Colloidal Chitin Prep and Assay

Weekly Sorted Experiments:

September

Week 1:

1-09-21: Colloidal Chitin preparation

3-09-21: DNS Assay

Week 2:

7-09-21: Colloidal Chitin Preparation

Week 4:

19-09-21: DNS Assay

21-09-21: DNS Assay

Week 5:

30-9-2021: Protein Estimation with BCA,
DNS Assay

31-09-21: Plotting NAG Standard Curve

October

Week 1:

1-10-21: DNS Assay

Week 2:

3-10-21: Preparation of DNS

4-10-21: DNS Assay

5-10-21: DNS Assay

DNS Assay (Reduced reaction volume)

Protein Estimation using BCA

7-10-21: Plotting NAG Standard Curve

8-10-21: OD vs Temperature Assay

9-10-21: OD vs Substrate Assay (Michaelis Menten)

Preparation of Colloidal Chitin

Experimental Aim: To prepare colloidal chitin using a protocol provided in the literature [1] .
Observation: Colloidal Chitin was obtained as a pasty white substance which was then dried off completely using a roto-evaporator.
Conclusion : The obtained colloidal chitin powder was stored in 4°C refrigerator for further use.

1-09-2021

DNS Assay

Experimental Aim: To check the activity of crude enzyme at different intervals of time.
Method: The crude enzyme was incubated at 37 °C for at intervals of 1, 2,4,6,8,19,30 and 41 hours. Appropriate controls (enzyme control, substrate control and heat denatured enzyme) were chosen to compare the values of OD540 while performing the DNS assay.

Detailed Method: The reaction mixtures are made accordingly in 15 mL falcon tubes.

- Keep tubes for incubation at 37 C for various time intervals.
- Add 250 uL of DNS to 500 uL of reaction mixture and heat in a water bath at 100 C for 10 mins.
- Centrifuge the samples to collect the supernatant
- Measure the OD (Optical Density) at 540 nm (UV spectrophotometer or Nanodrop).
- This procedure is repeated at various time intervals like 1hr, 2hr, 4 hr, 6 hr etc

Observation: The test sample and substrate control did show an increasing OD540 values with time.

3-09-2021

Observation

	Colloidal Chitin (Substrate)	PBS	Crude Enzyme	Milli Q
Substrate Control	-	2.5 mL	0.5 mL	2.5 mL
Enzyme Control	2.5 mL	2.5 mL	-	0.5 mL
Test Sample	2.5 mL	2.5 mL	0.5 mL	-
Denatured Enzyme Control	2.5 mL	2.5 mL	0.5 mL	-

Note:

Enzyme was heat denatured at 100 C for 10 mins in water bath.

Result:

Optical Density (OD)				Time (hrs)
Substrate Control	**Enzyme Control**	**Test Sample**	**Denatured Enzyme Control**	
0.03	0.02	0.00	0.05	0
0.06	0.01	0.05	0.04	1
0.06	0.04	0.08	0.04	2
0.06	0.02	0.11	0.04	4
0.08	0.03	0.18	0.05	6
0.12	0.02	0.20	0.04	8

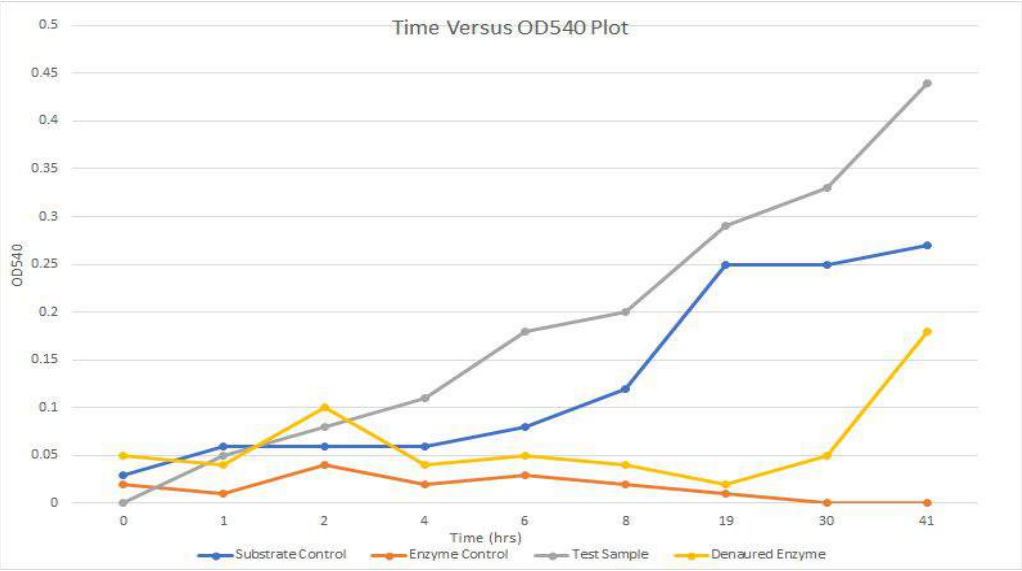
Results (Continued.)

Optical Density (OD)				Time (hrs)
Substrate Control	**Enzyme Control**	**Test Sample**	**Denatured Enzyme Control**	
0.25	0.01	0.29	0.02	19
0.25	0.00	0.33	0.05	30
0.27	0.00	0.44	0.18	41

Observation:

It was observed that when the chitin powder was mixed with water, we didn’t not obtain a colloidal solution. Instead it was more of a suspension. Later on when we had an expert interaction, we came to know that colloidal chitin wasn't supposed to behave as such and our procedure might be wrong.

Time Vs OD540 Plot



Conclusion:

The results are inconclusive as the controls also show an increase in OD540 with time.

