## A. Bagasse treatments

### Protocol Pretreatment thermo-hydrolysis:

**Materials:**

1. (3) Ball flask
2. (3) 10 - 200 °C Thermometer
3. (6) Finger clamps
4. (3) Condenser
5. (3) Rubber cork
6. (10) Hoses

**Reagents:**

1. Treated water
2. 500 g of *Agave tequilana* W. bagasse

**Equipment and its specifications:**

1. Drying oven
- *DHG-9145A / OP12119*
2. Vacuum bomb
- *Felisa/ Model: FE-1500/ Series: 1102078*
3. Digital gram balance
- *GR-200 / 14243017*
4. Timer
5. Mill
6. Cold water line
- *Active aqua/ Model: AAPW250/ Voltage: 120 V/ Power:16W*
7. Heating plate
- *Fisher Scientific/ Isotemp Termoplaca*

For the thermic hydrolysis the *Agave tequilana* Weber bagasse will be dried and set up to a $\frac{6\ ml\ water}{1\ g\ bagasse}$ of dry weight ratio, the mixture will be put in a refluxing apparatus and temperature will be fixed to 190 °C for 90 minutes. After that the bagasse will be filtered with a buchner funnel using a vacuum bomb to separate it from the hydrolysis liquor, which will be stored for further analysis, and then the bagasse will be cut with a mill (**Figure 3)** to reduce the the size of the fiber to 25 mm long pieces. The bagasse will then be dried at an oven and weighed (Abril, Medina, & Abril, 2012).

1. Dry in oven the bagasse sample until constant weight is achieved **Figure 1**.
2. Weigh the dried bagasse.
3. Set up refluxing apparatus as shown in **Figure 2**.

4. Pour in the dried bagasse in the ball flask.
5. Pour in the corresponding water $\frac{6\ ml\ water}{1\ g\ bagasse}$ of dry weight ratio.
6. Set the heating plate at 400°C until the temperature of the system reach 190 °C and maintain. After the temperature is reached let the hydrolysis take place for 90 minutes. Keep an eye on the system. Once the time has passed, let the system cool down enough to safely handle it.
7. Filter the bagasse and save the liquor.
8. Cut the bagasse in the mill (**Figure 3**) while it's still hot. Recover as much as you can from the fiber and the remaining liquor.
9. Dry the bagasse thermo hydrolysed until constant weight is achieved.
10. Weigh the bagasse and register the data.
11. Take a 100 g sample for analysis.
12. Save the bagasse and the liquor for further use.

**Figure 1.** Bagasse in the oven

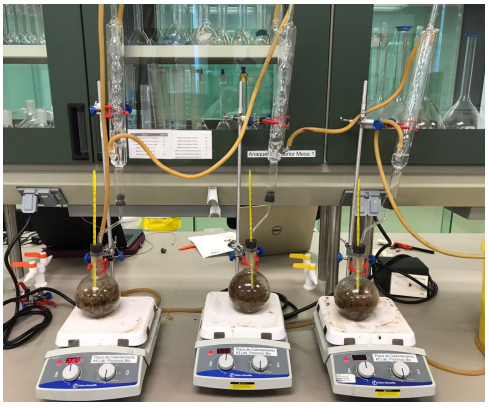

**Figure 2.** Refluxing apparatus set up.

**Figure 3.** Mill.

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***Lab Notebook:***

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Thermal hydrolysis (Pre-treatment of bagasse)

14 g of bagasse were weighed and placed into the ball flask.
84 mL of water was added to the flask.
This was done in triplicate.
The plates were preheated to 530 ° C, thermometers recorded a temperature of 92 ° C.
The system was connected and the process was done for 90 minutes.
The bagasse was vacuum filtered with a buchner funnel and allowed to dry for 24 hours at 60 ° C.

## Alkaline treatment:

**Materials:**

1. (3) Ball flask
2. (3) 10 - 200 °C Thermometer
3. (6) Finger clamps
4. (3) Condenser
5. (3) Rubber cork
6. (10) Hoses

**Reagents:**

1. Distilled water
2. 3 % w/v NaOH
3. 10 % w/v $H_2SO_4$

**Equipment and its specifications:**

1. Potentiometer
- *Hanna- HI 2214*
2. Mill
3. Digital gram balance
- *GR-200 / 14243017*
4. Drying oven
- *DHG-9145A / OP12119*
5. Heating plate
- *Fisher Scientific/ Isotemp Termoplaca*
6. Cold water line
- *Active aqua/ Model: AAPW250/ Voltage: 120 V/ Power:16W* -

The previously thermo hydrolysed bagasse will be set to a 7.83 % w/v solution of 3 % w/v NaOH in a refluxing apparatus for 32.5 minutes at 121 °C in a gas extraction hood The

hydrolysed solid will be filtered and its pH will be set to 7 with $H_2SO_4$ (10 % w/v) (Sánchez *et al*, 2015), then the bagasse will be cut with a mill (**Figure 3**) to reduce the the size of the fiber to 1 mm long pieces. The bagasse will then be dried at a dried oven.

1. Set up refluxing apparatus as shown in **Figure 4**. and set it in the gas extraction hood.
2. Pour in the previously thermo hydrolysed bagasse in the ball flask.
3. Pour in the corresponding NaOH in the ball flask.
4. Turn on the heating plate at 400°C and wait until the temperature of the system reach 121 °C. After the temperature is reached let the hydrolysis take place for 32.5 minutes. Keep an eye on the system. Once the time has passed, let the system cool down enough to safely handle it.
5. Filter the bagasse and save the liquor.
6. Neutralize the pH from the liquor and the bagasse separately.
7. Cut the bagasse in the mill. Recover as much as you can from the fiber.
8. Dry the hydrolysed bagasse until constant weight is achieved (**Figure 5)**.
9. Take a 100 g sample for analysis.
10. Save the bagasse for further use.

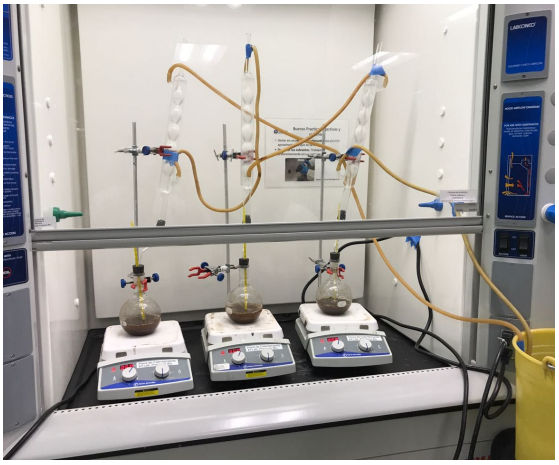

**Figure 4.** Refluxing apparatus up in extraction hood.

**Figure 5.** Dried and milled bagasse set after alkaline treatment

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*Lab Notebook:*

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**Alkaline treatment**

This treatment was performed within the exhaust hood by triplicate.

1. 12,699 g and 162.1839 mL of NaOH were placed in a 500mL ball flask
2. 10.5409 g and 134 622 mL NaOH were placed in a 500mL ball flask.

3. 13.3833 g and 170 926 mL NaOH were placed in a 500mL ball flask.

It was heated to 100 ° C with the use of thermoplates for 35 minutes to achieve effective hydrolysis.
Then it was placed to pH 7 and filtered. Finally it was set to dry in an oven for 24 hours at 60 ° C.

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Weight dried and ground recovered from alkaline hydrolysis processes.

1. Sample 1: 10.46 g
2. Sample 2: 10.13 g
3. Sample 3: 7.53 g

## Cellulose quantification:

### Materials

1. (6) beaker (250 mL)
2. (6) Buchner funnel
3. (6) Kitasato flask
4. (2) Vacuum pump
5. (2) spatula
6. (6) volumetric flask (200 mL, 500 mL)

### Reagents

1. SLS
2. EDTA
3. Na2B4O7 *10H2O
4. Na2HPO4
5. PG
6. Na2SO3
7. H2SO4
8. CTAB
9. alpha-amylase
10. Distilled water
11. acetone

### Equipment and its specifications

1. (6) Stirring heating plate

- *Fisher Scientific/ Isotemp Termoplaca*

2. (2) Vacuum pump

- *Felisa/ Model: FE-1500/ Series: 1102078*

3. Analytical balance
- *GR-200 / 14243017*
4. Drying Oven
- *DHG-9145A / OP12119*
5. Extraction hood

*Solutions preparation*

***Table 1:*** *Preparation of 500 mL of Neutral detergent*

| | |
|---|---|
| Distilled water | 0.5 L |
| SLS | 15 g |
| EDTA | 9.3 g |
| (Na2B4O7*10H2O) | 3.4 g |
| (Na2HPO4) | 2.3 g |
| PG | 5.0 mL |

1. Add 0.2 L of distilled water in a beaker on a stirring heating plate.
2. Add each reactant one by one except for TG.
3. Heat if necessary to dissolve ingredients. Be careful not to create much foam.
4. Add remaining water and TG, leave it overnight to eliminate foam.

***Table 2:*** *Solution preparation for 50 mL of 2% alpha-amylase solution*

| | |
|---|---|
| | 50 mL |
| Distilled water | 49 mL |
| Saliva samples | 1 mL |

1. 3 Saliva donors had a mouthwash with saline solution for 30 seconds.
2. Collect saliva samples in a 250 mL beaker with filter paper and swirl gently.
3. Take 1 mL of saliva mix and add 49 mL of distilled water.

***Table 3:*** *Solution preparation of 800 mL of acid detergent*

| | |
|---|---|
| | 800 mL |

| | |
|---|---|
| H2SO4 1N | 800 mL |
| CTAB | 16 g |

1. In extraction hood add 21 mL de H2SO4 98% and then add distilled water until reaching 800 mL.
2. Add 16 g of CTAB and mix in a stirring plate.
3. Leave it overnight to eliminate foam.

*FND determination*

1. Weight 0.55 g of pre treated and non pre treated bagasse (by triplicate = 1.65 g of each type of bagasse) and add them to 250 mL beakers.
2. Pre heat stirring plates at 300°C.
3. Add 0.5 g of Na2SO3 to each sample.
4. Add 50 mL of neutral detergent to each sample.
5. Heat samples at 300°C during 5 min as shown in **Figure 6.**
6. Remove beakers from plate and add 2 mL of alpha-amylase solution.
7. Place them on the plate again and continue heating at 100°C for 1 hour.
8. Boil distilled water at the same time to use it for the filtration step.
9. Preheat buchner funnels at 60°C for the filtration step.
10. Weight 6 filter papers and register data.
11. Decant samples on funnels with filter papers, wash beakers with boiling water and decant them again on the funnels. Filter with vacuum.
12. Add 30 mL of boiling water and 2 mL of alpha-amylase solution. Let solution react for a minute and filter again as shown in **Figure 7.**
13. Add 30 mL of boiling water to each funnel for the first wash, let it settle for 2 minutes and then filter. Repeat one more time.
14. Repeat step 14 with acetone.
15. Recover samples on filter papers and let them dry overnight in the oven at 105°C.
16. Weight dry papers and register data.

*FAD determination*

1. Weight 1.1 g of pre treated and non pre treated bagasse (by triplicate each type of bagasse) and add them to 250 mL beakers.
2. Pre heat stirring plates at 300°C inside the extraction hood.
3. Add 100 mL of acid detergent solution to each sample.
4. Place the beakers on the stirring plates at 200°C inside the extraction hood.
5. Let it boil for 5 minutes and then reduce temperature to avoid foam.
6. Heat samples for 1 hour.
7. Preheat funnels at 60°C.

8. Remove samples from plates and let them cool.
9. Decant samples on funnels with filter papers, wash beakers with boiling water and decant them again on the funnels. Filter with vacuum as shown in **Figure 8.**
10. Add 30 mL of boiling water and 2 mL of alpha-amylase solution. Let solution react for a minute and filter again.
11. Add 30 mL of boiling water to each funnel for the first wash, let it settle for 2 minutes and then filter. Repeat one more time.
12. Repeat step 14 with acetone.
13. Recover samples on filter papers and let them dry overnight in the oven at 105°C.
14. Weight dry papers and register data.

*Calculus for %cellulose*

$$\% \text{ of cellulose} = \frac{W_2 - W_3}{W_1} \times 100$$

*$W_2$: Dried weight of digested residue of bagasse of FAD process*
*$W_3$: Dried weight of digested residue of bagasse of FND process*
*$W_1$: Dried weight of the bagasse sample*

**Figure 6.** Heated bagasse samples samples with neutral detergent at 300°C

**Figure 7.** Filtration system of FAD solution

**Figure 8.** Heated bagasse samples with acid detergent at 200°C

**Figure 9.** Filtration system of FND acid solution

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The crucibles were baked to achieve constant weight (60 ° C).
Bagasse weight after heat treatment, drying and grinding.

