Unit 4: Microbial enzymes of industrial interest and enzyme immobilization

Hands-on screening for cellulose hydrolysis

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- This lecture is in continuation to what was covered in the class.
- This is being posted in pursuance of the notification on continued online learning during class suspension due to Covid-19.

Unit 4: Microbial enzymes of industrial interest and enzyme immobilization

- Microorganisms for industrial applications
- Hands –on screening for casein hydrolysis
- Hands-on screening for starch hydrolysis
- **Hands-on screening for cellulose hydrolysis**
- Methods of immobilization
- Advantages and applications of immobilization
- Large-scale applications of immobilized enzymes (glucose isomerase and penicillin acylase)

Introduction

- The cellulases are a group of enzymes that synergistically hydrolyze cellulose into glucose.
- They are of 3 major types: endo-β-1,4 glucanase, cellobiohydrolase and β-D-glucosidase.
- Cellulases are commonly produced by bacteria and fungi.

Reactions: endo-β-1,4 glucanase, cellobiohydrolase and β-D-glucosidase

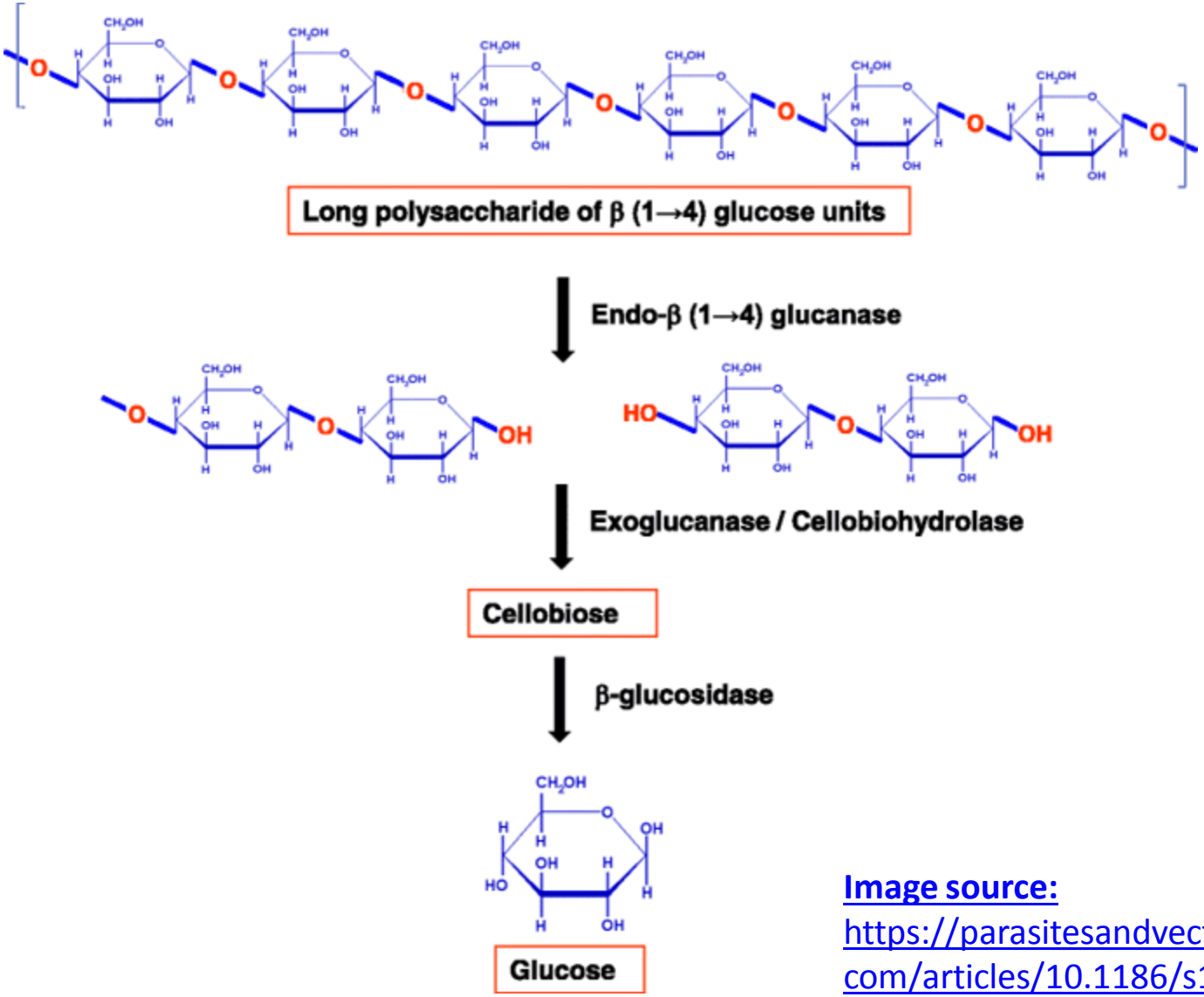


Image source:
https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-015-0642-7

Cellulases: Introduction contd.

- Their substrate is cellulose, which is a polymer of D-glucose units linked by β-1,4 bonds that is the most abundant biomass and a major structural component of plants and a renewable source of energy in the biosphere.
- Importance of cellusases: They have diverse application in textile, detergent, leather, food, feed and paper industries. They are also used in biomass fermentation, fiber modification and in pharmaceutical purposes.
