International Rules for Seed Testing
Annexe to Chapter 7: Seed Health Testing Methods

7-021: Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* on *Phaseolus vulgaris*

Published by: International Seed Testing Association (ISTA), Bassersdorf, Switzerland
2007

Crop: *Phaseolus vulgaris* (bean)

Pathogen: *Xanthomonas axonopodis* pv. *phaseoli* (common blight) and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans*

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Revision History: Version 1.0, 22 February 2006.
Version 2.0, 18 August 2006.

Background

This method is derived from the validation studies carried out by ISTA in 2003, in collaboration with the International Seed Health Initiative for Vegetables (ISHI-Veg) (Sheppard and Remeeus, 2005). For routine testing of bean seed a combination of two complementary semi-selective media, MT and XCP1, is recommended with a pathogenicity test to confirm suspect isolates.

The two media, XCP1 and MT, have been chosen for their ease of use and selectivity for *X. axonopodis* pv *phaseoli*. In addition both media can be used to detect both *X. axonopodis* pv *phaseoli* and *X. axonopodis* pv *phaseoli* var *fuscans*. Although initially the morphology of fuscans and non-fuscans strains of *X. axonopodis* pv *phaseoli* appear to be similar on the media after a longer incubation the fuscans colonies are distinguished by a distinct brown pigmentation. A further advantage of MT medium is that it can also be used for identifying other bacterial seed-borne pathogens of beans, e.g. *Pseudomonas savastanoi* pv. *phaseolicola* and *Pseudomonas syringae* pv. *syringae*.

Validation Studies

Sheppard, J.W. and Remeeus, P.M. (2005)

Copies are available: by E-mail from ista.office@ista.ch; by mail from the ISTA Secretariat, Zürichstrasse 50, 8303 Bassersdorf, Switzerland.

Detection limit 15 cfu/ml (theoretical, P=0.95).

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat

Safety Precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during preparation of media, autoclaving, and weighing out of ingredients. It is assumed that this procedure is being carried out in a microbiological laboratory by persons familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic technique. Dispose of all waste materials in an appropriate way (e.g. autoclave, disinfect) and in accordance with local health, environmental and safety regulations.

Treated Seed

Seed treatments may affect the performance of this test. It must only be performed on untreated seed.

Sample and sub-sample size

The sample (total number of seeds tested) and sub-sample size to be tested depends on the desired tolerance standard (maximum acceptable percentage of seeds infested) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected); in any case the maximum sub-sample size should be 1000 seeds.

Materials

Reference material	-	A known strain for both fuscans and non fuscans types of *X. axonopodis* pv. *phaseoli* or standardised reference material.
Plates of MT medium	-	9.0 cm Petri dishes (3 plates of each medium per sub-sample + controls)
Plates of XCP1 medium	-	9.0 cm Petri dishes (3 plates of each medium per sub-sample + controls)
Plates of YDC	-	for sub-culture (at least 1 per sub-sample).
Polythene bags or containers	-	of sterile saline (0.85% NaCl) plus Tween 20 (0.02% - 0.2 ml per litre) for soaking seeds (volume (ml) required is equivalent to 2.5 x TSW (g)).
Dilution bottles	-	containing 4.5 ml of sterile saline (2 per sub-sample). Other volumes may be acceptable, see General Methods.
70% ethanol	-	for disinfection of surfaces, equipment
Incubator	-	operating at 28°C ± 2°C
Balance	-	capable of weighing to the nearest 0.001 g
Automatic pipettes	-	check accuracy and precision regularly
Bean seedlings	-	susceptible to all races of the pathogen for pathogenicity test (e.g. Michelet, Contender)

Sample Preparation

1. This can be done in advance of the assay.
2. It is vital to exclude any possibility of cross-contamination between seed samples. It is therefore essential to disinfect all surfaces, containers, hands, etc. both before and after handling each sample. This can achieved by swabbing/spraying equipment and gloved hands with 70% ethanol.
3. If the submitted sample is received in several packets, these should be combined by emptying into a new, clean polythene bag and mixing by hand to give a composite sample.
4. Count the number of seeds in a known weight. Estimate the Thousand Seed Weight (TSW) as:

$$TSW = \frac{Weight\ of\ seed}{Number\ of\ seed} \times 1000$$

5. Based on the estimated TSW, weigh out sub-samples of the required size into new, clean polythene bags or containers.

Method

[Critical control points are indicated by **CCP**]