**Figure 10.** Crucibles at constant weight.

**<u>Procedure for cellulose quantification</u>**
**Preparation of solution**

1. Prepare Neutral detergent (500 mL)

***Table 4:*** *Components of neutral detergent (500mL)*

| | |
|---|---|
| Distilled water | 0.5 L |
| Sodium lauryl sulfate | 15 g |
| EDTA | 9.3 g |
| Borate Sodium (Na2B4O7 * 10H2O) | 3.4 g |
| dibasic sodium phosphate (Na2HPO4) | 2.3 g |
| Triethylene | 5.0 mL |

2. Add 0.2 L of distilled water in a beaker and place a magnetic stirrer to homogenize the sample. Add the reagents gradually with the exception of TEG. Heat if necessary to further dilution. Add remaining water carefully slowly to avoid foaming. When three quarters of water have been added, add the TEG. Allow to stand overnight or until foam disappears.

***Table 5:*** *Components of acid detergent (500mL)*

| | |
|---|---|
| H2SO4 1N | 800 mL |
| CTAB | 16 g |

**NOTE:** To prepare the solution of H2SO4, 21 mL of 98% H2SO4 were placed in 800 mL of H2O.

**Precautions**
Acid vapors mask should be used, work with gloves, safety glasses and under hood when preparing acid and neutral detergent solution. Digestions are performed in the same safety conditions. CTAB is a mucosal, eye and skin irritant use adequate protection (gloves, lenses, mask). Acetone is flammable. Work in extraction hood.

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**Preparation of amylase from saliva:**

1. Prepare a beaker of 250 mL and place a filter over it.
2. Rinse mouth with saline solution for 30 seconds.
3. Discard saline solution into the sink, and chew a piece of cotton during 1 minute.
4. When sufficient saliva has been produced, remove cotton from mouth and dispose of saliva on the filter.
5. Remove 1 mL of saliva and put into a 250 mL flask with 49 mL of distilled water to prepare a 2% solution of amylase (assuming that saliva is 40% amylase).
6. Shake the flask slightly to homogenize the mixture and store at room temperature.

**determination FND**

1. Weigh 0.55 g of bagasse (in triplicate = 1.65 g) and transfer to a 250 mL container.
2. Preheat stirring plate at 300 ° C
3. Add 0.5 g of sodium sulfite (Na2SO3) to each sample.
4. Place 50 mL of neutral detergent solution to each glass.
5. Stop heating the samples at 300 ° C for 5 min.
6. Remove the plate and place 2 mL of thermoresistant amylase.
7. Reposition over thermplate at 250 ° C and leave for one hour refluxing. After 7 minutes of heating, wash vessel walls with neutral detergent solution using the same volume for each glass.
8. The temperature of the plates was reduced to 100 ° C because the boling of P3 was causing sample loss.
9. Distilled water is heated to its boiling point for use during filtration.

10. Filter paper weights:

***Table 6.*** *Weight of filter paper*

| **Test Number** | **Treated Sample** |
|---|---|
| P1 | 0.8122 g |
| P2 | 0.8090 g |
| P3 | 0.8028 g |
| | |
| | **Untreated Sample** |
| S1 | 0.8314 g |
| S2 | 0.8266 g |
| S3 | 0.8189 g |

11. Decant gooch crucibles that have been previously dried and tared.
12. Wash the glasses with boiling water to transfer contents of the vessels to the crucible and vacuum filter.
13. Add to the sample crucible 30 mL of boiling water and add 2 mL of alpha-amylase. Set to react for 45-60 seconds and filter vacuum.
14. Pour 30-40 ml of boiling water to each pot. Let stand 2 minutes soaking and vacuum filter. Repeat wash once again.
15. Fill pot with 30-40 mL of reagent grade acetone, allow to stand for 2 minutes and vacuum filter. Repeat wash once again.
16. Vacuum fiilter sample. To remove most of the liquid, filter each sample for 20-30 seconds.
17. Set bagasse filters to bake at 105 ° C overnight. Cool in desiccator and weigh.

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**Weights FND**

***Table 7****. Weight of bagasse after treatment (FND)*

| **Test Number** | **Weight filter** | **Weight of filter + bagasse** | **Bagasse Weight** |
|---|---|---|---|
| **Untreated sample** | | | |
| S1 | 0.8122 g | 1.1545 g | 0.3423 g |
| S2 | 0.8090 g | 1.2387 g | 0.4297 g |

| | | | |
|---|---|---|---|
| S3 | 0.8028 g | 1.1688 g | 0.3668 g |
| **Treated Sample** | | | |
| P1 | 0.8314 g | 1.0403 g | 0.2089 g |
| P2 | 0.8266 g | 1.2566 g | 0.4300 g |
| P3 | 0.8189 g | 1.2251 g | 0.4062 g |

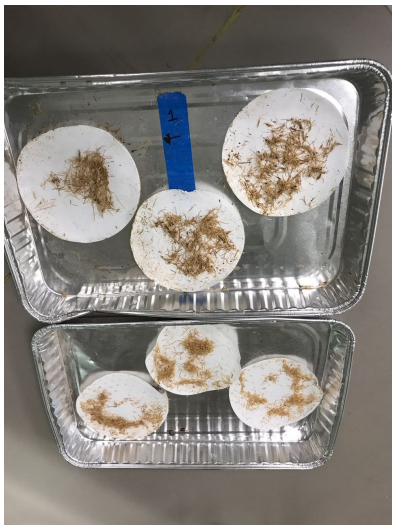

**Figure 11**. Dried bagasse after FND treatment.

**Determination of FAD**

1. Weight 1.1 g of bagasse and place in beaker 250 mL (by triplicate).

***Table 8.*** *Bagasse weight*

| **Untreated bagasse samples.** | **Sample treatment bagasse** |
|---|---|
| 1.117 g | 1.1407 g |
| 1.1109 g | 1.1103 g |
| 1.115 g | 1.1441 g |

2. Weight filter paper

***Table 9.*** *Weight of filter paper*

| **Test No.** | **Untreated Sample** |
|---|---|

| | |
|---|---|
| P1 | 0.8407 g |
| P2 | 0.7957 g |
| P3 | 0.8093 g |
| | **Treated Sample** |
| S1 | 0.8024 g |
| S2 | 0.8267 g |
| S3 | 0.8318 g |

3. Add 100 mL of detergent acid solution,at room temperature.
4. Place the cups on a plate preheated to 200 ° C in a laminar flow hood.
5. Heat to boil for 5-10 minutes. Reduce heat if excessive foaming.
6. Keep warm for 60 minutes from start to boil.
7. After 30 minutes of boil wash vessel walls with detergent acid solution. Remove the cups of the plate and decant for 30-60 seconds.
8. Preheat the previously tared crucibles with boiling water.
9. Decant content of the vessels in the Gooch crucibles and vacuum filter.
10. Quantitatively pass content with boiling water.
11. Wash with 30-40 mL of boiling water, soak for 2 minutes and filter with vacuum. Perform rinse with water twice.
12. Wash with 30 mL of acetone, soak for 2 minutes and vacuum filter. Repeat this wash 2 times.
13. Dry bagasse in oven at 105 ° C overnight.
14. Place in desiccator and weigh.

## Enzymatic treatment with Aspergillus niger and enzymatic activity:

*A. niger growth*

### Materials

1. (1) 125 ml Erlenmeyer flask
2. (1) 500 ml Erlenmeyer flask
3. (2) Cotton swab
4. Kraft paper
5. (4) Petri dish
6. (1) Inoculation loop
7. (2) Spatula
8. Weighing boats

**Reagents**

1. 70 % (v/v) Ethanol
2. Agar
3. Distilled water
4. $KNO_3$
5. $KH_2PO_4$
6. $MgSO_4 \cdot 7H_2O$
7. $FeCl_3$
8. Sucrose
9. HCl 1M
10. NaOH 1M

**Equipment**

1. Autoclave
- *Yamato Sterilizer M5510*
2. Biosafety cabinet
- *Esco class II BSC- AC2-4S2*
3. Digital analytical balance
- *GR-200 / 14243017*
4. Digital gram balance
- EK-610i
5. Loop sterilizer
- *McCormick / Bacti-cinerator IV*
6. Potentiometer
- *Hanna- HI 2214*
7. Incubator at 28 °C
- *Thermo Scientific/ 30M-3971/ 315647-308*
8. Shaking incubator at 28 °C and 120 rpm
- *Thermo scientific / 4342*

**Microorganisms**

- *Aspergillus niger*

*Preparation of Richard's Broth:*

Richard's Broth will be prepared for the *A. niger* biomass that will be employed for the cellulase production on the basal medium.

1. Weight Richard's Broth components according to **Table 10**.

2. Transfer broth components to an 250 ml Erlenmeyer flask, and add 250 ml of distilled water.
3. Set medium's pH to 4.5
4. Seal medium with cap and kraft paper on top.
5. Sterilize in autoclave for 15 min at 121 °C and 15 psi.
6. Transfer broth medium and PDA plate with *A. niger* to biosafety cabinet.
7. Approximately cut a 1 cm wide circle with sterile bisturi and transfer to broth medium.
8. Incubate at 28 °C for 7 days.
9. After incubation period, store culture at 4 °C

***Table 10.*** *Richard's Broth Components*

| Component | g/l |
|---|---|
| $KNO_3$ | 10.000 |
| $KH_2PO_4$ | 5.000 |
| $MgSO_4 \cdot 7H_2O$ | 2.500 |
| Sucrose | 50.000 |

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*Enzyme production, extraction, and quantification.*

**Materials**

1. 2,500 ml Erlenmeyer flasks
2. (1) Büchner funnel with rubber cork
3. (1) Büchner flask
4. (1) Spatula
5. Weighing boats
6. Whatman No. 1 (Filter papers)

**Equipment**

1. Autoclave
- *Yamato Sterilizer M5510*
2. Biosafety cabinet
- *Esco class II BSC- AC2-4S2*
3. Falcon tube centrifuge
- *Eppendorf / 5804 R / 5805YQ136367*
4. Digital gram balance

- EK-610i

5. Digital analytical balance
- *GR-200 / 14243017*

6. Loop sterilizer
- *McCormick / Bacti-cinerator IV*

7. Potentiometer
- *Hanna- HI 2214*

8. Shaking incubator at 32 °C and 200 rpm
- *Thermo scientific / 4342*

9. Vacuum bomb
- *Felisa/ Model: FE-1500/ Series: 1102078*

10. Heating plate
- *Fisher Scientific/ Isotemp Termoplaca*

**Reagents**

1. 70 % (v/v) Ethanol
2. Treated water
3. Carboxymethylcellulose
4. Pretreated *Agave tequilana* Weber
5. HCl 1 M
6. NaOH 1 M
7. $(NH_4)_2SO_4$
8. $KH_2PO_4$
9. $CaCl_2$
10. $MgSO_4 \cdot 7H_2O$
11. Peptone
12. Yeast extract
13. Tween 80
14. Salt solution:
    a. $FeSO_4 \cdot 7H_2O$
    b. $MnSO_4 \cdot H_2O$
    c. $ZnSO_4 \cdot 7H_2O$
    d. $CoCl_2$

**Microorganisms**

7. *A. niger* culture grown for 7 days in Richard's broth.

The liquid state fermentation medium will be prepared with pretreated *Agave tequilana* Weber as sole carbon source, with known cellulose composition with the nutrients and salts concentrations (**Table 11** and **12** respectively). After the digestion, the broth will be filtered to obtain the crude enzyme extract.

***Table 11.*** *Liquid state fermentation medium composition (Cunha, Esperança, Zangirolami, Budino and Farimas, 2012).*

| **Component** | **(w/v) %** |
|---|---|
| $(NH_4)_2SO_4$ | 0.14 |
| $KH_2PO_4$ | 0.20 |
| $CaCl_2$ | 0.03 |
| $MgSO_4 \cdot 7H_2O$ | 0.02 |
| Peptone | 0.50 |
| Yeast extract | 0.20 |
| Tween 80 | 0.10 |
| Salt solution (**Table 4**) | 0.10 |

***Table 12.*** *Salt solution composition (Cunha, Esperança, Zangirolami, Budino and Farimas, 2012).*

| **Component** | **mg/l** |
|---|---|
| $FeSO_4 \cdot 7H_2O$ | 5.0 |
| $MnSO_4 \cdot H_2O$ | 1.6 |
| $ZnSO_4 \cdot 7H_2O$ | 1.4 |
| $CoCl_2$ | 2.0 |

In order to obtain a comparison of cellulase production with a known cellulose source a second flask of broth with liquid fermentation medium and CMC with the same cellulose amount as the sample.

