_4{\cdot}5H_2O$ (0.005 g/L), agar (20 g/L) in 1 L ddH_2O. Autoclave for 15 min, at 121°C on liquid cycle (44).
6. Raulin Thom Oatmeal (RTO) agar: $(NH_4)H_2PO_4$ (0.4 g/L), K_2CO_3 (0.4 g), $ZnSO_4{\cdot}7H_2O$ (0.07 g/L), $CuSO_4{\cdot}5H_2O$ (0.005 g/L), $FeSO_4{\cdot}7H_2O$ (0.06 g/L), $(NH_4)_2SO_4$ (0.16 g/L), $MgCO_3$ (0.25 g), tartaric acid (2.6 g/L), di-ammonium tartrate (2.6 g/L), glucose (50 g/L), oatmeal (30 g/L), agar (20 g/L) in 1 L ddH_2O. pH adjusted to 6.5 with 1 N NaOH before autoclaving. Autoclave for 15 min, at 121°C on liquid cycle (36).
7. Rice Corn steep (RC) agar: Rice meal (50 g/L), corn steep solids (40 g/L), $ZnSO_4{\cdot}7H_2O$ (0.01 g/L), $CuSO_4{\cdot}5H_2O$ (0.005 g/L), agar (20 g/L) in 1 L ddH_2O. Autoclave for 15 min, at 121°C on liquid cycle (45).
8. Oatmeal Agar (OA): Oatmeal (30 g/L), $ZnSO_4{\cdot}7H_2O$ (0.01 g/L), $CuSO_4{\cdot}5H_2O$ (0.005 g/L), agar (20 g/L) in 1 L ddH_2O. Autoclave for 15 min, at 121°C on liquid cycle (5, 41).
9. MEA (see above) (5).
10. Czapek Yeast Autolysate Salt (CYAS) agar: CYA agar with 50 g/L NaCl (46) (see Note 4).
11. CY20 or CY20S (Czapek Yeast autolysate 20% Saccharose agar): CYA agar with 170 g/L saccharose added, but ddH_2O only to 1 L (46) (see Note 4).
12. Pharma Medium (PM): Maltose (30 g/L), polypeptone (10 g/L), $(NH_4)_2SO_4$ (2 g/L), Cotton seed flour (Pharmamedia) (40 g/L), KH_2PO_4 (2.5 g/L), K_2HPO_4 (7.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $ZnSO_4{\cdot}7H_2O$ (0.01 g/L), $CuSO_4{\cdot}5H_2O$ (0.005 g/L), agar (15 g/L) in 1 L ddH_2O. Autoclave for 15 min, at 121°C on liquid cycle (see Note 5) (47).

13. Yeast Extract (YE) agar: Yeast extract (20 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $ZnSO_4 \cdot 7H_2O$ (0.01 g/L), $CuSO_4 \cdot 5H_2O$ (0.005 g/L), agar (15 g/L) in 1 L ddH_2O. Autoclave for 15 min, at 121°C on liquid cycle (see Note 6) (36).
14. LB (Lysogeny Broth, "Luria Broth," "Lennox Broth," "Luria-Bertani medium") agar: Tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L), agar (15 g/L) in 1 L ddH_2O. pH 7.0. Autoclave for 15 min, at 121°C on liquid cycle (see Note 6) (48, 49).
15. Dichloran Rose bengal Yeast Extract Sucrose (DRYES) agar: Sucrose (150 g/L), yeast extract (20 g/L), dichloran (0.002 g/L), rose bengal (0.025 g/L), chloramphenicol (0.05 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $ZnSO_4 \cdot 7H_2O$ (0.01 g/L), $CuSO_4 \cdot 5H_2O$ (0.005 g/L), agar (15 g/L) in 1 L ddH_2O. Autoclave for 15 min, at 121°C on liquid cycle. After autoclaving add 0.05 g/L chlortetracycline aseptically. pH 5.6 (41) (see Note 7).

2.3. Extraction of Fungal Cultures

1. Extraction liquid: Ethylacetate:dichloromethane:methanol = 3:2:1, this mixture containing 1% formic acid (see Note 8).
2. Organic solvent for redissolving fungal plug extract: Methanol.

2.4. Ultrahigh-Performance Liquid Chromatography Procedure

1. Elution liquid I: Acetonitrile with 50 ppm trifluoro acetic acid (TFA).
2. Elution liquid II: Water with 50 ppm TFA.
3. Alkylphenone retention standard mixture: Acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, octanophenone, decanophenone, 0.1% of each dissolved in acetonitrile. Keep in −18°C freezer until use.
4. If available dissolve secondary metabolite authentic standards in methanol, and keep in −18°C freezer until use.

