Starch Agar Protocol

Created: Thursday, 01 November 2012

Author

- Archana Lal
- Naowarat Cheeptham

Information

History

In 1812, the Russian chemist Gottlieb Kirchhoff hydrolyzed starch into glucose by boiling its suspension with sulphuric acid. In 1814, J. J. Colin and H. F. Gaultier de Claubry showed that iodine develops blue color with starch (6). In 1815, S. S. Stromeyer confirmed this test. In 1833, Payen and Persoz (12) isolated a white, water soluble substance from germinating barley by ethanol precipitation. The substance was capable of hydrolyzing starch and was named diastase. In 1883, Duclaux introduced the custom of designating an enzyme by the substrate on which its action was first observed and adding the suffix, "-ase." In 1835, the Swedish chemist Jons Berzelius called the hydrolysis process "catalysis" and demonstrated that the germinating barley extract catalyzed starch hydrolysis more efficiently than sulphuric acid (11; http://www.scienceclarified.com/everyday/Real-Life-Physics-Vol-3-Biology-Vol-1/Enzymes-How-it-works.html). The α-amylases were named by Kuhn in 1925, because the hydrolysis products are in the alpha configuration (17).

Starch agar was first developed by Vedder (13) as a suitable medium for cultivating *Neisseria*. Before the development of starch agar, salt-free veal agar (salt-free veal broth with 2% agar, neutralized to phenolphthalein with or without 5% defibrinated rabbit blood (15)) was used to grow cultures of *Neisseria*. However, one of the disadvantages of veal agar was that transfers had to be made every 2 to 3 days in order to keep the cultures alive.

Starch agar was developed as a medium on which *Neisseria* could remain alive for a longer period (20 to 40 days, initially in an incubator at 35 to 37^{o}C for several days and then at room temperature) without the need of frequent transfers. Vedder (13) developed starch agar that consisted of beef infusion agar (beef infusion with 1.5% to 1.75% agar) to which 1% of cornstarch was added. Other kinds of starch (tapioca, potato, and wheat) in the beef infusion agar were also tested to determine whether starch from any source was equally suitable. *Neisseria* grew on all of these starch agars better than on the ordinary media but the most luxuriant growth was observed with cornstarch. Cornstarch appeared to be more suitable for other organisms as well.

Vedder noticed that *Neisseria* grew densely on this medium after 24 hours of incubation and continued to increase growth for up to 3 or 4

days, after which there was no apparent increase in the density of the growth. He also found the starch agar suitable for routine use as most of his stock cultures grew as well or better than on plain agar. In addition, the cultures grown on starch agar were suitable for antigen preparation as starch did not have any adverseeffect on the cultures and the growth was dense. Furthermore, it is known that *Neisseria* are fastidious, capnophilic, and susceptible to cool temperatures, drying, and fatty acids (http://www.life.umd.edu/classroom/bsci424/PathogenDescriptions/Neisseria.htm). Hence, *Neisseria* requires complex media that are normally prewarmed to 35 to 37°C. Soluble starch is at times added to neutralize fatty acid toxicity to enhance *Neisseria* growth (http://www.life.umd.edu/classroom/bsci424/PathogenDescriptions/Neisseria.htm). *Neisseria* grows best in a moist atmosphere supplemented with CO_2.

Some other organisms that did not grow well on other media grew rapidly on the starch agar (13). A number of strains of tubercle bacilli and one of the possible leprae bacilli grew freely on the starch agar medium (13). Dextrose starch agar was used by Wilkins, Lewis, and Barbiers (16) in an agar dilution procedure to test the activity of antibiotics against *Neisseria* species. Presently, starch agar is no longer used for cultivating *Neisseria* but with pH indicators it is used to isolate and presumptively identify *Gardnerella vaginalis*.

