PROTOCOL

INVESTIGATING PHOSPHORYLASE ACTIVITY

Extract an enzyme from biological material and catalyse a synthesis reaction

sserc

This investigation involves the synthesis of starch from glucose-1-phosphate by starch phosphorylase. In Part 1, the enzyme, starch phosphorylase is extracted from potatoes. In Part 2, the enzyme is combined with the substrate and sampled for product formation along a 6 minute time-course. The investigation also includes opportunity for progression in Part 3 and 4. This builds in aspects of the CfE Advanced Higher Biology curriculum, whereby learners should become familiar with different instruments for measurement and the production of a standard curve. Part 3 requires learners to produce a dilution series of starch to generate a standard curve. In Part 4, the standard curve is used to determine the concentration of starch synthesised by phosphorylase in the assay run in Part 2.
This investigation offers the opportunity to build laboratory technique and can be simply adapted for various independent variables.

Contents

AH Advanced Higher Course Specification

SQA

Advanced Higher Biology

BACKGROUND

In this investigation, starch phosphorylase is extracted from potato tuber and used to synthesise starch. Phosphorylase (α-1,4-D-glucosyltransferase; EC 2.4.1.1), an important enzyme found in several higher plants, catalyses the reversible transfer of glucosyl units from glucose-1-phosphate to the non-reducing end of α-1,4-D-glucan chains with the release of phosphate (1), forming starch (Figure 1).

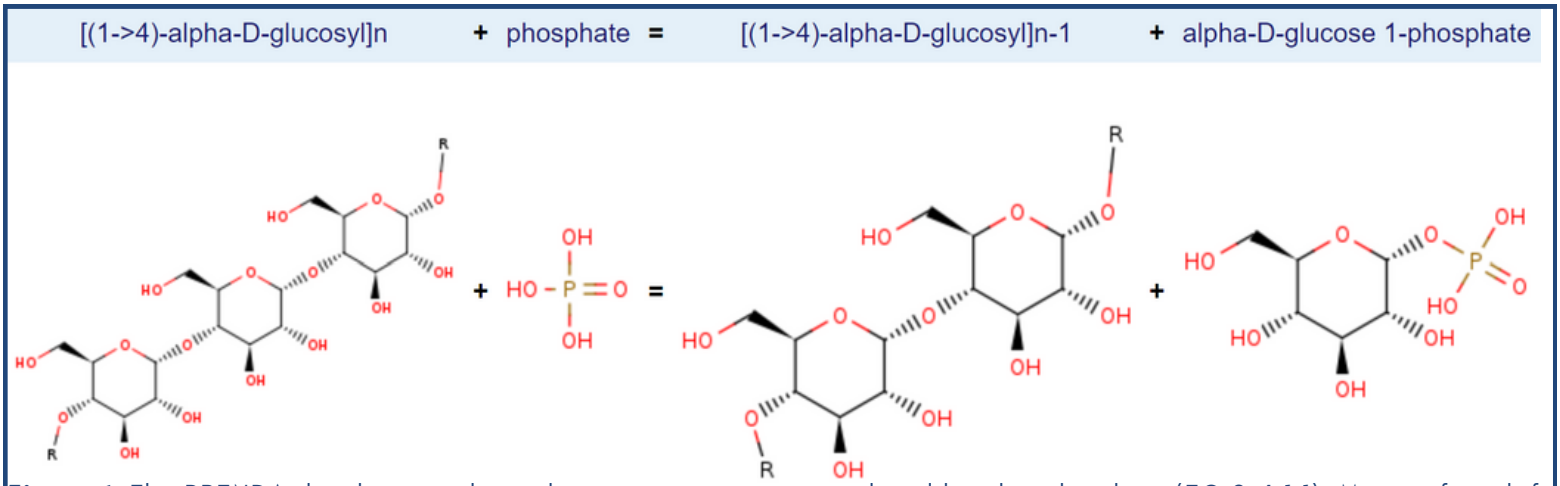


Figure 1. The BRENDA database outlines the enzyme reaction catalysed by phosphorylase (EC 2.4.1.1). Moving from left to right shows the degradation of starch; however, this equilibrium can be shifted in the absence of inorganic phosphate and the presence of glucose-1-phosphate.

The synthesis of starch can be observed if the enzyme is combined with glucose-1-phosphate, in the absence of inorganic phosphate. In this direction, starch is synthesised by the addition of successive glucose molecules to the substrate molecule. This reaction proceeds with an initial lag period. Colorimetry is used to monitor the progress of the reaction, through the addition of iodine which forms a complex with starch.

Starch phosphorylase is used in industry for the production of glucose-1-phosphate and for the development of engineered glucans and starch (2).

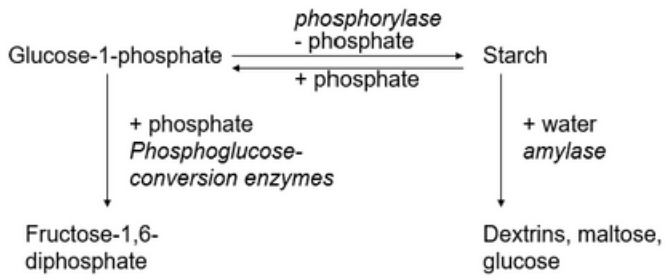


Figure 2. An overview of the role of phosphorylase in metabolism. Image adapted from (1).

References

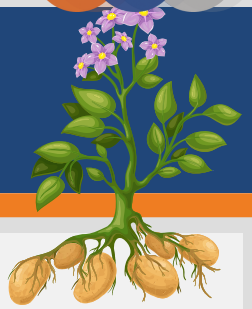
1. Hanes, C.S. (1940), The reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase, The Royal Society Publishing, available from https://royalsocietypublishing.org/doi/pdf/10.1098/rspb.1940.0035 (accessed August 2023).

