Isolation of predominant bacteria in marine molluscs

CONTENTS

Editions

Edition	Date	Updated part
N° 1	13/03/2015	Creation

1. Scope

This instruction describes a standardized procedure to isolate predominant bacteria from marine mollusc tissues.

2. References

- Stanier R.Y., Doudoroff M., Adelberg A., 1966. Microbiologie générale. Ed Masson, Paris, 638p.
- Garnier M., Labreuche Y, Garcia C., Robert M., Nicolas J-L., 2007. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. Microbial ecology 53 : 187-196.
- Saulnier D., De Decker S., Haffner P., Cobret L., Robert M,. Garcia C., 2010. A Large-Scale Epidemiological Study to Identify Bacteria Pathogenic to Pacific Oyster *Crassostrea gigas* and Correlation Between Virulence and Metalloprotease-like Activity. Microbial ecology 59 : 787-798.

3. Equipment and environmental conditions

This instruction requires classical equipments and conditions for bacteriology

- One Bunsen burner and/or one laminar flow cabinet
- One incubator at 20°C ± 2°C
- One set of pipettes (200 µL and 1000 µL)
- Sterile consumable : tips (200 εl et 1000 εl), sterile tubes (1.5 or 2 ml), sterile petri dishes, Pasteur pipettes sterilized with a Bunsen burner, platinium loops sterilized with a Bunsen burner or a sterile plastic loops, sterile plungers.
- Sterile piston
- One pHmeter
- FTA paper (Whatman)
- One vortex
- One autoclave

Manipulator must wear a lab coat during all the different steps described below and should wash and disinfect his hands. Most of these steps should be carried out in a Bunsen burner environment to guaranty sterility.

4. Procedure

4.1. Media preparation

Marine Agar

Commercial marine agar	27.55 g
Distilled water	500 mL

Medium have to be boiled before autoclaving.

Zobell medium

Pepton	2 g
Yeast extracts	0.5 g
Agar	7.5 g
Phosphate ferric	*50 mg (facultative)*
Filtered or artificial seawater	qsp 500 mL

Adjust pH to 7.4 before autoclaving.

Liquid Zobell medium

Pepton	2 g
Yeast extracts	0.5 g
Phosphate ferric	*50 mg (facultative)*
Filtered or artificial seawater	qsp 500 mL

Adjust pH to 7.4 before autoclaving.

Conservation medium

Glycerol	40 mL
Liquid Zobell medium	10 mL

Aliquote in 20 ou 10 mL-tubes and autoclave.

Artificial Seawater

NaCl	23 g (2.3%)
KCl	1.49 g (20 mM)
MgSO4	1.23 g (5 mM)
CaCl2	0.29 g (2 mM)
Distilled water	qsp 1000 mL

4.2. Sample preparation

Tissue sampling should be done near a Bunsen burner.

4.2.1. Larvae

For larva, pools of 50 mg of whole animals (including the shell) completed with 200 µL of sterile seawater are crushed in a 1.5 mL tube with a sterile piston.

4.2.2. Spat smaller than 5 mm

For spat smaller than or of 5 mm, pools of whole animals (including the shell) completed with 200µL of sterile seawater are crushed in a 1.5 mL tube with a sterile piston.

4.2.3. Spat between 0.5 and 1 cm

Whole soft tissues are individually sampled with sterile scalpels and crushed in 200µL of sterile seawater, in a 1.5 mL tube, with a sterile piston.

4.2.4. Spat between 1 and 3 cm

Between 40 and 50 mg of each of the following organs, gills, adductor muscle and mantle, are sampled with sterile scalpels and crushed in 200µL of sterile seawater, in a 1.5 mL tube, with a sterile piston.

4.2.5. Animal bigger than 3 cm

For animals bigger than 3 cm, it is recommended to collect 100µL of hemolymph from the adductor muscle sinus or from the cardiac cavity. However, for moribund animals, if no hemolymph can be sampled, proceed as described in 4.2.4.

4.3. Dilutions and plating

4.3.1. Dilutions

Dilute hemolymph or supernatant of crushed tissues in 1.5 mL or 2 mL sterile tubes containing artificial or filtered sterile seawater (generally, 100µL of sample plus 900µL of seawater). Prepare dilutions to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Homogenize by aspiration and vortex with moderation.