Starch agar and modified related media are also used to detect certain bacterial contamination in food industry. For instance, a modified selective medium containing sodium glutamate, starch, and phenol red was invented to select for*Pseudomonas,* both amylase positive and amylase negative species, (8, 10) and *Aeromonas* in milk and dairy products (7). In another study, starch ampicillin agar, which contains the antibiotic ampicillin for selection and uses starch fermentation as differentiation, was created for the isolation of *Aeromonas* spp. from food products (4).

Purpose

Starch agar is a differential medium that tests the ability of an organism to produce the extracellular enzymes (exoenzymes) α-amylase and oligo-1,6-glucosidase that are secreted out of the bacteria and diffuse into the starch agar. These enzymes hydrolyze starch by breaking the glycosidic linkages between glucose subunits and allow the products of starch hydrolysis to enter the cell.
Starch agar is also used in differentiating members of various genera which have both amylase-positive and amylase-negative species, including *Streptococcus*, *Clostridium,*
Corynebacterium, *Fusobacterium*, *Enterococcus,*
Pseudomonas, and *Bacillus* (8, 10).

Theory

Starch, a polysaccharide, is made up of α-D-glucose subunits bonded to each other by α-glycosidic linkages. It exists as a mixture of linear α-amylose form and branched amylopectin form, the latter being

predominant (8). Alpha-amylose is a linear polymer of several thousand α-D-glucose linked by 1,4-α-glycosidic bonds. The amylopectin is larger than amylose. It consists mainly of α-D-glucose linked by 1,4-α-glycosidic bonds but is a branched molecule with 1,6-α-glycosidic branch points every 24 to 30 glucose residues (14).

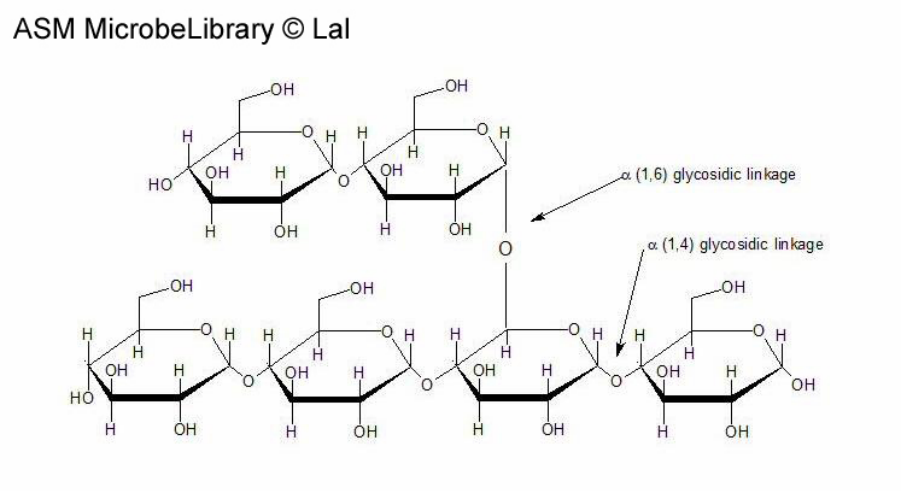

FIG 1 Structure of starch molecule showing the 1,4-α-glycosidic and 1,6-α-glycosidic linkages.

Polymers such as starch molecules are too large to be transported into the bacterial cells through the plasma membrane. Some bacteria produce and secrete the extracellular enzymes α-amylase and oligo-1,6-glucosidase and hydrolyze starch molecules outside the cell by breaking the glycosidic linkages between glucose subunits. The resulting dextrin, maltose, or glucose molecules are more readily transported into the bacterial cell to be used inmetabolism.