Preparation of New Colloidal Chitin

Experimental Aim: To prepare colloidal chitin using the protocol suggested by Dr Binod Parameswaran. A detailed protocol has been provided in the protocol section.
Observation: Colloidal Chitin was obtained as a pasty white substance and the moisture content within it was determined.
Conclusion : The obtained colloidal chitin paste was stored in 4°C refrigerator for further use.

Note: The washing of colloidal chitin with water might have to be performed multiple times in order to increase the pH to 5.5. Each wash can be done with 1 L water, although we had used 300 mL in the initial stages.

7-09-2021

Washes	pH	Comments
0	0.59	Done with 300 mL wash
1	0.96	Done with 300 mL wash
2	1.55	Done with 300 mL wash
3	2.22	Done with 300 mL wash
4	2.85	Done with 300 mL wash
5	3.56	Done with 300 mL wash

6	3.57	Done with 300 mL wash
7	3.63	Done with 300 mL wash
8	3.83	Done with 300 mL wash
9	4.04	Done with 300 mL wash
10	4.22	Done with 300 mL wash
11	4.28	Done with 1L wash
12	4.73	Done with 1L wash
13	5.05	Done with 1L wash
14	5.50	Done with 1L wash

DNS Assay With Crude Protein

Since we couldn't estimate the concentration of our protein of interest, we wanted to analyse the volume of our crude protein to cause about a significant change in OD (Optical Density).

Experimental Aim: To establish presence of desired enzymatic activity by observing the following trend:

As the Enzyme**:**Substrate ratio increases, more degradation of the substrate takes place which corresponds to an increase in OD540.

Method:

The Temperatures ranges selected for the assay were 30°C, 40°C , 50°C, 60°C.

The ratio of enzyme to substrate selected for each temperature were:

1:1, 3:1, 5:1.

The control selected was crude lysate of BL21 hosting pET28a without our insert.

The blank was DNS with water.

The experiment was done in a 96 well plate and graph was plotted for the OD vs ratio of substrate to enzyme.

19-09-2021

OD540 RESULT (96 Well Plate)

S130 (0.2285)	S330 (0.2932)	S530 (0.3322)	BLANK (0.2115)
S140 (0.2316)	S340 (0.3079)	S540 (0.3523)	BLANK (0.211)
S150 (0.2222)	S350 (0.334)	S550 (0.33)	
S160 (0.2114)	S360 (0.2679)	S560 (0.3542)	
C130 (0.2302)	C330 (0.267)	C530 (0.3238)	
C140 (0.2279)	C340 (0.3185)	C540 (0.4881)	
C150 (0.2326)	C350 (0.2643)	C550 (0.464)	
C160 (0.2122)	C360 (0.3155)	C560 (0.3586)	

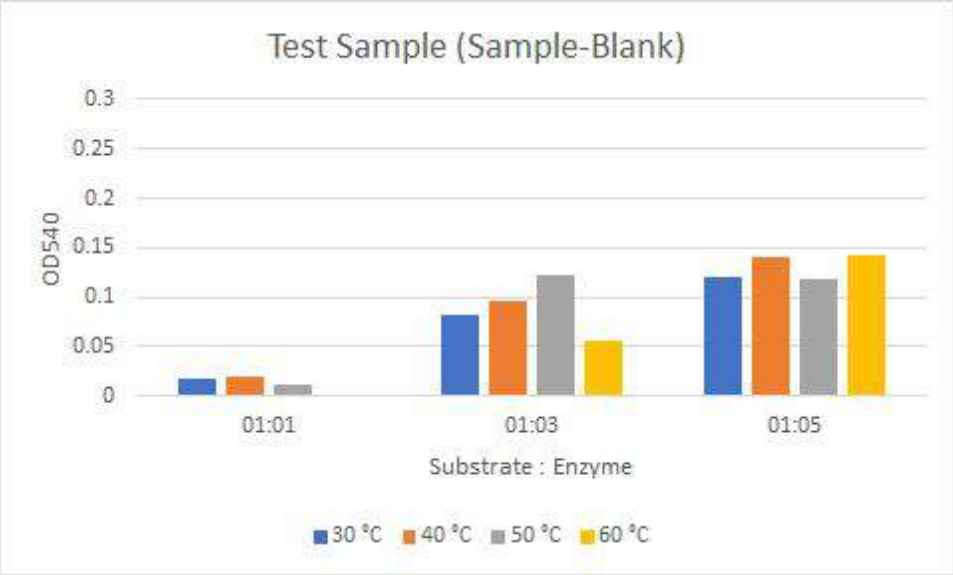
Annotation:

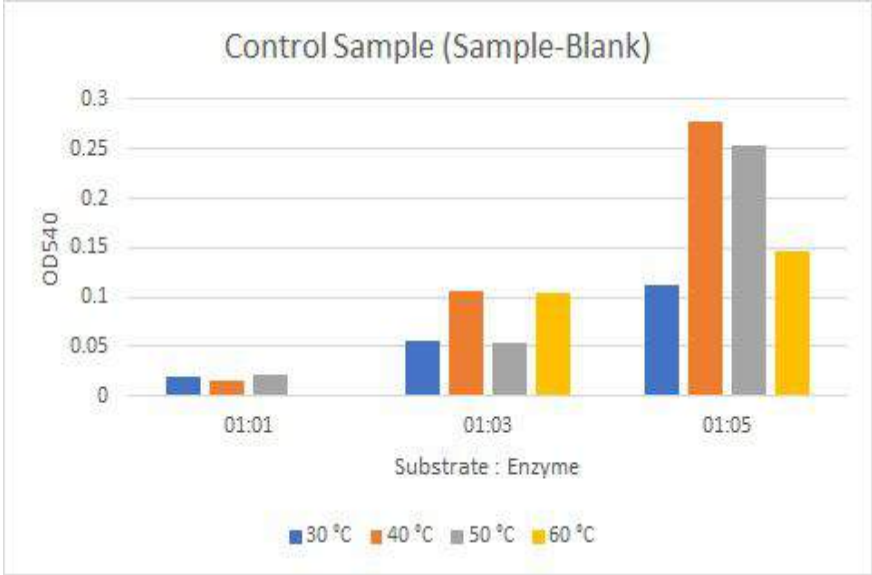
S360 stands for Test Sample where Enzyme:Substrate ratio is 3:1 and the reaction mixture is incubated at 60° C.