- Also cellulases for effective treatment and utilization of cellulose containing wastes as cheap carbon sources are of economic importance.
- Application of these enzymes in such industries demands the identification of stable enzymes that can active at high pH and increased temperature. Thus effective screening methods are very important.

Hands-on screening for cellulose hydrolysis

- **Microbe-Secreted Endoglucanase Assay on CMC-Agar Medium procedure:**

  This method is essentially based on the interaction of Congo red with intact P-(l-4)-~-glucans in carboxymethyl cellulose.

Reagents:

- CMC (1% w/v, low viscosity) in 1.5% agar medium. Dissolve CMC before adding agar and autoclave.
- Congo red solution (1 g/l) prepared by dissolving 100 mg Congo red in 99 ml water and 1% ethanol.

Procedure

1. Inoculate the endoglucanase-secreting microorganisms on the solid Carboxymethyl cellulose (CMC) medium. The growth time depends on the growth rate of the microorganism and enzyme activity.
2. Stain a 9-cm Petri dish by adding 20 ml of Congo red solution at room temperature for 30 min.
3. Rinse the residual dye on the dish using distilled water.
4. Destain Congo red with ~20 ml of 1 M NaCl for 30 min. If the halos are not clear, destain the dish by another ~20 ml of NaCl solution.
5. Detect the clear, weak yellow halos for endoglucanase activity with the red background.
6. **Optional:** In order to increase halo contrast, add ~20 ml of 5% acetate acid or 1 M HCl to the plate at room temperature for 10 min, and pour off. The background of the plate will turn blue.

- If CMC inhibits microorganism growth, a second layer of CMC solid medium can be applied to the primary medium containing other carbon sources or nutrients.