Enzymatic hydrolysis of starch occurs at the α-1,4- and α-1,6-glycosidic linkages that hold the starch polymer together. Alpha-amylase hydrolyzes the α-1,4-glycosidic linkages of starch. It attacks the interior of polysaccharide chains resulting in the formation of a mixture of fragments of 5 to 9 units of the alpha configuration (10). Amylase completely splits amylose into glucose subunits. The enzyme oligo-1,6-glucosidase acts on 1,6-branch points as well as α-1,4-glycosidic linkages in starch. It cleaves glucose units from the nonreducing ends of the polysaccharide starch and results in the formation of linear or branched dextrins and maltose. Dextrins and maltose are transported into the bacteria and are hydrolyzed by specific intracellular enzymes (10).

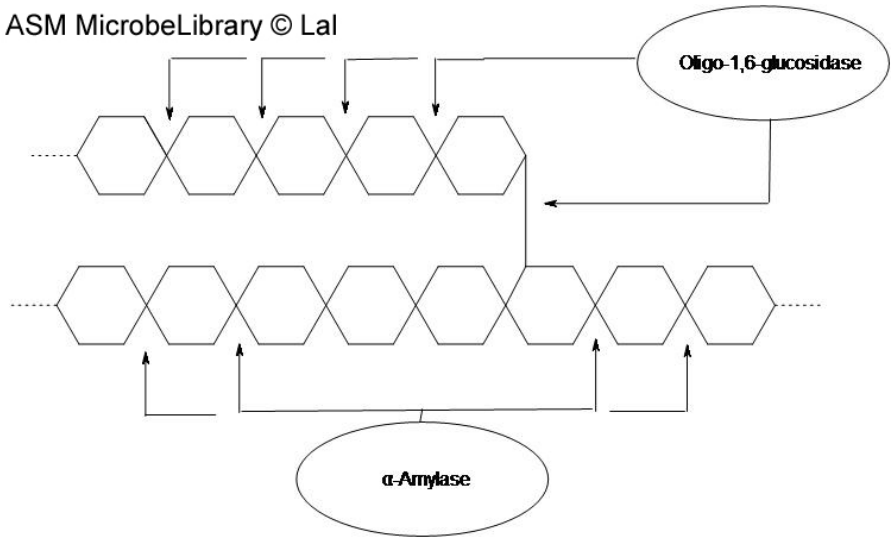

FIG 2 Starch hydrolysis by α-amylase and oligo-1,6-glucosidase. Alpha-amylase hydrolyzes α-1,4-glycosidic linkages of starch whereas oligo-1,6-glucosidase acts on 1,6-branch points as well as α-1,4-glycosidic linkages in amylopectin.

When bacteria capable of producing α-amylase and oligo-1,6-glucosidase are grown on starch agar, they secrete these enzymes into the surrounding areas and hydrolyze the starch (8). To detect the hydrolysis of starch, Gram's iodine (I_2KI solution or Lugol's iodine) is used. Gram's iodine reacts with starch to form a dark blue, purple, or black complex depending upon the concentration of iodine. The α-linkages give the amylose chain a spiral conformation which is responsible for a soluble dark blue starch-iodine complex with iodine reagent (10) and when triiodide ions in the iodine reagent slip into this spiral structure, the complex becomes blue. If this spiral conformation disintegrates, the blue color is lost. Highly branched chains of amylopectin form a red insoluble complex with iodine because they do not coil effectively (10). Upon hydrolysis of amylopectin, the first dextrin formed is erythrodextrin, which gives a color progressing from blue to violet to red-brown after the addition of iodine. With further hydrolysis, the iodine color is not produced because of the formation of colorless achroodextrins.

