FOOD OR FUEL?

production of second generation biofuel

Certified NLT module for secondary school

B-Basic

natuur leven technologie

WAGENINGEN UNIVERSITY
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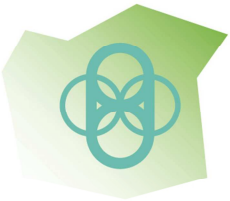
Colophon

The ‘Food or Fuel’ module is intended for the Nature, Life and Technology (NLT) lessons. The module was certified on 10 June 2009 by the NLT Steering Committee for use on the VWO in domain F (Biomedical Technology and Biotechnology). The certification number of the module is X225-050-VF.

The original certified module is available as a pdf download from http://www.betavak-nlt.nl.



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Materials and websites that students require for this module are available from the NLT digital classroom:
►http://vaklokaal-nlt.nl

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Table of contents

1. Introduction

This module is all about bioethanol. Ethanol is the official name for the liquid we refer to at home as 'alcohol'. It is frequently used as a solvent (for example, in cleaning products), as a flavouring (in wine and other alcoholic drinks) and as a fuel. We are familiar with ethanol as the fuel used in meths burners, but these days we are more likely to come across it in car fuel.
Bioethanol is no different from ethanol, except that, as the name indicates, it is made from biomass, usually plants.

The theoretical part of the module consists of Chapters 2 to 6. Chapter 7 is an extended practical in which you are going to make your own bioethanol from a surprising raw material: vegetable waste. There will also be an opportunity for you to prepare properly for the different chapters by running through the **digital background module** (►URL 1). This digital module can only be done in Dutch.

Chapter 2. Biofuels

This chapter deals with the industrial use of fossil fuels, such as oil and natural gas, and the relationships with the greenhouse effect. It also explains why biofuels give us the opportunity to counter this greenhouse effect.
In this chapter you can read about how existing biofuel production competes with food production. New production methods should facilitate the production of second generation biofuels, but the technology needed to do so is still in the development stage. It will soon be possible to make biofuels on an industrial scale from crops grown on so-called marginal ground, i.e. agricultural land not suitable for food crops. Another possibility is to produce biofuel from vegetable waste; you are going to investigate this yourself later in this module.

In this chapter you will learn about:

- the different kinds of biofuels;
- the area of tension between food and fuel and how the future of so-called second generation biofuels can resolve this dilemma;
- how to calculate the amount of energy gained from biofuel per hectare of agricultural land;
- how to calculate the amount of land required for a certain transport need.

Chapter 3. Raw materials

This chapter describes how ethanol is extracted from plants.

In this chapter you will learn about:

- how to make a calculation using the combustion reaction of glucose;
- the composition of sugar, starch and lignocellulose;
- the composition of lignocellulose;
- the four basic steps of ethanol production.

Chapter 4. Preprocessing

This chapter takes a more in-depth look at why it is so difficult to produce ethanol from the non-edible parts of plants. It also deals with the theory behind enzyme kinetics.

In this chapter you will learn about:

- why it is so difficult to produce ethanol from lignocellulose;
- what the difference is between acid catalysed and enzymatic hydrolysis;
- the theory behind enzyme kinetics;
- how to do calculations using the theory of enzyme kinetics.

Chapter 5. Fermentation

This chapter is about fermentation. It begins by taking a look at the history of fermentation. Then the composition of the yeast cell is more closely scrutinised. The chapter offers an explanation of how ethanol production is related to the metabolisation of the yeast cell. Finally, the fermentation processes and the mathematical models used to accurately monitor fermentation are dealt with.

In this chapter you will learn about:

- how to recognise fermentation;
- the composition of the yeast cell and the structure of several biomolecules;
- what glycolysis involves and the significance of glycolysis for ethanol production;
- why genetic modification of the yeast cell is important;
- two types of bioreactors;
- how to do calculations using mathematical models of unlimited growth and logistical growth.