Prepare broth in two 250 Erlenmeyer flask, add the corresponding amount cellulose source to each medium. Sterilize and inoculate the flask with fungal spores from *A. niger*. Measure pH, adjust to 6, with either HCl or NaOH solutions. Maintain the flask at agitation speed of 200 rpm and 30C° in the rotary shake incubator. After 7 days, filtrate culture and centrifuge to 6000 rpm for 15 minutes. The supernatant of the centrifuged exhausted broth will be recovered and saved for further steps.

1. Weight the Liquid state fermentation medium components according to **Table 11**.
2. Transfer medium components to an 500 ml Erlenmeyer flask, and add the corresponding amount of water using the thermo hydrolysis liquor, if more water is required use treated water.
3. Set medium's pH to 6.
4. Seal medium with cap and kraft paper on top.
5. Sterilize medium and the filtration system components (**Figure 11**) in autoclave for 15 min at 121 °C and 15 psi.
6. Transfer broth medium, the filtration system components and *A. niger* in Richard's broth to biosafety cabinet.
7. Assemble filtration system.
8. Filter the microorganism from the broth and transfer it to the liquid state fermentation medium.
9. Incubate at 30 °C for 7 days at 200 rpm.
10. After incubation period, filter the microorganism and the remaining bagasse fibers in the biosafety cabinet from the exhausted broth, preserve the broth and dose it to Falcon tubes for centrifugation. Sterilize the remaining biomass at 121 °C and 15 psi and save it for the enzymatic hydrolysis.
11. Centrifuge the exhausted broth in the Falcon tubes at 6000 rpm for 15 minutes, recover the supernatant and store it for further analysis and usage.
12. Weight 1 g of BSA powder and dilute it in 1 mL of mili Q water to obtain stock solution 100 mg/mL in mLQ water.
13. Dilute stock solution to obtain 1 mL of 1000 ug/mL BSA solution.
14. Prepare BSA dilutions of 5, 10, 20, 30, and 40 ug/mL to obtain the calibration curve in eppendorf tubes by duplicate. A blank tube was also prepared. They were all labeled.
15. Each tube must contain 800 uL of the corresponding BSA dilution and then 200 uL of Bradford solution were added. Each tube was briefly vortex.
16. Each reaction was read by spectrophotometer 15 minutes after at 600 nm.
17. A sample of 800 uL was taken from the enzyme culture and mixed with 200 uL of Bradford solution in an eppendorf tube, by duplicate.
18. 15 minutes after, reactions were read by spectrophotometer at 600 nm.
19. Enzymatic concentration was determined.

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*Lab Notebook:*

Day: September 27, 2017
Study:biotechnological
**Preparation fermentation broth and inoculation with Aspergillus niger**

1. Preparation of fermentation broth to inoculate 500 mL and 100 mL for white.

***Table 13.*** *Components and measurements for fermentation broth (500mL)*

| Components | Amount (grams) |
|---|---|
| $(NH4)_2SO_4$ | 0.7 |
| $KH_2PO_4$ | 0.10 |
| CaCl2 | 0.15 |
| $_{MgSO4} \cdot 7H_2O$ | 0.1 |
| Peptone | 2.5 |
| Yeast extract | 1 |
| Tween 80 | 0.5 |
| Salt solution (Table #) | 0.5 |

***Table 14.*** *Composition of saline*

| components | Amount (grams) |
|---|---|
| $_{FeSO4} \cdot 7H_2O$ | 0.0025 |
| $MnSO_4 \cdot H_2O$ | 0.008 |
| $ZnSO_4 \cdot 7H_2O$ | 0.0007 |
| $CoCl_2$ | 0.001 |

***Table 15.*** *Components and measurements of fermentation broth (100mL)*

| Components | Amount (grams) |
|---|---|
| $(NH4)_2SO_4$ | 0.14 |
| $KH_2PO_4$ | 0.2 |
| $CaCl_2$ | 0.3 |
| $MgSO_4 \cdot 7H_2O$ | 0.2 |
| Peptone | 0.5 |

| | |
|---|---|
| Yeast extract | 0.2 |
| Tween 80 | 0.1 |
| Salt solution (Table # ) | 0.1 |

***Table 16.*** *Composition of saline (100mL)*

| Component | Quantity (grams) |
|---|---|
| $FeSO4 \cdot 7H_2O$ | 0.0005 |
| $MnSO_4 \cdot H_2O$ | 0.00016 |
| $ZnSO_4 \cdot 7H_2O$ | 0.00014 |
| $CoCl_2$ | 0.0002 |

2. NaOH was added to place at pH 6.
3. Broth was placed in the autoclave .
4. Broth was inoculated with *Aspergillus niger* grown in Richards broth along with 26.47 gr. of treated bagasse.
5. Broth with *Aspergillus niger* and bagasse was placed in a shaking incubator at 200 rpm and 30 ° C for 7 days.

**Glucose quantification:**

**Materials**
Test tubes 25 mL
Cell for spectrophotometer
Micropipette

**Reagents**
Randox Glu-HK

**Equipment**
Walter bath
Spectrophotometer

1. Number the test tubes as Blank, and Test 1, Test 2, Test 3 and calibrator
2. Transfer 10uL of de liquor obtained from the filtration after the enzymatic hydrolysis and 1000uL of R1a reagent in one test tube, do it by triplicate
3. Transfer 1000uL of R1a reagent in the test tube named as blank
4. Transfer 10uL of cal reagent and 1000 uL reagent in the test tube named as calibrator
5. Mix correctly and incubate the samples for 10 minutes at 37C in a water bath
6. Measure the absorbance at 500 mm
7. Calculate the concentration of sugar

Glucose concentration (mg/dl) = Absorbance sample/ Absorbance patron x 1000(mg/dl)

*Lab Notebook:*

Thursday 05 October

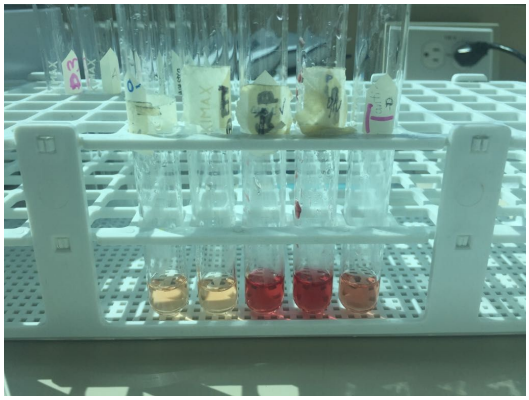
1. Number the test tubes as Blank, negative control, Test 1, Test 2, and calibrator
2. Transfer 10uL of de liquor obtained from the filtration after the enzymatic hydrolysis and 1000uL of R1a reagent in one test tube, do it by duplicate
3. Transfer 1000uL of R1a reagent in the test tube named as blank
4. Transfer 10uL of destilated water and 1000 uL of R1a reagent in the test tube named as negative control
5. Transfer 10uL of cal reagent and 1000 uL of R1a reagent in the test tube named as calibrator
6. Mix correctly and incubate the samples for 10 minutes at 37C in a water bath
7. Measure the absorbance at 500 mm
8. Calculate the concentration of glucose

Glucose concentration (mg/dl) = A sample x 1000(mg/dl)

Negative control
Glucose concentration (mg/dl) = -0.5 / .181 x 1000(mg/dl) = 0mg/dl

Test 1
Glucose concentration (mg/dl) = 0.905/0.181 x 1000(mg/dl) =0.0501g/ml

Test 2
Glucose concentration (mg/dl) = 0.911/0.181 x 1000(mg/dl) =0.0512g/ml



## Agave's leaves pre-treatment

### Materials

- 1 *Agave tequilana* Weber leave
- 1 knife
- 1 ziploc bag
- 1 aluminium tray

### Equipment

- Oven
- Mill

### Methods

1. Cut the leave in four big pieces, and let it dry under the sun for 24 hrs.
2. Cut the dry pieces into approximately 2 cm x 2 cm squares and place them in the aluminium tray.
3. Put the oven at 60°C, place the tray with the leaves inside, and let them dry for another 24 hrs.
4. Take the tray out of the oven and let it cool for about 10 minutes, then put the pieces into the mill and take the powder that results.
5. Store the final powder (should be cellulose) in the ziploc bag until needed.

## Glucose calibration curve

### Materials

- 1 Falcon tube of 50 mL
- 11 Falcon tubes of 15 mL
- 1 Erlenmeyer flask of 50 mL
- 1 micropipette of 1000 µL
- 1 pipette of 5 mL
- 2 cuvettes
- Latex gloves
- Kimwipes

### Equipment

- Spectrophotometer
- Vortex
- Heating plank

### Reagents

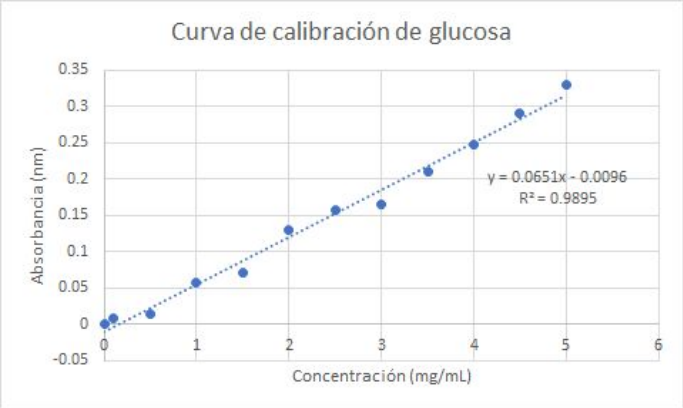
- Stock glucose
- Distilled water
- DNS reagent

### Methods

1. Prepare a 1% (w/v) glucose solution in the Erlenmeyer flask.
2. Do a serial dilution from 5 mg/mL, to 0.5 mg/mL in the Falcon tubes. It results in 10 solutions at different concentrations and the blank.

3. Mix 200 µL of each dilution with 200 µL of DNS, and boil it for 5 minutes.
4. After the 5 minutes, add 5 mL of cold distilled water to stop the reaction.
5. Measure absorbance in the spectrophotometer at 540 nm.
6. Register results.

**Result**




**Enzymatic hydrolysis of A. tequilana cellulose**

**Materials**

- *A. Tequilana* leaves' powder
- 2 amber flasks
- 11 test tubes
- 1 micropipette of 100 µL
- 1 micropipette of 1000 µL
- 1 micropipette of 10 µL
- 1 cuvette
- 1 spatula
- 1 test tube rack
- 2 beaker (100 mL)
- 1 graduated cylinder

**Equipment**

- Incubator
- Spectrophotometer
- pH meter
- Analytical balance

**Reagents**

- Cellulase from *Aspergillus niger*
- DNS solution
- Citric acid
- Sodium phosphate dibasic

**Methods**

Citrate-phosphate buffer

1. Weigh g of citric acid and g of sodium phosphate dibasic.
2. Pour 100 mL of water in the graduated cylinder.
3. Dissolve both reagents in some distilled water of the graduated cylinder. Then, pour the remaining water
4. Measure the pH. If necessary, add more sodium phosphate dibasic until reach a pH of 6.
5. Reserve the solution inside and amber flask.

Cellulase solution

1. Weigh 1 g of cellulase from *Aspergillus niger.*
2. Dissolve in 10 mL of water and mixed.
3. Reserve the solution inside a test tube and store in the fridge.

Cellulose solution

1. Weigh 1 g of *A. Tequilana* leaves' powder.
2. Pour 100 mL of distilled water in the graduated cylinder.
3. Dissolve the *A. Tequilana* leaves' powder in some distilled water of the graduated cylinder. Then, pour the remaining water.
4. Mix the solution and reserve inside an amber flask.

