الجمهورية الجزائرية الديمقراطية الشعبية
وزارة التعليم العالي والبحث العلمي
PEOPLE'S DEMOCRATIC REPUBLIC OF ALGERIA
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Academic year 2023/2024

Higher School of Biological Sciences of Oran

Second cycle Department

Educational handout

Subject: Practical Work

ENZYME ENGINEERING 2:
Immobilization of Biological Systems

Level: 2nd year of the second cycle
Option: Enzyme engineering
Specialty: Biotechnology
Field: Biological Sciences
Domain: Natural and Life Sciences

Prepared by:

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Subject taught during the Academic Years:

2023/2024

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Foreword

Practical work is a cornerstone of scientific education, offering students invaluable hands-on experience to bridge the gap between theoretical knowledge and real-world applications. The **Practical Works in Enzyme Engineering 2** is specifically designed to introduce students to advanced methodologies in enzymology while fostering a deeper understanding of enzyme behavior, manipulation, and industrial applications.

This course emphasizes the importance of experimental precision, analytical thinking, and teamwork. It also prepares students to address challenges in enzyme immobilization, activity assays, and production processes. Through these laboratory exercises, students will acquire critical skills that are indispensable for careers in biotechnology, pharmaceuticals, food sciences, and environmental engineering.

We encourage students to approach each experiment with curiosity and rigor, as these qualities are integral to scientific discovery. The accompanying guide has been meticulously crafted to provide clarity and support throughout your laboratory experience.

We hope this practical work inspires your enthusiasm for enzymology and empowers you to explore its vast potential.

Practical Work Details

Institution: Higher School of Biological Sciences of Oran

Department: Second Cycle

Specialization: Enzyme Engineering

Target Audience: 2nd Year Enzyme Engineering Students

Unit: EUF 1

Credits: 4

Coefficient: 2

Duration: 12 weeks

Practical Leader: Dr. BENYOUCEF Amel

Practical Instructor: Dr. ASFOURI. Yasmine. Nadia

Recommended prerequisites: Foundational understanding of microbiology, biochemistry, biotechnology, and analytical chemistry.

Introduction

Enzyme Engineering is a fundamental discipline within the fields of biotechnology and biochemistry. It involves the manipulation and exploitation of enzymes—extraordinary biological catalysts—for a variety of industrial, medical, and environmental applications. One of the most widely used techniques in this field is enzyme immobilization, which offers numerous advantages over free enzymes, including increased reusability, enhanced stability, and ease of separation.

The process of immobilizing biological systems involves attaching or binding biological materials, such as enzymes, cells, or biomolecules, onto a solid surface. This allows for more efficient and controlled reactions, often leading to more sustainable and cost-effective industrial processes.

Immobilized enzymes have a wide range of applications across various industries, including food production (e.g., the conversion of glucose to high-fructose corn syrup), biopharmaceuticals (e.g., the production of drugs), environmental cleanup (e.g., wastewater treatment), and in biosensors for detecting specific substances. These applications demonstrate the broad potential of enzyme immobilization in addressing real-world challenges.

A deep understanding of system biology, the chemical properties of immobilization support, and the optimal environmental conditions for this process is essential for achieving successful outcomes. This knowledge is key to ensuring the stability, activity, and efficiency of immobilized enzymes in various biotechnological applications.

Objectives of the Practical Work

This practical work program consists of sessions designed to explore enzyme and yeast immobilization techniques and their biotechnological applications.

The main objectives of these sessions are:

- To provide students with hands-on experience in enzyme and yeast immobilization, enabling them to apply theoretical concepts to real-world scenarios.
- To deepen students' understanding of the significance of immobilized enzymes and yeast in advancing biotechnology and industrial applications.
- To explore the properties and applications of immobilized yeast in processes such as fermentation, alongside enzyme-based applications.
- To enhance students' skills in experimental design, problem-solving, and data analysis, all of which are essential for success in the biotechnological field.

By the end of these sessions, students will have a comprehensive understanding of the role of immobilized enzymes and yeast in various industries and will be better equipped to tackle challenges in enzyme and yeast engineering.

Educational Tips for Students

Here are some helpful tips to support students in succeeding in practical work. These strategies will enhance focus, organization, and understanding, making your practical sessions more effective and enjoyable.

1. **Before the Practical Work:**
   - **Read the protocol**: Go through the practical steps and related theories beforehand. This helps you understand what you'll be doing and why.
   - **Know the objectives**: Be clear about what you must learn or demonstrate (e.g., understanding an enzyme mechanism, measuring enzyme activity).
   - **Prepare your tools**: Bring your lab notebook, pen, calculator, and any other materials you need.
   - **Review safety rules**: Ensure you know safety guidelines for handling chemicals and lab equipment.

2. **During the Practical Work:**
   - **Arrive on time**: Being punctual helps keep things organized.
   - **Listen carefully**: Pay attention to instructions and take notes to avoid mistakes during experiments.
   - **Follow the protocol**: Stick to the steps and record your observations and measurements accurately in your lab notebook.
   - **Stay safe**: Always wear your lab coat, gloves, goggles, or protective gear. Handle chemicals and equipment carefully.
   - **Work together**: Communicate with your teammates to share tasks and improve efficiency.

3. **After the Practical Work:**
   - **Organize your results**: Present data clearly, using tables or graphs where possible. Look for any errors or unusual results.
   - **Reflect on your findings**: Think about why the results might differ from what you expected. Compare your results with your initial hypotheses.
   - **Discuss with others**: Take the opportunity to ask teachers and classmates for feedback and clarification to deepen your understanding.

4. General Guidelines: Best Practices in the Lab

- **Keep things tidy**: Clean your workspace after each experiment and stay organized.
- **Handle equipment with care**: Be gentle with tools and reagents to avoid damaging them.
- **Stay curious**: Ask questions, suggest ideas for improving experiments, and engage with the concepts.
- **Learn from mistakes**: Mistakes are part of the learning process, so use them to improve and keep going.

5. Writing the Lab Report

It is essential to structure your lab report clearly and logically. A well-organized report helps to effectively communicate your findings and demonstrate your understanding of the experiment.

1. **Abstract**:

   The abstract should provide a concise summary of the entire experiment, including the objectives, methods, key results, and conclusions. It should be written in three languages: French, Arabic, and English.
2. **Introduction**:

   In the introduction, briefly explain the theoretical background relevant to the experiment and state the objectives of the study. You should provide enough context to help the reader understand the purpose of the experiment and why it is important.
3. **Materials and Methods**:

   Describe the materials, equipment, and techniques used in the experiment. Outline the procedure step-by-step, ensuring that someone else can replicate the experiment based on your description. Be specific about the quantities and conditions used.
4. **Results**:

   Present your findings in an organized manner, using tables, charts, or graphs as necessary. Each piece of data should be clearly labeled, and concise descriptions should be included to explain what the data shows. Avoid interpreting the results in this section; simply present them as they are.
5. **Discussion**:

   In the discussion, analyze your results by comparing your findings with those from other relevant studies or literature to help contextualize your results. For example, if previous

studies report similar outcomes under similar conditions, consider whether your results align with or differ from those and explore why. If your results contradict other studies, think about possible reasons for these differences, such as variations in experimental conditions, methods, or sample sizes. Also, address any discrepancies, errors, or unexpected findings, and suggest possible reasons for them.

This section should also include an assessment of the limitations of your experiment. Consider factors such as measurement accuracy, instrument reliability, and potential sources of error. Discuss how these factors might have influenced your results and suggest improvements for future studies. Finally, propose any changes to the experimental design that could help achieve more reliable or informative results in future experiments.

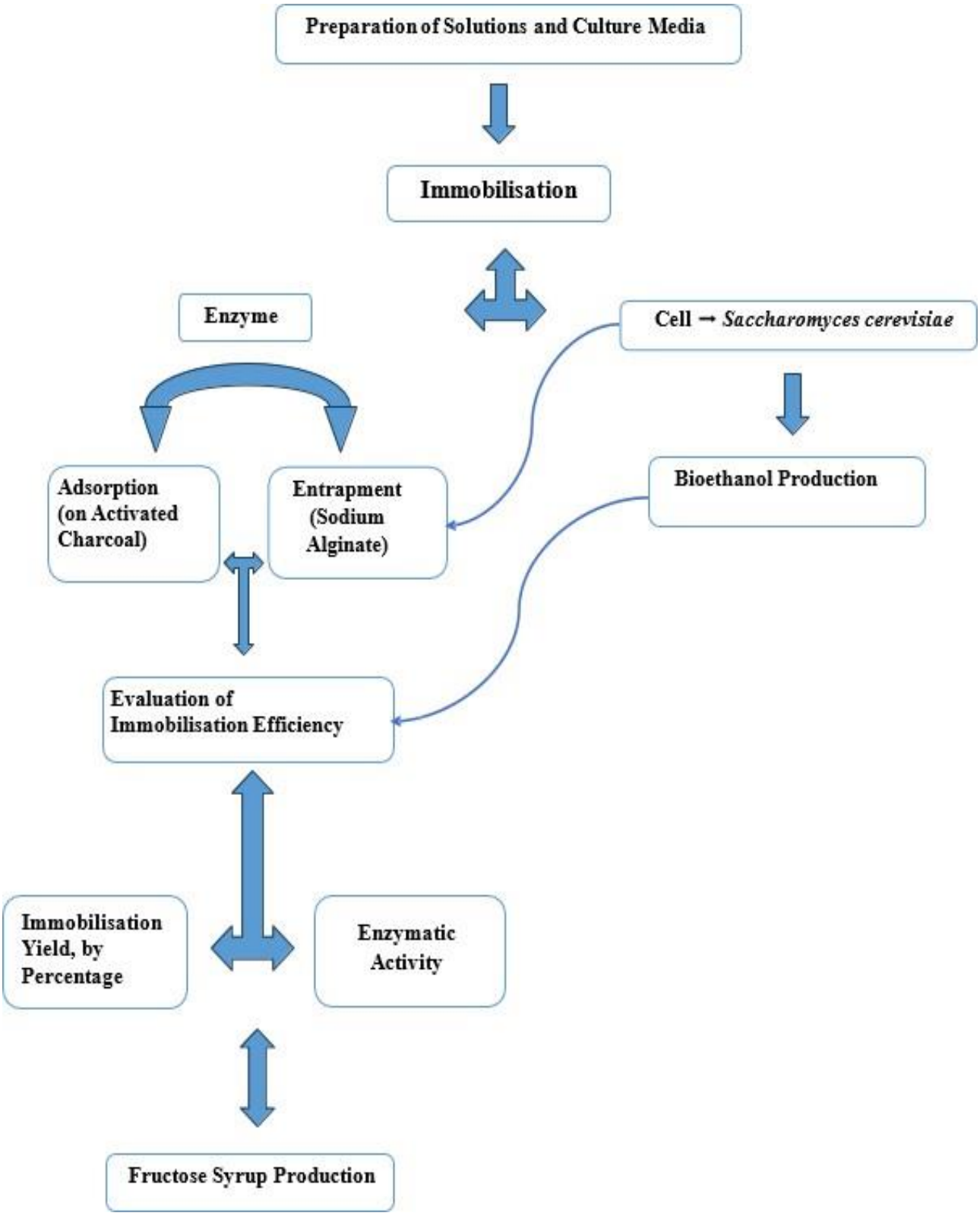
6. **Conclusion**:

   Summaries the key takeaways from the experiment. Highlight the main findings and their implications. It's important to relate your conclusions to the objectives stated in the introduction and provide a final reflection on the experiment's success or failure.

