

LABORATORY PRODUCTION AND ASSAY OF AMYLASE BY FUNGI AND BACTERIA.

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What are amylases?

Amylases are enzymes that break down starch or glycogen.

Amylases are produced by a variety of living organisms, ranging from bacteria to plants and humans. Bacteria and fungi secrete amylases to the outside of their cells to carry out extra-cellular digestion. When they have broken down the insoluble starch, the soluble end products such as (glucose or maltose) are absorbed into their cells.

Amylases are classified based on how they break down starch molecules

- i. α -amylase (alpha-amylase) - Reduces the viscosity of starch by breaking down the bonds at random, therefore producing varied sized chains of glucose
- ii. β -amylase (Beta-amylase) - Breaks the glucose-glucose bonds down by removing two glucose units at a time, thereby producing maltose
- iii. Amyloglucosidase (AMG) - Breaks successive bonds from the non-reducing end of the straight chain, producing glucose

Many microbial amylases usually contain a mixture of these amylases.

Why bother about amylases?

Humans exploit microbial amylases for the following purposes:

1. High Fructose Corn syrup preparation
2. Additives to detergents for removing stains
3. Saccharification of starch for alcohol production
4. Brewing

What organisms are responsible for amylase production?

Although many microorganisms produce this enzyme, the ones most commonly used for their industrial production are *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquifaciens* and *Aspergillus niger*

General Lab Requirements:

- Autoclave or pressure cooker
- Hot Plate or Microwave oven
- Nutrient Agar powder
- Potato Dextrose Agar powder
- Soluble starch
- Weighing scales
- Shaker
- Spectrophotometer or colorimeter
- Water bath (Temperature controlled)

Materials per group of 4 students

- Hand trowel or disposable spoons
- Sterile pipettes (One each of 10 mL, 5 mL and 1 mL)
- Pipette pumps
- Six bottles of sterile water, containing 90 mL each
- Sterile Glass Petri dishes or Pre-sterilized disposable Petri-dishes
- Wire loop
- Dissecting needle or Cork borer
- Bunsen burner and matches
- Glass spreader
- 95% ethanol.
- Dialysis tube

SECTION 1.

Isolation of Amylase producers from the environment

The soil contains a rich deposit of both bacteria and fungi, which produce amylases. Starch hydrolyzing fungi or bacteria could be isolated from the soil, foods or could be purchased. Buying saves time and ensures a high yielding strain. However, isolating could be fun, and constitutes an additional lab.

Isolation Procedure

- i. Select a moist part in a wood or park (only in warm climates, during the warm season in colder climates)

EITHER: Sweep off the debris from the top of the soil, use a hand trowel to collect a sample of the top soil (about 100 grams), into a "Ziploc" bag

OR:

Bury a cut piece of cut potato about four inches deep, and cover with soil. After about 8 days, dig the potato out, scrape the soil off and take it to the lab in a "Ziploc" bag or a plastic bag. (This is called baiting)

- i. Suspend about 10 grams of either soil or rotten potato, in 90 mL sterile distilled water, mix properly
- ii. Pipette 10 mL of the above and transfer to another 90 mL of water
- iii. Dilute further in two more 90 mL sterile water blanks
 - For fungi, spread 0.1 mL from the dilutions on Potato Dextrose Agar plates (fortified with 0.1 mg/mL streptomycin sulfate) with a glass spreader. (The glass spreader is quickly sterilized by dipping in 95% ethanol and putting in the flame, so that the alcohol burns off) Incubate at room temperature for about 3 days
 - For bacteria, spread 0.1 mL of the diluted samples on Nutrient Agar plates containing 1 % w/v soluble starch and incubate at 30°C for 24 hours
- iv. Starch-producing colonies will have an area of clearing around them.

- v. Confirm by flooding plates with Gram's iodine. Parts of the plant still containing starch will stain ink-black.

Transfer distinguishable, amylase-producing fungi to fresh plates of Potato Dextrose agar containing 1 % starch, using a sterilized dissecting needle. For bacteria, streak on a fresh plate of Nutrient Agar containing 1 % starch.

Transfer your isolated amylase-producing fungi to Potato Dextrose Agar slants, and the bacteria to Nutrient Agar. Allow bacteria to grow for 24 hours and fungi to grow for 72hours, then store in the refrigerator until needed.