2. Rathore, R.S, et al. (2009), Starch phosphorylase: role in starch metabolism and biotechnological applications, Critical Review of Biotechnology, 29(3), 214-24. Available from https://pubmed.ncbi.nlm.nih.gov/19708823/ (accessed August 2023).

3. Glycogen phosphorylases, https://www.brenda-enzymes.org/enzyme.php?ecno=2.4.1.1

MATERIALS

The materials listed per are "per pair", with the exception of the blender and centrifuge. Leaners will extract starch phosphorylase from potatoes and then use the extract to synthesis starch from glucose-1-phosphate. The progress of the reaction can be monitored visually or using colorimetry.



AIM

The aim of this experiment is to extract an enzyme from biological material and investigate an enzyme synthesis reaction.

METHODOLOGY AT A GLANCE

This experiment involves the isolation of a synthesis enzyme, called starch phosphorylase, from potato. Potato must be blended and then centrifuged to isolate the enzyme. The enzyme extract is combined with the substrate, glucose-1-phosphate and the synthesis of starch is monitored using iodine solution.

HEALTH AND SAFETY

When starting an experiment with a class, you must ensure you are comfortable and familiar with the risk assessment. What are the hazards, the level of risk and control measures put in place? Is the current risk assessment appropriate for your class? Do you need to make adjustments. This page of the SSERC website provides a Risk Assessment template and information about Dynamic Risk Assessments.
Click the download icon to view our model risk assessment for this activity.

PART 1: EXTRACTION OF PHOSPHORYLASE FROM POTATO

Materials:

- potato
- knife, chopping board, vegetable peeler
- blender
- muslin
- beaker
- centrifuge
- centrifuge tubes & rack
- container to store extract
- 3 cm^3 plastic pipettes
- 0.01 M iodine solution
- dimple tile

Method:

1. Peel and cut a medium-sized potato into small chunks. Combine 50 g of chopped potato and enough water (approximately 20 cm^3) in a blender to produce a "slurry".
2. Pass the potato slurry through a single layer of muslin, into a clean beaker.
3. Divide the filtered extract equally between centrifuge tubes. The volume of extract must be approximately equal to ensure the centrifuge rotor is balanced.
4. Transfer the centrifuge tubes to a centrifuge rotor, ensuring tubes are placed opposite each other in the rotor. Tubes opposite each other should be approximately equal in mass. Centrifuge the extract for 5 minutes at about 6000 rpm. This will separate the starch granules (form pellet) from the enzyme-containing supernatant.
5. The supernatant must be tested to check that starch granules have been removed. Using a plastic pipette, take one drop of supernatant and combine with iodine on a dimple tile. If a blue colour appears, centrifuge the contents for a further 5 minutes. Repeat this test until no blue colour is observed.
6. Transfer the supernatant, using a plastic pipette, to a clean container. The enzyme is light-sensitive - cover the bottle with foil or black paper to omit light.

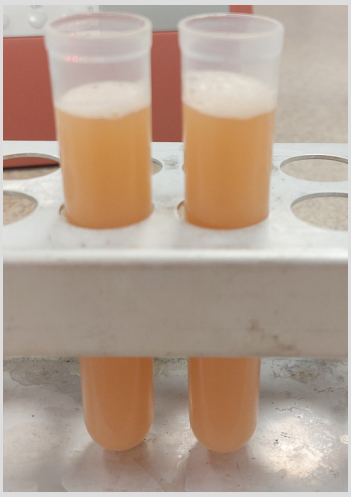
The extract does not keep for long. The experiment should be planned well to allow triplicate experiments to be carried out within about 2 hours. The extract will discolour, affecting absorbance values.

