Sep 15, 2024

Clostridia cultivation

DOI
dx.doi.org/10.17504/protocols.io.yxmvm76eov3p/v1

Eva Petrova[1], Roey Angel[1]

[1]Soil and Water Research Infrastructure

Eva Petrova
Soil and Water Research Infrastructure

DOI: dx.doi.org/10.17504/protocols.io.yxmvm76eov3p/v1

Protocol Citation: Eva Petrova, Roey Angel 2024. Clostridia cultivation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.yxmvm76eov3p/v1

Protocol status: Working
We use this protocol and it's working

Created: July 04, 2019

Last Modified: September 15, 2024

Protocol Integer ID: 25512

Abstract

adopted from Dr Tomasz Grenda (2018)

Pictures of *C. perfringens* strains grown on Willis – Hobbs agar:
http://www.medycynawet.edu.pl/images/stories/pdf/pdf2018/00-artwait/2019016161.pdf

Pictures of *C. botulinum* type A (proteolytic) strain on Willis – Hobbs agar:

https://www.researchgate.net/publication/280042476_Contamination_of_honey_produced_in_the_Republic_of_Kazakhstan_with_Clostridium_botulinum

	16S fw	fw 5'-TGGCTCAGATTGAACG CTGGCGGC-3'	Vaneechoutte, 1996
	16S rev	rev 5'-TACCTTGTTACGACTT CACCACA-3	

sequencing primers for Clostridia species

Vaneechoutte M, Cartwright CP, Williams EC, Jäger B, Tichy HV, De Baere T, De Rouck A, Verschraegen G. Evaluation of 16S rRNA gene restriction analysis for the identification of cultured organisms of clinically important *Clostridium* species. Anaerobe 1996, 2, 249-256.

Cultivation

1

- Inoculate about 1 g of sample into two tubes with preregenerated (by thermal deoxygenation or conditioning in anaerobic conditions) TPGY broth.
- Submit half of inoculated tubes to pasteurisation process at 70°C for 15 min and then incubate all of them in an anaerobic jar/chamber at 30°C or 37°C (optionally) for 48h, under anaerobic conditions.
- After incubation, check turbidity of broth and gas production abilities of each culture.
- Subsequently, strike about 10µl liquid culture (collected from the bottom of each tube) onto surface of Willis – Hobbs or FAA agar.
- Subject stroked agar plates into incubation process at 30°C or 37°C (optionally) for 48h, under anaerobic conditions.
- After incubation evaluate change of agar colour, proteolytic properties (uncoloured zones surrounding colonies), lecythynolytic abilities (precipitation zones around colonies), lipolytic properties (characteristic pearl, shining layer of colonies surface).
- Subject isolates to Gram staining and molecular analyses.

Characteristic properties of clostridia growth on Willis – Hobbs and FAA agar:

	Species	Precitipitation zone	Pearl layer – lipolytic properties	Lactose fermentation	Proteolytic properties
	C. perfringens	+	-	+	-
	C. bifermentas	+	-	-	+
	C. botulinum group I; C. sporogenes	+	+	-	+
	C. botulinum group II;	+	+	-	-
	C. botulinum group III; C. novyi type A;	+	+	-	+/-
	C. beijerinckii and C. butyricum	+	-	-	-
	C.botulinum group IV C. subterminale, C. schirmacherense, C. hastiforme	+	-	-	+
	C. novyi type B	+	-	-	-
	C. bifermentans	+	-	-	+

Gram staining

2 Make a thin film of the material on a clean glass slide, using a sterile loop. Air dry, then heat fix the slide by passing it several times through a flame (the slide should not become too hot to touch). Failure to follow these directions may cause staining artifacts and disrupt the normal morphology of bacteria and cells.

1. Flood slide with crystal (or gentian) violet- 60 seconds.

2. Flood with Gram's iodine - 180 seconds.

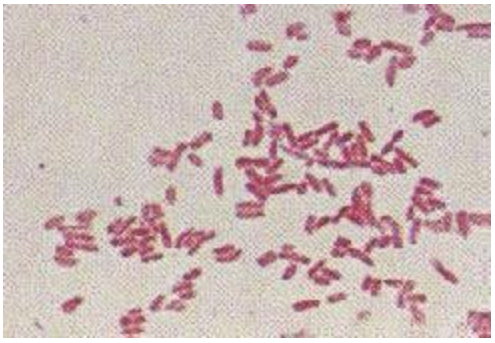
3. Carefully decolorize with 95% ethanol until thinnest parts of the smear are colourless. (Wash with water).

