INTERNATIONAL BIOLOGY OLYMPIAD e. V. IBO

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27th International Biology Olympiad

July 17-23, 2016
Hanoi, Vietnam



Practical Exam 3

BIOCHEMISTRY and MICROBIOLOGY

Total points: 100
Duration: 90 minutes

DEAR PARTICIPANTS,

This exam consists of three experiments:

- **Experiment 1: Expression, purification and characterization of proteins (40 points)**
- **Experiment 2: Antioxidant activity of coffee extract (30 points)**
- **Experiment 3: Lactic acid fermentation (30 points)**

Please note the following:

- Please remember to write your **Country** and **Student code** in the given box.
- Write your answers in the separate **Answer Sheet**. Only the answers given in the **Answer Sheet will be evaluated**.
- Make sure that you have received all the materials and equipment listed. If any of these items are missing, please raise the **Red card** immediately.
- During experiments, ensure to handle equipment properly. Any spilled solutions or broken equipment will not be replenished.
- Gel electrophoresis in Experiment 1 must not be performed in the last 30 minutes. You are recommended to do Experiment 1 first.
- Ensure to obtain spectrophotometer readings to answer the questions in Experiment 2.
- Stop answering and put down your pen immediately when the bell rings at the end of the exam. Enclose the **Question Paper**, **Answer Sheet**, and **Data printout** in the provided envelope.
- No paper, materials or equipment should be taken out of the laboratory.

Materials & Equipment

Equipment and Materials for 3 experiments

Name	**Quantity**
Micropipette P1000 (100-1000 µl)	1 piece
Micropipette P200 (20 – 200 µl)	1 piece
Micropipette P20 (2 – 20 µl)	1 piece
Pipette tips for micropipette P1000	1 box
Pipette tips for micropipette P20 and P200	1 box
Deionized water (**dH_2O**)	1 bottle
Microcentrifuge rack	1 piece
Round plastic container for liquid waste (**Liquid waste**)	1 piece
Square plastic container for solid waste (**Solid waste**)	1 piece
Timer	1 piece
Gloves	1 pair
Tissue paper	1 box
Glue	1 tube
Labels of student code	5 pieces
Red card	1 piece
Green card	1 piece
Calculator	1 piece
Marker	1 piece
Goggles	1 piece

For Experiment 1

Name	**Quantity**
SDS-PAGE electrophoresis gel tank and power supply	1 set
Gel comb	1 piece
Gel container (with student code)	1 piece
Microcentrifuge tubes 1.5mL	10 piece
Polyacrylamide gel cassette assembled in the electrophoresis gel tank	1 piece
Magenta microcentrifuge tube with 2X SDS-PAGE loading buffer (**Buffer**)	1 piece
Yellow microcentrifuge tube with 8 µl of protein marker (**M**)	1 piece
Microcentrifuge tube with 30 µl of cells without IPTG (**NO_IPTG**)	1 piece
Microcentrifuge tube with 30 µl of cells with IPTG (**IPTG**)	1 piece
Microcentrifuge tube with 30 µl containing pellet of cell extract from cells with IPTG (**Pellet)**	1 piece
Microcentrifuge tube with 30 µl of supernatant of cell extract from cells with IPTG (**Super**)	1 piece
Microcentrifuge tube with 30 µl of purified proteins (**Puri-P**)	1 piece
Falcon tube (green cap) with 40 mL of SDS-PAGE staining solution (**STAIN**)	1 piece

For Experiment 2

Name	Quantity
96-well microplate with student code (**do not touch the bottom of the plate**)	1 piece
Blue microcentrifuge tube with 300 µl of 1 mg/mL ascorbic acid solution (**AA**)	1 piece
Blue microcentrifuge tube with 300 µl of 5 mg/mL coffee extract (**CC**)	1 piece
Brown bottle with 15 mL of 0.2 mM DPPH solution (**DPPH**)	1 piece

For Experiment 3

Name	Quantity
Burette containing 25 mL of 0.1M NaOH solution with a stand and a clamp	1 set
Magnetic stirrer	1 piece
Stirring bar	1 piece
Forceps	1 piece
Measuring cylinder (10 mL)	1 piece
Measuring cylinder (25 mL)	1 piece
Beakers (100 mL)	8 piece
Hanna portable pH meter and a screw driver	1 piece
Rinsing water bottle (**H_2O**)	1 piece
Bottle with pH 4.01 buffer (**pH 4.01**)	1 piece
Bottle with pH 7.01 buffer (**pH 7.01**)	1 piece
Falcon tubes with 15-30 mL supernatant of culture broth (samples **A0, A2, A3 and A5**)	4 piece

EXPERIMENT 1. EXPRESSION, PURIFICATION AND CHARACTERIZATION OF PROTEINS (40 POINTS).

