EXPERIMENTAL STUDY ON ALPHA(α)-AMYLASE AND PROTEASE ENZYME ACTIVITY OF GERMINATING BARLEY SEEDS AT DIFFERENT TEMPERATURE

A thesis submitted towards the partial fulfilment of the requirements for the degree of ***Master of Technology in Food Technology And Biochemical Engineering*** *Course affiliated to*
Faculty of Engineering and Technology,
Jadavpur University

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CERTIFICATE OF RECOMMENDATION

We hereby recommend that the thesis entitled " ***EXPERIMENTAL STUDY ON ALPHA(α)-AMYLASE AND PROTEASE ENZYME ACTIVITY OF GERMINATING BARLEY SEEDS AT DIFFERENT TEMPERATURE*** " carried out under my supervision by Sanjib Das may be accepted in partial fulfilment of the requirement for awarding the Degree of Master in Food Technology And Biochemical Engineering of Jadavpur University. The project, in our opinion, is worthy for its acceptance.

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The forgoing thesis is hereby approved as a creditable study of an engineering subject carried out and presented in a manner satisfactory to warrant its acceptance as a prerequisite to the degree for which it has been submitted. It is understood that by this approval the undersigned do not necessarily endorse or approve any statement made, opinion expressed or conclusion drawn therein but approve the thesis only for the purpose for which it is submitted.

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of his Master of Engineering in Biomedical Engineering studies during academic session 2019-2021.

All information in this document has been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by this rules and conduct, I have fully cited and referred all material and results that are not original to this work.

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ABSTRACT

The germination process increases enzymatic activity. However, this does not occur in the same way in all cereals. It depends on the type of enzyme, the cereal, and the conditions of germination. During germination, most enzymes are localized in the aleurone layer and the scutellum. Some of them, such as xylanases, proteases, and β-glucanases, are also localized in the endosperm while β-glucanases and lipases have been identified in the embryo. Germination mobilizes and increases the activity of some enzymes. Temperature, steeping time, and variety are determining factors in the activation time. At higher temperatures, the enzymatic activation is generally faster; however, there are some exceptions. The use of germination could be a promising resource for modifying grain properties and increase enzymatic activity; also, this process is simple and economical.

Significance of Work

Enzymatic activity in cereals increases with germination, which may have advantages or disadvantages depending on the application. It is important to know how the enzymatic activity is modified, since it can have a negative effect on the quality of the processing of the flours in the milling and the cooking. On the other hand, the enzymatic activity can improve functional properties. For example, the greater enzymatic activity allows hydrolysis of macromolecules that can be easier to digest and also generate bioactive compounds. Enzymes such as amylases, proteases, and xylanases are generally added to modify rheology of the dough, gas retention, and softness of the crumb. However, the time of enzymatic activation varies depending on the cereal type, growing conditions, and germination, as well as the presence of hormones such as gibberellins, which are produced in embryos of the grains that stimulate the synthesis and production of enzymes, particularly α-amylases. embryos of the grains that stimulate the synthesis and production of enzymes, particularly α-amylases. These hormones are also able to induce the expression of genes necessary for the production of proteases and glucanases. Therefore, the process of germination is viable to modify the action of enzymes.

On the other hand, enzymatic activity is important during germination because the hydrolysis of compounds that provide energy to the plant for growth needs to occur. However, enzymes are not always activated as soon as germination starts. In the early stages of germination, α-amylases are less active and the energy generated for the plant usually comes from the hydrolysis of sucrose and maltose by β-amylases , while α-amylases are activated over time.

In the production of malt based beverages and malted milk food, barley grain is first converted into malt. Malted barley gives beer its color, malty sweet flavor, dextrins to give the beer body protein to form a good head, and perhaps most important, the natural sugars needed for fermentation. Barley’s role in beer making is equivalent to grapes’ role in wine making.

INTRODUCTION

Barley is used for a wide range of traditional and novel end-uses. In most countries, the major portion of barley is fed to animals, particularly cattle and pigs. Human food uses of barley are more limited , although recent trends in the use of barley varieties, high in dietary fibre, have been identified. A significant high-value use is to produce malt as a raw material for the brewing industries, including beer and whiskey.

Improved barley production requires barley breeding programs to provide varieties with the combination of reliable and efficient production characteristics and grain quality attributes suited to these uses. Selection of varieties, with the correct range of traits, is a difficult process. It is necessary to produce high quality malt for beer and other fermented beverages. However, a number of factors influence the final quality of the barley grain as well as the malt . Testing of end product quality for each breeding line is not only expensive but requires the availability of larger quantities of barley than is available from single plants or lines at an early stage in barley breeding. Biochemical or molecular tests that predict likely feed or malting and brewing quality are therefore needed to allow rapid development of barley cultivars.

Research continues to unravel the biochemistry behind areas of grain development (gene to protein to product) which relates to end product quality. Research into well-established components such as starch and protein, improves our knowledge of the relation between barley, malt and beer. However, the genetic, chemistry and synergistic relationship between some components that are important in malt and beer quality are still unknown.

Many of the traditional testing and evaluation methods aim to ensure a consistency of quality without a link between the attribute measured and the end-use quality being known. This results in attributes of uncertain value being assessed to reduce the risk of adopting or using barley that causes difficulties in processing or end product quality. This process may discriminate against barley with superior processing traits and will only be overcome by improved understanding of the basis of barley quality. This is highlighted when comparing our understanding of barley carbohydrates or proteins and the enzymes that degrade them being tested in conditions that do not duplicate the conditions of the in-situ reaction . A number of

these key enzymes have inhibitors and some aspects of the role and function of these inhibitors also are still unclear .

A number of quality attributes for malt barley are critical for the identification and release of malt varieties. These are hot water extract, viscosity, Kolbach index, wort ,B-glucan, fermentability, and diastatic power. Additional parameters include a-amylase, ,B-amylase, free amino nitrogen, friability, and ,B-glucanase. However, the value of parameters currently used to measure malt quality has been questioned (MacGregor, 1996;Palmer, 1983). The limitations of some of these traits in predicting brewery performance have led to suggestions that specific brewery tests were also needed to adequately describe malt quality (Axcel, 1998). More recently, Evans et al. (2007) demonstrated that the sum of individual starch degrading enzymes (as well as the thermostability of these enzymes) was a better indicator of diastatic power and that these enzymes also correlated better to ferment ability.

The barley grain is comprised of three main components, namely the germ or embryo, the outer layers (husk, pericarp, testa and aleurone layers), and the endosperm. Fig.1 shows the overall structure and individual components of the grain , with the endosperm being the dominant component. However, the main living organ in the grain is the embryo, with the aleurone layers (also a living tissue) surrounding the endosperm.

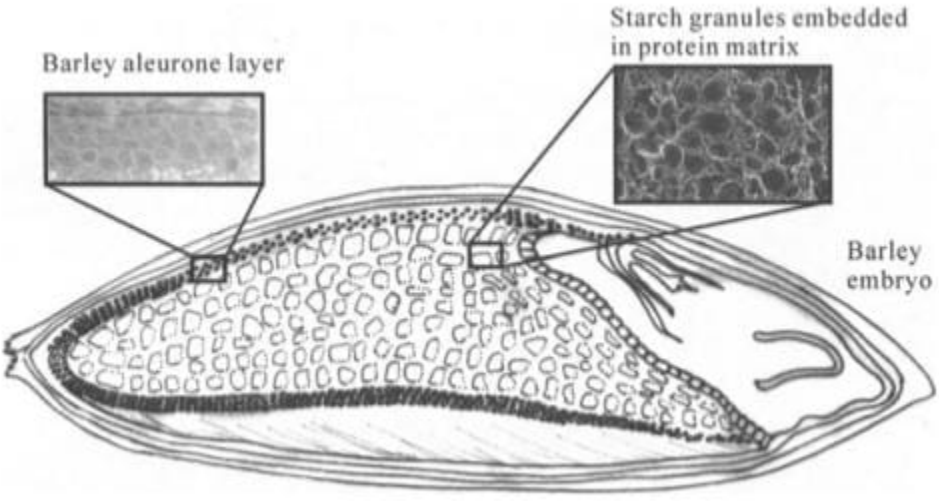


Fig 1 : Structure of Barley

The aim of this work is to examine α-amylases and proteases enzymes activities of germinating barley seeds at different temperatures.

- **Germination and Emergence**

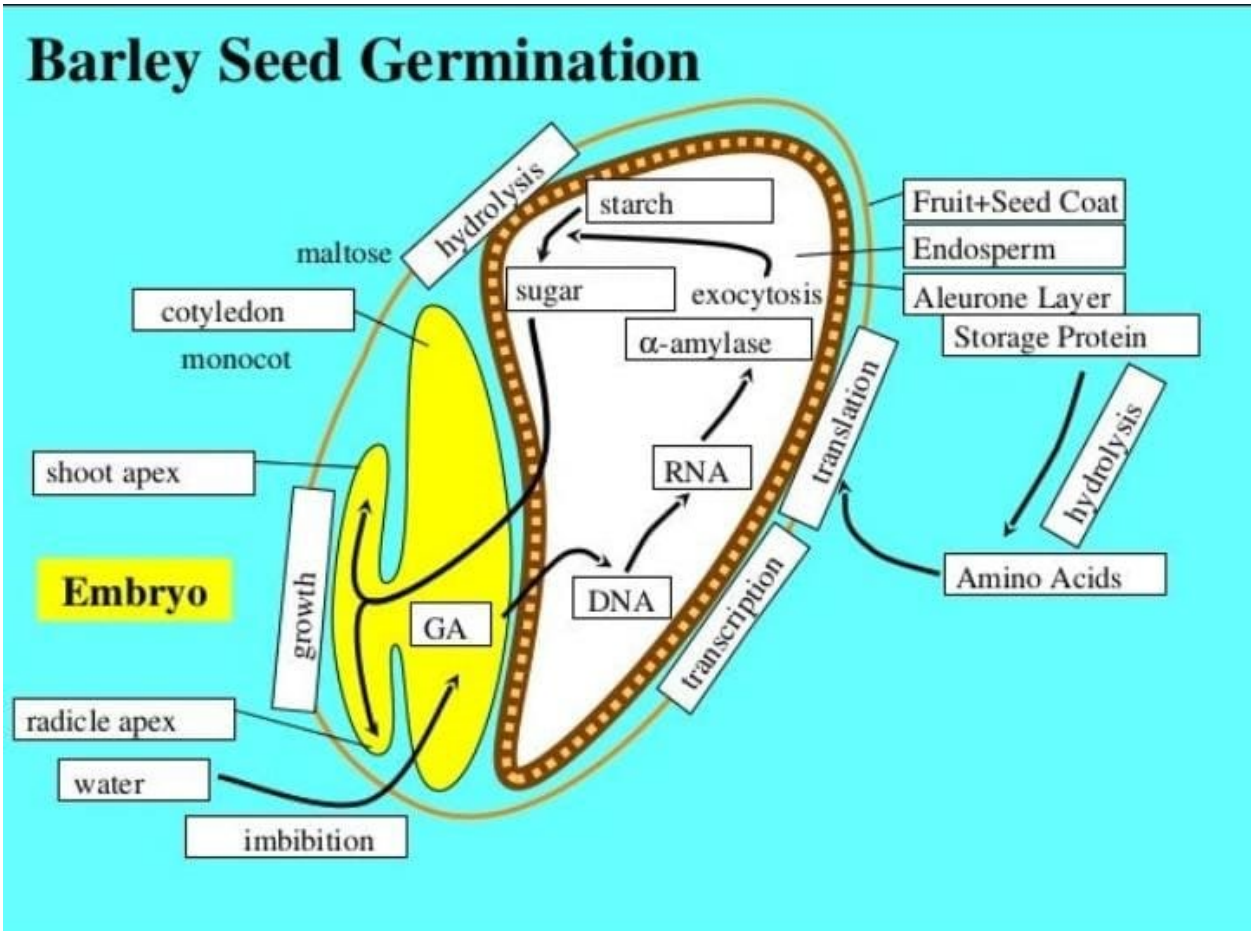
Germination

Germination begins when the seed absorbs water and ends with the appearance of the radicle. Germination has three phases:

• water absorption (imbibition)
• activation
• visible germination

Phase 1: Water absorption

Phase 1 starts when the seed begins to absorb moisture. Generally, a barley seed needs to reach a moisture content of around 35–45% of its dry weight to begin germination. Water vapour can begin the germination process as rapidly as liquid can. Barley seeds begin to germinate at a relative humidity of 97.7%. Soil so dry that roots cannot extract water still has a relative humidity of 99%, much higher than that of a dry seed. So even in dry conditions, there can be enough moisture for the seed to initiate germination, albeit at a slower pace than in damp conditions.



Phase 2: Activation

Once the embryo has swollen it produces hormones that stimulate enzyme activity. The enzymes break down starch and protein stored in the seed to sugars and amino acids, providing energy to the growing embryo. If the seed dries out before the embryo starts to grow it remains viable. Phase 2 continues until the rupture of the seed coat, the first visible sign of germination.

Phase 3: Visible germination

In Phase 3 the embryo starts to visibly grow. The radicle emerges, followed soon after by primary roots and the coleoptile. The enzymes produced in Phase 2 mobilise sugars and amino acids stored in the seed and enable their transfer to the growing embryo.

Emergence

As the first primary roots appear, the coleoptile bursts through the seed coat and begins pushing towards the surface. Emergence is when the coleoptile or the first leaf becomes visible above the soil surface.

● Factors affecting germination and emergence

Dormancy

In a barley seed, germination begins after a very short period of dormancy. Some level of seed dormancy is necessary to help prevent ripe grain from germinating in the head before harvest. However, excessive dormancy can be a problem in malting barley, forcing maltsters to store the grain for an extended period after harvest before it can be successfully malted.

Moisture

Soil moisture influences the speed of germination. Germination is rapid if the soil is moist. When the soil dries to near the permanent wilting point, the speed of germination slows.

The germination process in a seed may stop and start in response to available moisture. Seeds that have taken up water and entered Phase 2, but not reached Phase 3, remain viable if the soil dries out. When the next fall of rain comes, the seed resumes germinating, taking up water and moving quickly through Phase 2. This

ability to start and stop the germination process in response to conditions before the roots and coleoptile have emerged is an important consideration when dry sowing. If the seedbed dries out before the coleoptile has emerged, the crop needs to be monitored to determine whether it will emerge, so that the critical decision to re-sow can be made.

Soil moisture also affects emergence. Sowing into hard-setting or crusting soils that dry out after sowing may result in poor emergence. Hard soil makes it difficult for the coleoptile to push through to the surface, particularly in varieties with short coleoptiles. In some crusting soils, gypsum and/or lime may improve soil structure and assist seedling emergence.

Fig 2 : Different varieties of Barley seeds

CHAPTER 1

1.1 INTRODUCTION

Enzyme production is an increasing field of biotechnology. Traditionally enzymes are produced by submerged fermentation. However, it requires expensive medium components, thus increasing the production cost of enzymes. The enzyme amylase is usually found in seedlings of many species during germination. It is produced during germination to mobilize storage macromolecules in the endosperm because simpler molecules are needed to provide energy to the seed during germination. It is synthesized during germination & is one of the few enzymes present in the barley grain during germination that can initiate native starch hydrolysis.
Enzymatic activity is important during germination because the hydrolysis of compounds that provide energy to the plant for growth needs to occur. However, enzymes are not always activated as soon as germination starts. In the early stages of germination, α-amylases are less active. The energy generated for the plant usually comes from the hydrolysis of sucrose and maltose by β-amylases, while α-amylases are activated over time.

This study examines α-amylase activities in the germinated barley seeds at different temperatures in two conditions, one is covered with cotton cloths with limited oxygen availability & another is uncovered.

