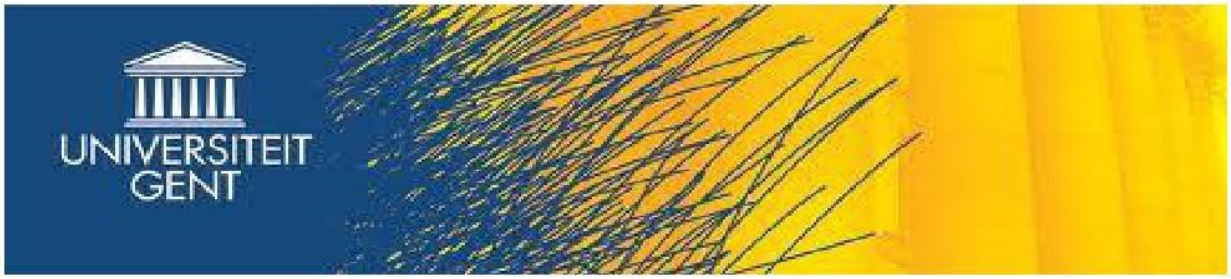

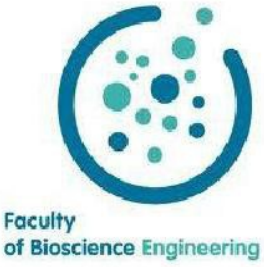

the Laboratory of Microbial Ecology and Technology

Final internship report in metter of:

"Study of the growth as a function of the carbon source of thirteen environmental isolates"

Giovanni Rossi

Promoter: Dr. Ramiro Vilchez-Vargas

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Chapter 1
INTERNSHIP FRAMEWORK

The European Commission's Lifelong Learning Programme enables people to take part in stimulating learning experiences, as well as helping to develop the education and training sector across Europe (European commission, Education & Training; 2007). Indeed, into Lifelong Learning Programme there are many projects such as *Comenius* for schools, Erasmus (European Region Action Scheme for the Mobility of University Students) for higher education, *Leonardo da Vinci* for vocational education and training and Grundtvig for adult education. Besides, in one deepest overview about Erasmus project there is the concept of student mobility for placement which is allowing to students the visit to a higher education institutions for (internship) period of 3 months in an company or organisation in another participating country (European commission, Education & Training, ERASMUS Student mobility for higher education and placement, 1987).

The project performed was the *Erasmus placement*, within which I was resulting as scholarship winner under University of Bologna announcement in January. The selected place was LabMET (Laboratory of Microbiology, Ecology and Technology) which is a part of the Faculty of Bioscience Engineering at Universiteit Gent, Belgium. Such programme started on 1st of April 2013 until 1st of July 2013 thank to accordance between the two parts. During this period, under supervision of Dr. Ramiro Vilchez-Vargas, I performed some basic microbiology techniques and several approaches to build the logic experimental design, studying bacterial growth patterns for further ecological researches.

At the beginning of this experience, it was followed the safety course for work at LabMET. Also it was part of internship programme to follow every Tuesday several seminars about the results of each other LabMET mates in a interdisciplinary framework between applied engineering and microbiology.

1.1 - Introduction

The work performed during the internship period was to make the growth curves in mineral medium, with 3 different carbon sources (Glucose, Succinate, Glycerol) for 13 strains:

- *Brachybacterium* (Fig.1a) → Kingdom: Bacteria - Division:Actinobacteria - Class: Actinobacteria - Order: Actinomycetales - Family: Dermabacteraceae - Genus *Brachybacterium* (8).

  It is a genus which contains non-filamentous actinobacteria species (25). As an example, the species *B. sacelli* is changing the morphology from short rods in the exponential phase to coccoid forms (single or in agglomerates) in the stationary phase. Cells are non-motile, do not form endospores and are Gram-positive (34).

- *Acidovorax* (Fig.1b) → Kingdom: Bacteria - Division: Proteobacteria - Class: β-Proteobacteria - Order: Burkholderiales - Family: Comamonadaceae - Genus: *Acidovorax* (36).

  This genus of β-Proteobacteria includes only aerobic species, and some of them pathogens *such as A.citrulli*. This specie is pathogen to various species of plants (Cucurbitaceae).
  It is a Gram-negative, strictly aerobic, rod-shaped, motile with a polar flagellum and its optimal temperature for growth is around 28 °C (5).

- *Comamonas* (Fig.1c)→ Kingdom: Bacteria - Division: Proteobacteria - Class: β-Proteobacteria - Order: Burkholderiales - Family: Comamonadaceae - Genus: *Comamonas* (11).

  This genus of Gram-negative bacteria includes only aerobic organisms and motile with bipolar or polar tufts of 1-5 flagella (32). In *Comamonas* genus it has also been seen that a β–subclass of Proteobacteria seems to proliferate at both aerobic and anoxic conditions (1).

- *Bacillus* (Fig.1d)→ Kingdom: Bacteria - Division: Firmicutes - Class: Bacilli - Order: Bacillales - Family: Bacillaceae - Genus: *Bacillus* (7).

  It is Gram-positive, rod-shaped bacteria. It is ubiquitous in nature and its habitat is mainly in soil. Many species of *Bacillus* are associated with plants as well as rhizosphere bacteria and endophytic bacteria (22). This genus includes free-living and pathogenic species (21).

- *Enterococcus* (Fig.1e)→ Kingdom: Bacteria - Division: Firmicutes - Class: Bacilli - Order: Lactobacillales - Family: Enterococcaceae - Genus: *Enterococcus* (31).

  It is a genus of Gram-positive able to produce lactic acid as major metabolic end-product of carbohydrate fermentation; often are living in pairs (Diplococci) or short chains (12). Enterococci is frequently associated with prickypear and fieldpoppy flowers and also some species are pathogens (29). .

- *Staphylococcus* (Fig.1f) → Kingdom: Bacteria - Division: Firmicutes - Class: Bacilli - Order: Bacillales - Family: Staphylococcaceae - Genus: *Staphylococcus* (27).

  The most relevant specie of this genus is *S. aureus*, it is a facultative anaerobic Gram-positive coccal bacterium also known as "golden staph" (26). *S. aureus* is also known to be an important pathogen that underlies various acute and chronic infectious diseases (35).

- *Shewanella* (Fig.1g) → Kingdom: Bacteria - Division: Proteobacteria - Class: γ-Proteobacteria - Order: Alteromonadales - Family: Shewanellaceae - Genus: *Shewanella* (19).

  The genus comprises several Gram-negative species which are widely distributed in marine and freshwater environments. Also, this genus seems to play an important role in the turnover of organic matter coupled to anaerobic respiration electron acceptors (28).

- *Pseudomonas* (Fig.1h) → Kingdom: Bacteria - Division: Proteobacteria - Class: γ-Proteobacteria - Order: Pseudomonadales - Family: Pseudomonadaceae - Genus: *Pseudomonas* (24).

  One member of this genus is *P. aeruginosa* that is a Gram-negative bacterium that causes a number of diseases in immunocompromised individuals such as infants, hospital patients, and elderlies. In recent years, *P. aeruginosa* has become an important health issue as it has been identified as being responsible for a number of opportunistic and nosocomial infections. Because *P. aeruginosa* exhibits extremely high metabolic versatility and adapts to a variety of conditions, it can survive in a variety of habitats, for example in different types of soils, rivers, lakes, and wastewaters (33).

- *Enterobacter* (Fig.1i) → Kingdom: Bacteria - Division: Proteobacteria - Class: γ-Proteobacteria - Order: Enterobacteriales - Family: Enterobacteriaceae - Genus *Enterobacter* (16).

  This is a genus of Gram-negative, rod-shaped, non-spore-forming bacteria includes several pathogenic strains and could be the responsible of some infections in the urinary and respiratory tracts. *Enterobacter* is a coliform, however it is not belonging to the fecal coliforms, being unable to grow at 44.5°C (6).

- *Beijerinckia* (Fig.1j) → Kingdom: Bacteria - Division: Proteobacteria - Class: α-Proteobacteria - Order: Rhizobiales - Family: Beijerinckiaceae - Genus: *Beijerinckia* (10).