3. Methods

Qualitative and semiquantitative screening for secondary metabolites in filamentous fungi can be done efficiently using a microscale agar plug method. In combination with ultrahigh-performance liquid chromatography (UPLC), this method will give very reproducible separation in 10 min for each sample, and many analyses can be done in a short time. It is recommendable to take plugs from agar cultures, as it is easily seen if there are any contaminants in the cultures, and such 9 cm agar plates are used for fungal identification anyway. Furthermore it can be determined if different parts of the fungal colony have different chemical profiles and

which metabolites are secreted into the medium. Some of these advantages are lost when using either small-scale 96-well plates (9) or at the other extreme, large fermentations. However, other screening methods have been suggested, for example one based on still rice cultures and a shaken liquid medium (28, 50).

Regarding growth conditions, usually filamentous fungi are grown at 25–30°C and will produce their secondary metabolites at their growth optimum, and often less when they are approaching their growth maximum or minimum. Of course thermophilic fungi will produce secondary metabolites at high temperatures. Chu et al. (51) found that *Talaromyces thermophilus* produced talathermophilins A and B at temperatures from 35 to 65°C. Likewise psychrotolerant fungi produce secondary metabolites optimally at 15–20°C (52). Concerning light, most fungi produce most of their secondary metabolites in darkness, but there are some exceptions. For example *Talaromyces stipitatus* produces some additional anthraquinones only when exposed to light (24), and in general the perfect and imperfect states of filamentous fungi may contain different secondary metabolites. As some of these fungi produce the imperfect state mostly when exposed to light and the perfect state preferentially when grown in darkness, secondary metabolite profiles have to be different in light and darkness (8). Furthermore fungi have a circadian regulation of secondary metabolite production and different kinds of light (blue light, yellow to green) have a pronounced effect on secondary metabolism (22). Furthermore light has a direct influence on carotene production (14, 15), and if these compounds are to be considered, incubation in light may be important. Low water activity will usually mean a lower production of secondary metabolites (17, 53), but Schmidt-Heydt et al. (21) have shown that while large amounts of secondary metabolites are produced close to the growth optimum, there is another smaller production optimal peak of secondary metabolite production at more extreme conditions. This was also the case for temperature and pH. Oxygen and carbon dioxide can also have a positive effect on mycotoxin production, but usually secondary metabolite-producing fungi produce most mycotoxins at high oxygen and low carbon dioxide levels (54). At high CO_2 levels or anaerobic conditions fungi will turn their metabolism to production of alcohol, esters, and other small volatile molecules as is known in yeasts.

3.1. Fungal Inoculation

Obtain fungal isolates directly from a substrate or via direct or dilution plating from the same substrate or order cultures from culture collections. Purify the fungal isolates by streaking on CYA, or similar agar media, or by single spore inoculation. Identify the fungal isolates to species level, using a polyphasic approach, using morphology, physiology, and molecular methods, and accession the cultures in one or preferably two international culture collections (i.e., CBS, the Netherlands; ATCC, the USA; NRRL, the USA;

IMI, Great Britain; IHEM, Belgium; CCF, the Czech Republic; CECT, Spain; VKM, Russia; DSMZ, Germany; IBT, Denmark; ITEM, Italy; IFO, Japan; IITA, Nigeria). Most of these culture collections also have a fungal identification service. Make a literature search on the known compounds from the species identified. For each fungal isolate make a conidium suspension (2×10^6 conidia per mL) in 0.2% agar in water with 0.05% Tween 80 and three point inoculate (39) onto the agar media chosen. Incubate at 25°C for 7 days in darkness.

3.2. Media for Secondary Metabolite Production

Three point inoculate the media YES, CYA, MEA, and OA (for *Penicillium, Aspergillus, Talaromyces, Rasamsonia, Hamigera*, and *Paecilomyces*); DRYES and PDA for *Alternaria*, *Cladosporium*, and other dematiaceous hyphomycetes; and YES and PDA for *Fusarium, Trichoderma, Verticillium, Stachybotrys*, and other genera with spores produced in slime. For a greater secondary metabolite profile, inoculate each fungal isolate on YE, LB, MME, WATM, RTO, RC, CYAS, CY20, and PM.

3.3. Extraction of Fungal Cultures

1. Take three 6 mm diam. agar plugs from one of the colonies from each agar medium used (one plug in the center of the colony and one from the periphery closest and furthest away from the other colonies) (55) and place in a 1.5 mm autosampler dram vial with a screw cap.
2. Extract secondary metabolites with ethyl acetate/methanol/dichloromethane (3:2:1) with 1% formic acid in 1.5 mL autosampler screw-cap vials.
3. Ultrasonicate for 50 min and transfer extract to a new 1.5 mL vial. Evaporate the contents and redissolve in 400 μl methanol by ultrasonication in 10 min, filter through a PTFE 0.45 μm filter, and keep at −18°C prior to analysis.