1.2 MATERIALS & METHODS

1.2.1 MATERIALS

1. Germinated barley seeds
2. 3,5 dinitro salicylic acid powder
3. Sodium Hydroxide (NaOH)
4. Sodium potassium tartrate
5. Sodium Thiosulphate
6. Phenol crystals
7. Distilled water

8. Seed Germinator
9. Spectrophotometer
10. Hot Water Bath
11. Test Tubes
12. Petri Dishes
13. Beakers
14. Mortar-Pestle

1.2.2 METHODOLOGY

- **Barley seeds Germination**
    1. Wash whole barley grain until the water clears, and the seeds are divided into Ten Petri dishes.
    2. Add 100ml of water to the washed grain of each Petri dish and keep it soaked for 8 hrs.
    3. After 8hrs, drain the water & remove the grain into Ten different cotton cloths and, tie the cloths, add it to a bowl of water, and squeeze the water by hand.
    4. Sprinkle water in between the seeds on the cloth, five cotton cloths are covered by the cotton cloth itself and remaining five cotton cloths are uncovered.
    5. Ten cotton cloths were stored in a seed germinator at five different temperatures, such as 5°C, 10°C, 15°C, 20°C, and Room Temperature, for a few minutes.
    6. After a few minutes, some seeds were taken (both Covered and Uncovered) as a DAY-0 barley seeds sample.
    7. After 24 hrs, some seeds were taken as a DAY-1 barley seeds sample.
    8. After 48 hrs, some seeds were taken as a DAY-2 barley seeds sample.
    9. After 72 hrs, some seeds were taken as a DAY-3 barley seeds sample.
    10. After 96 hrs, some seeds were taken as a DAY-4 barley seeds sample.
    11. After 120 hrs, some seeds were taken as a DAY-5 barley seeds sample.
    12. After 144 hrs, some seeds were taken as a DAY-6 barley seeds sample.
    13. After 168 hrs, some seeds were taken as a DAY-7 barley seeds sample.

- **DNS Reagent Preparation**

For 100ml of DNS reagent,

1. Take 1g NaOH in 70ml distilled water, and mix well.
2. Add 30g of Sodium Potassium Tartrate to it and mix till it dissolves fully.
3. Add 1g of DNS powder, and continuously stir till it dissolves.
4. Add 0.05g of Sodium Thiosulphate to it and dissolve fully.
5. Add 0.2g of Phenol crystals.
6. Make up the volume to 100ml.
   DNS reagent is ready and stored in a cool and dark place.

- **α-amylase ASSAY**

α-Amylase activity was assayed according to the method of Anto et al. (2006). The amount of reducing sugar liberated was estimated according to the method of Miller (1959) using DNSA reagent. The absorbance was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm. One unit (U) of α-amylase activity was defined as the amount of enzyme that releases 1μmol of reducing sugar per min under the assay conditions.

The α-amylase assay is a simple, direct, and automation-ready procedure for measuring the α-amylase activity and is, therefore, very desirable.

1. Make a smooth paste from DAY 0 to DAY 7 collected samples by mortar-pestle.
2. Mix the 1g paste in 10ml distilled water.
3. Take 3ml stock solution and add 3ml Water [T1]
4. Take 0.5ml T1 and add 4.5ml Water [T2]
5. Take 0.5ml T2 and add 4.5ml Water [T3]
6. Take 0.5ml T3 and add 4.5ml Water [T4]
7. Take 3ml T1 and add 1ml DNS reagent [S1]
8. Take 3ml T2 and add 1ml DNS reagent [S2]
9. Take 3ml T3 and add 1ml DNS reagent [S3]
10. Take 3ml T4 and add 1ml DNS reagent [S4]
11. Take 3ml Water and add 1ml DNS reagent [S5; Blank]
12. Put S1, S2, S3, S4, and S5 in a boiling water bath for 5 minutes.
13. Cool it at Room Temperature (Incubation Time 10 minutes).

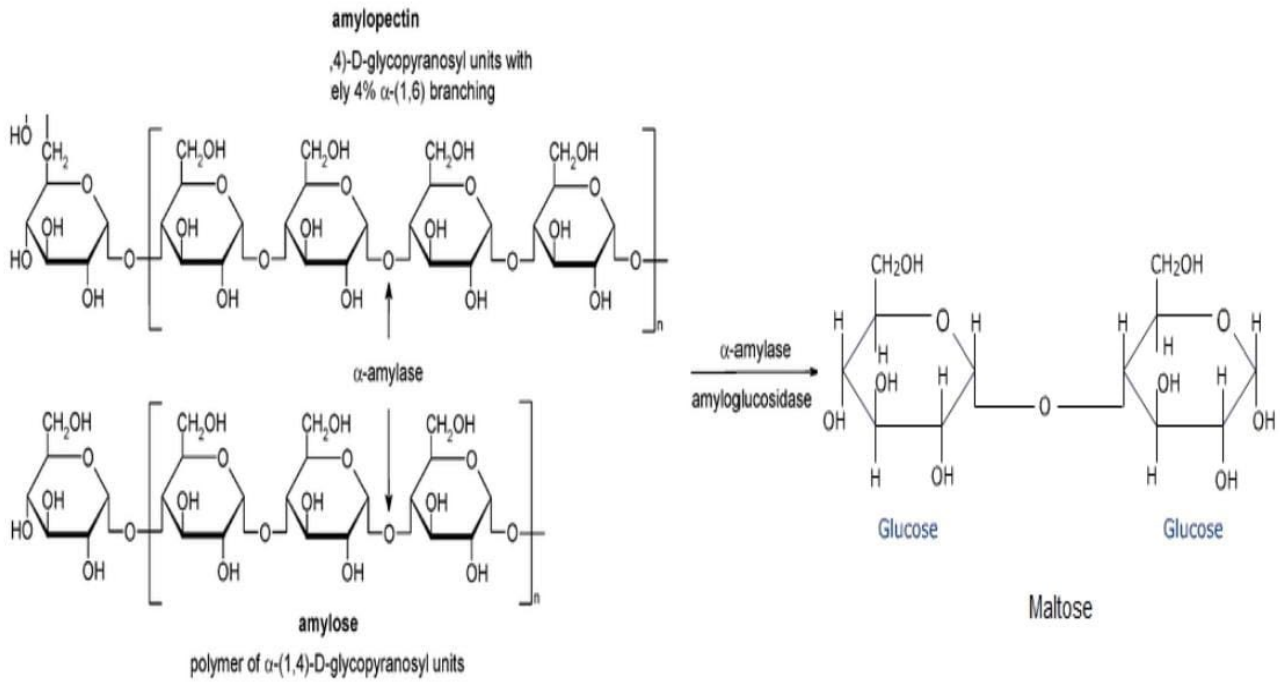
14. Measure each sample's Optical Density at 540nm in Spectrophotometer, using S5 as Blank.

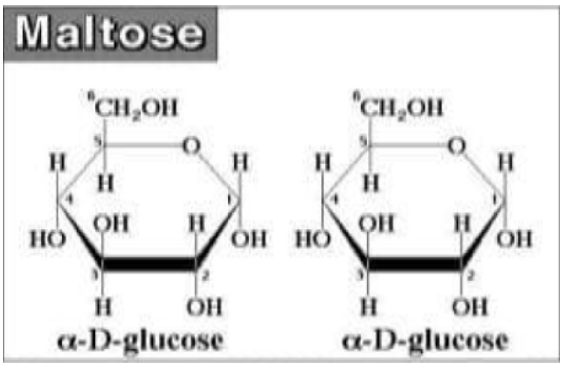
1.3 RESULTS & DISCUSSION

When we want to measure enzyme activity either we measure the decrease in the substrate concentration or the increase in the product concentration.

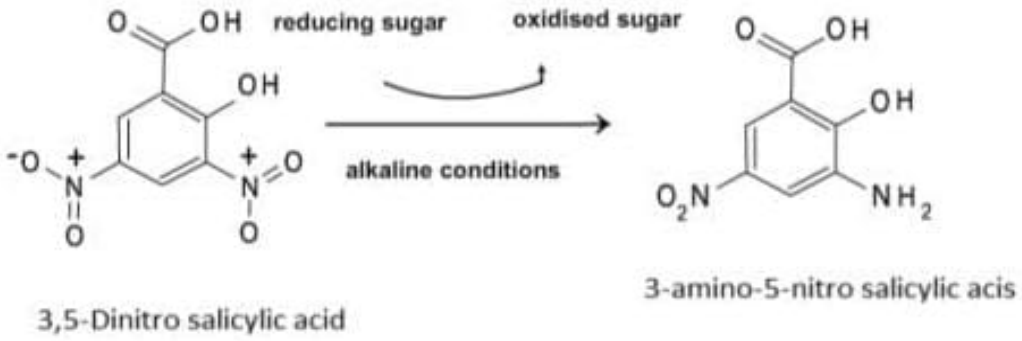
The α -amylase activity is measured using a colorimetric method with 3,5-dinitrosalicylic acid (DNS) reagent. In this method, starch by α-amylase is converted into maltose. Maltose released from starch (Barley grain) is measured by the reduction of 3,5-dinitrosalicylic acid.

Starch + H2O $\xrightarrow{\text{α-Amylase}}$ Maltose (reducing agent)





Maltose reduces the pale yellow coloured alkaline 3, 5-Dinitro salicylic acid (DNS) to the orange- red colored. The intensity of the color is proportional to the concentration of maltose present in the sample.



This intensity change in color is measured using a spectrophotometer as the absorbance at 540nm wavelength. Wave length is set to 540 nm because it is the region where orange-red color absorbs.This procedure applies to all products that have a specification for α-amylase.

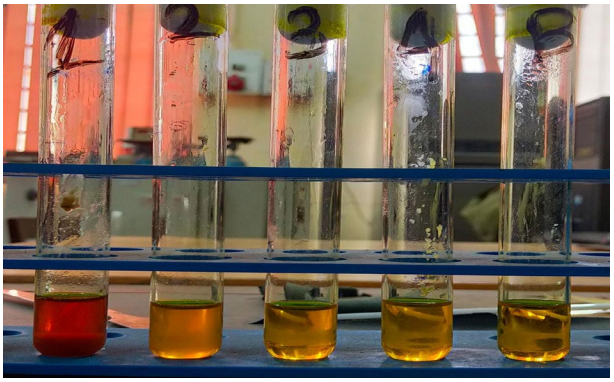


Fig 1.1 : Experimental Image for α -amylase activity measurement using a colorimetric method with 3,5-dinitrosalicylic acid (DNS) reagent.

- **OPTICAL DENSITY MEASUREMENT**

Sample ↓| Temperature →

DAY-0	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.179	1.207	1.239	1.326	1.625
S2	0.149	0.160	0.181	0.211	0.245
S3	0.031	0.032	0.034	0.036	0.037
S4	0.007	0.009	0.011	0.013	0.016
S5 (Blank)	0	0	0	0	0

Table 1.1 : DAY-0 Cotton cloths **covered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

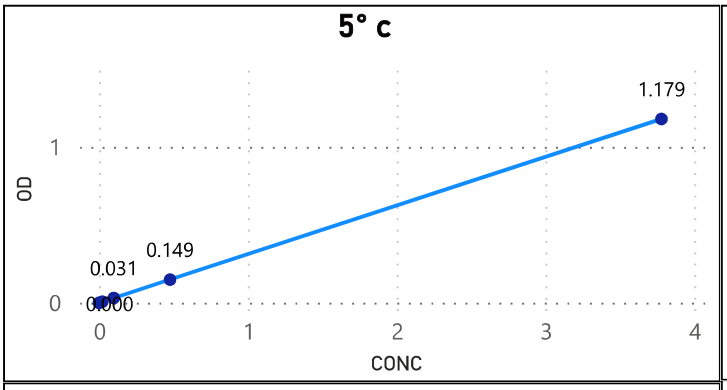
Sample ↓| Temperature →

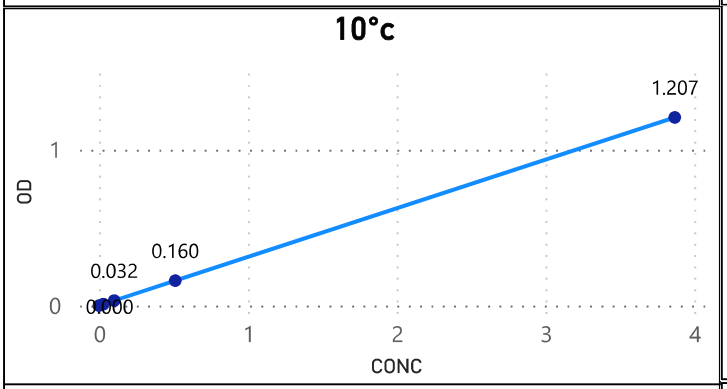
DAY-0	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.152	1.194	1.229	1.326	1.588
S2	0.136	0.152	0.175	0.189	0.227
S3	0.021	0.025	0.029	0.033	0.035
S4	0.004	0.007	0.010	0.012	0.016
S5 (Blank)	0	0	0	0	0

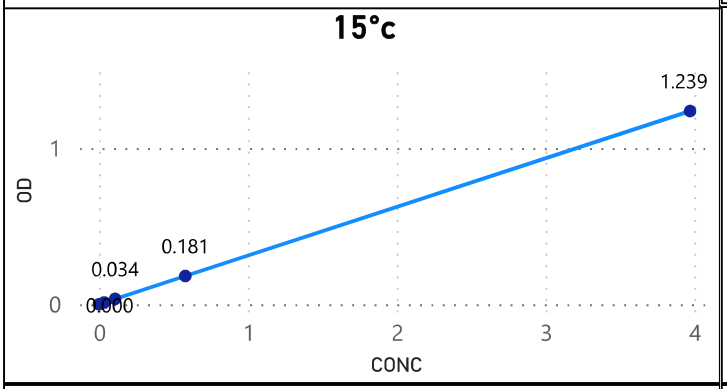
Table 1.2 : DAY-0 Cotton cloths **uncovered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