4.3.2. Plating on agar plates

Vortex tubes before taking 50µl of 10^{-2} and 10^{-4} dilutions and plate it on Marine Agar or Zobell plates.
Incubate petri dishes in an incubator at 20°C for 48 hours minimum.

4.4. Analysis and isolation of predominant bacterial strains

4.4.1. Analysis of petri dishes

Analysis is generally done after 48 hours or 72 hours if necessary.
Examples of table that can be used for results reporting are given in Annex 1.

1 ï For each dilution, count the number of colonies (if this number is smaller than 200).

2 ï Define the morphological types (size, form, color é) for each animal

3 ï Count the number of colonies of each type on each dilution (if <200 colonies)

Remark: For one individual, colony types are noted T1, T2, T3 é For a second individual, numbering will also begin at T1, meaning that type 1 is generally different for each individual.

4 ï Specify colony types that are predominant for each dilution. Note that on a same dilution, two or three types can be defined as predominant.
A predominant colony is defined as a colony observed on the two dilutions (10^{-2} and 10^{-4}) and present at least at 5.10^{4} CFU/mL (meaning 50 colonies on the 10^{-2} dilution).

5 ï Describe predominant types: mean size in mm (measured on at least 5 colonies), color, form (plate, round, invaginated), contour (regular or irregular), surface (smooth or rough) and appearance (opaque, translucent, mucosa).

6 ï Specify if one type of predominant colonies can be found on different individuals (i.e. type T1 of ind1 appears morphologically similar to type T3 of ind4).

Remark: Bacteria can be identified by Real time PCR from this step (http://www.eurl-mollusc.eu/SOPs)

4.4.2. Isolation of predominant bacteria colonies

Isolate on Marine agar or Zobell medium the predominant different types observed by taking with a sterile Pasteur pipette or a sterile loop a representative colony and spread it on agar.
Incubate petri dishes at 20°C for 48 hours, check bacterial growth and purity or isolation step should be done again from the same plate (if purity problem) or from another representative colony (if growth problem).

Remark: Petri dishes of the different dilutions are kept in a fridge until isolation is finished.
Remark: Bacteria can be identified by PCR or QPCR from this step (http://www.eurl-mollusc.eu/SOPs)

Pictures of Vibrio splendidus and V. aestuarianus strains isolated on different bacteriological media are given in Annex 2.

4.5. Conservation of strains

4.5.1. Culture in a liquid medium

A pure colony from the isolated bacteria is re-suspended in a cryotube containing 865µL of liquid Zobell medium and incubated for 24 to 48 hours under gentle agitation at 20°C, to favor growth.

Remark: Petri dishes with the pure isolated colonies are kept in a fridge until conservation is done.

If no growth can be noticed, culture should be done again from a new colony of isolated bacteria.

4.5.2. Conservation of bacterial DNA on FTA paper

This step is done under laminar flow cabinet Gloves are recommended in this step to limit contamination.
65 µL of bacterial liquid culture are deposited on FTA paper. After 1 hour of drying under one laminar flow cabinet, FTA paper can be conserved at room temperature in a desiccator.

4.5.3. Conservation of bacterial strains at -80°C

200 µL of conservation medium are added to cryotube. After 15 minutes, cryotubes are transferred to -80°C freezer.

5. Elimination of wastes

All disposable instruments (tips, tubesé) and contaminated media are destroyed in the autoclave before going to household wastes.

