

Amylase – from Molecules to Systems

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Amylase enzymes breakdown starch into simpler sugars and are produced by plants, fungi, bacteria and mammals. This pair of laboratory modules addresses two questions. The first, “Do amylase enzymes from different organisms have similar reaction conditions?” examines the pH optima for alpha amylases from several organisms, using a quantitative starch-iodine reaction to measure starch concentration and determine the rate of reaction for each enzyme. The second module examines the question: “How similar are amylase enzymes from different organisms at the nucleic acid and amino acid levels?” This module uses PCR to examine DNA sequence specificity and simple bioinformatics tools such as NCBI and DNA subway to compare nucleotide and amino acid sequences.

Keywords: amylase, PCR, DNA sequencing

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Introduction

One of our goals as biology teachers is to train students to think like biologists and to do biology rather than to simply *learn* biology. Part of this journey is to make connections between molecules, cells, organisms and systems. This pair of Introduction to Biology modules form part of a project-based laboratory course at RIT. The two modules take students from starch detection assays, through enzyme assays to simple bioinformatics.

The first module is presented to freshmen life sciences majors early in their first semester. Students examine starch-degrading amylase enzymes from various organisms. In this module we ask the question:

“Do the amylase enzymes from different organisms have similar reaction conditions?”

We use a simple quantitative assay based on the reaction of starch with iodine to determine differences in pH optima for amylase enzymes from different sources and determine concentrations of unknown starch solutions using standard curves. Once the class is familiar with the basic assay techniques, groups of students devise their own experiments using similar experimental techniques, and a limited list of reagents.

In Module 2, we ask the question:

“If all of these amylase enzymes break down starch to maltose, how similar are they at the nucleotide and amino acid level?”

Students use PCR to amplify human salivary alpha amylase from their own DNA and then use simple bioinformatics tools (NCBI and DNA subway) to explore differences in gene structure and sequences among amylase enzymes from different organisms.

Student Outline

Module 1: Determining the Optimum pH For Amylase Enzymes

Week 1: Measuring starch and maltose concentrations and constructing standard curves.

1. Add 25 μ l IKI to 10 ml 0.1% starch. Label this tube #1.
2. Make serial dilutions to obtain 0.05%, 0.025% and 0.0125% starch.
3. Measure the absorbance of these solutions at 620 nm, using 10 ml water + 25 μ l IKI as a blank.

Plot your results on a graph, with concentration (the thing you are changing, the independent variable) on the x-axis, and absorbance (the thing you are measuring, the dependent variable) on the y-axis.

What do you notice about the color of the tubes and their absorbance readings?

This kind of graph is called a **standard curve**, and we can use this to find the concentration of unknown solutions. We'll be using standard curves a lot in the next few weeks.

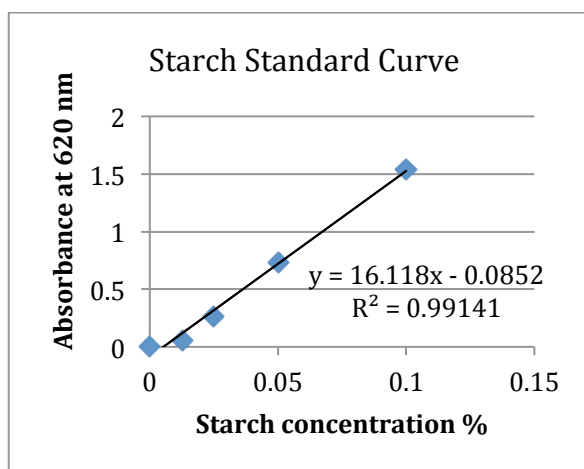


Figure 1. Starch standard curve.

Maltose Standard Curve

We can use IKI to detect starch, but in this experiment we want to see the product of the reaction too. In the case of starch and amylase, the product is maltose. Maltose is a disaccharide consisting of two glucose molecules. It can be detected using a reagent called dinitrosalicylic acid, or DNSA.

1. Prepare dilutions of 1% maltose as follows: all volumes are in microliters.

Tube #	0	1	2	3	4
Maltose	0	50	200	400	600
DI Water	2000	1950	1800	1650	1400

2. Add 1 ml DNSA reagent to each tube. Label each tube with a sharpie marker, and boil for 15 minutes.
3. Allow to cool for 5 minutes, then add 9 ml water to each tube.
4. Use tube 0 as blank and measure the absorbance at 540 nm for each sample.
5. Make a scatter plot of absorbance and maltose concentration and fit a linear trend line. Display r^2 and the equation of the line.