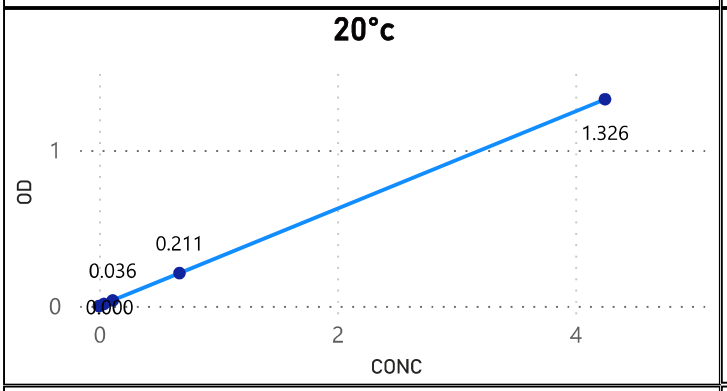
DAY 0

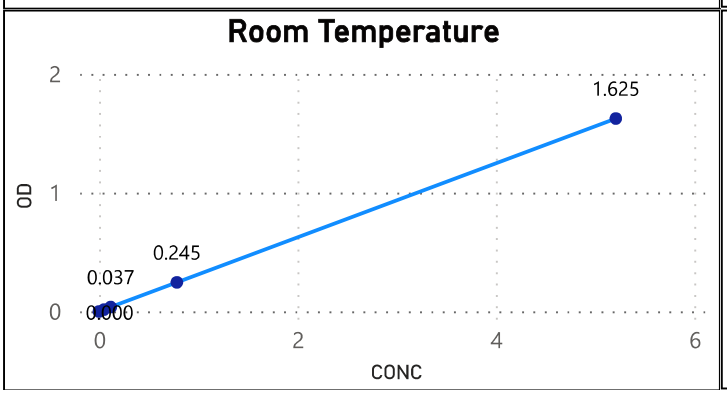
COVERED



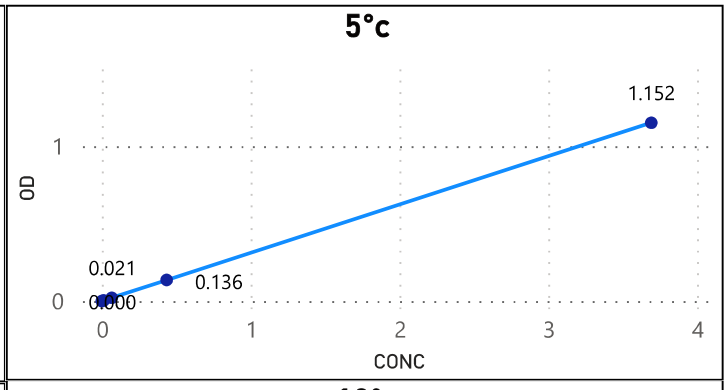


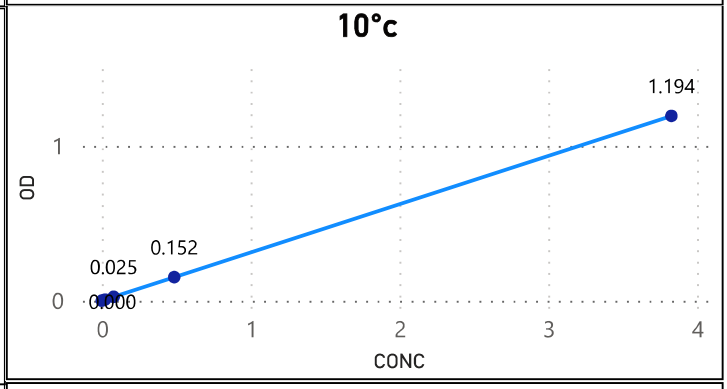


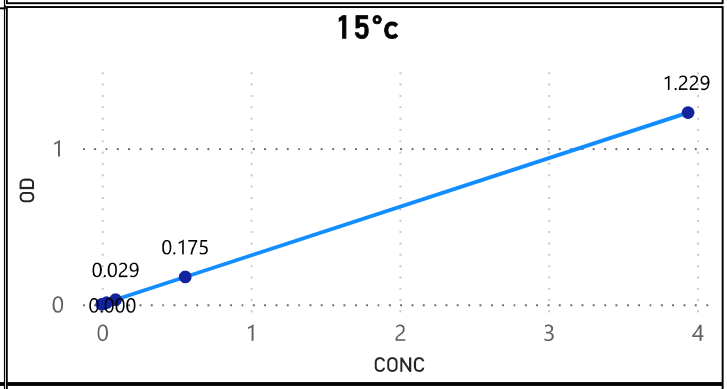


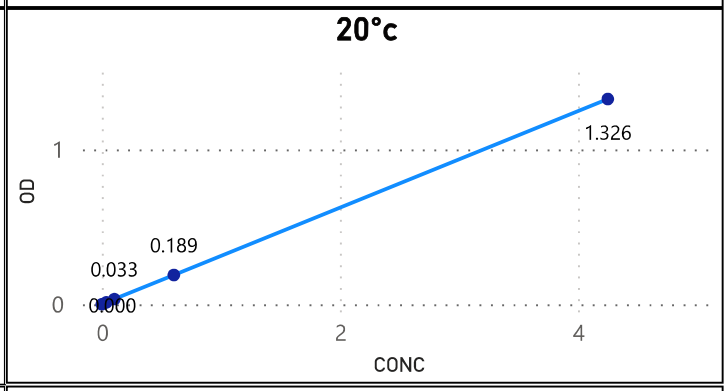


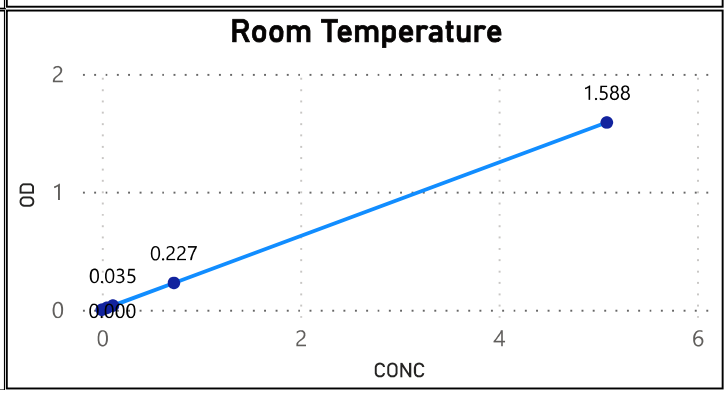
UNCOVERED











Sample ↓| Temperature →

DAY-1	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.179	1.207	1.239	1.714	2.355
S2	0.208	0.297	0.316	0.391	0.477
S3	0.021	0.032	0.039	0.049	0.060
S4	0.011	0.016	0.017	0.022	0.027
S5 (Blank)	0	0	0	0	0

Table 1.3 : DAY-1 Cotton cloths **covered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

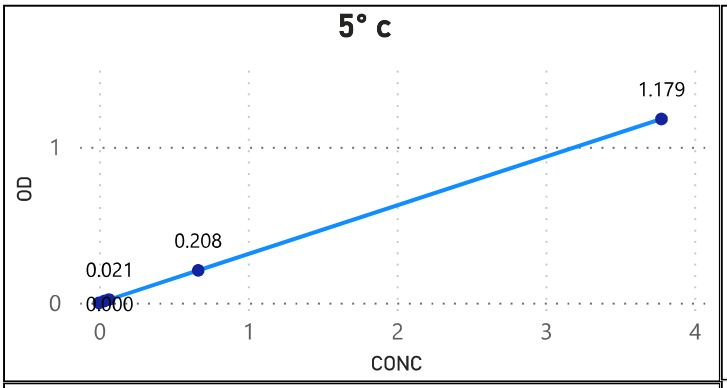
Sample ↓| Temperature →

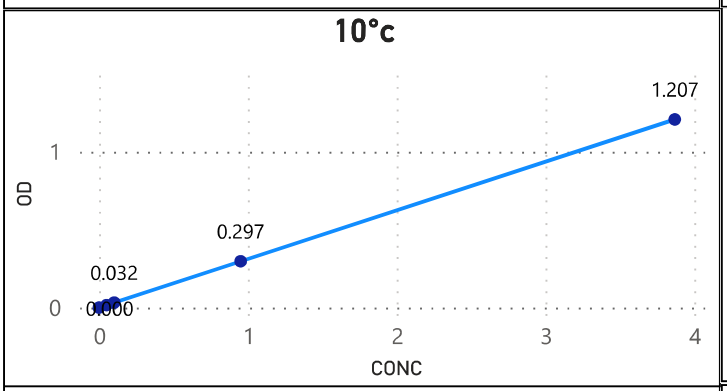
DAY-1	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.275	1.392	1.425	1.615	2.274
S2	0.173	0.212	0.228	0.257	0.366
S3	0.021	0.028	0.033	0.039	0.041
S4	0.009	0.014	0.016	0.020	0.025
S5 (Blank)	0	0	0	0	0

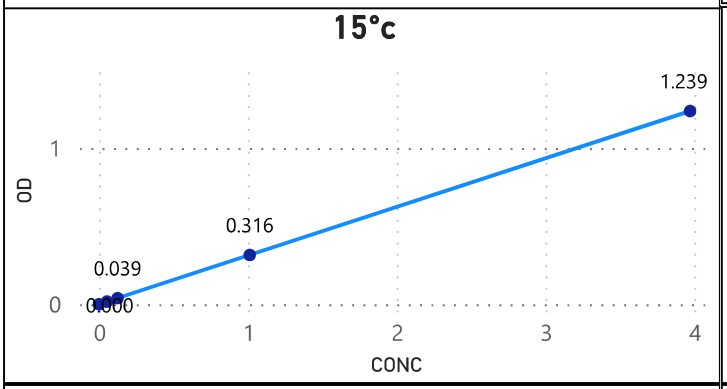
Table 1.4 : DAY-1 Cotton cloths **uncovered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

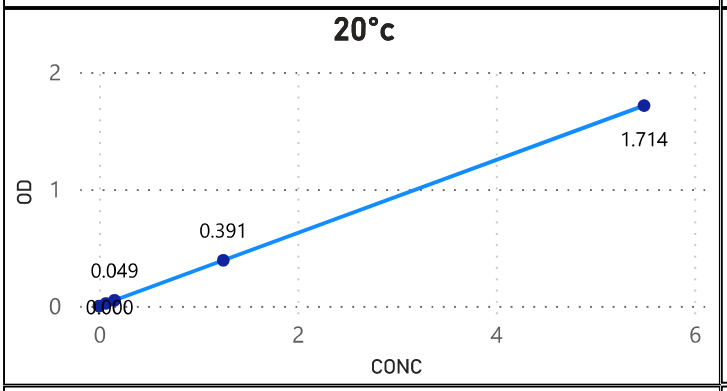
DAY 1

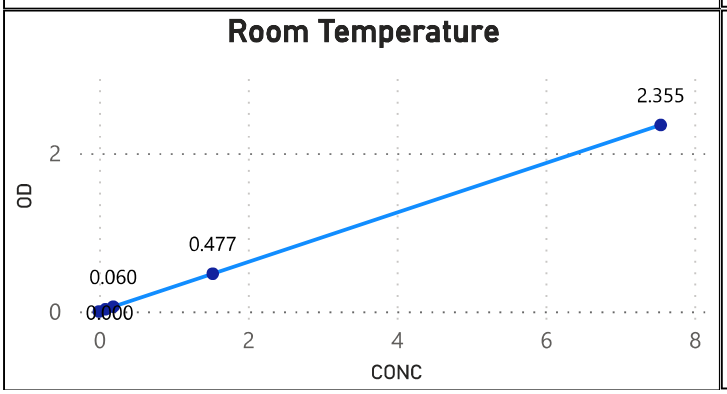
COVERED











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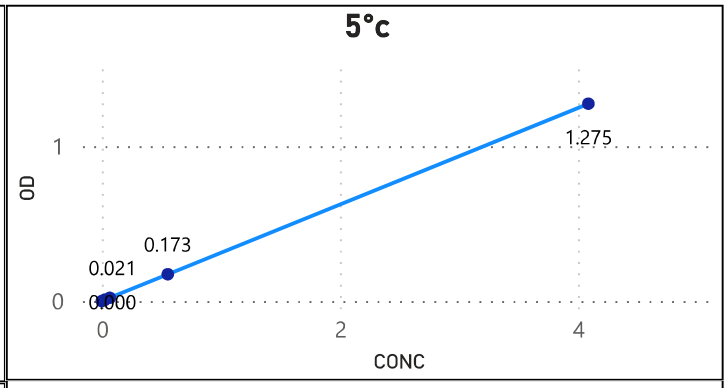
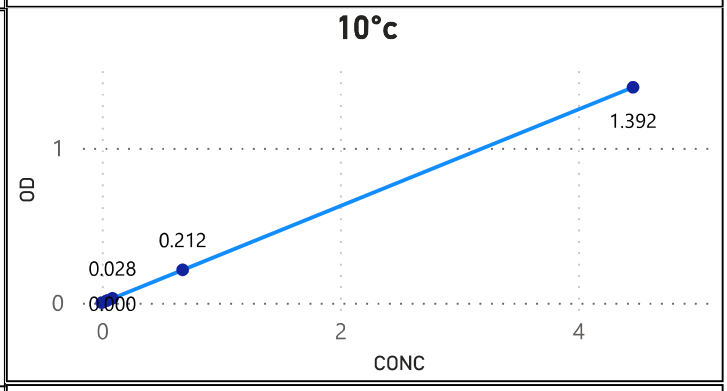
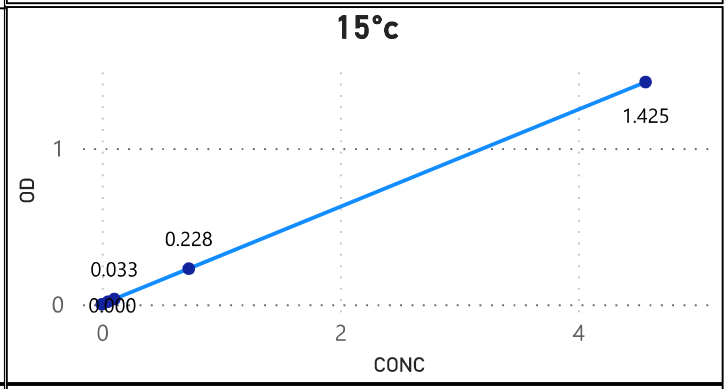
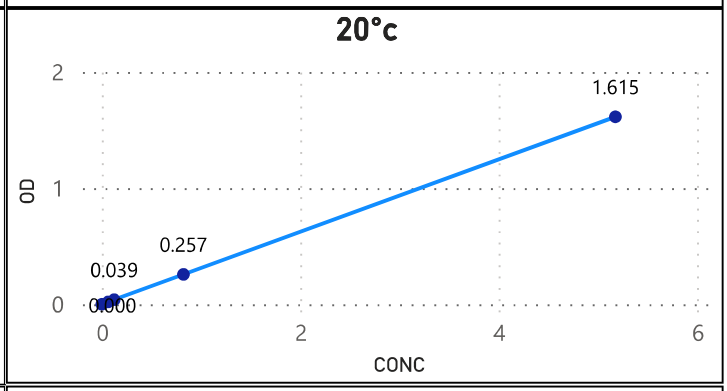
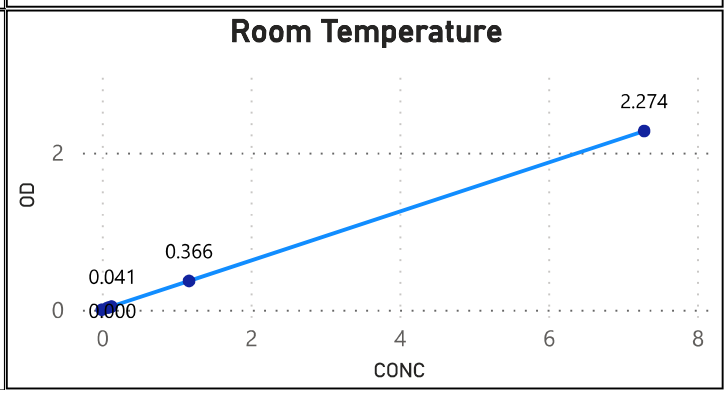










Sample ↓| Temperature →

DAY-2	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.294	1.415	1.637	1.876	2.355
S2	0.267	0.348	0.397	0.486	0.542
S3	0.035	0.039	0.043	0.052	0.069
S4	0.015	0.018	0.022	0.025	0.032
S5 (Blank)	0	0	0	0	0

Table 1.5 : DAY-2 Cotton cloths **covered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

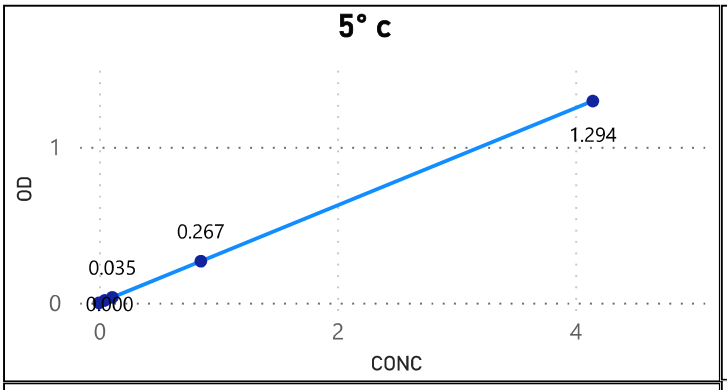
Sample ↓| Temperature →

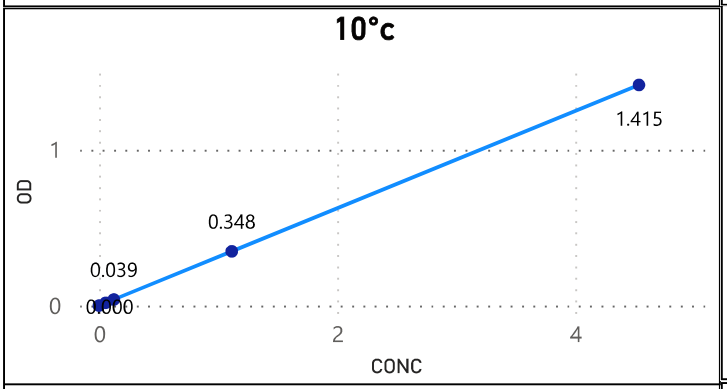
DAY-2	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.253	1.351	1.513	1.787	2.288
S2	0.211	0.248	0.267	0.317	0.443
S3	0.031	0.035	0.038	0.044	0.053
S4	0.014	0.017	0.018	0.021	0.029
S5 (Blank)	0	0	0	0	0