1. *Extraction*
   1.1. Suspend seeds in sterile saline plus Tween 20 (0.02% v/v) in a polythene bag or container. The volume of saline required in ml should be equivalent to 2.5 x TSW (g) e.g. TSW = 300 g, therefore volume of saline required is 2.5 x 300 = 750 ml (Olivier and Remeeus, 2004).
   1.2. Soak sub samples overnight (16-18 h) at 5°C (± 4°C).
2. *Dilution and plating*
   2.1. Shake containers or polythene bags to obtain a homogenous extract before dilution.
   2.2. Prepare a tenfold dilution series from the seed extract. Pipette 0.5 ml of the extract into 4.5 ml of sterile saline and vortex to mix (10^1 dilution). Pipette 0.5 ml of the 10^1 dilution into another 4.5 ml of sterile saline and vortex to mix (10^2 dilution) (see General Methods).
   2.3. Pipette 100 µl of each dilution and the un-diluted seed extract onto plates of each of the selective media (MT and XCP1) and spread over the surface with a sterile bent glass rod (see General Methods).
   2.4. Incubate inverted plates at 28°C ± 2°C and examine after 4-5 d.
3. *Positive control (culture or reference material)*
   3.1. Prepare a suspension of a known strain of *X. axonopodis* pv. *phaseoli*, fuscans and non-fuscans, in sterile saline or reconstitute standardised reference material according to the supplier's instructions.
   3.2. Dilute suspension sufficiently to obtain dilutions containing approximately 10^2 to 10^4 cfu/ml. This may require up to seven ten-fold dilutions from a turbid suspension.
   3.3. Pipette 100 µl of appropriate countable dilutions onto plates of each of the selective media (MT and XCP1) and spread over the surface with a sterile bent glass rod.
   3.4. Incubate plates with the sample plates.
4. *Sterility check*
   4.1. Prepare a dilution series from a sample of the extraction medium (i.e., saline

plus Tween 20), containing no seeds, and plate on each of the media as for samples.

5. Examination of the plates

5.1. Examine sterility check and recovery of positive control on semi-selective medium (**CCP**).

5.2. Examine the sample plates for the presence of typical *X. axonopodis* pv. *phaseoli* colonies by comparison with the positive control plates.

5.3. After 4-5 d on MT, *X. axonopodis* pv *phaseoli* colonies are yellow distinguished by two zones of hydrolysis; a large clear zone of casein hydrolysis and a smaller milky zone of Tween 80 lysis (Fig. 1a and b). The fuscans of *X. axonopodis* pv *phaseoli* colonies produce a brown diffusible pigment. If not visible after 4 d inclubate for an additional day. Often the fuscans type colonies show Tween 80 lysis.

5.4. After 4-5 d on XCP1, *X. axonopodis* pv *phaseoli* colonies are yellow, glistening and surrounded by a clear zone of starch hydrolysis (Fig. 2b). The fuscans of *X. axonopodis* pv *phaseoli* colonies produce a brown diffusible pigment after 5 d of incubation (Fig 2a). Often the fuscans type colonies show Tween 80 lysis.

5.5. The colony size and colour can differ within a sample.

5.6. Estimate the number of suspect and other colonies (see General Methods).

6. *Confirmation/identification of suspect colonies*

6.1. Sub-culture suspect colonies to sectored plates of YDC. To avoid the potential for cross-contamination of isolates, use a new sectored plate for each sub-sample. The precise numbers of colonies sub-cultured will depend on the number and variability of suspect colonies on the plate: if present, at least six colonies should be sub-cultured per sub-sample (**CCP**).

6.2. Sub-culture the positive control isolate to a sectored plate for comparison (Fig. 3).

6.3. Incubate sectored plates for 24-48 h at 28°C ± 2°C.

6.4. Compare appearance of growth with positive control. On YDC *X. axonopodis* pv *phaseoli* colonies are yellow and mucoid in appearance (Fig. 3) (**CCP**).

6.5. Confirm the identity of isolates by pathogenicity on bean seedlings of known susceptibility (**CCP**).

6.6. Record results for each colony sub-cultured.

7. *Pathogenicity* (Saettler, 1971)

7.1. Grow seedlings of a bean cultivar known to be susceptible to all races of *X. axonopodis* pv. *phaseoli* (e.g. Michelet or Contender at 20-30°C in small pots or modules until the first leaf is just visible (usually about 10 days after sowing).