Chapter 6

This chapter takes a closer look at the techniques for obtaining pure ethanol: filtration and distillation.

In this chapter you will learn about:

- how to separate ethanol from water by distillation;
- how vapour pressure is created and what boiling is;
- the theory of distillation;

- how repeated distillation leads to ever higher concentrations of ethanol;
- why you cannot obtain pure ethanol from distillation.

2. Biofuels

In the future biofuels will replace petroleum in an increasing number of applications. Petroleum is becoming scarce and expensive and the global demand for liquid fuel is on the rise. The graph below shows why. More and more people want to own a car. In the Netherlands alone, the car fleet just keeps on growing (Figure 1). In Asia too, the demand for cars is increasing. In countries like India and China, the increase as witnessed in the Netherlands in the second half of the last century, has yet to materialise.

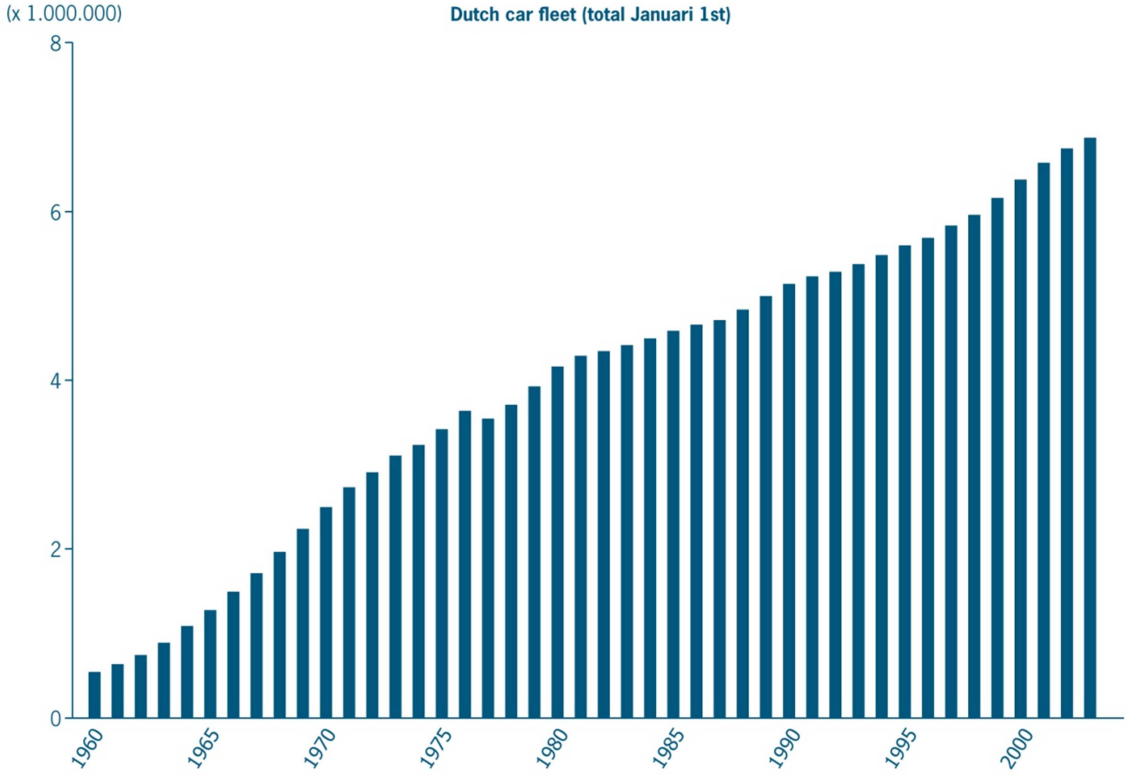


Figure 1 The growth of the Dutch car fleet between 1960 and 2004

Leaving aside the economic factors, there are other reasons that render the transition to sustainable solutions such as biofuels more likely. More and more people are concerned about CO_2 emissions. Many scientists believe that these emissions are responsible for climate change. Substituting fossil fuels with biofuels may be a way of reducing these CO_2 emissions.
In 2003, it was decided in an EU directive that by 2010 almost 6% of the energy content of fuel at the pump should be from biofuel.