7. **Submit on Time**:

   Adhere to the submission deadlines. Follow any specific guidelines provided for formatting, file type, and presentation. Ensure that your report is well formatted, clearly written, and free of errors before submitting.

Laboratory Exercises



Practical Work ❶ Preparation of solutions and culture media & Enzyme production

I. Introduction

In microbiology and biotechnology, the successful execution of experiments and production processes relies heavily on properly preparing solutions and culture media. Solutions provide the necessary environment for enzyme reactions, ensuring optimal conditions for their stability and activity. Culture media supply the essential nutrients required for microbial growth, allowing us to cultivate organisms crucial for enzyme production. Understanding the principles of preparing culture media ensures optimal growth conditions for the target microorganisms, leading to successful fermentation and enzyme secretion.

II. Objectives

In this practical work, we will focus on two key objectives:

- Learn to prepare accurate solution concentrations and various culture media types.
- Explore cultivating microorganisms (e.g., bacteria, fungi) to produce industrial enzymes like amylase, glucose isomerase, and laccase.

III. Materials

Products	Equipment/ instruments
▪ Distilled water ▪ HCl ▪ NaOH ▪ Iodine crystals ▪ Potassium Iodide (KI) ▪ LB media (liquid and solid) ▪ Agar ▪ Starch ▪ Potassium phosphate buffer ▪ Potassium sodium tartrate tetrahydrate ▪ DNSA ▪ Peptone ▪ Yeast extract ▪ Sodium chloride ▪ $MgSO_4.7H_2O$ ▪ KH_2PO_4 & K_2HPO_4 ▪ $CaCl_2.2H_2O$ ▪ Sodium Alginate	▪ pH meter ▪ Precision balance ▪ Refrigerator ▪ Magnetic stirrer + magnetic stirring bar ▪ Watch glass ▪ Test tubes ▪ Petri dishes ▪ Spatulas ▪ Erlenmeyer flasks ▪ Bottles ▪ Whatman filter paper ▪ Alcohol wash bottle ▪ Bunsen burner ▪ Yellow pipette tips, ▪ Micropipettes ▪ Beakers ▪ Autoclave ▪ Incubators

IV. Methods

IV.1. Preparation of solutions and cultural media

Reagents and buffers		Culture medias	
Compound	**Amount**	**Compound**	**Amount**
Reagent DNS • DNSA • NaOH (2M) • Potassium sodium tartrate tetrahydrate • Distilled water	**(100mL)** 10g 16g 300g 1L	**Production of media Bacteria for Amylase** Soluble starch Peptone Yeast extract KH_2PO_4 $MgSO_4 \cdot 7H_2O$ NaCl $CaCl_2$ Distilled water pH 7.0	**(200mL)** 10 g 5g 2g 1g 0.5g 1g 0.2g 1L
Potassium Phosphate Buffer, 50 mM, pH 7,0	**(100mL)**		
Potassium Phosphate Buffer, 0.1M, pH 7	**(150 mL)**	**Production media Bacteria for Laccase** • Glucose • Peptone • Yeast extract • KH_2PO_4 • $MgSO_4 \cdot 7H_2O$ • $CaCl_2$ • $FeSO_4 \cdot 7H_2O$ • $CuSO_4 \cdot 5H_2O$ • $ZnSO_4$ • Guaiacol	**(200mL)** 20 g 5 g 2g 0.5g 0.5g 0.2 g 0.01 g 0.2 mM 0.001 g 0.3 Mm
1% buffered starch solution • Starch • Potassium phosphate buffer, 50 mM, • pH 7	**(100mL)** 1g 100mL		
1% iodine solution • Iodine • Potassium Iodide (KI) • Distilled Water	**(100mL)** 1 g 2 g 100 mL		
3% the sodium alginate solution	**(200mL)**	**GYP medium (For fungi)** • Glucose • Yeast extract • peptone • Starch • Agar • Distilled water • pH 6.0.	1 g 0.1 g 0.5 g 0.2% 16 g 1L
0.03 M calcium chloride ($CaCl_2$) solution	**(400mL)**		
0.3 M calcium chloride ($CaCl_2$) solution	**(500mL)**	**Fermentation medium (for amylase fungi)** • Raw potato peel powder • Minimal salt solution. • pH 6.0 or • Malt extract • Starch • Minimal salt solution • pH 5.5- 6	**(200mL)** 500g 1L 20 g 10 g 1L
Minimal salt solution • KH2PO4 • MgSO4.7H2O • NaCl • CaCl2 • Peptone • Distilled water	**(200mL)** 2 g 0.2 g 0.1 g 0.1 g 0.2 g 1 L		

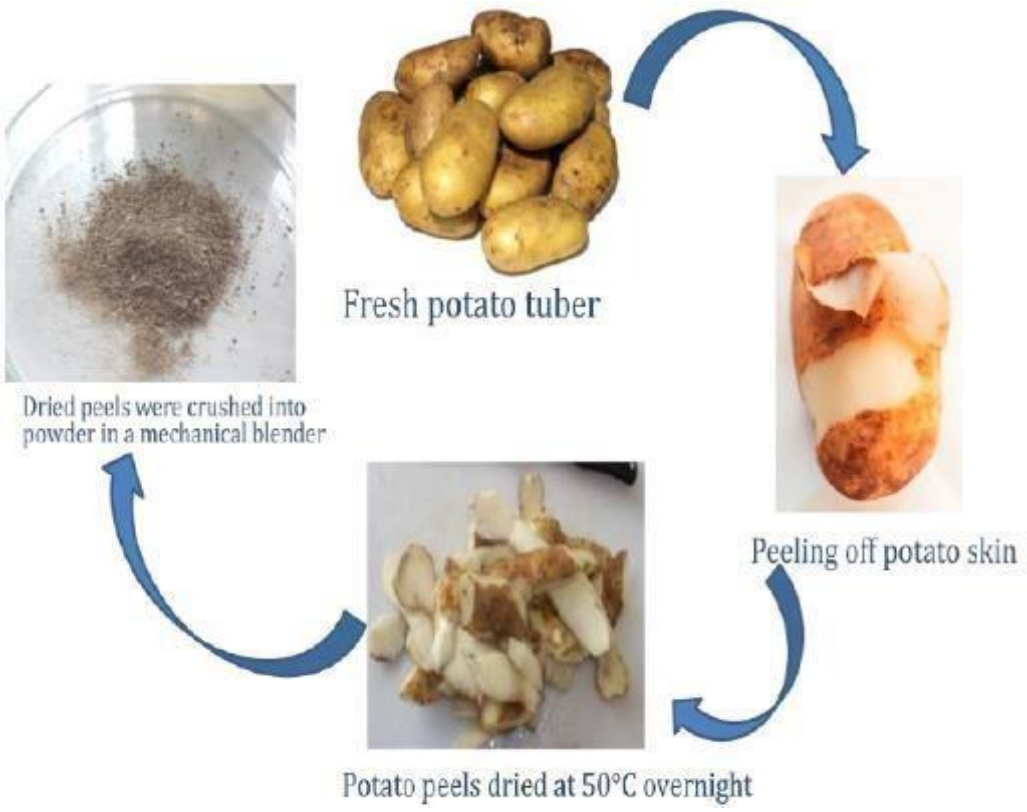


Figure 1: Preparation of potato peel powder (PPP) (Tabassum Mumtaz *et al.,* 2014).

Note: Store the prepared solutions at 4°C if not used immediately.

IV.2. Production of enzyme

IV.2.1. Screening of Amylase Activity (Starch hydrolysis test)

2. 1. 1. Principle

Amylase breaks down amylose, a straight-chain polymer, and amylopectin, a branched polymer that makes up starch, into simpler sugars. The process involves the breaking of α-1, 4 glycosidic bonds within the starch molecules, resulting in the production of smaller sugar units. There are two main types of amylases:

α-amylase breaks the internal 1,4 bonds in starch, resulting in a mixture of maltose and glucose.

β-amylase cleaves starch at the non-reducing ends, releasing maltose units.

Amylase activity is commonly measured by evaluating its ability to degrade starch. Incubating amylase with a starch substrate and detecting it using iodine, which reacts with starch to form a blue-black complex, is a common method. When amylase breaks down starch, the color fades, and clear zones form, indicating enzyme activity. The larger the clear zone, the higher the amylase activity.

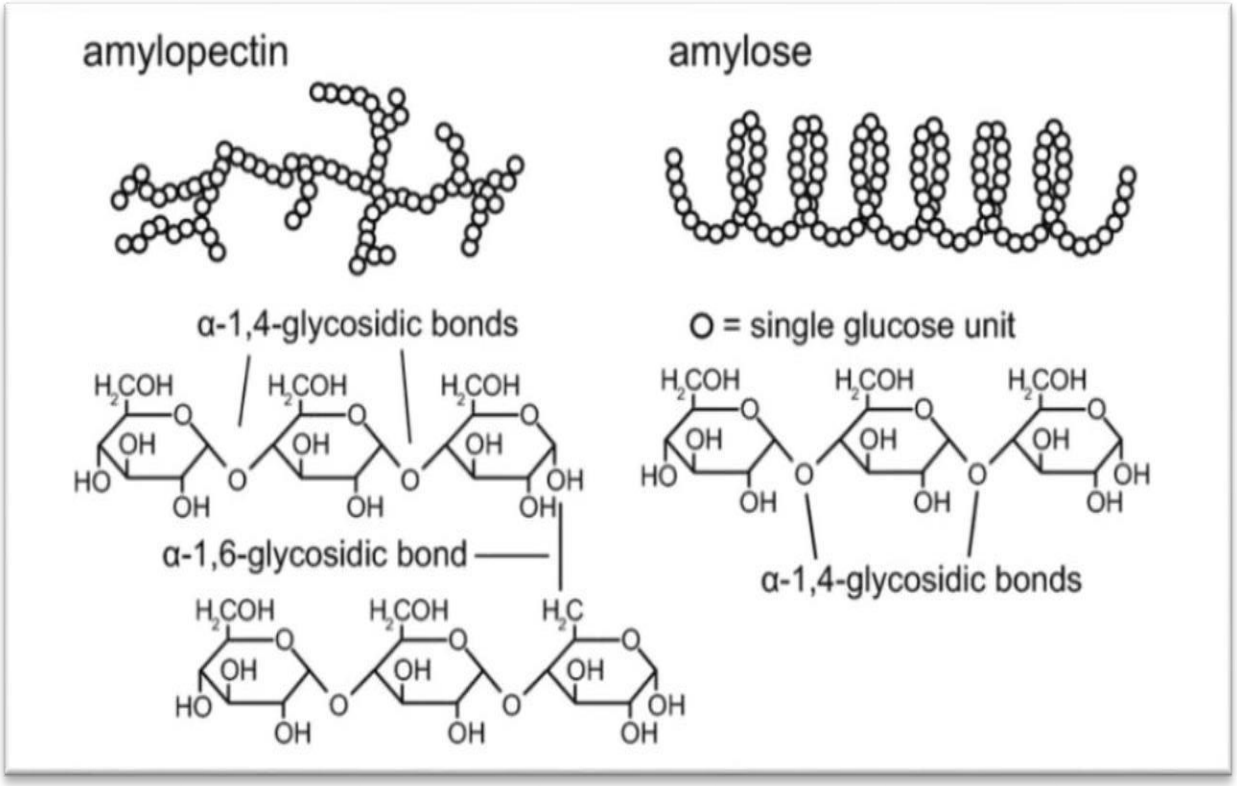


Figure 2: Chemical structure of the corn starch constituting natural polymers amylopectin and amylose. Amylopectin is a branched polymer, whereas amylose is a linear polymer forming a helix (Willfahrt, 2019)