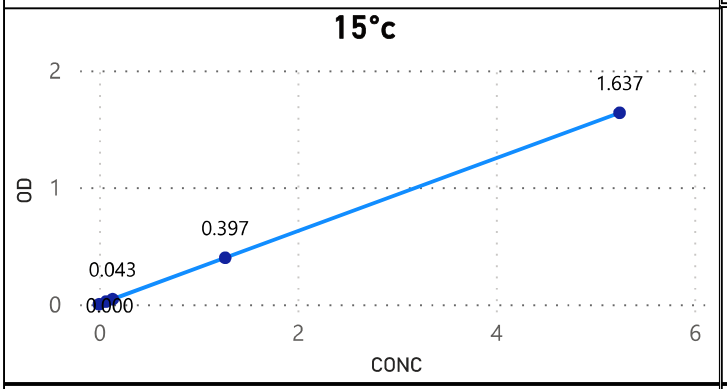
Table 1.6 : DAY-2 Cotton cloths **uncovered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

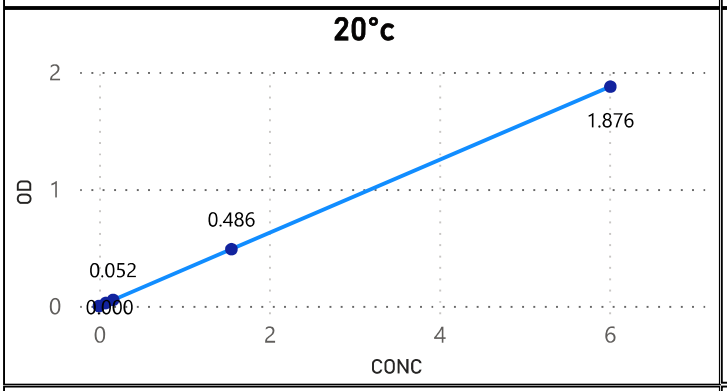
DAY 2

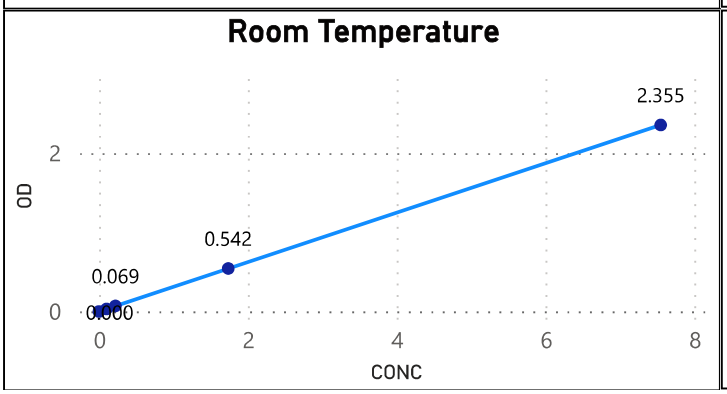
COVERED











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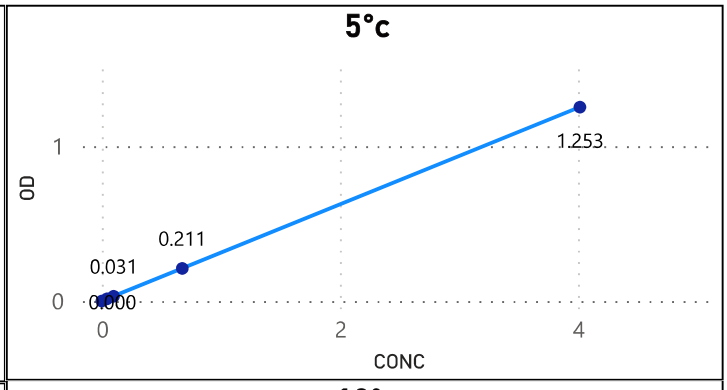
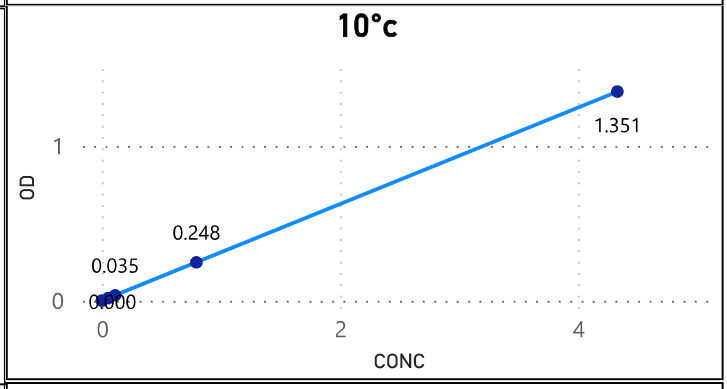
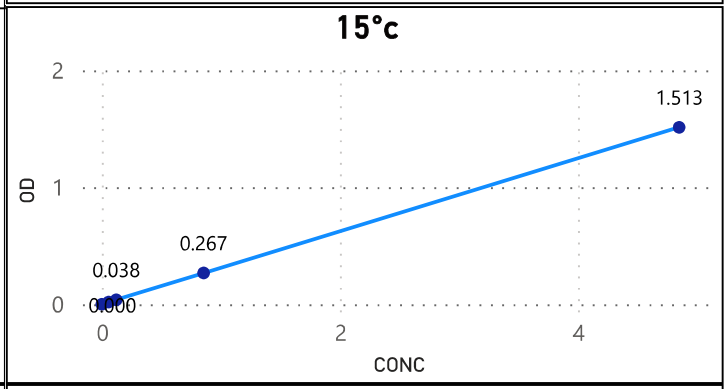
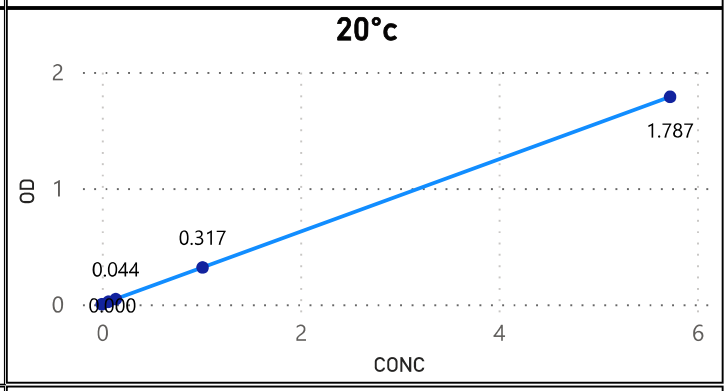
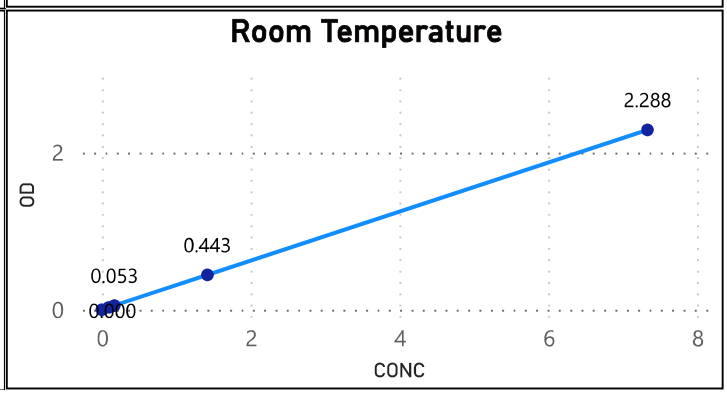










Sample ↓| Temperature →

DAY-3	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.357	1.523	1.738	2.125	2.667
S2	0.317	0.412	0.473	0.548	0.659
S3	0.038	0.046	0.058	0.096	0.140
S4	0.025	0.029	0.033	0.038	0.049
S5 (Blank)	0	0	0	0	0

Table 1.7 : DAY-3 Cotton cloths **covered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

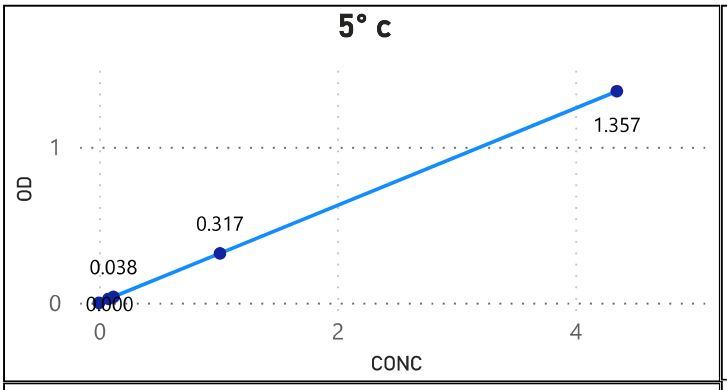
Sample ↓| Temperature →

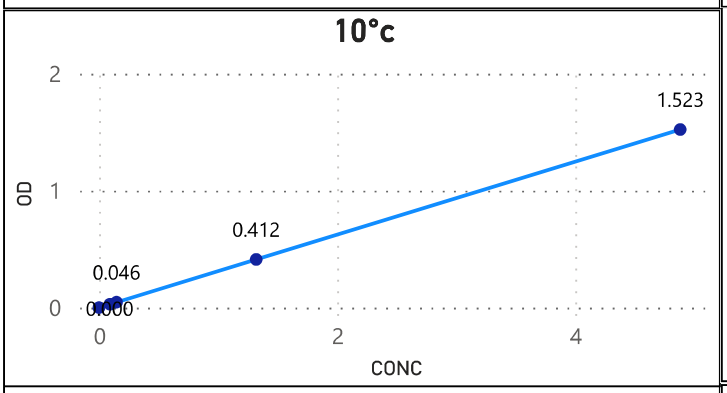
DAY-3	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.301	1.392	1.649	2.036	2.587
S2	0.277	0.335	0.356	0.403	0.553
S3	0.031	0.041	0.051	0.063	0.095
S4	0.017	0.020	0.025	0.029	0.038
S5 (Blank)	0	0	0	0	0

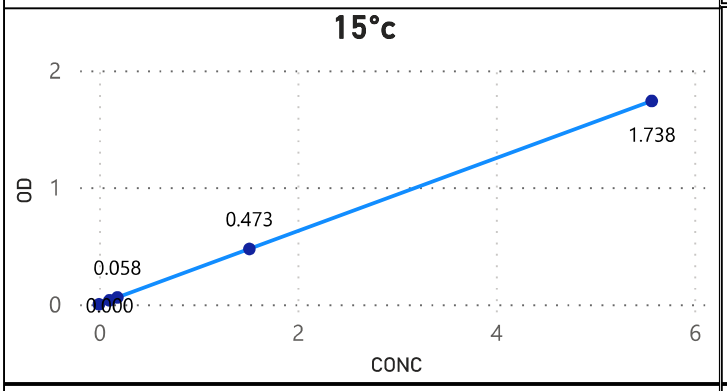
Table 1.8 : DAY-3 Cotton cloths **uncovered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

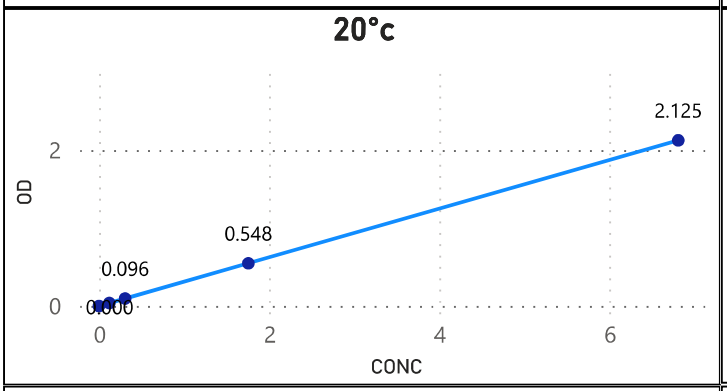
DAY 3

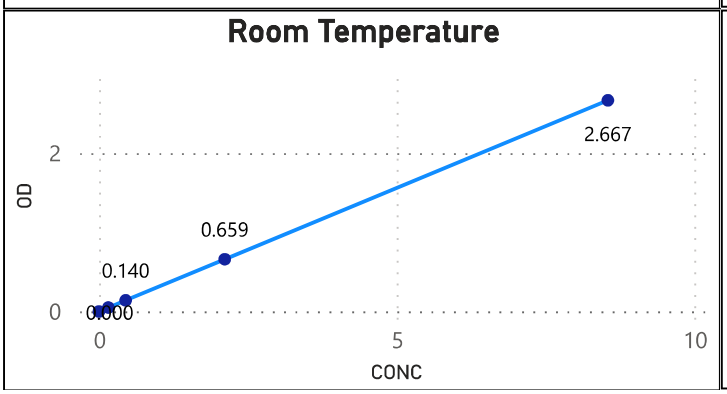
COVERED











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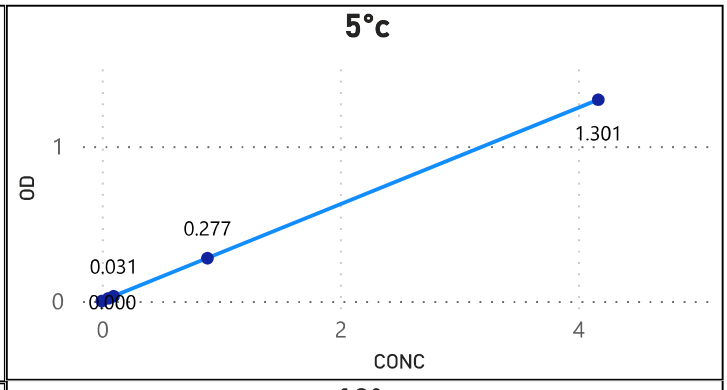
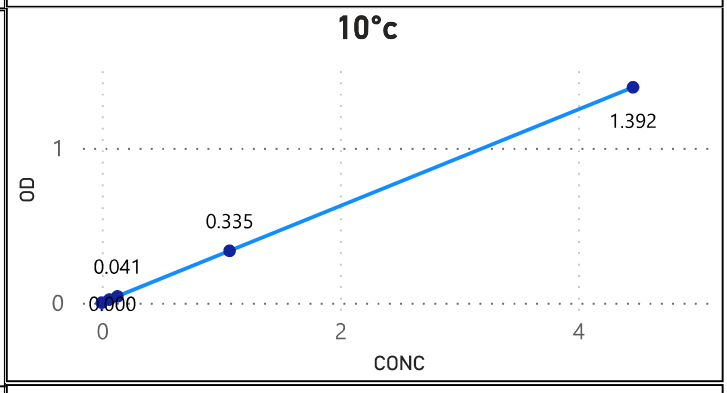
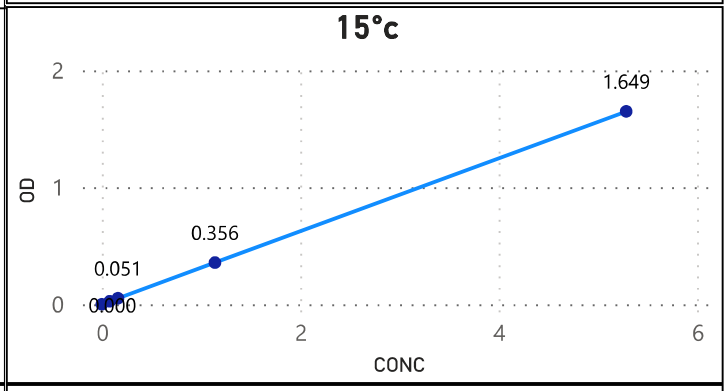
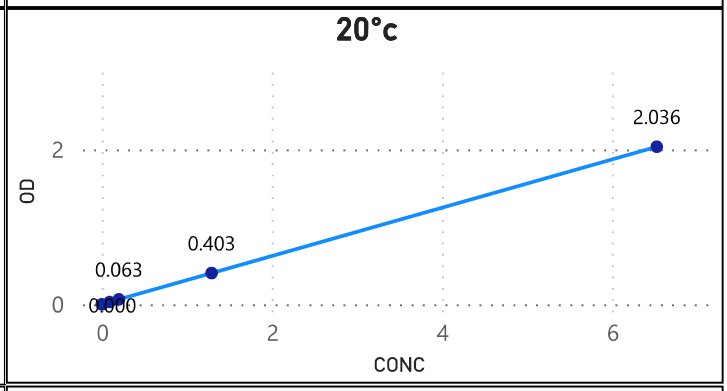
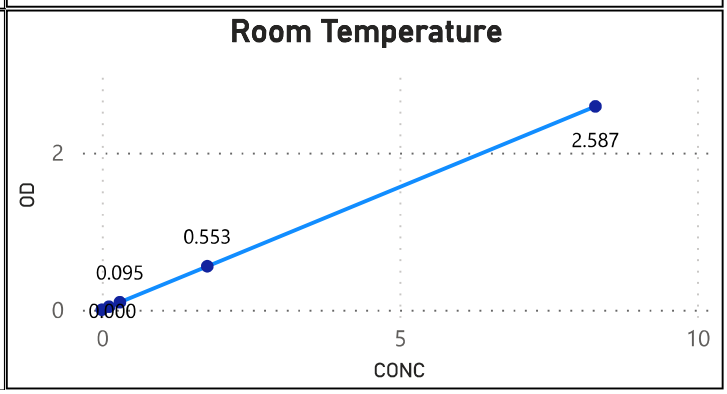