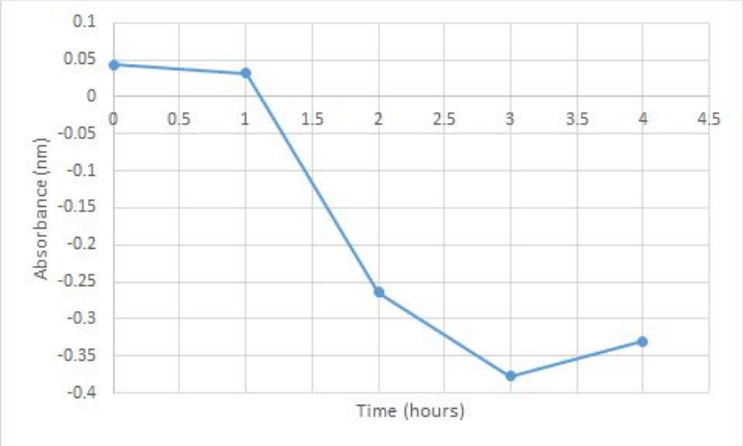
Enzymatic hydrolysis

1. Place the test tubes in the test tubes rack: five test tubes in the first row and the other five in the second row, just behind the others.
2. Pour 950 µL of buffer to the tubes in the first row.
3. Add 50 µL of cellulase solution to the buffer tubes and mix.
4. Finally, add 1 mL of cellulose solution and mixed.
5. Incubate tubes at 28°C at times 0, 15, 30, 45 and 60 minutes.
6. After incubation, add 3 mL of DNS solution and immediately place the test tubes on ice in order to stop the reaction.
7. Pour 1 mL of the new solution into the corresponding test tubes of the second row. Then, add 9 mL of distilled water and mixed.
8. Read each solution's absorbance at 540 nm.

**Results**

Measurements were made by triplicate; the reported results below are an average of them.

| **Time** | **Absorbance** |
|---|---|
| 0 | 0.043 |
| 1 | 0.032 |
| 2 | -0.264 |
| 3 | -0.377 |
| 4 | -0.33 |




**Discussion**

Results obtained in the hydrolysis of agave leaves were not as expected. Negative absorbances are reported, showing some inaccuracy in the performance. This could be caused by a mistake in the procedure, the impossibility of the experiment, or another kind of imprecisions.

The qualitative method used was a reducing sugars analysis, in which DNS was added to the solution for the staining of sugars. It was expected growing results, meaning that the *A. niger* was hydrolyzing correctly the cellulose, and liberating the glucose to the medium. As the microorganism uses the glucose for its metabolism, the broth should have enough cellulose for the *A. niger* to provide itself, but also to let free glucose in the medium.

Low absorbances may be the result of the lack of glucose present in the agave leaves dust obtained in the pretreatment. The cellulose solution was prepared using just 1 g of the agave leaves dust in 100 mL of distilled water; considering that there were no chemical pretreatment for the leaves, the amount weighed may not have been enough to provide the *Aspergillus niger* with the necessary amount of cellulose to cover its needs of glucose and hence, to release to the environment the glucose it didn't need. The dust, besides, was not entirely finite due to the process in which it was milled, so small (but big, compared to finite dust) parts of agave leaves were still in the solution, making it harder to dissolve in water.

Low absorbances may be derived from the fact that *Aspergillus niger* was directly used in the samples instead of using the cellulase itself, and is harder to control a microorganism than a enzyme, because they are more complex and we could ignore a biological process in that specific strain that affects the results of the experiment.

# Protocol  for detecting PHA-producing bacteria

**Materials:**

(2) 100 ml Erlenmeyer flask
(1) 250 ml Erlenmeyer flask
(7) Measuring plates (for the A. balance)
Spatule
(4) Petri dish
Bunsen burner /
PH measuring stick (or pH meter)
50 ml Volumetric flask
Flask for storing  trace element solution SL6

**Equipment:**
Analytical balance
Autoclave
(2) Magnetic stirrer hot plate
(2) Magnetic stirrer
Microbiology Lab Extractor hood
Microbial incubator
PH meter (or pH measuring sticks)
UV light

***Reagents:***

*For Mineral Salt Medium:*

$KH_2PO_4$
$MgSO_4 \times 7\ H_2O$
$(NH_4)_2HPO_4$
$CaCl_2 \times 2\ H_2O$

$FeCl_3 \times 6\ H_2O$
Trace element solution SL6
Agar
Glucose

*Alternative medium, carbon rich nutrient agar medium:*

Glucose
Beef extract
Peptone
Sodium chloride
Agar
Nile Blue solution DMSO

*For trace element solution SL6:*
$ZnSO_4\ x\ 7\ H_2O$
$MnSCl_2\ x\ 4\ H_2O$
$H_3\ BO_3$
$CoCl_2\ x\ 6\ H_2O$
$CuCl_2\ x\ 2\ H_2O$
$NiCl_2\ x\ 6\ H_2O$
$Na_2MoO_4\ x\ 2\ H_2O$
Distilled water

*For Nile blue solutions:*

a. For staining

Nile blue
Ethanol (70%)

b. For Agar,

Nile blue
Dimethylsulfoxide (DMSO)

# Methodology

## A. *Trace element solution SL-6 for Medium option 1*

**In case there is no stock in the laboratory, this preparation is for 50ml (preparation for 1000ml will be attached at the end of the protocol).

| Reagent | Quantity (for a 50 ml preparation) |
|---|---|
| Distilled water | 50 ml |
| $ZnSO_4 \, x \, 7 \, H_2O$ | 0.005 g |
| $MnCl_2 \, x \, 4 \, H_2O$ | 0.0015 g |
| $H_3 \, BO_3$ | 0.015 g |
| $CoCl_2 \, x \, 6 \, H_2O$ | 0.01 g |
| $CuCl_2 \, x \, 2 \, H_2O$ | 0.0005 g |
| $NiCl_2 \, x \, 6 \, H_2O$ | 0.001 g |
| $Na_2MoO_4 \, x \, 2 \, H_2O$ | 0.0015 g |

1. Weight the shown quantities in the analytic balance
2. Prepare the solution with 50 ml in the volumetric flask
3. Homogenize and store the solution

## B. *Mineral Salt Medium (MSM) Agar, as described by Schlegel et al. (1961)*

**The current preparation is for 100 ml of Agar, approximately 4-5 petri dishes
Based on Schlegel et al (1961) and Spiekermann, et al. (1998) with modification of glucose adding.

| Reagent | Quantity |
|---|---|
| Distilled water | **100 ml** |
| $KH_2PO_4$ | **0.21 g** |
| $MgSO_4 \times 7 \, H_2O$ | **0.02 g** |
| $(NH_4)_2HPO_4$ | **0.2 g** |
| $CaCl_2 \times 2 \, H_2O$ | **0.01 g** |

| | |
|---|---|
| $FeCl_3 \times 6\ H_2O$ | **0.0006 g** |
| Trace element solution SL6 | **0.01 ml** |
| Agar | 2 g (2% of total) |
| Glucose or fructose | **0.05 g**- 0.1 g |

Composition: 2.0 g $L^{-1}$ $(NH_4)_2HPO_4$, 2.1 g $L^{-1}$ $KH_2PO_4$, 0.2 g $L^{-1}$ $MgSO_4 \times 7\ H_2O$, 0.1 g $L^{-1}$ $CaCl_2 \times 2\ H_2O$, 0.006 g $L^{-1}$ $FeCl_3 \times 6\ H_2O$, 0.1 ml $L^{-1}$ of trace element solution SL6 (Schlegel et al. 1961)

1. Weight the reagents in the given quantities
2. In an Erlenmeyer flask (250 ml) add distilled water and the materials, ensure homogenization of the materials before adding agar. Use the magnetic agitator.
3. After homogenization, add 50 ml to a 100 ml E. flask and do the same with the remaining 50 ml.
4. In one of the flasks add 0.002 vol of a solution of 0.25 mg of Nile Blue A per ml dimethylsulfoxide (DMSO) and add to the medium so its final concentration on the medium is 0.5 µg dye /ml medium.
5. Seal and autoclave both flasks at 121°C.
6. Label 4 petri dishes, two will act as a negative control, one that has the dye included and one without it. The other two serve as the test for the *P. putida KT2440* strain obtained.
7. In sterile conditions serve the mediums into the plates.
8. Let them solidify.

## *B.1 Medium alternative*

Carbon rich nutrient agar
For 100 ml of agar, composition:
Glucose 1%, beef extract 0.3%, peptone 0.5%, sodium chloride 0.8%, and agar 1.5% and adding of 0.5 µg/mL solution of Nile blue A dye. For the rest distilled water.

## *C. Culture and test of the Microorganism*

1. After solidification inoculate the plates, for the dyed ones, one will be for the P. *putida KT2440* and the other for a non-PHA producing strain. The same for the petri dishes without dyes.
2. Incubate the petri dishes for 48 hrs or until sufficient growth.

## D. *Staining method for the non-dyed plates*

*Before staining take 4 samples of the bacteria (2 of each plate) for heat-sealing and staining with sudan B and Blue Nile A solution (0.5 g Nile blue in 100ml ethanol)*

1. Prepare a solution of 0.5 g of nile blue in 100 ml ethanol
2. Stain both agar plates with 5 ml of staining solution and shake gently
3. After 20 min remove stain and drie
4. Irradiate with UV light

5. PHA producing bacteria should appear fluorescent

## E. *Protocol of staining methods for PHA detection in optical microscopy*

### *A. Sudan black stain for detection of PHA*

***Materials:***
(2) Volumetric flasks (100ml)
(2) Amber flasks (100ml)
Cristal mixing stick
Measuring plates
Microscope slides

***Equipment:***
Analytical balance
Microscope

***Reagents:***
Sudan Black B
Ethanol 60%
Safranin
Distilled water

## Methodology

1. Prepare a solution of 0.3 g of Sudan Black and 100 ml of 60% ethanol
2. Prepare a solution of 0.5 g of safranin powder and 100 ml of distilled water
3. Using the Sudan solution air dry smears for 10 min, adding more stain if slide dries before, rise for 1 second afterwards
4. Shake off excess water and stain with safranin solution for 10 sec, rinse well with water and dry before observing
5. Examine the prepared specimen through a bright-field objective lens (400-1000x magnification). Blue/black granules in red-ish coloured background will indicate presence of lipophilic material usually PHA or PHB.

### *F. Protocol for Nile Blue A staining*

***Reagents:***
Nile Blue A 1% solution in distilled water
Acetic acid solution 8%

***Equipment:***
Heat oven
Microscope
Fluorescence microscope

***Materials:***
Heat-fixed smears of the bacteria in slides

## Methodology

1. Incubate heat-fixed smears with nile blue solution at 55°C for 10 min, wash with water to remove excess stain and add acetic acid solution for 1 min.
2. Wash with water and blot dry
3. After dry add coverslip (immersion oil extracts the fluorescent dye, contact between both should be avoided)
4. View with epifluorescence microscopy with wavelength of 360-400 nm
5. PHA or PHB granules should appear bright orange

**Sources:**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***Adjoints:***

| *Trace element solution SL-6:* | | |
|---|---|---|
| $ZnSO_4 \times 7\ H_2O$ | 0.10 | g |
| $MnCl_2 \times 4\ H_2O$ | 0.03 | g |
| $H_3BO_3$ | 0.30 | g |
| $CoCl_2 \times 6\ H_2O$ | 0.20 | g |
| $CuCl_2 \times 2\ H_2O$ | 0.01 | g |
| $NiCl_2 \times 6\ H_2O$ | 0.02 | g |
| $Na_2MoO_4 \times 2\ H_2O$ | 0.03 | g |
| Distilled water | 1000.00 | ml |

**Fig. 1 Trace element solution for a 1000 ml stock (DSMZ, 2007)**

# Laboratory log for for PHA-producing bacteria detection protocol

## Microbiology lab

***Date: 08/28/17***

Preparations made for the **Protocol for detecting PHA producing bacteria**

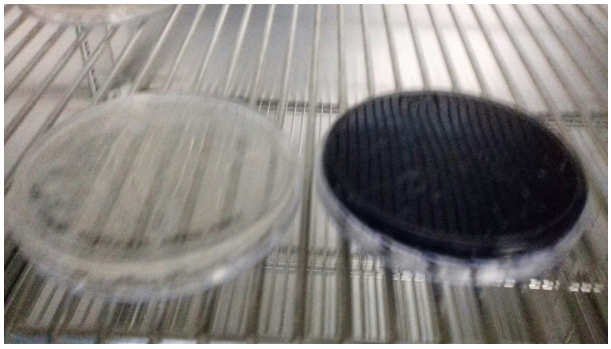
60% ethanol sol.