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Annex 1

Analysis of Petri dishes

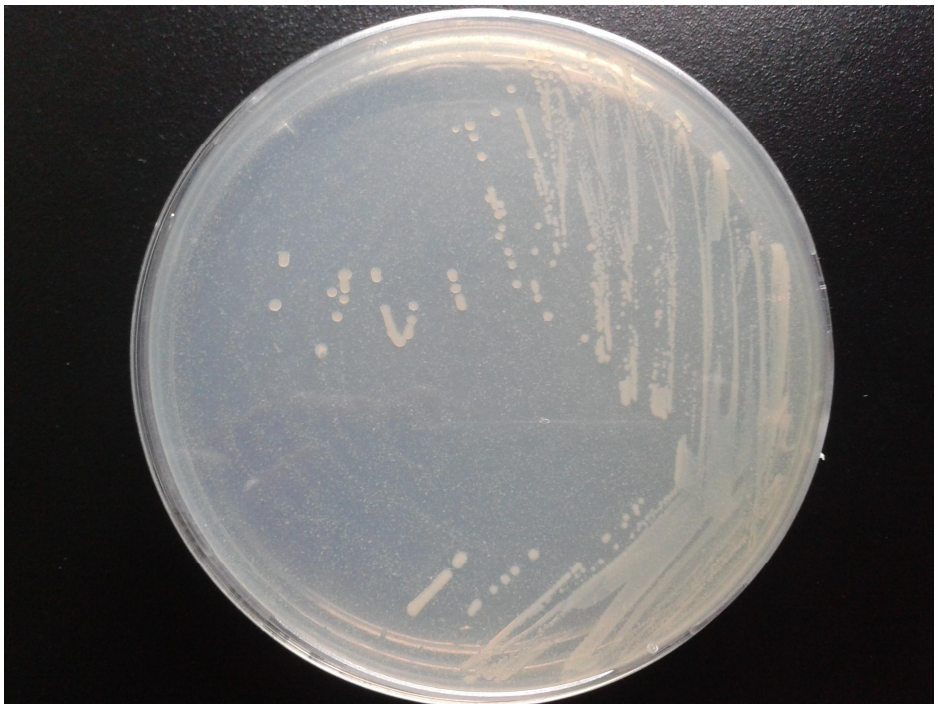
Sample code	Dilution 10^{-2}			Dilution 10^{-4}		
	Number of colonies	Present types and number of colonies per type	Predominant type	Number of colonies	Present types and number of colonies per type	Predominant type

Description of bacterial strains

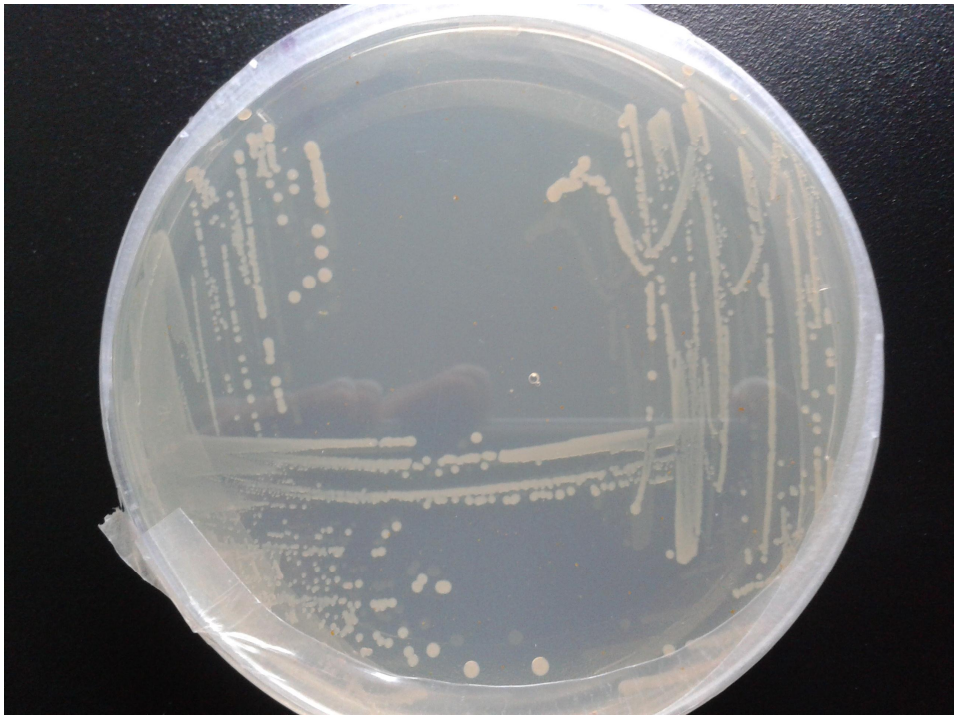
Sample code	Strain code	size (mm)	Colour	Form			Contour		Surface		Appearance			Comments
				Plate	Round	Invaginated	Regular	Irregular	Smooth	Rough	Translucent	Opaque	Mucosa	

