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| ALKEN-MURRAY CORPORATION | TITLE: RHODAMINE B AGAR PREPARATION PROCEDURE | NO. QC-99 |
| MICROBIOLOGICAL DIVISION | QUALITY CONTROL | REV: 3 |
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ALKEN-MURRAY CORPORATION

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QUALITY CONTROL METHOD - 99

Preparation and Use of Rhodamine B Lipase Agar

Description:

This quality control procedure is designed to verify that Rhodamine B Agar is properly prepared, used and stored. This procedure demonstrates the ability of some bacteria to produce lipase and degrade food oils, such as olive oil, in a clear and easily interpreted manner. We found that the Spirit Blue method (QC-94) was harder to interpret, leading to misinterpreted results. This procedure should be performed by a trained laboratory technician.

Ingredients:

For lipoidal emulsion (50 ml per 450 ml of base medium) - enough lipoidal emulsion for 1 liter:

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| Olive oil | 30 ml |
| Tween 80 | 250 μ l |
| Distilled water | 50 ml |
| Rhodamine B ¹ | 20 mg/20 ml sterile water (0.02% w/v total media prepared) |

1. Add filter sterilized Rhodamine B solution, after autoclaving other ingredients

For base medium:

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|-----------------|---------------------------------|
| Nutrient Broth | 4.5 g (alternate Peptone water) |
| Yeast extract | 1.25 g |
| Agar | 10 g |
| Distilled water | 450 ml |

Final pH 7.0 \pm 0.2

Equipment and Supplies:

- 500 ml Graduated cylinder
- 50 ml Graduated cylinder
- 1 ml sterile syringe with 18 gauge needle
- 10 ml sterile syringe with 18 gauge needle
- 10 ml sterile serological pipet
- Drummond* Pipet-Aid® or rubber bulb
- 2 - 50 ml sterile serological pipets

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1 Liter Erlenmeyer flask with ground glass stopper(double the volume of media being prepared)
 250 ml Erlenmeyer flask (for lipoidal emulsion)
 250 ml Sterile Media Storage bottle (to store excess lipoidal emulsion)
 Laboratory balance sensitive to 0.001 g
 Heated magnetic stirrer plate
 Stir bar
 Autoclave
Oster or *Waring* blender
 pH meter
 Calibrated pH solutions to verify pH meter (we prefer disposable pouches (pH 4, 7 & 10 for accuracy)
 Wash bottle with 500 ml of distilled water
 Sterile disposable 10 µl inoculating loop-needles (yellow) or sterile 3 mm platinum loop
 Thermometer
 rubber stoppers
Green Steri-Wrap II
 rubber bands for closure
 Rapid-Flo double gauze Milk Filter (from Filter Fabrics Inc.) to seal flasks (with *Steri-Wrap II*)
 Sterile gloves
 Sterile disposable 100 mm petri dishes
 Weighing dish
BBL GasPak 100 or *150 Anaerobic System*
BBL GasPak Plus carbon dioxide and hydrogen gas envelope with Palladium catalyst
 0.2 µm syringe filter and sterile 25 ml disposable syringe

Procedure:

1. Purchase ingredients and supplies from approved sources.
2. Place a label on packages with expiration date marked in bold RED letters
3. Discard unused media by expiration date marked on the package.
4. Add 250µl (0.25 ml) of Tween 80 to 50 ml of distilled water in 250 ml Erlenmeyer flask. Disposable sterile 1 cc syringe may be easier to use than pipet, due to viscosity of solution.
5. Add 30 ml of olive oil. If you are not using a *Drummond* Pipet-Aid®, rubber bulb will not offer sufficient suction to hold olive oil in pipet, so use disposable 50 cc sterile syringe instead.
6. Emulsify mixture in *Waring* or *Oster* blender and test pH. Adjust to 7.0 ± 0.2.
7. Autoclave solution separately from completed base medium, but in same autoclave cycle.

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8. Alternative preparation of lipoidal emulsion: sterilize olive oil in 200 deg. C oven for 4 hours, then add aseptically to sterile water and Tween in sterilized *Waring* blender jar. Then keep warm in sterile double boiler and wait for bubbles to subside before adding to base medium. We found this more complicated and harder to keep ingredients sterile through this procedure.
9. Fill 1 liter Erlenmeyer flask with 200 ml distilled water.
10. Weigh base medium ingredients and add to flask.
11. Add 250 ml more of distilled water, washing down the sides of the container.
12. Test pH at 7.0 ± 0.2. Adjust if necessary.
13. Insert stirbar in flask and apply closure.
 - 11.1 A 1-2 liter flask closure consists of a double layer of steri-wrap (or equivalent) with a double gauze Rapid-Flo milk filter (or equivalent) sandwiched in between, secured with rubber bands
14. Place flask over heated stir plate, and heat gently until it reaches the boiling point. Boil as briefly as possible to obtain solution. Avoid excess boiling so as to minimize foaming and water evaporation. To avoid loss of flask contents, be prepared for possible foaming of the medium and immediately remove the flask from the heat source when this begins.
15. Autoclave medium and emulsion for 15 minutes at 121°C at 15 psi.
16. Allow media to cool to 60°C and maintain temperature in waterbath.
17. Aseptically add 50 mg of Rhodamine B to 50 ml of sterile water. Mix thoroughly.
18. Using 25 cc sterile disposable syringe and 0.2 µm syringe filter, add 20 ml of Rhodamine B solution to lipoidal emulsion and stir thoroughly.
19. Add 50 ml of lipoidal emulsion and dye to base medium and mix thoroughly.
20. Pour 10 - 12 ml volumes into sterile 100 mm petri dishes immediately.
21. Inoculate with 1 loopful from yellow sterile individually wrapped 10µl loops or two loopfuls from 3 mm platinum loop, sterilized.
22. Place in GasPak 100 or 150 Anaerobic System and activate GasPak Plus envelope. Seal jar by hand tightening screw. Do not over tighten or you will crack lid and ruin it.
23. Incubate at 35°C for 48 to 96 hours

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24. Check for lipase enzyme by viewing culture plates under UV lamp at 350 nm. Those strains that produce strong levels of lipase will glow brilliant pink-red under UV lamp.
25. Read and record results.

References:

1. E-mail dated 7/16/2001 from Yves Hurtubise of Choisy Laboratories in Toronto, Canada