C540 stands for Control Sample where Enzyme:Substrate ratio is 5:1 and the reaction mixture is incubated at 40°C.

And the pattern follows

Bar Graph (OD540 Vs Enzyme-Substrate Ratio)





The control was showing a similar trend and had even higher values at OD540 for some temperatures

Note: The Blank ODs were averaged and subtracted from the Sample ODs to get final OD measurement.

Experiment 2:
To check if the OD540 increases without the Substrate.

Experimental Aim :The previous experiment data was inconclusive of the enzymatic activity. Hence we carried out another experiment where we would prevent reaction between enzyme and substrate by selectively adding only crude enzyme. If the OD540 results do come similar, it would imply that the quantitative assays with Crude Enzyme is not possible and the data is very inconclusive.

Method: Similar reaction was repeated as previously mentioned. The only change made was that 1X PBS buffer was to be added instead of substrate solution. Only the higher ratios 3:1 and 5:1 were chosen and the reaction was carried out at 50 °C

21-09-2021

OD540 vs Substrate-Enzyme Ratio

C350 (0.2643)	S350 (0.334)	BLANK (0.2115)
C550(0.464)	S350 (0.33)	BLANK (0.211)

Conclusion:

The new results (without substrate) are almost comparable to the previous data (with substrate). This implies that quantitative assays with crude enzymes will not be conclusive for proving the activity of our enzyme.

Protein Estimation using BCA (Bicinchoninic acid)

Experimental Aim: To estimate the concentration of the purified protein using BCA kit.

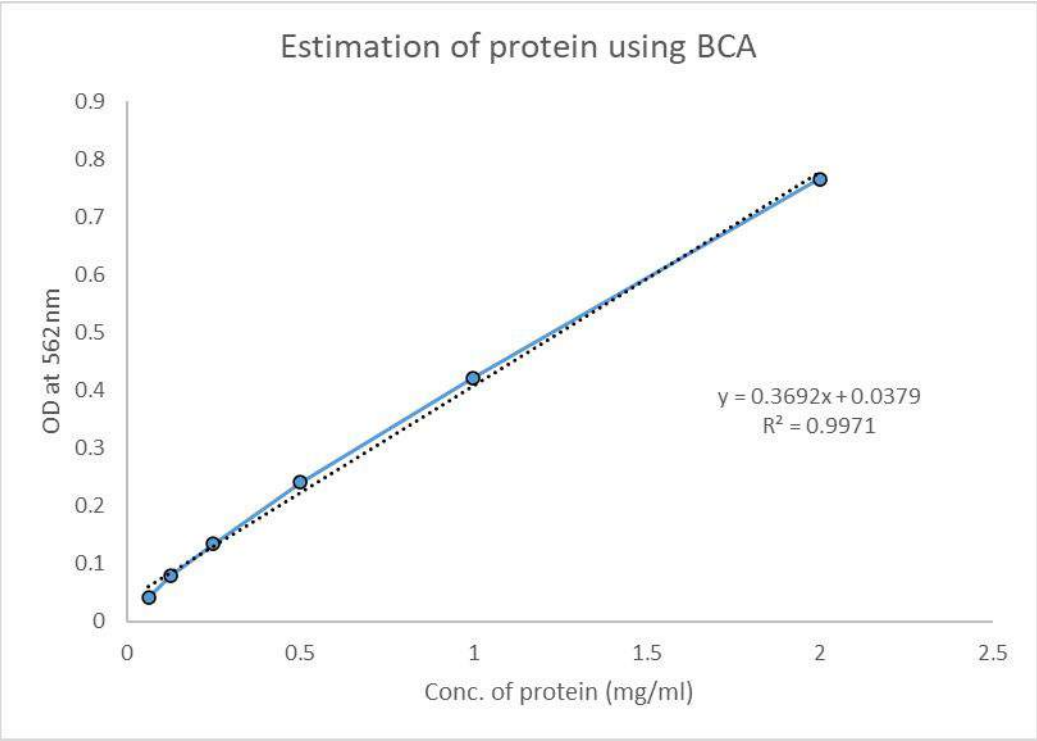
Method: 5 µL of each sample was mixed with 20µL distilled water and 200 µL of freshly prepared BCA reagent (50 parts of BCA and 1 fractions of $CuSO_4$) and incubated at 37°C for 30 mins. A standard curve was also plotted using BSA (Bovine Serum Albumin) protein. Triplicates were performed with appropriate blanks. The OD was measured at 562 nm.

30-09-2021

Observation

Standard Curve		
Sample	**Conc.**	**OD**
2mg/ml	2	0.7653
1mg/ml	1.00	0.42
0.5mg/ml	0.50	0.24
0.25mg/ml	0.25	0.13
0.125mg/ml	0.13	0.08
0.0625mg/ml	0.06	0.04

Standard Curve of BCA



Result

Protein Sample	Avg OD	Conc (mg/mL)
Protein Extracted (SDS in buffer) (28-09-2021)	1.02	3.2
Protein Extracted without SDS (28-09-2021)	0.13	-0.06
Protein Extracted on (27-09-2021) IC50 one	0.45	1.09

DNS Assay

Experiment Aim: To check for activity of enzyme using DNS method. The assay was performed for 5ug and 100 ug of protein sample.