  It is a genus comprising free-living (non-symbiotic) Gram-negative, aerobic, chemoheterotrophic bacteria which can fix N_2 Rod-shaped in juvenile stage; pear, or dumbbell shaped cells with aging; polar lipoid bodies on each end of the cell. These bacteria are extremely acid tolerant and have been shown to fix more N₂ as the acidity levels increase (up to 2.7 pH). The microbe is more abundance in acidic soils and is also found in alkaline soils as well (4).

- *Ochrobactrum* (Fig.1k) → Kingdom: Bacteria - Division: Proteobacteria - Class: α-Proteobacteria - Order: Rhizobiales - Family: Brucellaceae - Genus: *Ochrobactrum* (15). These bacteria can be found in several habitats including soils, plants and their rhizosphere, (waste)waters, animals and humans. *O. anthropi* and *O. intermedium* appear to be particularly ubiquitous colonizers (18).

- *Rhizobium* (Fig.1l) → Kingdom: Bacteria - Division: Proteobacteria - Class: α-Proteobacteria - Order: Rhizobiales - Family: Rhizobiaceae - Genus: *Rhizobium* (17). It is Gram-negative soil bacteria which can fix N_2 forming an endosymbiotic partnership with roots of some legumes plants (32). It has been proven that some bacterial consortia, of *R. tropici*, in presence of chrysene was directly proportional to bacterial biomass, so this may indicate that this genus could use the chrysene as carbon source (23).

- *Paracoccus* (Fig.1m) → Kingdom: Bacteria - Division: Proteobacteria - Class: α-Proteobacteria - Order: Rhodobacterales - Family: Rhodobacteraceae - Genus: *Paracoccus* (9).

  Genus *Paracoccus* comprises Gram-negative bacteria, including *P. denitrificans* that is a coccoid, non-motile and denitrifer. It is a rod-shaped bacterium but adopts spherical morphology during the stationary phase (13). Members of the genus *Paracoccus* are constituting an important part of the autochthonous flora of the denitrifying biofilms (1).

Chapter 2
OBJECTIVES

The aim of this internship programme in LabMET was to learn lab methodologies and techniques in Microbiology.

1. Media preparation.
2. Pure cultures.
3. Inocula preparation at defined optical density.
4. Cell quantification by flow cytometry.

Chapter 3
MATERIALS AND METHODS

3.1.1 - Media preparation

Media are used in microbiology to isolate, cultivate, identify and keep the bacteria (20). Three kinds of media were use in this study: Lysogeny broth (LB) and LB agar which are nutritionally rich media, first time prepared and used by Bertani Giuseppe (4) and Mineral Medium (MM) with no carbon sources and only the minimal nutrients for bacteria growth, without amino acids and it is often used to obtain the growth from wildtype microorganisms.

All media and solutions were made in 1L Scott's bottle and after sterilizing by autoclaving at 120°C during 15 minutes or filtering by 0.22µm membrane if some component were thermolabile.

3.1.2 - LB broth liquid medium

For inocula preparation was used LB broth medium. 12 g of LB broth were solved in 600 ml of distillate water (made according to the manufacturer) and after homogenizing the solution by magnetic stirrer, sterilized as mentioned above.

3.1.3 - LB agar solid medium

This medium was used to grow the isolate in solid medium. The protocol was as follow: 12g of LB broth and 12g of Agar powder were added to 600 ml of distillate water. Suspension was homogenized the solution by magnetic stirrer before to sterilize by autoclaving. Afterwards, it was placed at 45°C to cool down the media before adding 25 ml per Petri plate. The solidification was done at room temperature.

3.1.4 - Mineral medium.

Composition per liter:

- 900 ml of sterile distillate water,
- 100 ml of sterile Phosphate Buffer Solution (PBS) from the stock, constituted by:
  1. 1L of dist. H_2O
  2. 140g of Na_2HPO_4 x$12H_2O$
  3. 20g of KH_2PO_4
- 10 ml of filtrated, by 0.22µm mebrane, Trace Element Solution (TES) from the stock constituted by:

- 900 ml of salt solution (SS) from the stock constituted by:
  1. 1L of dist. H_2O
  2. 5g of $Ca(NO_3)_2$
  3. 100g of $(NH_4)_2SO_4$
  4. 20g of $MgSO_4$ x$7H_2O$
  5. 1g of $Fe(NH_4)C_6H_8O_6$

- 100 ml of metal solution (MS) from the stock prepared as follow:
  1. 1L of dist. H_2O
  2. 900µl of HCl 37%
  3. 70mg of $ZnCl_2$
  4. 100mg of $MnCl_2$ x$4H_2O$
  5. 62mg of H_3BO_3
  6. 190mg of $CoCl_2$ x$6H_2O$
  7. 17mg of $CuCl_2$ xH_2O
  8. 24mg of $NiCl_2$ x$6H_2O$
  9. 36mg of $NaMoO_4$ x$2H_2O$

3.1.5 - Carbon sources

The carbon sources used were added to the MM in these concentrations: 2mM of glucose $C_6H_{12}O_6$ (Glucose stock 100mM), 2mM of succinate $C_4H_6O_4$(Succinate stock 100mM) and 2mM of glycerol $C_3H_4O_3$ (Glycerol 100mM stock).

3.2.1 - Glycerol stock isolates activation

Each bacterium was kept at LabMET glycerol collection at -78°C.10µl of each suspension were transferred to Petri plates with LB agar 2% and was streaked by dilution and incubated for 24 to 48h (depending on the isolate) at 28°C.

3.2.2 - Streaking

The streaking is applied in microbiology to obtain single colony. It was used an inoculation loop, also named microstreaker, useful to dilute amount of cells from the inoculum (e.g. 10µl from the glycerol stock or one colony from the Petri plates) across the quadrants of one plate to get single colonies. This technique is useful because allows to see the morphology of the colonies and to check if a contamination occurred during the experiment.

For example, if there are colonieswith different morphologies.

3.2.3 - Isolates

All isolates were incubated by streaking method on Petri plates with LB agar 2% solid medium. Afterwards, Petri plates were incubated during 24 hours at 28°C (Fig. 1). However, longer incubation times were required for some isolates in order to get single colonies. Finally, isolates were kept at 4°C. Fresh isolates were prepared weekly in the same way.