Introduction

H and B proteins are two important proteins of *Aeromonas hydrophilas.* To study them, a scientist wanted to co-express them in *E. coli*. For this, gene *b* was cloned into multiple cloning site 1 (MCS-1) and gene *h* in MCS-2 of expression vector p1 (**Fig.1.1**). The obtained p1-*b*-*h* vector was transformed into *E. coli* and protein expression was induced by IPTG (isopropyl *β*-D-1-thiogalactopyranoside). The proteins were then purified by affinity chromatography, in which a protein containing 6xHis-tag binds to the nickel column. Expression and purification were finally evaluated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), a method for separation of proteins based on their molecular weight. Note that H is smaller than B.

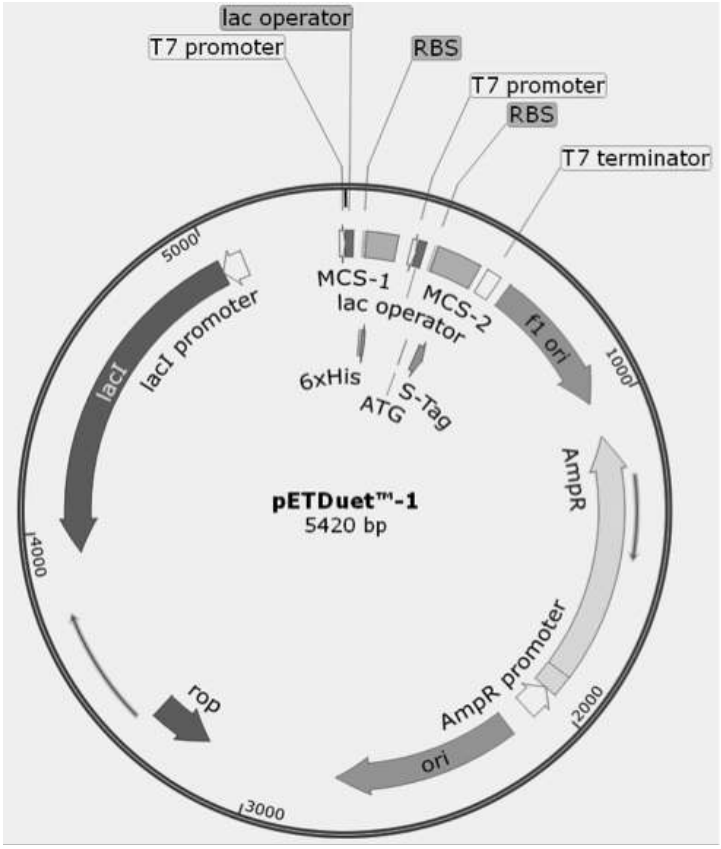


Fig.1.1. Overview map of plasmid p1 expression vector
(ori: origin, rop: repressor of primer, AmpR: ampicillin resistant)

A single colony of *E. coli* containing p1-*b*-*h* vector was cultured in 50 mL of LB medium. The culture was grown at 37ºC until the OD600 reached 0.6. In order to analyze the expression and purification of the recombinant proteins, the scientist has collected the following cell and protein samples:

- **NO_IPTG**. One mililiter of the culture was transferred to a – tube, grown at 2θC for 16 hours (OD_{600} = 2.4) and then centrifuged. The supernatant was discarded and the cell pellet was resuspended in 50 µl H_2O and then mixed with 50 µl 2X SDS-PAGE loading buffer to yield 100 µl sample.

In the remaining 49 mL culture, protein expression was induced by adding IPTG. The culture was further grown at 20oC for 16 hours.

- **IPTG**: One milliliter of the culture with IPTG (OD_{600} = 1.4) was centrifuged. The supernatant was discarded and the cell pellet was resuspended in 50 µl H_2O and then mixed with 50 µl of 2X SDS-PAGE loading buffer to yield 100 µl sample.

The remaining 48 ml culture was centrifuged, the supernatant was discarded and the cell pellet resuspended into 2 mL of nickel binding buffer. The cell suspension was lysed and subsequently centrifuged. Both pellet and supernatant were collected.

- **Pellet**: The pellet obtained from the IPTG-cell lysate was resuspended into 2 mL buffer and then mixed with 2 mL of 2X SDS-PAGE loading buffer (pellet stock solution).
- **Super**: 10 µl of supernatant obtained from IPTG-cell lysate was mixed with 10 µl of 2X SDS-PAGE loading buffer
- **Puri-P:** The rest of the supernatant was loaded onto the nickel column for protein purification. The purified proteins were eluted from the column using 2 mL elution buffer. 10 µl of purified protein was mixed with 10 µl of 2X SDS-PAGE loading buffer.

All samples for SDS-PAGE analysis were boiled at 100ºC for 5 min.

Design your SDS-PAGE experiment to analyze protein expression.

The standard final concentration of total protein for SDS-PAGE analysis must be equal to $5x10^6$ cells/µl. At first, calculate the concentration of cells in each sample, knowing that OD_{600} value of 1 corresponds to $8x10^8$ cells/mL and take into consideration the dilution of each sample during the procedure.