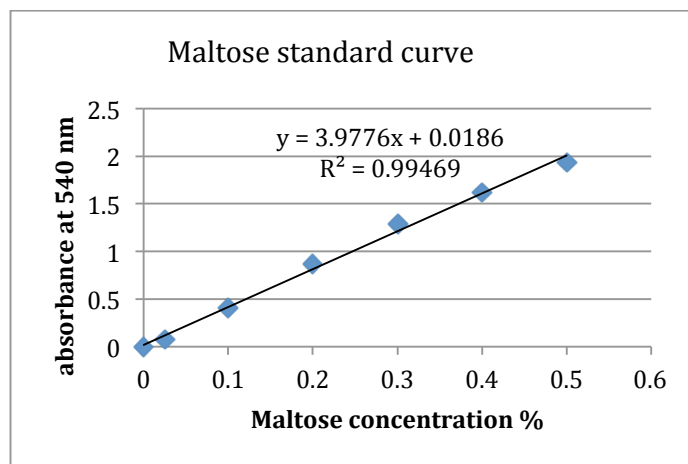


Figure 2. Sample Maltose standard curve from DNSA reaction

Week 2: Enzyme reactions and pH

Last week you learned how to detect and quantify starch and a reducing sugar (maltose). In this lab you will look at the optimum conditions for two different amylase enzymes. Most organisms produce amylase enzymes, and many organisms produce more than one form. Humans, and many other mammals, produce alpha amylase in their mouths (salivary amylase) and in their pancreas (pancreatic amylase). Many plants produce two different amylases: alpha and beta amylases. Plants produce starch, store it in roots and leaves and often seeds, and then break it down again into glucose for metabolism and growth. Bacteria and fungi also produce amylase enzymes that enable them to break down starch.

So we might ask the question: Do these amylase enzymes all work at the same pH? How can we find out?

You already know how to measure the amount of starch (the substrate for amylase) and the amount of reducing sugar, (the product of starch breakdown). Today we'll use a series of buffers to change the pH of the reactions and measure the rate of starch breakdown for several amylase enzymes. We'll use the starch / iodine reaction to measure the rate of starch breakdown and measure the amount of reducing sugar produced at the end of the reaction time.

Procedure

Each team will test two enzymes: human pancreatic amylase and EITHER alpha amylase from *Bacillus* species or alpha amylase from *Aspergillus oryzae*.

Setting up the reaction tubes:

1. Label one large test tube for each pH buffer: pH 4.0, 5.0, 5.8, 7.0, and 8.0.
2. Add 8 ml 0.1% starch and 2 ml of the appropriate buffer to each tube (1-5).
3. Now prepare the iodine tubes. You need a total of 21 tubes (small ones work best for this). Each tube should contain 1 ml of distilled water and 10 μ l IKI. To save pipetting 34 x 10 μ l IKI, make a single batch of water + IKI and pipette 1 ml into each tube. (So you will need 23 ml water and 230 μ l IKI- be sure to mix it well!). Prepare ALL the tubes before you start the enzyme reactions- you need to be super organized for this! I suggest you put the tubes in a rack with the enzyme tubes along the front and the corresponding water IKI tubes behind. You need 4 IKI tubes per pH. The extra tube is the blank.
4. Before you start the reactions by adding the enzyme, PLEASE READ THE NEXT PART.
5. To determine the amount of starch at the start of each reaction, take 0.5 ml of the starch/buffer solution for each pH and add this to the time zero IKI tube.
6. To determine the amount of maltose at the beginning of the experiment, take 1 ml from each tube, place in separate large test tubes and set aside until the end of this experiment.

7. You will start the reaction in all 5 tubes at the same time. Start with tube 1 and add 0.1 ml of enzyme, cover the open end with parafilm and invert to mix. Then do tube 3, 4 5. **START YOUR TIMER.** One person could be adding the enzyme whilst the other one covers the tubes and mixes.
8. After 3 minutes take 0.5 ml from tube 1 and add it to one of the IKI tubes. Repeat with tubes 2 to 5, using a clean tip each time and adding to the IKI tube directly behind the pH / enzyme tube. Take the samples in the same order as you started the reactions.
9. Repeat at 6, and 9 minutes, taking samples in the same order each time, using clean tips and adding to the next IKI tube.
10. Prepare a blank by adding 0.5 ml water to one of the IKI tubes (contains 1.5 ml water + 10 μ l IKI).
11. Set the spectrophotometer to 620 nm and set zero with the blank.
12. Now measure the absorbance of each tube and record the results in a table.