Annex 2

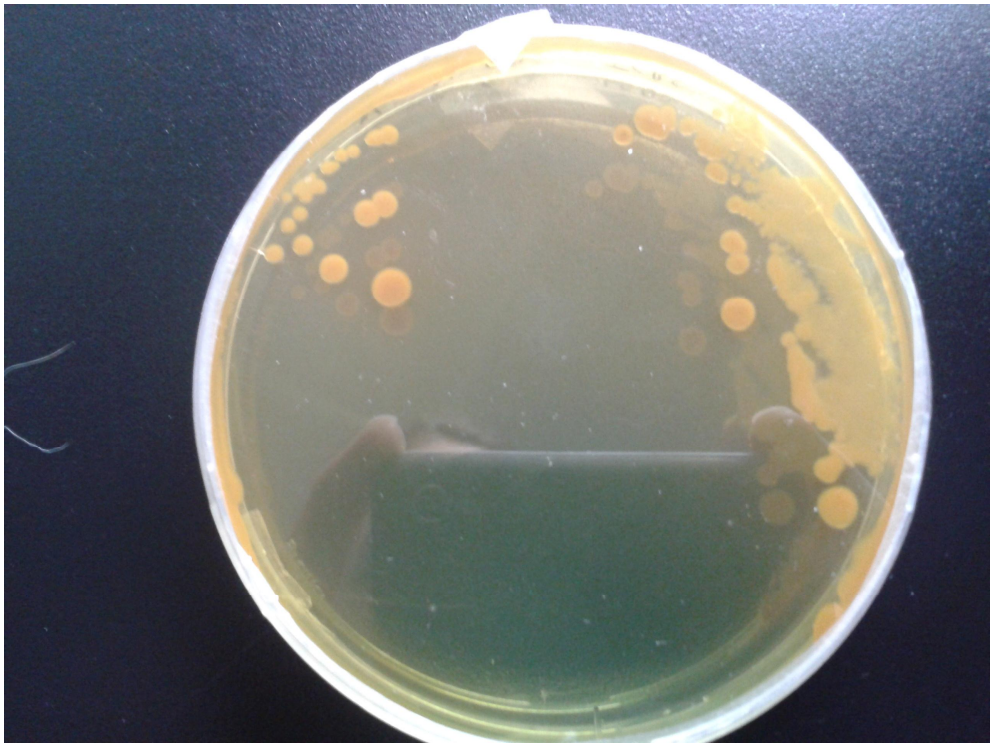
Plate with *Vibrio aestuarianus* subspecies *aestuarianus*



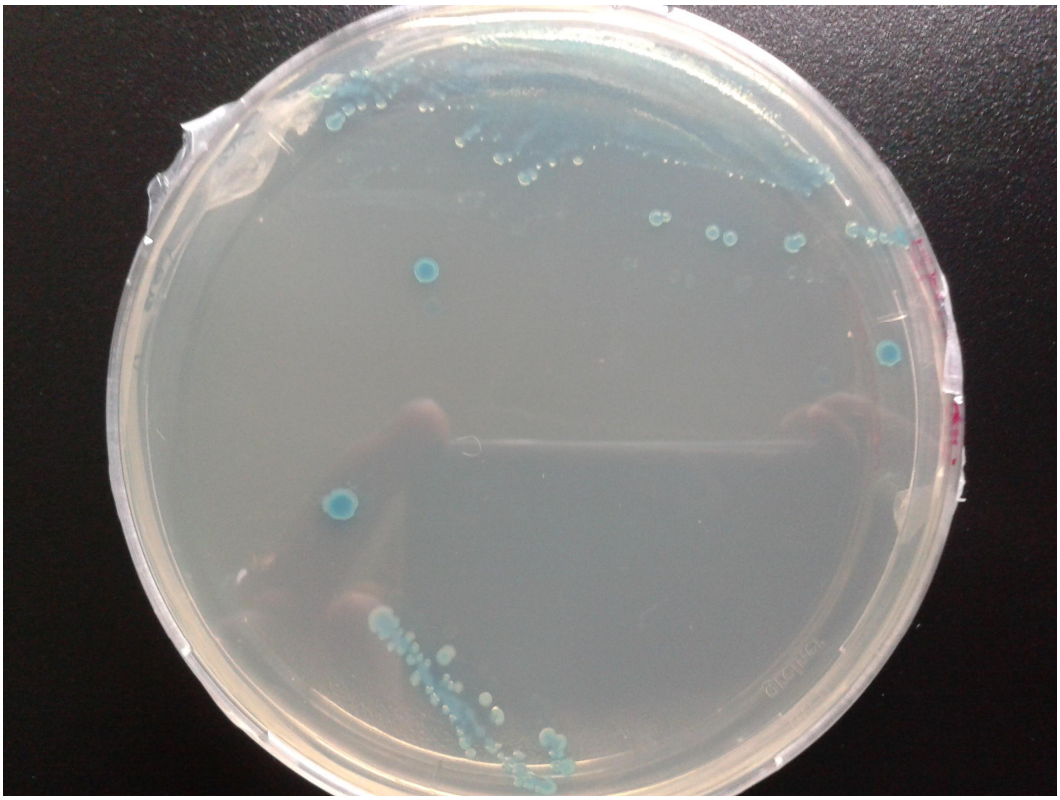
Vibrio aestuarianus subspecies *aestuarianus* on Marine agar