Q.1.1 (6 POINTS)

Calculate and fill the volume (µl) of samples in the table in the **Answer Sheet**. Use one decimal place.

Procedures

1. Based on the table above, prepare all SDS-PAGE samples in empty microcentrifuge tubes provided. Mix each sample by pipetting up and down 4-5 times.

   After completing this step please raise the Green card. An assistant will guide you to the loading area and help to stick your student code to the gel tank.
2. Load 20 µl of each sample on SDS-PAGE gel. Samples must be loaded in order from tube 1 to 6. To load a sample, use the P20 micropipette with tip to withdraw 20 µl of a sample, and carefully place the tip on the top of the well (**Fig.1.2**).

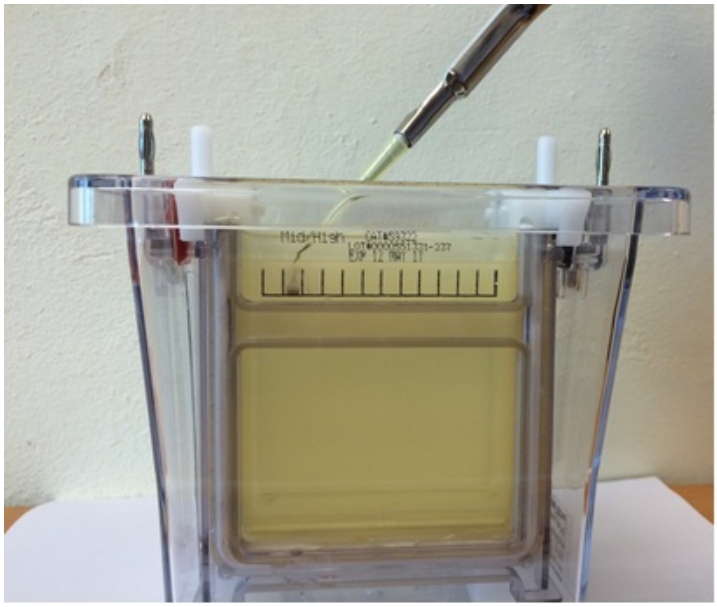


Fig.1.2. Sample loading on SDS gel

3. The assistant will run the SDS-PAGE for 20 min and tell you to set your timer for 20 min.

   You can do another experiment while running SDS-PAGE. After 20 min, please raise the Green card to inform an assistant to return the SDS-PAGE gel to you.

4. Remove the SDS-PAGE gel from the plastic cassette using the gel comb as in the diagram below (**Fig.1.3.)** and put the gel into the gel container.

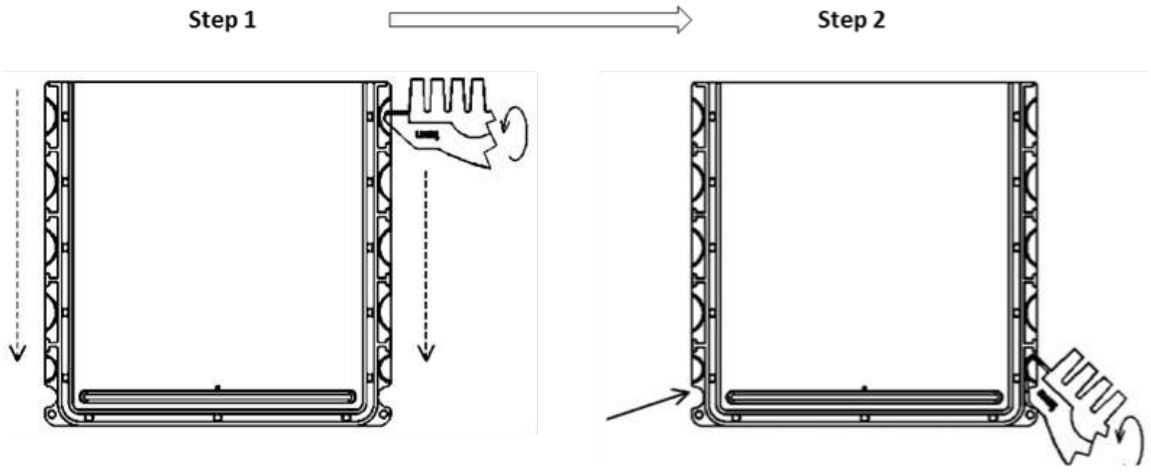


Fig.1.3. Remove the SDS-PAGE gel from the plastic cassette

Step 1. Crack open cassette sides by inserting the comb's slanted edge into each of the notches around the cassette and twisting firmly. Starting with the notches at the top, move down each side of the cassette.

Step 2. After the sides are open, place the comb's slanted edge at a 45-degree angle between the plates at each bottom corner and twist firmly.

Step 3. Gently separate the two pieces of the cassettes.

5. Add 40 mL of staining solution (**STAIN**) into the gel container and rock on the rocker for 10 min.
6. Discard the staining solution from the gel container and rinse the gel 3 times using deionized water.