To determine the amount of maltose produced:

At the end of the 9 minutes, take a 1 ml sample from each enzyme reaction and add it to a new tube. Add 1 ml of DNSA reagent and boil for 15 minutes. Do the same with the tubes you prepared at the beginning of the experiment. Cool for 5 minutes and add 9 ml water. Measure the absorbance at 540 nm.

Calculation of starch concentration from absorbance measurements

Now return to the starch absorbance table above. Use the standard curve you made last week to work out the starch concentration at each time point. Make a new table showing the amount of starch remaining at each time point for each pH. You now have enough information to find the rate of reaction for amylase at each pH.

Plot a graph of starch concentration vs. time (use Excel) and use this to find the rate of reaction for each pH.

You can also use the DNSA / maltose standard curve you made last week to find the amount of maltose produced from the starch by amylase at each pH.

- Which pH gave the greatest amount of maltose? At which pH was the reaction rate greatest?
- Does your experimental data support or refute your hypothesis?
- Compare the rates of reaction at different pH for the two enzymes studied by your team. Are they similar or different? Can you explain why?
- Write a summary your results and conclusions using the questions above as an outline.

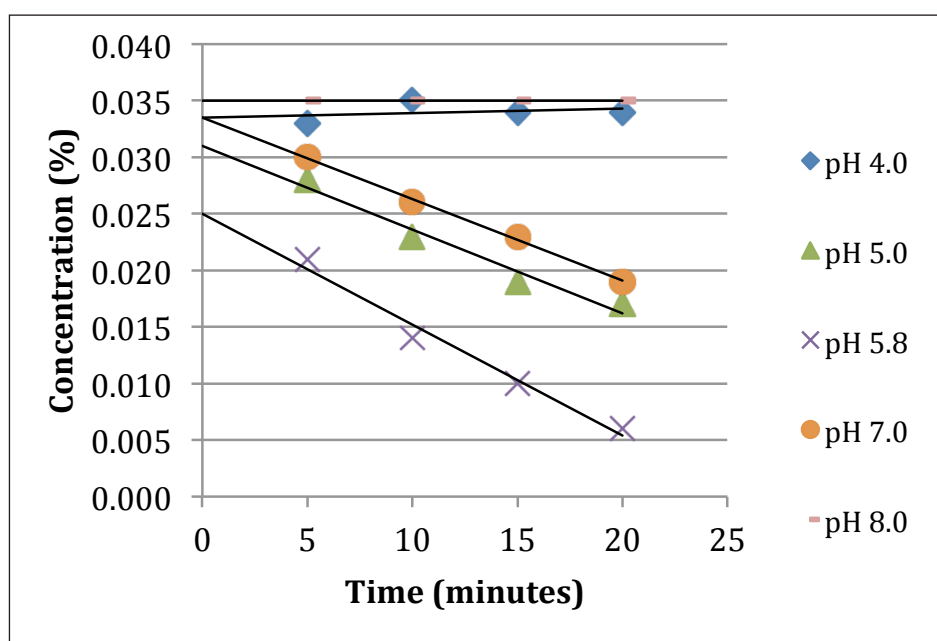


Figure 3. Sample data. Rates of reaction of porcine pancreatic amylase under different pH conditions.

Week 3: Student-Designed Experiments

Using the tools and concepts learned so far, devise an experiment to answer a question about amylase enzymes. Propose a reasoned hypothesis for your experiment. Plan and conduct your experiment with suitable controls.

Here are some examples:

- Can cellulase break down starch?
- What is the pH optimum for human salivary enzyme?
- Determine the temperature optimum for one or more of the amylase enzymes available.
- Can we extract amylase enzymes from plants or mushrooms?
- Can amylase break down glycogen?
- Can amylase break down cellulose?

Module 2: Sequencing and Comparison of the Amylase Gene

As part of our investigation into amylase enzymes we'll ask the following question:

“If most organisms make amylase enzymes, how similar are they at the DNA level?”

Week 1: PCR

In this lab, we will use PCR to amplify a small region of the alpha amylase gene from human DNA, plant DNA and bacterial DNA. I will supply you with the plant and bacterial DNA samples, but you will need to extract human DNA from yourselves- don't worry, we don't need blood- just some cheek cells that you shed quite easily!