2. 1. 2. Procedure

Two methods are commonly used to evaluate bacterial amylase activity. Both methods involve the use of starch agar as a substrate to determine the ability of bacteria to hydrolyze starch, which is an indicator of amylase production:

❶ the first method, described by (Yassin *et al.,* 2021):

- ❖ Inoculate Bacteria onto starch agar plates.
- ❖ Incubate the plates at 37°C for 48 hours (2 days) to allow bacterial growth.
- ❖ After incubation, flood the plates with 1% iodine solution.
- ❖ Let the iodine solution sit on the plates for 5-10 minutes without disturbance.
- ❖ Remove the iodine solution from each plate by carefully decanting it.
- ❖ Look for clear zones around the bacterial colonies on the plates, which indicate the hydrolysis of starch.
- ❖ Measure the diameter of the clear zones around each colony.

❷ The second method, described by (Choubane *et al.,* 2016).

- ❖ Inoculate Bacteria onto starch agar plates.
- ❖ Incubate the plates at 37°C for 48 hours.
- ❖ After incubation, invert the plate (after removing the lid) over a beaker containing iodine crystals.
- ❖ Observe:
  - The hydrolysis of the starch indicates a positive test for amylase activity if the colony's surrounding area stays clear.
  - If the medium turns blue (without a clear zone), hydrolysis of the starch has not occurred, indicating a negative test result.
- ❖ Measure and record the diameter of the clear zone around the colony to quantify the amylase activity.

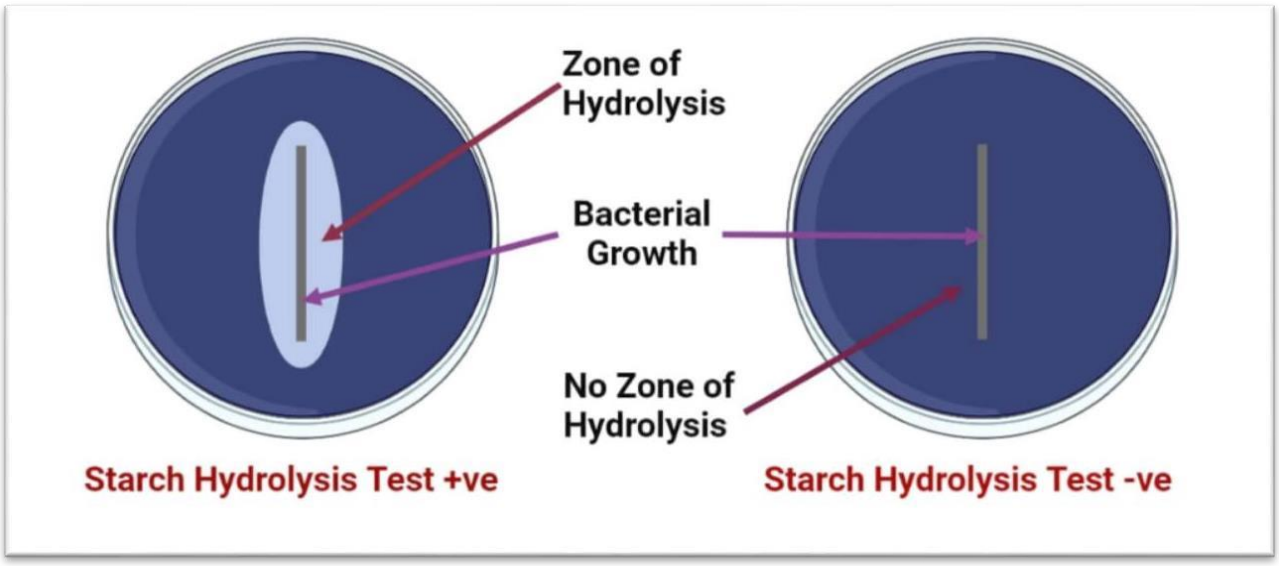


Figure 3: Starch Agar plate with amylase activity showing clear zone formation (+) and no amylase activity showing no clear zone formation (-) (Aryal, 2023).

Note:

- ❖ Use the same procedure for fungis, but adjust the agar medium, incubation time, and temperature as appropriate.
- ❖ The formula calculates the amylolytic index according to (Satrimafitrah *et al*., 2020)

$$\text{Amylolytic Index} = \frac{CZ - CD}{CD}$$

Where: CZ was the Clear Zone Diameter (mm), and CD was the Colony Diameter (mm)

IV.22. Screening of Laccase Activity

2. 2.1. Principle

Laccase is an oxidative enzyme that catalyzes the oxidation of phenolic and non-phenolic compounds by reducing molecular oxygen to water. For screening laccase activity, use substrates that undergo visible color changes upon oxidation. The basic principle involves detecting this enzymatic oxidation through colorimetric, spectrophotometric, or plate-based assays. Use plates containing specific substrates, such as guaiacol or ABTS, for solid medium screening. We spot or streak the microorganism onto the plates, allowing laccase-producing organisms to create distinct color zones around their growth through substrate oxidation.

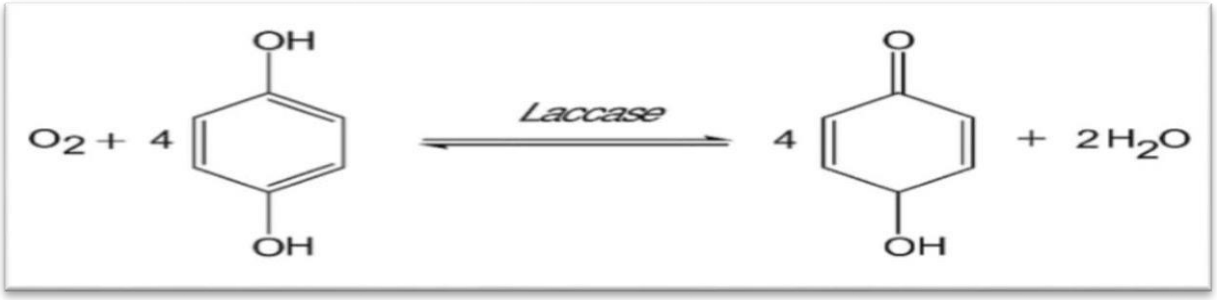


Figure 4: Oxidation of Hydroquinone to Benzoquinone by Laccase (Peralta *et al.,* 2017).

2. 2. 2. Procedure

To investigate laccase activity in fungi as described by Senthivelan *et al.,* 2019**:**

- ❖ Prepare the PDA agar and sterilize it in an autoclave.
- ❖ After sterilization, mix the substrate solutions in the agar. For each substrate, prepare separate plates:
  - **ABTS Plates:** Add 3 mM ABTS to the PDA agar.
  - **Guaiacol Plates:** Add 4 mM guaiacol to the PDA agar.
- ❖ Pour the PDA agar and substrates into sterile Petri dishes and allow them to solidify.
- ❖ Transfer a small amount of fungal culture to the center of each substrate-containing agar plate.
- ❖ Incubate the plates at room temperature (typically 25-30°C) for 7 days.
- ❖ Observe the plates after incubation for color changes around the fungal colonies.
  - **ABTS:** A blue-green color indicates laccase activity.
  - **Guaiacol:** A brown to reddish-brown color indicates laccase activity.

Note: Use the same procedure for bacteria, but adjust the agar medium, incubation time, and temperature as appropriate.

IV.2.3 *Screening of Glucose Isomerase Activity*

2. 3.1. Principle

Glucose isomerase is an enzyme that catalyzes the conversion of D-glucose (a six-carbon sugar, an aldose) to D-fructose (a six-carbon sugar, a ketose). This reaction involves rearranging the carbonyl group from the aldehyde position (C1) in glucose to the ketone position (C2) in fructose. TTC (2, 3, 5-triphenyl tetrazolium) acts as an electron acceptor in this assay. When glucose isomerase is active, the glucose it converts generates reducing sugars (like fructose). These reducing sugars, or intermediates, produced in the reaction facilitate the reduction of TTC to formazan, a red-colored compound. The formation of the color red on the plate indicates glucose isomerase activity.

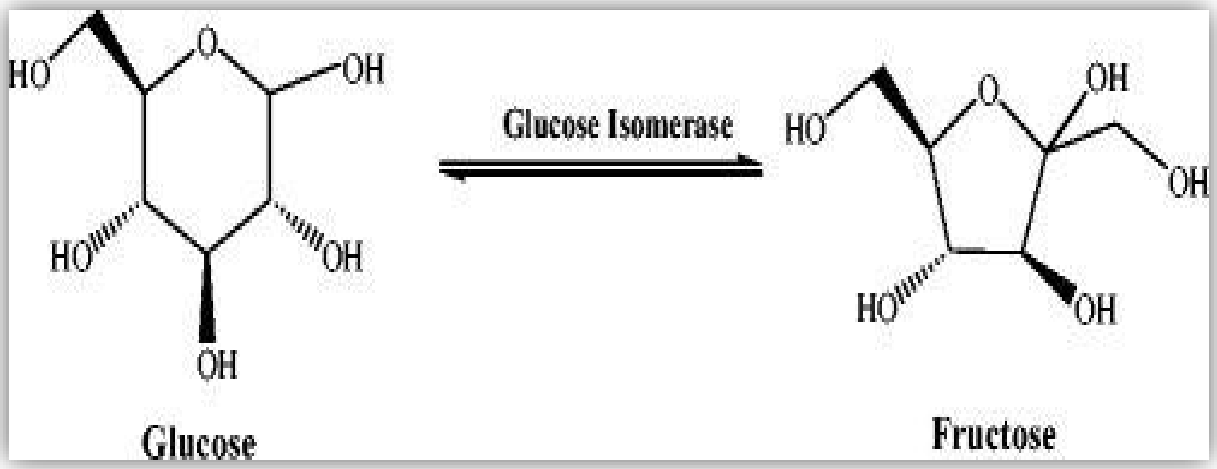


Figure 5: The isomerization of glucose to fructose catalyzed by glucose isomerase (Jin *et al.,* 2017)

2. 3. 2. Procedure

To investigate the activity of glucose isomerase as described by (Sathya and Ushadevi, 2014):

- Grew bacteria on peptone-yeast agar with 0.5% glucose as a substrate and incubated them at 37°C for 48 hours.
- Treat the plates with a reaction mixture after the incubation period is over. The reaction mixture is made up of 0.1% 2, 3, 5-triphenyltetrazolium solution in 1 M NaOH, which is a chemical that shows redox reactions.
- Expose the plates to this mixture in the dark at 37°C for 1 minute. Keeping it in the dark prevents light-induced reactions that could interfere with the results.