When finished, raise the Green card to ask an assistant to take photo of the gel.

Q.1.2. SDS-PAGE RESULT (10 POINTS)

After obtaining the photo of SDS-PAGE gel, stick it to the place given in the **Answer Sheet**.

Q.1.3 (4 POINTS)

Based on the information provided in **Fig.1.4A** below, plot the molecular weight of at least five marker proteins versus their relative migration-Rf values on the graph paper provided in the **Answer Sheet**. (Rf: distance migrated by protein/distance migrated by dye front)

Q.1.4. (4 POINTS)

Use the graph from **Q.1.3** and the SDS-PAGE gel to estimate the molecular mass of H and B proteins.

Q.1.5 (4 POINTS)

Based on the SDS-PAGE result, indicate if each of the following statements is true or false. Mark "√" for True or False statements in the **Answer Sheet**.

A H protein is over expressed in LB media with IPTG.

B B protein is completely soluble in the nickel binding buffer.

C H and B protein interact with each other.

D Majority of recombinant proteins was bound to the nickel column.

Q.1.6. (4 POINTS)

Based on detailed restriction map of the p1 expression vector (**Fig.1.5**), indicate if each of the following statements is True or False. Mark "√" for True or False statements in the **Answer Sheet**.

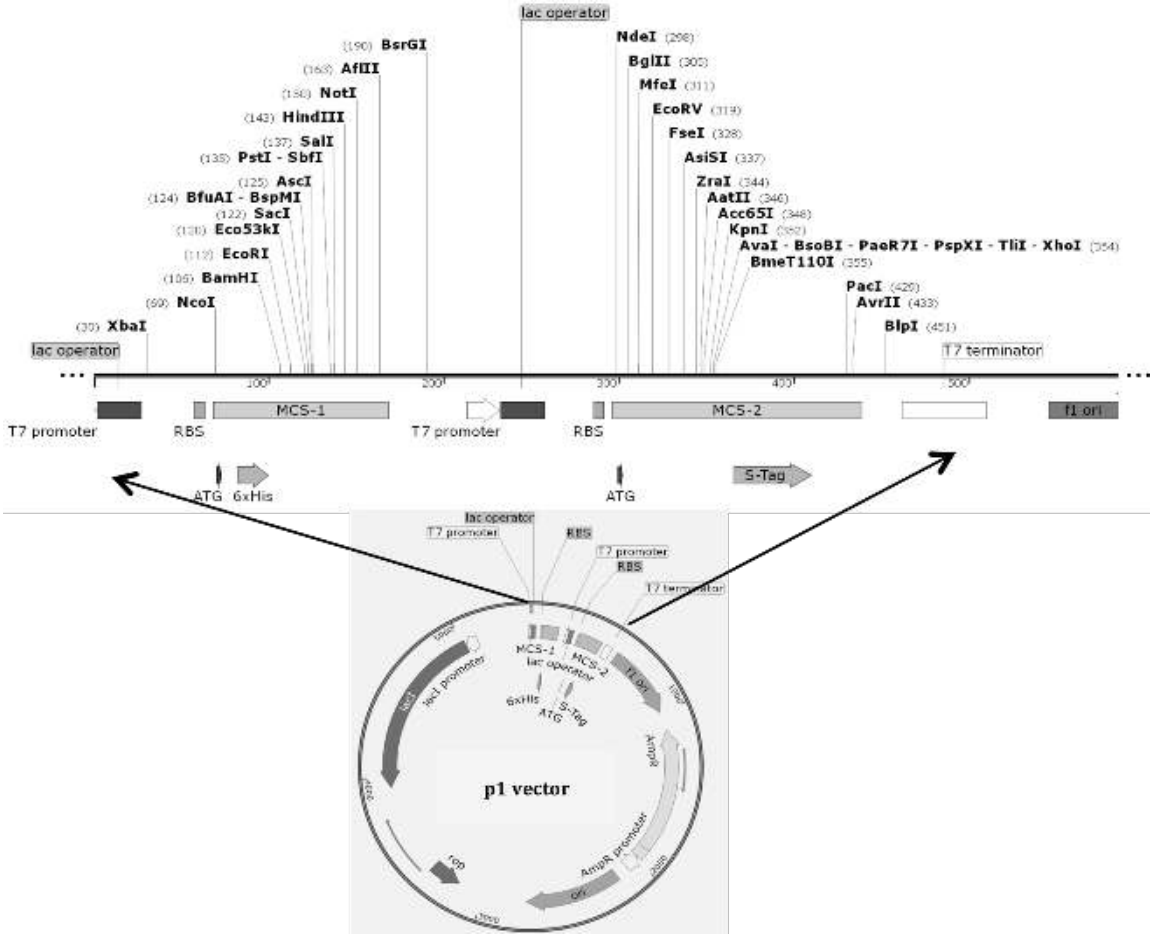


Fig.1.5. Detailed restriction map of p1 expression vector(RBS: ribosome binding site)

A *Sal*I and *Bam*HI can be used to insert *b* gene into MCS-1.
B Gene *h* and *b* should be cloned in the same orientation to be expressed simultaneously.
C Gene *h* and *b* should be in the same reading frame to be expressed simultaneously.
D To maintain the plasmid, ampicillin should be added to the culture medium.

