

<b>Experiment</b>	<b>Hydrolytic Enzymes: Amylases, Proteases, Lipases</b>
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<b>Reading</b>	Chapters in BBOM 9 <sup>th</sup> : 15.24, 15.26 Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9 <sup>th</sup> Edition, Prentice Hall, 1999.
<b>Objectives</b>	<ul style="list-style-type: none"> <li>Assay bacterial cultures and natural samples for the presence of exoenzymes</li> <li>Learn the metabolic products of hydrolysis</li> </ul>
<b>Background</b>	<p>Polysaccharides, proteins and lipids (fats) are degraded into smaller molecules by bacterial exoenzymes. The monomers are then accessible for the transport into the cell, where they are used in biosynthetic or energy-yielding reactions. Hydrolytic exoenzymes are secreted by the bacteria into the environment, or they are released after cell lysis. The process of hydrolysis involves a biochemical reaction in which H<sub>2</sub>O is added when a chemical bond is opened. These exoenzymes can be detected in environmental samples, enrichment cultures and pure cultures.</p> <p><b>Common hydrolytic enzymes are:</b></p> <ul style="list-style-type: none"> <li> <b>AMYLASE</b>  <b>Starch-hydrolyzing enzymes</b>            The polysaccharide starch, is a polymer of amylose (linear polymer of glucose molecules linked by 1,4-glycosidic bonds) and amylopectin (branched polymer of glucose molecules linked by 1,4- and 1,6-glycosidic bonds). A chain consists of a few hundred to a few thousand glucose units which form a helical structure in which iodine can be trapped. Bacteria that produce amylase (amyloglucosidase) can hydrolyze the starch into shorter polysaccharides (dextrins) and finally into disaccharides and monosaccharides (Fig. 1a).         </li> <li> <b>PROTEASE</b>  <b>(PROTEINASE, PEPTIDASE): Protein-hydrolyzing enzymes</b>            Proteolytic enzymes catalyze the hydrolysis of proteins into smaller peptide fragments and amino acids. The bonds are broken by the addition of water between the adjacent carboxyl and amino groups (Fig. 1b).            Casein is a mixture of phosphoproteins (a complex polymer of essential amino acids) found in cow milk to the extent of about 2.5% by weight. It exists in milk as the water-soluble calcium salt of a phosphoprotein and can be hydrolyzed by a series of enzymes collectively called Caseinases (Fig. 1b).         </li> <li> <b>LIPASE</b>  <b>Lipid-hydrolyzing enzymes</b>            Fats are broken down into fatty acids and glycerol by an enzyme called lipase (Fig. 1c).         </li> </ul>