SECTION 2

Set up for fungal amylase production

1. Pour 10 mL of sterile distilled water on the slant containing fungal spores
2. Scrape with a wire loop to loosen the spores
3. Inoculate medium with 0.5 mL spore suspension of fungi

Medium composition (grams per liter)

KH_2PO_4	1.4
NH_4NO_3	10
KCl	0.5
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01
Soluble starch	20
Adjust pH to 6.5	

(Distribute in 30-40 mL of medium into 50 mL Erlenmeyer flasks and sterilize by autoclaving at 121°C for 15 minutes) Allow to cool down to room temperature

Incubate with shaking (If shaker is available) for 72 hours. 200 rev/min is adequate if grown on a shaker. However, it possible to obtain a good yield with intermittent shaking with hand if a shaker is not available.

Set up for Bacterial Amylase production

1. Grow bacteria in Nutrient Agar, or Tryptic Soy agar slants
2. Add a loopful of bacterial culture into the amylase production medium

Medium for bacterial amylase production (g/l)

Bacteriological Peptone	6
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5
KCl	0.5
Starch	1

Mix and distribute in 30-40 mL volumes into 100 mL Erlenmeyer flasks Sterilize by autoclaving at 121°C for 15 minutes

Extraction of Enzyme from Fungi

It is very easy to remove the fungal mycelium from the enzyme production medium.

Pour the whole content of the flask containing the growing fungus through a funnel fitted with Whatman number 1 filter paper. The filtrate contains the crude amylase.

Extraction of Enzyme from bacteria

You will need a high speed, refrigerated centrifuge for this. Pour the bacterial culture into centrifuge tubes, and spin for 20 minutes at 5000 rpm. Decant the supernatant, which is the crude enzyme extract.

Dialysis (Optional)

Dialysis will remove residual sugars from the enzyme mixture

- i. Tie one end of a dialysis tube
- ii. Pour enzyme mixture into dialysis tube
- iii. Tie the other end securely
- iv. Put dialysis tube in distilled water in a beaker
- v. Change the water several time for 24hours
- vi. Pour crude enzyme into clean Universal bottles or screw cap tubes and store in a freezer at about 0°C until needed

Demonstration of Enzyme Activity

- i. Pipette 1 mL of culture extract "enzyme" into a test tube
- ii. Add 1 mL of 1% soluble starch in citrate-phosphate buffer (pH6.5)
- iii. Incubate in a water bath at 40 °C for 30minutes
- iv. Set up a blank consisting of 2mL of the enzyme extract that has been boiled for 20 minutes (boiling inactivates the enzyme), added to the starch solution and treated with the same reagent as the experimental tubes.
- v. Stop the reaction by adding 2 mL of *DNS reagent (1.0 g of 3, 5, dinitrosalicylic acid, 20 mL of NaOH and 30 grams of sodium potassium tartarate in 100 mL)
- vi. Boil for 5 minutes
- vii. Cool and add 20 mL of distilled water
- viii. Determine color intensity at 540 nm

A concentration glucose calibration curve is used to convert color to reducing sugar equivalent.

Enzyme activity may be defined as the amount of glucose produced per mL in the reaction mixture per unit time.