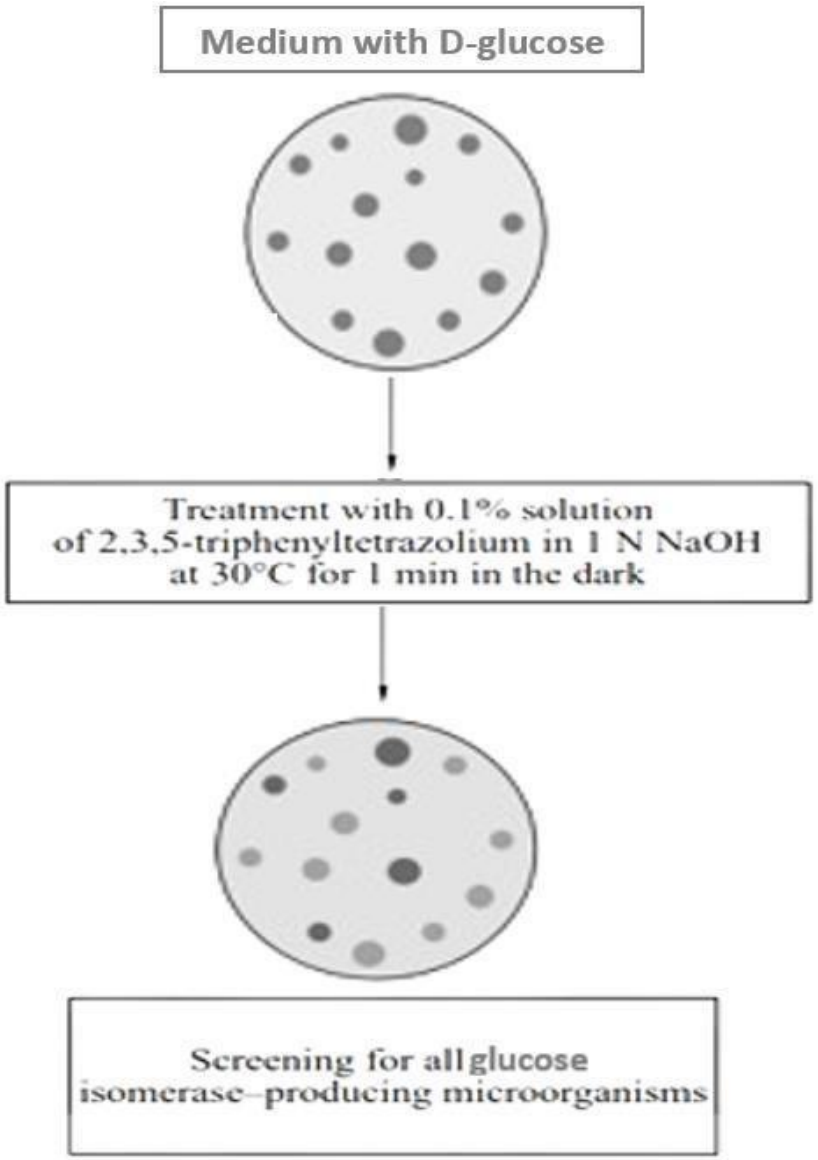


Figure 6: The scheme of screening for glucose isomerase-producing microorganisms (Sapunova *et al.,* 2004).

IV. 4. Fermentation and Extraction of the enzyme extracellular

4.1. Principle

During the growth phase of a culture in a nutrient-rich medium that contains carbon sources like starch for amylase production, nitrogen sources like peptone or yeast extract, and essential minerals, the microorganisms secrete enzymes into the surrounding medium. pH, temperature, oxygen availability, and substrate concentration all influence the fermentation process.

The first step in enzyme recovery is to separate the microbial cells from the liquid medium containing the enzyme, usually done by filtration or centrifugation.

4. 2. Procedure

The bacterial culture protocol is based on the work of Sanjaya *et al.* (2024):

- Inoculate 100 mL of production media with 1mL of bacterial culture
- Incubate the culture at 37°C for 48 h with shaking if necessary.
- After 48 hours of incubation, centrifuge the culture at 4°C for 15-20 minutes at 6000 rpm.
- Carefully transfer the supernatant (the liquid on top) to a new container, ensuring it does not disturb the bottom pellet containing cell debris and insoluble components.
- Store the supernatant at -20°C until further use.

The fungal culture protocol follows the methods detailed by Sunitha *et al*. (2012):

- Inoculate 100 mL of fermentation medium with 5 mm discs from a 5-day-old fungal culture.
- Incubate the cultures at 30°C under static conditions (without shaking).
- Filter the fungal culture through Whatman's No. 1 filter paper.
- Centrifuge the filtrate at 6000 rpm for 15 minutes. Collect the cell-free supernatant as the crude enzyme for subsequent analysis.
- Store the filtrate at -20°C until further use.

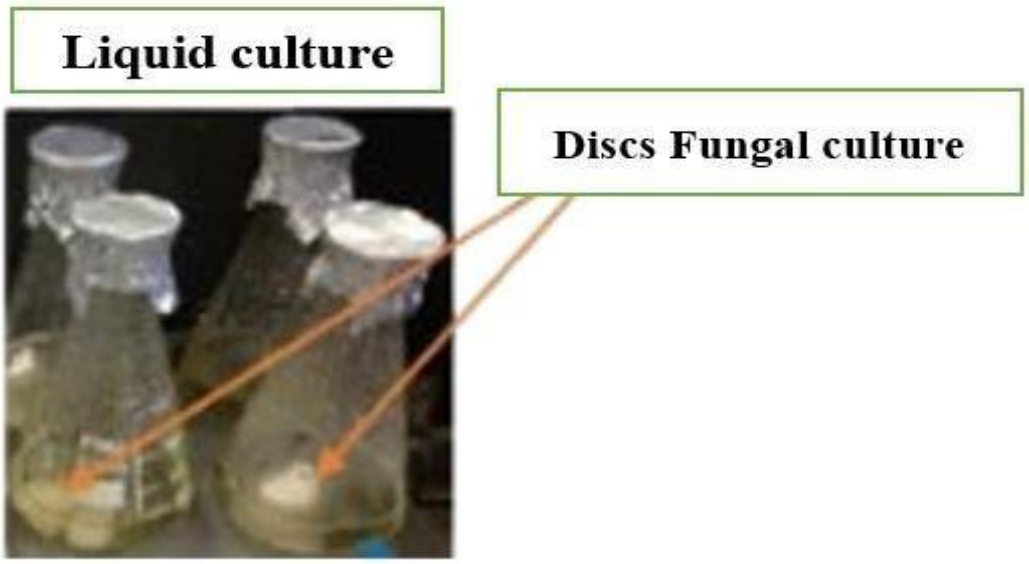


Figure 7: Inoculation of Fungal Culture in Fermentation Medium

Practical Work ❷ Immobilization of the enzyme

I. Introduction

The practical application of soluble enzymes as green catalysts is often limited by several drawbacks, including their non-reusability, high sensitivity to denaturing agents, high production costs, instability during large-scale processing, and conformational changes that affect activity. Additionally, soluble enzymes are unsuitable for fixed-bed reactors and cannot be easily recycled (Badgujar *et al.,* 2022). Over the past century, numerous enzyme immobilization strategies have been developed to address these limitations and enhance enzyme performance. Immobilization techniques have proven effective in improving catalytic activity, stability, storage stability, and reusability while reducing downstream processing costs (Cardoso Pinto *et al.,* 2023). Enzyme immobilization is now a cornerstone of large-scale applications due to its advantages, including ease of catalyst recycling, continuous operation, simplified separation processes, compatibility with a wide range of reactors, and reduced costs of product purification (Parandi *et al.,* 2023). Immobilized enzymes are generally more stable and efficient than their soluble counterparts, making them highly valuable for industrial applications. Researchers have explored various immobilization strategies, such as affinity adsorption, covalent binding, cross-linking, and entrapment or encapsulation. These methods have been applied to diverse materials, including organic polymers like polyallylamine, activated carbon, and chitosan, as well as inorganic polymers like nanographene oxide, nano silica, and nanogold. These advancements continue to make enzyme immobilization a reliable and efficient approach for industrial biocatalysis (Mohidem *et al.,* 2023).

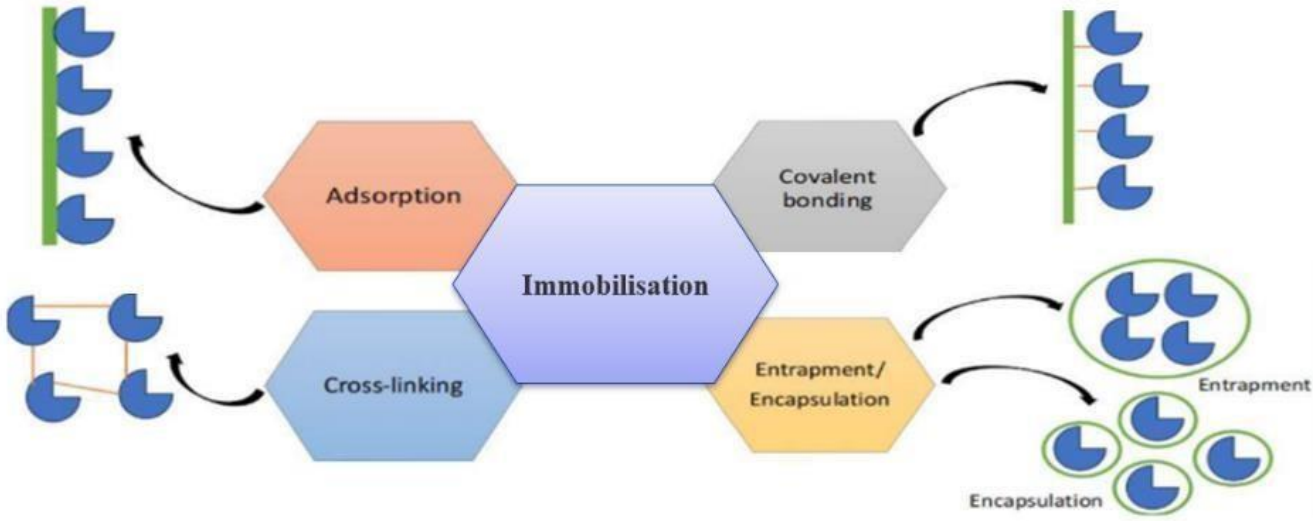


Figure 1: An illustration of enzyme immobilization methods using agrowaste nanocarrier. Clarification: = enzyme; or = carriers; = linker (Mohidem *et al.,* 2023).