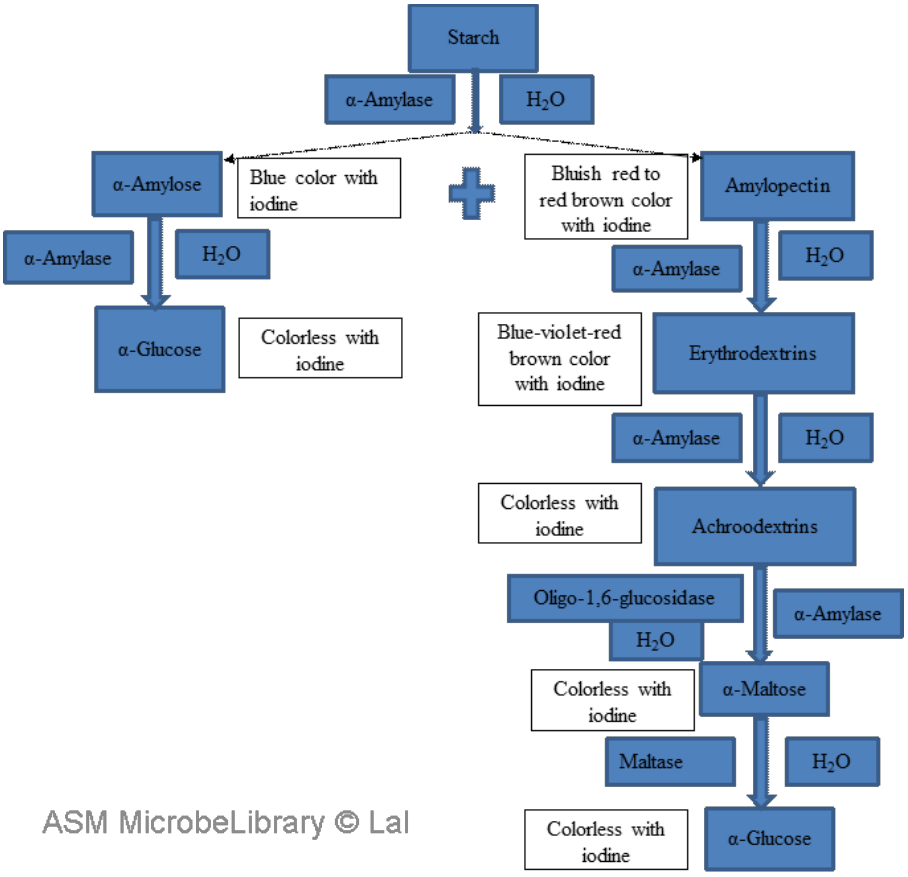

FIG 3 Diagrammatic representation of complete hydrolysis of starch showing all the intermediates and their reaction to iodine.

RECIPE (18)

Starch agar composition (g/liter)

Beef extract	3 g
Soluble starch	10 g
Agar	12 g
Distilled water	1 liter

Suspend the first three ingredients in 1 liter of distilled water. Mix thoroughly. Heat with frequent agitation and carefully bring to just boiling. Do not allow to boil as excessive boiling may hydrolyze the starch (5). Autoclave at 121°C for 15 min at 15 psi. Final pH of the medium should be 7.5 $\pm$ 0.2 at 25°C. After sterilization, pour the melted medium into sterilized petri plates (approximately 20 to 30 ml per plate) and let it solidify before use. Prepared medium is light amber to slightly opalescent. The prepared starch agar plates become opaque if refrigerated (10). The prepared medium can be dispensed into screw-

cap tubes and stored for up to 2 weeks. After 2 weeks the starch changes and reddish purple spots may develop upon addition of iodine (1). The stored medium in the tubes should be melted in a boiling water bath, poured into individual plates, and brought to room temperature before use.
Starch agar medium is also commercially available as premixed dehydrated powder from biological supply companies. The manufacturer's instructions should be followed to prepare the plates. This medium can also be purchased as premade agar plates from biological supply companies.

PROTOCOL

Inoculation. Use a fresh (16- to 18-hour) pure culture of test bacteria as an inoculation source. Pick a single isolated colony and either single streak or spot inoculate the surface of the agar medium. A single starch agar plate can be divided into four quadrants for four different inoculations, except when using motile organisms.

Incubation. Incubate plates for 24 to 48 hours or longer (3 to 5 days) (3) at 35 ± 2^{o}C in an incubator.