To ensure that our PCR reaction is successful, we need to ensure the following:

1. We put in the correct ingredients- the mix you will use has the dNTPs (nucleotides A,C,G and T), Taq polymerase and buffers. It also contains a red dye so that you can check you put the mixture into each tube. The red dye is also a gel loading dye.
2. We remember to add our DNA sample, and the DNA is reasonably pure.
3. We need to make sure that our primers are complementary to the DNA we want to amplify. In our experiment, I designed the primers to be complementary to *human salivary amylase*.

Harvesting Cheek Cells

Either: use a sterile stick (wooden end of the swab) to scrape cells from the inside of your cheek. Transfer the cells to a tube of sterile saline. Alternatively, swish 5 ml of sterile saline around your mouth for 1-2 minutes, and then transfer it to a clean cup.

1. Take 1 ml of the saline containing cheek cells and transfer it to a microfuge tube. It should look cloudy. Centrifuge the cells for 10 minutes.
2. You should see a white, match-head sized pellet at the bottom of the tube. If you don't see a pellet, add more saline (containing cells) or scrape again and re-centrifuge.
3. Pour off the saline and re-suspend the cells in 50 μ l saline (using the vortex machine).
4. Add 20 μ l of cells to a tube containing 200 μ l Instagene matrix.
5. Incubate the tube containing Instagene and cells at 56°C for 10 minutes (water bath).
6. Vortex to mix, then incubate at 100°C (heat block) for 5 minutes.
7. Cool slightly, then centrifuge for 5 minutes to pellet the Instagene matrix.

Setting Up the PCR Reaction

1. Use the 0.2 ml PCR tubes with the domed caps. Be careful, they are very thin walled (for better heat penetration), and quite fragile!
2. Using a P-20 micropipette and filter tips, transfer 20 μ l of JumpStart Red Taq Master mix to each of 6 tubes- one per team member + one negative control + either plant or bacterial DNA.

3. Add 20 μ l sterile distilled water to the control tube. Mark this tube “B” and close the tube. Add 20 μ l of your DNA sample to your tube. Be very careful to take liquid from the top of the tube. Do not transfer ANY of the Instagene matrix to the PCR tube. Even a tiny bit will inhibit the reaction and you won’t get a product!
4. In addition to your cheek cell DNA samples and the negative control, I will give you another DNA sample- either plant DNA or bacterial DNA. In the final (6th) tube, pipette 20 μ l of the DNA sample. Mark this tube “P” for plant or “B” for bacteria.
5. Label your tube with your initials and put it into the thermocycler when instructed.
6. When all of the tubes are in the machine:

95°C	5 minutes	denature	}	1 cycle
95°C	30 seconds	denature		30 cycles
52°C	30 seconds	anneal		
72°C	3 minutes	extend		
72°C	5 minutes	extend		1 cycle

What is your prediction for the result of this experiment: which DNA will amplify with the primers we included?

- Human cheek cell DNA?
- Plant DNA?
- Bacterial DNA?

Week 2: Gel Electrophoresis

We’ll continue with our question about similarities and differences between amylase enzymes and alpha amylase genes from different organisms. Last week you set up PCR reactions using primers designed to the human salivary alpha amylase, and attempted to amplify (or replicate) a small part of the alpha amylase DNA from human DNA (you), plant DNA and bacterial DNA (supplied).

1. Perform gel electrophoresis to visualize the PCR products (DNA)
2. Think about controls for this experiment, and what a “negative” result really means
3. Look at the size and structure of alpha amylase genes from different groups of organisms.

Setting-up Gel electrophoresis

Gel electrophoresis is a technique for separating molecules such as DNA, RNA by size. Using specific stains, we can then visualize the DNA (or RNA) and estimate the size of the DNA fragments and the concentration. The gel is usually made from agarose, and the concentration of the agarose determines the pore size- more agarose, smaller pore size. The gel also contains a buffer that allows an electric charge to be applied whilst minimizing pH changes in the DNA or RNA. DNA and RNA are negatively charged (phosphate backbone), and will travel towards the anode (positive electrode). Smaller molecules will travel through the gel more rapidly than larger molecules, so the DNA will form a gradient of bands through the gel. These bands can be visualized using nucleic acid specific stains.

Prepare a 1% agarose gel in 1 x TAE and run out the PCR samples. Stain with gel Red and visualize on a UV light box.

Interpreting your gel

If you were successful in amplifying the human alpha amylase gene, you should see a band corresponding to about 900 bp. You may also see a smaller band at around 500 bp. You may also see a bright band running ahead of the 100 bp marker- this is likely a primer dimer- the primers have amplified off each other giving a 40-50 bp band.