In order to characterize the oligomeric states of H and B proteins, 3 protein samples were prepared: (1) H protein; (2) H and B proteins obtained from the above experiment; (3) B protein. Samples 1 and 2 were transparent but in sample 3 most of the protein was precipitated. Samples 1 and 2 were then loaded on a gel-filtration column. The obtained profiles are shown in **Fig.1.6A**. The sizes of reference molecule on a gel-filtration column are shown in **Fig.1.6B**.

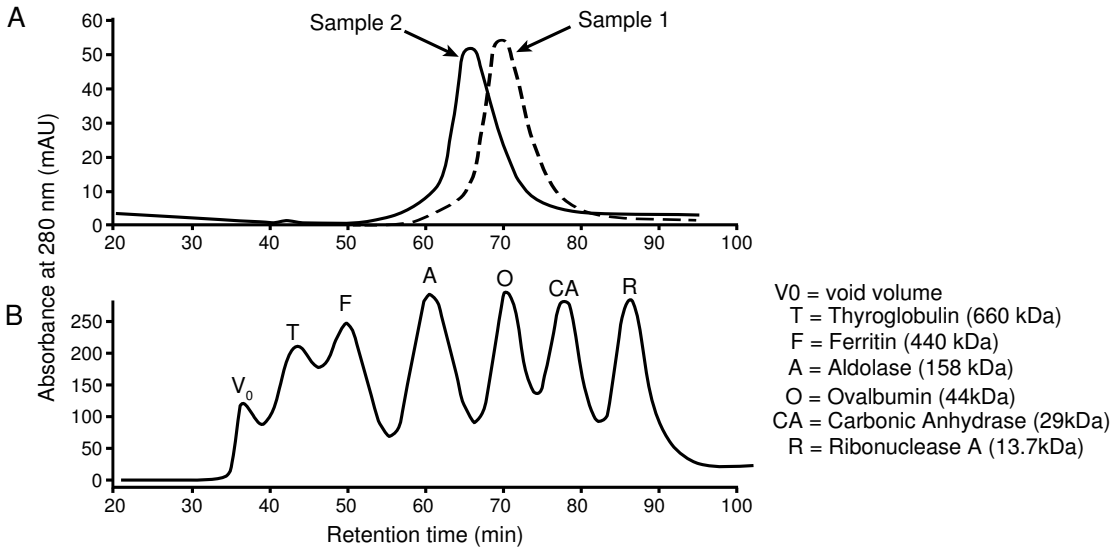


Fig.1.6. Gel filtration analysis of H and B proteins
(A) Chromatogram of sample 1 (dashed line) and 2 (solid line)
(B) Chromatogram of reference molecules

Q.1.7. (4 POINTS)

Calculate and report the relative size of the proteins corresponding to gel filtration peaks from sample 1 and 2 on the table in the **Answer Sheet**.

Q.1.8. (4 POINTS)

Indicate if each of the following statements is True or False. Mark "√" for True or False statements in the **Answer Sheet**.

A H protein exists as monomer
B H and B probably exist as heterodimer
C H protein helps to stabilize B protein.
D In native gel-filtration column analysis, retention time of a protein is proportional to their molecular weight.

EXPERIMENT 2. ANTIOXIDANT ACTIVITY OF COFFEE EXTRACT (30 POINTS)

Introduction

Biological oxidation produces reactive oxygen radicals that can cause serious damage to cells. Antioxidants are molecules that can scavenge radicals and thus inhibit oxidative reactions. Antioxidants include reducing agents such as thiol compounds, ascorbic acid and phenolics. Coffee, prepared from roasted coffee beans, is a potential source of antioxidants.

In this experiment, a 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, in which DPPH is reduced and looses its purple color, is performed. SC_{50} value (scavenging capacity) is commonly used for evaluation of antioxidant activity. This value represents the concentration of sample which scavenge 50% of DPPH radicals. Absorbance of DPPH will be measured at the wavelength of 517 nm. Absorbance of blank is assumed negligible. Absorbance of control (without scavenger, Ac) and sample (As) will be used to calculate scavenging percentage (SC%) for each concentration of samples as:

$SC\% = (Ac - As) \times 100/Ac$

A plot will be created based on the logarithm of concentration series of samples and corresponding scavenging percentage, from which SC_{50} value will be calculated.

In this experiment, beans of a Vietnamese coffee variety (*Coffea canephora*) will be investigated for antioxidant activity. Coffee bean powder (1g) was suspended in deionized water at 80°C for 30 min, then filtered and water was added to a final volume of 200 mL of extract solution.