Method:

1 mL of Enzyme Solution was added to 1 mL (1%) Colloidal Chitin (suspended in acetate Buffer pH 5.6.

Appropriate blank (Buffer with colloidal chitin) was considered and triplicates were made.

The solution was incubated for 10 mins at 40°C.

After incubation 3 mL DNS was added and heated for 10 mins at 100°C.

The resultant was centrifuged at 5000 x g for 10 mins and 200 uL of each sample was loaded on a 96 well plate.

30-09-2021

OD540 Readings

Samples	OD540	OD540	OD540	Mean OD	Mean Sample OD-Mean Blank OD
Control	0.2766	0.2839	0.2707	0.2771	0.0000
100 ug	0.2832	0.2801	0.2857	0.2830	0.0059
5 ug	0.2805	0.2783	0.2809	0.2799	0.0028

Conclusion:

The OD540 readings of the sample was comparable to the readings of the control hence not much can be said about the activity of the enzyme. However, we still wanted to check how much activity is present by plotting a Standard curve of N-Acetylglucosamine (NAG).

Plotting Standard Curve for NAG

Experimental Aim: To plot a standard curve for NAG and correlate the amount of NAG released in the previous experiment.

Method :

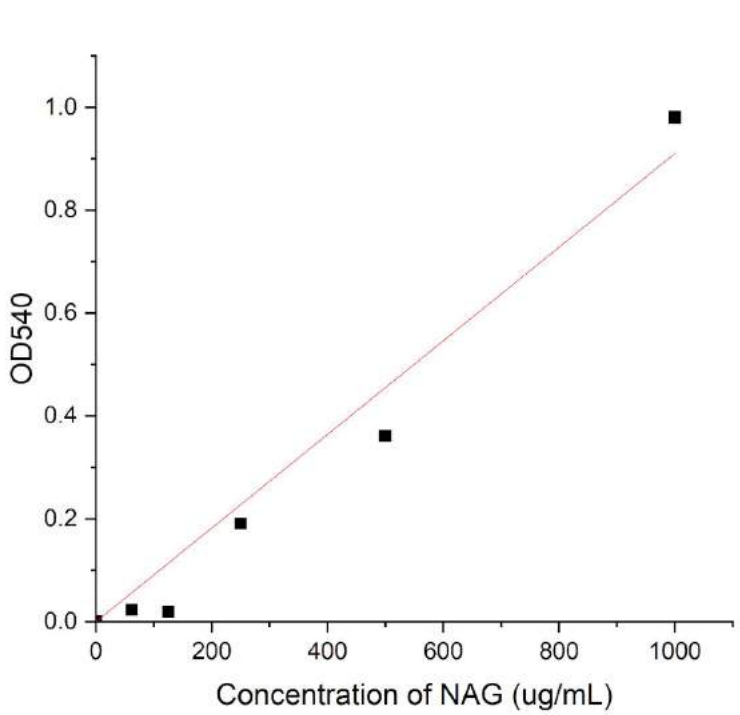
NAG (1mg/mL) was serially diluted to the following concentrations (by maintaining total volume in each tube to be 700 uL):

Conc. Of NAG (mg/ml)	1	0.5	0.25	0.125	0.0625	0.03125	0.01562	0.00781

1.4 mL DNS was added to the tubes and heated to 100°C for 10 mins. After heating the samples were loaded on a 96 well plate in triplicates.
(DNS with water was kept as the blank.)

31-09-2021

NAG Standard Curve



Equation	y = a + b*x
Plot	OD540
Weight	No Weighting
Intercept	0 ± --
Slope	9.1025E-4 ± 6.14
Residual Sum of	0.02519
Pearson's r	0.98878
R-Square (COD)	0.97769
Adj. R-Square	0.97322

Enzyme Activity Calculation

$$Specific\ activity(U/mg) = \frac{(Micromoles\ of\ NAG\ released)}{(Conc.\ of\ enzyme(mg/mL)) \cdot (Incubation\ time(min))}$$

Enzyme Sample	Specific Enzymatic Activity (U/mg)
100 ug	$9.03 * 10^{-5}$
5 ug	$1.87 * 10^{-4}$

DNS Assay

Experiment Aim: To perform DNS assay with purified protein. The assay was performed with 5 µg and 100 µg of protein.

Method:

1 mL of Enzyme Solution was added to 1 mL (1%) Colloidal Chitin.
Appropriate blank (1X PBS Buffer with Colloidal Chitin) was considered and triplicates were made.
The solution was incubated for 60 mins at 40 C.
After incubation 3 mL DNS was added and heated for 10 mins at 100 C.
The resultant was centrifuged at 5000 xg for 10 mins and 200 µL of each sample was loaded on a 96 well plate.

1-10-2021

Observations:

Samples	OD540	OD540	OD540	Mean OD	Mean Sample OD-Mean Blank OD
5 ug	0.2499	0.244	0.2448	0.2462	-0.0069
100 ug	0.2397	0.2457	0.2427	0.2427	-0.0104
Control	0.2622	0.2515	0.2456	0.2531	0

Observation: The OD540 readings of the samples is lower than the control.

Results

Since the OD540 results are lower than the control, the results of the experiment are inconclusive and hence the protocol for DNS assay had to be modified.

Preparation of Fresh DNS Reagent

Aim: To prepare fresh DNS reagent for assays.