Sample ↓| Temperature →

DAY-4	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.611	1.814	2.131	2.626	3.125
S2	0.389	0.496	0.533	0.654	0.735
S3	0.042	0.049	0.064	0.111	0.194
S4	0.029	0.031	0.038	0.056	0.060
S5 (Blank)	0	0	0	0	0

Table 1.9 : DAY-4 Cotton cloths **covered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

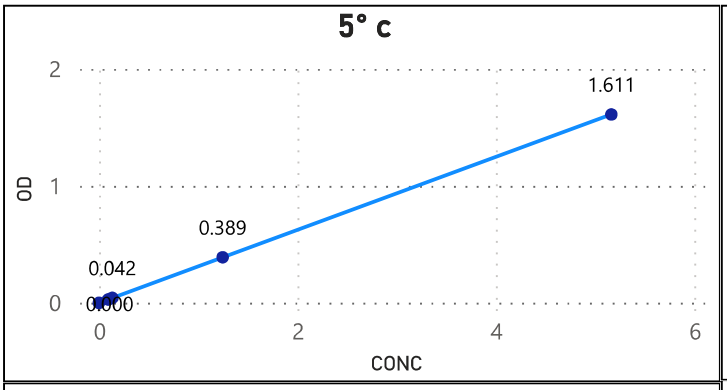
Sample ↓| Temperature →

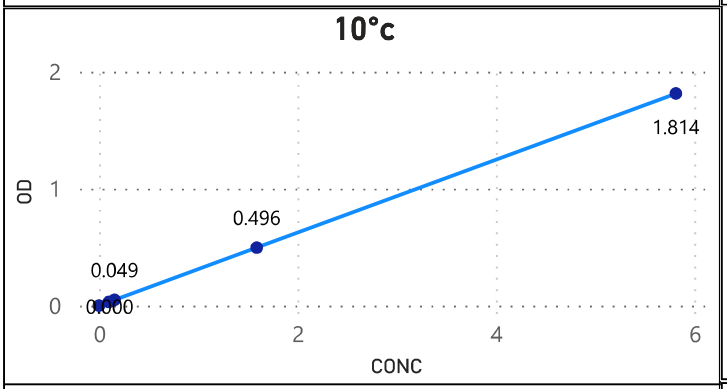
DAY-4	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.406	1.598	1.878	2.339	2.597
S2	0.352	0.426	0.492	0.540	0.648
S3	0.035	0.048	0.056	0.087	0.119
S4	0.023	0.027	0.029	0.036	0.040
S5 (Blank)	0	0	0	0	0

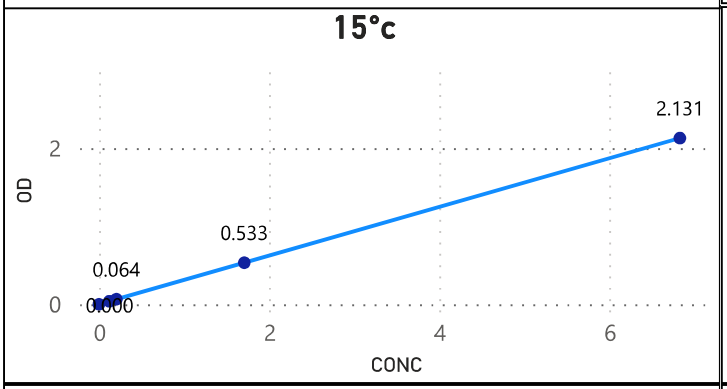
Table 1.10 : DAY-4 Cotton cloths **uncovered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

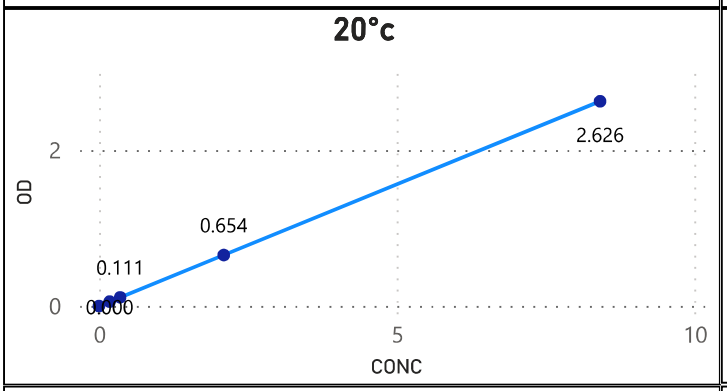
DAY 4

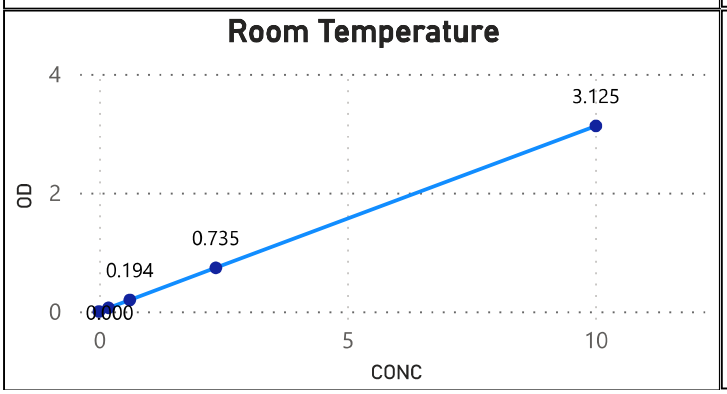
COVERED











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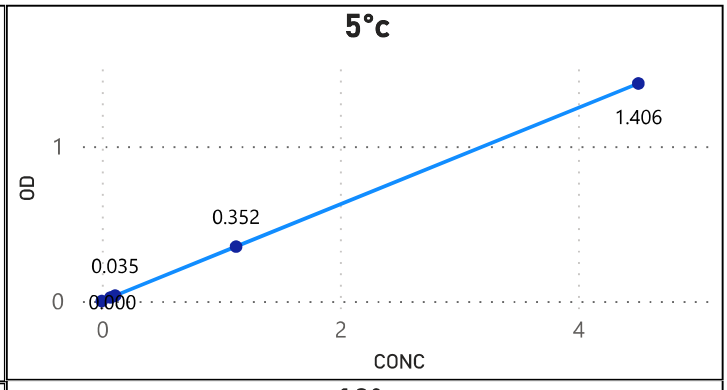
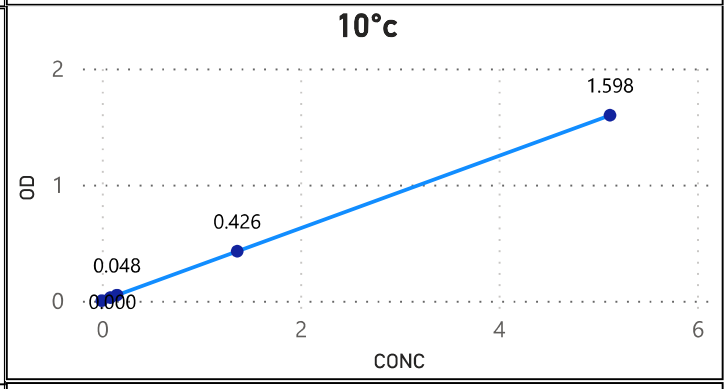
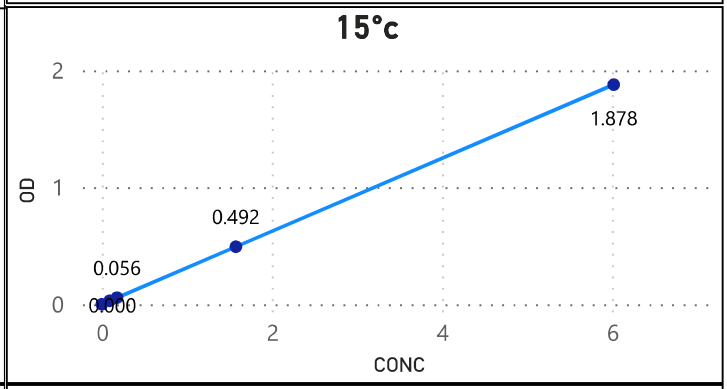
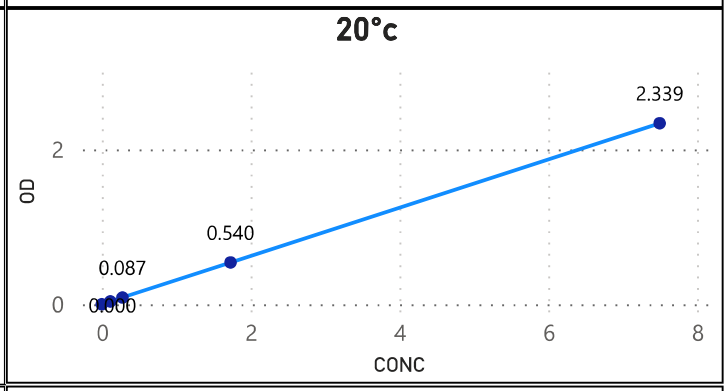
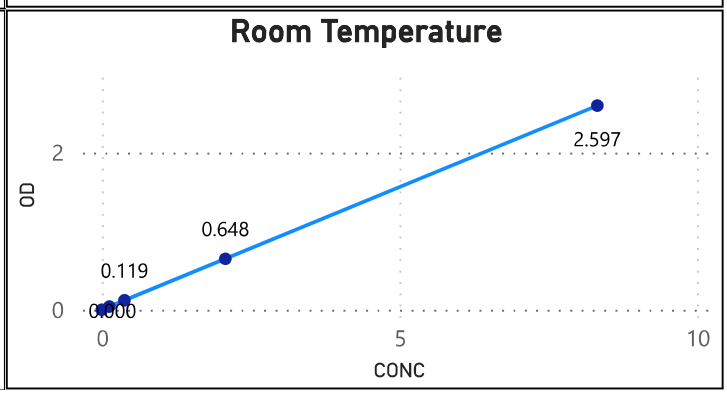










Sample ↓| Temperature →

DAY-5	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.973	2.157	2.430	2.913	3.822
S2	0.446	0.542	0.605	0.714	0.817
S3	0.046	0.053	0.079	0.136	0.201
S4	0.032	0.036	0.041	0.056	0.061
S5 (Blank)	0	0	0	0	0

Table 1.11 : DAY-5 Cotton cloths **covered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

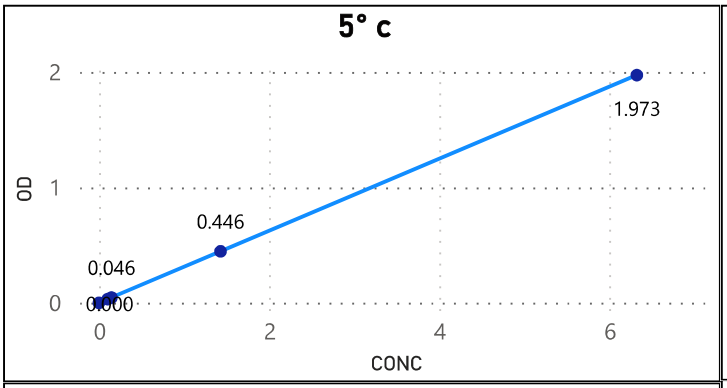
Sample ↓| Temperature →

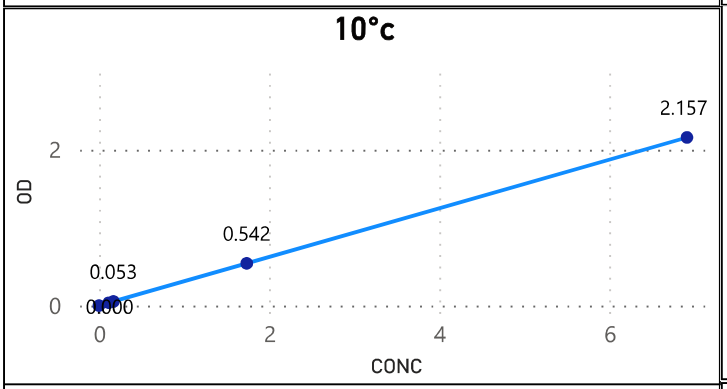
DAY-5	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.621	1.891	2.113	2.751	3.188
S2	0.399	0.467	0.542	0.629	0.785
S3	0.038	0.051	0.060	0.092	0.126
S4	0.025	0.028	0.035	0.039	0.052
S5 (Blank)	0	0	0	0	0

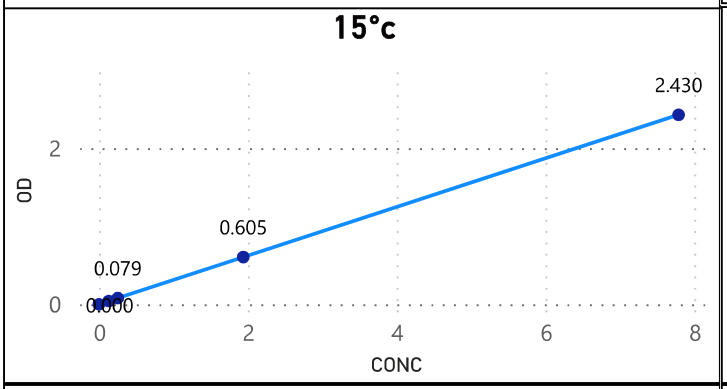
Table 1.12 : DAY-5 Cotton cloths **uncovered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