7.2. Water plants 1-3 h before inoculation to provide better conditions for infection.

7.3. Dip a sterile toothpick or needle into the bacterial culture of a 2-4 d old YDC culture (i.e. sectored plate).

7.4. Inoculate the seedling by stabbing the toothpick or needle through the primary node at an angle of about 45°. Stop stabbing as the needle emerges from the opposite side of the node. Turn the toothpick or needle slightly while drawing to release bacteria. Support the seedling with one hand during the process.

7.5. The number of plants which should be inoculated will depend on the variability of the cultivar and experience of the operator, but 1-3 plants per isolate should usually be sufficient. It is better to inoculate more isolates with 1 plant per isolate than one isolate with 3 plants.

7.6. Inoculate seedlings with the two positive control isolates fucans and non-fucans (following 7.3-7.5) (**CCP**) and a negative control with only a sterile toothpick or needle (**CCP**).

7.7. Grow on plants at 20-25°C.

7.8. Record symptoms after, 8-10 d and again at 14 d. Compare with positive control (**CCP**). Typical *X. axonopodis pv. phaseoli* symptoms include dark green water-soaked lesions at the point of entry of the toothpick or needle. Lesions can become red-brown, elongate extending into the stem causing slight to severe stem cracking (Fig. 4). Symptons for fuscans and non-funscans are the same. Should the time from inoculation be extended wilting and flagging of the top foliage followed by necrosis can occur 14-18 d after inoculation.

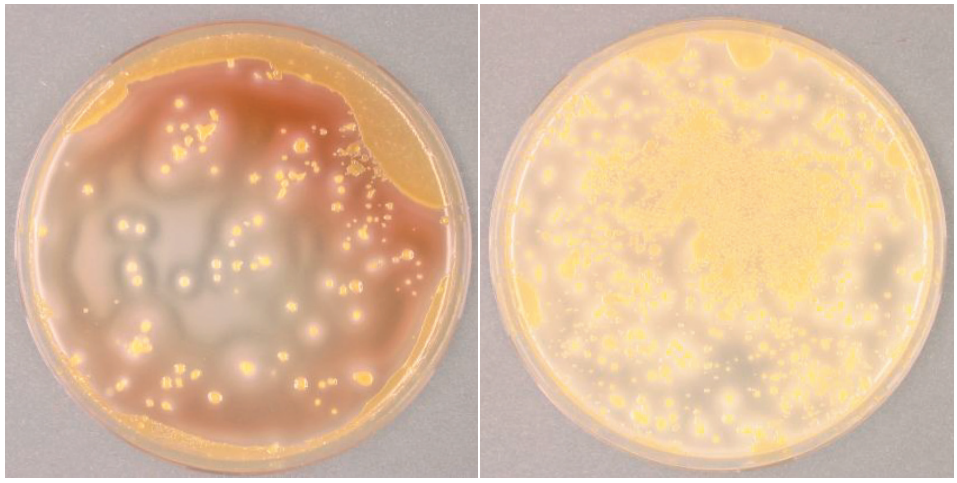

Fig. 1a **Fig. 1b**

Fig. 1. *X. axonopodis* pv. *phaseoli* colonies on MT plates after 4 d indicated by a large clear zone of casein hydrolysis (a) and a smaller milky zone of Tween 80 lysis (b).

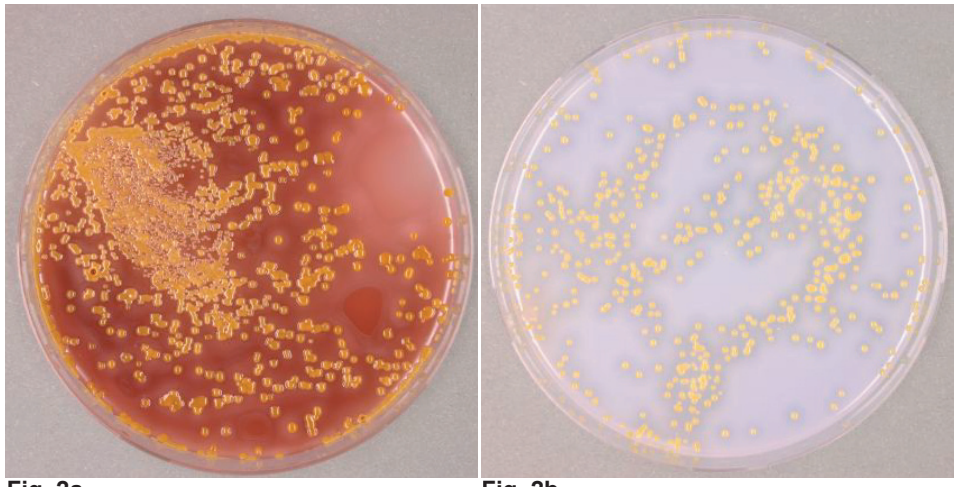

Fig. 2a **Fig. 2b**

Fig. 2. *X. axonopodis* pv. *phaseoli* colonies, fuscans (a) and non-fuscans (b), on XCP1 plates showing a clear zone of starch hydrolysis and fuscans on XCP1 showing a milky zone, after 4 d