II. Objectives

The practical work aims to provide students with the opportunity to:

- Understand the concept of enzyme immobilization.
- Learn and apply different methods of enzyme immobilization.
- Develop practical laboratory skills necessary for working with enzymes and immobilization techniques.

III. Materials

Products	Equipment/ instruments
▪ Activated carbon ▪ 0.1 M phosphate buffer pH 7 ▪ 3% sodium alginate solution ▪ 0.3 M calcium chloride ($CaCl_2$) solution ▪ 0.03 M calcium chloride ($CaCl_2$) solution	▪ Refrigerator ▪ Magnetic stirrer + magnetic stirring bar ▪ Vortex ▪ Yellow pipette tips, ▪ Micropipettes ▪ Beakers ▪ Latex gloves

IV. Methods

IV. 1. Immobilization of Amylase

IV. 1.1. Adsorption method

1. 1. 1. Principle

To immobilize enzymes using the adsorption method, enzymes are attached to a solid support through weak, non-covalent bonds. These interactions include van der Waals forces, hydrophobic forces, hydrogen bonding, and ionic interactions. The support material, which can be organic (such as cellulose) or inorganic (such as silica or activated charcoal), physically adsorbs the enzyme onto its surface. This method is straightforward and cost-effective, requiring no chemical reactions or harsh conditions, making it ideal for preserving enzyme activity.

1. 1.2. Procedure

The method, as described by Chan, Liu *et al.* (2008), consists of the following steps:

- ❖ Mix 1 g of activated carbon with 10 ml of enzyme solution in the beaker and stir gently.
- ❖ Incubate the mixture at 4°C for 1 hour.

- Filter the mixture using filter paper.
- Wash the immobilized enzyme with 0.1 M phosphate buffer to remove any unbound enzyme and maintain enzyme activity.
- Store the immobilized enzymes in 0.1 M phosphate buffer, pH 7, at 4 °C until they are ready for use.

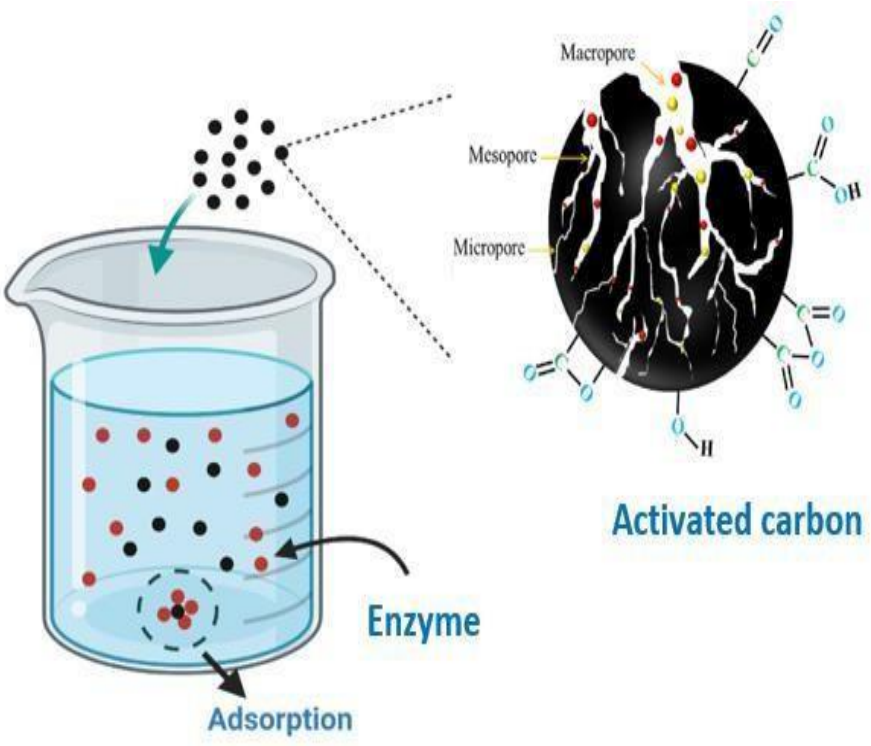


Figure 2: Adsorption of enzymes using activated carbon (Sultana, M., *et al.,* 2021).

IV. 1.2. Entrapment method

1.2.1. Principle

The entrapment method of enzyme immobilization involves physically enclosing enzymes within a porous matrix, such as hydrogels, polyacrylamide gels, or natural polymers like alginate or carrageenan. The matrix forms a three-dimensional network that restricts the movement of the enzyme while permitting the free diffusion of substrates and products through its pores. This approach ensures the enzyme remains confined within the matrix while preserving its catalytic activity. Entrapment provides a stable microenvironment, shielding the enzyme from external stress factors such as pH fluctuations, temperature variations, or mechanical forces, thereby improving its operational stability and reusability (Hasan *et al.,* 2021).

1.2.2. Procedure

The following procedure, adapted from (Tumturk *et al.,* 2007), outlines the step-by-step:

- Slowly mix 1 mL of the enzyme solution into 25 mL of 3% sodium alginate solution while stirring gently.
- Drop the mixed solution into a 0.3 M calcium chloride ($CaCl_2$) solution using a syringe or micropipette. This step causes the sodium alginate to gel and entrap the enzyme.
- Remove the formed gel beads and place them in a dilute $CaCl_2$ solution (0.03 M) for 1 day at 4°C.
- After curing, wash the beads with distilled water and store them at 4°C.

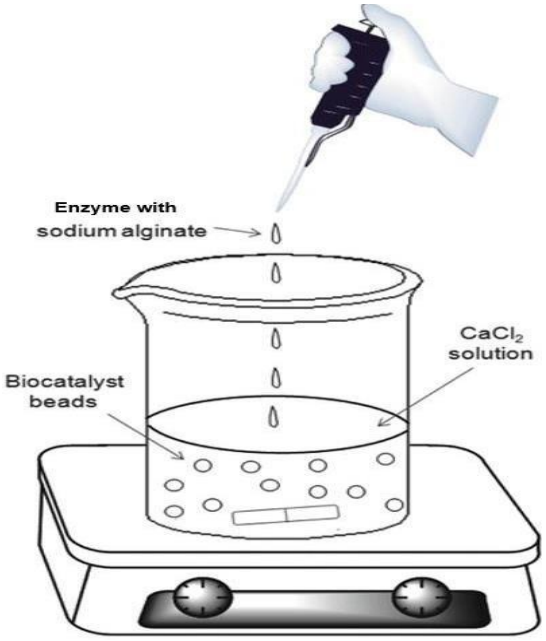


Figure 3: Entrapment of enzymes using sodium alginate **(**Trelles and Rivero, 2013)

Note:

To evaluate the efficiency of enzyme immobilization after completing the provided protocol, it is important to measure the initial activity of the free enzyme solution before immobilization. Next, measure the enzyme activity in the filtrate (for the adsorption method) or in the $CaCl_2$ solution (for the encapsulation method), which represents the amount of enzyme that was not immobilized and remained in the solution. Finally, measure the activity of the immobilized enzyme to assess the effectiveness of the immobilization process.

Practical Work ❸ Evaluation of immobilization efficiency

I. Introduction

The efficiency of immobilization is an important parameter to consider, as it directly affects the performance of the immobilized biomolecule. The percentage of the biomolecule that successfully attaches to the solid support is known as immobilization efficiency.

The evaluation of immobilization efficiency is typically performed by measuring the activity of the immobilized biomolecule before and after immobilization. The difference in activity is then divided by the initial activity to calculate the immobilization efficiency.

Several different methods can be used to evaluate immobilization efficiency. One common method is to measure the enzyme activity of the immobilized enzyme. You can achieve this by adding a substrate to the immobilized enzyme and tracking the amount of product it produces over time.

II. Objectives

The practical work aims to provide students with the opportunity to:

- Define immobilization efficiency and understand its role in the functionality and performance of immobilized biomolecules.
- Measure the activity of both free and immobilized enzymes using appropriate assays.

III. Materials

Products	Equipment/Instruments
• 1% starch solution (prepared in 50 mM phosphate buffer, pH 7), • DNS reagent • Maltose • Glucose • phosphate buffer (200 mM, pH 7), • $MgSO_4$ • $CoCl_2$ • Hydrochloric acid • L-cysteine hydrochloride • sulfuric acid • carbazole solution • Fructose • ABTS • 0.1 M citrate-phosphate buffer, pH 2.6)	• UV/Vis spectrophotometer, • centrifuge, • water bath, • incubator, • analytical balance • shaking water bath, • Column reactor setup,

IV. Methods

IV. 1. Free and adsorbent amylase

IV.1. 1. Amylase assay

1.1. 1. Principle

Amylase activity is typically measured by quantifying the reducing sugars released during the hydrolysis of starch. Colorimetric methods, such as the DNS (dinitrosalicylic acid) assay, detect these reducing sugars by producing a color change in their presence.

1.1.2. Procedure (Adapted from Baltas, N. *et al.,* 2016, with modifications)

1. Combine 1 mL of 1% starch in 50 mM phosphate buffer (pH 7) with 1 mL of free α-amylase enzyme solution or 100 mg of adsorbent containing immobilized α-amylase.
2. Prepare a blank by mixing 1 mL of 50 mM, pH 7-buffered starch 1% with 1 mL of distilled water.
3. In a water bath, incubate the reaction mixture and the blank at 70°C for 30 minutes.
4. After incubation, centrifuge the reaction mixture to separate the adsorbent (with immobilized enzyme) from the liquid.
5. Mix 0.5 mL of DNS reagent with 0.5 mL of the free amylase reaction mixture, 0.5 mL of the supernatant (from the immobilized enzyme), and 0.5 mL of the blank for each respective tube.
6. Place the tubes in a boiling water bath for 5 minutes to develop the color and stop the enzymatic reaction.
7. Allow the tubes to cool to room temperature.
8. Measure the absorbance of the reaction mixtures at 540 nm.

Maltose Calibration Curve

1. Prepare standards containing a maltose concentration range of 0.2 to 1mg/ml in a final volume of 1 ml.
2. Mix 0.5 ml of each standard with 0.5 ml of DNSA reagent.
3. Prepare the blank by mixing 0.5 ml of distilled water with 0.5 ml of DNSA reagent.
4. Incubate the standards and the blank at 100 °C for 5 minutes.
5. Read the absorbance at 540 nm.