Starch hydrolysis test. After proper incubation, flood the surface of the agar with Gram's iodine solution. Record results immediately as the blue-black color formed with starch may fade (1) giving a false-positive result of absence of starch. Appearance of a clear zone surrounding the bacterial growth indicates starch hydrolysis (+) by the organism due to its production of the extracellular enzymes. The zone will start out yellow (from the iodine) and becomeprogressively lighter yellow and then clear. The lack of a clear zone surrounding the growth indicates that starch is present and has not been hydrolyzed (-) and the organism did not produce the extracellular enzymes.

Flooding plates with iodine reagent does not contaminate the plates. Plates can be incubated further and retested if necessary (9). (Please refer to the Tips & Comments section).

Iodine reagent should be a rich yellow-gold to brownish color and stored in a dark bottle to avoid any light exposure. It should be tested with known positive and negative cultures before use.

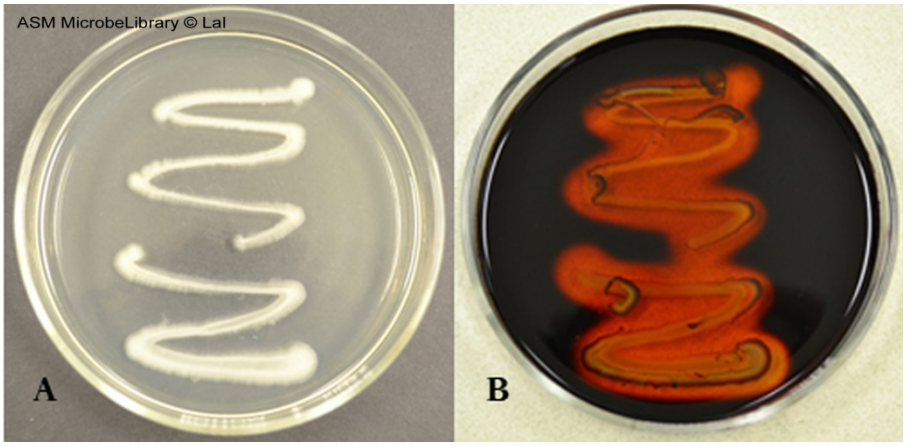

FIG 4 Growth of *Bacillus subtilis* on starch agar plate before the addition of iodine solution (A) and after the addition of iodine solution (B). After the addition of iodine the clearing surrounding the bacterial growth indicates starch hydrolysis (+).

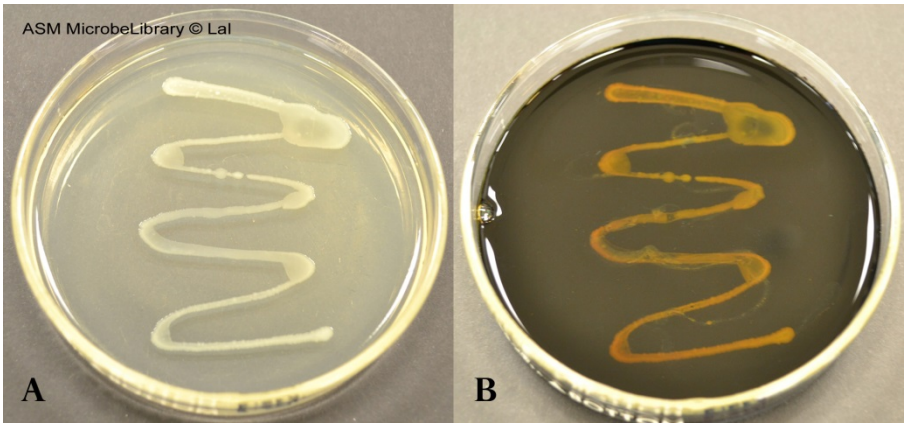

FIG 5 Growth of *Escherichia coli* on a starch agar plate before the addition of iodine solution (A) and after the addition of iodine solution (B). After the addition of iodine the dark blue or black color surrounding the bacterial growth (lack of a clear zone) indicates absence of starch hydrolysis (-).