PART 1: INTEGRATED INSTRUCTIONS

1. Blend potato

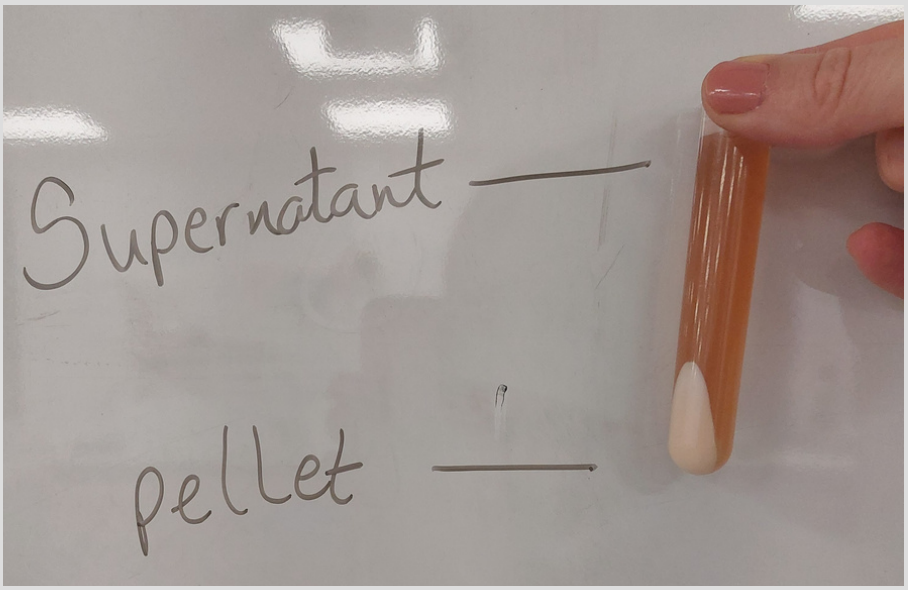
2. Pass through muslin

3. Divide between centrifuge tubes



4. Centrifuge at 6000 rpm for 5 minutes

5. Test the supernatant with iodine to check if starch is present.



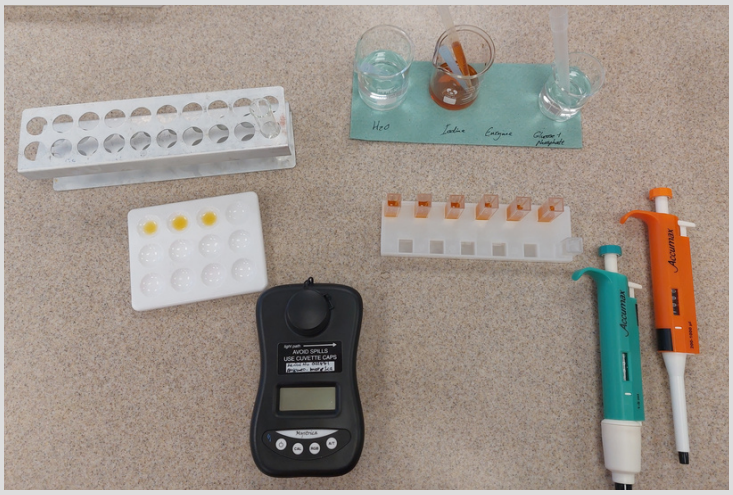
6. Transfer supernatant to a clean container and wrap in foil to omit light. The supernatant contains enzyme and lack starch (which will be present in the pellet).

Discard the pellet.

PART 2: PHOSPHORYLASE ASSAY

Materials:

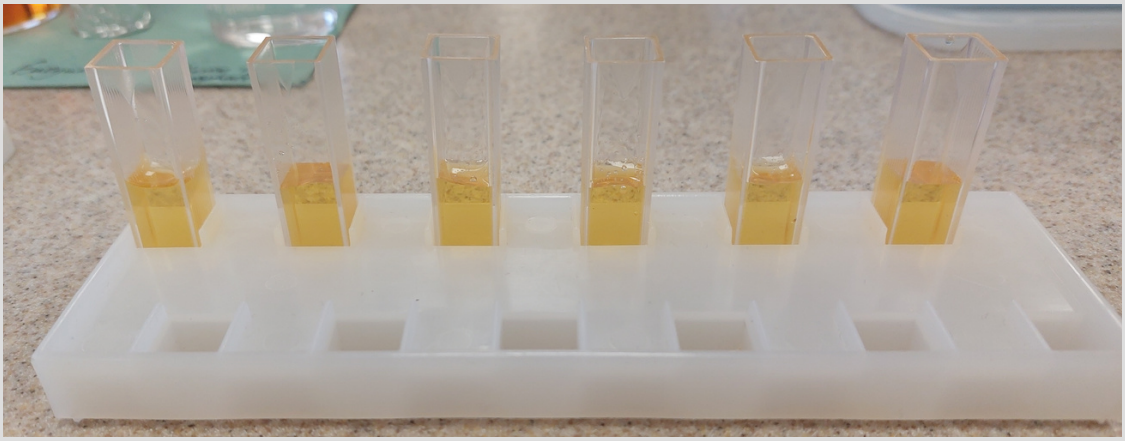
- enzyme extract
- 1% glucose-1-phosphate
- 2x test tubes and rack
- 7x cuvettes and rack
- colorimeter (red diode or approx. 600 nm)
- beaker of distilled water
- 3 cm^3 plastic pipettes
- 0.01 M iodine solution
- dimple tile
- 1 cm^3 automatic pipette & tips
- 5 cm^3 syringes or automatic pipette & tips



1

Set up 7 cuvettes in the cuvette rack.
Cuvette 1: Add 3 cm^3 water. This will be used to zero the colorimeter.
Cuvette 2 - 7: Add 1 cm^3 water, 1 cm^3 iodine.

Use the automatic pipette and fresh tips to complete this step.



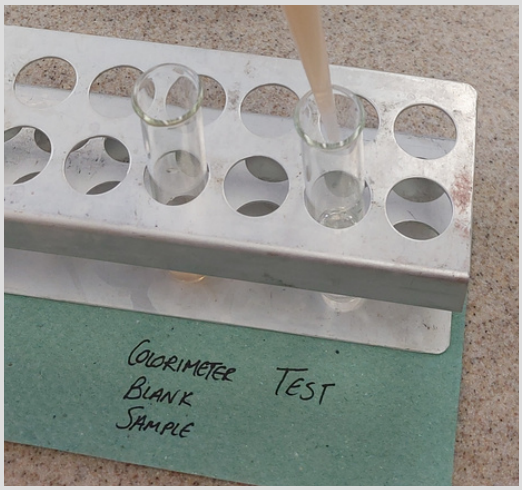
2

Collect 2 test tubes and position in the test tube rack. Test tube 1 will contain the contents for the colorimetric blank, which will account for the colour of the enzyme. Test tube 2 will contain the assay mixture.

Test tube 1: Add 1 cm^3 enzyme + 1 cm^3 water (because this has the same absorbance as glucose-1-phosphate).

Test tube 2: Add 5 cm^3 enzyme + 5 cm^3 1% glucose-1-phosphate. Immediately start the stopwatch.