100 ml Sudan B staining (0.3 g in 100 ml 60% ethanol)

1% in distilled water Blue A Nile solution

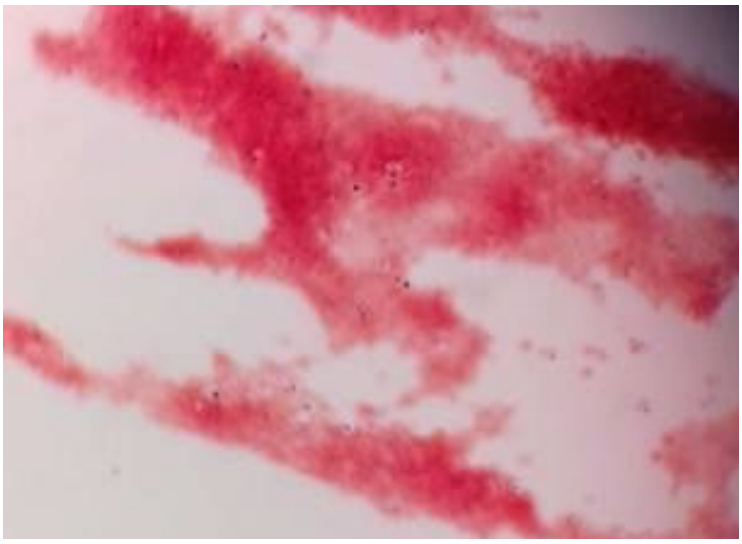
Made the option 2 of Agar for detection (due to lack of materials in lab)

***Date: 08/29/17***

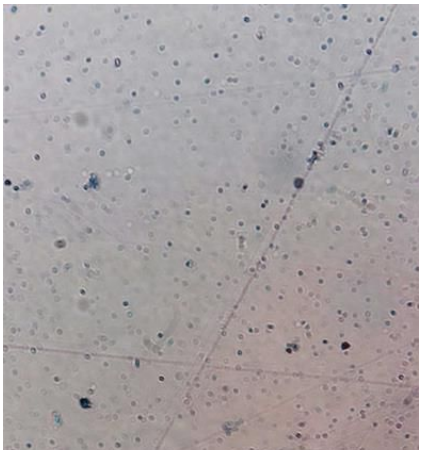


*Culture of P. putida KT2440 in carbon rich agar medium from the protocol  Left in incubator at 30°C.*

For establishing a comparison after growing the putida in a medium for PHA production, we made sudan B and Nile blue A stainings from previously ketp P. putida KT2440 in Eosin methylene blue agar.



*Sudan B staining of P. putida KT2440 with safranin as contrast. Could observe some black spots (PHA) within the red spots at 100x. The team did not expect a high level of PHA  due to the medium conditions (not nitrogen restricted).*



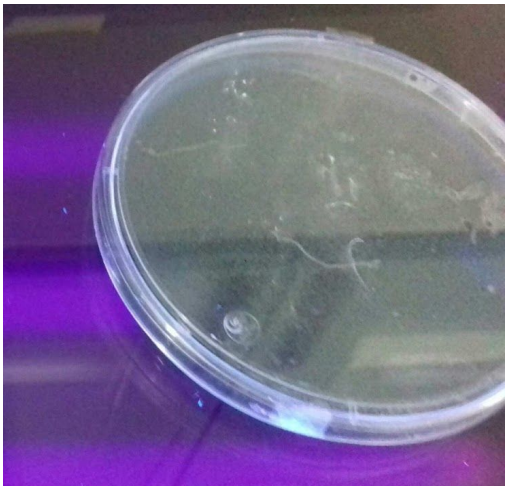
*Blue Nile A staining of Putida Kt2440, without fluorescence (Fluorescent microscope not available).*

***Date: 09/01/17***

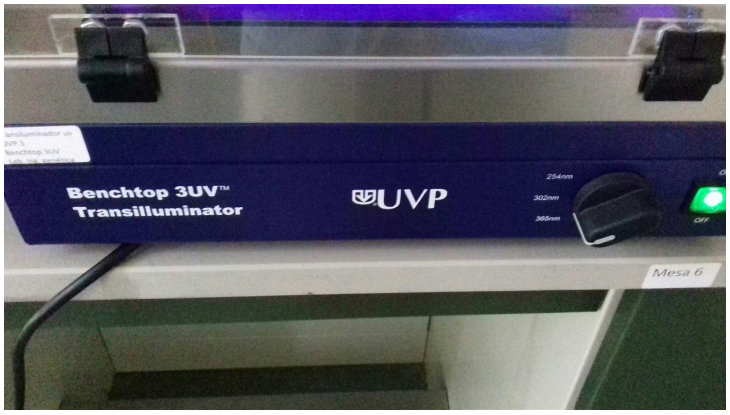
Photos of the medium after 72 hrs of growth:

*P. putida Kt2440 in carbon rich nutrient agar and Nile blue A in DMSO. Decided to leave for more time to increase cellular growth.*

*Carbon rich agar with P.putida KT2440 growth for later plate staining according to the protocol.*



*First fluorescence test at 365 nm of P. putida KT2440 (no significant fluorescence observed)*




*Equipment Benchtop transilluminator 3UV.*

***Date: 09/04/17***

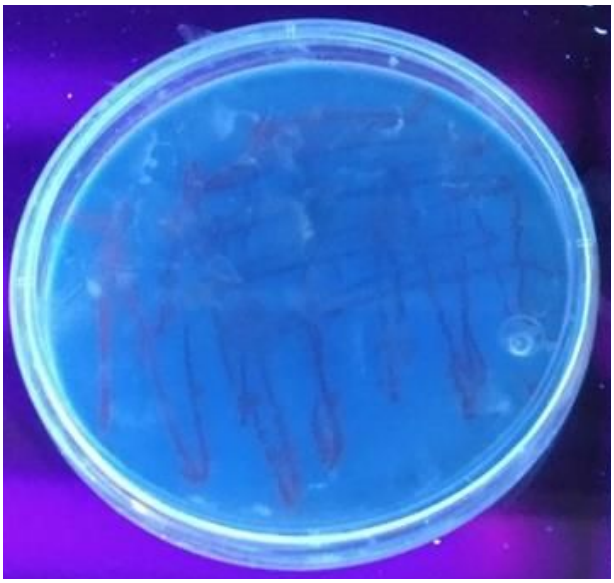


*Image of fluorescence of P. putida KT2440 (not very notorious in the photos). At the human eye, peers could observe a red intense slightly dark fluorescence on the colonies.*

***Date: 09/06/17***

Sudan B & safranin staining after growth in medium for PHA production:

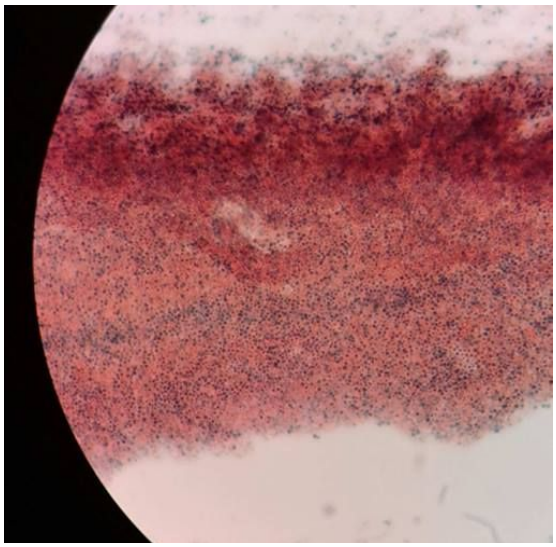
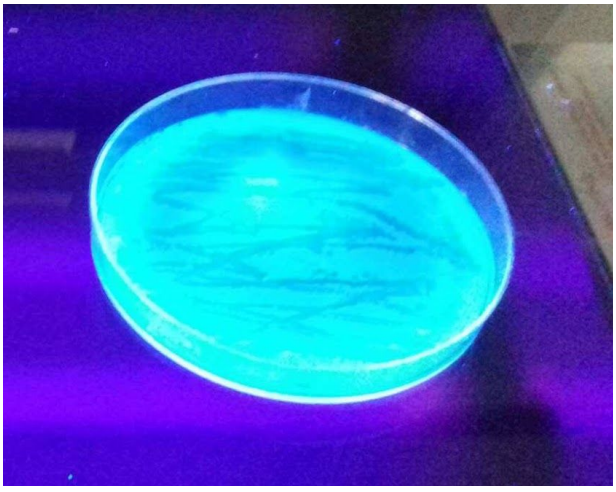


*Image of the staining of P. putida after being grown on the medium for PHA production (obtained from the plate without DMSO and nile blue) with Sudan B and safranin as contrast as established in the protocol. We could observe deep dark dots within the areas where bacterias were stained with safranin as contrast, thus confirming PHA production.*

**Nile blue staining for fluorescent microscope was left out because none was available.*

Staining of the Agar Plate with Blue Nile A, the staining was made into the plate in a medium where no nile blue in DMSO was added to the medium.



*P. putika KT2440 plate stained with Blue Nile according to the protocol. Notorious fluorescence was observed thus confirming PHA production of our strain.*

The other plate with Nile Blue in DMSO and included in the medium


*Images of the same plate of Nile Blue in DMSO and in the medium where bacteria was grown, and fluorescence of P. putida KT2440 (not very notorious in the photos). At the human eye, peers could observe a red intense slightly fluorescence on the colonies, and medium.*

### Conclusion:

PHA production was detected thanks to the different methods written in the protocol. This was crucial step for a comparative study on yields of P. putida KT2440 strain vs our genetically engineered *E.coli.*
*This also serves if DNA extraction is planned to be done in a PHA producing bacteria

# Protocol for PHA extraction

**Montserrat Villegas - Esmeralda Azano**

**Equipment**

- Centrifuge
- Incubator
- Autoclave

**Reagents**

- NaOH 0.1N
- Carbon rich nutrient alternative medium
- Distilled water

**Material**

- (1) 100 mL Erlenmeyer Flask
- (4) 15 mL Centrifuge tubes
- (1) 10 mL Volumetric pipette
- (1) Suction bulb
- (1) 1000 µL Micropipette
- 1000 µL Micropipette tips

**Methodology**

1. Reactor preparation (Based on the protocol of Choi et al., 1999)
    a. Incubate the *Pseudomona putida* KT2440 cells for 72 hours at 37°C with continuous agitation in the carbon rich saline medium.
2. Homogenize the reactor, take 1 mL of it with the micropipette, and put them in one centrifuge tube. Repeat three more times.
3. Collect the cells by centrifugation at 3600g for 20 minutes at 25°C.
4. Carefully, dispose of the supernatant without losing any of the pellet.
5. Resuspend the cells in 1 mL of distilled water.
6. Add 10 mL of NaOH (0.1 N) to each centrifuge tube, and incubate the digestion reaction for 1 hour at 37°C.
7. Centrifuge at 2500g for 20 minutes, and dispose of the supernatant.
8. Rinse the recovered granulates with distilled water.
9. Centrifuge again in the same conditions as before, throw away the pellet, and let dry at room temperature.

**Sources**

Choi, J. and Lee, S. (1999). *Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant Escherichia coli by simple digestion with chemicals.* Biotechnology and bioengineering. 62(5). Retrieved from: http://vh2ds7jt9c.search.serialssolutions.com/?ctx_ver=Z39.88-2004&ctx_enc=info%3Aofi%2Fenc%3AUTF-8&rfr_id=info%3Asid%2Fsummon.serialssolutions.com&rft_val_fmt=info%3Aofi%2Ffmt%3Akev%3Amtx%3Ajournal&rft.genre=article&rft.atitle=Efficient+and+economical+recovery+of+poly%283-hydroxybutyrate%29+from+recombinant+Escherichia+coli+by+simple+digestion+with+chemicals&rft.jtitle=BIOTECHNOLOGY+AND+BIOENGINEERING&rft.au=Choi%2C+JI&rft.au=Lee%2C+SY&rft.date=1999-03-05&rft.pub=WILEY-BLACKWELL&rft.issn=0006-3592&rft.eissn=1097-0290&rft.volume=62&rft.issue=5&rft.spage=546&rft.epage=553&rft.externalDBID=n%2Fa&rft.externalDocID=000078140300006&paramdict=en-EN

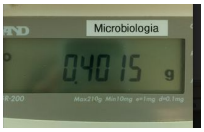
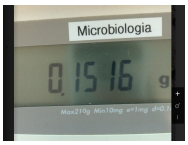
# Laboratory log for PHA extraction

## Microbiology lab

***Date: 09/08/17***

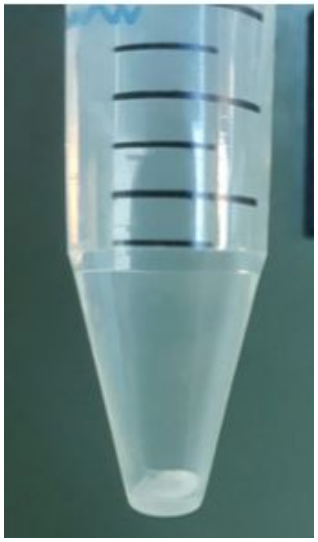
Preparations made for the **Protocol for PHA extraction**

50 mL of carbon rich broth special for PHA production (established in the Protocol PHA detection/visualization, Agar & staining) inoculated with *Pseudomona putida* KT2440 from a carbon rich agar Petri dish culture inoculated 08/28/2017, maintained at 4°C.

| | Theoretical value | Experimental value | Photo |
|---|---|---|---|
| Glucose | 0.50 g | 0.5009 g |   |
| NaCl | 0.40 g | 0.4015 g |   |
| Beef extract | 0.15 g | 0.1516 g |   |
| Peptone | 0.25 g | 0.2506 g |   |

***Date: 09/11/17***

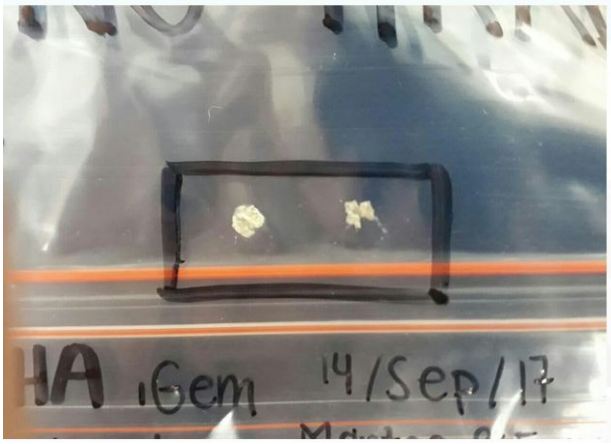
Two different volumes of cells were used, 1 mL and 7 mL, two centrifuge tubes for each volume.



