PMF
Pharmaceutical Microbiology Forum

PMF NEWSLETTER

A Publication of the Pharmaceutical Microbiology Forum
DISTRIBUTED INTERNATIONALLY

Volume 10, Number 2 May - June 2003

Inside this issue:

President's Message

One of the areas in pharmaceutical microbiology that has received great attention lately is Automation/Rapid Methods, especially in the field of microbial identification. Knowing the species of microorganisms present in a sample is key to a successful evaluation of possible adverse impact on product quality and safety. Traditionally, identification of filamentous fungi (molds) has been carried out via classical macroscopic and microscopic techniques. To this day, the identification of molds to the species level is still an art not pursued by many microbiologists, a fact that leaves the quality group begging for skilled resources or a rapid method that can provide accurate and reliable data. The good news is that some manufacturers of automated microbial identification systems have heard our cry for help and started developing systems that can perform mold identification. In this issue of the PMF newsletter we feature an article by Biolog, a company that that now offers fungal identification using the Biolog technology of carbon source utilization. I am sure you will find this article most interesting.

I would also like to alert our readers of the new USP Chapter <1117>, Microbiological Good Laboratory Practices, which was published in the Pharmacopeial Previews section of PF Vol.29(3) [May-June 2003]. Be sure to get acquainted with the proposed practices.

Finally, as you have noticed, our newsletter has been reformatted and we hope to go virtual sometime this year. So, it is very important that you provide us with/confirm your e-mail address as soon as possible. This information should be submitted to emsource@aol.com.

Enjoy!

Lucia Clontz

Using Biolog Technology for Fungal Identifications

DR. JOHN MURPHY

The identification of microbes collected during routine microbial sampling/ environmental monitoring has become a well-established practice in the Pharmaceutical laboratory. Pressures to assure product quality has led to an increased level of microbiological testing. However, the methods chosen to identify such samples vary considerably from traditional biochemical methods, phenotypic classification, and by the use of molecular techniques.

Biolog Inc. (http://www.biolog.com/microID.html) offers a method of microbial identification based on carbon source utilization. In simple terms, a microbe is isolated into pure culture and suspended in an inoculating fluid. This suspension is pipetted to a 96 well microtitre plate, which contains 95 carbon sources, as well as a negative control well. If a particular carbon source is utilized, redox chemistry will indicate the associated metabolic activity by means of well color changes. The resulting metabolic data (series of positive/ negative reactions generated) is interpreted by Biolog software and a match is made against the appropriate database. Currently Biolog has approximately 2,000 microbial species in its databases. The focus of this article will be to look at how the Biolog System is used to identify Filamentous Fungi.

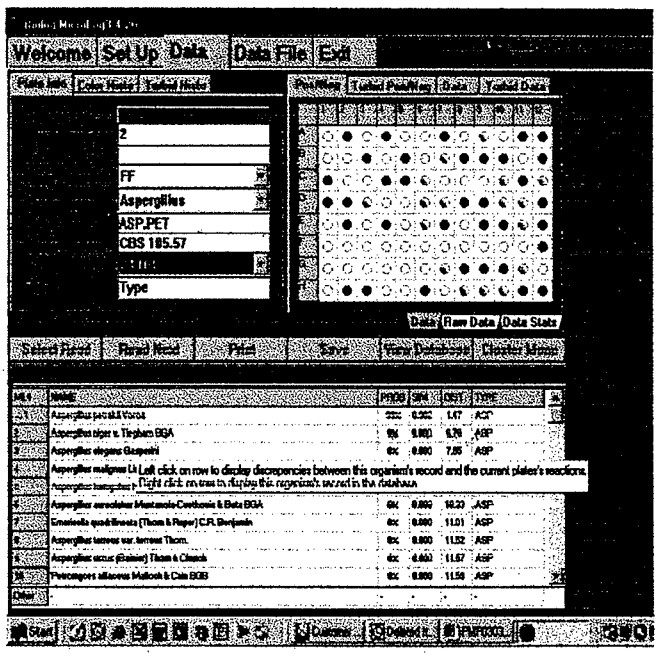
> **Classical methods:** A majority of fungal identifications are carried out by examining fungal structures both macroscopically and microscopically. Fungi behave very differently than bacteria and require special handling techniques. Fungi typically produce strands called hyphae (singular hypha). How these hyphae branch, whether or not they contain cell walls, and the types of sporulating structures (if applicable) produced play a large role in how fungi are classified and subsequently identified. Methods such as growing on selective media and observation of culture characteristics over time also help the mycologist to determine the identity of a fungus.