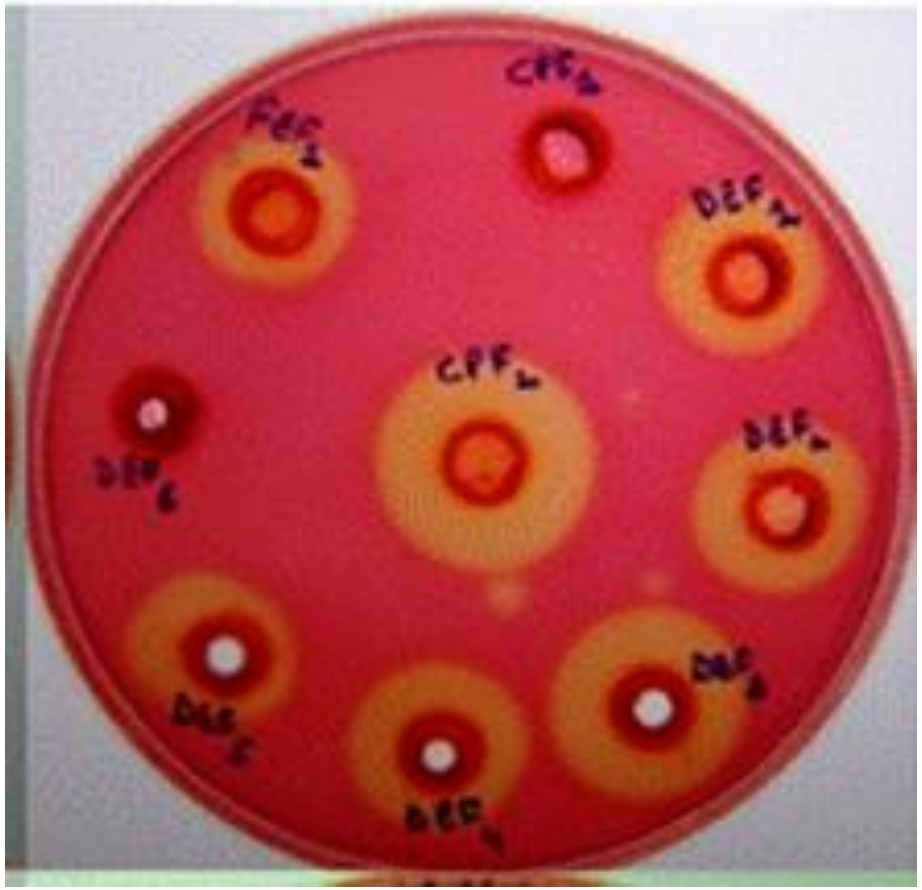


Image source: http://pubs.sciepub.com/ajmr/1/4/4/index.html

- The above figure shows varying endoglucanase activities of various colonies. Larger halos imply greater enzyme activity than smaller halos.

Screening fungi for cellulose hydrolysis using CMC

- Isolated fungal cultures can be screened for their ability to produce cellulases complex following the method of **Teather & Wood (1982).**
- Medium used: Czapek-Dox. Composition is as follows (pH adjusted to 5):

Component (g/L)	Sucrose	$NaNO_3$	K_2HPO_4	$MgSO_4$	KCl	$FeSO_4$	CMC	Agar
Amount	30	2	1	0.05	0.5	0.01	1%	20

- The medium is autoclaved (at 121°C and 15 lbs. pressure) & poured into Petri plates and allowed to solidify.
- Cavities of 6 mm size are made in the solidified medium and inoculated with 0.1 ml of fungal suspension prepared from 7 day old slants.
- The plates are then incubated at RT (28 ± 2 °C) for three days to allow fungal growth, then again incubated for 18 h at 50 °C which is the optimum temperature for cellulases activity.
- After incubation, 10 ml of 1% Congo - Red staining solution is added to the plates that are shaken at 50 rev/ min for 15 min.
- The Congo - Red staining solution is then discarded & 10 ml of 1 N NaOH is added to the plates and shaken again at 50 rev/min for 15 minutes.
- Finally 1 N NaOH is also discarded and the staining of the plates can be analyzed by observing the formation of clear or yellowish zones around the fungal spore inoculated wells

Screening fungal isolates in liquid medium for cellulase activity using CMC

- The isolated fungal cultures can used to know their potential for cellulase production and activities in the following way:

A volume of 100 ml of Czapek-Dox broth medium amended with 1% cellulose is distributed into separate 250 ml conical flasks (pH 5) & autoclaved.

↓

The fungal spore suspensions are inoculated into the conical flasks.

↓

The flasks are incubated at 32 °C on a rotary shaker at 120 rpm for 3 days.

↓

After 3 days, culture filtrate is collected, centrifuged at 6000 rpm for 15 min. The supernatant was used to the estimation extracellular protein content .

- Not only this, it also used as a crude source of cellulase.

