Proof of Concept proposal-

Enzymatic degradation of Cellulose Acetate

PROFESSOR IN-CHARGE

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CONCEPT-

Lytic polysaccharide monooxygenase (LPMO9B) degrades the polymeric unit into oligomeric units. It also acts as a cellulase when cellulose is freely available (1). Glucomannan deacetylase (AXE2C) catalyzes the de-acetylation reaction of the cellulose acetate polymer and its efficiency is so much that it can deacetylate 60% in few minutes [2].

References

1. https://www.sciencedirect.com/science/article/pii/S1367593116300229
2. https://pdfs.semanticscholar.org/5210/c9682a141ea5931fb9f8ad9626d3a1a63948.pdf

Experiments Planned:

We will use commercially produced **LPMO9B and Axe2c enzymes from NZYTECH**. We will test the catalytic activity of these readymade enzymes individually and then together in three types of system. We will use pure cellulose acetate, diaper piece, and cigarette butt as substrates for these enzymes respectively. The above experiment serves two purposes. First, it **establishes an idea about the primary activity of these industrially purified enzymes to prove our concept**. Secondly, it will allow us to optimize the conditions like **pH,temperature, substrate concentration etc** under which these reactions will occur and to accelerate the rate of degradation of cellulose acetate.

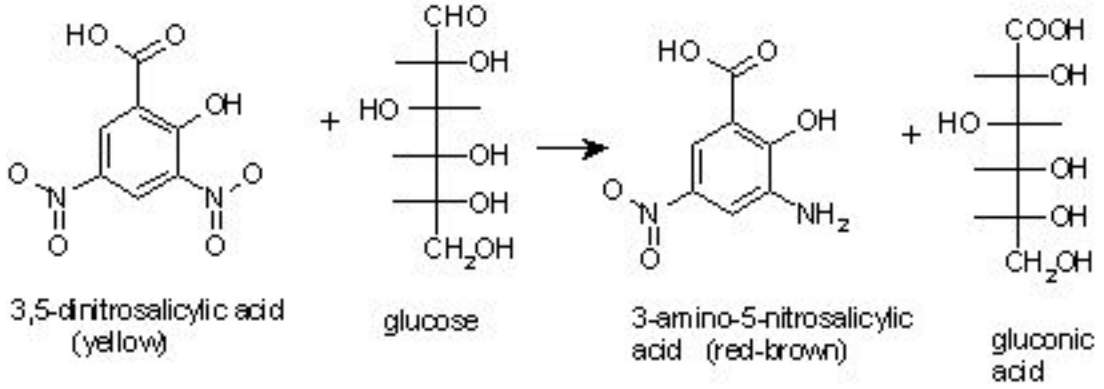
S No.	Enzyme	Substrate
1.	LPMO9B	Cellulose Acetate
		Diaper Piece
		Cigarette Butt
2.	Axe2c	Cellulose Acetate
		Diaper Piece
		Cigarette Butt
3.	LPMO9B and Axe2c	Cellulose Acetate
		Diaper Piece
		Cigarette Butt

During the combined action of LPMO9B and AXE2C on cellulose acetate,it will cause deacetylation of cellulose acetate,converting it cellulose and free acetic acid. LPMO9B will act as as a cellulase which ultimately degrades the cellulose into reducing sugars. To quantify how much of cellulose is degraded into reducing sugar , DNSA assay can be used.

DNSA assay protocol is discussed below

Principle:

3,5-Dinitrosalicylic acid (DNSA, IUPAC name 2-hydroxy-3,5-dinitrobenzoic acid) is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm (In case of glucose). This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions.[3]



Chemicals Required:

1. DNSA(2-hydroxy-3,5-dinitrobenzoic acid)
2. Sodium potassium tartrate ($KNaC_4H_4O_6.4H2O$)
3. 2 N sodium hydroxide (2N NaOH)
4. Dinitro salicylic acid (DNSA)
5. Distilled water
6. Sample

Equipments Required:

1. Test tubes
2. Pipette
3. Water bath
4. Spectrophotometer
5. Cuvette
6. Vortex mixer
7. Measuring cylinder
8. Beaker (50ml)