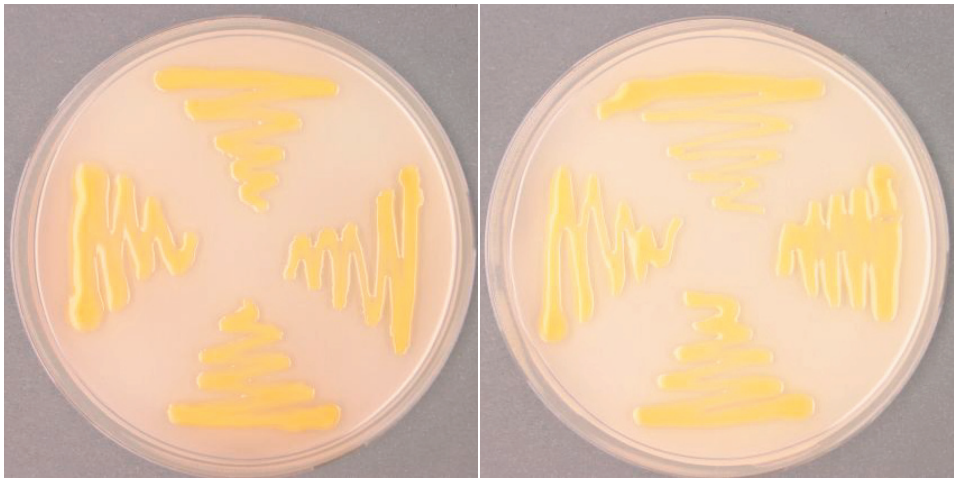

Fig. 3a **Fig. 3b**

Fig. 3. *X. axonopodis* pv. *phaseoli* colonies, fuscans (a) and non-fuscans (b), on YDC plates after 2 d are brown and yellow in appearance.

Fig. 4a **Fig. 4b**

Fig. 4. *Phaseolus vulgaris* stem 10 days after inoculation with typical lesion of *X. axonopodis* pv. *phaseoli* (a), and a negative control (b).

General Methods (common to many test procedures)

1. *Preparation of ten-fold dilution series*
   Each dilution should be prepared by pipetting 0.5 ml (± 5%) from a well-mixed seed extract or previous dilution into a universal bottle (screw-capped) or similar container containing 4.5 ml (± 2%) of sterile diluent and then vortexing to mix prior to the next dilution step. A new sterile pipette tip should be used for each dilution step. Pipettes should be checked regularly for accuracy and precision and re-calibrated as necessary. It is acceptable to prepare ten-fold dilutions using other volumes provided that the laboratory can demonstrate that the required accuracy and precision can be achieved.
2. *Plating of dilutions.*
   This should be done as soon as possible after dilutions have been prepared and certainly within 30 min. Working from the highest (most dilute) dilution to the undiluted extract, 0.1 ml is pipetted onto the centre of a surface-dry, labelled agar plate. The liquid should then be spread evenly over the entire surface of the medium with a bent glass rod. If care is taken to work from the highest to the lowest dilution (or undiluted extract) a single pipette tip and a single bent glass rod can be used for each sample. Ensure that all liquid has been absorbed by the agar before inverting and incubating plates. If necessary allow plates to dry under a sterile air-flow in a microbiological safety cabinet or laminar flow hood.
3. *Recording of dilution plates*
   Record the results for all dilution plates. The most accurate estimate of bacterial numbers should be obtained from spread plates with total number between 30 and 300 colonies. However this may be further complicated depending on the relative numbers of suspect pathogen and other colonies. In order to minimise effort, start recording with the highest dilution (most dilute) and count the number of suspect and the number of other colonies. If the total number of colonies on a plate greatly exceeds 300 there is little value in trying to make a precise count if a more reliable count has already been obtained from a more dilute plate, in which case it is sufficient to record the number of colonies as ‘m’ (many) if they are still separate or ‘c’ (confluent) if they have run together.
4. *Sectored Plates*
   Using a laboratory marker pen draw lines on the base of a standard 9 cm plate (Petri dish) to divide it into six equal sectors. Sub-culture single colonies from dilution plates and make a single zigzagged streak within a single sector on the plate. Take care to leave sufficient space between each isolate to ensure the growth does not coalesce. Thus six suspect colonies can be sub-cultured to each sectored plate. Separate plates should be used for each sample/sub-sample. If the purity of sub-cultured isolates is doubtful, they should be further streaked out on whole plates.
5. *Reporting Results*
   The result of a seed health test should indicate the scientific name of the pathogen and the test method used. When reported on an ISTA Certificate, results are entered under *Other Determinations*.