This third step is the most critical and also the one most affected by technical variations in timing and
reagents.

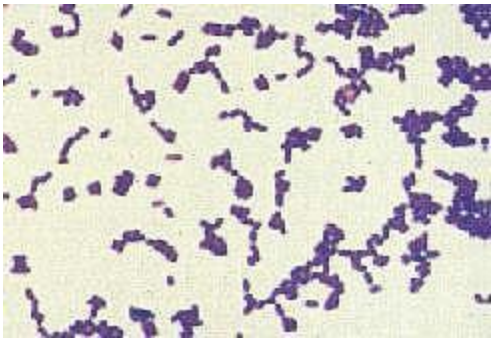
4. Flood with safranin (pink colour) (10% Fuchsine) - 60 seconds. (Wash with water).

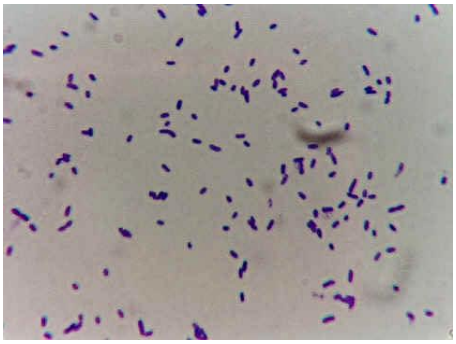
5. Air dry, or blot with absorbent paper.

A. Example of “G-“ preparation

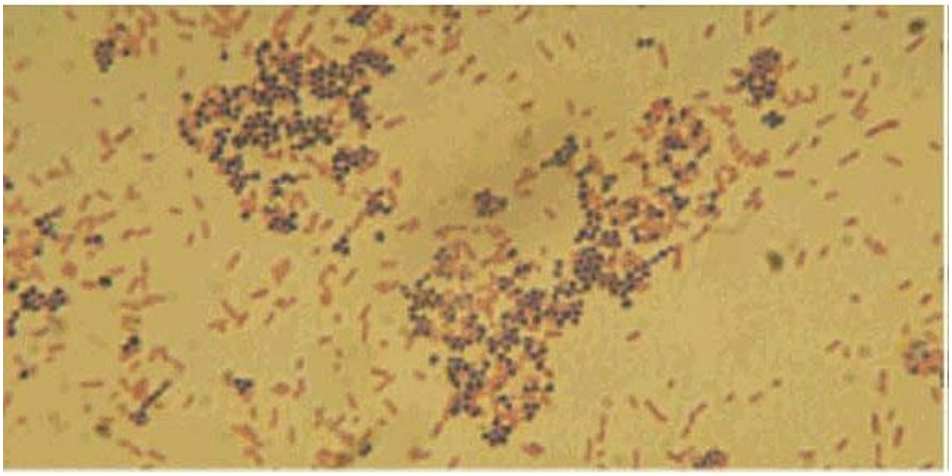


B. Example of “G+” preparation





C. Example of mixed preparation



DNA preparation

3 Isolate DNA from 24h culture of purified strains suspected of belongings to *Clostridium* sp.

- Prepare, the suspension of analysed isolates in 0.5 – 1ml PBS or saline solution in dense of 3.5 McFa.
- Subject prepared suspension to boiling for 15 min.
- After thermal treatment insert the tubes with suspension into ice.
- Spin down prepared suspensions at 11000 – 14000 rpm for 10 min.
- Before PCR performing, dilute DNA solution 10 times

Amplification and Sequencing of Clostridia 16S rDNA

4 For identification of 16S rDNA from unidentified anaerobic strains suspected of being *Clostridium* sp., primers previously described by Vaneechoutte et al.1996 could be used.

Reactions should be performed in the volume of 25 µL with the following reagent constituents:

3 µL of DNA matrix,
2.5 µL 10× Taq buffer with KCl,
4 mM MgCl2,
200 µM dNTP, and
1.25 U per 25 µL Taq polymerase.

The reaction was staged as follows:

initial denaturation at 95°C for 5 min,
35 cycles of denaturation at 95°C for 45 sec,

annealing at 55°C for 45 sec,
extension at 72° for 1 min,

and a final extension at 72°C for 10 min.