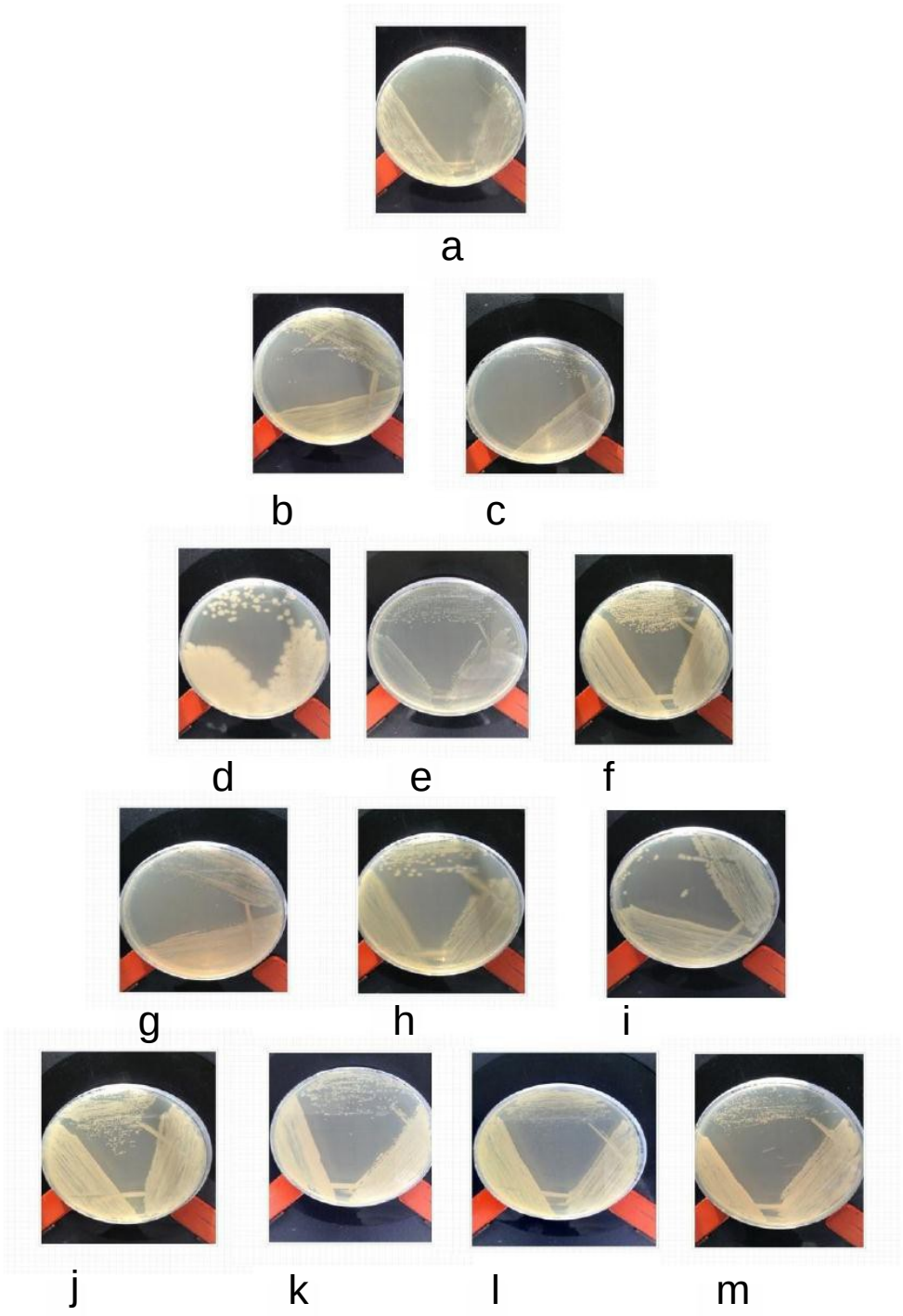


Fig.1 - Single colonies on LB agar 2% after 24-48 hours at 28°C: Actinobacteria: a; β-Proteobacteria: b, c; Bacilli: d, e and f; γ-Proteobacteria: g, h and i; α-Proteobacteria: j, k, l and m.

3.2.4 - Inocula preparation

Once all of the isolates were grown in LB agar (24-48h) at 28°C, one colony was taken from each solid culture, using the inoculation loop and it was inoculated into test tubes with 10 ml of LB broth liquid. Test tubes were incubated 24 hours at 28°C in a shaker-incubator at 110 rpm to increase the oxygenation (Fig.2). After the incubation it was calculated which dilution was required per inoculum (Fig.3) in order to reach an OD of 0.05.

Afterwards, it was transferred 10 ml from the inoculum in 10 eppendorf tubes. Each eppendorf was centrifuged at 5.000 rpm, at 28°C during 30 minutes. The supernatant (LB) was discarded and the pellet was resuspended in MM. Dilutions were done in 50 ml falcons (13 per carbon source) and when OD = 0.05 was reached, carbon source was added.

Fig. 2 -Shaker-incubator at 28°C.

Fig. 3 -Inocula.

3.3.1 - Biomass/Cells count

An estimation of the biomass (OD) and the number of cells in liquid cultures (n. of cells/ml) were done with different methods.

3.3.2 - Optical Density

The Optical density by spectrophotometer (Fig.4) was the first method applied. It is a useful measure to estimate the biomass in a liquid medium. It was taken 1 ml of the sample and transferred into a cuvette of 1.5 ml. The wavelength was set at 610 nm. The first sample was the blank (only the medium without bacteria) to calibrate the instrument and after the samples.

3.3.3 - Flow Cytometry

This method allows to have a large amount of informations about cell sizes and about their number in a high throughput manner because it is possible to combine this technique with 96-well plates. Flow cytometry was used to obtain accurate estimation about the number of cells in the inoculum. The equipment (BD Accuri c6 Flow cytometer) is shown in Fig.5. To calibrate this machine it was followed the cleaning protocol using 24-tubes plate format by decontamination solution (sodium hypochlorite 1%), detergent solution, beads solution (solution with chemicals to check the machine signal during the cleaning protocol) and 2 water EVIAN solutions (commercial water previously filtrated). Afterwards, the analysis of the samples.

A new workspace was set where parameter such as, flow speed, size signal, fluorescent filters, quantity and samples position in the template were fixed.

Samples, in triplicates, were prepared as follow: 20µl from the inoculum was added to 180µl of PBS solution filtrated (in order to diminish the background noise) and 2µl of fluorescent life-stain.

Fig. 4 - Spectrophotometer

Fig. 5 - Flow cytometer BD Accuri c6.

Chapter 4

RESULTS AND DISCUSSIONS

4.1.1 - Growth data overview

The results of bacterial growth curves were according to the theoretical expectation: lag phase, exponential phase and stationary phase. All of the isolates, with the exception of *Acidovorax* that it was growing faster in MM (2mM of succinate), were growing faster in MM (2mM of glucose).

It was observed little decreases in biomass with MM (2mM of succinate), suggesting lower metabolic rate. Succinate is a intermediate metabolite downstream in the catabolic pathway of the glucose (14).

Regarding MM (2mM of glycerol), the strains were growing much slower. It is possible to see these differences on the OD values range in glycerol between some isolates.

4.1.2 - Growth curves and phases

Ochrobactrum and *Shewanella* (Fig.6) were growing with the same pattern in glucose: lag phase was during the first 9 hours, exp phase was comprised between first 10 and 30 hours after inoculation and reached the stationary phase (OD = 0.6) after 36 hours.

Ochrobactrum was growing in succinate showing this pattern: lag phase during 6 hours, exp phase between 6 and 33 hours after incubation, reached the stationary phase after 36 hours (OD = 0.3).

Ochrobactrum was growing in glycerol with this pattern: lag phase during 6 hours, exp phase between 6 and 30 hours after incubation, reached the stationary phase after 36 hours (OD = 0.15).

Shewanella was growing in succinate showing this pattern: lag phase during 9 hours, exp phase between 9 and 36 hours after incubation, reached the stationary phase after 36 hours (OD = 0.2).

Shewanella was growing in glycerol with this pattern: lag phase during 9 hours, exp phase between 9 and 33 hours after incubation, reached the stationary phase after 36 hours (OD = 0.12).