Procedure and questions

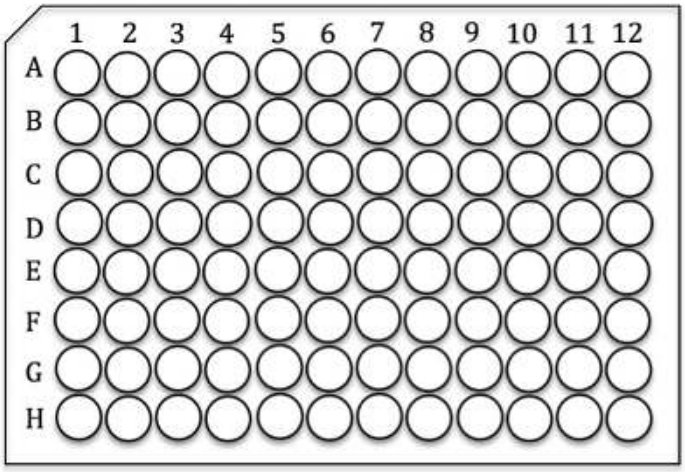


Fig 2.1 96-well microplate

The 96- well microplate above can be used to perform a serial dilution. Positions on this plate are indicated by a letter (A – H) and a number (1 – 12) specifying rows and columns respectively.

1. Use your micropipette to prepare 4 solutions of ascobic acid (AA1-AA4 in wells A1 – A4 in the 96-well microplate) and 4 solutions of coffee extract (CC1-CC4 in wells A6 – A9 in the 96-well microplate) by **serial dilution** with a dilution factor of 2, to achieve a lowest concentration of 0.025 mg/mL and 0.625 mg/mL, respectively. The volume of each solution produced should be 200 uL before further dilution is performed.
   Note: If an error is made in loading any of these wells use wells H1-H4 for ascobic acid solutions AA1 to AA4 and/or wells H6 – H9 for coffee extract solutions CC1 – CC4.

Q.2.1 (4 POINTS)

Fill in the table in the **Answer Sheet** what you calculated for preparing ascorbic acid and coffee extract dilutions.

2. Pipette 20 uL of ascorbic acid solution and/or coffee extract solution from each well in row A to the corresponding wells in rows B, C and D. If an error is made during this step, the procedure may be repeated using the corresponding wells in rows E, F and G.

3. Pipette an aliquot of 20 µl H_2O into wells B11, C11 and D11.
4. Pipette 180 µl of DPPH solution into all wells prepared in steps 2 and 3.
5. Cover the plate with the lid and incubate at room temperature for 10 min and set the timer.

After completing this step, raise the Green card for the assistant to help you in measuring absorbance on a microplate reader and return your data printout.

Q.2.2 (5 POINTS)

Calculate the logarithm (log_{10}) of ascorbic acid and coffee extract concentration and fill in the table in the **Answer Sheet** (all numbers are rounded to 2 decimal places). You can use your calculator to calculate common logarithm value following steps below:

- Press the **ON** key to turn on the your calculator
- Press the four keys **SHIFT, CLR, 2,** and **=** to return to calculation mode.
- Press the **log** key
- Enter the number
- Press the **=** key

Calculate the mean absorbance for each dilution, the scavenging percentage for each sample and fill in the table in the **Answer Sheet**.

Q.2.3. (5 POINTS)

Use the calculated value to plot a linear curve on scavenging percentage versus logarithm (log_0) of ascorbic acid concentration in the grid lines given in the **Answer Sheet**.

Q.2.4. (5 POINTS)

Calculate SC_{50} value of ascorbic acid and coffee extract and fill in the table in the **Answer Sheet** (You can plot a linear curve for coffee extract in the grid lines given in Q.2.3 but this curve will not be scored).

Q.2.5. (3 POINTS)

Using the same protocol, SC_{50} value of extracts of some coffee varieties were collected as follows:

Coffee extract	SC_{50}
X	3.8 mg/mL
Y	2.6 mg/mL

Compare antioxidant activity of different coffee bean types including the one in this experiment (Z) and arrange them in order from the strongest to the weakest and fill in the given space in the **Answer Sheet**.

Q.2.6. (4 POINTS)

Assume that in your experiment the absorbance of all the mixtures of different diluted coffee extracts and DPPH were similar and negligible. Indicate if each of the following statements is true or false.

Mark "√" for True or False statements in the **Answer Sheet**.

A Antioxidant activities of the diluted coffee extracts are negligible.

B To obtain more accurate determination of antioxidant activities, another experiment with higher concentration samples needs to be carried out.

C Activity of antioxidant enzymes in coffee extract resulted in the above result.

D If NADH is added in the wells, there will be no change in the assumed absorbance value.

Q2.7. (4 POINTS)

A student measured antioxidant activity of a sample using the same protocol as you have done (Protocol A) and a different protocol (Protocol A*) which was modified from Protocol A. The result is shown in the following table.

	Protocol A	**Protocol A***
SC_{50} (mg/mL)	1.95	3.9

Which of the following changes could result in a higher value of SC_{50}?