   In the case of a negative result (pathogen not detected in any sub-samples), the results should be reported in terms of the tolerance standard and detection limit. The tolerance standard depends on the total number of seeds tested, n, and is approximately 3/n (P=0.95) (see Roberts *et al*., 1993); the detection limit per sub-sample is equal to the detection limit per ml multiplied by the volume of extract.

   In the case of a positive result, the report should indicate the mean number of pathogen

propagules (cfu) per seed and either the number of positive sub-samples out of the total number tested and the sample size or the maximum likelihood estimate of the proportion of infested seeds (Most Probable Number).

Quality Assurance

General

A record should be kept of the date and results of pipette calibration checks.

It is essential that operators have received appropriate training and use automatic pipettes correctly.

Critical Control Points

[Identified by **CCP** in the methods]

Dilution plates prepared from positive control isolate(s) or reference material, should give single colonies with typical morphology (Step 5.1 and 6.4).

The numbers of colonies on dilution plates prepared from the positive control isolate(s) or reference material should be similar on both media (Step 5.1). Note that recovery of the fuscans type *X. axonopodis* pv. *phaseoli* is in general lower on MT than on XCP1.

Numbers of bacteria on dilution plates should be consistent with the dilution (i.e. should decrease approximately ten-fold with each dilution) (Step 5.1).

There should be no growth on dilution plates prepared as a sterility check (Step 5.1).

Due to the potential for non-pathogenic isolates to be present in seed lots together with pathogenic isolates, it is essential to sub-culture (Step 6.1), if present, at least the minimum number of suspect colonies specified (six per sub-sample) and to test all *Xanthomonas*-like sub-cultured isolates for pathogenicity (Step 6.5).

Positive control isolates should be included in every pathogenicity test (Step 7.6).

The positive control isolate should give typical symptoms in a pathogenicity test (Step 7.8).

The quality of milk powder is vital to develop the hydrolysis of starch in MT medium. Two milk sources that work well are Oxoid and Sigma brands.

The activity (g) of some antibiotics may vary between batches. It may be necessary to adjust the weight or volume added to ensure that the final number of units per litre of medium is consistent (MT and XCP1 media).

Preparation of sterile saline (van Vuurde *et al.*, 1989)

Compound	**g/l**
Sodium chloride (NaCl)	8.5
Distilled/de-ionised water	1 000 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1 000 ml of distilled/de-ionised water.
3. Dissolve and dispense into final containers.
4. Autoclave at 121°C, 15 psi for 15 min.
5. For extraction of seeds, add 0.2 ml of sterile Tween 20 per 1 000 ml.

Storage

Provided containers are tightly closed, may be stored for several months before use.

Preparation of MT (Milk Tween) agar medium
(adapted from Goszczynska and Serfontein, 1998)

Compound	g/l
A	
Proteose peptone no. 3	10.0
$CaCl_2$	0.25
Tyrosine	0.5
Agar	15.0
Distilled/de-ionised water	500 ml
B	
Skim milk powder (Oxoid, Sigma) CCP	10.0
Distilled/de-ionised water	500 ml
C	
Tween 80	10.0 ml
D	
Nystatin[a]	40 mg (1 ml)
Cephalexin[b]	80 mg (1 ml)
Vancomycin[c]	10 mg (1 ml)

[a, b, c] Added after autoclaving

Preparation

1. Weigh out all ingredients in section A into a suitable container.
2. Add 500 ml of distilled/de-ionised water.
3. Dissolve ingredients.
4. In a separate container, dissolve skim milk powder in 500 ml distilled water.
5. Separately prepare 10 ml Tween 80.
6. Sterilise preparations from section A, skim milk solution (section B) and Tween 80 (section C) separately at 121ºC, 15 psi for 15 min.
7. After sterilisation, of all components aseptically add sterilised skim milk preparation and sterilised Tween 80 to sterilised ingredients in section A.
9. Prepare antibiotic solutions (section D).
10. Allow medium to cool to approximately 50ºC and add antibiotics.
11. Mix gently to avoid air bubbles and pour plates 22 ml per 9.0 cm plate.
12. Leave plates to dry in laminar flow bench or similar before use.