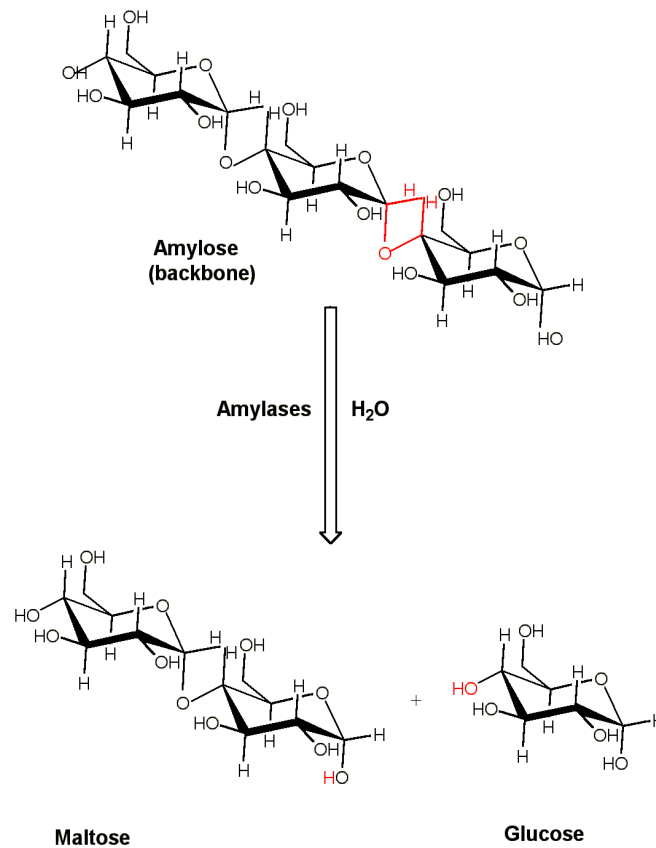


Fig. 1a: Starch hydrolysis by amylase

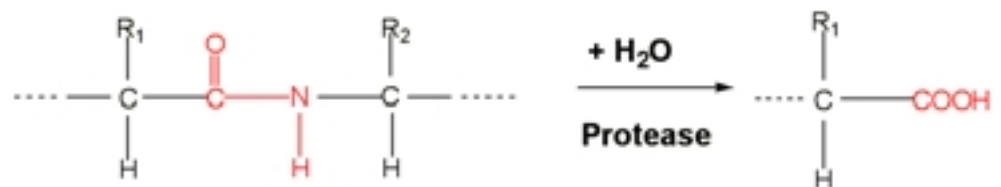


Fig. 1b: Protein hydrolysis by protease

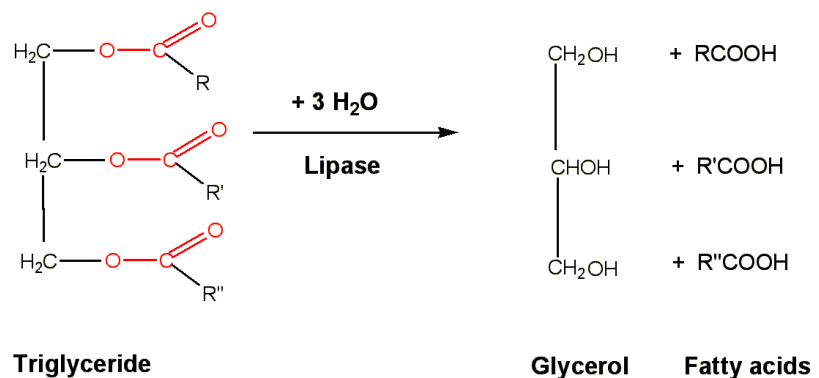


Fig. 1c: Fat hydrolysis by lipase

<b>Literature</b>	<ul style="list-style-type: none"> <li>• Hauer, B., Breuer, M., Ditrich, K., Matuschek, M., Ress-Loschke, M. and Sturmer, R. 1999. The development of enzymes for the preparation of chemicals. <i>Chimia</i> 53: 613-616.</li> <li>• Coolen, M.J.L. and Overmann J. 2000. Functional exoenzymes as indicators of metabolically active bacteria in 124'000-year-old sapropel layers of the eastern Mediterranean Sea. <i>Applied &amp; Environmental Microbiology</i> 66: 2589-2598.</li> <li>• McGrath, C.C.S. and R.A. Matthews. Cellulase activity in the freshwater amphipod <i>Gammarus lacustris</i>. <i>Journal of the North American Benthological Society</i> 19: 298-307, 2000.</li> </ul>
<b>www. Links</b>	<p>Visual lab study guide:  <a href="http://styx.pitt.cc.nc.us/sci/faculty/ta/metab.htm">http://styx.pitt.cc.nc.us/sci/faculty/ta/metab.htm</a></p> <p>Practical uses of lipases:  <a href="http://www.mahidol.ac.th/abstracts/annual2000/0692.htm">http://www.mahidol.ac.th/abstracts/annual2000/0692.htm</a></p>
<b>Practical Work</b>	<p>The students will learn to:</p> <ul style="list-style-type: none"> <li>• Detect some exoenzymes with specific tests in environmental samples and in enrichment cultures</li> <li>• Write a short laboratory report</li> </ul>
<b>Materials and Experimental Protocols</b>	<hr/> <p><b>AMYLASE-TEST PROCEDURE</b></p> <hr/> <p>“Starch agar”  Composition of starch agar medium in g/l (prepared):  KNO<sub>3</sub> 0.5, K<sub>2</sub>HPO<sub>4</sub> 1, MgSO<sub>4</sub> · 7H<sub>2</sub>O 0.2, CaCl<sub>2</sub> 0.1, FeCl<sub>3</sub> traces, potato starch 10, Agar 15, dH<sub>2</sub>O 1000 ml. Mix, check the pH (should be 7.2) and autoclave.  Lugol solution: 1 g crystalline iodine, 2 g KI, 300 ml dH<sub>2</sub>O (prepared)</p> <ol style="list-style-type: none"> <li>1. Pour 14 ml of sterile starch agar medium into a Petri dish</li> <li>2. Let the agar solidify</li> <li>3. Label the starch agar plate with the name of the bacterium to be inoculated</li> <li>4. Streak a drop of a bacterial culture onto the starch agar plate</li> <li>5. Incubate inverted at 30°C for 48 hrs</li> <li>6. When colonies are visible, flood the plate with Lugol solution</li> <li>7. Let the iodine react for at least 1 min</li> <li>8. Pour off the iodine from the plate</li> <li>9. Wash the plate with dH<sub>2</sub>O.</li> </ol> <p><b>Results:</b></p> <p>➔ If starch is present in the agar, a blue-black color will appear: the test result is <b>negative</b> (i.e. hydrolysis of the starch did not take place).</p> <p>➔ If the starch has been hydrolyzed by the excreted amylase, a <b>clear zone</b> around the bacterial colony will appear. The test result is <b>positive</b>.</p> <hr/> <p><b>CASEINASE TEST PROCEDURE</b></p> <hr/> <p>“Skim milk agar”  Mix 1 g of agar suspended in 50 ml dH<sub>2</sub>O with 5 g skim milk powder suspended in 50 ml dH<sub>2</sub>O to make 100 ml “skim milk agar”; pH = 7.2. Autoclave and pour plates</p> <ol style="list-style-type: none"> <li>1. Pour 14 ml of sterile skim milk agar medium into a Petri dish</li> <li>2. Let the agar solidify</li> <li>3. Label the agar plate with the name of the bacterium to be inoculated</li> <li>4. Inoculate the plates with one streak of inoculum</li> <li>5. Incubate the plates inverted at 30°C for 48 hrs</li> <li>6. When colonies are visible inspect the plates for clear zones around and below caseinase-positive colonies.</li> </ol>