Indicate if each of the following statements is true or false. Mark "√" for True or False statements in the **Answer Sheet**.

A The student has used 0.1 mM DPPH for Protocol A*.

B The student has loaded 10 µl of sample in each well for Protocol A*.

C After adding DPPH, the student has incubated the 96-well plate for a shorter time than in Protocol A.

D The student has used better solvent for antioxidants.

EXPERIMENT 3. LACTIC ACID FERMENTATION (30 POINTS)

Introduction

Recently, a scientist isolated a homolactic acid *Lactobacillus* strain (*Lactobacillus* sp. VN156) from traditionally fermented mustard in Vietnam. In this experiment, *Lactobacillus* sp. VN156 was grown in MRS medium. The initial pH of the culture medium was 5.6. Samples were taken at different times during the cultivation for measuring the optical density (OD) of bacterial cells at 600 nm (**Fig.3.1**). An OD_{600} value of 1 corresponds to $2x10^8$ cells/mL. The samples A0, A2, A3 and A5 are supernatants of collected samples which will be used for analysis of lactic acid production.

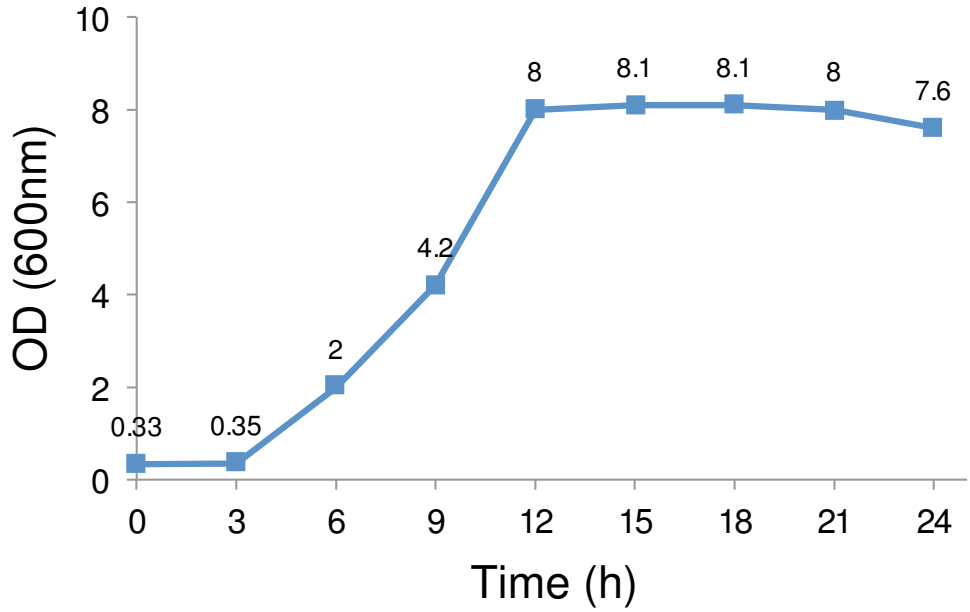


Fig.3.1. Growth curve of *Lactobacillus* sp. VN156

Q.3.1. (3 POINTS)

Assume that the **Fig 3.1** represents the real course of the growth. Calculate the generation time (h) of *Lactobacillus* sp. VN156 during exponential phase and record the value in the **Answer Sheet**.

Q.3.2. (3 POINTS)

If 1 mL of the culture at 9 h is diluted into fresh MRS medium, calculate the number of bacterial cells after 6 h of cultivation and record the value in the **Answer Sheet**.

Calibration of the pH meter

Use Hanna portable pH meter **(Fig 3.2)** for measuring the pH

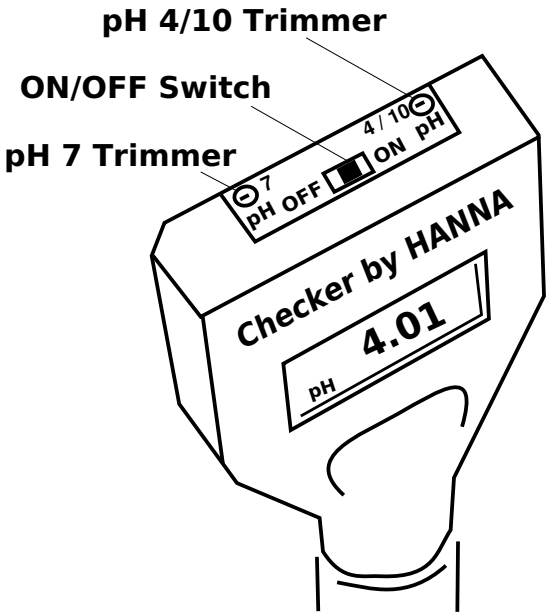


Fig 3.2.Hanna portable pH meter

Calibrate the pH meter according to the following procedure**Fig 3.3.**

- Turn the pH meter on by pressing ON/OFF button.
- Remove the protective cap and rinse the tip of the electrode with water, gently wipe with tissue paper.
- Dip the tip of the electrode in pH 7.01 buffer solution. Ensure the electrode tip and junction are fully immersed in the solution (about 2 cm of the tip is in the solution). Allow the reading to stabilize.
- Use a screwdriver to adjust the pH 7 trimmer until the display reads pH**7.01**
- Rinse the pH electrode with water, gently wipe with tissue paper.
- Dip the tip of the electrode in pH 4.01 buffer solution. Allow the reading to stabilize.
- Use the screwdriver to adjust the pH 4 trimmer until the display reads pH**4.01**.
- *Calibration is completed.*
- **Note:** if you switch off the pH meter you should calibrate it again

