ANEXO A

MEIOS DE CULTURA

IDENTIFICAÇÃO DA BACTÉRIA

Meios de cultura

1. Bactéria

A bactéria utilizada foi *Bacillus cohnii* da colecção DSMZ 6307. A cultura inicial foi feita a partir da bactéria liofilizada e todas as outras a partir da inoculação em glicerol a -80ºC.

2. Meios de Cultura

Bacillus cohnii foi cultivada em condições aeróbias com os meios recomendados pelo laboratório: 5g de peptona, 3g de extracto de carne, 0,42g $NaHCO_3$ e 0,53g $NAHCO_3$ por cada litro de água destilada (pH=9,7) e também em meio alcalino suplementado com manganês para aumentar a capacidade de esporulação por parte da bactéria.

O meio alcalino contém por cada litro de água destilada 0,2g de NH_4Cl, 0,02g de KH_2PO_4, 0,225g de $CaCl_2$, 0,2g de KCl, 0,2g $MgCl_2.6H_2O$, 0,01g $MnSO_4.2H_2O$, 1ml de SL12B, 0,1g de extracto de levedura, 5,16g de ácido cítrico (*citric acidum trisodium salt*), 4,2g de $NaHCO_3$ e 5,3g de Na_2CO_3 (pH próximo de 10). A cultura foi inoculada em tubos "*Falcon*" a 150rpm e 31ºC e quantificada em microscópio óptico com lente de imersão.

As culturas já esporuladas após 24h foram lavadas em centrifugação repetida a 7500rpm durante 10minutos e diluídas novamente em água da torneira para introdução no betão.

1. NUTRIENT AGAR

Peptone	5.0	g
Meat extract	3.0	g
Agar, if necessary	15.0	g
Distilled water	1000.0	ml

Adjust pH to 7.0. For Bacillus strains the addition of 10.0 mg $MnSO_4$ x H_2O is recommended for sporulation.

28. PFENNIG'S MEDIUM I (modified 1988, for purple sulfur bacteria)

Solution A:

$CaCl_2$ x 2 H_2O	1.25	g
KH_2PO_4	1.70	g
NH_4Cl	1.70	g
KCl	1.70	g
$MgSO_4$	2.50	g
Distilled water	4000.00	ml

(For marine or estuarine isolates add 100.0 g NaCl to this solution and increase the $MgSO_4$ x 7 H_2O to 15.0 g).

Solution B:

Distilled water	860.00	ml

Autoclave in a cotton-stoppered Erlenmeyer flask and cool to room temperature under an atmosphere of N_2 in an anaerobic jar.

Solution C:

Vitamin B_{12} solution (0.002% in H_2O)	5.00	ml

Filter sterilize.

Solution D:

Trace element solution (SL-12 B)	5.00	ml

Autoclave at 121°C for 15 min.

Solution E:

$NaHCO_3$	7.50	g
H_2O	100.00	ml

Bubble with CO_2 and, after saturation, filter sterilize under CO_2 pressure into sterile, gas-tight, 100 ml screw-cap bottle.

Solution F:

Na_2S x 9 H_2O (10 g in 100 ml)	20.00	ml

Prepare in a screw-cap bottle, bubble with N_2 to replace air, close tightly and autoclave.

Continued on next page

Trace element solution SL-12 B:

Distilled water	1000.00	ml
Na_2-EDTA	3.00	g
$FeSO_4$ x 7 H_2O	1.10	g
$CoCl_2$ x 6 H_2O	190.00	mg
$MnCl_2$ x 2 H_2O	50.00	mg
$ZnCl_2$	42.00	mg
$NiCl_2$ x 6 H_2O	24.00	mg
Na_2MoO_4 x 2 H_2O	18.00	mg
H_3BO_3	300.00	mg
$CuCl_2$ x 2 H_2O	2.00	mg

Adjust pH to 6.0.

Autoclave solution A for 45 min. in 5-litre special bottle or flask (with four openings at the top) at 121°C, together with a teflon-coated magnetic bar. In this 5-litre bottle, two openings for tubes are in the central, silicon rubber stopper; a short, gas-inlet tube with a sterile cotton filter; and an outlet tube for medium, which reaches the bottom of the vessel at one end and has, at the other end, a silicon rubber tube with a pinch cock and a bell for aseptic dispensing of the medium into bottles. The other two openings have gas-tight screw caps; one of these openings is for the addition of sterile solutions and the other serves as a gas outlet.