Requirements :

- 2M NaOH
- DNS (3,5-dinitrosalicylic acid) Powder
- Sodium Potassium Tartrate

3-10-2021

Method:

- Solution I:

Dissolve 2g DNS in 40 mL 2M NaOH. Warm up the solution on a heat block with constant stirring to dissolve DNS completely.

- Solution II:

Dissolve 60 g of Sodium Potassium tartrate in 100 mL warm distilled water.
Mix solution I and solution II and make up volume to 200 mL with H_2O. Let the final solution cool down to room temperature and then store it light sensitive bottle at room temperature.

Experiment : Background Noise Data

Aim:

To check if the new DNS has a lower OD540 read than the old DNS. We also wanted to check if any of the buffer components had significant OD540 readings before and after heating it to 100°C.

Before Heating				
Sample	**OD540**	**OD540**	**OD540**	**Mean OD**
Old DNS	0.4179	0.413	0.4099	0.4136
New DNS	0.2578	0.2798	0.2813	0.272967

4-10-2021

Observations

After Heating				
Sample	**OD540**	**OD540**	**OD540**	**Mean OD**
Old DNS	0.6128	0.544	0.6492	0.602
New DNS	0.2505	0.2854	0.2905	0.275467
Old DNS + Water	0.215	0.2706	0.4771	0.3209
New DNS + Water	0.1322	0.1437	0.1469	0.140933
PBS	0.0381	0.0385	0.0391	0.038567
Water	0.0376	0.038	0.0375	0.0377

Conclusions:

New DNS did show lower OD540 value than the old DNS. Both of them showed lower values after heating to 100°C for 10 mins. The buffer and water did not show any significant OD540 values. This implies that the buffer and water are not contributing significantly to the OD540 values during actual experimentation.

Plotting NAG Standard Curve with new DNS Reagent

Experimental Aim: To plot a standard curve for NAG and correlate the amount of NAG released in the previous experiment.

Method :

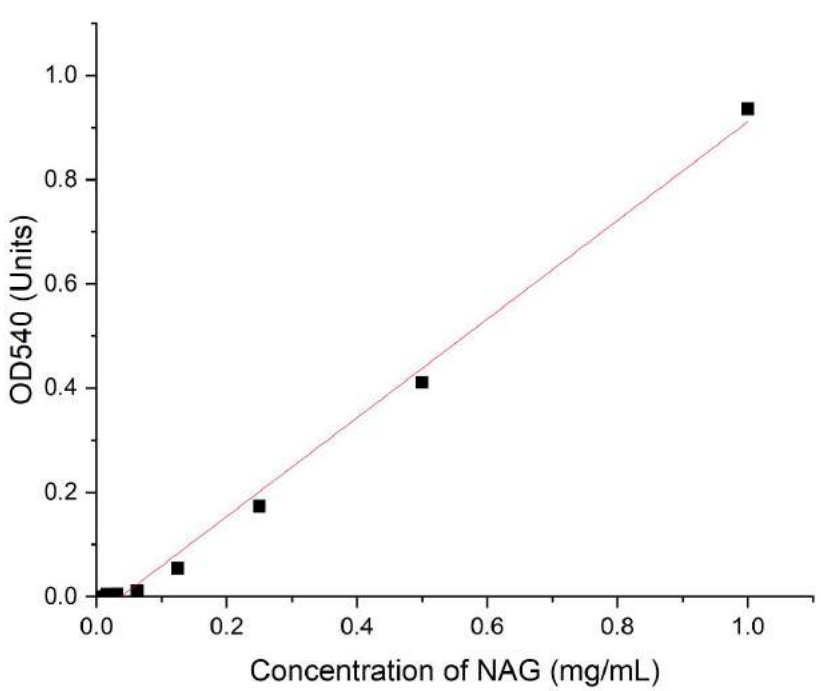
NAG (1mg/mL) was serially diluted to the following concentrations (by maintaining total volume in each tube to be 700 uL):

Conc. Of NAG (mg/ml)	1	0.5	0.25	0.125	0.0625	0.03125	0.01562	0.00781

1.4 mL DNS was added to the tubes and heated to 100°C for 10 mins. After heating the samples were loaded on a 96 well plate in triplicates.
(DNS with water was kept as the blank.)

5-10-2021

NAG Standard Curve with New DNS



Equation	y = a + b*x
Plot	OD540
Weight	No Weighting
Intercept	-0.03522 ± 0.012
Slope	0.94656 ± 0.0318
Residual Sum of Squar	0.0051
Pearson's r	0.99663
R-Square (COD)	0.99327
Adj. R-Square	0.99215

DNS Assay

Experiment Aim: To perform DNS assay with purified protein. The assay was performed with 5 µg and 100 µg of protein.

Method:

1 mL of Enzyme Solution was added to 1 mL (1%) Colloidal Chitin.
Appropriate blank (1X PBS Buffer with Colloidal Chitin) was considered and triplicates were made.
The solution was incubated for 60 mins at 40 C.
After incubation 3 mL DNS was added and heated for 10 mins at 100 C.
The resultant was centrifuged at 5000 xg for 10 mins and 200 µL of each sample was loaded on a 96 well plate.

5-10-2021

Observation:

Sample (ug of protein)	Mean Sample OD- Mean Blank OD	OD540	OD540	OD540	Mean OD540
5	-0.018733333	0.1411	0.1441	0.134	0.139733333
100	-0.018633333	0.1434	0.1383	0.1378	0.139833333
200	-0.0228	0.1414	0.1362	0.1294	0.135666667
Blank (CC+Protein Buffer+DNS)	0	0.1634	0.1604	0.1516	0.158466667

Results :

- The OD540 values of test samples were lower than the control samples. Hence the results are inconclusive. We might have to improve on our method of assay.
- We believed that the small amount of NAG released gets diluted so much that the compound responsible for absorbing light at 540 nm is not able to be detected by the machine properly. Hence it gives comparable readings between test and control sample.

