UNIVERSITÀ DEGLI STUDI DI TERAMO

Faculty of Bioscience and Technology for Food, Agriculture and Environment

Food Microbiology

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MEET THE MICROORGANISMS

The microorganisms we will study:

Pseudomonas fluorescens strains isolated from dairy products

✓Survive at a wide range of temperatures (**4**-42°C)

✓Produce different pigments

✓Produce exopolysaccharides and readily form **BIOFILMS**

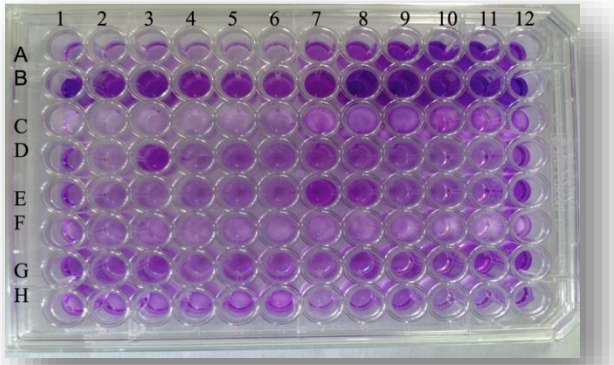
✓Responsible for spoilage of different food matrices

✓Persist in food processing environments

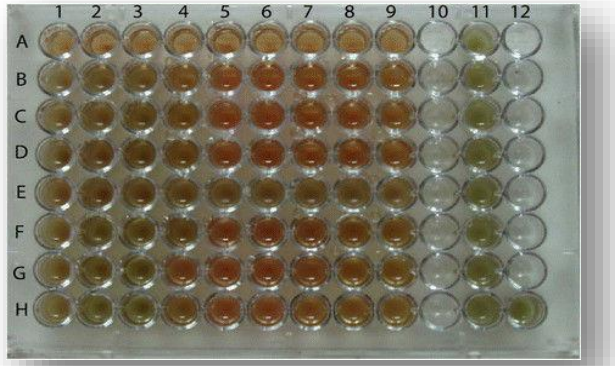
(*Scales et al.,* 2014)

TODAY’s LAB: EXPERIMENTAL PLAN

1- Determination of biofilm formation capacity
2:30 pm – 3:45 pm



2- Determination of antibacterial activity of essential oils according to the microdilution method
2:45 pm – 4:15 pm

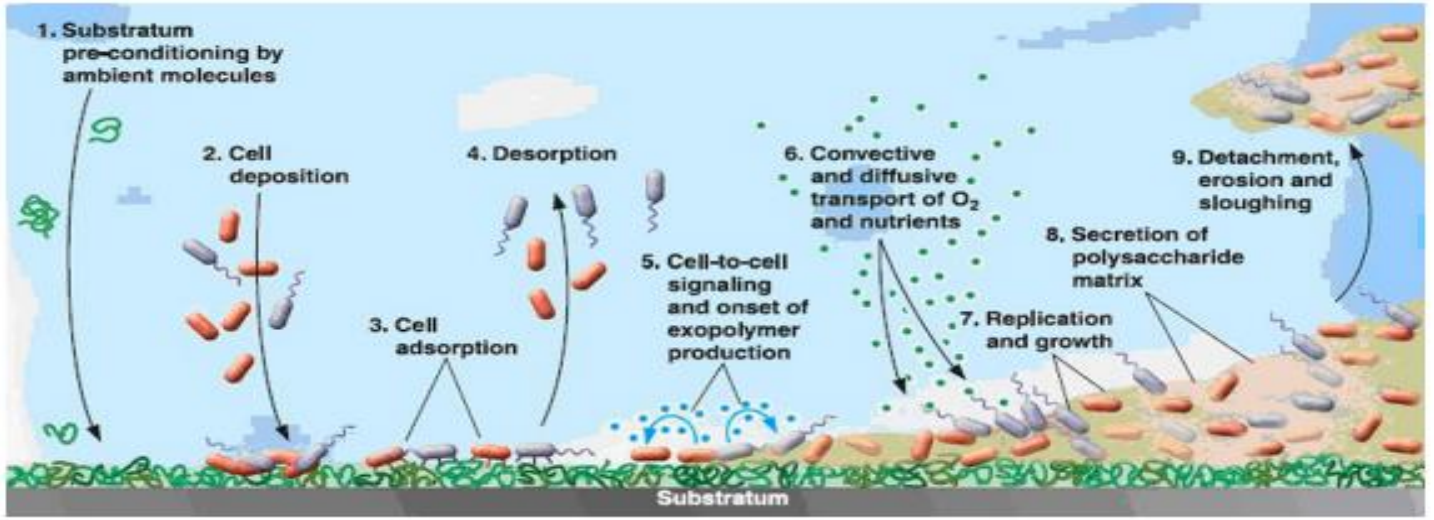


3- Determination of sensitivity to different concentrations of sodium hypochlorite
4:15 pm – 5:30 pm

1- DETERMINATION OF BIOFILM FORMATION CAPACITY

What is a biofilm?

