<table>
<thead>
<tr>
<th>Title of Experiment</th>
<th>Fermentation of sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assistant</td>
<td>Thomi Horath, e-mail: <a href="mailto:horath@botinst.unizh.ch">horath@botinst.unizh.ch</a>, phone: 01 634 82 86</td>
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<tr>
<td>Objectives</td>
<td>1. We will explore how ethanol fermentation works. 2. We will determine which of the sugars glucose, lactose, or sucrose and whether starch can be fermented by <em>Saccharomyces cerevisiae</em> (baker's yeast). We will follow gas production employing the &quot;Durham tube&quot; method.</td>
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<tr>
<td>Background</td>
<td>Many microorganisms are able to ferment, that means to grow anaerobically in the presence of a suitable organic compound, such as a sugar or an amino acid, and in the absence of an external electron acceptor. During fermentation, the cell extracts some oxidation energy from the organic compound and excretes one or more products as a way of disposing of the electrons derived from substrate oxidation. Typical examples of fermentation products are CO₂ (leavening of bread), ethanol (beer and wine production), lactic acid (formation of dairy products) and propionic acid (in cheese production). In contrast to the process of respiration in which an external electron acceptor is used and the organic compound is often fully oxidized to CO₂ and H₂O, during fermentation the organic compound serves as both an electron donor and, after partial oxidation, as an electron acceptor. Fermenting organisms get less ATP from each molecule of food oxidized than aerobically respiring organisms. They excrete large quantities of only partially oxidized products during growth. These products can be used as indicators when searching for fermenters. Many of the products are acids which can be detected by a change in pH; other products are gases, collectable in an inverted glass tube inside the liquid medium (Durham tube). Many fermentable compounds belong to the sugars. In this exercise we will look for the ability of for instance <em>Saccharomyces cerevisiae</em> (baker's yeast) to ferment three different sugars; sucrose, glucose, lactose and the polysaccharide starch. Whereas sucrose and glucose are more abundant in nature, lactose is found predominantly in milk; hence the number of organisms evolved to ferment it is less than for the other sugars. The Durham tube is a convenient device for studying gas production during fermentation by microorganisms. Our tubes contain a low concentrated nutrient broth</td>
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supplemented with a particular sugar (0.5 % w/v). In addition, an indicator dye is used (phenol red) which turns its colour from red to yellow if the pH falls below 7. A small inverted vial filled completely with medium is used to demonstrate gas production.

Remark: Since there are other nutrients present, many organisms can grow even if they cannot ferment the sugar. Nevertheless, only an organism able to ferment the sugar will produce larger amounts of acid and / or gas. No special precautions are needed in this experiment to exclude air. O₂ is poorly soluble in H₂O, and it will be used up quickly as organisms begin to grow. Once micro-oxic or anoxic conditions have been established, fermentation will predominate. Recording the results of fermentation tests on a daily basis for the first 2-3 days of growth give results of maximum validity. After fermentative growth has ceased, O₂ might slowly diffuse into the medium, allowing further oxidative metabolism of the fermentation products. If these are acids, they may be further oxidized to CO₂ and H₂O, causing the indicator dye to change back from yellow to red (alkaline reversion). Slow deamination of amino acids produces for example ammonia (NH₃, basic) which can contribute to alkaline reversion as well, and in the absence of fermentation to pH increase indicated by a deeper red of the indicator dye.

### Literature

### www. Links
(examples)
- [http://medic.med.uth.tmc.edu/path/TESTS.HTM](http://medic.med.uth.tmc.edu/path/TESTS.HTM)
- [http://medic.med.uth.tmc.edu/path/00001458.htm](http://medic.med.uth.tmc.edu/path/00001458.htm)

### Material
Material needed for each student:
- 6 x LB Durham tube with low concentrated LB-medium and about 0.2 ml of 0.04% in water dissolved phenol red sodium salt (Fluka 77665)
- 1 Pipette (1 ml), 1 Pipette (100 µl)
- 1 tube rack

Available:
- some boxes with blue and yellow tips
- *Saccharomyces cerevisiae* (baker's yeast) liquid culture in flask
- unknown strains: consortium of enrichments obtained from a compost heap, liquid culture in flask
- yoghurt strain consortium
- 11 x concentrated sucrose stock solution (5.5 %: 5.5 g/100 ml)
- 11 x concentrated lactose stock solution (5.5 %: 5.5 g/100 ml)
- 11 x concentrated glucose stock solution (5.5 %: 5.5 g/100 ml)
- 11 x concentrated starch stock solution (5.5 %: 5.5 g/100 ml)
• vortex apparatus
• 32°C incubating room or box

The Durham tube:
A tube, containing a low concentrated nutrient broth (10% LB e.g.) supplemented with a high content (0.5 % w/v) of a particular sugar. Phenol red is used to indicate pH changes and a small inverted vial filled completely with medium serves to demonstrate gas production.