IV.1. 2. Glucose isomerase assay

1.2.1. Principle

Glucose isomerase (GI) catalyzes the conversion of glucose to fructose. The activity is determined by measuring the fructose produced. A colorimetric assay using carbazole quantifies fructose, with absorbance at 566 nm correlating with fructose concentration. One unit of GI activity produces 1 µmol of fructose per minute under specified conditions.

1.2.2. Procedure (Adapted from Seyhan Tükel & Dilek Alagöz, 2008; Tumturk *et al.,* 2007)

1. Prepare 1 mL of 2.6 M glucose solution. Buffer this solution by adding 200 mM phosphate buffer (pH 7.0). To support the enzymatic reaction, add 20 mM magnesium sulfate ($MgSO_4$) and 1 mM cobalt chloride ($CoCl_2$).
2. Add 0.25 mL of free glucose isomerase solution or 5mg of immobilized glucose isomerase (adsorbent) to the glucose mixture. This enzyme will catalyze the conversion of glucose to fructose.
3. Incubate the reaction mixture at 60°C for 30 minutes. This elevated temperature accelerates the enzymatic conversion.
4. After incubation, stop the reaction by adding 0.25 mL of 20% (v/v) hydrochloric acid (HCl). This acidic environment will halt the enzyme activity, preserving the amount of fructose produced.
5. Take 1.0 mL of the solution; add 0.1 mL of 2% (w/v) L-cysteine hydrochloride, and 5.0 mL of 75% (v/v) sulfuric acid (H2SO2). Shake the mixture in a 40°C bath.
6. Add 0.15 mL of 12% (w/v) carbazole solution, shake the tube in an ice bath to cool, and allow the color to develop at room temperature.
7. Measure the color intensity at 566 nm using a UV/Vis spectrophotometer.
8. Determine the fructose concentration from the glucose conversion using a fructose calibration curve.

Fructose Calibration Curve

1. Prepare standard fructose solutions (2 to 20 mg/mL) in a 2 mL final volume.
2. For each standard:
   - Add 0.1 mL of 2% L-cysteine hydrochloride.
   - Add 5.0 mL of 75% H_2SO_4.
   - Heat at 40°C.
   - Add 0.15 mL of 0.12% carbazole solution.

3. Cool in an ice bath, and allow to develop at room temperature.
4. Measure the absorbance at 566 nm.
5. Plot absorbance vs. fructose concentration to create a standard curve.

IV.1.3. Laccase assay

1.3.1. Principle

The ability of the laccase enzyme to oxidize ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic- sulfonic acid) and cause a color change is a measure of its activity. Laccase's oxidation of ABTS yields a colored radical cation, which we can quantify by measuring the absorbance at 420 nm. We use the rate of oxidation to determine the laccase activity. The enzyme activity is expressed as the amount of enzyme required to oxidize 1 micromole (µM) of ABTS per minute.

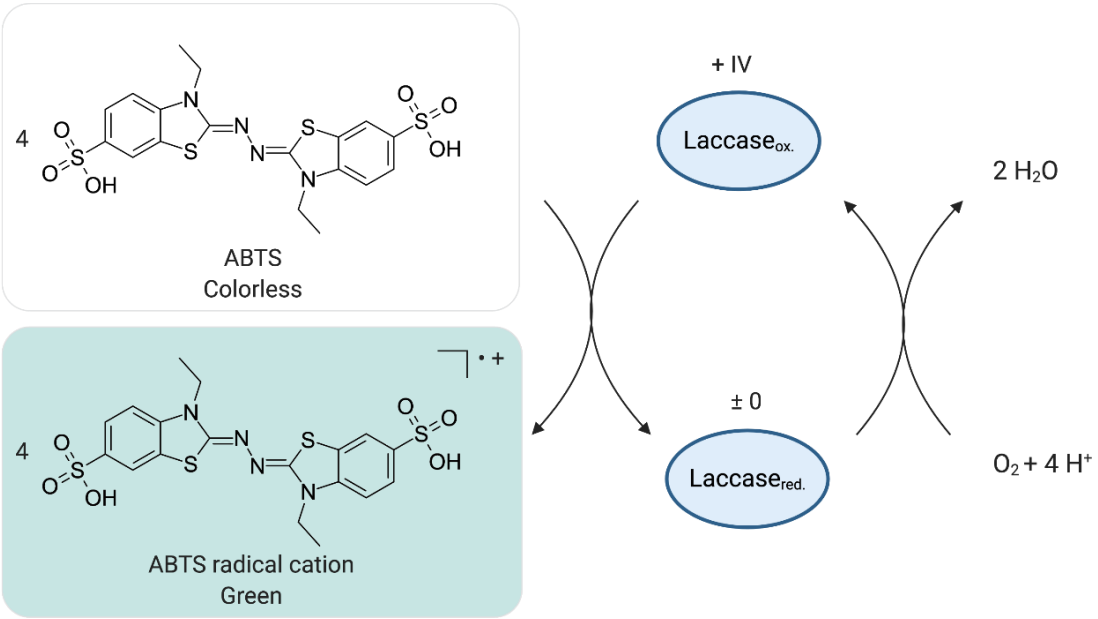


Figure 1: Oxidation of ABTS by Laccase and Formation of ABTS Radical Cation

1.3.2. Procedure (Dhakar and Pandey, 2013)

2. Mix 1 mL of free laccase (or 100 mg of adsorbent containing immobilized laccase) with 1 mL of 2 mM ABTS solution (in 0.1 M citrate-phosphate buffer, pH 2.6).
3. Incubate at room temperature for 2 minutes.
4. Measure absorbance at 420 nm.
5. Calculate activity using the extinction coefficient (36,000 M^{-1} cm^{-1}).
6. Express activity as the amount of enzyme required to oxidize 1 µmol of ABTS per minute.

IV. 2. Immobilized Enzyme Assay (Alginate Beads)

2.1. Principle of column reactor

A column reactor for immobilized enzyme assays constantly moves a substrate solution along a column filled with beads containing immobilized enzymes. This setup creates an environment where substrate molecules are drawn toward the enzyme surfaces on the beads due to a concentration gradient. The immobilized enzymes catalyze the conversion of the substrate into specific products as it moves through the column. The column then releases the reaction products and the effluent, allowing for their collection and enzymatic activity measurement. This stable and reusable system is ideal for continuous or repeated processing.

The porous nature of the alginate beads allows substrate molecules to penetrate the bead's interior, increasing the surface area available for interaction with the immobilized enzymes.

Once the substrate molecules reach the enzyme's active sites within the beads, they undergo catalysis, converting the substrate into specific products.

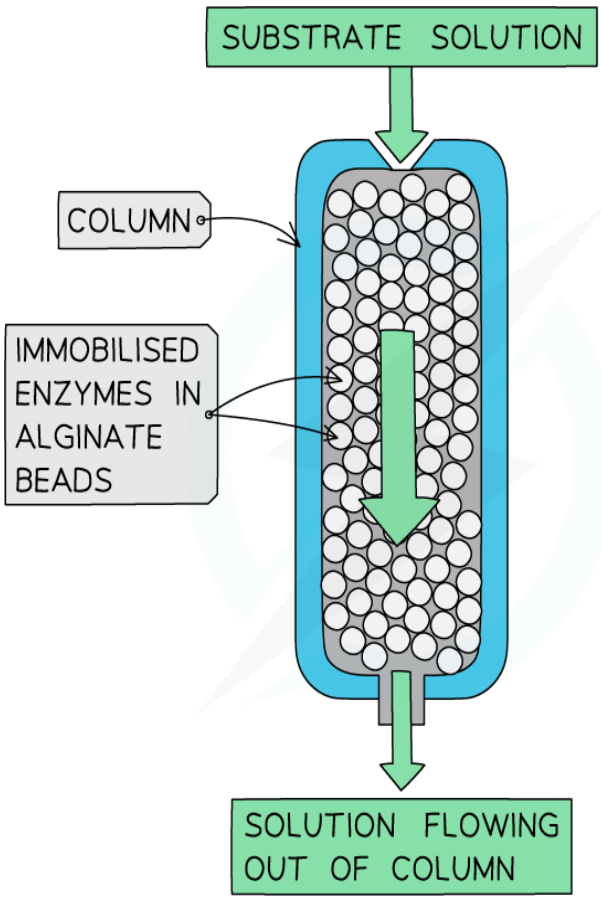


Figure 2: The immobilized enzymes are contained within a column

2.2. Procedure:

2.2.1. Amylase Assay

1. Pack immobilized amylase beads into a column.
2. Flow starch solution through the column at a constant rate.
3. Collect effluent at 0, 15, and 30 minutes.
4. Add 0.5 mL DNS reagent to 0.5 mL effluent.
5. Heat at 100°C for 5 minutes, then cool to room temperature.
6. Measure the absorbance at 540 nm using a UV/Vis spectrophotometer to determine the amylase activity.

2.2.2. Glucose Isomerase Assay

1. Pack immobilized glucose isomerase beads into a column.
2. Flow glucose solution (2.6 M glucose) through the column at a constant rate.
3. Collect effluent at 0, 15, and 30 minutes.
4. Add 0.1 mL of 2% L-cysteine hydrochloride and 5.0 mL of 75% (v/v) sulphuric acid to 0.5 mL effluent.
5. Heat the mixture at 40°C to help the reaction.
6. Add 0.15 mL of 12% carbazole solution to the mixture.
7. Allow the color to develop at room temperature and cool in an ice bath.
8. Measure the absorbance at 566 nm to quantify fructose production, reflecting the glucose isomerase activity.

2.2.3. Laccase Assay

1. Pack immobilized laccase beads into a column.
2. Flow 2 mM ABTS solution (in 0.1 M citrate-phosphate buffer, pH 2.6) through the column at a constant rate.
3. Collect effluent at 0, 15, and 30 minutes.
4. Measure the absorbance at 420 nm to monitor the oxidation of ABTS by laccase and calculate the enzyme activity.

Note: For calculating enzyme activity for amylase and glucose isomerase is:

1. **Calculation of Massive Concentration (Cm)** can be determined directly by the equation of the line from your standard curve: $Y = mX + b$

Where:

- Y = Absorbance (measured value).
- m = Slope of the line.
- X = Concentration (Cm).
- b = Y-intercept (this is sometimes zero if the line passes through the origin).

After determining the concentration (X), convert to mol/L by dividing by the molecular weight of the substance and multiplying by 1000 to convert from mg/mL to mol/L (M).

1. **Calculation of Moles (n): The molar concentration (Cm)** is given in moles per liter (mol/L). To calculate the number of moles (n), use the formula: $n = \mathrm{Cm} \times V$

Where:

- V is the volume of the solution in liters.

2. **Calculation of Enzyme Activity (Z)**

$$Z = \frac{n}{T}$$

Where:

- n is the number of moles.
- T is the incubation time, which is the period during which the enzyme is active.