You should print off a photograph of your gel and stick it in your notebook. Label the wells, and note the contents of each well. Then record the size of the band(s) you see in each lane

Summarize your results. Which samples show a PCR product of 900 bp? Which samples do not show a product? If there was no product, why not? (Think about what is needed for PCR- is anything missing? Did your DNA sample contain high quality DNA?). What other factors do we need to take into account?

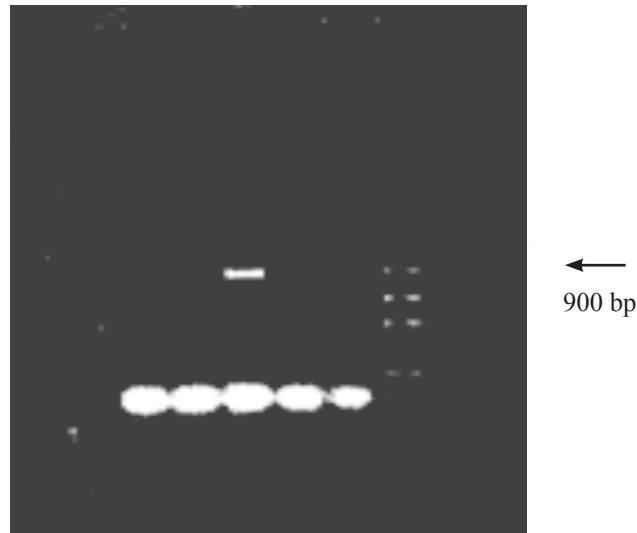


Figure 4. PCR amplification of a 900 bp amplicon from genomic DNA from (l to r) barley, *Aspergillus*, human, and *Bacillus* sp. with primers designed to the human salivary alpha amylase sequence.

Week 3: BLAST

This week we will conclude project 2 by asking some questions and looking for the answers in the National Center for Biotechnology Information (NCBI).

How specific are our PCR primers?

- Go to <http://ncbi.nlm.nih.gov>.
- On the welcome page, right hand side, click BLAST
- BLAST stands for Basic Local Alignment Search Tool. It is a very powerful set of programs that allow us to search for sequence strings in the NCBI database.
- We will use Primer BLAST to look for sequences that are the same or similar to the primers we used for our PCR reactions.
- *Remember, the primers will anneal or stick to complementary DNA sequences when we heat up and cool down our DNA during PCR. If the DNA and primer sequences match exactly, the primers will anneal to the DNA and PCR can occur. If there is a small mismatch, then some PCR can occur. If there is very little similarity (a lot of mismatch) between primers and DNA, it is unlikely that the primers will be able to anneal, and PCR won't happen.*
- Scroll down to Specialized BLAST and click Primer BLAST
- In the PCR template box, type (or copy/paste) [NM_001008221.1](#)
- In the primer parameters box:
 - Forward Primer is 5' - TGACATTGGTGTTCAGGGT - 3'
 - Reverse Primer is 5' - AATTCTGTCACCCGCCATT - 3'
- In Primer Pair Specificity Checking Parameters
 - Database Select "Genome (chromosomes from all organisms)" from the drop-down menu
 - Organism Homo sapiens
 - Click "Get Primers"
- You will have to wait a short time whilst the search is conducted.
- When the search results are returned, make a note of the matches in your lab notebook. You can save the pages for printing later, but you must remember to stick the relevant parts in your lab notebook.

Now repeat his exercise, same primers, but change the organism to:

- 1) Viridiplantae (just type Virid. The rest will be filled in for you!) It's Latin for green plants.
- 2) Bacteria
- 3) Fungi.

Questions:

1. How many matches did each search return?
2. What size product is predicted?
3. What is the identity of the match? Alpha amylase or something else?
4. How close is the match? (primer BLAST shows you differences- a line of dots means the sequences are identical).
5. Can you identify the origin of the bands on your gel?
6. Would you expect to amplify alpha amylase from plants or bacteria using the primers above? Why or why not?

How similar are the alpha amylase genes from humans, plants fungi and bacteria at the mRNA level?

Next, we will look at the similarity of the alpha amylase cDNAs from humans, barley *Aspergillus oryzae* and *Bacillus cereus*.