Biofilms are surface-attached groups of microbial cells encased in an extracellular matrix that are significantly less susceptible to antimicrobial agents than non-adherent, planktonic cells (*Hall and Mah*, 2017)



✓ Protection from hostile environments (defense)
✓Sequestration of nutrients in rich substrates (colonization)
✓Benefit from "collective welfare" (community)

1- DETERMINATION OF BIOFILM FORMATION CAPACITY

PROTOCOL

(*Rossi et al*., 2018; *Di Bonaventura*, 2008; *Stepanovic et al.,* 2004)

BACTERIAL SUSPENSION PREPARATION

- The bacterial strains were grown overnight in Tryptic Soy Broth (TSB), at 30°C.
- After 18 h of incubation, the fresh cultures were harvested by centrifugation at 13000 g for 5 min and washed three times with Phosphate Buffer Saline (PBS) 50 mM pH 7.0
- Inocula were standardized by means of absorbance measures at 590 nm
- The bacterial suspensions were adjusted to a corresponding absorbance value of about 10^5 log ufc/mL in TSB supplemented with 0.2% of glucose

INOCULATION OF 96-WELL MICROTITER PLATES

- Each strain was tested in 10 replicates after inoculation of the standardized culture. To set up the microtiter plates, 200 µL of growth medium (TSB with 0.2% of glucose) were added to each well of 11° and 12° columns, which represent the negative control
- The microplates were incubated at 10°C for 5 days

1- DETERMINATION OF BIOFILM FORMATION CAPACITY

WASHING TREATMENT

- **Remove the planktonic cells from each well and then rinse the microtiter plates three times with deionized water. Remove excess moisture by tapping the microplates on sterile napkins**
- **Air-dry the plates for 15 min**

CRYSTAL VIOLET STAINING

- **Stain with 200 µL of 0.1% crystal violet solution at room temperature for 30 min**
- **Wash the microplates four times with deionized water to remove the stain; remove the excess of water by tapping the microplates on sterile napkins**
- **Solubilize crystal violet with 250 µL of 30% glacial acetic acid solution**
- **Measure the absorbance at 590 nm**

EXPRESSION OF RESULTS

- **Group the strains into the following categories: no biofilm producers, weak, moderate or strong biofilm producers:**
  - **If OD ≤ ODc: no biofilm producers**
  - **If ODc < OD ≤ 2 ODc: weak biofilm producers**
  - **If 2 ODc < OD ≤ 4 ODc: moderate biofilm producers**
  - **If OD > 4 ODc: strong biofilm producers**

The cut-off O.D. (**O.D.c**) was defined as three standard deviations above the mean O.D. of the negative control

2- DETERMINATION OF ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS ACCORDING TO THE MICRODILUTION METHOD

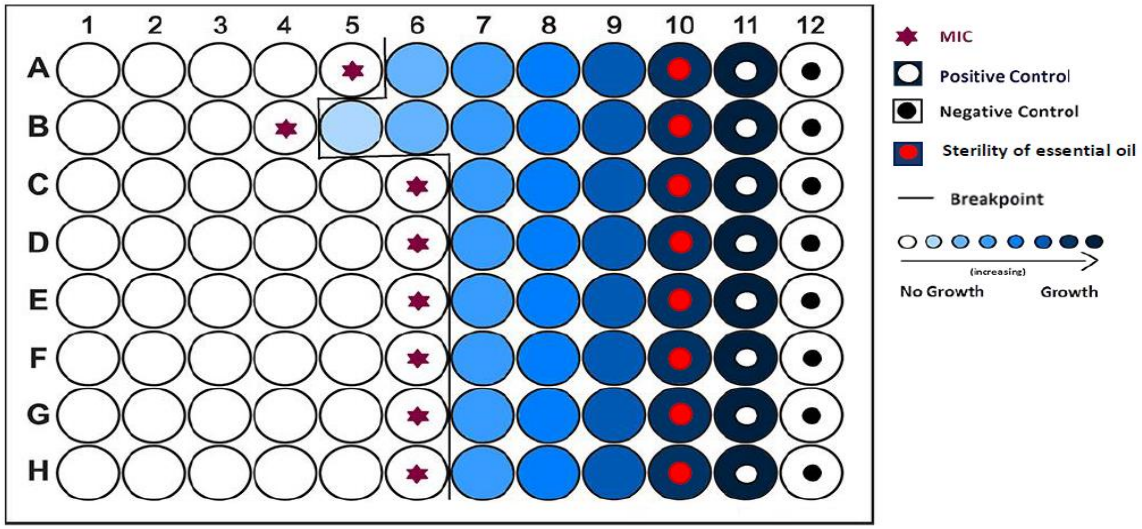
BIOPRESERVATION

What is an essential oil?