*Tube 1 and tube 2 (1 mL of initial cell content each), correspondingly, after the extraction procedure. A white viscous film was observed, too small for further characterization experiments.*

The experiments with 7 mL were unsuccessful, so, the results weren't taken into account.

***Date: 09/14/17***


*The extracted polymer over a plastic surface before and after the air-dry procedure, in that order from right to left.*

The recovered PHA was too light to be weighted, or measured in another way.

**Conclusion:**

- ★ Two centrifugations are needed after the chemical digestion for the correct PHA sedimentation.
- ★ For a 50 mL reactor with saline carbon rich broth and *P. putida* after 72 hrs of incubation, 1 mL is the ideal cell volume for the experiment.
- ★ 7 mL of cell volume is too much for the conditions in the procedure; hence, a higher NaOH volume or centrifugation time would be needed for the successful PHA extraction.

# Protocol for PHA extraction-control (with chloroform)

**Montserrat Villegas - Esmeralda Azano**

**Equipment**

- Centrifuge
- Incubator

**Reagents**

- Sodium hypochlorite 3% v/v
- Chloroform
- Distilled water
- Methanol 70%
- *Pseudomona putida* culture in saline broth, incubated for 72 hours at 37°C

**Material**

- (2) 100 mL Erlenmeyer Flask
- (1) 200 mL Beaker
- (2) 15 mL Centrifuge tubes

- (1) 100 mL Test Tube
- (1) 1000 µL Micropipette
- 1000 µL Micropipette tips
- (1) Wattman filter

**Methodology**

1. Place 1 mL of cell broth in each centrifuge tube; and centrifuge for 20 minutes at 3600g and 25°C.
2. Carefully discard the supernatant, and add 10 mL of the sodium hypochlorite/chloroform (5:5, v/v) solution to each tube.
3. Incubate the reaction at 32°C for 1 hour.
4. After the incubation, centrifuge the tube containing the sodium hypochlorite/chloroform at 3600g and 25°C for 10 minutes.
5. Remove the upper phase with a pipette; remove then the middle phase via filtration in order to keep the chloroform phase containing the PHA material.
6. Add 5 mL of the methanol/water (7:3, v/v) solution and let it rest for 2 hours.
7. After the nonsolvent precipitation, recover the PHA by filtration and dry.

**Sources**

Gamal, R., Abdelhady, H., Khodair, T., El-Tayeb, T., Hassan, E., & Aboutaleb, K. (2013). Semi-scale production of PHAs from waste frying oil by Pseudomonas fluorescens S48. *Brazilian Journal Of Microbiology*, *44*(2), 539-549. http://dx.doi.org/10.1590/s1517-83822013000200034
http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1517-83822013000200034

# Laboratory log for PHA extraction-control (with chloroform)

## Microbiology lab

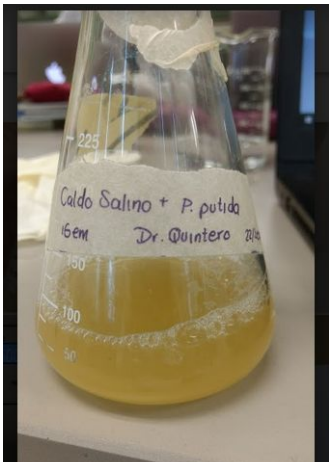
***Date: 09/22/17***

Preparations made for the **Protocol for PHA extraction.**

50 mL of carbon rich broth special for PHA production (established in the Protocol PHA detection/visualization, Agar & staining) inoculated with *Pseudomona putida* KT2440 from 1 mL of the broth prepared on september 08th.

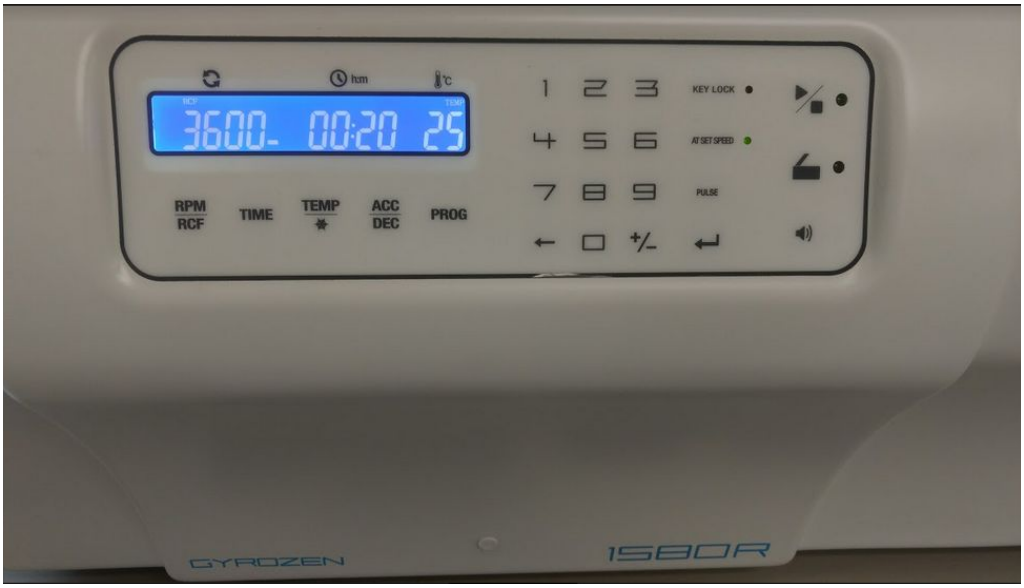

*Conditions of the thermoshaker for the incubation of the P. putida kt2440.*

***Date: 09/25/17***



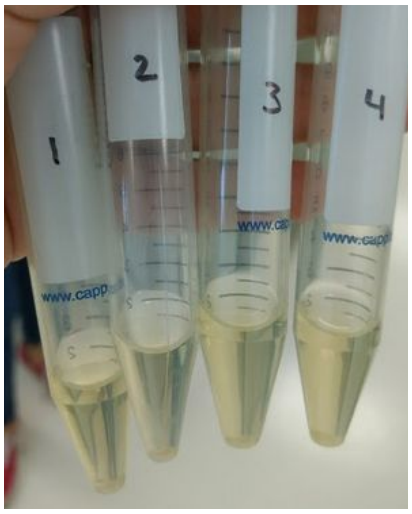

*Cell broth after 72 hrs of incubation at the mentioned conditions.*

This time, 2 mL of cells per tube were collected for the centrifugation.




*Conditions for the first centrifugation.*

4 tubes containing 1 mL of cell broth were centrifuged, but 2 were used in the PHA extraction by chemical digestion.




*The four tubes after the first centrifugation for the cell disruption.*

The 2 tubes were stored in the refrigerator at 4°C until the treatment.

## Bioprocesses lab

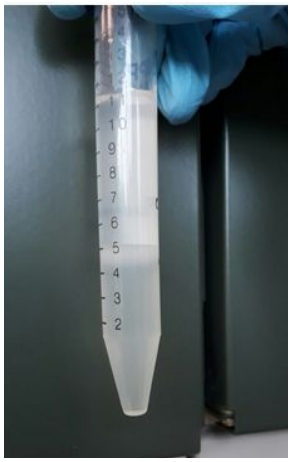
***Date: 09/27/17***

A sodium hypochlorite/chloroform solution was prepared, mixing in the extraction hood 5 mL of chloroform, with 5 mL of sodium hypochlorite 3% (v/v).

One corning tube was placed inside the thermoshaker at 32°C and 200 RPM for 1 hour.

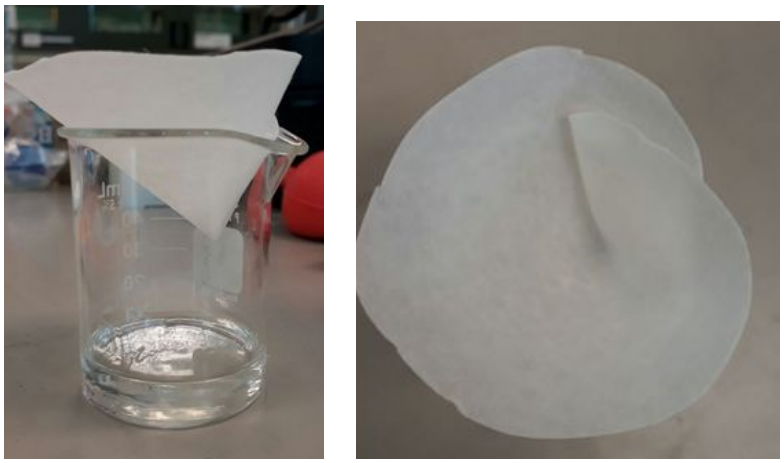
After the reaction hour, 5 mL of methanol 70% were added for the PHA precipitation.

The complete precipitation took 2 hours to accomplish.



*Precipitation of the PHA after the 5 mL of methanol 70% were added.*

The top phase was removed with a micropipette, and the rest was filtrated.



*Filtration of the solution for the PHA recovery.*

The filter paper was placed in the dry-air oven, but the PHA quantity was so low it was impossible to appreciate it.

**Conclusion:**

- ★ 2 mL of cells is not enough for the PHA recovery.
- ★ The centrifugation method is more efficient and faster than the one using chloroform.
- ★ In this procedure, more solvents are needed, making it more expensive and damaging for the environment.