Antibiotics (amounts for guidance only, **CCP**)

[a] Dissolve 400 mg nystatin in 10 ml 70% ethanol.

[b] Dissolve 800 mg cephalexin in 10 ml 70% ethanol.

[c] Dissolve 100 mg vancomycin in 10 ml 70% ethanol.

(Filter sterilise when antibiotics are dissolved in water rather than 70% ethanol.)

Note

Cycloheximide can be used as an alternative for nystatin to control fungi. Dissolve 500 mg of Cycloheximide in 10 ml 70% ethanol, add 1 ml to cool medium.

Storage

Store prepared plates inverted in polythene bags at 4-8°C and use within two weeks of preparation to ensure activity of antibiotics.

Preparation of XCP1 agar medium

(adapted from McGuire *et al.*, 1986)

Compound	g/l
A	
KBr	10.0
$CaCl_2$	0.25
Soluble Potato Starch	10.0
Peptone	10.0
Agar	15.0
Crystal violet (1% aqueous)	0.15 ml
Distilled water/de-ionised water	1 000 ml
B	
Tween 80	10.0 ml
C	
Nystatin[a]	40 mg (1 ml)
Cephalexin[b]	10 mg (1 ml)
Fluorouracil[c]	3 mg (1 ml)
Tobramycin[d]	0.16 mg (1 ml)

[a, b, c, d]Added after autoclaving

Preparation

1. Weigh out all ingredients in section A into a suitable container.
2. Add 1 000 ml of distilled water/de-ionised water.
3. Dissolve ingredients.
4. Add crystal violet.
5. Sterilise 121ºC, 15 psi for 15 min.
6. Sterilise 10 ml Tween 80 separately (section B) at 121ºC, 15 psi for 15 min.
7. Aseptically add Tween 80 to ingredients in section A.
8. Prepare antibiotic solutions.
9. Allow medium to cool to approximately 50ºC and add antibiotics.
10. Mix gently to avoid air bubbles and pour plates (22 ml per 9.0 cm plate).
11. Allow plates to dry in laminar flow bench or similar before use.

Antibiotics (amounts for guidance only, **CCP***)*

[a] Dissolve 400 mg nystatin in 10 ml 70% ethanol.

[b] Dissolve 100 mg cephalexin in 10 ml 70% ethanol.

[c] Dissolve 30 mg fluorouracil in 100 ml 70% ethanol.

[d] Dissolve 16 mg tobramycin in 100 ml 70% ethanol.

(Filter sterilise when antibiotics are dissolved in water rather than 70% ethanol.)

Note

Cycloheximide can be used as an alternative for nystatin to control fungi. Dissolve 500 mg of Cycloheximide in 10 ml 70% ethanol, add 1 ml to cool medium.

Storage

Store prepared plates inverted in polythene bags at 4-8°C and use within two weeks of preparation to ensure activity of antibiotics.

Depending on the source of starch, pre-storage in the refrigerator for several days before use may result in more easily visible zones of starch hydrolysis.

Preparation of Yeast Dextrose Chalk (YDC) agar medium

(Wilson *et al*., 1967)

Compound	g/l
Bacto Agar	15.0
Yeast Extract	10.0
$CaCO_3$ (light powder)	20.0
D-Glucose (Dextrose)	20.0
Distilled/de-ionised water	1 000 ml

Preparation

1. Weigh out all ingredients into a suitable oversize container (i.e. 250 ml of medium in a 500 ml bottle/flask) to allow swirling of medium just before pouring.
2. Add 1 000 ml of distilled/de-ionised water.
3. Steam to dissolve.
4. Autoclave at 121°C, 15 psi for 15 min.
5. Allow medium to cool to approximately 50°C.
6. Swirl to ensure even distribution of $CaCO_3$ and avoid air bubbles, and pour plates (22 ml per 9.0 cm plate).
7. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at 8-20°C.

Prepared plates can be stored for several months provided they do not dry out.

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