Vibrio aestuarianus subspecies *aestuarianus* on Zobell medium

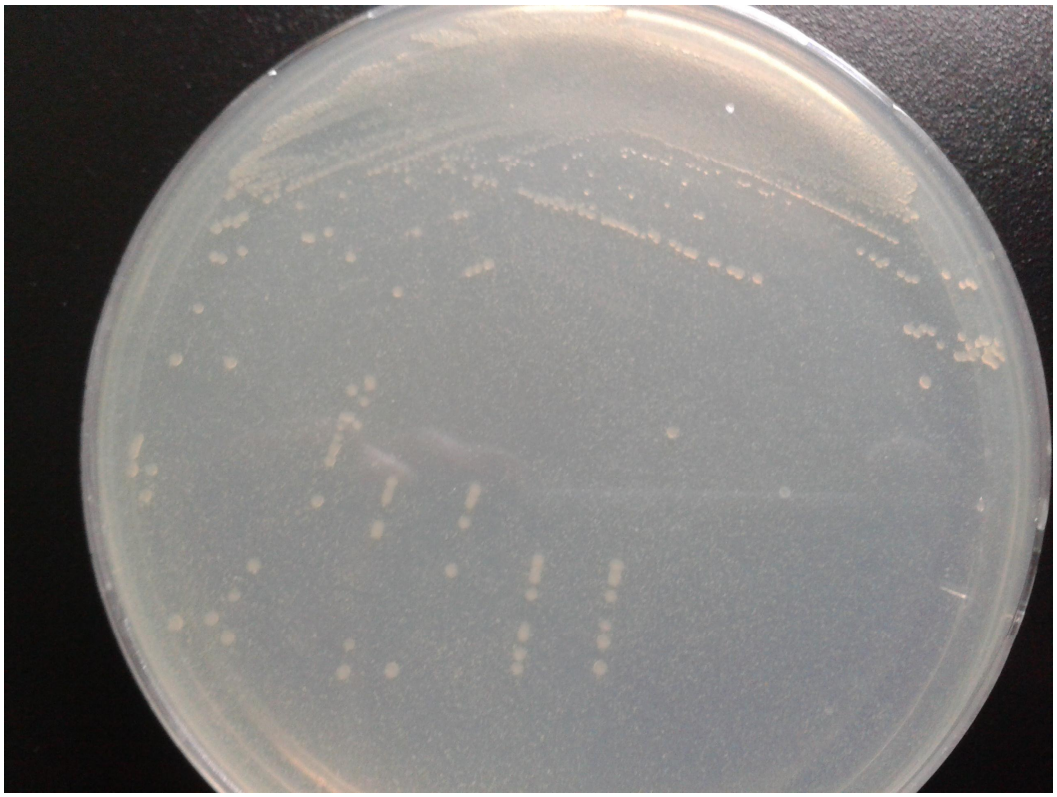


Vibrio aestuarianus subspecies *aestuarianus* on TCBS



Vibrio aestuarianus subspecies *aestuarianus* on ChromAgar