	<p><b><u>LIPASE-TEST PROCEDURE</u></b></p> <p>The procedure is demonstrated.</p> <ol style="list-style-type: none"> <li>1. Autoclave 70 ml of trypticase soy agar growth medium</li> <li>2. Keep the medium at 50°C</li> <li>3. Prepare the fat stained with Nile blue sulfate (see below)</li> <li>4. Add 3.5 ml of melted fat to 70 ml of trypticase soy agar medium; mix well and dispense into 5 petri dishes</li> <li>5. Label the agar plate with the name of the bacterium to be inoculated</li> <li>6. Let solidify and streak the plates with 3 pure cultures and 1 'natural' sample</li> <li>7. Keep one plate uninoculated.</li> </ol> <p><b>Staining of a fat with Nile blue sulfate</b> (for fats which are liquid at room temperature).</p> <ol style="list-style-type: none"> <li>1. Prepare a saturated solution of Nile blue sulfate in dH<sub>2</sub>O</li> <li>2. Add 1 N NaOH until complete precipitation</li> <li>3. Filter and wash the precipitate → Nile blue sulfate oxazine base</li> <li>4. Dry the precipitate</li> <li>5. Prepare a saturated solution of the Nile blue sulfate oxazine base</li> <li>6. Mix 10 ml of any fat (e.g., Tween 80, polyoxyethylene sorbitan monooleate) with 1 ml of the Nile blue sulfate oxazine base solution</li> <li>7. The fat must be kept liquid, if necessary, in a heated water bath</li> <li>8. Add 2 volumes of diethyl ether to the dye-fat mixture in a separatory funnel</li> <li>9. Separate the red ether-fat layer from the water layer and wash it several times with water</li> <li>10. Separate the ether-water layer and evaporate the ether</li> <li>11. Autoclave for sterilization</li> <li>12. Store refrigerated.</li> </ol> <p><b>Results:</b> → A <b>positive</b> test is indicated by the formation of a blue halo around the colonies, e.g., positive for Tween 80: <i>Pseudomonas aeruginosa</i></p>
<b>Laboratory Rules &amp; Precautions</b>	Ether is flammable; use it in a fume hood.
<b>Goals &amp; Experiences gained</b>	<ul style="list-style-type: none"> <li>• Exoenzymes are widespread in environmental samples.</li> <li>• Tests for Amylase, Proteinase and Lipase can be performed on agar plates containing the compound to be degraded and made visible by indicator reactions</li> </ul>
<b>Timing</b>	15-30 min (step 1: inoculation, step two: enzyme test)
<b>Reporting</b>	What results do you have? Discuss and explain!
<b>Questions to be answered</b>	<p><a href="http://www.smccd.net/accounts/case/biol240/bprd.html">http://www.smccd.net/accounts/case/biol240/bprd.html</a> :</p> <ul style="list-style-type: none"> <li>• To which class of enzymes does Caseinase belong to?</li> <li>• Design a test for fat-digesting enzymes, different from the one demonstrated!</li> </ul>