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.

COMMENTS AND TIPS

By placing a white sheet under the plate, the yellow zone going clear will be more easily observed.

If reincubation is anticipated, do not flood the plate with the iodine reagent. Apply a few drops of iodine reagent to a small area of the agar around the growth. Then if needed, the plate can be reincubated and retested. There are two reasons for this variation: (i) iodine, as an antiseptic, may kill or inhibit further growth of the bacteria and (ii) flooding may cause the bacterial growth to lift off of the agar surface.

This protocol can lead to a class discussion on the biological and ecological significance of amylase production or other related metabolic exoenzymes. As questions to the students, (i) what is the purpose of the beef extract in the medium? (ii) If starch agar were prepared without beef extract, what would you expect to happen to the organisms you have tested?

Microbial amylase has many important applications in industry, including food processing (e.g., high fructose corn syrup production), pharmaceuticals (e.g., digestive aids), and textiles (e.g., laundering products and detergents) (2). The isolation of novel microbial amylases (e.g., that tolerate cold, alkaline conditions, etc.) is an ongoing discoveryprocess. Instructors are encouraged to consider such issues for challenge problems, scientific inquiry, or libraryresearch papers.

TABLE 1 Suggested BSL1 bacteria to use for starch agar protocol

Organism	ATCC numbers	Results[a]
Bacillus subtilis	ATCC 6051 or ATCC 465	(++)
Bacillus cereus	ATCC 2 or ATCC 13061	(+)
Staphylococcus epidermidis	ATCC 155 or ATCC 14990	(-)
Escherichia coli	ATCC 23724 or ATCC 33876	(-)
Serratia marcescens	ATCC 264 or ATCC 43862	(-)

[a] (++), strong positive reaction for starch hydrolysis; (+), positive reaction for starch hydrolysis; (-), negative reaction for starch hydrolysis.

REFERENCES

1. **Allen PW.** 1918. A simple method for the classification of bacteria as to diastase production. J. Bacteriol. **3:**15–17.
2. **Madigan MT, Martinko JM, Stahl DA, Clark DP.** 2012. Brock biology of microorganisms, 13th ed. Benjamin Cummings, San Francisco, CA. http://www.scribd.com/doc/74592238/Biology-of-Microorganisms-13th-Ed-M-Madigan-Et-Al-Pearson-2012-BBS.
3. **Collins CH, Lyne PM, Grange JM.** 1995. Collins and Lyne's microbiological methods, 7th ed, p 117. Butterworth-Heinemann, Oxford, United Kingdom.
4. **Corry JEL, Curtis GDW, Baird RM.** 2003. Handbook of culture media for food microbiology, 2nd ed. Elsevier Science, Amsterdam, The Netherlands.
5. **Cowan ST.** 1974. Cowan & Steel's manual for the identification of medical bacteria, 2nd ed, vol 12, p 148, 162. Cambridge University Press, Cambridge, England.
6. **Delly JG.** 2004. The literature of classical microchemistry, spot tests, and chemical microscopy. http://www.modernmicroscopy.com/main.asp?article=69&page=2.