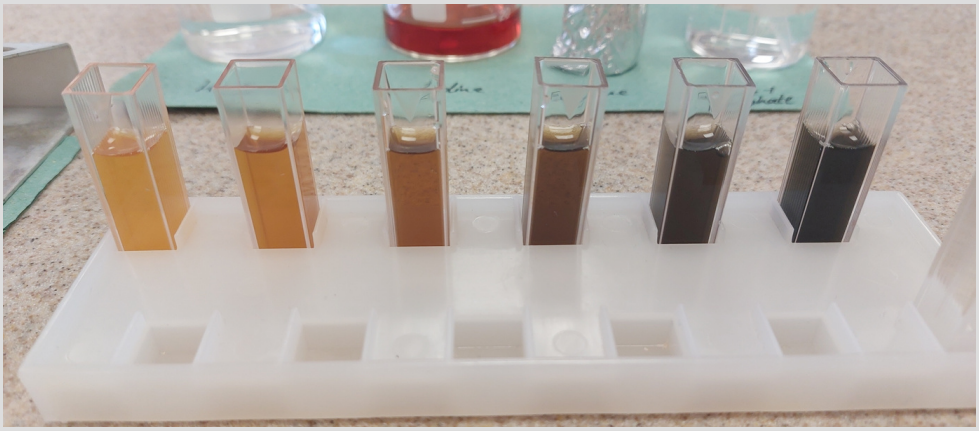
These will be incubated at room temperature, which should be recorded using a thermometer at regularly points during the assay. Alternatively, use a thermostatically-controlled waterbath at 25 °C.

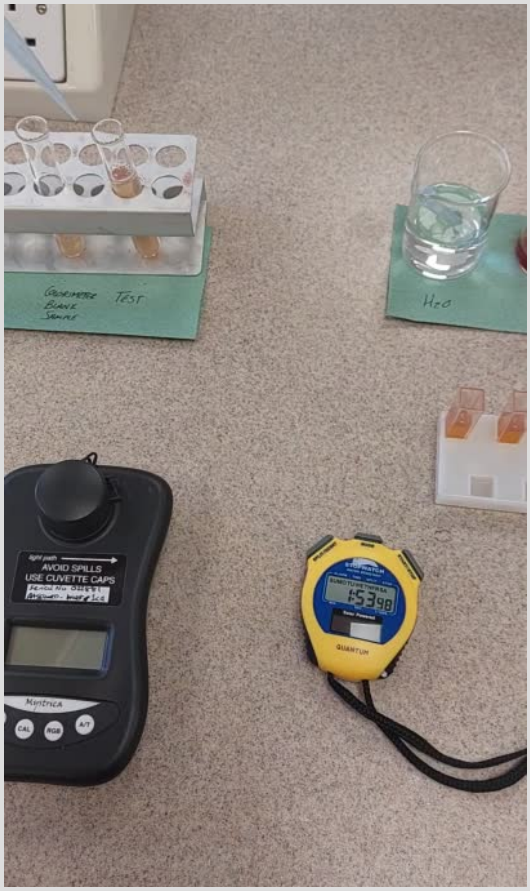


3

After 2 minutes, use the automatic pipette to transfer 1 cm^3 assay mix to cuvette 3. Watch this video to see the process.

4 Repeat this process every 60 s, removing a 1 cm^3 sample from the assay mixture and transferring it to the next cuvette.





5 To measure the aborbance of the samples, turn on the colorimeter and select the red diode. Using a cuvette of water (cuvette 1) to zero the absorbance reading.

To cuvette 2, add 1 cm^3 of the colorimeter blank sample set up in Step 1. This should have 1 cm^3 water and 1 cm^3 enzyme in the test tube. This provides the absorbance reading of enzyme in the presence of iodine and can be recorded as 0 s time point.

6 Measure the absorbance of each sample. The absorbance from cuvette 2 can be substracted from each reading. Record your results in the table below.

We will record results and plot these just now. After this, a standard curve will be produced for starch so the absorbance values can be related to a starch concentration.

RESULTS

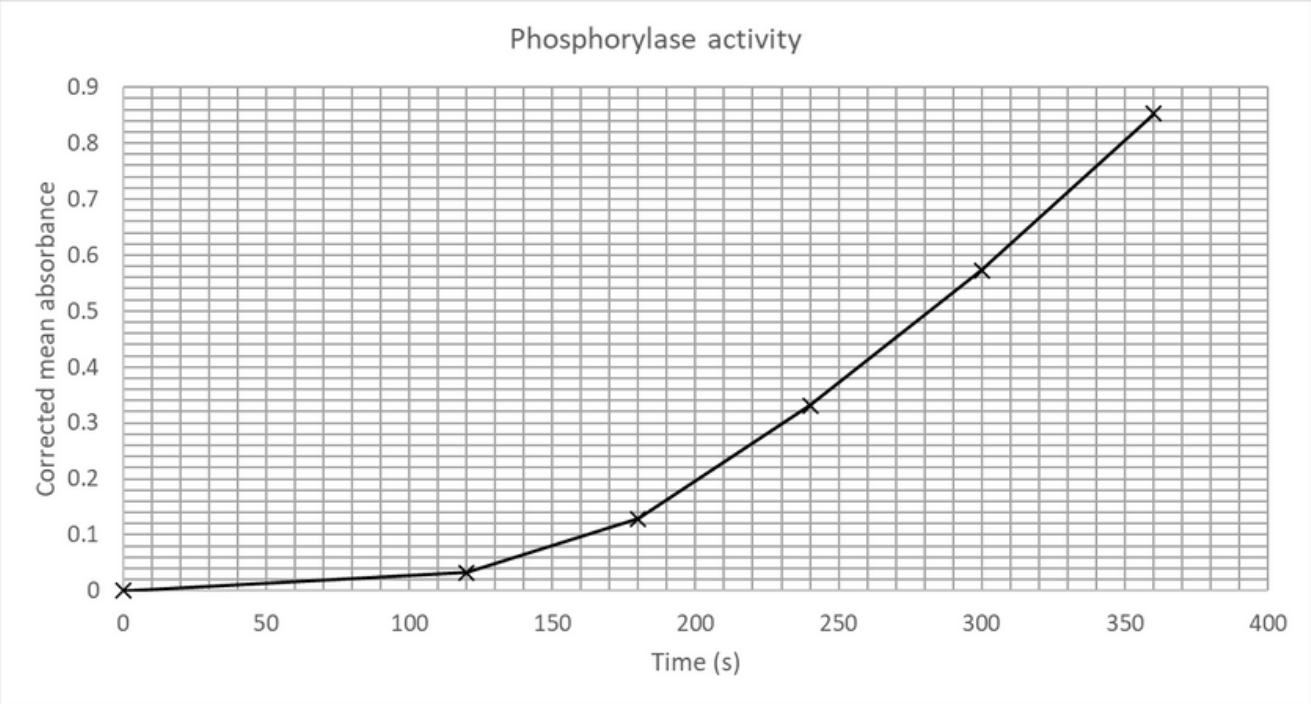
Record the absorbance readings in the table below. The experiment should be carried out in triplicate. Each time, the colorimetric blank should be repeated as the colour of the enzyme will change over time.