3.4. UPLC

1. Arrange all samples in a rack (for the autosampler of the liquid chromatograph) and analyze in the following order: (a) a blank sample, (b) the alkylphenone mixture, (c) any standards, (d) plugs extracts of the media without fungi as controls, (e) the actual samples with fungal secondary metabolites, and (f) the alkylphenone mixture once again.
2. Inject 1 μl extract or standard into a liquid chromatograph with two pumps and with diode array detection, fluorescence detection, and electrospray MS detection (56). Analyze by UPLC using alkylphenone retention indices and diode array UV–VIS detection as described by Frisvad and Thrane (57, 58).
3. Separate the secondary metabolites on a C_{18} (150×2.1 mm) column with 2.6 μm particles. Hold column temperature at 60°C. Inject 1 μl of fungal extract into the UPLC. Use a

gradient based on water with 50 ppm TFA and acetonitrile (AcCN) with 50 ppm TFA, at a flow rate of 0.8 mL/min: Start conditions 85% H_2O with TFA and 15% AcCN with TFA. Gradient: 15–25% AcCN in ½ min, from 25 to 65% AcCN in 5½ min, from 60 to 100% AcCN in 1 min, at 100% AcCN in 1 min, back to 15% AcCN in 1 min, and then 1 min constant at 15% AcCN. The run-time is therefore 10 min.

4. Record UV chromatograms at 210 and 280 nm, and record the full diode array spectra in a range from 190 to 600 nm.
5. Print or examine chromatograms, DAD spectra, and MS spectra on the screen. Identify secondary by comparison of the UV-Visible DAD spectra, MS spectra, and retention times of the secondary metabolites with authentic standards (56).
6. Record the peak area of each chromatographic peak for semi-quantitative measurements. In that case make biological replicates of the extracts of the fungal cultures to calculate the average and standard deviation of the results.

3.5. Conclusions

No single medium under standardized growth conditions can secure expression of the full potential for producing secondary metabolites (the secondary metabolome) of a fungal culture. Despite a plethora of media and growth conditions for fungal secondary metabolite production used by natural product scientists, secondary metabolite profiling can be done using a simple compromise method. YES agar, PDA (or equivalent media such as MEA or oatmeal agar), and a carbohydrate-poor medium such as Lysogeny Broth agar incubated at 25°C for 1 week in darkness will give a relatively good representation of major secondary metabolites of a fungal species. Extracted agar plugs from the fungal agar cultures can then be analyzed by HPLC-DAD-MS in order to identify as many secondary metabolites as possible. However there are many possibilities to have fungal cultures express even more biosynthetic families of secondary metabolites, including adding histone methylation inhibitors, adding plant parts in the medium, adding other microorganisms, using light, using longer incubation periods, using other temperatures, using low or high pH, etc.

4. Notes

1. Many other media are excellent for production of large numbers of fungal conidia: PDA, Potato Carrot Agar (PCA), Spezieller Nährstoffarmer Agar (SNA) agar, and several other media (see (41) for medium composition of those). For genera like *Fusarium*, *Trichoderma*, and *Alternaria*, an alternate light cycle will promote sporulation (41).

2. YES agar appears to be optimal when made with Difco yeast extract, but can also be made from Sigma yeast extract and many other yeast extracts. The addition of magnesium sulfate will make YES agars made from different yeast extracts less variable concerning secondary metabolite production (32). Sometimes a modification of YES agar, DRYES (59), will give even better secondary metabolite production (60).
3. This medium is especially suited for non-ribosomal peptides, but alternative media, such as ALK and ABE, have also been proposed for alkaloids (61).
4. CYAS and CY20 are especially suited for secondary metabolite production by osmophilic and halophilic fungi such as *Eurotium* (*Aspergillus* section *Aspergillus*), but have also been shown to be optimal for certain secondary metabolites in less osmophilic and halophilic species, for example fumonisin production by *Aspergillus niger* (46).
5. PM is especially suited for Zygomycetes, such as *Rhizopus* (47).
6. These two media contain no or very little direct carbon source and are supportive of production of certain non-ribosomal peptides such as penicillin, gliotoxin, and aspergillic acids. Other media that have been used for this purpose are Tryptone Glucose Yeast (TGY) extract agar (62) and Aspergillus Flavus Parasiticus Agar (AFPA) (63). There have as yet not been any systematic comparisons of these media for their efficiency in metabolite profiling, but many known secondary metabolites, such as aflatoxins, are not produced on these low-carbon-source media.
7. Secondary metabolites can be produced in still or shaken broth cultures, in agar media, on other kinds of solid support, for example Lightweight Expanded Clay Aggregates (LECA) "nuts" (64), and on other gelling agents such a pluronic polyol F-127 (65, 66), but solid agar media have given consistently good results.
8. Other alternative extraction liquids are, for example, ethyl acetate with 1% formic acid, isopropanol, 75% methanol (excellent for fumonisins (46)), and hexane, for apolar sclerotial metabolites (30).

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