KA2 - Cooperation for Innovation and the Exchange of Good Practices

KA229 - School Exchange Partnerships

Debrecen

2019.

The **pharmaceutical industry** discovers, develops, produces, and markets drugs or pharmaceutical drugs for use as medications to be administered (or self-administered) to patients, with the aim to cure them, vaccinate them, or alleviate the symptoms. The dosage form for a pharmaceutical contains the active pharmaceutical ingredient (API) which is the biologically active component. Active pharmaceutical ingredients can be manufactured by chemical synthesis, extraction, biological processes, by recovery from natural sources, or by any combination of these processes.

Biological Processes predominantly involve fermentation where microorganisms are cultured in a substrate to produce useful substances e.g. antibiotics, immunosuppressive agents, vitamins, enzymes etc. Sometimes API's are produced by genetically modified organisms.

During the first part of the laboratory period students will work with an enzyme (bacterial amylase) which is produced by bacterial fermentation. They will measure enzymatic activity and determine enzyme concentrations. Students will study cells of the producing microorganism with microscopes.

The second part of the laboratory work is about an API, namely ascorbic acid which is manufactured mainly by bacterial fermentation. Students will determine the ascorbic acid concentration of an unknown solution by titration.

Students will visit the R&D Division of the TEVA Pharmaceutical Works. They will see the strain development and pilot fermentations. They will also visit the University of Debrecen where much basic research (e.g. pharmaceutical research) is carried out.

Task 1.

Assaying for Amylase Activity (G Biosciences kit designed by Ellyn Daugherty)

Objectives: What is the difference between the behavior of a natural bacterial amylase and a protein engineered version of the same enzyme?

Background: Amylase is an enzyme that catalyzes starch digestion. Amylose is one type of plant starch. The amylose molecule is very long, composed of hundreds of glucose molecules linked together (Fig. 1). Human amylase breaks the bond between glucose molecules in the amylose (starch) chain to produce the disaccharide, maltose. In this case amylose is the substrate of the enzyme maltose is the product of the enzyme. Some bacterial amylases (*e.g. Bacillus subtilis* amylase) and fungal amylases produce glucose from starch. In this case amylose is the substrate of the enzyme glucose is the product of the enzyme. Bacterial amylase activity can be measured by the velocity of decrease in starch concentration (Starch Breakdown Assay) or by the velocity of increase in glucose concentration (Glucose production assay). In this experiment we compare two amylases. A natural *B. subtilis* amylase (this enzyme is produced by natural, not transformed *B. subtilis* cells) and a protein engineered *Bacillus subtilis* amylase (in this case amylase is made by *Bacillus* cells that have been transformed with recombinant DNA (rDNA)). Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. Pharmaceutical and clinical sectors require high purity amylases.
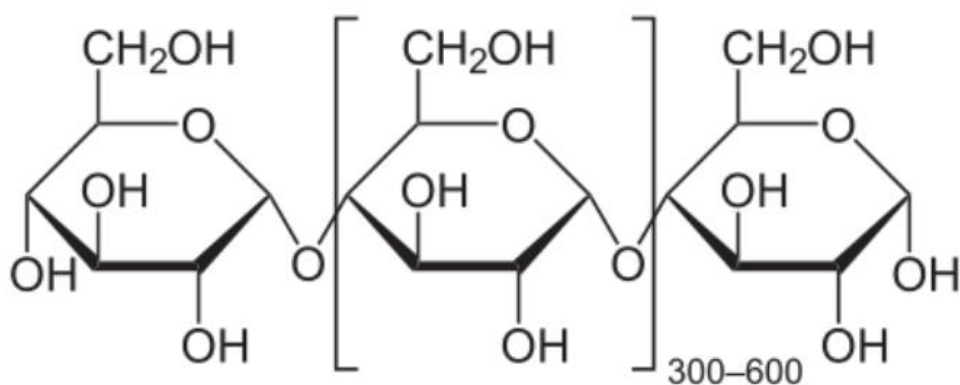