Time (s)	Absorbance				
	Trial 1	Trial 2	Trial 3	Mean	Corrected mean
0					
120					
180					
240					
300					
360					

SAMPLE RESULTS

The following results were obtained for this experiment. The corrected mean values were plotted as a line graph.

Time (s)	Absorbance				
	Trial 1	Trial 2	Trial 3	Mean	Corrected mean
0	0.206	0.203	0.207	0.207	0.000
120	0.229	0.232	0.240	0.240	0.033
180	0.327	0.334	0.336	0.336	0.129
240	0.567	0.514	0.533	0.538	0.331
300	0.769	0.769	0.783	0.780	0.573
360	1.082	1.065	1.032	1.060	0.853

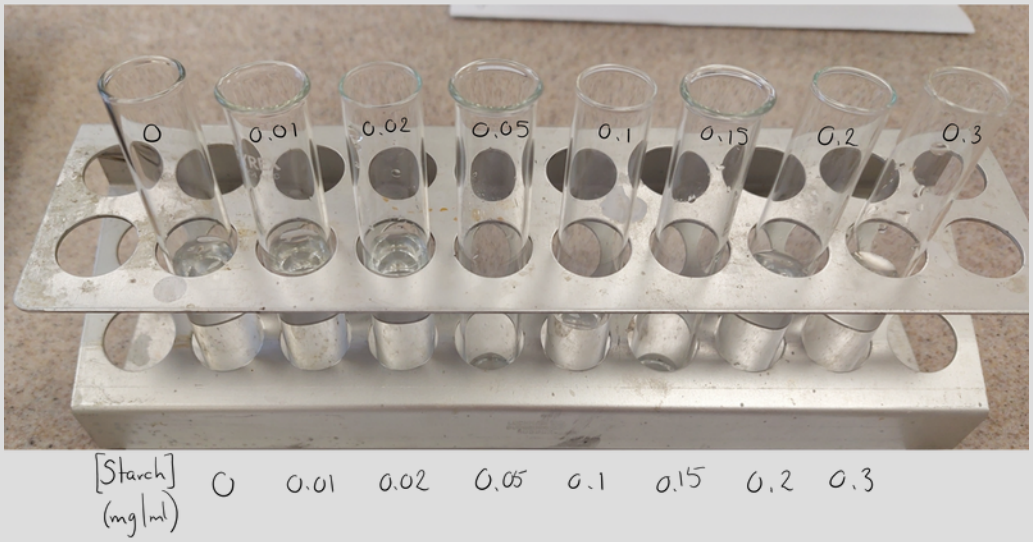


PART 3: STARCH STANDARD CURVE

In the final (optional) part of this investigation, a standard curve for starch will be plotted. This involves calculating a linear dilution series, carefully measuring volumes to produce starch suspensions of known concentration and measuring their absorbance in the presence of iodine. The standard curve will then be used to determine the concentration of starch in each of the reactions from Part 2.

Materials:

- 0.01 M iodine
- colorimeter (red diode)
- 9x cuvettes and rack
- 8x test tubes and rack
- 1 mg/ml starch suspension
- 2 mg/ml starch suspension
- 3 mg/ml starch suspension
- beaker of distilled water
- 1 cm^3 automatic pipette and tips
- 10 cm^3 measuring cylinder
- 3 cm^3 plastic pipettes



Dilutions

Using the various stock starch suspensions provided, use the C1V1 = C2V2 calculation (or your preferred alternative) to produce 10 cm^3 of each working starch suspensions listed in the left column. The table towards the bottom of this page provides possible solutions to this. Store the prepared solutions in labelled test tubes.

Concentration (C1) of solution required (mg/ml)	Volume (V1) of solution required (ml)	Concentration (C2) of stock to be used (mg/ml)	Volume (V2) of stock required (ml)	Volume of water required (ml)
0	10	0	0	10
0.01	10			
0.02	10			
0.05	10			
0.1	10			
0.15	10			
0.2	10	2	1	9
0.3	10	3	1	9

Worked example

To produce 10 cm^3 0.3 mg/ml starch. The logical option would be to start with a 3 mg/ml starch stock.

C1V1 = C2V2

0.3 X 10 = 3 X V2

3 = 3V2

V2 = 1 ML

Add 1 cm^3 3 mg/ml stock solution, adding water up to the 10 cm^3 mark. This will give a 0.3 mg/ml working starch suspension.