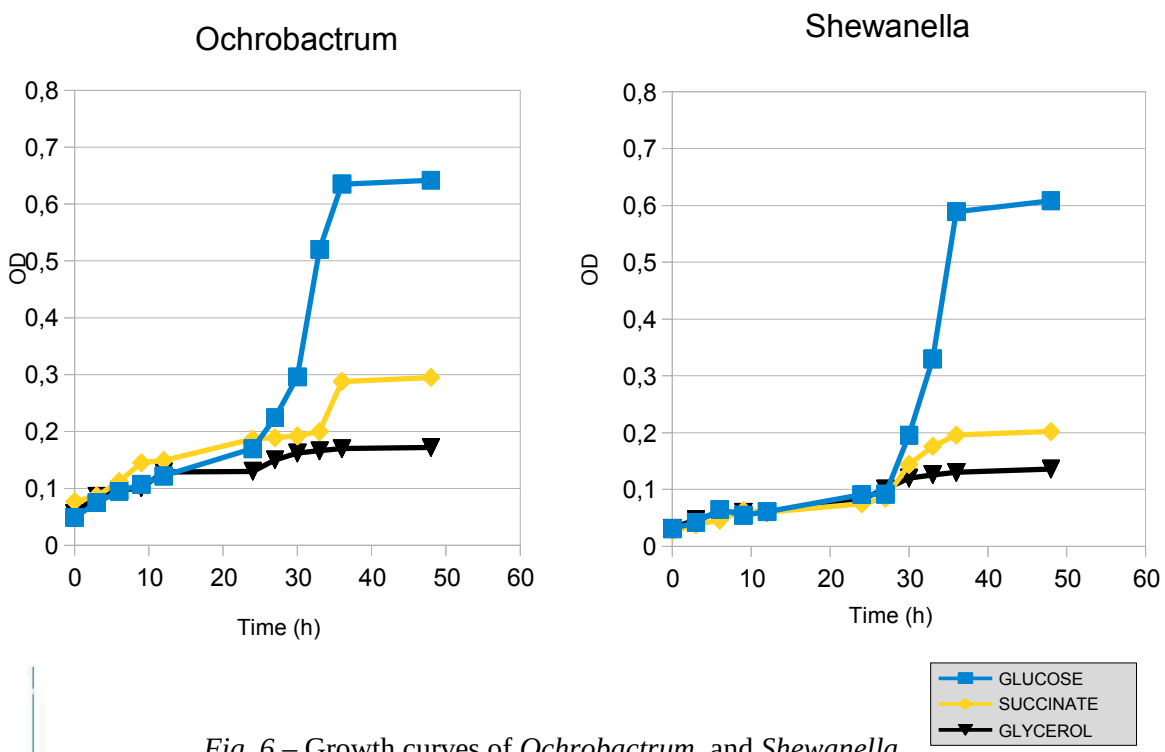


Fig. 6 – Growth curves of *Ochrobactrum* and *Shewanella*.

Bacillus and *Enterococcus* (Fig.7) were growing with similar pattern in glucose: lag phase during the first 3 hours for *Bacillus* and 6 hours for *Enterococcus*, exp phase had the same common point after 24 hours, reached the stationary phase of *Enterococcus* after 33 hours (OD = 0.4), then of *Bacillus* after 36 hours (OD = 0.35).

Bacillus was growing in succinate and glycerol showing the same pattern: lag phase during 3 hours, exp phase between 6 and 24 hours after incubation, reached the stationary phase after 27 hours (OD ~ 0.18).

Enterococcus was growing in succinate with this pattern: lag phase during 6 hours, exp phase between 9 and 33 hours after incubation, reached the stationary phase after 36 hours (OD = 0.22).

Enterococcus was growing in glycerol showing this pattern: lag phase during 9 hours, exp phase between 12 and 27 hours after incubation, reached the stationary phase after 30 hours (OD = 0.15).

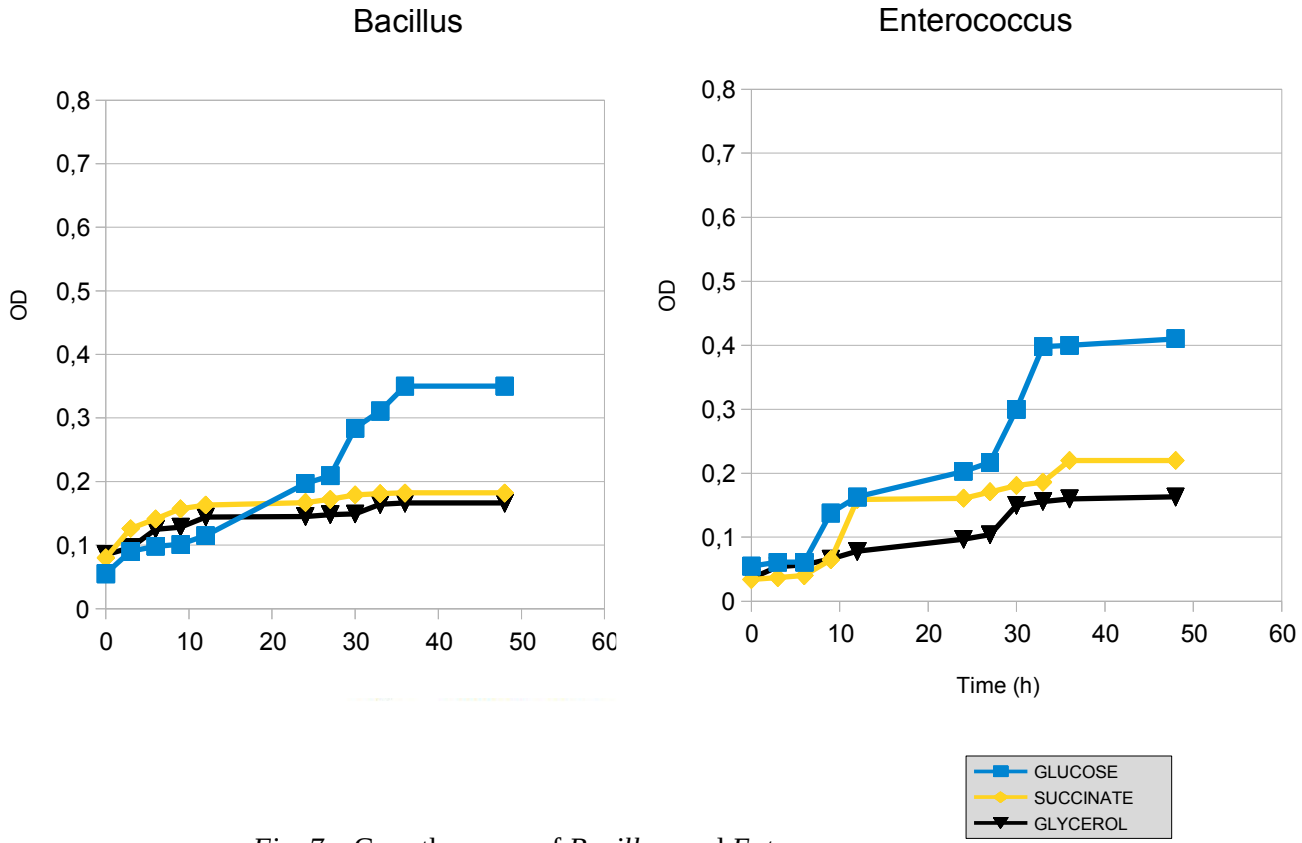


Fig. 7 – Growth curves of *Bacillus* and *Enterococcus.*

Enterobacter (Fig.8) was growing in glucose and glycerol with the same pattern: lag phase during 6 hours, exp phase between 9 and 24 hours after incubation, reached the stationary phase after 27 hours (OD glucose = 0.4; OD glycerol = 0.2).

Enterobacter was growing in succinate showing this pattern: lag phase during 9 hours, exp phase between 12 and 24 hours after incubation, reached the stationary phase after 27 hours (OD = 0.2).

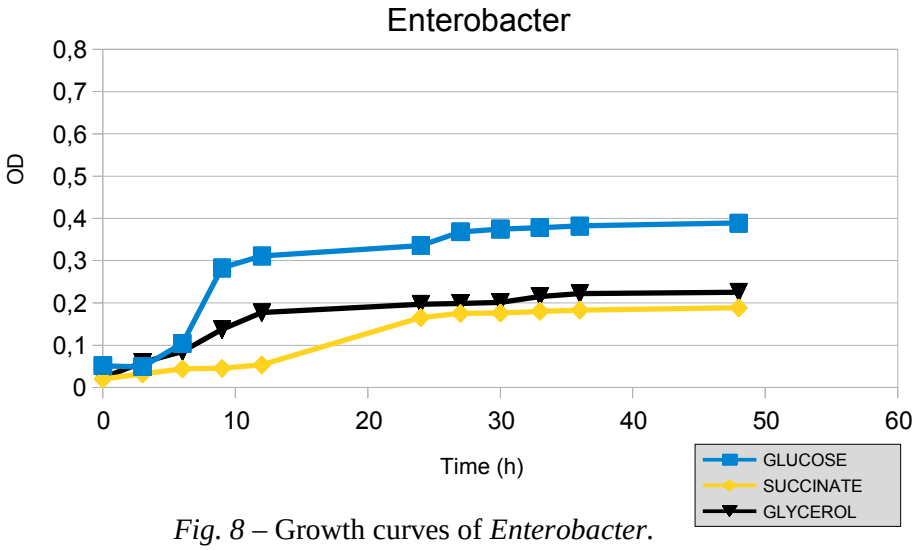


Fig. 8 – Growth curves of *Enterobacter*.