Fig. 1: Molecular Structure of amylose

Procedure:

1. In a 24-well-plate place 1 ml of starch solution in each well of the first three rows of six wells (Fig. 2). The bottom row will not be used.
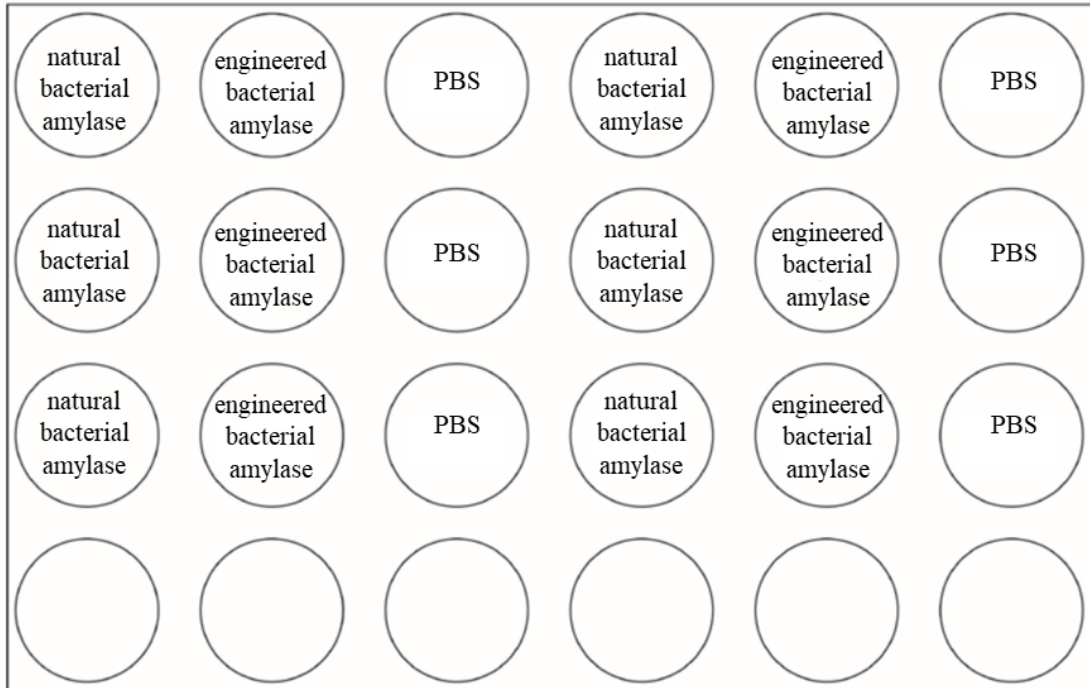

Fig. 2

2. Add 300 μl of natural bacterial amylase solution to the first and fourth wells of each row (see figure 2). Mix thoroughly for 2 seconds using the micropipette tip. Change tips before going to Step 3.

3. Add 300 μl of protein engineered bacterial amylase solution to the second and fifth wells of each row (see figure 2). Mix thoroughly for 2 seconds using the micropipette tip. Change tips before going to Step 4.

4. Add 300 μl of 1X PBS, pH 7.0 to the third and sixth wells of each row (see figure 2). Mix thoroughly for 2 seconds using the micropipette tip.

The fourth, fifth and sixth wells in each row will be used for starch breakdown assay, the first, second and third wells in each row will be used for sugar production assay.

Starch Breakdown Assay:

5. Add 20 µl Starch Indicator Solution (Lugol's Iodine Solution) to all the fourth, fifth and sixth wells in each row (see figure 2). Mix thoroughly for 2 seconds using the micropipette tip. Change tips after each use.

6. Identify the negative controls in this experiment.

7. Place the 24-well plate on a piece of white paper, out of direct light as iodine decolorizes in light. Wait until a noticeable color change occurs in the natural amylase containing wells (4th column).