FIGURE 1. OUTLINE PROCEDURE FOR ISOLATION OF AMYLASE-PRODUCING FUNGI

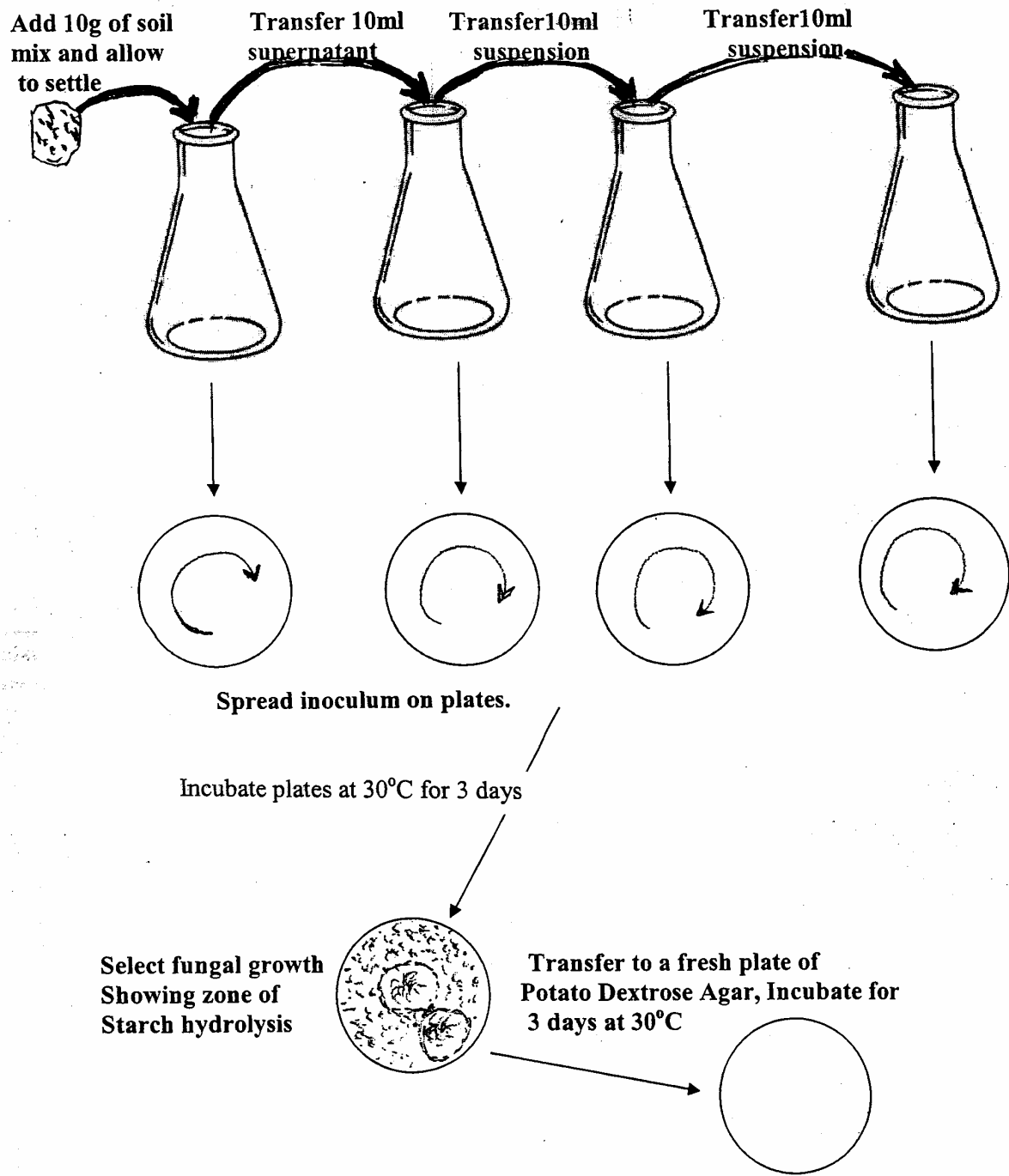


FIGURE 2: OUTLINE PROCEDURE FOR GENERATING FUNGAL AMYLASE FROM FUNGAL SPORES.