Screening for cellulase using Dinitrosalicyclic acid reagent method

- Cellulolytic activity can also be screened by measuring the amount of reducing sugar (glucose) produced with the dinitrosalicyclic acid reagent method.
- This assay is based on a fixed degree of conversion of substrate, i.e. a fixed amount (2 mg) of glucose (based on reducing sugars measured by the DNS assay) released from 50 mg of filter paper (i.e., both amorphous and crystalline fractions of the substrate are hydrolyzed) within a fixed time (i.e., 60 min).
- Total cellulase activity is described in terms of “filter-paper units” (FPU) per milliliter of original (undiluted) enzyme solution.
- It is a redox reaction. Glucose is reducing sugar & has free carbonyl group which is oxidized to carboxyl group and in turn DNS is reduced to ANS (3-amino-5-nitrosalicylic acid), a brown color compound which absorbs light mostly at 540 nm.

DNS (3,5-dinitrosalicylic acid) reagent preparation

Completely dDissolve 10.6 g of DNS and 19.8 g of NaOH in 1,416 ml of distilled water.

↓

Add 360 g of Rochelle salts (sodium potassium tartrate) + 7.6 ml of melted phenol (at 50°C) + 8.3 g of sodium metabisulfite, and then mix well.

↓

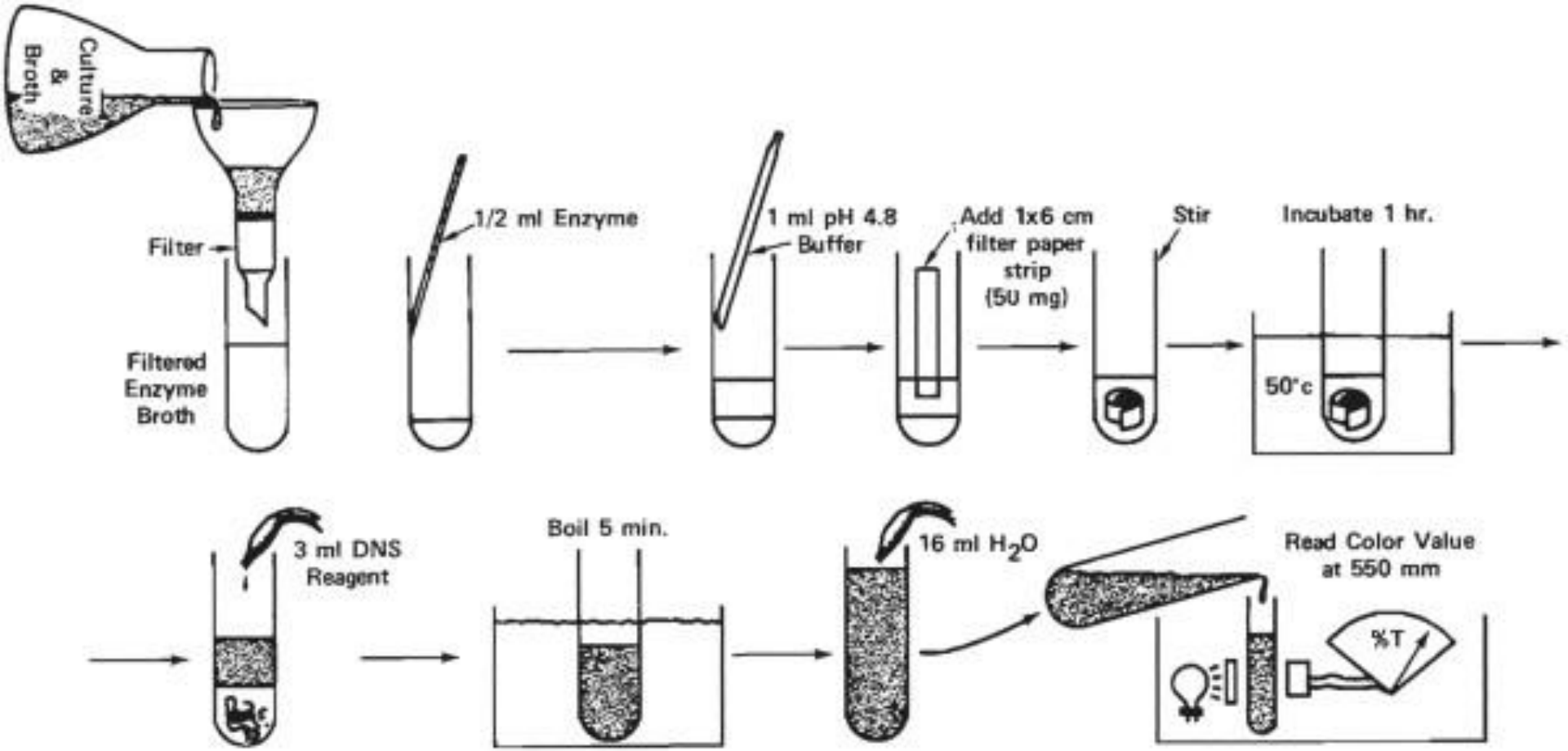
Titrate 3 ml of the DNS reagent using 0.1 M HCl using the phenolphthalein endpoint pH check. It should take 5–6 ml of HCl for a transition from red to colorless.