Essential oils are complex volatile compounds, synthesized naturally in different plant parts during the process of secondary metabolism. The presence of different types of aldehydes, phenolics, terpenes, and other antimicrobial compounds means that the essential oils are effective against a diverse range of pathogens (*Swamy et al.,* 2016)

What is the MIC?

The Minimum Inhibitory Concentration is the lowest concentration of antibiotic that is effective for the inhibition of bacterial growth (*Onishi et al.,* 2018)



2- DETERMINATION OF ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS ACCORDING TO THE MICRODILUTION METHOD

PROTOCOL

(CLSI, 2011)

BACTERIAL SUSPENSION PREPARATION

-Bacteria were cultured overnight on agar plates (TSA)

-After 24 hours, one colony was transferred to 1 mL of TSB broth and incubated at 30°C for 18 hours to obtain a fresh working culture

-The fresh cultures were harvested by centrifugation at 13000 g for 5 min and were washed for three times with Phosphate Buffer Saline (PBS) 50 mM pH 7.0

-The inocula were standardized by means of absorbance measures at 590 nm

-The bacterial suspensions were adjusted to a corresponding absorbance value of about 10^5 log ufc/mL

FORMULATION OF ESSENTIAL OIL (EO) EMULSION

- The EO was dissolved in sterile PBS (Phosphate Buffer Saline) 50 mM pH 7.0 and Tween 80 (1%) to reach the initial concentration of 4.0%.

2- DETERMINATION OF ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS ACCORDING TO THE MICRODILUTION METHOD

INOCULATION OF 96-WELL MICROTITER PLATES

- To set up the microtiter plate, add 100 µL of suitable growth medium (TSB) to each well
- Add 100 µL of EO emulsion in the first well and mix. Take 100 µL of this and inoculate the next well, continue until the 10° well, where you have to release the 100 µL
- Starting from the 11° well, add the standardized bacterial suspension (100 µL) to all wells, excluding the 10° well
  - 12° well: Negative control, substrate sterility
  - 11° well: Positive control, activity of bacterial suspension
  - 10° well: Essential oil sterility

EXPRESSION OF RESULTS

- The plates are automatically scanned and recorded every 15 min for two days by the OmniLog incubator/reader (Biolog Inc., Hayward, USA) and microbial growth curves as well as MIC values are evaluated

3- DETERMINATION OF SENSITIVITY TO DIFFERENT CONCENTRATIONS OF SODIUM HYPOCHLORITE

Why sodium hypochlorite?

The use of sodium hypochlorite (NaClO) has shown in several studies a strong antimicrobial activity against pathogens and reduction of the microbial count
(*Mir et al*., 2017, *Chandra et al.,* 2012, *Char et al.,* 2012, *Issa-Zacharia et al*., 2011)

Why sodium thiosulphate?

This chemical can rapidly and effectively neutralize NaClO. This is important because failure to neutralize would effectively prolong contact times with NaClO, determining false results.
(*Radcliffe et al*., 2004)

3- DETERMINATION OF SENSITIVITY TO DIFFERENT CONCENTRATIONS OF SODIUM HYPOCHLORITE

PROTOCOL

(*Erkmen*, 2004 with some modifications)

PREPARATION OF TEST SOLUTIONS

- Sodium hypochlorite solutions were diluted from a initial solution containing an active chlorine concentration of about 4%
- Sodium thiosulphate ($Na_2S_2O_3$) buffer (0,1 M) was prepared using 1 mL of sodium thiosulfate 0,1 M in 49 ml of phosphate buffer 0,1 M pH 6.5
- Phosfate buffer was prepared using 3,81 mL of K_2HPO_4 1 M and 6,19 ml of KH_2PO_4 1 M bringing to volume of 100 ml with distilled water
- Saline solution of bovine serum albumin (BSA) was prepared by mixing 15 g/L of BSA and 8,5 g/L of sodium chloride

BACTERIAL SUSPENSION PREPARATION

- Starting from pure cultures on a solid substrate TSA, one colony was sub-cultured into TSB and was incubated at 30°C for 24 h
- Bacteria were harvested by centrifugation and washed three times with physiological solution
- Inocula were standardized at about 10^5 log ufc/mL

3- DETERMINATION OF SENSITIVITY TO DIFFERENT CONCENTRATIONS OF SODIUM HYPOCHLORITE

CHLORINE INACTIVATION PROCEDURE

- Put 500 µL of BSA solution in 500 µL of standardized inocula and incubate at 20°C for 2 min
- Add 500 µL of previous suspension in 12 mL of sodium hypochlorite at the established concentrations and incubate at room temperature for 5 min
- Add 500 µL of suspension with hypochlorite in 4.5 mL of neutralizing solution at room temperature
- After 5 minutes harvest, wash the cells and resuspend in physiological solution
- Dispense each suspension of hypochlorite-treated cells into wells of microtiter plate (50 µL) (5 replicates each concentration), with 150 µL of TSB, and incubate at 30°C into the OmniLog incubator/reader (Biolog Inc., Hayward, USA)

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Thank you for
your attention!