Biolog launched its FF (Filamentous Fungi) MicroPlate in 2000 as a response to a growing demand for a rapid and less subjective means of identifying spore forming filamentous fungi. The FF Database contains the identification patterns for over 618 taxa from 120 plus genera, as well as a macroscopic and microscopic photo library for many of the species.

Typical Procedure

1. The mold/ fungus is isolated into pure culture on 2% Malt Extract Agar for 5-10 days until sporulation occurs.
2. A cotton swab is used to remove spores/ hyphae for transfer to an inoculation fluid. This fluid is a saline solution containing a wetting agent and a gelling agent for even suspension of spores (this suspension is inoculated at 75% transmittance).
3. The spore suspension is pipetted (100µl/well) into the Biolog FF MicroPlate and incubated at 26^0 C. The inoculated MicroPlate is read at 24, 48, 72, 96 hours and at 7 days by an automated plate reader until identification is called. Once an ID is determined there is no need to continue reading the plate.

What type of data is generated?



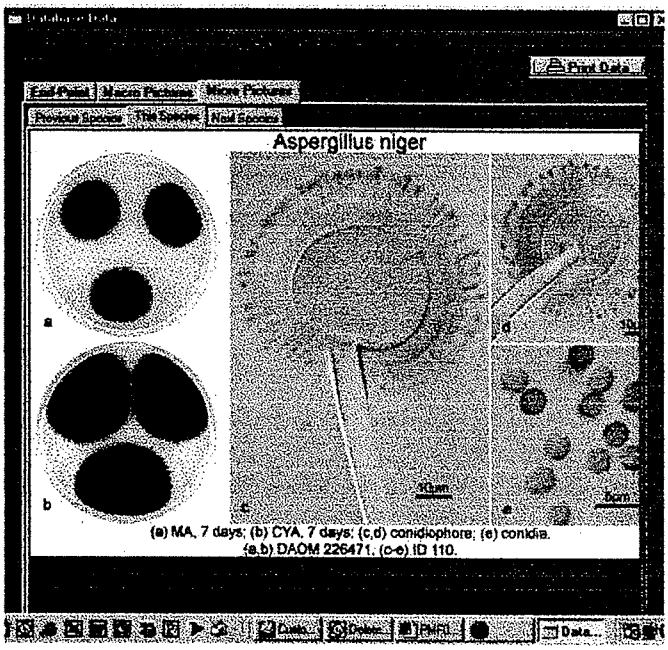
The FF MicroPlate uses a special dye (iodonitrotetrazolium violet, which is non toxic to filamentous microorganisms) that is colorless in the oxidized state. If reduced during metabolic activity a red/orange color is generated. The MicroPlate is read at 490 nm wavelength to generate 96 color data points. The MicroPlate is also read for turbidity at 750 nm wavelength, which yields an additional 96 turbidity data points (total = 192 data points per microplate) on any given day. Turbidity is an important indicator of fungal growth.

(Continued from page 2)

How is this data interpreted?

Biolog software matches the observed pattern against the FF Database, as well as a User Defined Database if applicable. A Users Database, containing prevalent microbes or those important to a particular site, can be easily created by saving your observed patterns. The software displays species in ranked order of closest match. Identification scores of PROB (% probability), SIM (similarity index value), and DIST (distance) are given as measures of how closely they match.

- % PROB – allows you to compare our Ids to other systems that use this type of calculation
- SIM – tells you how good each match is



(range: 0.0 - 1.0; 1.0 = perfect match)

- DIST – tells you number of mismatches between your MicroPlate results and the database pattern for that species

How do I decide on which ID to call?

The ID scores generated give a good indication of how confident one can be in the top choice generated. While this technology greatly reduces the search and is easy to use, the following factors must be borne in mind:

(1) When selecting a database it is important to narrow the search by choosing the most appropriate strain type. These are Air, Food, *Aspergillus*, *Colletotrichum*, *Fusarium*, *Penicillium*, *Trichoderma*, Yeast or User Defined. The strain types "Food" and "Air" are linked to where the sample is most likely to have been isolated from.

(2) Verify identifications by comparing the macroscopic and microscopic morphologies against the photo library. It is important to look at the top three choices displayed on a daily basis.

How does this product assist me if I have to search the top choices generated and read the plates over 2-3 days?

The data points generated are not as subjective as visual observation alone. Once a MicroPlate is prepared it only takes a few seconds to reread it. The choices generated reduce the necessity of going to many textbooks or prepared slides to determine the ID. In short, it greatly reduces the search effort and yields quantitative data for later review. This is particularly useful for microbiologists who have only minor training in Mycology, and for more established users who wish to reduce the amount of time taken to obtain results.