Pseudomonas (Fig.9) was growing in glucose and glycerol with the same pattern: lag phase during 24 hours, exp phase between 27 and 33 hours after incubation, reached the stationary phase after 36 hours (OD glucose = 0.3; OD glycerol~0.1).

Pseudomonas was growing in succinate showing this pattern: lag phase during 3 hours, exp phase between 6 and 33 hours after incubation, reached the stationary phase after 36 hours (OD = 0.2)

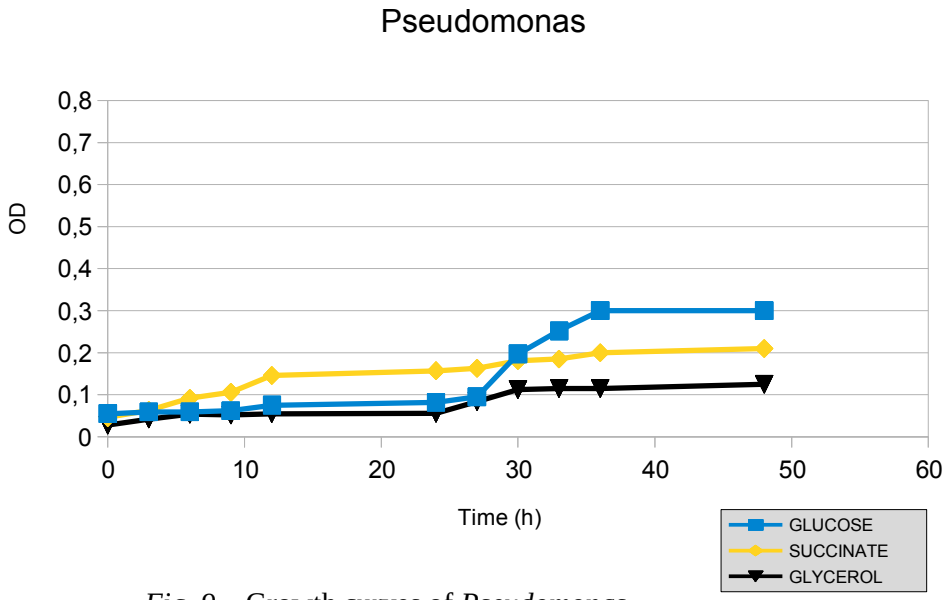


Fig. 9 – Growth curves of *Pseudomonas*.

Acidovorax (Fig.10) was growing in glucose and glycerol with the same pattern: lag phase during 6 hours, exp phase between 9 and 27 hours after incubation, reached the stationary phase after 30 hours (OD glucose ~0.1; OD glycerol~0.1).

Acidovorax was growing in succinate showing this pattern: lag phase during 3 hours, exp phase between 6 and 24 hours after incubation, reached the stationary phase after 27 hours (OD = 0.

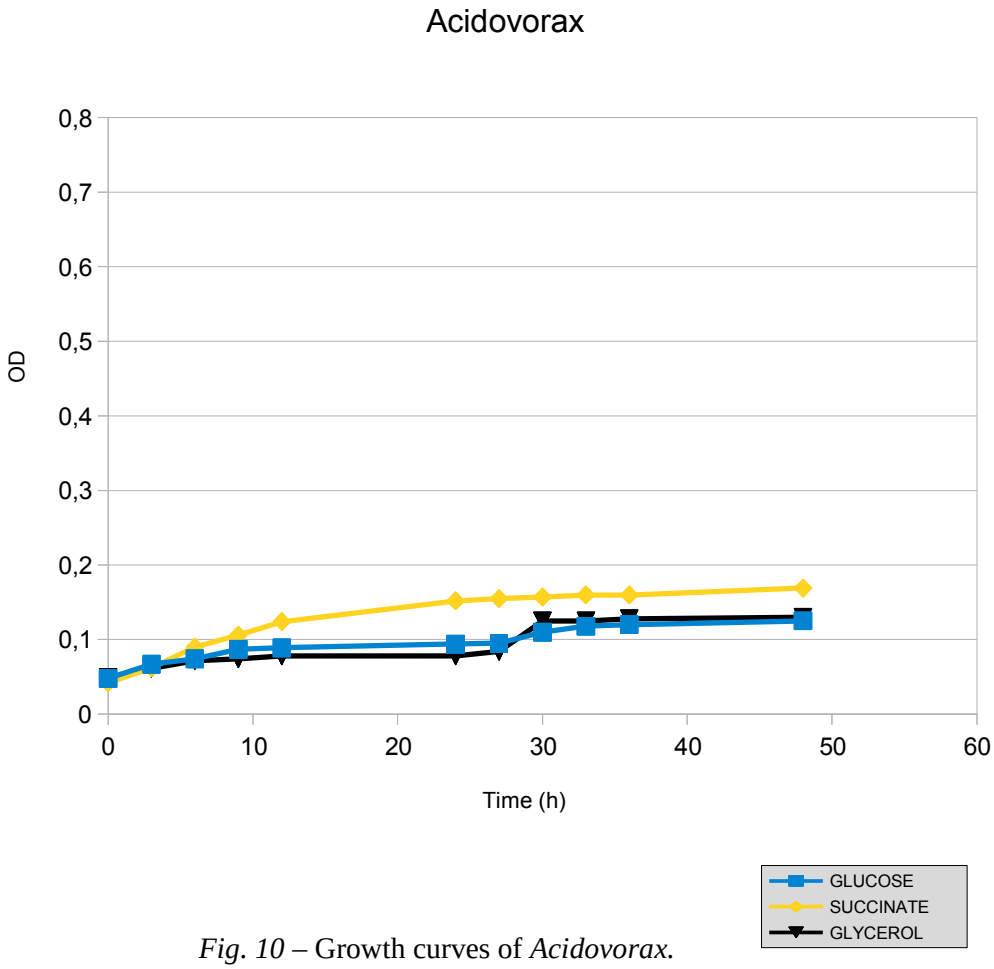


Fig. 10 – Growth curves of *Acidovorax.*

Beijerinckia (Fig.11) was growing in any carbon sources with the same pattern: lag phase during 3 hours, exp phase between 6 and 33 hours after incubation, reached the stationary phase after 36 hours (OD glucose = 0.25; OD glycerol = 0.15; OD succinate = 0.18).