Procedure

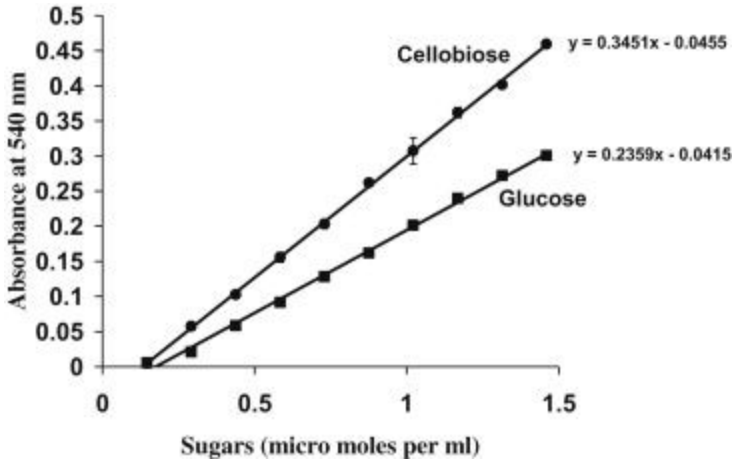
1. Prepare 20 mL of 2N NaOH.
2. Weigh 1 g DNSA and dissolve in 20 mL NaOH with the help of a magnetic stirrer
3. Weigh 30 g of sodium potassium tartrate and dissolve in 50 mL dH2O.
4. Slowly pour sodium potassium tartrate solution in the DNSA and NaOH solution and made the volume up to 100 mL (Note: Wait for the two to mix properly).
5. Decant the contents in a brown bottle. Filter if necessary.

Protocol

1. Take eight tubes and label them as Blank and 1 to 7.
2. Make dilutions of glucose standards
3. Add 3 ml of DNSA reagent to all the eight test tubes. Mix well.
4. Keep in boiling water bath for 15 minutes.
5. After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer at 540 nm.
6. First, take the absorbance (OD) of Blank and make it zero.
7. Take the OD of all the tubes (No. 1-7). Wash the cuvettes each time after taking OD.

Expected Results

We can find out from the above assay that how much cellulose acetate is degrading and in different time point we can check the activity of the enzyme through this process. The concentration of reducing sugars can be estimated by comparing this in the following graph:



The above protocols targets the formation of reducing sugar by cellulose acetate degradation There is another method to check the rate of degradation of cellulose acetate by **measuring its acetyl content using Near Infrared Spectroscopy .**

Determination of Acetyl Content of Cellulose Acetate by Near Infrared Spectroscopy:

Principle:

The degradation of cellulose acetate produces cellulose and acetic acid.It means the acetyl content of cellulose acetate decreases in its degradation .By measuring the acetyl content of cellulose acetate at different intervals of time we can check its rate of degradation.
We can determine the acetyl content of cellulose acetate by Infrared spectroscopy. The absorption due to the residual hydroxyl groups can be measured in the near infrared region.The sample has pyrrole as a suitable solvent,which permits the determination of the acetyl content of cellulose acetate having an acetyl content from 35 to 44.8% .This method can be calibrated accurately.For comparison the concentration of acetyl part we can use the below table:

Materials and equipment:

1.Spectrophotometer
2.Quartz cell
3.Micro pipettes and pipettes sucker for solution transfer,
4. Eppendorfs,tarson tube,
5. measuring cylinder
6. Microtips
7. tissue roll(for wiping of cuvette)
8. Incubator (for incubating at sufficient temperature)
9. Ground glass-stoppered bottle(60 ml).

Chemicals Required:

Cellulose acetate
Pyrrole
Carbon tetrachloride(CCl_4)

Working buffer:

Solution of pyrrole containing 5% carbon tetrachloride

Procedure:

1. Dry samples of cellulose ester (here cellulose acetate) for 2 hours at 105° C
2. Weigh 1.25 grams of sample into a ground glass-stoppered bottle of 60 ml. capacity and redry for 30 minutes at 105°C.
3. Add 25 ml of pyrrole from a calibrated pipet and tumble or shake until the sample is dissolved.
4. Place the sample in a constant temperature bath until the samples are at 25°C.
5. Place the sample solution in a 5-cm. stoppered quartz cell ,and place pyrrole containing 5%carbon tetrachloride in a matched cell.
6. Scan the spectrum from 1300 to 1700 μ with the pyrrole carbon tetrachloride solution in the reference compartment.Measure the absorbance at 1445 μ and determine percent acetyl by referring to the calibration curve.

Observation and Calibration curve

The resulting curves gives maximum absorbance at 1445 μ . When the maximum absorbance from these curves is plotted against percent acetyl , a reasonably straight line is obtained which indicates that the absorbance is inversely proportional to the percent acetyl .Such a curve is used as a working calibration curve for the experiments.