8. Starch mixed with iodine gives a very dark blue-black color. Iodine is a golden red-brown when no starch is present. Starch breakdown is measured by a decrease in the starch-iodine reaction (a lessening of the blue-black color).

9. Measure the amount of starch breakdown (5 → 0 ratings) by recording the degree of lightening of the iodine solution from black (0) to a light red-brown or clear color (5) in the wells of columns 4, 5, and 6.

Results of starch breakdown assay:

natural bacterial amylase (4th column)	protein engineered bacterial amylase (5th column)	PBS (6th column)
Average results		

10. After ……minutes measure glucose concentration in Columns 1, 2, and 3 using supplied sugar (glucose) test strips.

11. Handling the test strip by the plastic end and dip the test indicator end into the sample. Immediately withdraw drawing the edge of the strip against the rim of the well to remove excess sample.

12. Compare the test strip to the color chart below (Fig 3.) and record the results in mg/dl. The mg/dl is a common unit of glucose concentration.
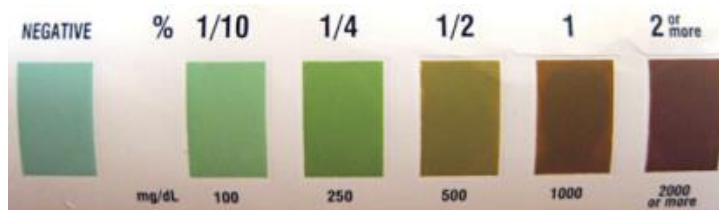
Fig. 3.

Results of glucose production assay:

natural bacterial amylase (1st column)	protein engineered bacterial amylase (2nd column)	PBS (3rd column)
Average results		

Data analysis and conclusions:

Discuss the results of the experiment, including your observations of the behavior of the natural bacterial amylase as compared to the behavior of the protein engineered bacterial amylase solution.

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Task 2.

Determining the Concentration of Amylase in a Solution (G Biosciences kit designed by Ellyn Daugherty)

Objectives: What is the concentration of two unknown amylase solutions?

Background: The pharmaceutical company wants purified proteins that are active, stable, and of a relatively high concentration. When proteins are purified from solutions (cytoplasm, fermentation broth, etc.) the product must be checked to see how much protein is present in it. Protein concentration is usually measured in milligrams per milliliter (mg/ml) or micrograms per milliliter (µg/ml). Since the protein molecules are submicroscopic, we must measure the proteins using indirect methods. First, we prepare solutions of known concentrations and read their absorbance at a given wavelength. Then we produce a "best-fit" curve, representing how the concentration affects the absorbance by the molecule being tested. The absorbance of an unknown solution is then determined. From the "best-fit" standard curve, the concentration of the unknown solution is determined based on where the absorbance value intersects the standard curve.

Procedure:

Preparation of the Standard (known) Samples by serial dilution of the known samples starting with the 0.5 mg/ml of amylase stock solution.

1. Label five microcentrifuge tubes No. 1 through 5.

2. Add 500 µl of 0.5 mg/ml of amylase stock solution to Tube No. 1.

3. Add 500 µl of 1XPBS Buffer to each of the test tubes No. 2 through 5.

4. Take 500 µl of the 0.5 mg/ml of amylase stock solution and add it to tube No. 2. Gently pipette it up and down to mix. What is the concentration of protein in this tube?

5. Take 500 µl of the solution in test tube No. 2 and add it to test tube No. 3. Gently pipette it up and down to mix. What is the concentration of protein in this tube?

6. Continue making these dilutions with the remaining tubes. After preparing test tube No. 5, discard the extra 500 µl so that all tubes No. 1 through 5 contain the same volume of solution (500 µl). Record the concentration of tubes 1-5 in the table below.

7. These 5 tubes contain the "known" standard samples of known concentration that are used to create the standard curve used in calculating the unknown samples' concentrations.