For calculating Laccase activity is:

$$\text{Enzyme Activity (U/ml)} = \frac{\Delta A \times V_{\text{total}}}{t \times \varepsilon \times V_{\text{enzyme}}}$$

Where:

- ΔA is the absorbance at 470 nm (which reflects the amount of ABTS oxidized).
- V is the total volume of the reaction mixture (ml).
- v is the volume of the enzyme used in the reaction (ml).
- t is the incubation time (minutes).
- Ɛ is the extinction coefficient of ABTS (36,000 $M^{-1}cm^{-1}$), which is a constant that relates absorbance to the concentration of the oxidized product.

Laccase activity can also be determined spectrophotometrically at 465 nm using 2 mM guaiacol as a substrate. The reaction mixture contains 50 mM phosphate buffer (pH 6.5), and the molar extinction coefficient used for the calculation is $\varepsilon_{465} = 48{,}000\ M^{-1}\ cm^{-1}$.

Calculating Immobilization Yield

Use the following formula to calculate the yield or efficiency of immobilization:

$$\text{Immobilisation Yield (\%)} = \left(\frac{\text{Activity of Immobilised Enzyme}}{\text{Activity of Free Enzyme}}\right) \times 100$$

Where:

- **Activity of Free Enzyme** is the activity measured before immobilization.
- **Activity of Immobilized Enzyme** is the activity measured after immobilization.

Practical Work ❹ Production of fructose syrup from hydrolyzed starchy substrates using immobilized enzyme

I. Introduction

The increasing demand for sugar in the food and beverage industry has led to growing interest in alternative sweeteners. One such alternative is fructose syrup, a liquid sugar with a higher sweetness level than glucose and sucrose, making it a desirable substitute. Fructose syrup, typically produced from corn starch through enzymatic isomerization, offers a healthier and more efficient option compared to traditional sugar cane. The sweetness of fructose is significantly higher than that of glucose and sucrose (White, 2008). Fructose syrup is made by converting glucose, which itself is derived from starch, into fructose using the enzyme glucose isomerase (Bray *et al.,* 2004). Starch, a polysaccharide composed of amylose and amylopectin, can be sourced from various materials for the production of fructose syrup. The production process involves first obtaining a high-concentration glucose syrup through enzymatic hydrolysis using α-amylase and glucoamylase, or a combination of acid and enzymatic hydrolysis, followed by isomerization to fructose (Permanasari & Yulistiani, 2015; Triyono, 2008).

II. Objectives

In this practical work, we will focus on:

- Investigating the enzymatic hydrolysis of starches (e.g., rice or potato powder) using immobilized amylase and glucose isomerase to produce fructose syrup.
- Quantifying the reducing sugars, including glucose and fructose, produced during the hydrolysis and isomerization using the DNSA and carbazole tests.
- Comparing the efficiency of fructose production between free and immobilized enzyme systems.

III. Materials

Products	Equipment/Instruments
• Rice or potato starch powder • Distilled water • Hydrochloric acid (HCl) • Sodium hydroxide (NaOH) • α-amylase • Glucoamylase • Glucose isomerase • DNSA reagent • Resorcinol reagent • Fructose standard	• Beakers • Glass stirring rods • pH meter or pH strips • Heating plate or water bath • Thermometer • Centrifuge • Boiling water bath • Spectrophotometer • Pipettes and micropipettes • Analytical balance

IV. Methods

IV. 1. Syrup fructose production

1.1 Principle:

The production of fructose syrup involves enzymatic hydrolysis, which occurs in four main stages: gelatinization, liquefaction, saccharification, and isomerization. Gelatinization is the first stage, where starch grains are heated in excess water to increase the amorphous regions of amylopectin and improve enzyme accessibility. This process causes the starch granules to swell, breaking the hydrogen bonds within the starch glycosidic bonds. Once gelatinized, the swelling of the granules is irreversible, and they cannot return to their original shape. Liquefaction follows gelatinization, and if performed without this step, it requires a longer processing time. In the liquefaction stage, bacterial α-amylase hydrolyses the starch into shorter chains known as dextrins or maltose. This step is conducted under neutral pH and moderate temperatures. During saccharification, glucoamylase further hydrolyses the dextrins and maltose into glucose, typically at an acidic pH and lower temperatures. To produce fructose syrup, the glucose undergoes isomerization, where glucose isomerase converts it into fructose. This process is carefully controlled to optimize conversion efficiency by maintaining appropriate pH and temperature conditions (Permanasari *et al.,* 2018).

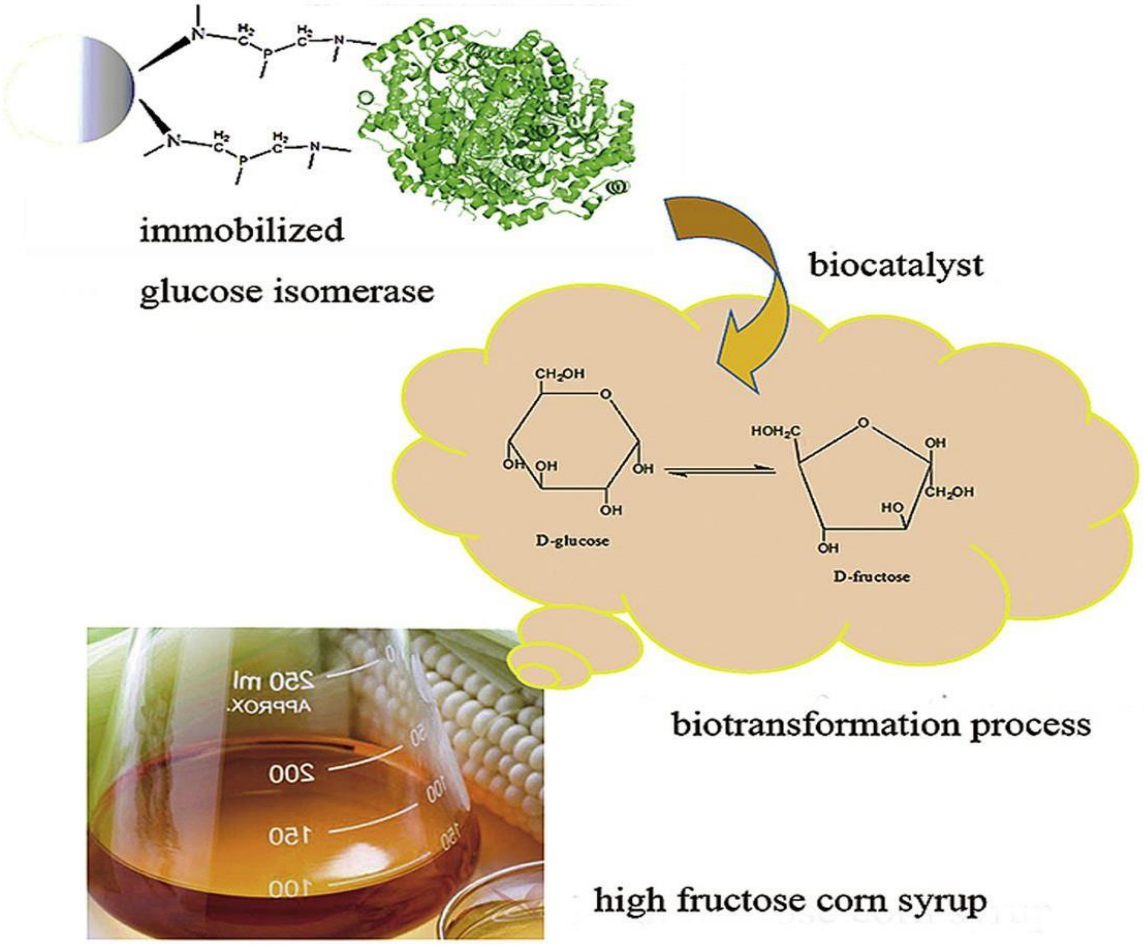


Figure 1: Enzymatic Biotransformation of Glucose to Fructose Using Immobilized Glucose Isomerase for High-Fructose Corn Syrup Production

1.2. Procedure (Yulistiani *et al.,* 2019*)*:

A. Gelatinization of Starch

- Dissolve 10 g of rice or potato powder in 100 mL of distilled water in a clean beaker.
- Stir thoroughly to ensure the starch is fully suspended.
- Heat the starch solution to 76°C for rice or 65°C for potato, maintaining the temperature for 20 minutes.

B. Dextrinization (Liquefaction)

- Adjust the pH of the gelatinized starch solution to 7 using hydrochloric acid or sodium hydroxide.
- Add 1 mL of α-amylase.
- Incubate the solution at 50°C for 30 minutes, stirring continuously.

C. Saccharification

- Adjust the pH to 4.5 using hydrochloric acid.
- Add 1 mL glucoamylase
- Incubate the mixture at 37°C for 1 hour with gentle stirring.

D. Isomerization

- Add 1% (v/v) glucose isomerase to the glucose solution.
- Incubate at 60°C, pH 8.2, for 24 hours.

E. Filtration and Concentration

- After saccharification, filter the solution by centrifugation to remove any insoluble residues or retrograded starch.
- Heat the filtrate to evaporate water and thicken the fructose syrup until the desired viscosity is achieved.

F. Analysis and Storage

- Analyze 1 mL of the digested product at 15-minute intervals using the DNSA and Resorcinol Method.
- Store the fructose syrup in a sterilized container at room temperature or refrigerate to prevent microbial contamination.

IV.2. *Determination of Reducing Sugar and Fructose Concentration* (Yulistiani *et al*., 2019)

2.1. Reducing Sugar Determination (DNS Method)

For each sample from the hydrolysis and isomerization process:

- Add 2 mL of the sample to 3 mL of DNS solution.
- Heat the mixture in boiling water for 15 minutes, then rapidly cool it in iced water.
- Measure the absorbance of the cooled sample at 540 nm.

2.2. Fructose Concentration (Resorcinol Method)

For the isomerization samples, determine the fructose concentration as follows:

- Add 1 mL of the sample to 0.5 mL of resorcinol reagent and mix thoroughly.
- Add 3.5 mL of hydrochloric acid solution (prepared by mixing 5 mL HCl with 1 mL distilled water) and mix again.
- Heat the sample in a water bath at 80°C for 10 minutes.
- Measure the absorbance of the solution at 520 nm.

Practical Work ❺ Decolorization of synthetic dyes by immobilized Laccase

I.Introduction

Synthetic dyes are a major source of pollution, causing significant environmental issues across various industries, including textiles, cosmetics, photography, and paper production (Samuchiwal *et al.,* 2021). A large portion, nearly 10-15%, of dyes from manufacturing processes is discharged into wastewater (Dauda and Erkurt, 2020). Traditional treatment methods, such as adsorption and chemical degradation, are often ineffective and generate harmful by-products (Osma *et al.,* 2010).