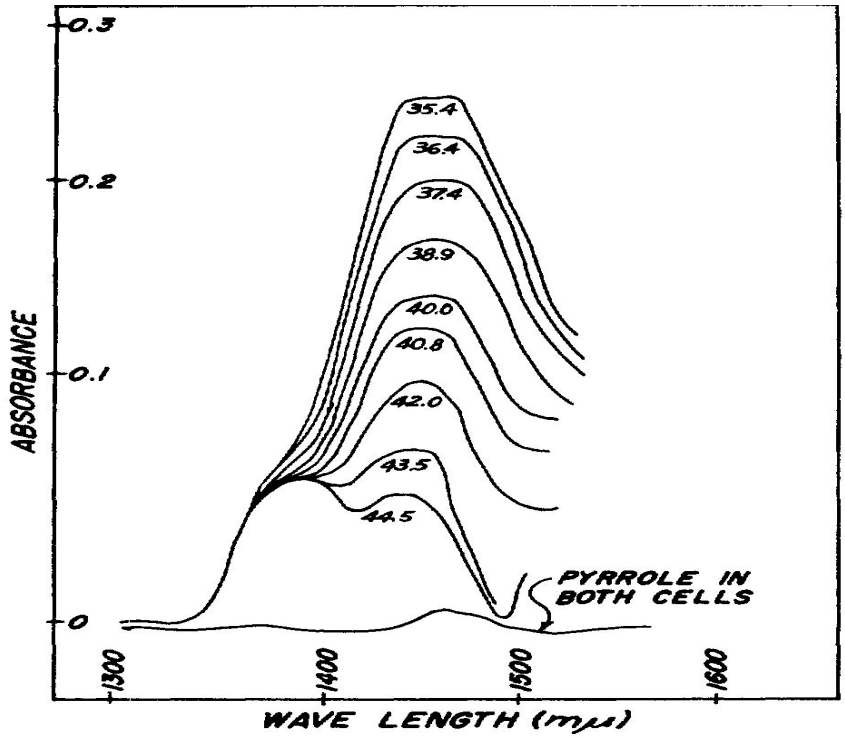


Figure 3. Spectra of 5% cellulose acetate in pyrrole showing effect of acetyl content on absorbance
Reference, 5% carbon tetrachloride in pyrrole

.Source:J.A.MITCHELL,C.D.BOCKMAN,Jr.,andA.V.LEE Tennessee Eastman Co.,aDivision of Eastman Kodak Co.,Kingsport,Tenn.

Precautions:

- Use of the 5-cm. Cell should be limited to the area between 700 and 1500 μ , because of the strong absorbance of pyrrole beyond this region causes the slit to open too wide for best results.
- After preparing the solution of pyrrole containing 5% carbon tetrachloride cover the solution immediately to prevent contact with air.
- Cellulose easter sample should be dried properly.The method could be affected by changes in the primary to secondary hydroxyl ratio.So ensure only pyrrole is added in sample,alcohol impurities should not be present.
- The sample should be standardized at 25 degrees to ensure lack reproducibility.

(Reference :https://pubs.acs.org/doi/pdf/10.1021/ac50162a023)
[1]https://www.sciencedirect.com/science/article/pii/S1367593116300229
[2]https://pdfs.semanticscholar.org/5210/c9682a141ea5931fb9f8ad9626d3a1a63948.pdf
[3]https://orbitbiotech.com/estimation-of-reducing-sugars-by-dnsa-method

COST ESTIMATION:

Tables :

S. No.	Enzyme	Company	Reference No.	Singular cost	Quantity	Total Cost
1.	Lytic cellulose monooxygenase 9B	nzytech	CZ09591	€129.00 (10,179.33 INR)	1	€129.00 (10,179.33 INR)
2.	Acetyl xylan esterase 2C	nzytech	CZ0210	€129.00 (10,179.33 INR)	1	€129.00 (10,179.33 INR)
	Total	**-**	**-**	**-**	**-**	**20358.66 INR**

S. No.	Material and equipment	Company	Reference No.	Singular cost	Quantity	Total Cost
1.	Pyrrole	SIGMA-ALDRICH	131709-500 ML	11,854 INR Per 500ml	500ml	11,854 INR
2.	Cellulose acetate	CDH PL-36	027654	1413 INR Per 500gm	1kg	2,826 INR
3.	Carbon tetrachloride	Thermo fisher Scientific	REVOC111	7,652 INR Per ml	1ml	7,652 INR
4.	Spectro photometer	Institute funded	-	-	1	-
5.	Incubator	Institute	-	-	1	-
	Total	-	-	-	-	**22,332 INR**

Total Cost estimate for proving concept = 42690.66 INR