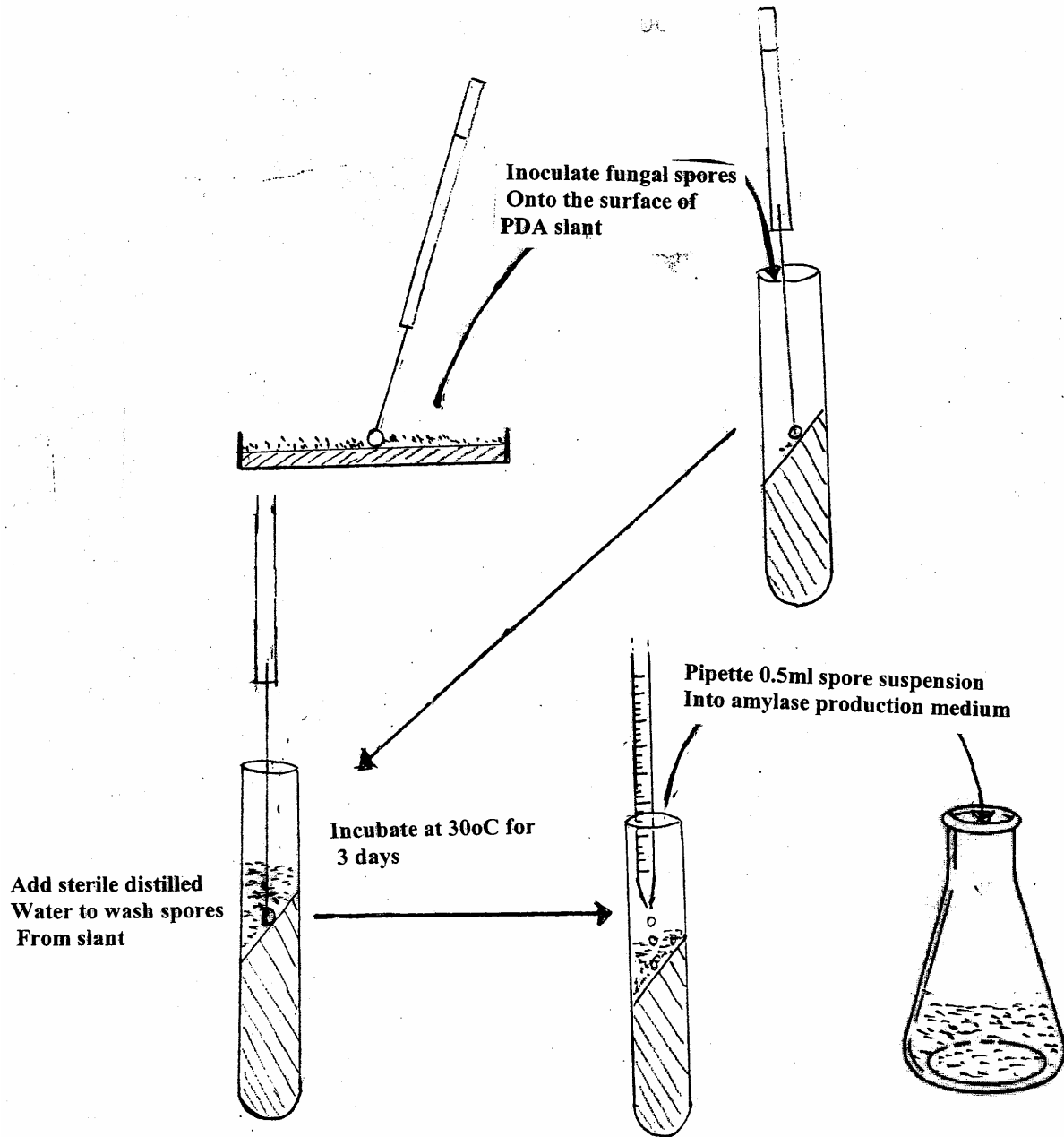
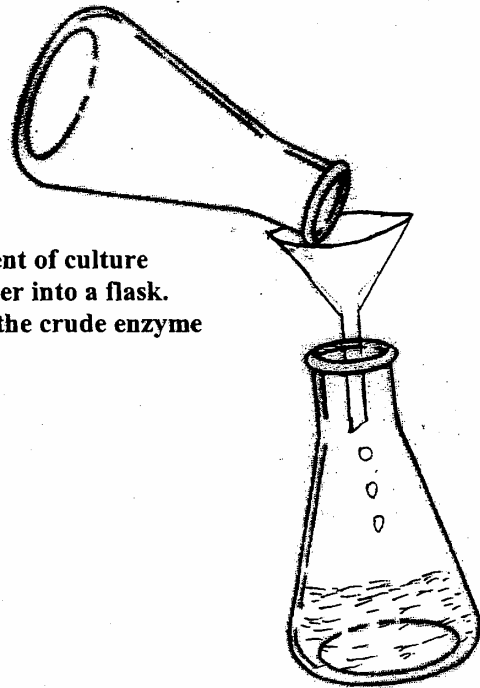


Figure 2: (continued)

Grow (if possible with shaking) at 25-30°C for 72 hours



Pour entire content of culture through filter paper into a flask. Culture filtrate is the crude enzyme



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