Accessed 29 March 2012.
7. **Flint S, Hartley N.** 1996. A modified selective medium for the detection of *Pseudomonas* species that cause spoilage of milk and dairy products. Int. Dairy J. **2:**223–230.
8. **Leboffe MJ, Pierce BE.** 2010. Microbiology: laboratory theory and application, 3rd ed. Morton Publishing Company, Englewood, CO.
9. **Lennette EH, Balows A, Hausler WJ, Jr, Shadomy HJ.** 1985. Manual of clinical microbiology, 4th ed, p 918–919, 945. American Society for Microbiology, Washington, DC.
10. **MacFaddin JF.** 2000. Biochemical tests for identification of medical bacteria, 3rd ed, p 412–423. Lippincott Williams and Wilkins, Philadelphia, PA.
11. **Marini I.** 2007. Two hydrolytic enzymes and an epistemological-historical approach. School in Science Issue 4. http://www.scienceinschool.org/repository/docs/issue4_enzymes.pdf.
12. **Payen A, Persoz JF.** 1833. Memoire sur la diastase, les principaux produits de ses reactions et leur applicationsaux arts industriels. Annales de chimie et de physique **53:**73–92.
13 . **Vedder EB.** 1915. Starch agar, a useful culture medium. J. Infect. Dis. **16:**385–388.
14. **Voet D, Voet JG.** 1990. Biochemistry, p 256. John Wiley & Sons, New York, NY.
15. **Warden CC.** 1915. Studies on the Gonococcus, III. J. Infect. Dis. **16**(3)**:**426–440.
16. **Wilkins JR, Lewis G, Barriers AR.** 1956. Streptonivicin, a new antibiotic. III. In vitro and in vivo evaluation. Antibiot. Chemother. **6:**149–156.
17. **Worthington Biochemical Corporation**. 2012. Amylase alpha, I.U.B.: 3.2.1.1, 1,4-α-D-Glucanohydrolase. http://www.worthington-biochem.com/AA/default.html. Accessed 10 May 2012.
18. **Zimbro MJ, Power DA, Millwer SM, Wilson GE, Johnson JA.** 2009. Difco and BBL manual: manual of biological culture media, 10th ed, p 879–880. Becton Dickinson and Co., Sparks, MD.

REVIEWERS

This resource was peer-reviewed. Participating reviewers:

Thomas Edison dela Cruz
University of Santo Tomas, Manila, Philippines

Anne Hanson
University of Maine, Orono, ME

Roxana B. Hughes
University of North Texas, Denton, TX

Min-Ken Liao
Furman University, Greenville, SC

Karen A. Reiner

Andrews University, Berrien Springs, MI

Patricia Shields
University of Maryland, College Park, MD

Erica Suchman
Colorado State University, Ft. Collins, CO

Jeremy Martin O. Torres
Ateneo de Manila University, Quezon City, Philippines

2012 AD HOC PROTOCOL REVIEW COMMITTEE

Sue Katz Amburn
Rogers State University, Claremore, OK

Benita Brink
Adams State University, Alamosa, CO

Elaine Brunschwig
Cuyahoga Community College, Cleveland, OH

Laura Cathcart
University of Maryland, College Park

Deborah V. Harbour
College of Southern Nevada, Las Vegas, NV

Jackie Reynolds
Richland College, Dallas, TX

Kate Rodgers
Virginia Tech, Blacksburg, VA

CONTRIBUTORS

The following contributed to the Comments and Tips section at the ASM Conference for Undergraduate Educators2012.

Participating contributors:

Jason C. Baker
Missouri Western State University, Saint Joseph, MO

Sarah Boomer
Western Oregon University, Monmouth, OR

Joseph Caruso
Florida Atlantic University, Boca Raton, FL

William Courctiesne
University of Nevada, Reno, NV

Michael Hanophy
St. Joseph's College, Brooklyn, NY

Zoe Hawk
Arizona Western College, Yuma, AZ

Geoffrey Holm
Colgate University, Hamilton, NY

Norrenna Hubbard
Hondros College, West Chester, OH

Tamara McNealy
Clemson University, Clemson, SC

Elizabeth Mitchell
Central Piedmont Community College, Charlotte, NC

Jeffrey Pommerville
Glendale Community College, Glendale, AZ

S. N. Rajagopal
University of Wisconsin, Lacrosse, WI

Jackie Reynold
Richland College, Dallas, TX

Harlan Scott
Howard Payne University, Brownwood, TX

Lisa Spring
Central Piedmont Community College, Charlotte, NC

Jacqueline Washington
Nyack College, Nyack, NY