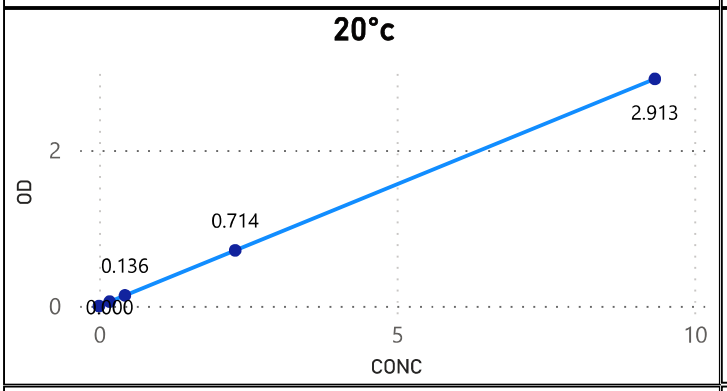
DAY 5

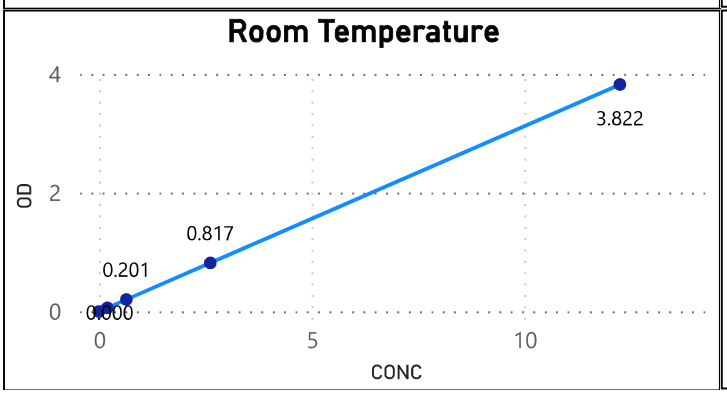
COVERED











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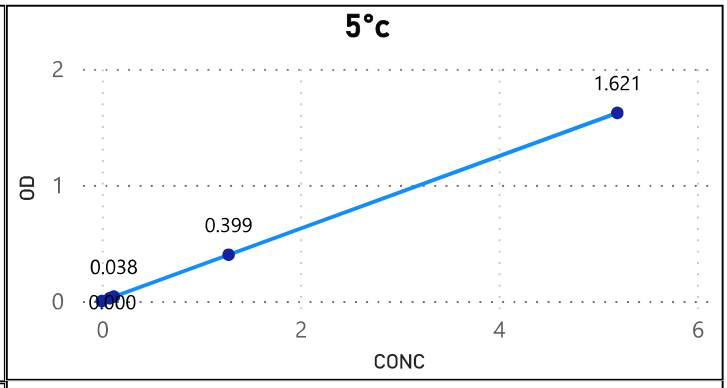
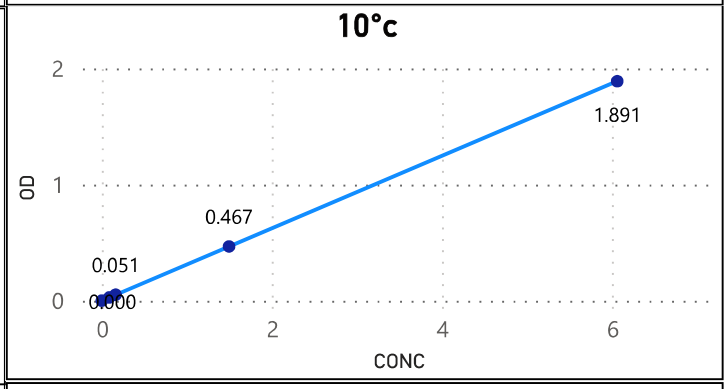
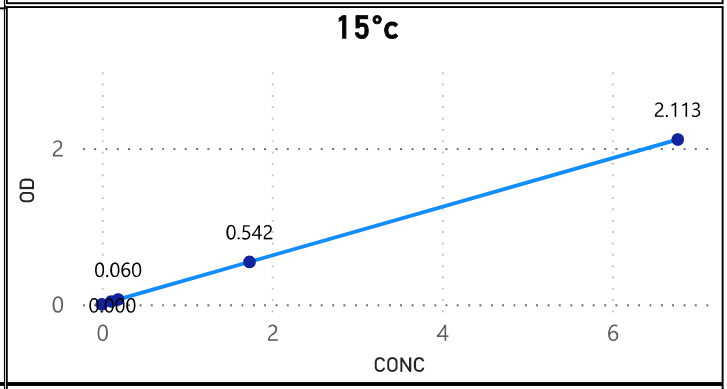
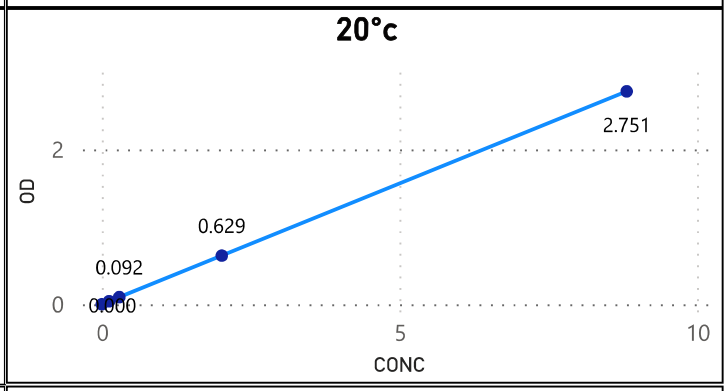
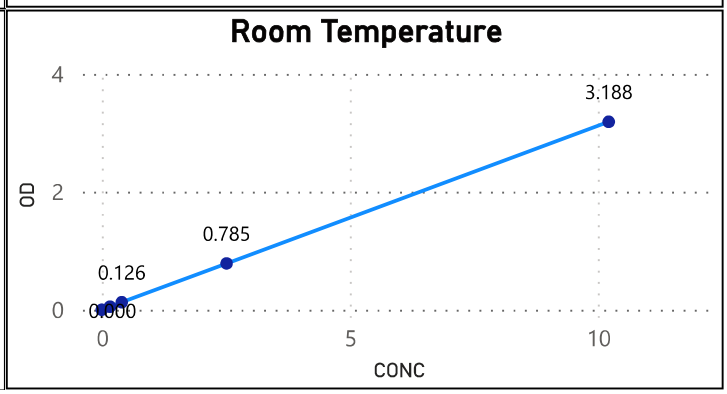










Sample ↓| Temperature →

DAY-6	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	2.109	2.476	2.891	3.285	X (where, X > 5)
S2	0.505	0.593	0.668	0.782	0.896
S3	0.049	0.061	0.097	0.185	0.263
S4	0.036	0.040	0.046	0.060	0.068
S5 (Blank)	0	0	0	0	0

Table 1.13 : DAY-6 Cotton cloths **covered Samples** Optical Density (O.D.) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

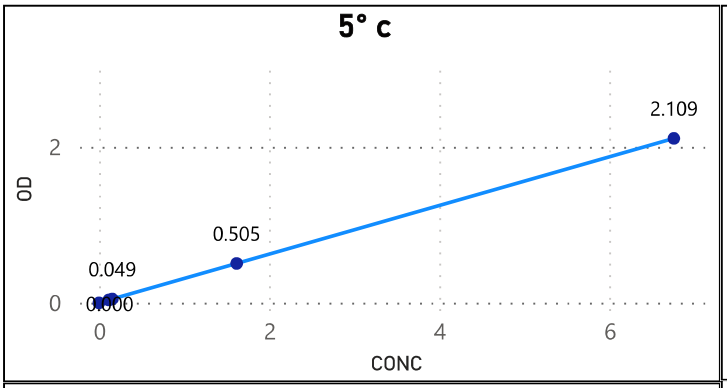
Sample ↓| Temperature →

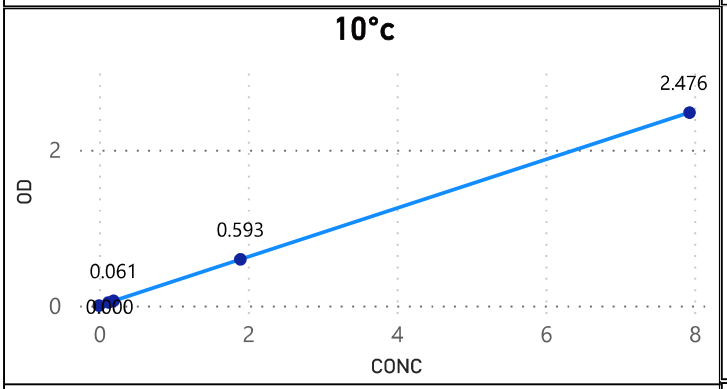
DAY-6	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	1.958	2.194	2.452	2.968	3.916
S2	0.438	0.513	0.616	0.719	0.827
S3	0.041	0.055	0.072	0.103	0.148
S4	0.028	0.030	0.038	0.040	0.060
S5 (Blank)	0	0	0	0	0

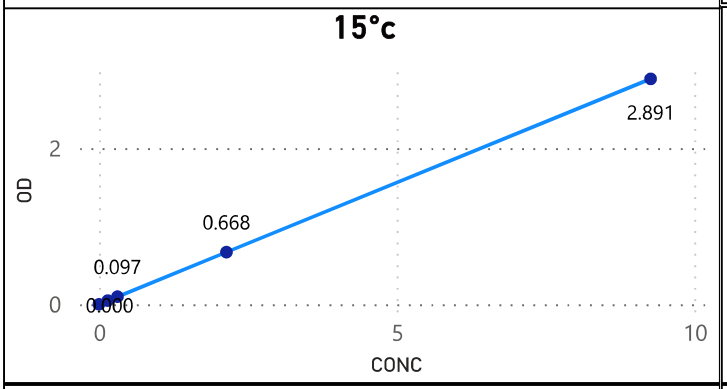
Table 1.14 : DAY-6 Cotton cloths **uncovered Samples** Optical Density (O.D.) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

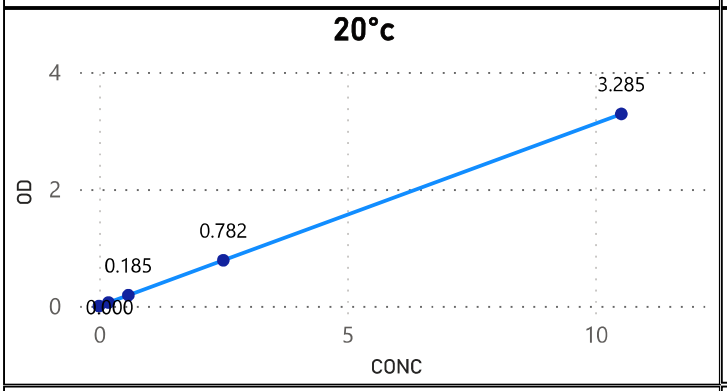
DAY 6

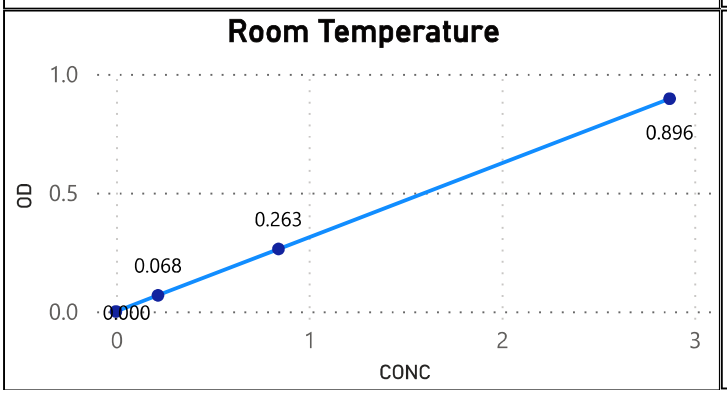
COVERED











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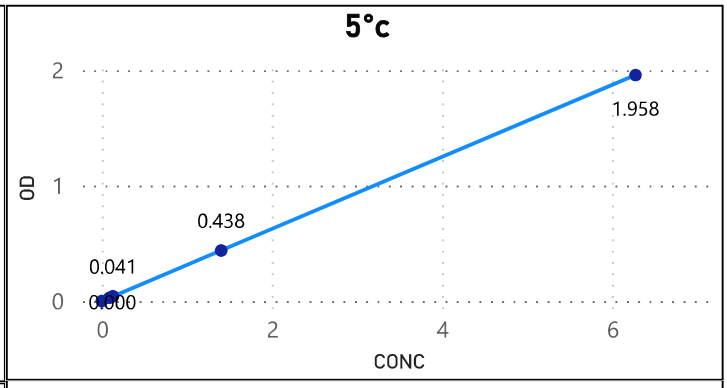
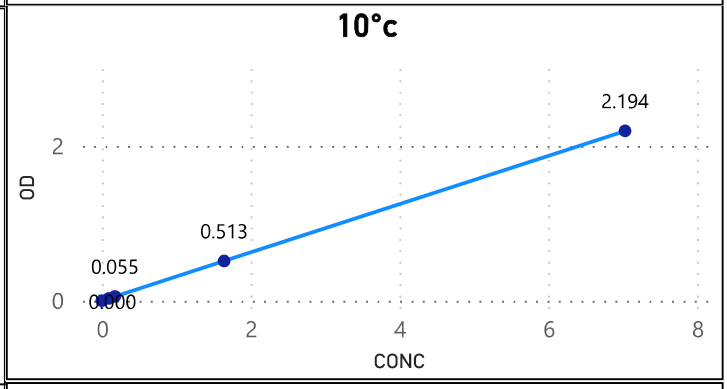
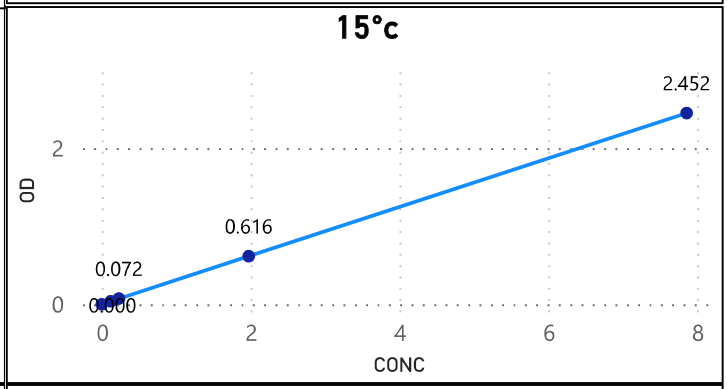
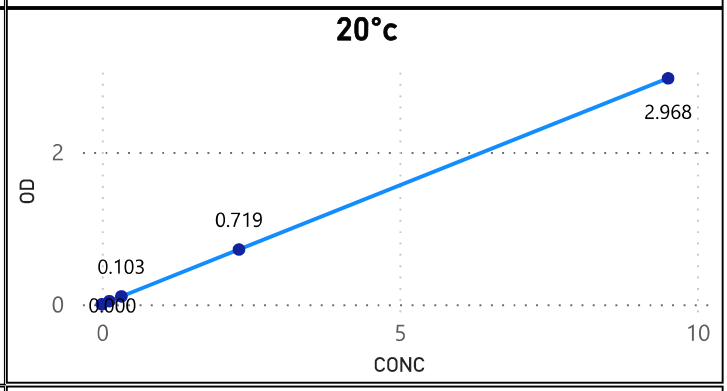
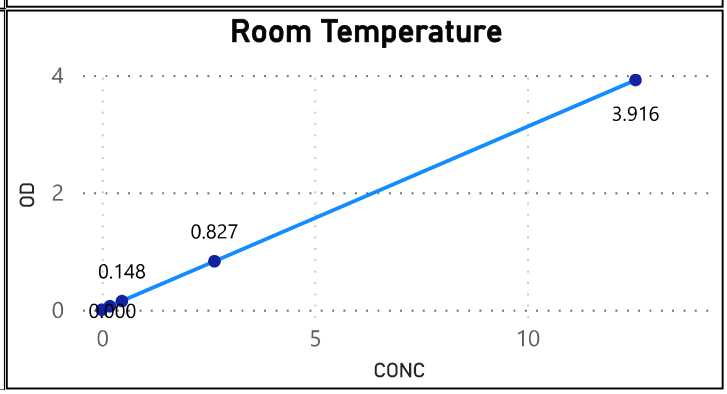










Sample ↓| Temperature →

DAY-7	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	2.394	2.708	3.114	3.862	4.735
S2	0.568	0.651	0.725	0.842	0.872
S3	0.051	0.066	0.112	0.202	0.251
S4	0.038	0.042	0.053	0.063	0.065
S5 (Blank)	0	0	0	0	0