References

1. Evaluation, Validation and Implementation of New Microbiological Testing Methods, Technical Report No. 33, PDA, Vol. 54, No. 3, May/June 2000, Supplement TR33.
2. Bochner, BR, "Sleuthing out Bacterial Identities", *Nature*, Vol. 339, 1989, pp. 157-158.
3. Bochner, BR, "Breathprints at the Microbial Level", *ASM News*, Vol. 55, 1989, pp. 536-539.
4. C. J. Alexopoulos, Charles W. Mims, and M. Blackwell, Introductory Mycology, John Wiley and Sons, 4th edition, 1996, ISBN 0471522295.

About the Author

Dr. John Murphy has worked as Technical Services Supervisor at Biolog for more than 2 years. Dr. Murphy completed his undergraduate and graduate degrees in plant pathology/ mycology at University College Cork Ireland before furthering his education in Quality Assurance Management. Prior to working with Biolog, Inc. Dr. Murphy worked in the Quality Control (microbiology) Department at Schering Plough (Brinny) Inc., with a focus on Environmental Monitoring during the aseptic manufacture of parenteral drugs. Dr. Murphy can be contacted by telephone at (510) 785-2564 x328; or by e-mail at jmurphy@biolog.com.

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USP Corner

The PMF recommends that you *write directly to the USP with your comments on all proposals*. You can write representing your company, or as an individual scientist.

Any questions concerning USP documents should be sent to David Porter, Ph.D. You can reach Dr. Porter at: (706) 353-4514, via mail at United States Pharmacopeia, 126 Twinbrook Parkway, Rockville, MD 20852 or via e-mail at DAP@USP.org. When communicating with Dr. Porter, let him know you are a PMF member.

Current Compendia

US Pharmacopeia (USP) 26/National Formulary 21
First Supplement

European Pharmacopoeia (EP) 4
Supplement 4.4 April 2003
Supplement 4.5 July 2003
Supplement 4.6 January 2004

Japanese Pharmacopoeia (JP) XIV 2001

Chinese Pharmacopoeia (1995)

* If you use any other compendia, let us know for inclusion in this corner.

Alert Corner — Warning Letters and 483s (issued in 2002)

"Your firm failed to conduct quality audits at the intervals listed in your quality audit procedures"

"...no validation has been performed on the equipment cleaning and sanitizing procedure..."

"Your cleaning procedure for product contact equipment surfaces has not been shown capable of reducing microbial and endotoxin contamination to acceptable levels"

"Written procedures designed to prevent microbiological contamination of sterile drug products were inadequate and resulted in inadequate controls and procedures for gowning, and inadequate validation of the sterilization process in no microbiological qualification or determination of the resistance of the biological indicator challenge system was performed."

Internet Address	Description
http://www.hc-sc.gc.ca/hpfb-dgpsa/inspectorate/risk_clas_gmp_obs_tc_e.html	Canadian Health Products and Food Branch Inspectorate audit observation classifications
http://www.health.gov.au/tga/docs/pdf/sterilit.pdf	TGA Guideline for Sterility Testing of Therapeutic Goods

If you have found an Internet site that contains information of relevance to pharmaceutical microbiology, please let us know.

PMF THREAD - Microbiological Method Validation for Total Viable Aerobic Count

Question 1
What is the acceptance criteria in terms of percentage recovery for Microbiological Method Validation for Total Viable Aerobic Count as per European Pharmacopoeia?

Answer 1
I thought a recovery factor of +/- 5 is the specification.
If the inoculum was 100 cfu, the recovery should be within 20 and 500 cfu.

Question 2
Can anybody explain to me why EP acceptance criteria are so wide please?
I understand that in PharmEuropa they are considering tightening the spec to +/- 2, but that's still a massive range.
I prefer to use the US acceptance criteria of the test sample having to be within 70% of the control. Not only is this a tighter acceptance criterion, I think that it helps to ensure good practices in the lab - personnel executing such tasks have to develop practices which result in optimization of accuracy and precision.
In addition, the fact that so many labs are able to comply with the USP requirements negates the reasoning that a wide range is necessary due to the inherent variability of biological systems.
I'd be interested in the other people's opinions regarding this matter.

Answer 1
That acceptance is generally accepted in Europe for Microbial Limits specifications.
I have heard two reported for validation - not less than 70% of control (USP guidance) and not less than 50% of control (Microbial Limits).

Answer 2
The limit of >70 % can give more precision & accuracy.

Answer 3
We have struggled with the obviously poor language chosen for the EP criteria, as well ("not differing by more than a factor of five"). Our different EU colleagues have responded with both interpretations (20% - 500% of control and >80% of control) and some have taken the safe route of "I'm not sure what they mean by that". We used to use the >50% of the control as our recovery % but have changed to 70% - 130% of control.