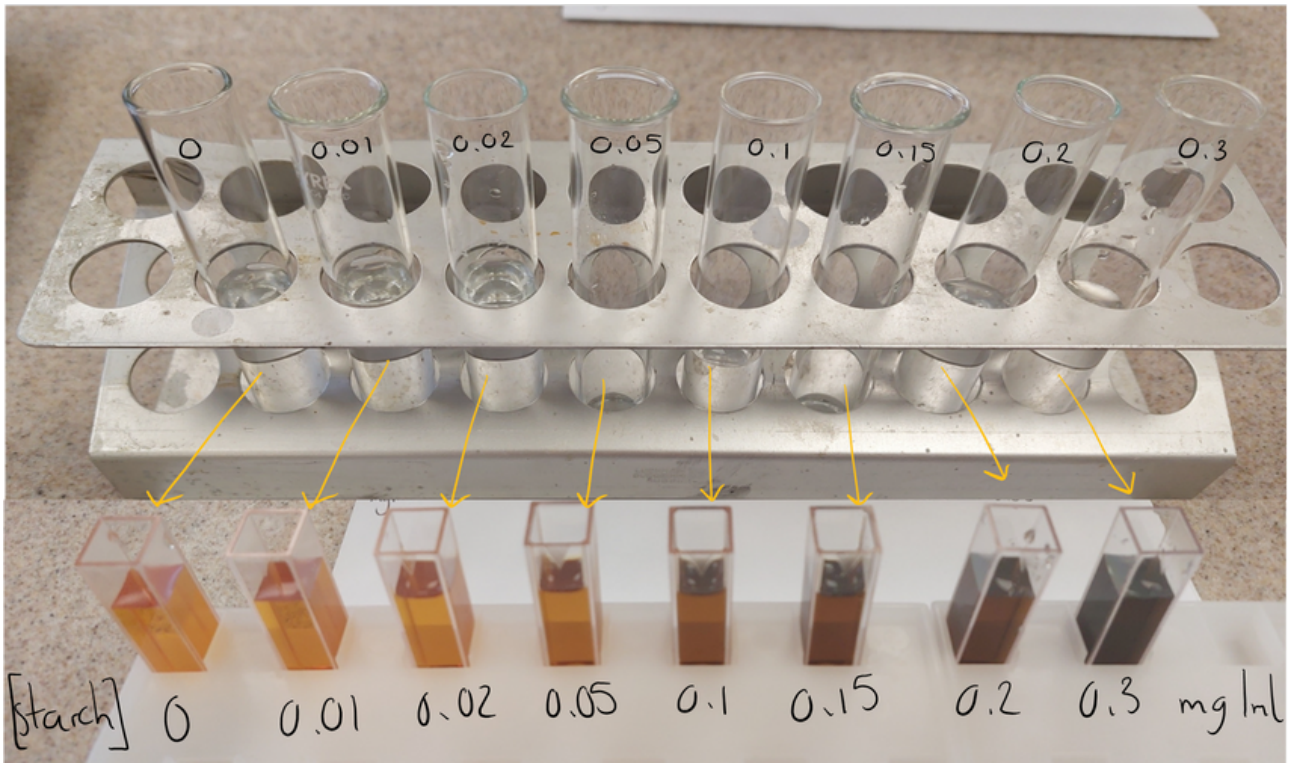
Concentration (C1) of solution required (mg/ml)	Volume (V1) of solution required (ml)	Concentration (C2) of stock to be used (mg/ml)	Volume (V2) of stock required (ml)	Volume of water required (ml)
0	10	0	0	10
0.01	10	0.1	1	9
0.02	10	0.2	1	9
0.05	10	0.1	5	5
0.1	10	1	1	9
0.15	10	0.3	5	5
0.2	10	2	1	9
0.3	10	3	1	9

1

Set up 8 cuvettes in the cuvette rack.
Cuvette 1: Add 3 cm^3 water. This will be used to zero the colorimeter.
Cuvette 2 - 8: Add 1 cm^3 water, 1 cm^3 iodine.

Use the automatic pipette and fresh tips to complete this step.

2 To Cuvette 2 – 7: Add 1 cm^3 respective starch suspension, working from the most dilute to the most concentrated. This means the tip for the automatic pipette does not need to be changed between each.



3 Set the colorimeter to the red diode and to measure absorbance.
Zero the colorimeter using a cuvette of water.
Measure the absorbance of each solution.
Record values in a table similar to the one below.

RESULTS

Starch concentration (mg/ml)	Absorbance
0	
0.001	
0.002	
0.005	
0.01	
0.015	
0.02	
0.03	
0.04	

Plot the results on graph paper or using a graphing program, such as Excel. Add a line of best fit (hand-drawn graph) or trendline (Excel). If using Excel, display the equation of the trendline. This equation will be used to determine the starch concentration of the enzyme assay in Part 2 of this investigation.

