**Ampicillin Synthesis Steps**

**(Corrected protocol)**

* Preparation of 500ml Potassium Phosphate Buffer (KH2PO4) (0.1M; PH=7):
* Weigh 4.672g Potassium phosphate dibasic and 3.154g Potassium Phosphate Monobasic
* Dissolve them in 250 ml of distilled water then put them in a volumetric flask of 500 ml then continue to fill the volumetric flask with distilled water up to the gauge mark
* Pour the contents of the volumetric flask into a suitable container after homogenization and then place this container in the autoclave
* Preparation of the solution of ampicillin synthesis:
* Weight 0.143 g commercial penicillin G (C=0.04g/ml) and dissolved it in 10 mL of potassium phosphate solution (pH=7 and C= 0.1 mol/L) in a volumetric flask
* Measure a volume of 7.5 mL from this mixture, using a graduated cylinder
* Add 0.8g of penicillin acylase (PGA)
* Agitating for 1h
* Add 7.5ml of the ester D-(-)-a-Phenylglycine methyl ester hydrochloride, D-PGMEH (0.24g ester in 10 ml potassium phosphate buffer) to the mix
* Add 0.24g enzyme (PGA) again
* Adjust the PH for about 6.4-7 by adding NaOH
* Put the beaker containing the bar magnet on the magnetic stirrer for 22.5 hours
* Ampicillin purification and harvesting:
* Add few drops of H2SO4 to stop the enzymatic reaction
* Filtrate the mix using a funnel fitted with filter paper

(Take 1ml of the filtrate for the bacterial sensibility test later)

* Add butyl acetate solvent (1Vsolvant/2Vfiltrate)
* Let rest for 2 minutes, then the organic phase is removed and the aqueous phase is discarded after decantation

(Take 1ml from each phase to test the presence of ampicillin later)

{We will carry out a qualitative, non-quantitative antibiogram, just to see the appearance or not of an inhibition zone following the application of: filtrate, the organic phase and the aqueous phase, stored in the eppendorfs tubes (as already explained) on petri dishes containing Muller-Hinton medium. So, we are going to test the effectiveness of the antibiotic on the bacteria.}

* Add 2% phosphate buffer (V/V) to the organic solution
* Adjust the PH to 7.5 by adding NaOH
* Crystallization:
* Add 2% (W/V) NaHCO3 to the aqueous phase medium
* Cool them at 4oC for about 7 days
* Filtrate them to harvest ampicillin sodium salt

**Ampicillin Quantification (Standard Protocol)**

* The one bacterial strain which can be used: E. coli
* Regeneration of Escherichia coli Bacteria
* Place the isolated colony of E. coli on a new standard agar petri dish and then spread it
* Incubate the bacteria for approximately 18 hours in an incubator at 37°C
* Qualitative antibiogram, (non-quantitative antibiogram) using disc diffusion method

**To test the effectiveness of the antibiotic on the bacteria**

* Take 3 to 5 colonies of the isolated colonies of E. coli with a loop
* Add them in 2ml sterile saline (NaCl 0.9%)
* Vortex the saline tube to create a smooth suspension
* Adjust the turbidity of this suspension to a 0.5 McFarland standard by adding more organism if the suspension is too light or diluting with sterile saline if the suspension is too heavy.
* Inoculate the surface of Mueller Hinton agar plate by streaking the swab 3 times over the entire agar surface
* Sit the plate at room temperature at least 3 to 5 minutes (but no more than 15 minutes) to let the surface of the agar plate dry before proceeding to the next step
* Reverse the plate and divide it into 4
* Deposit 3 disc in each quadrant with an empty quadrant used as control
* Add 20µl of each simple on a disc
* Reverse the plate and incubate it at at 37o for 18 to 24h
* Preparation for commercial ampicillin quantification

In order to carry out an antibiogram, from which the diameters of the zones of inhibition can be measured and the standard curve established

* Weigh 1g of commercial ampicillin
* Add 5 ml of potassium phosphate buffer to the ampicillin
* Filtrate the mix using a sterile funnel and filter paper
* Store the mix (C standard ampicillin=200mg/ml) in the fridge for later use
* Prepare the sterile tubes with different concentration **20, 15, 12, 10, 8 et 5 mg/mL**

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| **TUBES** | **Solution ml** | **Distilled water ml** |
| Tube1  20mg/ml | 1 | 9 |
| Tube2  15mg/ml | 7.5 | 2.5 |
| Tube3  12mg/ml | 6 | 1.5 |
| Tube4  10mg/ml | 5 | 1 |
| Tube5  8mg/ml | 4 | 1 |
| Tube6  5mg/ml | 2.5 | 1.5 |

* Preparation of the turbidity calibration 0.5McFarland:
  + Mix 0.05 mL of 1.175% barium chloride dihydrate (BaCl2•2H2O), with 9.95 mL of 1% sulfuric acid (H2SO4) and shook vigorously
* Quantification of the produced ampicillin using the disc diffusion method:
* Take 3 to 5 colonies of the isolated colonies with a loop, and we added them in 2ml sterile saline (NaCl 0.9%)
* Vortex the saline tube to create a smooth suspension.
* Adjust the turbidity of this suspension to a 0.5 McFarland standard
  + by adding more organism if the suspension is too light or diluting with
  + sterile saline if the suspension is too heavy.
* Use this suspension within 15 minutes of preparation.
* Inoculate the surface of 3 Mueller Hinton agar plate by streaking the swab 3 times over the entire agar surface, we rotated the plate approximately 60֯ each time to ensure an even distribution of the inoculum (use a control plate with E. coli on muller Hinton agar)
* Leave the plates at room temperature at least 3 to 5 minutes (but no more than 15 minutes) for the surface of the agar plate to dry before proceeding to the next step
* Reverse the plates and divide it into 4
* Deposit a disc in each quadrant
* Add 20µl of each concentration on a disc with the unknown one
* Reverse the plate and incubate it at at 37o for 18 to 24h
* After the growth time, measured the zone of inhibition that had appeared using a ruler.
* Draw a graph showing the concentration of ampicillin as a function of the diameter in order to be able to quantify the produced ampicillin (diameter as a function of Log C).