Reducing Reaction Volume from 4 mL to 400 uL

Experimental Aim: To perform DNS assay of our enzyme with reduced reaction volume.

Method: 100 µL of our enzyme (1.153 mg/ml) was mixed with 100 µL of 1%(w/v) colloidal chitin and incubated the solution at 40°C for 10 mins. Appropriate control was also set up accordingly. After incubation the solution was treated with 200 µL DNS and heated to 100°C for 10 mins in a boiling water bath. After heating 200 µL of each sample was loaded in a 96 well plate and its absorbance was measured at 540 nm.

5-10-2021

Observation

Sample (ug of protein)	Mean Sample OD- Mean Control OD540	OD540
160	0.0808	0.2407
Control (CC+PBS+NaCl+Glycerol+SDS)		0.1599

Observations:

We observed that as we reduced the reaction volume, we obtained higher OD540 than before when compared to the control.

Protein Estimation using BCA (Bicinchoninic acid)

Experimental Aim: To estimate the concentration of the purified protein using BCA kit.

Method: 5 µL of each sample was mixed with 20µL distilled water and 200 µL of freshly prepared BCA reagent (50 parts of BCA and 1 fractions of CuSO4) and incubated at 37°C for 30 mins. A standard curve was also plotted using BSA (Bovine Serum Albumin) protein. Triplicates were performed with appropriate blanks. The OD was measured at 562 nm.

5-10-2021

Observation

Sample	OD 1	OD 2	OD Mean	OD-Blank	Con. mg/ml
Blank	0.0998	0.0988	0.10	0.00	**0.00**
Sample 1	1.0179	0.9774	1.00	0.86	**2.22**
Sample 2	0.8046	0.8552	0.83	0.69	**1.77**
Sample 3	1.0467	0.9861	1.02	0.88	**2.27**
Sample 4	0.3095	0.3291	0.32	0.18	**0.38**

New Standard Curve with Reduced Volume

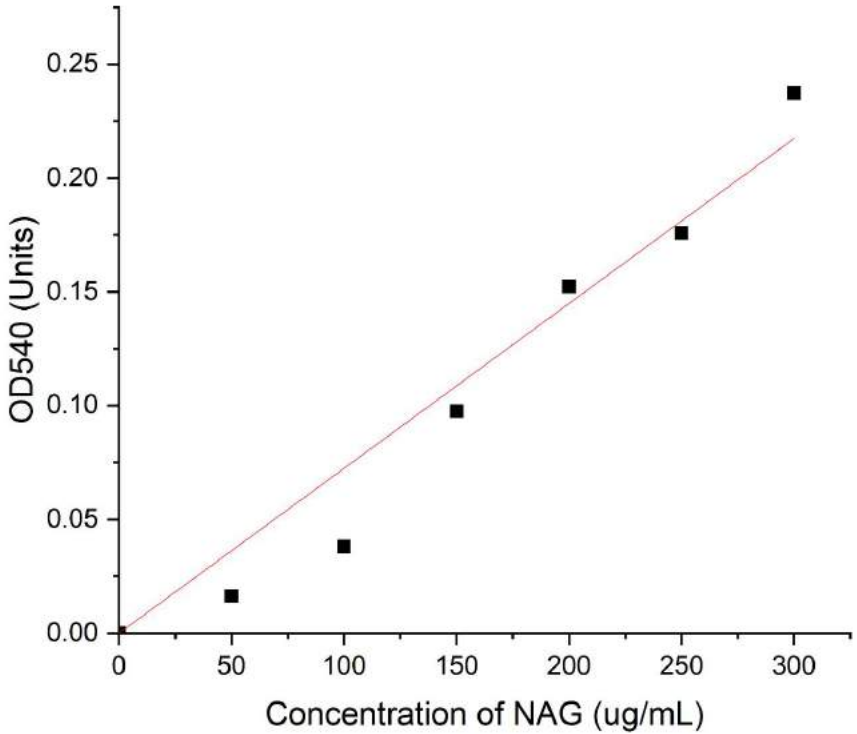
Experimental Aim: To plot a standard curve of NAG against OD540 after treatment with new DNS and correlate the OD540 readings from previous experiment to calculate the enzymatic activity.

Method: Prepare stock NAG solution of 5 mg/mL. Make the following dilution as provided in the table below. Treat the solutions and the control (water) with DNS and heat at 100°C for 10 mins. Measure the absorbance at 540 nm.

NAG from Stock (uL)	Buffer (uL)	Total Volume (uL)	Final NAG Conc. (ug/mL)
0	200	200	Control (0)
2	198	200	50
4	196	200	100
6	194	200	150
8	192	200	200
10	190	200	250
12	188	200	300

7-10-2021

New NAG Standard Curve



Equation	y = a + b*x
Plot	OD540
Weight	No Weighting
Intercept	0 ± --
Slope	7.24549E-4 ± 4.008
Residual Sum of Sq	0.00219
Pearson's r	0.99094
R-Square (COD)	0.98196
Adj. R-Square	0.97896

Calculating Enzyme Units (DNS Assay)

Sample (conc.)	Mean Sample OD- Mean Blank OD
1.1535 mg/mL	0.0982

$$Specific\ activity(U/mg) = \frac{(Micromoles\ of\ NAG\ released)}{(Conc.\ of\ enzyme(mg/mL)) \cdot (Incubation\ time(min))}$$

Sample (conc.)	Mean Sample OD- Mean Control OD	Enzymatic Units (U/mg)
1.1535 mg/mL	0.0982	0.5635