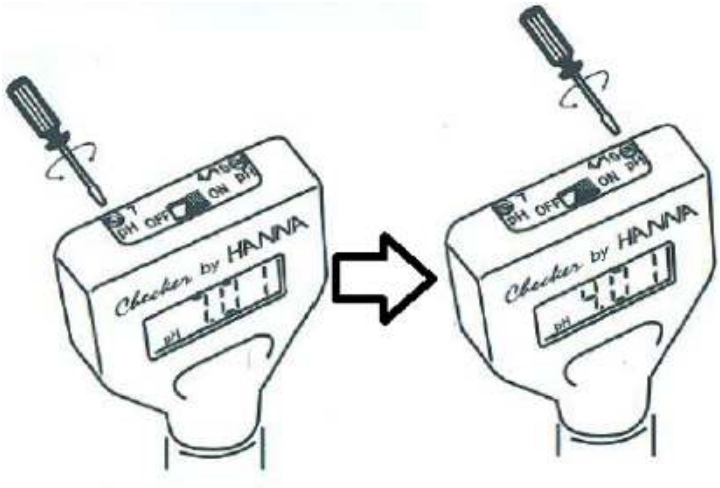


Fig 3.3. pH Calibration

Lactic acid titration

Titration is an analytical technique, which allows the quantitative determination of a specific substance (analyte) dissolved in a solution. It is based on a complete chemical reaction between the analyte and a reagent (titrant) of known concentration, which is added to the solution. In this part, titration method will be used to determine lactic acid concentration in the samples by titration with 0.1M sodium hydroxide solution as shown in **Fig.3.4**.

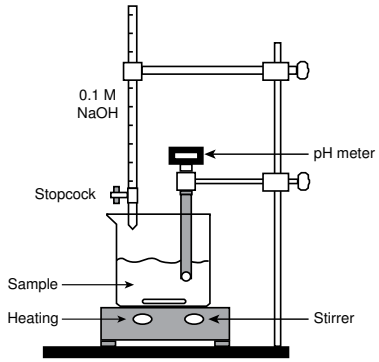


Fig.3.4. Titration setup

Q.3.3. (2 POINTS)

Calculate the volume of sample (mL) and water (mL) needed to be used according to the table in **Q.3.2** and record the values in the table provided in the **Answer Sheet**.

1. Based on your calculation, prepare sample dilutions for each sample in 100 mL beakers. Prepare two replicates for each sample using 25 mL measuring cylinder for deionized water, micropipette and 10 mL measuring cylinder for samples.
2. Carefully place a magnetic stirring bar into the diluted sample solution. Clamp the pH meter and position the pH electrode deep in the solution (about 2 cm of the tip is in the solution) so that the stirring bar will not hit the pH electrode while stirring. Begin stirring slowly and record the starting volume of 0.1M NaOH solution.
3. Open the stopcock of the burette to allow the NaOH solution from the burette to slowly run into the sample in the beaker. Stop adding NaOH when the pH of sample changes to neutral (6.95-7.05). Record the final volume of 0.1M NaOH solution.
4. After each titration, carefully remove the pH electrode from the solution, rinse it with water. Remove the stir bar by using a pair of forceps and rinse it with water.
5. Repeat steps 2–4 with for each sample.

Q.3.4. (9 POINTS)

Record the volume of 0.1 M NaOH used to titrate each sample in the table provided in the **Answer Sheet**.

Q.3.5. (10 POINTS)

Calculate the mean volume of 0.1 M NaOH needed to be used to titrate 30 mL of each stock sample and the concentration of lactic acid in each sample based on the final volume of base titrated. Record all values in the table provided in the **Answer Sheet**.

Note: NaOH (Mw=40) and lactic acid -$C_3H_6O_3$ (Mw=90)

Q.3.6. (3 POINTS)

Based on **Fig 3.1**, assuming that the OD_{600} of 1.0 equals to the densitiy of 2×10^8 cells/mL. The concentration of lactic acid will be increased 1g/L if the number of bacterial cells increases 2×10^8 cells/mL. If at 11 hours of cultivation the lactic acid concentration is 6 g/L, calculate the number of bacterial cells (cells/mL) and record the value in the **Answer Sheet**.

Record the value in the **Answer Sheet**.

END OF PRACTICAL 3