Plate with *Vibrio aestuarianus* subspecies *francensis*

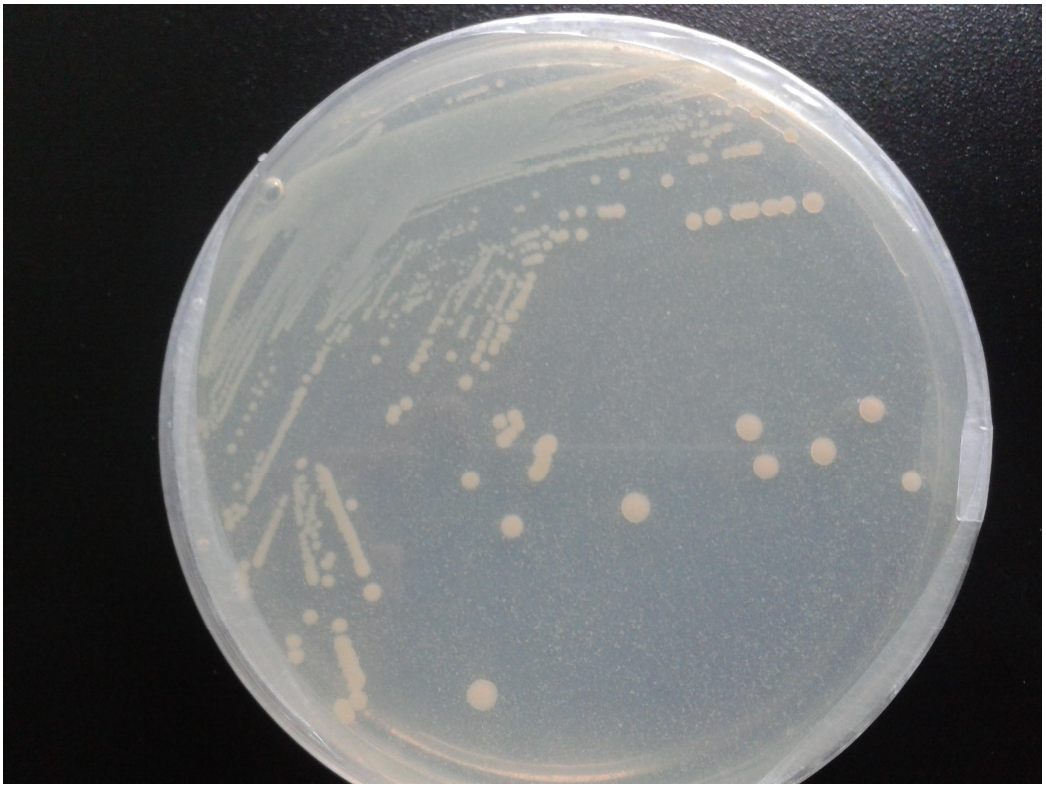


Vibrio aestuarianus subspecies *francensis* on Marine agar

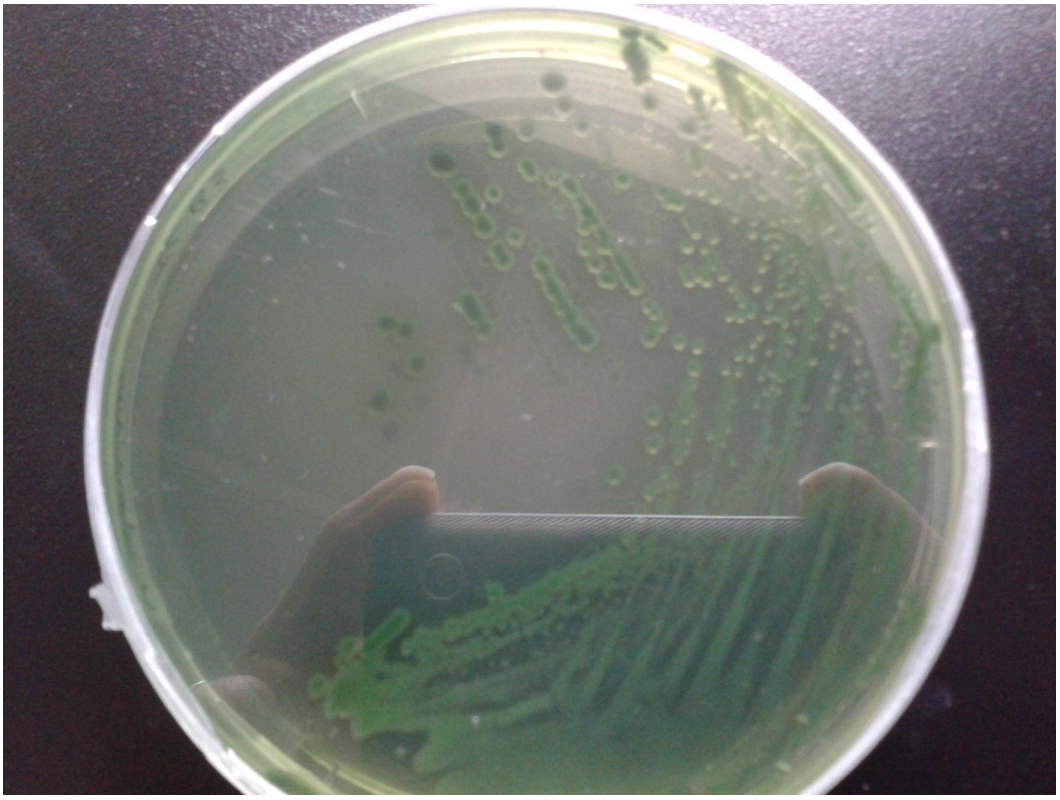
Vibrio aestuarianus subspecies *aestuarianus* on Zobell medium