# Protocol for PHA characterization

**Montserrat Villegas - Esmeralda Azano**

**Equipment**

- Vertical gel electrophoresis chamber
- Power supplier
- (2) Glasses for electrophoresis
- Gel agitator
- White light transilluminator
- Potentiometer

**Reagents**

- PHA solution obtained in *Protocol for PHA extraction*
- Molecular weight marker
- Acrylamide/bisacrylamide 30%
- Tris-HCl 0.5 M, pH 6.8
- Tris-HCl 1.5 M, pH 8.8
    - Pure Tris buffer
    - HCl 5 M
- SDS solution 10% (w/v)
- TEMED
- Ammonium persulfate 10% (w/v)
- Load buffer 1X
- Running buffer 1X
- Coomassie solution 2.5% (w/v)
    - Coomassie brilliant blue
    - Ethanol 96%
    - Glacial acetic acid
    - Distilled water
- Destaining solution
    - Ethanol 96%
    - Glacial acetic acid
    - Distilled water

**Material**

- (5) 50 mL Beakers
- (2) 250 mL Beakers

- (1) 100 mL Test tube
- (1) 1000 µL Micropipette
- 1000 µL Micropipette tips
- (1) 200 µL Micropipette
- 200 µL Micropipette tips
- (1) 20 µL Micropipette
- 20 µL Micropipette tips
- Staining tray
- Filter paper

**Methodology**

1. Previous preparations:
    a. For Tris-HCl 0.5 M, pH 6.8: Weight 0.30 grams of base Tris, and dissolve in 5 mL of distilled water. Check the pH with the potentiometer, and add HCl until the desired value.
    b. For Tris-HCl 1.5 M, pH 8.8: Weight 0.91 grams of base Tris, and dissolve in 5 mL of distilled water. Check the pH with the potentiometer, and add HCl until the desired value.
    c. For SDS 10%: Weight 0.1 grams of SDS, and dissolve in 1 mL of distilled water.
    d. For PSA 10%: Weight 0.1 grams of PSA, and dissolve in 1 mL of distilled water.
    e. For Coomassie 2.5%: Inside the extraction hood, put 125 mL of distilled water in one of the 250 mL beaker, add 12.5 mL of glacial acetic acid, then 112.5 mL of ethanol 96%, and finally, 0.625 grams of Coomassie brilliant blue stain. Stir on a heating plate for 4 hours.
    f. For Destaining solution: Inside the extraction hood, put 137.5 mL of distilled water in the other 250 mL beaker, add 100 mL of ethanol 96%, and finally, 12.5 mL of glacial acetic acid. Homogenize.
2. Assemble the vertical gel electrophoresis chamber for the gel construction.
3. Prepare the stacking gel:
    a. In a 5 mL baker mix: 4.045 mL of distilled water, 3.3 mL of acrylamide/bisacrylamide 30%, 2.5 mL of Tris-HCl 1.5 M, pH 8.8, 0.1 mL SDS 10%, 50 µL of PSA 10%, and 5 µL of TEMED.
4. With a micropipette, add slowly the solution between the glasses until ¾ of the chamber.
5. Cover the solution with water to avoid the oxygen presence, improving the gel solidification.
6. Wait until the gel is solid, for about 1 or 2 hours.
7. Eliminate the water between the glasses using a filter paper.
8. Prepare the running gel:
    a. In a 5 mL baker mix: 3.02 mL of distilled water, 0.65 mL of acrylamide/bisacrylamide 30%, 1.25 mL of Tris-HCl 0.5 M, pH 6.8, 50 µL of SDS 10%, 25 µL of PSA 10%, and 5 µL of TEMED.
9. With a micropipette, add slowly the solution between the glasses until the edge of the chamber.
10. Carefully put the comb (8 teeth) on the top of the chamber, and wait until the gel solidifies for about 45 minutes.
11. Take out the comb and wash the gel with distilled water.
12. Put the glasses in the chamber, and fill it with running buffer until the gel is completely covered.

13. With the 20 µL pipette, charge the first and the last pit of the gel with 5 µL of the molecular weight marker; in the consecutive pits, add 5 µL of the blank; and finally, in the lasting pits, add 5 µL of the sample.
14. Close the electrophoresis chamber, and apply a 120 V current.
15. When the colorant has migrated enough (about 45 min), turn off the power supply, and take the gel out of the chamber.
16. Put the gel inside the staining tray, and add Coomassie solution until it is completely covered.
17. Stir in the gel agitator for 30 minutes.
18. Remove the Coomassie solution, and add the destaining solution until the gel is completely covered, stir for another 30 minutes.
19. Repeat step 18 until the gel is transparent, and the electrophoresis lines are clear.
20. Visualize the result with the white light transilluminator.

**Sources**

Bravo de la Garza, A.; González de la Rosa, C.; Le Borgne, S. (2012). *Manual de prácticas de laboratorio de Biología Molecular*. Universidad Autónoma Metropolitana. Recovered from: http://www.cua.uam.mx/pdfs/conoce/libroselec/Manual_de_Prcticas_de_BM.pdf

# Laboratory log for PHA characterization

## Bioprocesses lab

***Date: 09/25/17***

One of the corning tubes containing PHA solution was labeled for its future use in electrophoresis.

*PHA containing tube for the electrophoresis.*

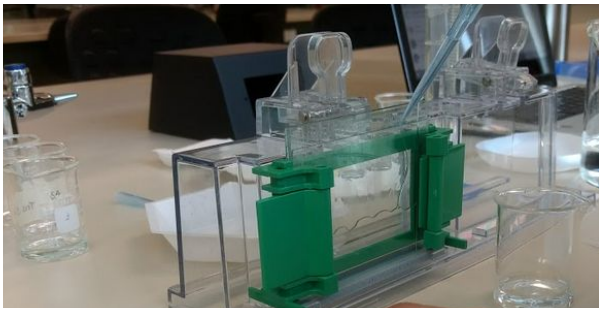
It was stored in the refrigerator at 4°C until needed.

## Genetic engineering lab

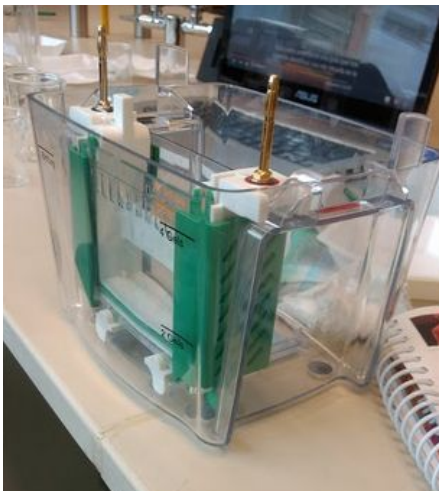
***Date: 09/26/17***

Preparations for the electrophoresis were made as stipulated in Protocol for PHA characterization.

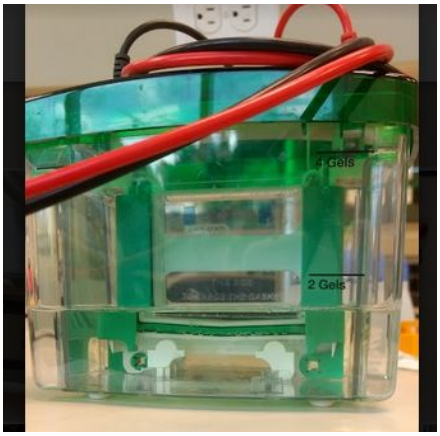
| | Theoretical weight | Experimental weight |
|---|---|---|
| Tris-HCl 0.5 M | 0.30 grams | 0.3025 grams |
| Tris-HCl 1.5 M | 0.91 grams | 0.9102 grams |
| SDS 10% | 0.1 grams | 0.1007 grams |
| PSA 10% | 0.1 grams | 0.1075 grams |



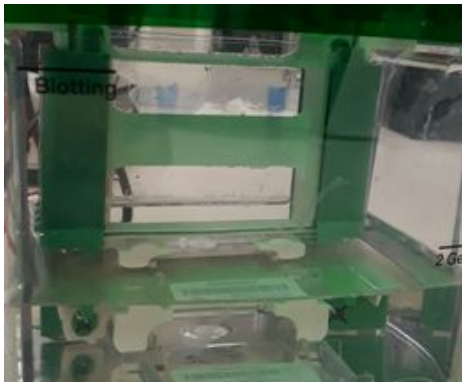
*Addition of the stacking gel between the glasses.*



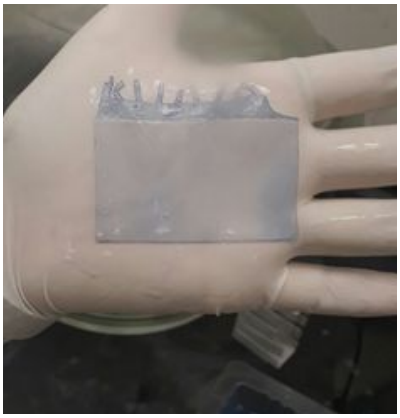
*Completed gel inside the electrophoresis chamber, before the addition of the running buffer.*




*Electrophoresis chamber with running buffer, prepared for the electrical current.*




*Electrophoresis gel inside the chamber after the running. The molecular weight markers are seen in the extremes, but nothing between them, where the samples were inserted. The fact that no sample runned could mean that no proteins were present, hence, the PHA is pure.*



*Gel after Coomassie staining, the lines disappeared due to mistakes in the procedure, but before the staining, no proteins were present.*

**Conclusion:**

- ★ Since the people in charge of this experiment were inexperienced, many mistakes could've happen.
- ★ The load buffer was prepared by us, and it only contained Bromophenol blue as a dye, so it could have not been enough.
- ★ No proteins were expected to be seen, because its presence would mean that the PHA is not pure.
- ★ No line was seen in the electrophoresis gel, due to the lacking of proteins in the solution. Hence, the PHA has a high level of purity.

# Protocol for PHA microscopy

**Montserrat Villegas - Esmeralda Azano**

**Equipment**

- Microscope

**Reagents**

- PHA obtained in *Protocol for PHA extraction*
- Nile Blue stain

**Material**

- (1) slide
- (1) coverslip
- (1) bacteriological loop

**Methodology**

1. Using the bacteriological loop, carefully grab a piece of the PHA obtained in *Protocol for PHA extraction* and place it in the slide.
2. Stain the PHA piece with a drop of Nile Blue and place the coverslip on top of it.
3. Observe the stained PHA under the microscope at 10x, 40x and 100x objectives.

# Lab log for PHA microscopy

## Microbiology lab

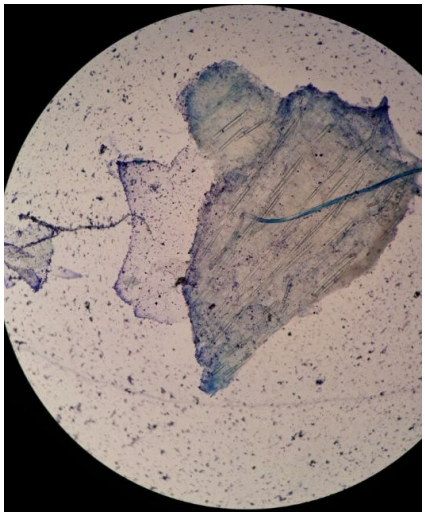
***Date: 09/25/17***

One Corning tube containing recovered PHA from **Protocol for PHA extraction** was reserved after the first centrifugation at 2500g and 25°C (before the second centrifugation for compression at the same conditions) in order to be able to visualize the semi-dissolved PHA.

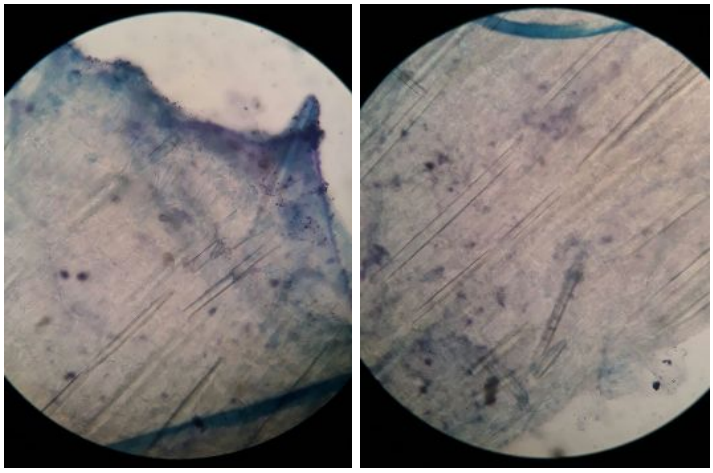
*Corning tube containing the uncompressed PHA, after the first centrifugation, used for microscopy .*

A small piece of that PHA was obtained and placed in a slide. One drop of Nile Blue was then added in order to stain any residual bacteria that may have been present in the sample at that time. A coverslip was placed on top of the sample to visualize it under the microscope.

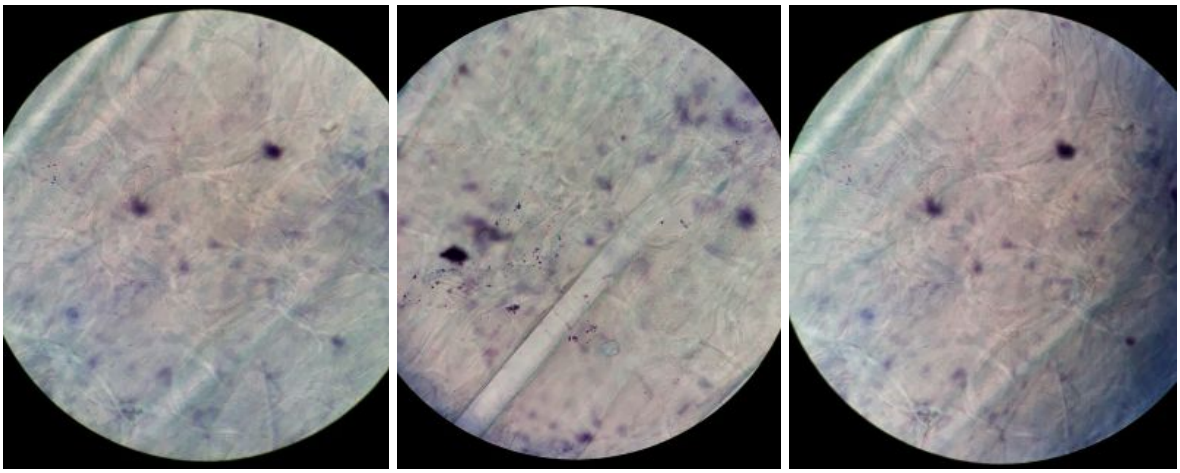
The rest of the PHA from the Corning tube was stored in the refrigerator for future procedures.