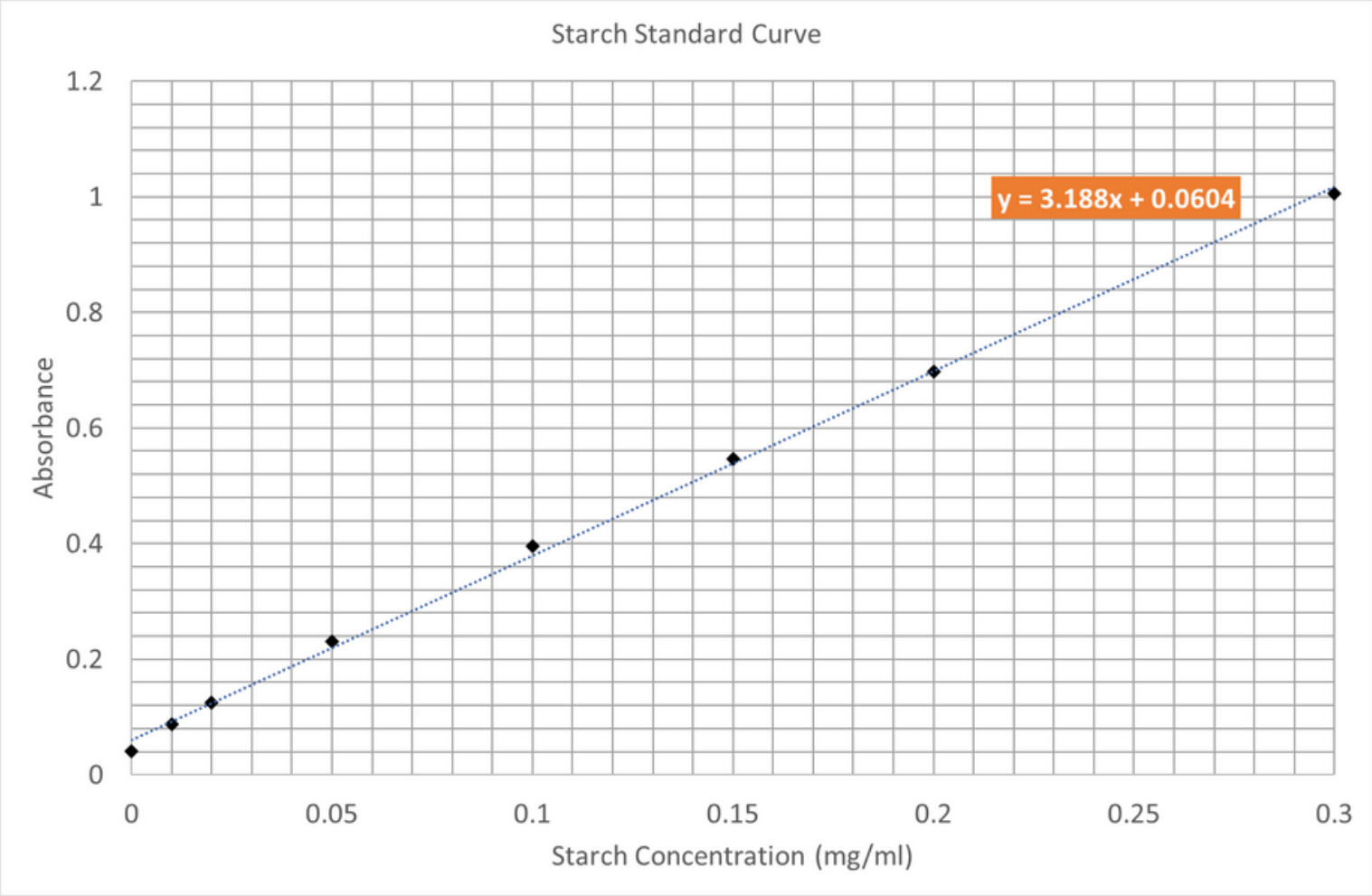
SAMPLE RESULTS

The following results were found at SSERC. These were then plotted using Microsoft Excel, with a trendline fitted to the data.

Starch concentration (mg/ml)	Absorbance
0	0.041
0.01	0.087
0.02	0.125
0.05	0.23
0.1	0.396
0.15	0.547
0.2	0.697
0.3	1.006

The equation of the fitted trendline is:

y = 32.439x + 0.0564



PART 4: DETERMINING THE STARCH CONCENTRATION OF PHOSPHORYLASE REACTIONS (PART 2)

Time (s)	Absorbance				
	Trial 1	Trial 2	Trial 3	Mean	Corrected mean
0	0.206	0.203	0.207	0.207	0.000
120	0.229	0.232	0.240	0.240	0.033
180	0.327	0.334	0.336	0.336	0.129
240	0.567	0.514	0.533	0.538	0.331
300	0.769	0.769	0.783	0.780	0.573
360	1.082	1.065	1.032	1.060	0.853

The data collected in Part 2 can be further processed to estimate the starch concentration in each sample, as a result of phosphorylase action on the substrate glucose-1-phosphate.

This can be done using the equation of the trendline generated in Part 3.

Worked example

Using the equation, y = 3.188x + 0.0604, we can estimate the starch concentration at the final time point in the assay (360 s).