Remark: *Vibrio aestuarianus* subspecies *aestuarianus* difficulty grows on TCBS.

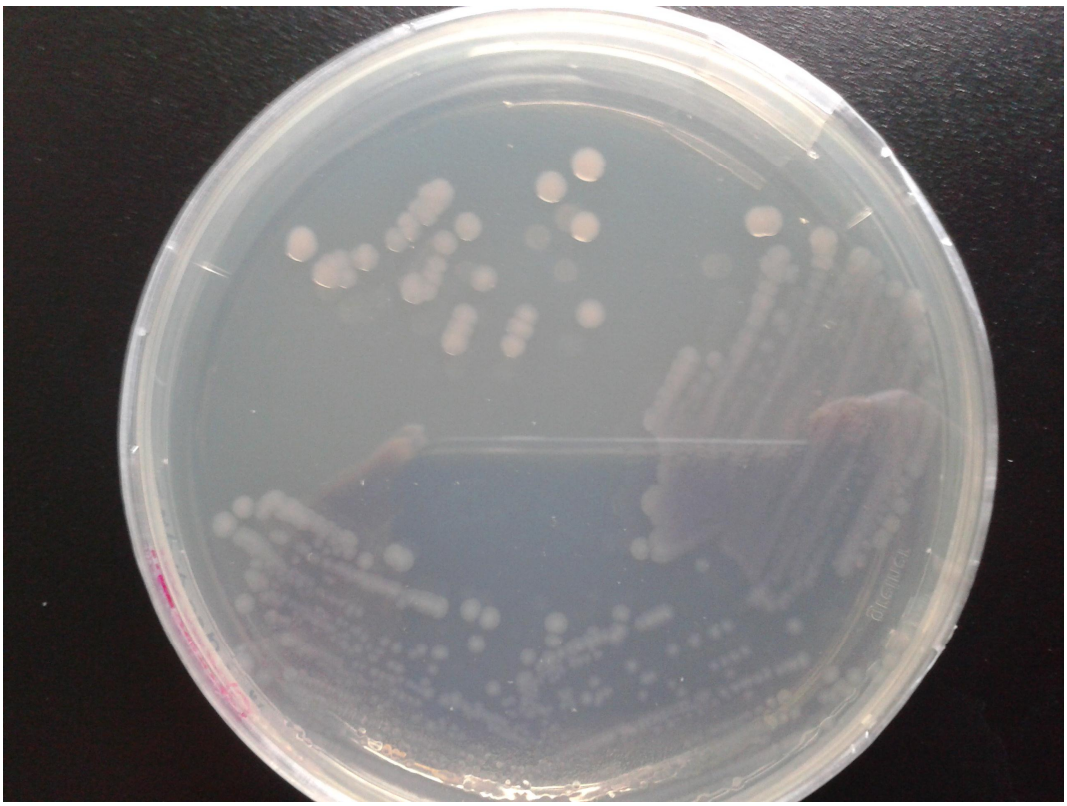
Plate with a bacteria of *Vibrio splendidus* group



Vibrio splendidus on Marine agar



Vibrio splendidus on TCBS



Vibrio splendidus on ChromAgar