Preparation of Samples for Spectrophotometry:

To take absorbance reading of all the samples, you must work quickly when using the spectrophotometer. For use in the visible spectrophotometer the samples (as well as buffer blank and the unknown samples) must be transferred to appropriate tubes for mixing with the colored protein indicator (Bradford Reagent). Each sample and Bradford Reagent is mixed in a separate test tube and then transferred to a cuvette for analysis.

1. Pipette all of the standard samples to labeled "mixing tubes". (Use a new tip for each sample.) Label these tubes with their actual concentration in mg/ml.

2. Warm-up your spectrophotometer, absorbance data will be taken at a wavelength of 595 nm. Set the spectrophotometer to a wavelength of 595 nm.

3. Place 500 μl of unknown sample 1 in a mixing tube. Label this tube "U1."

4. Place 500 μl of unknown sample 2 in a mixing tube. Label this tube "U2."

5. Place 500 μl of the 1X PBS Buffer in a mixing tube. Label this tube "B", for blank.

6. Do not go onto step 8 until the spectrophotometer you will be using is free and ready for you to use. Once you add Bradford Reagent to the samples the absorbance of each must be read in 3 minutes.

7. Using a transfer pipet and without touching the inside of each tube, add 2.5 ml of 1X Bradford Reagent to the blank, each of the standard known samples, and the two unknown samples. Be careful to not touch the solutions in the tubes, spill or accidentally contaminate one sample with another. Gently mix thoroughly without letting the mixture bubble. Set the spectrophotometer to zero using the blank sample. Within 3 minutes use the spectrophotometer to observe (take absorbance readings) of all the samples.

Creating a Protein Concentration Standard Curve:

1. After collecting the absorbance data, use the absorbance of the known standard samples to make a "standard curve" graph. Plot the concentration (mg/ml) on the x-axis and absorbance on the y-axis. Add the absorbance data points for the known standards. The points should "line up" since the absorbance is correlated to the number of molecules present to absorb the light passing through a sample. However, there is an optimal range of concentrations a spectrophotometer can detect. If a sample's concentration is "too high" the spectrophotometer will report it erroneously lower than it actually is. This data will skew the standard curve line. Pick the best 3-4 data points to use for the best-fit standard curve line and ignore samples that are obviously skewing the line.

2. Make a "best fit" straight line through the data points of the known standard samples. The best-fit straight-line estimates what a sample of amylase molecules will absorb within a range of concentrations. NOTE: If you are creating the graph using Microsoft® Excel®, pull down "Chart" on the "Menu," select "Add Trendline," and select "Linear Regression." It will add a "best-fit" straight line for you. Double-click on the "Best-fit Trendline." Select "Options." Click on "axis through 0" and "show equation of the line." This will determine the "equation of the line" to be used later.

Determining of Protein Concentration Using a Standard Curve:
Use the equation to determine the concentrations of the unknown samples.

Calculation of the average concentrations of the group:
Collect the concentration determinations for each unknown (y = mx + c) from the other groups in the class (multiple replications). Put these values into a new data table. Calculate the average concentration of each sample for all the replications of the experiment. These averages are the best guess of the true concentrations of the "unknowns."

Results:
Absorbance values

standard samples (mg/ml)					unknown sample		blank
0.5					1.	2.	
							0

Equation:

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Calculation of the concentration values of unknown samples (your own determination)

unknown sample	
1.	2.

Calculation of the average concentrations of the group:

	unknown sample	
	1.	2.
group 1		
group 2		
group 3		
group 4		
group 5		
Average		

Conclusion:

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Task 3.

Studying the cell morphology of an amylase producing *Bacillus* subtilis strain

Objectives: What is the shape of *Bacillus subtilis* cells?