*Recovered PHA piece observed under 10x objective.*



*Recovered PHA piece observed under 40x objective.*



*Recovered PHA piece observed under 100x objective. PHA granules appear colorless.*

**Conclusion:**

- ★ The centrifugation process produces bacteria-free PHA
- ★ PHA is compressed even after the first centrifugation at 2500g, resulting in a clean and viable plastic resin ready for its use.

# Protocol for PHA production analysis in *Pseudomona putida* KT2440

**Montserrat Villegas - Esmeralda Azano**

**Equipment**

- Centrifuge
- Shaker incubator
- Laminar flow hood
- Autoclave

**Reagents**

- NaOH 0.1 N
- Carbon rich nutrient alternative medium
- Distilled water

**Material**

- (1) 10 mL volumetric pipette
- (1) rubber pipette filler
- (1) 1000 µL Micropipette
- 1000 µL Micropipette tips
- (4) Corning tubes
- (1) 50 mL Beaker
- (1) 100 mL Erlenmeyer flask

**Methodology**

1. Autoclave the carbon rich nutrient alternative medium broth and let it cool.
2. Inoculate the broth with 1 mL of *P. putida* previously incubated in the Laminar flow hood to prevent contamination.
3. Take 1 mL of the recently inoculated broth and place it inside a Corning tube previously labeled as “Time 0” and immediately place the new inoculated medium in the shaker incubator at 37°C.
4. Collect the cells by centrifugation of the “Time 0” Corning tube at 3600g and 25°C for 20 minutes.
5. Carefully dispose of the supernatant in the beaker without losing any pellet.
6. Resuspend the cells in 1 mL of distilled water.
7. Add 10 mL of NaOH (0.1 N) solution, vortex the mixture and incubate for 1 hour at 37°C.
8. After the incubation, centrifuge the “Time 0” Corning tube at 2500g and 25°C for 20 minutes.

9. Carefully dispose of the supernatant in the beaker without losing any pellet.
10. Rinse the recovered PHA granulates with distilled water.
11. Centrifuge again the rinsed PHA granulates at the same conditions (2500g and 25°C) and dispose of the supernatant.
12. Recover the compressed PHA and let dry at room temperature.
13. Repeat steps 4 to 12 with “Time 1”, “Time 2” and “Time 3” Corning tubes (24, 48 and 72 hours, respectively).

**Sources**

Choi, J. and Lee, S. (1999). *Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant Escherichia coli by simple digestion with chemicals.* Biotechnology and bioengineering. 62(5). Retrieved from: http://vh2ds7jt9c.search.serialssolutions.com/?ctx_ver=Z39.88-2004&ctx_enc=info%3Aofi%2Fenc%3AUTF-8&rfr_id=info%3Asid%2Fsummon.serialssolutions.com&rft_val_fmt=info%3Aofi%2Ffmt%3Akev%3Amtx%3Ajournal&rft.genre=article&rft.atitle=Efficient+and+economical+recovery+of+poly%283-hydroxybutyrate%29+from+recombinant+Escherichia+coli+by+simple+digestion+with+chemicals&rft.jtitle=BIOTECHNOLOGY+AND+BIOENGINEERING&rft.au=Choi%2C+JI&rft.au=Lee%2C+SY&rft.date=1999-03-05&rft.pub=WILEY-BLACKWELL&rft.issn=0006-3592&rft.eissn=1097-0290&rft.volume=62&rft.issue=5&rft.spage=546&rft.epage=553&rft.externalDBID=n%2Fa&rft.externalDocID=000078140300006&paramdict=en-EN

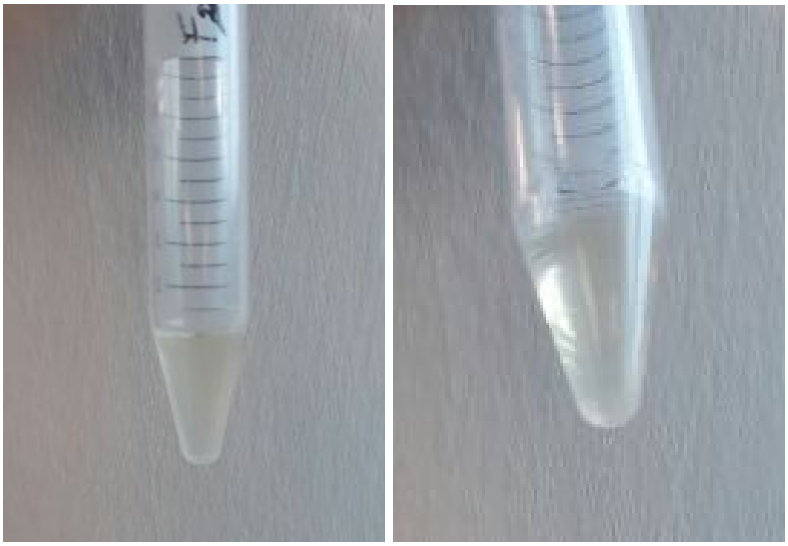
# Lab log for PHA production analysis in *Pseudomona putida* KT2440

## Microbiology lab

***Date: 09/26/17***

50 mL of carbon rich broth special for PHA production (established in the Protocol PHA detection/visualization, Agar & staining) was prepared, autoclaved and inoculated with *Pseudomona putida* KT2440 from 1 mL of the broth prepared on september 22nd.

“Time 0” sample was taken right after the inoculation and centrifuged in order to analyze the content of PHA presented in it.

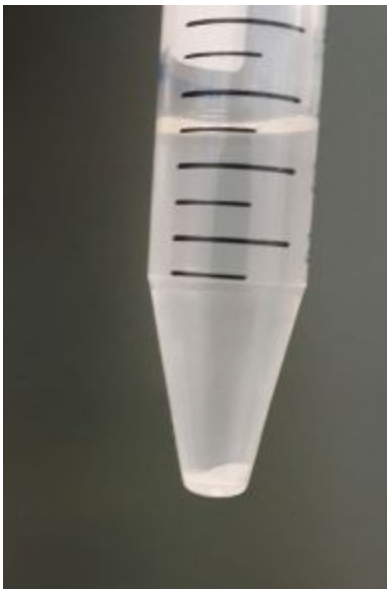


*Corning tube containing 1 mL of inoculated sample "Time 0" (0 hours of incubation) after centrifugation to recover PHA.*

*P. putida* in the carbon rich broth was placed in the shaker incubator at 37°C.

***Date: 09/27/17***

After 24 hours of incubation at the described conditions, "Time 1" sample was taken and centrifuged in order to analyze the production of PHA at that time.

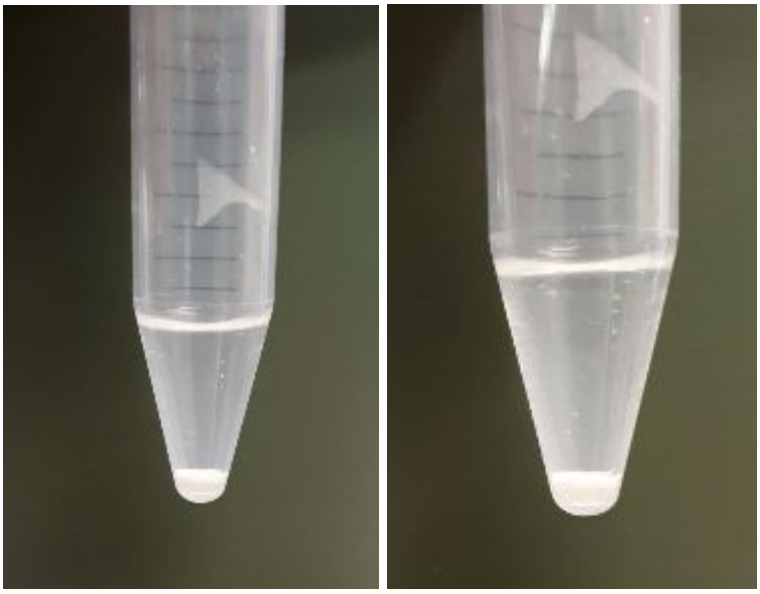


*Corning tube containing 1 mL of inoculated sample "Time 1" (24 hours of incubation) after centrifugation to recover PHA.*

*P. putida* in the carbon rich broth was placed again in the shaker incubator at 37°C to continue with the incubation.

***Date: 09/28/17***

After 48 hours of incubation at the described conditions, “Time 2” sample was taken and centrifuged in order to analyze the production of PHA at that time.

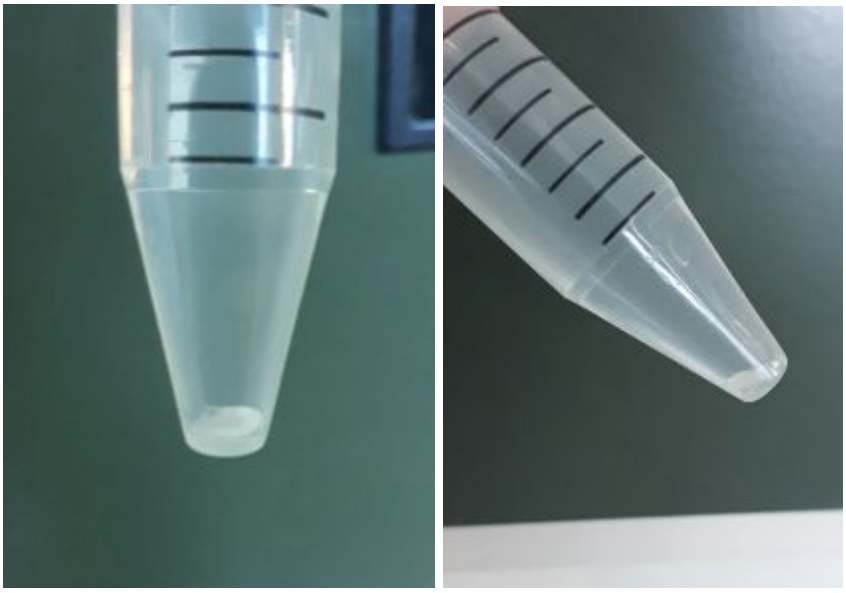


*Corning tube containing 1 mL of inoculated sample “Time 2” (48 hours of incubation) after centrifugation to recover PHA.*

*P. putida* in the carbon rich broth was placed again in the shaker incubator at 37°C to continue with the incubation.

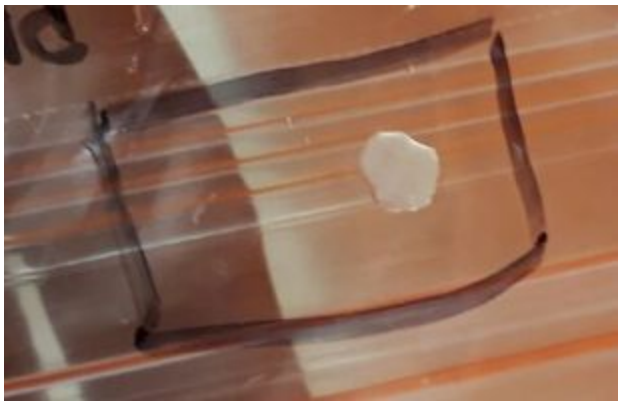
***Date: 09/29/17***

After 72 hours of incubation at the described conditions, “Time 3” sample was taken and centrifuged in order to analyze the production of PHA at that time.



*Corning tube containing 1 mL of inoculated sample "Time 3" (72 hours of incubation) after centrifugation to recover PHA.*

Recovered PHA from each sample (Time 0, 1, 2 and 3) was dried at room temperature and stored for later procedures.



*Recovered PHA of "Time 2" sample (48 hours of incubation) on a plastic bag to let dry at room temperature.*

## Conclusion:

- ★ A carbon rich broth is ideal for PHA production since it provides the perfect conditions for the bacteria to produce its internal carbon and energy storage.
- ★ *P. putida* produces the largest amount of PHA after 48 hours of incubation.
- ★ Even when the amount of PHA after 24 and 72 hours of incubation is lower, it's still stable and rigid.