OD vs Temperature Assay

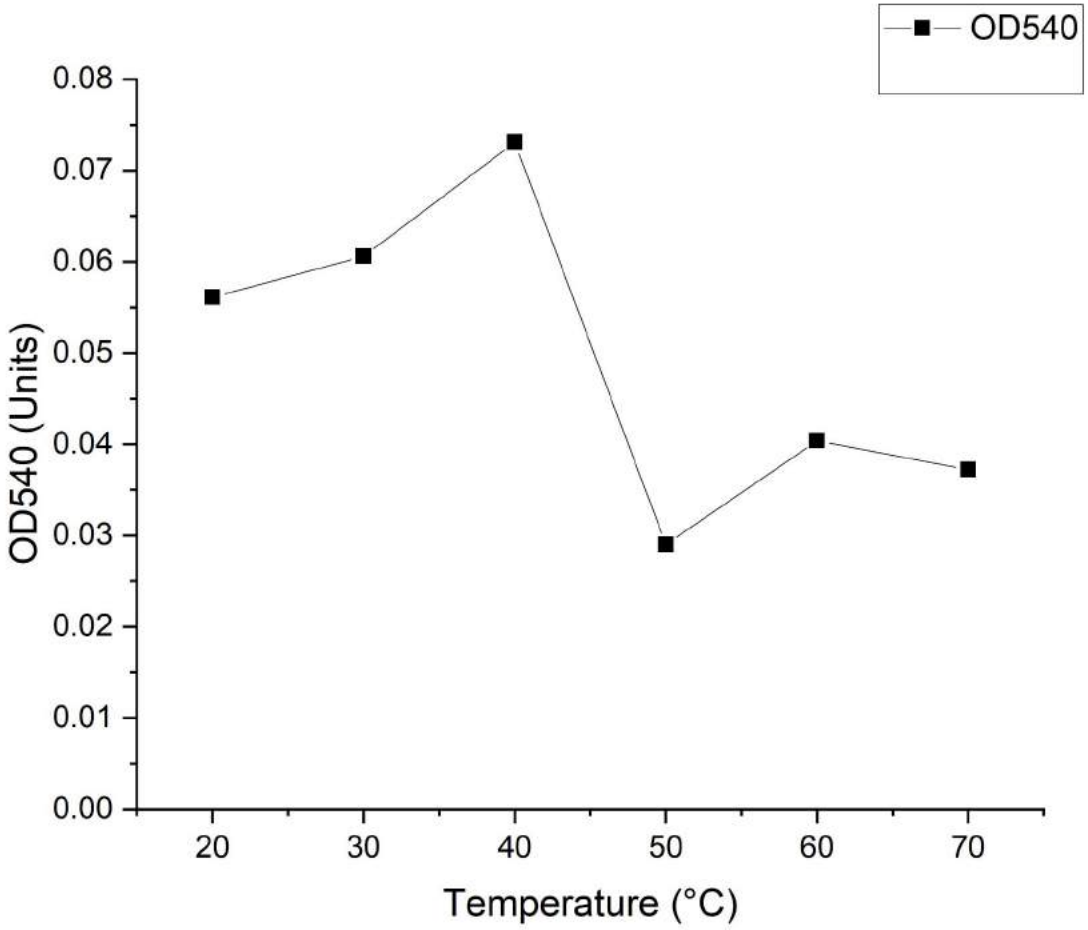
Aim: To check the activity of our chitinase with increasing temperatures.

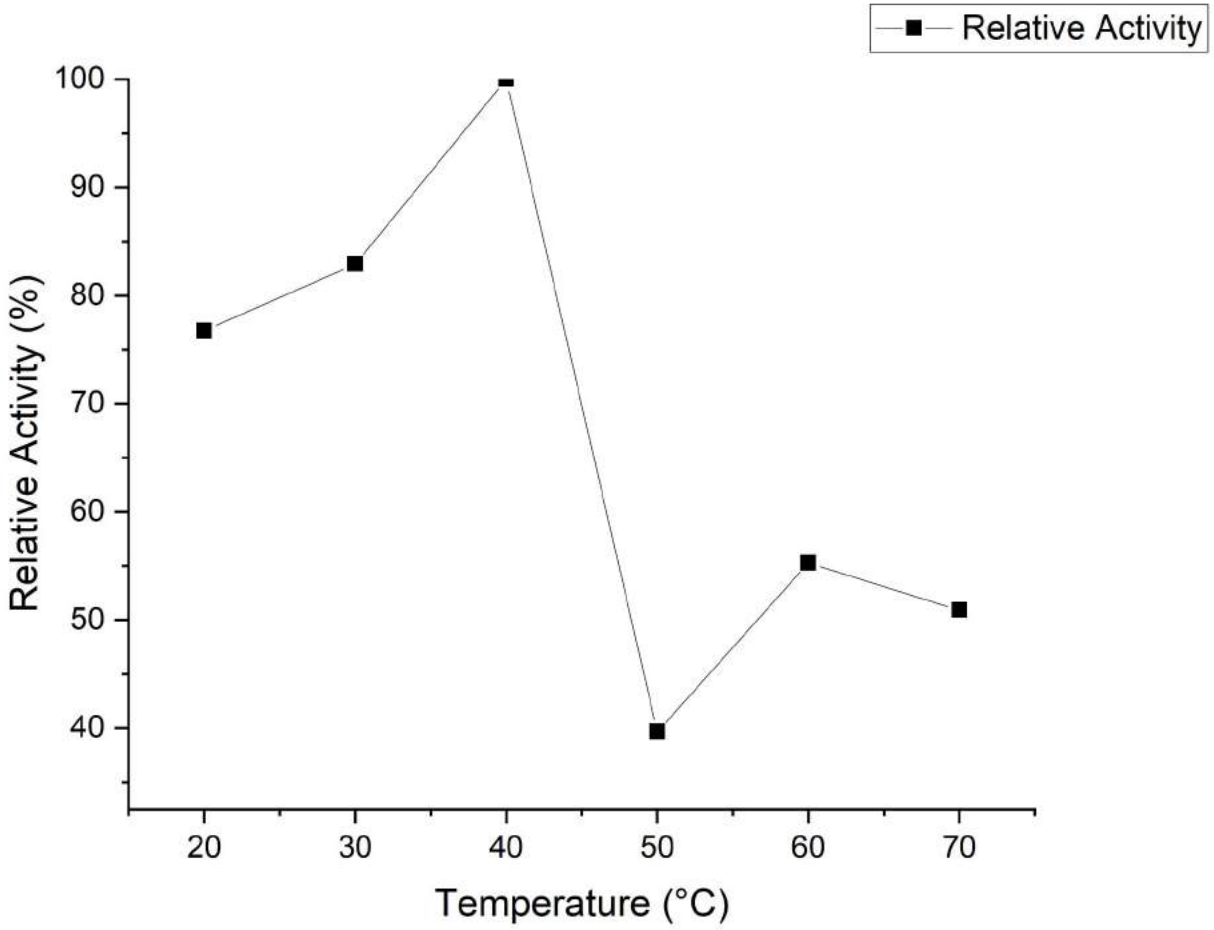
Method: 100 uL of the Enzyme was incubated with 100 uL of the substrate at different temperatures (20° C,30°C,40°C,50°C,60°C,70°C) for 10 mins. The amount of NAG released was calculated by DNS assay.