$$y = 3.188\,x + 0.0604$$

$$0.853 = 3.188x + 0.0604$$

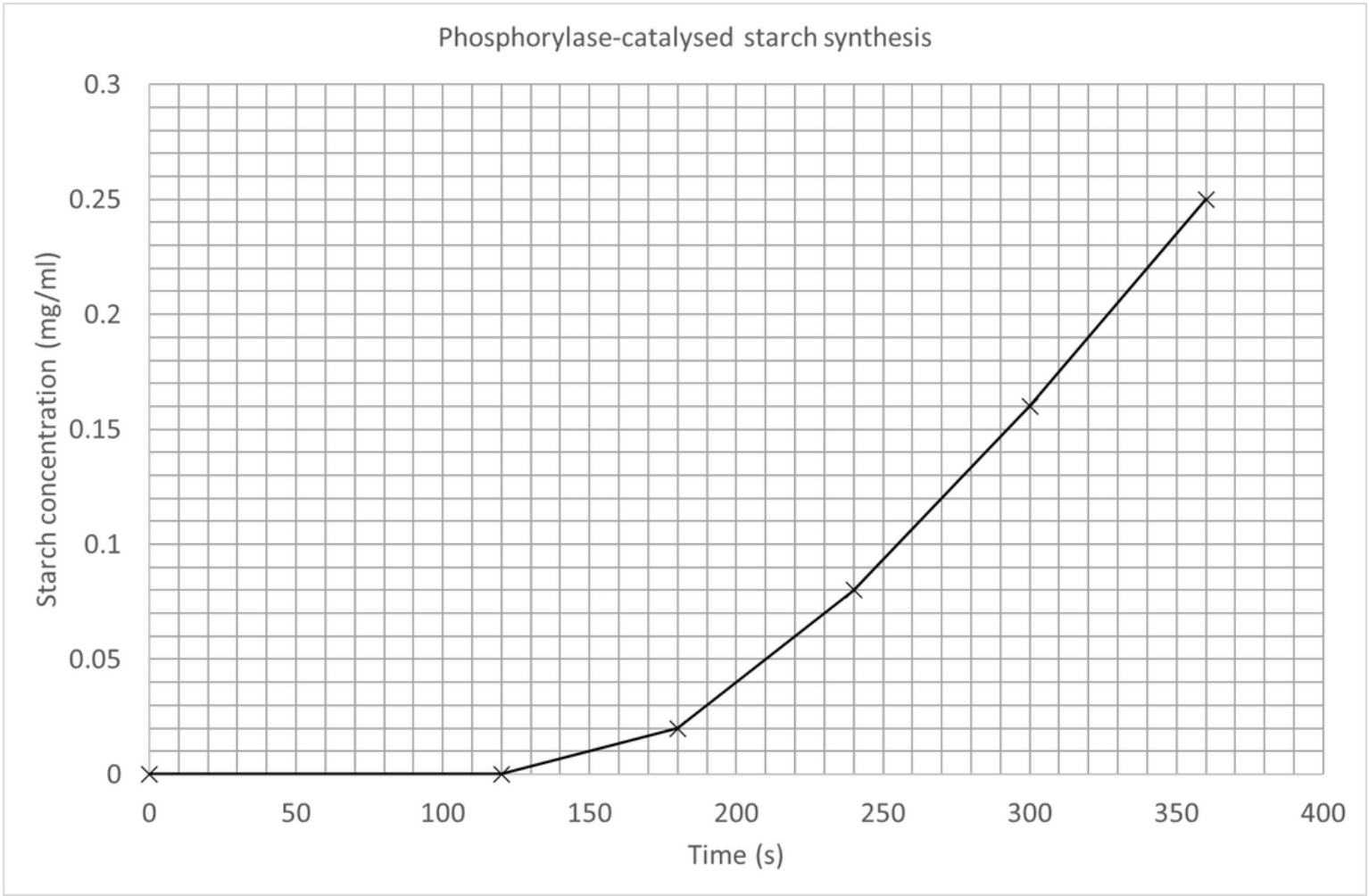
$$0.7926 = 3.188x$$

$$x = 0.25 \text{ mg/ml starch.}$$

At the end of the phosphorylase reaction, the enzyme had synthesised 0.25 mg/ml starch from the glucose-1-phosphate substrate.

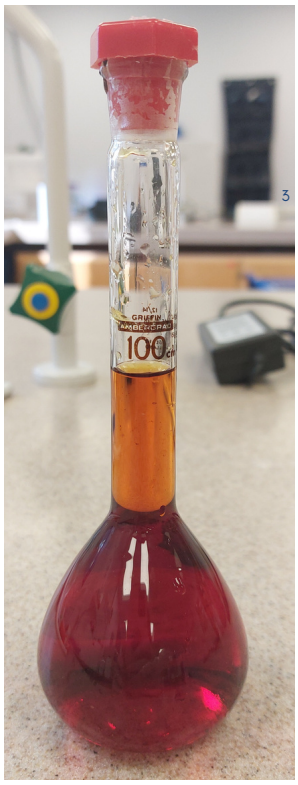
This calculation can be repeated to reveal the synthesis of starch over the course of the reaction and plotted.

Time (s)	Corrected mean absorbance	Starch concentration (mg/ml)
0	0.000	0
120	0.033	0
180	0.129	0.02
240	0.331	0.08
300	0.573	0.16
360	0.853	0.25



Preparation of 0.1 M iodine solution:

- The SSERC website has a downloadable excel sheet to quickly work out masses required for different volumes. You will require login details to access this.



To make 100 cm^3 0.1 M iodine solution, add 0.840g KI to a volumetric flask with a small volume of water. Mix using a magnetic flea and stirrer. Grind 0.254 g iodine and then add this to the aqueous KI. Add distilled water to the 100 cm^3 mark on the volumetric flask. Store in a reagent bottle suitable for light-sensitive chemicals.

Select substance →	Iodine	Mass of I_2 needed (g) =	0.254
RMM of substance	253.81	Mass of KI (g)	0.840
Molarity required	0.01	Enter data in boxes here ←	
OR Percent solution required			
Volume required (cm^3)	100		

If it is easier, you can simply make up the iodine solution in 0.5M potassium iodide

Name	RMM
Iodine	253.81

Screenshot of the SSERC website tool to assist with making iodine solutions.

Preparation of starch stock suspensions:

Start by preparing a 1% starch suspension (**10 mg/ml**). Weigh out 1 g soluble starch and transfer to a small beaker. Add about 0.5 cm^3 boiling water and stir, using a stirring rod, to form a paste. Add small additional volumes of boiling water until all solid material has been incorporated into the paste.

Add a magnetic flea to the beaker and place the beaker on a magnetic stirrer. Add about 30 cm^3 boiling water and allow to mix well. This suspension should go clear. Transfer to a 100 cm^3 volumetric flask and top up to the 100 cm^3 mark. Remove the magnetic stirrer and recheck the volume. The starch stock suspension can now be used to prepare an additional stocks required for learners. The starch suspension produced should be clear. This can be used to prepare the 1 mg/ml, 2 mg/ml and 3 mg/ml starch suspensions for the standard curve.

Glucose-1-phosphate solution

A 1% solution can be made by dissolving 0.5 g glucose-1-phosphate in 50 cm^3 of distilled water. The compound is relatively unstable in solution, hydrolysing to glucose and phosphoric acid at room temperature. This should ideally be prepared immediately before use, but up to 24 hours ahead of time if stored in the fridge at 4°C. There is no significant hazard associated with the powder or solution form.

Centrifuge requirements

Each group will require about 25 cm^3 of potato extract, allowing for triplicate results and pipetting inaccuracies. A centrifuge of sufficient size will be required to manage this. A speed of just under 3000 revolutions per minute (rpm) is sufficient to separate the starch grains from the supernatant.

Potatoes

Medium-sized potatoes work well. If too large, often there is a poor yield of enzyme. If too small, e.g. new potatoes, the starch grains are small and difficult to separate from the enzyme preparation by centrifugation. Typically, we have used medium-sized baking potatoes.