- To do this we'll use an application called DNA Subway <http://dnasubway.iplantcollaborative.org/>. You need to create a free account.
- In DNA subway, go to the Blue line- this allows you to compare DNA and protein sequences.
- In Select Project Type, click DNA.
- In Sequence type: Go to NCBI and find: **NM_001008221.1** (human alpha amylase)
- Click FASTA at the top of the sequence file, and copy and paste the FASTA file into the box in DNA sub way.
- Click return and repeat the above steps for the following sequences:
 XM_001823889.2 (*Aspergillus* alpha amylase)
 M17126.1 (Barley alpha amylase)
 EU735542.1 (*Bacillus cereus* alpha amylase)
- Give your project a title and click continue. On the DNA Subway page, click MUSCLE in the analyze sequences section.
- The pop-up window will show you the sequences lined up. If you click on the ACGT tab at top left, you will be able to see the nucleotides. Scroll along and see the differences between the sequences. How similar or different are they?
- If you click in the "show differences" box (top right), you will see the numerical percent similarity for the sequences you compared.
- Record the % similarity for each pair of sequences.
- Now go back to NCBI and find the human pancreatic amylase. NM_000699.2 Add that sequence to the comparison. What do you notice this time?
- How does this result relate to the PCR primer BLAST?
- On the Genbank page for either the human pancreatic or salivary amylase, scroll down to the "Related Information" list on the right. Click on **HomoloGene**.
- Here you will be able to see other alpha amylase sequences that are similar (or homologous) to the human alpha amylase. You will need to know the Latin names of the organisms. Add 2 or 3 sequences to your comparison in DNA Subway. Note how similar or different these species are at the DNA level.

How similar are the alpha amylase proteins (at the amino acid level)?

DNA subway also allows you to compare protein sequences.

- Create a new project in the Blue Line.
- This time, click protein, and add the following protein sequences (in FASTA format).
- Name the project and follow the directions for DNA.

Human salivary amylase	NP_001008222.1
Human pancreatic amylase	NP_000690.1
Barley alpha amylase	AAA32925.1
<i>Aspergillus oryzae</i> alpha amylase	XP_001823941.1
<i>Bacillus cereus</i> alpha amylase	ACF36878.1

Homologous Sequences from Other Animals

Add some of the protein sequences from your previous comparison- chimp (*Pan troglodytes*), cow (*Bos taurus*),... any others you want to add.

Questions:

1. How similar (or different) are these species at the protein (amino acid) level?
2. If these enzymes all do the same thing, why are the protein and DNA sequences so different?

Notes for the Instructor

These are stripped-down student outlines. I usually add an introduction to glucose, starch, cellulose, glycogen and amylases to module 1, and an introduction to PCR to module 2. This material may be covered outside of the laboratory.

Reagents

- *Soluble starch* (Sigma-Aldrich S9765). To make 1 L of a 0.1 % solution, mix 1 g of starch with 50 ml water to form a slurry. Add to 850 ml distilled water and heat to boiling, stirring continuously. Once the solution clears, switch off the heat and continue to stir until cool. Bring up to 1 liter with water.
- IKI (iodine in potassium iodide): 0.5 % iodine in 5 % potassium iodide.
- *DNSA reagent*: to make 500 ml 1% dinitrosalicylic acid, dissolve 300 g sodium potassium tartrate in 150 ml 2M NaOH. Warm to dissolve fully, make up to 200 ml with water. Mix 500 ml 1% dinitrosalicylic acid with 200 ml sodium potassium tartrate in sodium hydroxide (solution 2). Make to 1 liter with water (about 300 ml). Store at room temp in a dark glass or foil-covered bottle.
- Porcine pancreatic amylase (Sigma-Aldrich A3176). 0.5 g in 100 ml water. Keep on ice.
- Alpha amylase from *Aspergillus oryzae* (Sigma-Aldrich 10065). 100 mg in 100 ml water.
- Alpha amylase from *Bacillus* sp. (MP Bio 100447). 50 mg in 100 ml water.

- Buffers for pH 5.8, 7.0 and 8.0 are 0.1 M sodium phosphate. Buffers for pH 4.0 and 5.0 are 0.1 M sodium acetate.

PCR Reagents

- Instagene matrix (Biorad) – Ready to use. Be sure to resuspend the beads before pipetting.
- For PCR I used JumpStart RedTaq (Sigma-Aldrich). This product is designed for high-throughput PCR screens and is stable at room temperature. Whilst it is a little more expensive than other Taq polymerases, it is almost student-proof- they only need to add template DNA and primers.
- Primers were from idtdna.com.
Fwd: 5' - TGACATTGGTGTGCAGGGT - 3'
Rev: 5' - AATTCTGTCACCCGCCATT - 3'

About the Authors

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