Bioremediation, particularly through the use of microbial enzymes, offers an environmentally friendly alternative for dye decolorization (Qin *et al.,* 2019). Among these enzymes, laccase, an oxidative enzyme produced by white-rot fungi, is especially effective in degrading synthetic dyes (Sosa-Martíneza *et al.,* 2020). Due to its multicopper functionality, laccase can oxidize aromatic compounds in synthetic dyes, leading to non-toxic products (Singh *et al.,* 2021). The use of immobilized laccase has gained attention as a promising strategy to enhance the stability and reusability of the enzyme in dye decolorization processes, offering a more sustainable approach to textile wastewater treatment.

II. Objectives

By the end of this practical work, each student should be able to:

- Understand the role of laccase in the decolorization process.
- Comprehend how laccase catalyzes the breakdown of synthetic dyes.
- Design an experimental setup for the decolorization process.
- Apply appropriate analytical techniques to quantify dye decolorization.

III. *Materials*

Products	Equipment/Instruments
• Methylene blue • Crystal violet • Congo red • Glucose • Luria-Bertani (LB) medium • $MgSO_4$	• UV/Vis spectrophotometer, • centrifuge, • analytical balance • shaking water bath, • Erlenmeyer flasks (250 mL) • pH meter

IV. *Methods*

IV.1. Dyes decolorization by laccase immobilized

1.1. Principle

Laccase, an oxidoreductase enzyme, catalyzes the oxidation of various aromatic and non-aromatic compounds, including dyes, using molecular oxygen as an electron acceptor. During the reaction, laccase oxidizes dye molecules by transferring electrons from their chromophoric groups, leading to the breakdown of these groups and resulting in decolorization.

This enzymatic process typically involves the generation of reactive radicals, which can further degrade complex dye structures into smaller, less colored, or colorless molecules. Laccase is particularly effective against phenolic and non-phenolic dyes, making it a versatile tool for dye removal in wastewater treatment. The efficiency of decolorization depends on factors such as pH, temperature, and the structure of the dye.

1.2. **Procedure** (El-Bendary *et al.,* 2020)

1. Dye Selection

Three dyes will be used in this experiment, selected based on their distinct molecular structures and specific wavelengths of maximum absorbance (λmax):

- **Methylene blue** (λmax=660 nm)
- **Crystal violet** (λmax=590 nm)
- **Congo red** (λmax=497 nm)

2. Experimental Setup

❖ For each dye, prepare three sets of 250 mL Erlenmeyer flasks as follows:

Set 1 (Enzyme-Dye Mixture):

- Add 1 mL of laccase enzyme solution.
- Add 100 mg/L of dye in sterile tap water.

Set 2 (Enzyme-Dye with Nutrient Medium):

- Prepare the same setup as Set 1 but include a 5% LB (Luria-Bertani) medium to provide additional nutrients, which may enhance enzymatic activity.

Set 3 (Control – Dye Only):

- Add 100 mg/L of dye without any enzyme to observe if decolorization occurs naturally.

❖ Incubate all flasks at 30°C with shaking at 140 rpm for 24 hours**.**

3. Post-Incubation Procedure

1. After incubation, centrifuge the samples at 6000 x g for 30 minutes to separate the solids from the liquid.
2. Collect the supernatant (clear liquid) for analysis.
3. Measure the absorbance of the supernatant at the λmax of each dye using a UV-Vis spectrophotometer.

4. Calculating Decolorization Efficiency

The efficiency of dye decolorization is determined using the following formula:

$$\text{Decolourisation Efficiency } (\%) = \frac{A - B}{A} \times 100$$

Where:

- A is the absorbance of the untreated dye solution (initial absorbance).
- B is the absorbance of the treated dye solution (after enzymatic treatment).

This calculation quantitatively measures the enzyme's ability to degrade or remove the dye.

Practical Work ❻ Bioethanol production by *Saccharomyces cerevisiae* immobilized

I. Introduction

Bioethanol production is a biological process carried out by microorganisms under specific conditions and in an appropriate medium. Therefore, selecting the right microorganisms is crucial for efficient bioethanol production. The choice should depend on the composition of the raw material and the optimization of conditions for the selected production method. Yeasts and bacteria commonly used in industrial ethanol production each have their advantages and limitations. Current research is particularly focused on biocatalysts capable of efficiently fermenting mixed sugar fractions. Examples include *Pichia stipitis*, which facilitates xylose fermentation in ethanol production; *Escherichia coli*; *Kluyveromyces* species; and *Zymomonas mobilis*, which enables the utilization of pentoses. Other significant yeasts include *Saccharomyces cerevisiae* and related species. Additionally, thermophilic bacteria like *Clostridium thermocellum*, which can metabolize cellulose, and white rot fungi, known for their lignin-degrading abilities, are also being actively studied. These organisms hold promise for improving the efficiency and versatility of bioethanol production (Bayrakci Ozdingis & Kocar, 2017).

II. Objectives

This practical work aims to:

- Achieve yeast immobilization.
- Produce ethanol using immobilized yeast in the fermentation process.
- Understand methods for separating ethanol from the fermentation mixture.

III. Materials

Products	Equipment/Instruments
• YEPD media • Sodium alginate solution • Acetate buffer pH 5 • Calcium chloride (CaCl2) • Distilled water • Glucose • DNS reagent • Na-K tartrate solution • Potassium dichromate reagent	• UV/Vis spectrophotometer • centrifuge • analytical balance • shaking water bath • Rotary evaporator

IV. Methods

IV.1. Immobilization of *Saccharomyces cerevisiae* in sodium alginate (Ali & Khan, 2014).

- Preparation of the Yeast Suspension:
    a. Cultivate yeast in 10 ml of YEPD media for 48 hours at 30°C with stirring.
    b. Centrifuge the culture at 4°C (3,300 g for 15 minutes).
    c. Remove the supernatant.
    d. Wash the pellet with sterile distilled water.
- Suspend the pellet in 5 mL of acetate buffer, pH 5.
- Add 25 ml of 3% sodium alginate solution to 1 ml of the yeast suspension.
- Drip the alginate-yeast mixture into a 0.1 M calcium chloride solution (CaCl2), using a syringe or dropper
- Leave to stand for 1 hour.
- Separate the beads from the solution using filter paper.
- Wash them three times with distilled water.

IV. 2. Bioethanol production (Ali & Khan, 2014).

1. Fermentation Process

Use 500-ml flasks, each containing 250 ml of YEPD media with 15% glucose as the carbon source. Add the immobilized cells or free cells into each flask and place flasks on a rotary shaker, set to 100 rpm and maintained at 30 °C. Maintain this setup until the end of the fermentation process. After the fermentation process, retrieve the immobilized cells from each flask.

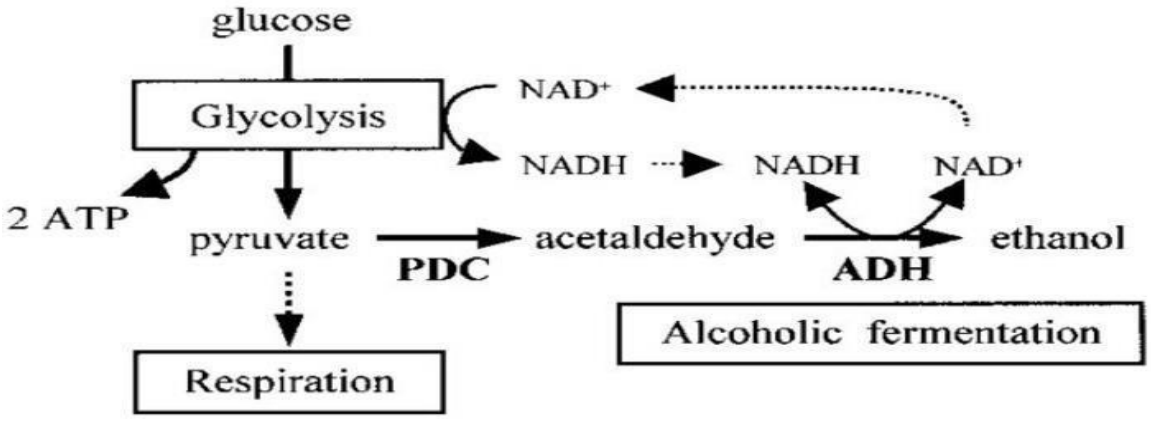


Figure 1: Diagram of the alcoholic fermentation pathway in plants. Alcoholic fermentation occurs in two reaction steps: the decarboxylation of pyruvate to acetaldehyde by PDC and the subsequent reduction of acetaldehyde to ethanol by ADH (Saika *et al.,* 2006).

2. Bioethanol Recovery Using a Rotary Evaporator

To recover bioethanol using a rotary evaporator, first assemble the evaporator, ensuring the flask is securely attached to the rotary shaft. Set the water bath to 40-50°C and connect the condenser to a cold-water source. Next, pour the filtered fermentation broth containing ethanol into the flask, filling it about one-third full. Begin rotating the flask to create a thin liquid film, enhancing evaporation efficiency. Activate the vacuum pump to lower the boiling point of ethanol, facilitating evaporation. Once the ethanol vapor condenses in the cooler condenser, collect it in a receiving flask. When evaporation is complete, stop the vacuum pump and turn off the evaporator. Finally, transfer the condensed ethanol into a clean, labeled container for storage or further analysis.

Figure 2: Rotary Evaporators for Ethanol Extraction

IV.3. Estimation of Glucose Using the DNSA Method

To estimate glucose according to (Joyce Hellen Sathya *et al*., 2020), take 0.8 ml and 1.0 ml of hydrolyzed pod samples in separate test tubes and adjust the total volume in each test tube to 1 ml using distilled water. Incubate the test tubes at room temperature for 5 minutes. Then, mix the contents thoroughly and add 1 ml of the DNS reagent to each test tube. Place the test tubes in a water bath at 95°C for 10 minutes, after which they should be transferred to a cold-water bath to cool the contents. Add a 40% Na-K tartrate solution to stop the reaction. For the blank, use 1 ml of distilled water instead of the sample and treat it similarly with the DNS reagent and tartrate solution. Finally, measure the absorbance of the red-colored solutions at 540 nm.

IV.4. Bioethanol assay

To perform the bioethanol assay (Caputi *et al.,* 1968), first prepare the blank by mixing 800 µl of distilled water and 1 ml of potassium dichromate reagent in a cuvette. Then, mix 200µl of the collected ethanol, 800µl of distilled water, and 1 ml of potassium dichromate reagent for each sample in a cuvette. Incubate the reaction mixtures at 60 °C for 20 minutes. After incubation, measure the absorbance of the reaction mixtures and the blank at 584 nm. Finally, a calibration curve using a 10% ethanol standard solution will be prepared, and the absorbance of each dilution will be measured at the same wavelength.

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Appendix

Use of AI Tools

This educational handout has been refined with the assistance of AI-based tools, including **ChatGPT**, **Quillbot**, **Gemini,** and **DeepL**, to enhance its clarity, coherence, and accessibility. These tools were used to paraphrase, optimize, and refine the text while maintaining the original intent and pedagogical objectives of the material. The integration of AI technologies ensured a more polished and reader-friendly presentation without compromising the academic rigor or educational purpose of the content.