After autoclaving cool solution A to room temperature under a N_2 atmosphere with a positive pressure of 0.05 - 0.1 atm (a manometer for low pressure will be required). Saturate the cold medium with CO_2 by magnetic stirring for 30 min. under a CO_2 atmosphere of 0.05 - 0.1 atm. Add solution B, C, D, E and F through one of the screw-cap openings against a stream of either N_2 gas or better, a mixture of 95% N_2 and 5% CO_2 while the medium is magnetically stirred.

Adjust the pH of the medium with sterile HCl or Na_2CO_3 solution (2 mol/liter each) to pH 7.3. Distribute the medium aseptically through the medium outlet tube into sterile, 100 ml bottles (with metal caps and autoclavable rubber seals) using the positive gas pressure (0.05 - 0.1 atm) of the N_2/CO_2 gas mixture: Leave a small air bubble in each bottle to meet possible pressure changes. The tightly sealed, screw-cap bottles can be stored for several weeks or months in the dark. During the first 24 h, the iron of the medium precipitates in the form of black flocks. No other sediment should arise in the otherwise clear medium. Incubate in the light using a tungsten lamp. Feed periodically with neutralized solution of sodium sulfide (see medium 27) to replenish sulfide and with other supplement solutions (see Ref. 3365).

31. ALKALINE NUTRIENT AGAR

Same as medium 1. After sterilization add sterile 1 M Na-sesquicarbonate solution (1 ml in 10 ml) to achieve a pH of 9.7.

Na-sesquicarbonate solution:

$NaHCO_3$	4.2	g
Na_2CO_3 anhydrous	5.3	g
Distilled water	100.0	ml

Reference: 064-TA6074 **Scharlau Microbiology - Technical Data Sheet**

Product: **MAXIMUM RECOVERY DILUENT (MRD)**

Specification

Isotonic diluent for the maximal recovery of stressed microorganisms according to ISO standards.

Presentation

20 Prepared tubes

Tube 16 x 113 mm

with: 9 ± 0.5 ml.

Packaging Details

1 box with 20 tubes, 16x112 mm glass tubes, ink labelled and metallic cap

Composition

Formula in g/l

Peptone.....1,00
Sodium chloride.....8,50

pH final 7,0 ±0,2 at 25ºC

Description

This formulation combines the osmotic pressure of the physiological saline solution with the protective action of the peptone to obtain good recovery of stressed microorganisms.
The sodium chloride ensures isotonic conditions and the low concentration of peptone does not allow cellular growth in the short period (2-4 hours) of time required for the preparation of the dilution bank of the sample.

Usage instructions

According to the ISO method, the sample is diluted in a ratio 1:10 with the Maximum Recovery Diluent and homogenized by a vortex mixer or Stomacher®. After a short period (10-15 minutes) of rest, a 1/10 dilution bank with the same diluent is prepared following standard procedures. Plates are inoculated using the range of different concentrations.

Quality control

Color : Colourless **pH:** (at 25 ºC) 7,0±0,2

Incubation temperature: 35ºC ±2,0 **Incubation time:** 24 h

Inoculum: 10-100 CFU. (Productivity) at 3 h. (20-25ºC)

Microorganism	Growth	Remarks
Staphylococcus aureus ATCC 6538	Good	Satisfactory
Pseudomonas aeruginosa ATCC 9027	Good	Satisfactory
Escherichia coli ATCC 8739	Good	Satisfactory
Candida albicans ATCC 10231	Good	Satisfactory
Bacillus subtilis ATCC 6633	Good	Satisfactory

Sterility Control

No growth within 48 h and 7 days at 20-25ºC and 30-35ºC

Storage/Shelf Life

Shelf Life	Storage
12 months	8-25ºC

Bibliography

· ISO 6887-1: 1999 Microbiology of food and animal feeding stuffs. Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions - Part 2 (2003): Specific rules for the preparation of meat and meat products.
· ISO 8261: 2001 Standard. Milk and milk products - General guidance for the preparation of test samples, initial suspension and decimal dilution for microbiological examination.
· ISO 21149: 2006 Standard. Cosmetics - Enumeration and detection of aerobic mesophilic bacteria.
· ISO 21150: 2006 Standard. Cosmetics - Detection of Escherichia coli.
· ISO 22717: 2006 Standard. Cosmetics - Detection of Pseudomonas aeruginosa.
· ISO 22718: 2006 Standard. Cosmetics - Detection of Staphylococcus aureus.