Background: Bacterial cells are about one-tenth the size of eukaryotic cells and are typically 0.5–5 micrometers in length. Most bacterial species are either spherical, called *cocci* (*sing. coccus*), or rod-shaped, called *bacilli* (*sing. bacillus*). Some bacteria, called vibrio, are shaped like slightly curved rods or comma-shaped; others can be spiral-shaped, called *spirillum*, or tightly coiled, called *spirochaete*.

Procedure:

Use rubber gloves! After preparation take off the gloves!

1. Take a microscope slide and place a drop of the given fermentation broth in the center of the slide.

2. At an angle, place one side of the cover slip against the slide making contact with the outer edge of the liquid drop.

3. Lower the cover slowly, avoiding air bubbles.

4. Remove excess water with a piece of paper towel.

5. Find the bacterial cells under the microscope and draw them.

How to use a microscope?

1. Turn the revolving turret (2) so that the lowest power objective lens (eg. 4x) is clicked into position.

2. Place the microscope slide on the stage (6) and fasten it with the stage clips.

3. Look at the objective lens (3) and the stage from the side and turn the focus knob (4) so the stage moves upward. Move it up as far as it will go without letting the objective touch the coverslip.

4. Look through the eyepiece (1) and move the focus knob until the image comes into focus.

5. Adjust the condenser (7) and light intensity for the greatest amount of light.

6. Move the microscope slide around until the sample is in the centre of the field of view (what you see).

7. Use the focus knob (4) to place the sample into focus and readjust the condenser (7) and light intensity for the clearest image (with low power objectives you might need to reduce the light intensity or shut the condenser).

8. When you have a clear image of your sample with the lowest power objective, you can change to the next objective lenses. You might need to readjust the sample into focus and/or readjust the condenser and light intensity. If you cannot focus on your specimen, repeat steps 3 through 5 with the higher power objective lens in place. **Do not let the objective lens touch the slide!**

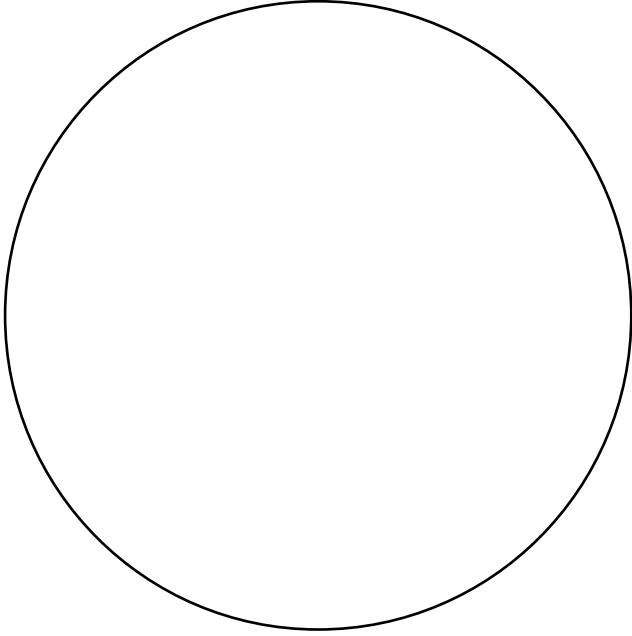9. When finished, lower the stage, click the low power lens into position and remove the slide.

After this experiment use the dezincification solution!

Results:

Magnification of the microscope:

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Conclusion:

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Task 4.

Determination of the ascorbic acid concentration of an unknown solution

Objectives: How much ascorbic acid is dissolved in the unknown sample?

Background:

Vitamin C, also known as ascorbic acid or L-ascorbic acid, is a vitamin found in various foods and sold as a dietary supplement. It is used to prevent and treat scurvy. Vitamin C is an essential nutrient involved in the repair of tissue and the enzymatic production of certain neurotransmitters. It is required for the functioning of several enzymes and is important for immune system function. It also functions as an antioxidant. Vitamin C was discovered in 1912, isolated in 1928, and in 1933 was the first vitamin to be chemically produced. Partly for its discovery, Albert Szent-Györgyi and Walter Norman Haworth were awarded the 1937 Nobel Prizes in Physiology and Medicine and Chemistry, respectively. Vitamin C is produced from glucose by two main routes. The Reichstein process uses a single pre-fermentation followed by a purely chemical route. The modern two-step fermentation process uses additional fermentation to replace part of the later chemical stages.