1 x LB ("Luria Bertani broth"):

<table>
<thead>
<tr>
<th></th>
<th>100% LB</th>
<th>10% LB</th>
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<tbody>
<tr>
<td>tryptone</td>
<td>10 g/l</td>
<td>1 g/l</td>
</tr>
<tr>
<td>yeast extract</td>
<td>5 g/l</td>
<td>0.5 g/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g/l</td>
<td>0.5 g/l</td>
</tr>
<tr>
<td>1M NaOH</td>
<td>1 ml/l</td>
<td>(→ pH 7)</td>
</tr>
</tbody>
</table>

In case you need LB solid medium: Add 15 g agar to 1l of liquid medium.

### Experimental Protocols

**Fermentation test:**

- Choose one of the inocula given (Saccharomyces cerevisiae, compost enrichments or yoghurt) and inoculate into a glucose, a lactose, a sucrose, a starch and a control tube. For comparison of the indicator colour, keep one tube without inoculating.
- Incubate at 32°C for 2-3 days.
- After 2-3 days (or one week) examine the tubes for growth of the cultures as indicated by turbidity of the medium, cell sediment at the bottom of the tube, or formation of a ring or pellicle at the air-liquid interface.
- Record the change in colour of the tubes as follows: A banana yellow colour indicates acid production; yellow-orange indicates production of slight acid; if there is no change in colour, i.e. if the colour is the same as that of the uninoculated control tube, it means that there was no more acid production than base production. If the colour changes from the original red to a more magenta colour, the alkalinity has increased. If the indicator turned colourless ("transparent") it means no acid was produced and the organism used the indicator as a hydrogen or electron acceptor, reducing it to the colourless form.
- Record the gas production as follows: A sizable bubble indicates gas production. A very tiny bubble does not indicate gas production by fermentation and can be recorded as ".". Gas produced by fermentation is always abundant and obvious. Remove the cap and sniff for odour. Is the odour familiar? Record your observations.
- Construct a table with your results. Continue incubating for a few more days. Then observe the tubes again. Did the colour of any of the acid tubes revert from yellow to red or pink? Record the changes.
- Observe the microbial community under a microscope.
**Experiment No. 5/B**

<table>
<thead>
<tr>
<th>tube #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB + indic.</td>
<td>10 ml</td>
<td>10 ml</td>
<td>10 ml</td>
<td>10 ml</td>
<td>10 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>sucrose</td>
<td>-</td>
<td>1 ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>lactose</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>glucose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>starch</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
<td>-</td>
</tr>
<tr>
<td>H₂O</td>
<td>1 ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.1 ml</td>
</tr>
<tr>
<td>inoculum *</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>-</td>
</tr>
<tr>
<td>volume total</td>
<td>11.1 ml</td>
<td>11.1 ml</td>
<td>11.1 ml</td>
<td>11.1 ml</td>
<td>11.1 ml</td>
<td>11.1 ml</td>
</tr>
<tr>
<td>incubation</td>
<td>32°C</td>
<td>32°C</td>
<td>32°C</td>
<td>32°C</td>
<td>32°C</td>
<td>32°C</td>
</tr>
</tbody>
</table>

**Results:**

<table>
<thead>
<tr>
<th>gas</th>
<th>colour</th>
<th>organisms</th>
<th>later changes</th>
</tr>
</thead>
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* choose one of the following inocula:
  - *Saccharomyces cerevisiae*
  - compost enrichments
  - yoghurt

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**Laboratory Rules & Precautions**

*Saccharomyces cerevisiae* is a harmless organism. But since we do not know what kind of organisms we grow up in the enrichments, we have to handle them cautiously. As a rule autoclave all tubes and plastic ware which have been in contact with cultures. Wash your hands from time to time.

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**Experiences gained**

Handling microorganisms, learning about anaerobic organotrophic life styles

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**Timing**

1 hour

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**Reporting**

Take notes on the experiment in your laboratory journal and report the results at the outcome on the 8th of February 2001 in class.

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**Questions to be answered**

1. How would you continue the experiment?
2. Try to write a stoichiometric sum equation which most closely describes the fermentation of one of the 4 offered substrates.
3. What is the difference between "substrate-level phosphorylation" during fermentation for instance and "electron transport phosphorylation" during respiration for example?
4. To what kind of pathway does glycolysis belong to; fermentation or respiration?
5. Which are the two (or three) most known fermentation products?
6. Besides photosynthesis, what are the main pathways to regenerate ATP for living organisms on Earth?