↓

Add NaOH if required (2 g of NaOH added = 1 ml of 0.1 M HCl used for 3 ml of the DNS reagent)

Procedure

1. Place a rolled filter paper strip into each test tube.
2. Add 1.0 ml of 50 mM citrate buffer (pH 4.8) to the tubes; the paper strip should be submerged in the buffer.
3. Prewarm the crude enzyme until equilibrium.
4. Add 0.5 ml of the enzyme to the tubes with filter paper substrate.
5. Incubate the tube in a 50°C water bath for exactly 60 min.
6. Add 3.0 ml of the DNS reagent to stop the reaction, and mix well.
7. Boil all tubes for exactly 5.0 min.
8. Transfer the tubes to an ice-cold water bath.
9. Withdraw ~0.5 ml of the colored solutions into 1.5-ml microcentrifuge tubes and centrifuge at ~10,000 g for 3 min.
10. Add 0.200 ml of the supernatant into 3-ml spectrophotometer cuvette tubes, add 2.5 ml of water, and mix well by using a pipette or by inversion several times.
11. Measure absorbance at 540 nm, where the absorbance of RB is used as the blank.



mg of Glucose produced is the FP activity

NOTE: Activity calculation

Prepare the dilute glucose standards (GSs) as below:

Prepare the enzyme dilution series, of which at least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (~2.1 mg) and one slightly less than 2.0 mg of glucose (1.9 mg).

GS1: 1.0 ml of glucose standard + 4.0 ml buffer = 2 mg/ml (1.0 mg/0.5 ml).

GS2: 1.0 ml of glucose standard + 2.0 ml buffer = 3.3 mg/ml (1.65 mg/0.5 ml).

GS3: 1.0 ml of glucose standard + 1.0 ml buffer = 5 mg/ml (2.5 mg/0.5 ml).

GS4: 1.0 ml of glucose standard + 0.5 ml buffer = 6.7 mg/ ml (3.35 mg/0.5 ml).

Add 0.5 ml of GS1–4 solutions to 13 × 100 mm test tubes, and add 1.0 ml of 0.050 M citrate buffer.

Prepare the blank and controls:

Reagent blank (RB): 1.5 ml of 50 mM citrate buffer. Enzyme controls (EC1–5): 1.0 ml of 50 mM citrate buffer + 0.5 ml enzyme dilution series whose enzyme concentrations are the same as those from E1 to E5**.** Substrate control (SC): 1.5 ml of 50 mM citrate buffer + filter paper strip.

References

- Pure & Appl. Chem., Vol. 59, No. 2, pp. 257—268, 1987.Printed in Great Britain.© 1987 IUPAC.
- **Y.H. Percival Zhang, Jiong Hong, and Xinhao Ye.** "Cellulase Assays" in *Biofuels: Methods and Protocols, Methods in Molecular Biology.* Jonathan R. Mielenz (ed.)*, Vol. 581.* DOI 10.1007/978-1-60761-214-8_14, © Humana Press, a part of Springer Science + Business Media, LLC 2009.