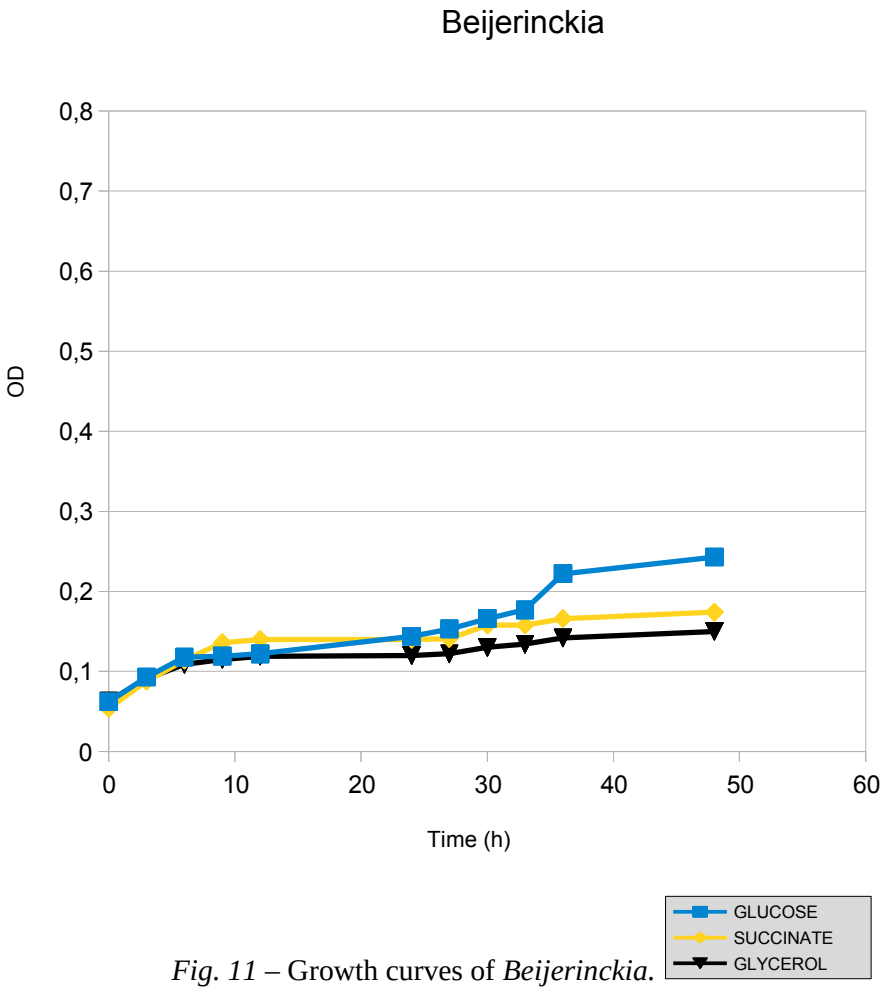


Fig. 11 – Growth curves of *Beijerinckia*.

Staphylococcus (Fig.12)) was growing in succinate and glycerol with the same pattern: lag phase during 12 hours, exp phase between 12 and 33 hours after incubation, reached the stationary phase after 36 hours (OD succinate = 0.15; OD glycerol = 0.15).

Staphylococcus was growing in glucose showing this pattern: lag phase during 12 hours, exp phase between 12 and 36 hours after incubation, reached the stationary phase after 48 hours, (OD~0.7)

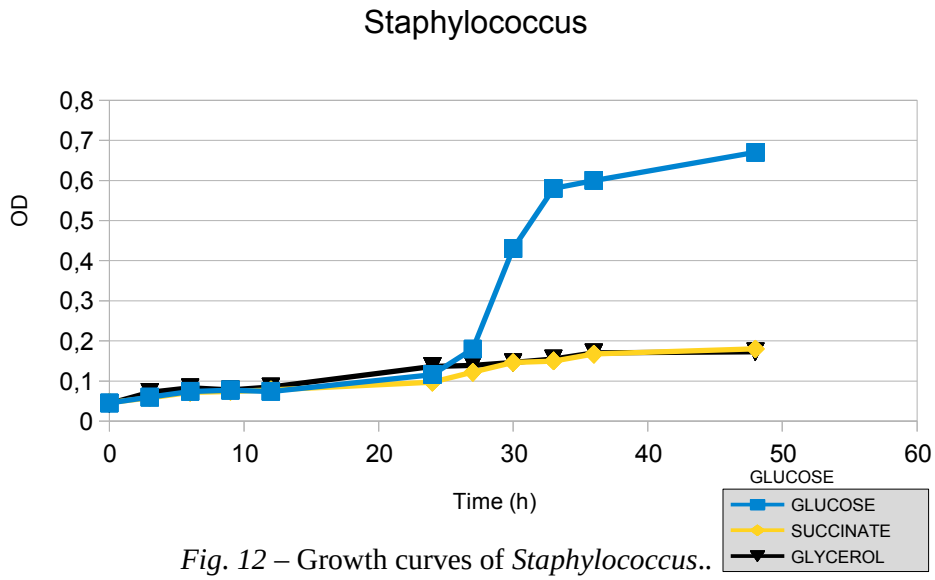


Fig. 12 – Growth curves of *Staphylococcus*..

Comamonas (Fig.13) was growing in succinate and glycerol with the same pattern: lag phase during 6 hours, exp phase between 9 and 24 hours after incubation, reached the stationary phase after 36 hours (OD glucose = 0.2; OD glycerol = 0.2).

Comamonas was growing in succinate showing this pattern: lag phase during 6 hours, exp phase between 9 and 33 hours after incubation, reached the stationary phase after 36 hours (OD = 0.5

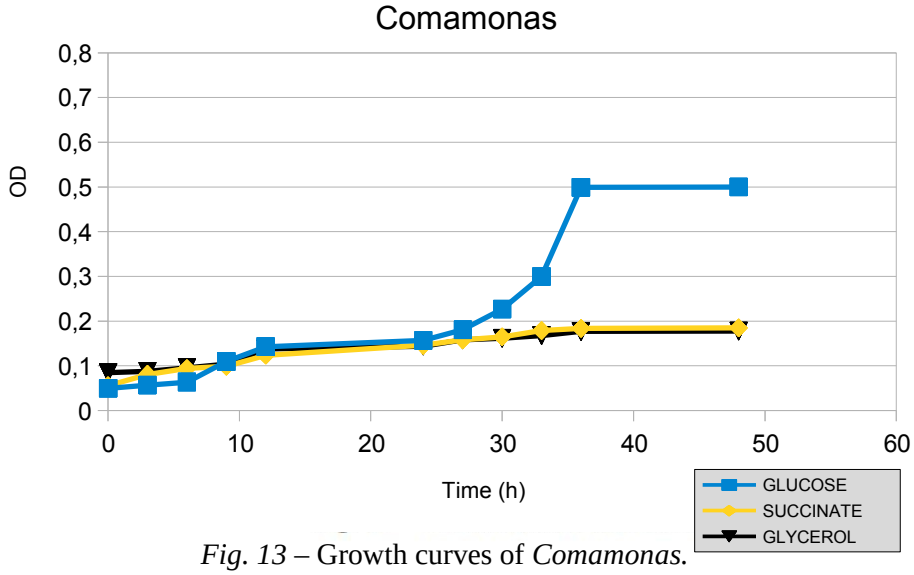
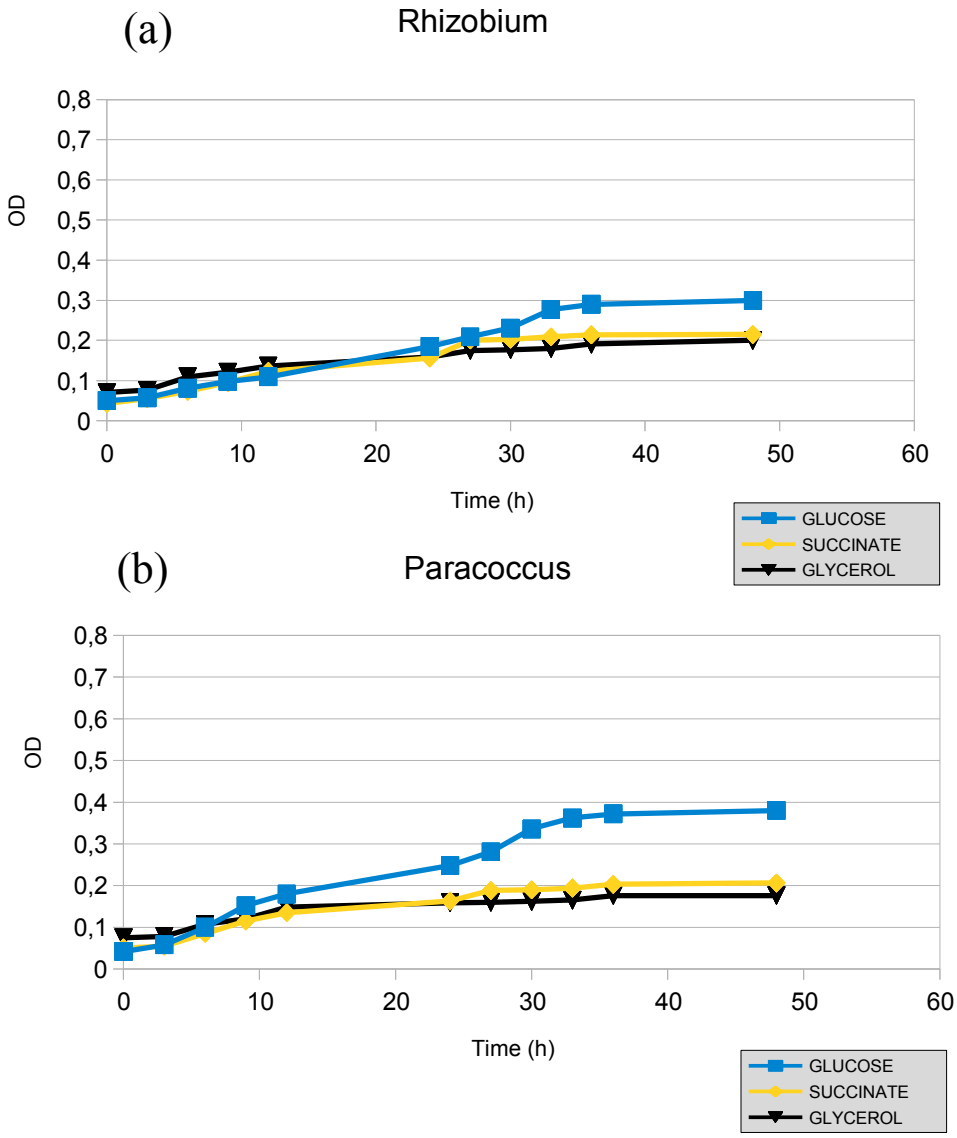