Table 1.15 : DAY-7 Cotton cloths **covered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

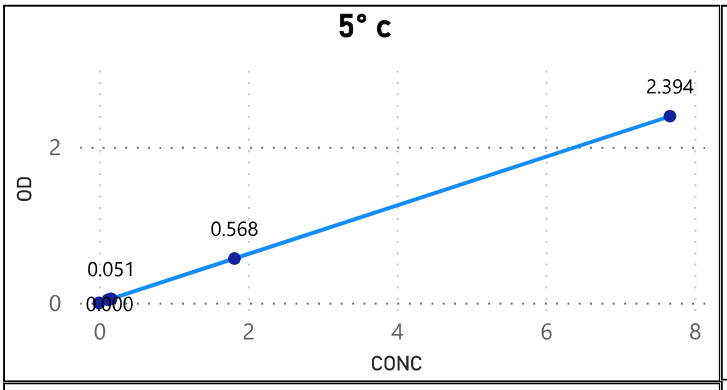
Sample ↓| Temperature →

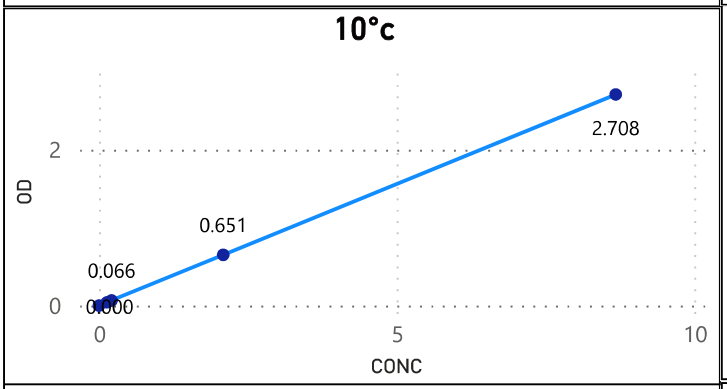
DAY-7	5°C	10°C	15°C	20°C	RT (Room Temp.)
S1	2.216	2.436	2.954	3.227	4.496
S2	0.475	0.553	0.672	0.788	0.839
S3	0.045	0.056	0.075	0.110	0.152
S4	0.030	0.032	0.040	0.042	0.062
S5 (Blank)	0	0	0	0	0

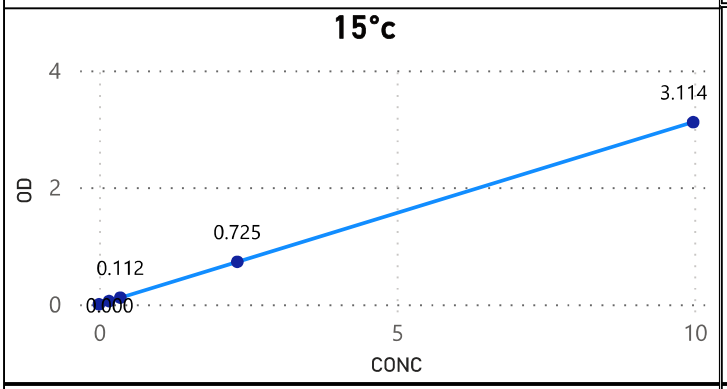
Table 1.16 : DAY-7 Cotton cloths **uncovered Samples** Optical Density (OD) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm.

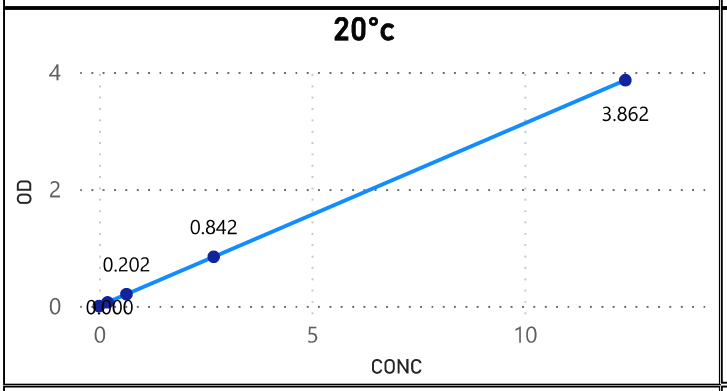
DAY 7

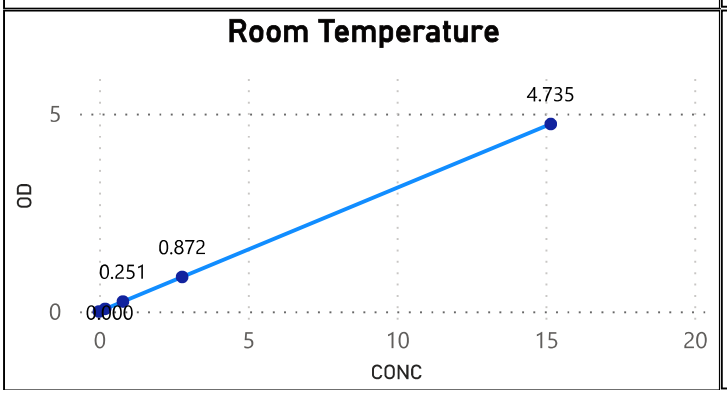
COVERED











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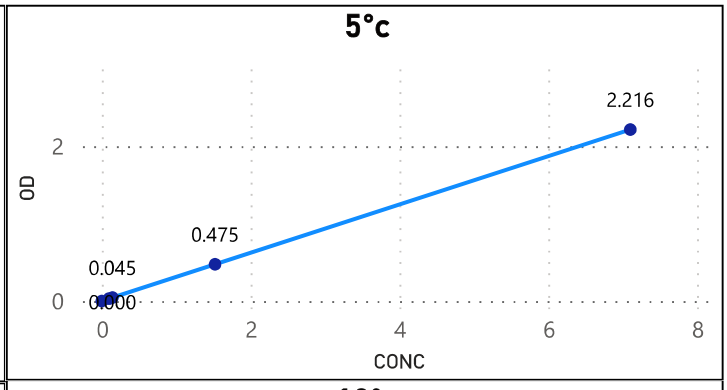
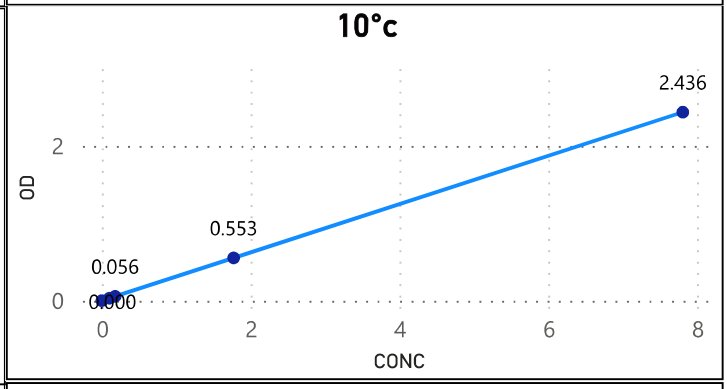
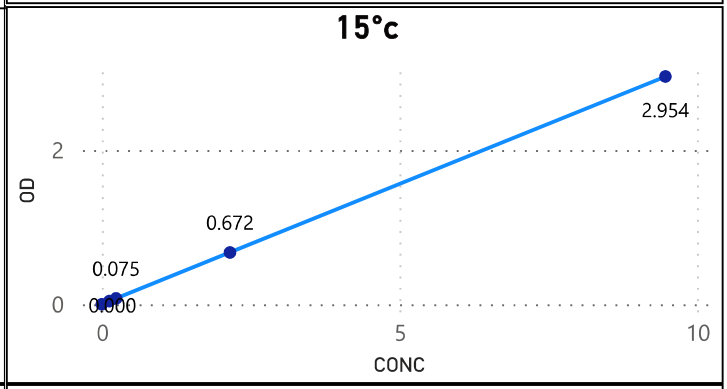
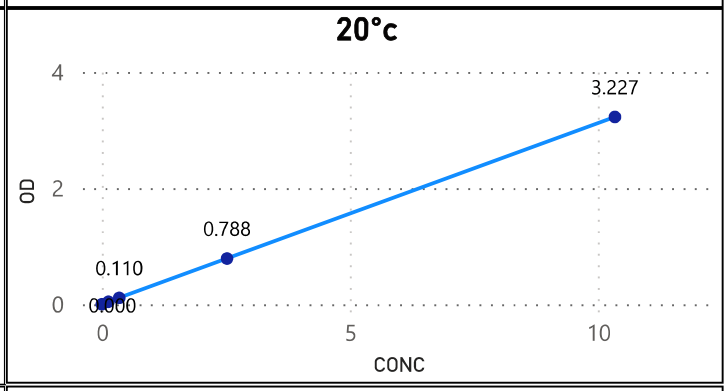
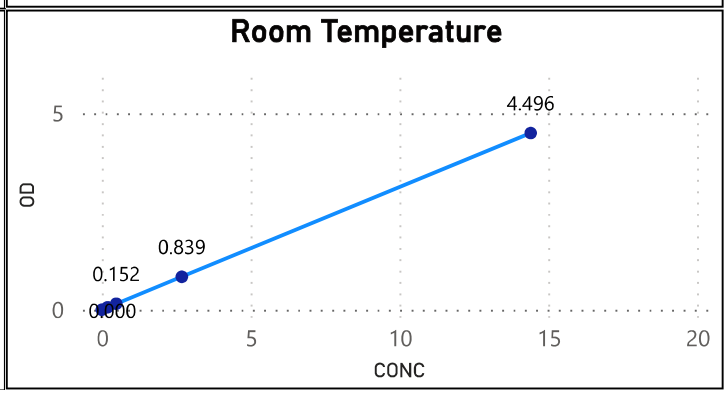










- **Enzymatic Activities**

Temperature (°C)	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
5	3.983	5.558	7.133	8.467	10.392	11.908	13.492	15.175
10	4.275	7.933	9.292	11.008	13.250	14.475	15.842	17.392
15	4.833	8.442	10.600	12.633	14.233	16.159	17.842	19.367
20	5.633	10.442	12.983	14.633	17.467	19.067	20.883	22.492
RT (Room Temp.)	6.542	12.742	14.475	17.600	19.633	21.825	23.934	23.292

Table 1.17 : DAY-0 to DAY-7 Enzymatic Activity Values(Unit/mol) for **covered** samples at different temperature in respect of standard O.D. value.

Temperature (°C)	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
5	3.633	4.617	5.633	6.067	6.733	7.983	9.025	10.008
10	4.058	5.658	6.625	7.608	8.708	9.800	11.033	12.100
15	4.750	6.092	7.134	8.175	9.133	10.733	11.908	13.142
20	5.050	6.867	8.467	9.425	10.417	11.458	12.608	13.650
RT (Room Temp.)	6.067	9.775	11.832	13.434	14.632	15.625	15.384	14.392

Table 1.18 : DAY-0 to DAY-7 Enzymatic Activity Values (Unit/mol) for **uncovered** samples at different temperature in respect of standard O.D. value.

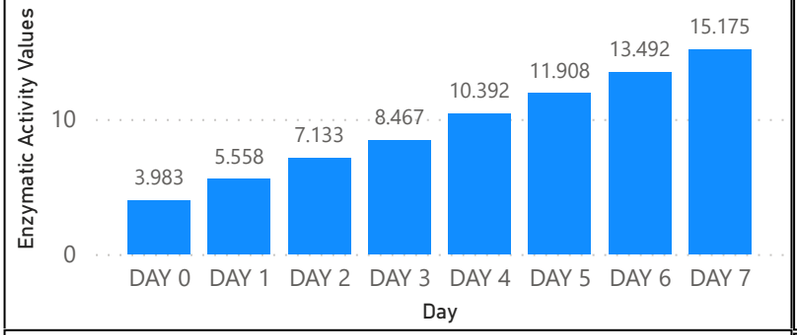
According to Table 1.17, we've seen that enzymatic activities are exponentially increasing day by day And as well as temperatures increased the enzymatic activities are also increased. At room temperature the highest enzymatic activity occurs at Day 6 and after that it's decreased on Day 7.

According to Table 1.18, we've seen that enzymatic activities are exponentially increasing day by day And as well as temperatures increased the enzymatic activities are also increased. At room temperature the highest enzymatic activity occurs at Day 5 and after that it's also decreased liked covered samples on Day 6 and On The Day 7 the enzymatic activity decreases from Day 6.