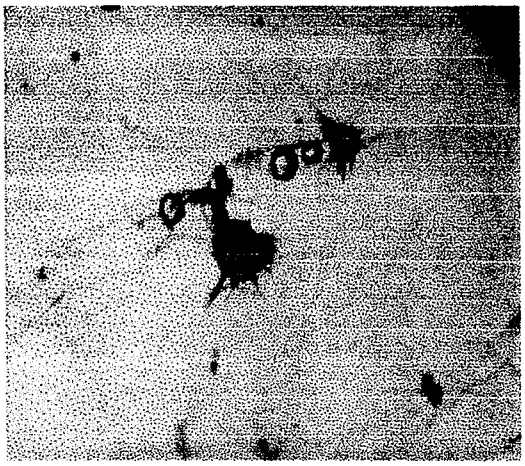
There is another issue that I've asked on this subject before and never received a response (anyone who has performed these will know what I'm talking about):
For a Total Count (Total Aerobic Microbial Count) Challenge Test:
If you challenge the material with six separate USP (or EP organisms), and you recover 70%-130% on 4 of them; and < 70% on the last two, do you keep repeating this until you get 70% on every organism? Figure you've already diluted to 1:100 and have neutralizers in the test.
If the goal is to recommend test methods for a given product, it would seem like you have the necessary information to make a recommendation when 4/6 organisms were recovered in proper amounts – endlessly repeating until you *have each organism 70-130% makes a nice tight complete package, but doesn't appear to add to what you already know.*
How does the group deal with this? Do you keep repeating until you get 70-130% for each organism?

Answer 4
Fact is, if we have gone through all the machinations and manipulations and additives and dilutions, on at least three lots of whatever material we're testing, and we get consistently below-par recovery on one or two organisms, we shrug our shoulders and write the method up as "best possible", explaining in the summary that the test material is somehow inhibitory to the organisms and therefore is unlikely to be a significant contaminant of this material.
I believe the USP and EP both have some provisions for this approach, even though it's sort of a 'qualified surrender'.

Answer 5
On the second issue, I wouldn't repeat the validation until I had 6/6 meet the validation "specification". I would first investigate:
1.) What are the two test species that don't meet? Are they both fungal, or GNR, or have some other similarity that might indicate ***true*** inhibition?
2.) What is my analyst's accuracy? Is she using calibrated micropipettes? Have I demonstrated that he can achieve 70 - 130% accuracy against himself?
3.) Is the control count in the upper regions of the specification? Greater accuracy occurs at 10^2 than 10^1.
After chemical neutralization and dilution, there is always membrane filtration. But continuous repeating until the 70 - 130% is reached doesn't get to the root cause of why it was missed in the first place.

Answer 6
Please, all of you, remember that the 70% or 50% (and we still use 50%) recovery rule applies only to standardized aqueous suspensions of cells in buffer or media, it in no way should apply to the recovery of challenge organisms from a raw material or a product formula. The mixing and binding properties of product materials with challenge cells is an unknown that definitely causes variability in challenge cell recovery results to vary by a lot more than 50%.
Keep your scientist hats on when you consider understanding the limitations of your test methods and do not stretch them to statistical areas in which they do not belong.
KNOW YOUR LIMITATIONS

Fungal ID Tips



Chaetomium

Hyphae septate and pale brown.

Chaetomium is readily recognized by its perithecia enveloped by long brown setae and its brown, lemon shaped ascospores.

The ostiole or perithecial opening is often concealed by the density of setae.

IMPORTANT ANNOUNCEMENT

PMF Newsletter will be distributed electronically in the near future.

All members need to provide their current e-mail address to Laura Valdes-Mora at Emsource@aol.com. Please mention PMF member e-mail in the subject line.

Article Review

Effect of Carrier Material on Resistance of Spores of *Bacillus stearothermophilus* to gaseous Hydrogen Peroxide

Volker Sigwarth and Alexandra Stark
PDA Journal of Pharmaceutical Science and Technology, Volume 57, Number 1, January/February 2003

The authors in this article have discussed the effect of different carrier materials to the resistance of the test organisms against H_2O_2.
The testing of the H_2O_2 decontamination process using spores of *Bacillus stearothermophilus* is widely accepted. Usually commercially available Biological Indicators (BIs) with a specific resistance to H_2O_2 are challenged to qualify the process. The authors have addressed the question that arises , whether the resistance of the test spores is dependant on the type of carrier material and whether the resistance is representative for the system under test.
The authors have extensively studied the effect of the carrier material using commercially available BIs, with various organisms and other issues related to the qualification of H_2O_2 decontamination process.
The authors have summarized their study on different resistances of *B. stearothermophilus* to H_2O_2 decontamination on different carrier materials in this article.
As a result of this study and the experience gained the authors indicate that such study should not be required for routine qualifications.

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