Fig. 13 – Growth curves of *Comamonas.*

Rhizobium (Fig.14a) was growing in any carbon sources with the same pattern: lag phase during 3 hours, exp phase between 6 and 33 hours after incubation, reached the stationary phase after 36 hours (OD glucose = 0.3; OD glycerol = 0.18;OD succinate = 0.2).

Paracoccus (Fig.14b) was growing in any carbon sources showing the same pattern: lag phase during 3 hours, exp phase between 6 and 30 hours after incubation, reached the stationary phase after 33 hours (OD glucose = 0.38; OD glycerol = 0.19;OD succinate = 0.2).

Brachybacterium (Fig.14c) was growing in any carbon sources with the same pattern: lag phase during 6 hours, exp phase between 9 and 27 hours after incubation, reached the stationary phase after 30 hours (OD glucose = 0.25; OD glycerol = 0.15;OD succinate = 0.18).



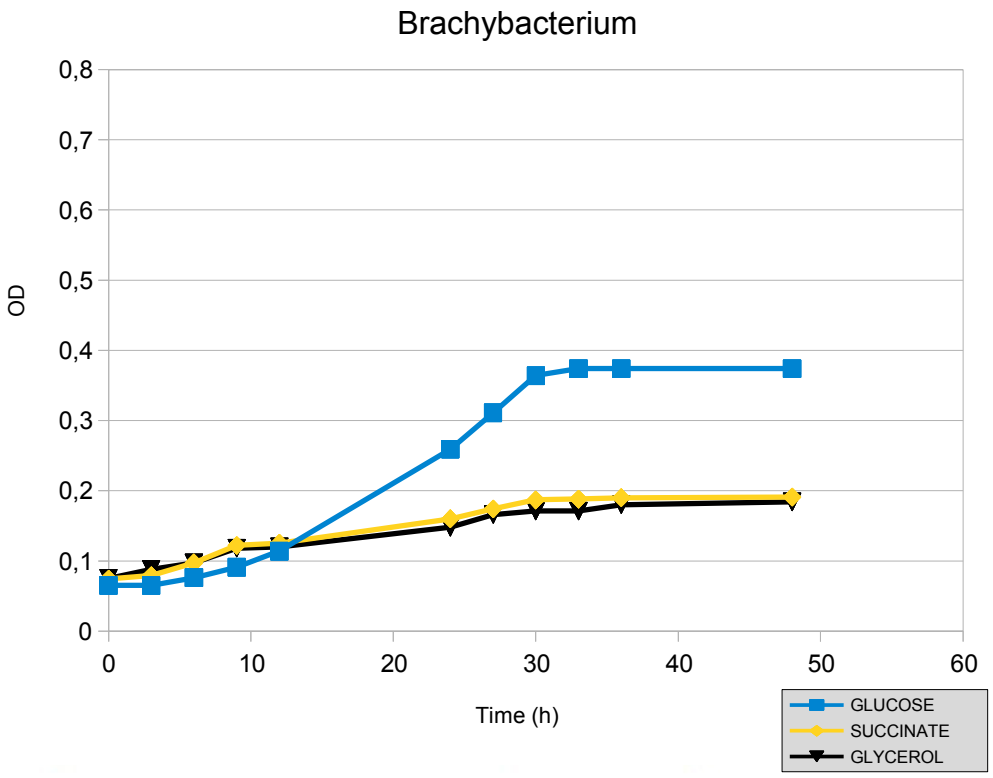


Fig. 14 – Growth curves of *Rhizobium* (a), *Paracoccus* (b) and *Brachybacterium* (c).

4.2.1 - Flow cytometry analysis on inocula.

These flow cytometry plots (Fig.15,16 and17) show the profile of inocula.

In this report only three profiles are shown although all inocula were analyzed by flow cytometry.

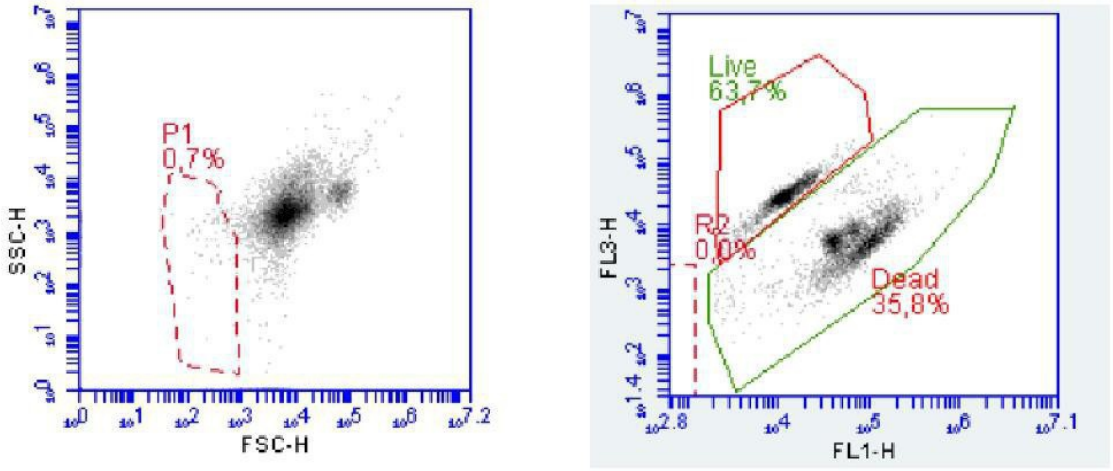


Fig. 15 – Flow cytometry plots of *Pseudomonas* inoculum.(Left, total n. of cells. Right, percentage of dead and living cells).

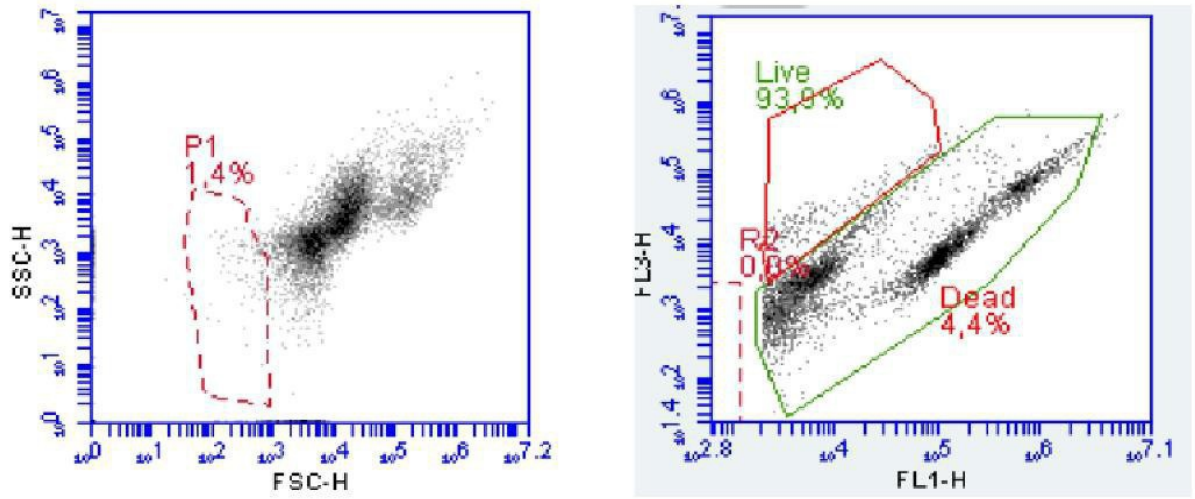


Fig. 16 – Flow cytometry plots of *Bacillus* inoculum.(Left, total n. of cells. Right, percentage of dead and living cells).