Ascorbic acid content of a solution is usually determined by iodometric titration.

The reaction of ascorbic acid with iodine:

Ascorbic acid
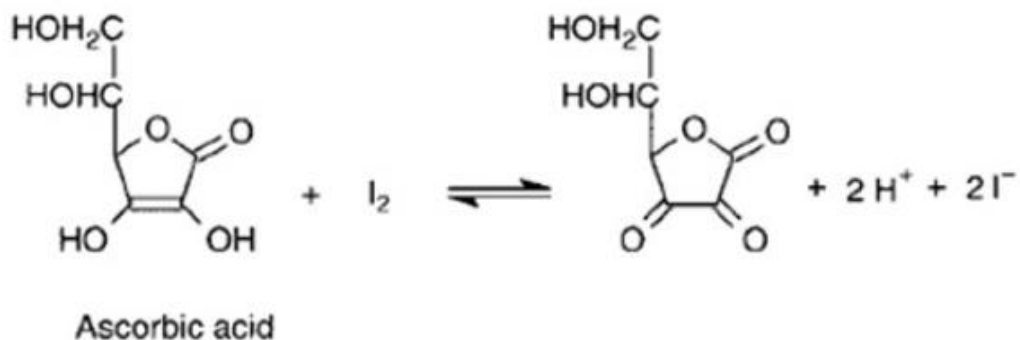
Procedure:

1. Fill a burette with the exactly 0.0500 mol/l iodine solution.

2. Take 10.0 ml of the unknown sample, placed in an Erlenmeyer flask 100 ml.

3. Add 5.0 ml 1% starch solution.

4. Titrate the solution drop by drop to reach a stable blue color.

5. Repeat the whole procedure 3 times.

Results:

	1st titration	2nd titration	3rd titration
volume of iodine solution (ml) V			
concentration of iodine solution (mol/l)	0.0500	0.0500	0.0500
iodine (mol)			
ascorbic acid (mol)			
concentration of ascorbic acid solution (mol/l)			
average concentration of ascorbic acid solution (mol/l)			

Calculation of iodine moles: $\dfrac{V*0.0500}{1000}$

Ascorbic acid moles are the same as iodine moles.

Calculation of the concentration of ascorbic acid solution (mol/l): ascorbic acid mol *100

References:

1. McGuire, John L.; Hasskarl, Horst; Bode, Gerd; Klingmann, Ingrid; Zahn, Manuel (2007). Ullmann's Encyclopedia of Industrial Chemistry. doi:10.1002/14356007.a19_273.pub2. ISBN 978-3527306732.

2. Assaying for Amylase Activity (G Biosciences kit designed by Ellyn Daugherty) https://www.gbiosciences.com/image/pdfs/protocol/BTNM-6C_protocol.pdf

3. Determining the Concentration of Amylase in a Solution (G Biosciences kit designed by Ellyn Daugherty) https://www.gbiosciences.com/image/pdfs/protocol/BTNM-7G_Amylase_Protein_Concentration.pdf

4. Yang DC, Blair KM, Salama NR (March 2016). "Staying in Shape: the Impact of Cell Shape on Bacterial Survival in Diverse Environments". Microbiology and Molecular Biology Reviews. 80 (1): 187–203. doi:10.1128/MMBR.00031-15. PMC *4771367*. PMID 26864431.

5. https://www2.mrc-lmb.cam.ac.uk/microscopes4schools/microscopes2.php

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7. Determination of Vitamin C Concentration by Titration: canterbury.ac.nz/media/documents/science-outreach/vitaminc_iodine.pdf