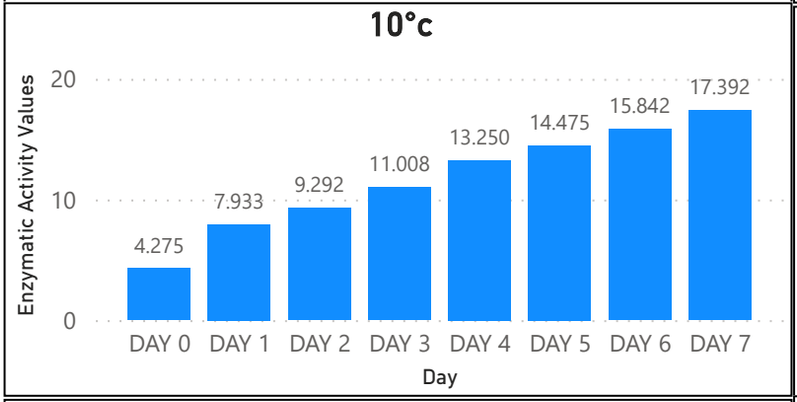
Enzymatic Activities

COVERED

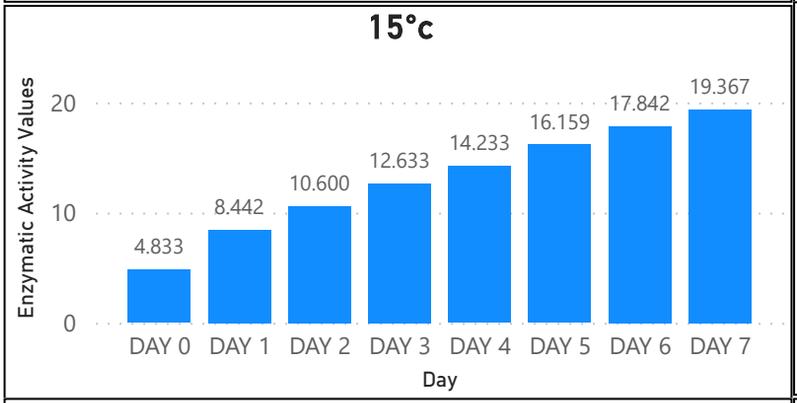
5°c



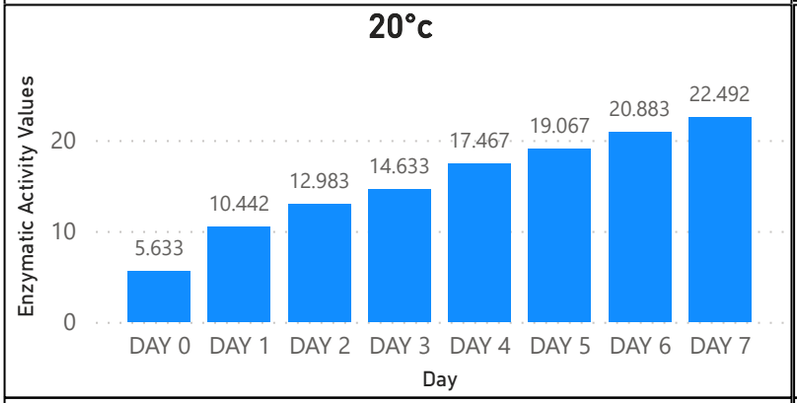
10°c



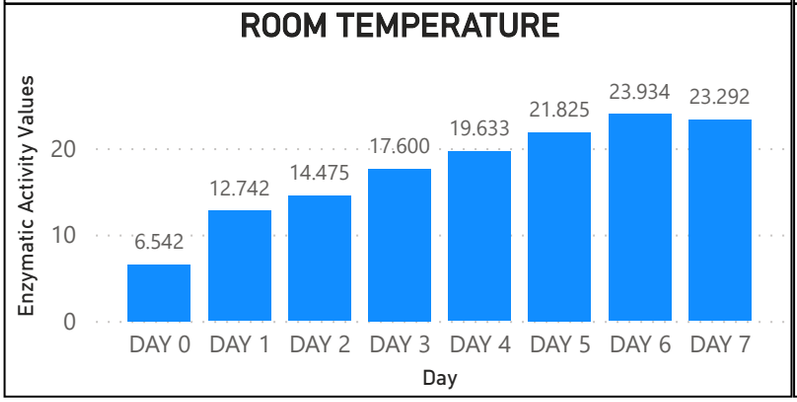
15°c



20°c

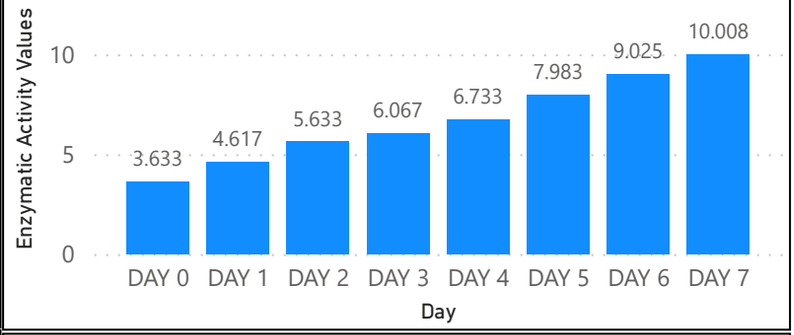


ROOM TEMPERATURE

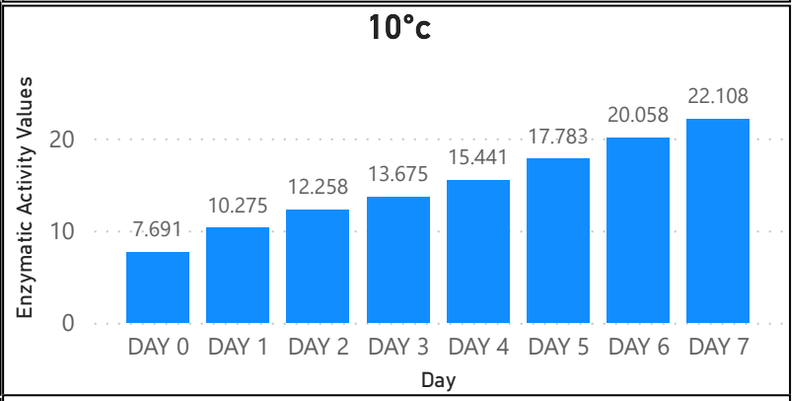


UNCOVERED

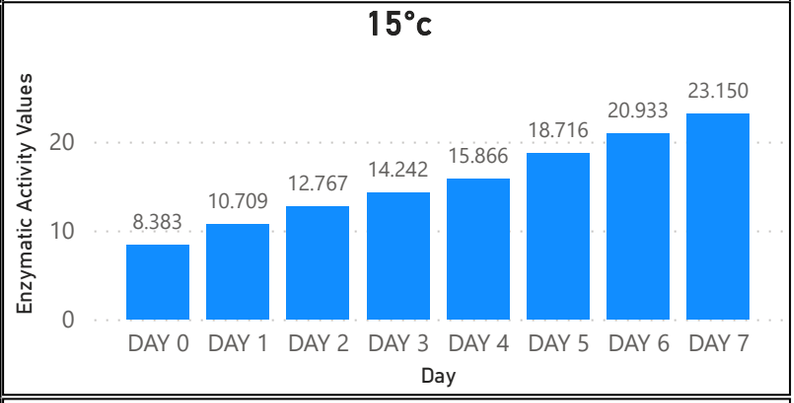
5°c



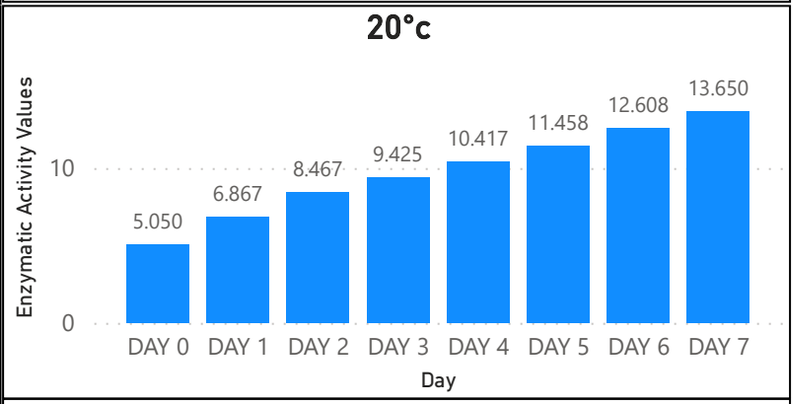
10°c



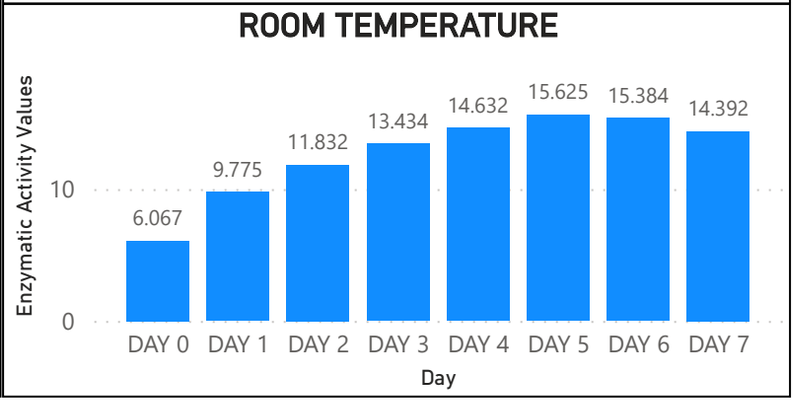
15°c



20°c



ROOM TEMPERATURE



1.4 CONCLUSION

α-amylase enzymatic activities are depends in term of Temperature of the germinating barley seeds and as well as it's depends on the oxygen availability on air. If temperature increased then the enzymatic activities are also increased till enzymatic activity reached it's highest limit. After that, temperature and germination day doesn't matter, it's gradually decreased.

CHAPTER 2

2.1 INTRODUCTION

Protein bodies are membrane-bound cellular organelles that contain storage proteins located in the cereal starchy endosperm. These macromolecules undergo modifications during the germination process due to the enzymatic activity of proteases. Protein degradation in plants involves a series of metabolic pathways, different subcellular compartments and different peptidases such as cysteine, serine, aspartic, and metalloproteases. During germination, the activity of endopeptidases is limited, which results in the formation of intermediates and then degrade in carboxypeptidases to amino acids, which are necessary for the growth of the seedlings. Later, proteins are synthesized during seed maturation and deposited in the protein bodies. Proteases are not only important in the hydrolysis of storage proteins, they are also responsible for the post-translational modifications, activation, and inactivation of enzymes and defense of plants.
This study examines Proteolytic activities in the germinated barley seeds at different temperatures.

2.2 MATERIALS & METHODS

2.2.1 MATERIALS

1. Germinated Barley Seeds
2. Tris-Hcl Buffer
3. Casein
4. Trichloroacetic acid
5. Whatman No. 1 filter paper
6. Seed Germinator
7. Spectrophotometer
8. Test Tubes
9. Petri Dishes
10. Beakers
11. Mortar-Pestle

2.2.2 METHODOLOGY

- **Barley seeds germination**

1. Wash whole barley grain until the water clears, and the seeds are divided into Five Petri dishes.
2. Add 100ml of water to the washed grain of each Petri dish and keep it soaked for 8 hrs.
3. After 8hrs, drain the water & remove the grain into Five different cotton cloths and, tie the cloths, add it to a bowl of water, and squeeze the water by hand.
4. Sprinkle water in between the seeds on the cloth, Five cotton cloths were stored in a seed germinator at five different temperatures, such as 5°C, 10°C, 15°C, 20°C, and Room Temperature, for a few minutes.
5. After a few minutes, some seeds were taken (both Covered and Uncovered) as a DAY-0 barley seeds sample.
6. After 24 hrs, some seeds were taken as a DAY-1 barley seeds sample.
7. After 48 hrs, some seeds were taken as a DAY-2 barley seeds sample.
8. After 72 hrs, some seeds were taken as a DAY-3 barley seeds sample.
9. After 96 hrs, some seeds were taken as a DAY-4 barley seeds sample.
10. After 120 hrs, some seeds were taken as a DAY-5 barley seeds sample.
11. After 144 hrs, some seeds were taken as a DAY-6 barley seeds sample.
12. After 168 hrs, some seeds were taken as a DAY-7 barley seeds sample.

- **Protease Assay**

Protease activity was measured by the method described by Kembhavi et al. Briefly, 0.5ml stock solution (1g germinated seeds paste suitably soluble in 100ml distilled water) was mixed with 0.5ml of 100mM Tris-Hcl Buffer (pH 8.0) containing 10g/l casein. The reaction mixture was incubated for 30 minutes at 37°C and stopped by adding 0.5ml of 20% trichloroacetic acid. The mixture was allowed to stand at room temperature for 15 minutes and then filtered through Whatsman no.1 filter paper. Then the O.D. of this filtrate was estimated spectrophotometrically at 280nm using HITACHI U-2000 spectrophotometer.

The proteolytic unit was defined as the amount of enzyme that released 1µ mol of tyrosine per minute under the assay conditions which expresses as U/gds (Unit per gram dry substrate).

1. Make a smooth paste of DAY-0 to DAY-7 collected samples.
2. Mix 1g of paste in 100ml distilled water.
3. Take 0.5ml of the mixed stock solution

Next steps were followed by the Protease ASSAY

2.3 RESULTS & DISCUSSION

- **Optical Density Measurement**

Temperature (°C)	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
5	0.013	0.014	0.014	0.015	0.015	0.016	0.016	0.018
10	0.016	0.016	0.017	0.018	0.019	0.019	0.020	0.021
15	0.018	0.018	0.020	0.021	0.022	0.023	0.025	0.027
20	0.021	0.021	0.022	0.024	0.025	0.026	0.028	0.030
RT (Room Temp.)	0.022	0.023	0.025	0.026	0.028	0.029	0.031	0.032

Table 2.1 : DAY-0 to DAY-7 Samples Optical Density (O.D.) Value was measured spectrophotometrically (Hitachi 2000, Japan) at 280 nm.

The change in absorbance in the test samples is determined by calculating the difference between the test sample absorbance and the absorbance of the test blank. Inserting the absorbance value for one of the test samples into the slope equation and solving will result in the micromoles of tyrosine liberated during this particular proteolytic reaction. Micromoles of tyrosine divided by time in minutes gives us our measurement of protease activity that we call units. We can cancel out the units for volume measurement in the numerator and denominator, and are hence left with a measurment of enzyme activity in terms of units/mL. To determine the activity in a solid protease sample diluted in enzyme diluent, divide activity in units/mL by the

$$\text{Units/mg solid} = \frac{\text{Units/mL enzyme}}{\text{mg solid/mL enzyme}}$$

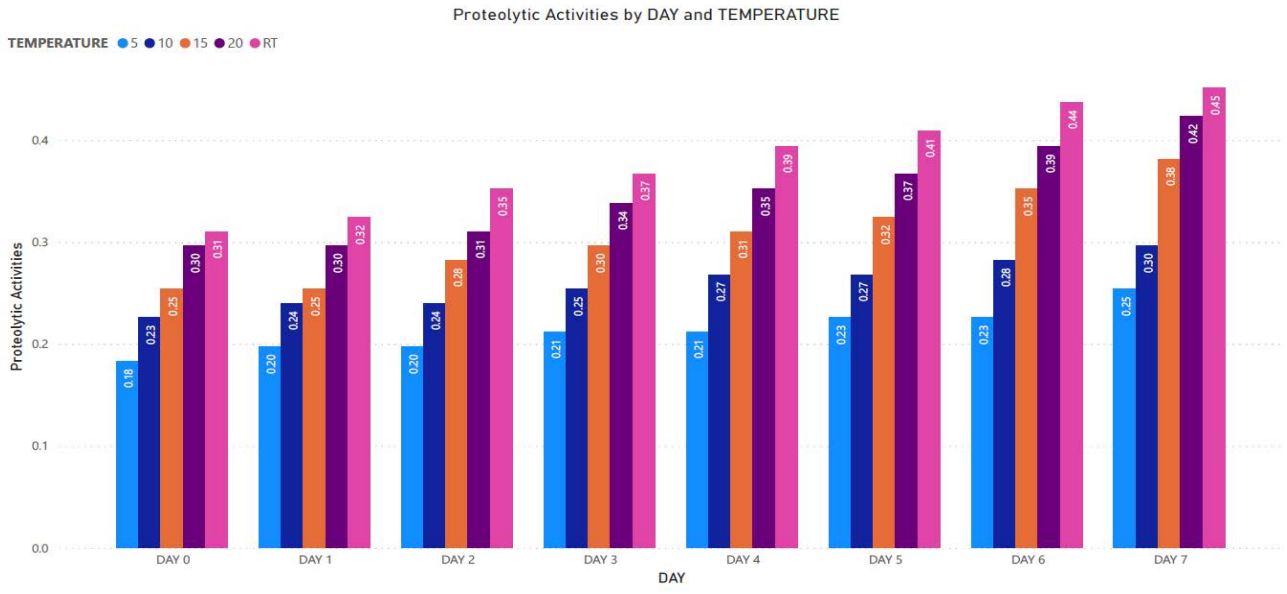
concentration of solid used in this assay originally in mg/mL, which gives activity in terms of units/mg.

- **Proteolytic Activities**

Temperature (°C)	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
5	0.183	0.197	0.197	0.212	0.212	0.226	0.226	0.254
10	0.226	0.240	0.240	0.254	0.268	0.268	0.282	0.296
15	0.254	0.254	0.282	0.296	0.310	0.324	0.352	0.381
20	0.296	0.296	0.310	0.338	0.352	0.367	0.394	0.423
RT (Room Temp.)	0.310	0.324	0.352	0.367	0.394	0.409	0.437	0.451

Table 2.2 : DAY-0 to DAY-7 Enzymatic Activity Values for samples at different temperatures in respect of O.D. value.

Proteases are not only important in the hydrolysis of storage proteins, they are also responsible for the post-translational modifications, activation, and inactivation of enzymes and defense of plants. During this process, proteolytic activity slowly increases from day-0 to day-7. Proteolytic Activities reached it's highest value at Room Temperature rather than other temperatures.



2.4 CONCLUSION

Proteolytic activities of geminating barley seeds is gradually increasing against temperature and the enzymatic activities are slowly increasing from Day-0 to Day-7. So, temperature plays the major role for increasing proteolytic activities of the germinating barley seeds.

CONCLUSION

Germination mobilizes and increases the activity of some enzymes. Temperature, steeping time, variety are determining factors in the activation time. At higher temperatures, enzymatic activation is faster. Germination represents an alternative process that allows us to modify the properties of grains of cereal. The advantages of the increase of the enzymatic activity with the germination presents many opportunities to be applied in the industry. Barley remains one of the world's economically important crops for its uses in food, feed and beverage production. While the main components are quite well understood, the minor components such as enzymes and inhibitors, their functions and roles and the synergistic interactions within the grain and when used in making value-added products remain to be completely unravelled. The opportunity to exploit wild and exotic barley germplasm, as well as possibly genetically enhanced barleys, will have two effects. This will allow researchers to continue to improve barley cultivars and gain future knowledge of the chemistry and biochemical processes of barley. Another aspect in gaining further understanding of barley will be the use of the most modern technologies, such as Nuclear Magnetic Resonance, Infrared Technologies, improved gene studies, i.e, Single Nucleotide Polymorphsims and Diversity Array Technologies.

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