8-10-2021

Temperature vs OD540 Data

	20°C	**30°C**	**40°C**	**50°C**	**60°C**	**70°C**
Sample	0.2	0.1931	0.1959	0.1666	0.1737	0.1828
Control	0.1439	0.1325	0.1228	0.1376	0.1333	0.1456
Sample-Control	**0.0561**	**0.0606**	**0.0731**	**0.029**	**0.0404**	**0.0372**


OD540
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
OD540 (Units)
20
30
40
50
60
70
Temperature (°C)


Relative Activity
100
90
80
70
60
50
40
Relative Activity (%)
20
30
40
50
60
70
Temperature (°C)

Conclusion

- Since the experiment was performed without duplications, we cannot rely on the data. But it does seem that our chitinase is more active around 40°C

Michaelis-Menten Assay

Aim: To plot the Michaelis-Menten curve for our chitinase and calculate it's Vmax and Km.

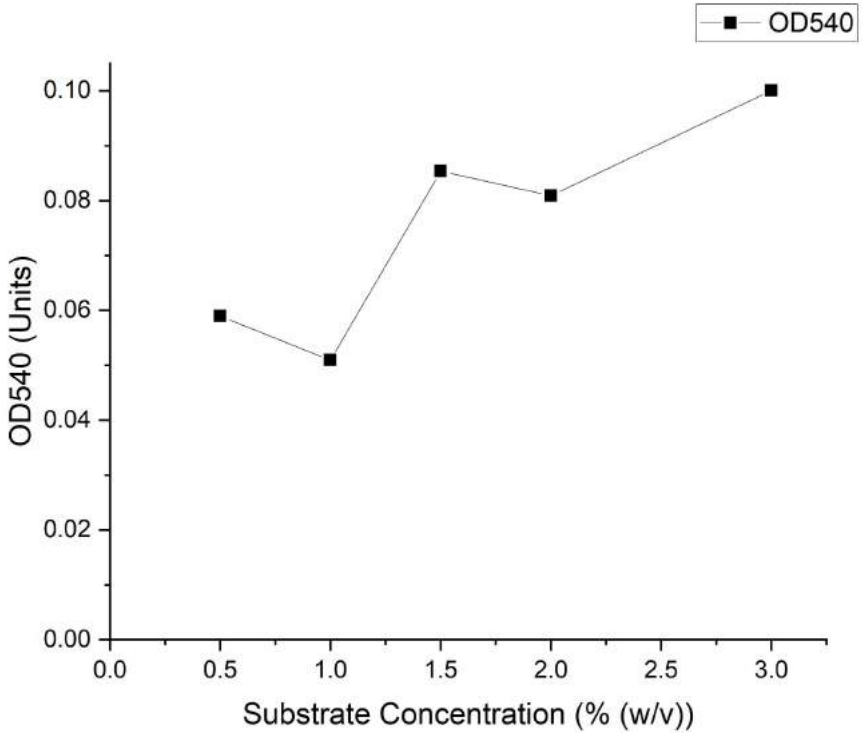
Method: 100uL of enzyme (0.842 mg/mL) was added to different concentrations of substrates (0.5%,1%,1.5%,2%,3% [w/v]). The solution was incubated at 40°C for 10 mins. The amount of NAG released was estimated by DNS Assay. The experiment was done in duplicates with appropriate controls (without enzyme).

Note: Stock concentration of Colloidal Chitin (5% (w/v)) was prepared. The solution components were added in the following manner:

9-10-2021

Concentration of substrate (%(w/v))	Vol colloidal chitin (from stock) (uL)	Vol of Buffer (uL)	Volume of Enzyme (uL)
0.5	10	90	100
1	20	80	100
1.5	30	70	100
2	40	60	100
3	60	40	100
Control 1	10	190	-
Control 2	20	180	-
Control 3	30	170	-
Control 4	40	160	-
Control 5	60	140	-

Substrate Conc. vs OD540



Conclusion

Since the OD540 values are within the error range of each other's reading, the data of this experiment is not reliable.