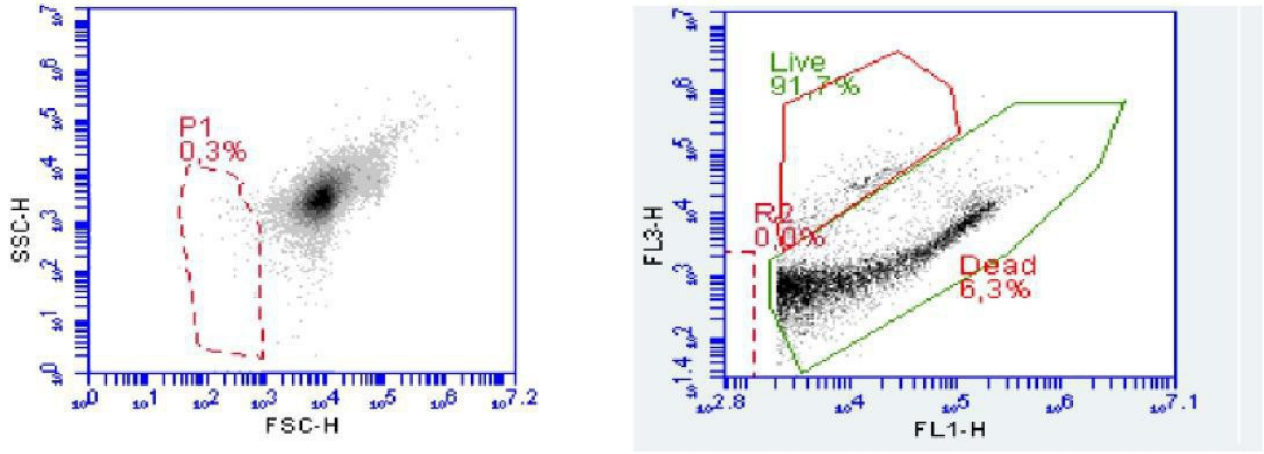


Fig. 17 – Flow cytometry plots of *Acidovorax* inoculum.(Left, total n. of cells. Right, percentage of dead and living cells).

The left graph of each figure shows the relation between size scatter (SSC-H) and fluorescent scatter (FSC-H) of the total amount of cells per inoculum. Moreover, the right graph of each figure shows the percentage of living cells (green plot) and dead cells (red plot, damaged membrane).

Figure 18 shows the total amount of living and dead cells of all the inocula.

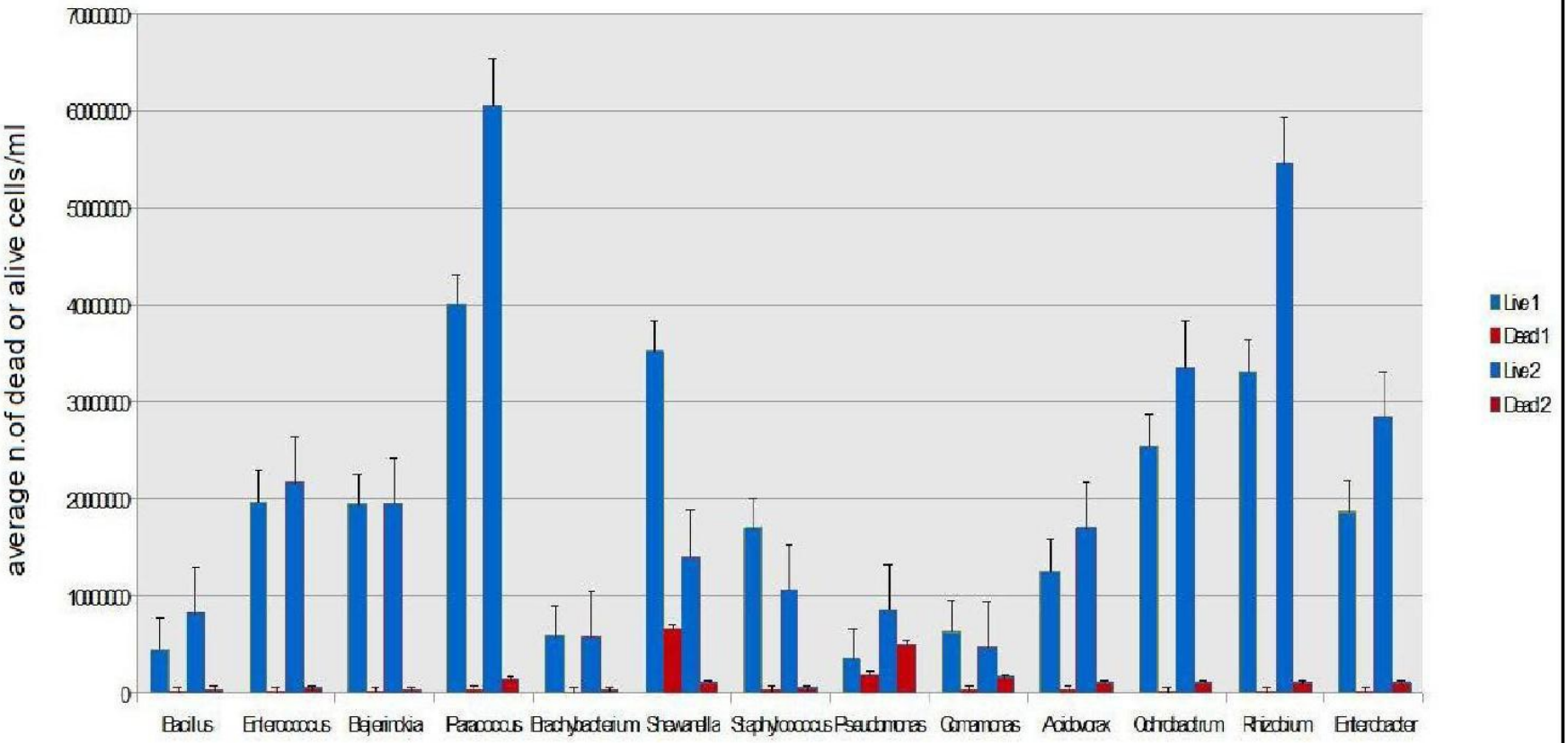


Fig.18 – This graph shows the total amount of living and dead cells of all the inocula (two different dilutions of the same inoculum and three technical replicas per dilution).

Chapter 5
CONCLUSIONS

1. All isolates grown in LB agar 2% medium after 48h of incubation at 28°C.
However, *Bacillus* grown after 24h.

2. Each isolate grown in LB broth liquid medium after 24-48 hours of incubation at 28°C

3. All isolates, except *Acidovorax*, grown in MM (2mM of glucose) reaching the maximum value of biomass.

4. All isolates grown in MM (2mM of succinate) and MM (2mM of glycerol), but reaching lower biomass values.

5. *Acidovorax* reached the maximum biomass value by MM (2mM of succinate) and also in MM (2mM og glycerol).

6. *Comamonas*, *Staphylococcus* and *Brachybacterium* did not show differences in MM (2mM of succinate) and MM (2mM of glycerol).

7. Flow cytometry is more accurate technique to quantify the amount of cells than OD. However, High standard deviation was observed due to dilution factors.

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