In addition to economic pressure, there is also political pressure to increase the availability of biofuels.
Now do the background module that accompanies Chapter 2 (►URL 1).

2.1 CO_2 neutral or not

The term biofuel is actually confusing, because the huge stocks of fossil fuels like oil, coal and natural gas also come from plants or animals. But there is an important difference between biofuels and fossil fuels. That difference becomes evident when you look at the time required for their creation and the speed with which they are used. Plants store energy from the sun. In fossil energy this process occurred millions of years ago, in biofuels it took place 'yesterday'.
If you use the biofuel wood to stoke your open fire, you are using energy that was made several decades ago. The amount of CO_2 emitted when you stoke the fire is equal to the amount of CO_2 needed to produce the wood. The CO_2 which was taken from the atmosphere a short time ago, is now returned to the atmosphere. Biofuel combustion is therefore CO_2 neutral.
The use of the fossil fuel petrol to make a car journey releases CO_2 which was taken from the air millions of years ago. The emission that takes place now causes CO_2 levels to rise. Fossil fuel combustion is therefore *not* CO_2 neutral.

2.2 Bioethanol and biodiesel

Biofuels come in all shapes and sizes. As solids (such as wood), as gases (e.g. methane released in the rotting process) and as liquids. The best known liquid biofuels are biodiesel and bioethanol. The latter is used in transport. This is a historical matter. In the industrialised world a choice was made to develop the combustion engines designed by Otto (petrol engine) and Diesel (diesel engine). Engines that, after more than a century's tinkering, now have an amazing reputation. In contrast to, for example, the heat engine designed by Stirling, these engines need liquid fuel. Biodiesel can be put straight into a diesel car tank. Bioethanol is designed for a petrol car. In order for a car to run entirely on bioethanol, a (minor) adjustment to the engine is required. However, petrol mixing is not a problem.

Bioethanol is just ethanol. It has been made for centuries using a biological process: fermentation, whereby the conversion takes place with the help of microorganisms.
Biodiesel is produced by a chemical process. Vegetable fat, combustible but too viscous for a car, is processed using methanol, to create a much more fluid biodiesel. Compared to bioethanol, biodiesel is relatively easy to make.

Task 1
Search the internet for the structural formulas for ethanol and biodiesel. Which molecule is the biggest? How many atoms does a molecule of ethanol contain? And a molecule of biodiesel?

Task 2
There is a reaction equation for the process in which biofuel is formed.

a. Using Google, search for the reaction equation for the formation of bioethanol. Consult at least two sites and note down the equation.
b. Do the same for biodiesel.

2.3 Food or fuel?

The transition to liquid biofuel is not without its problems. Current technology uses food crops to produce ethanol. Rising oil prices can therefore lead to a rise in food prices.

Task 3
Explain why the price of bread would rise in the event of a dramatic rise in the demand for biofuels.

Since biofuels are still being produced with crops like corn and grain, energy markets and food markets will both be affected when the petroleum price reaches a certain level. For many this is a nightmare.
The large-scale use of traditional food crops for transport fuel needs has unavoidable and far-reaching consequences. The American environmental thinker Lester Brown calculated that the amount of grain needed to fill the tank of an SUV would feed one person for a year.
Rather than make this sort of comparison, it is better to look at the energy yield per hectare. This is shown in Table 1.

Table 1 The energy yield per hectare of various crops

Crop	Yield per hectare in GJ
Sugar cane	104
Sugar beet	90
Corn	54
Wheat	45
Barley	20
Sunflowers	16
Rape seed	20
Palm oil	81
Source: Louise Fresco, Duisenberg lezing (2006): Biomass for food or fuel: is there a dilemma?	

For the sake of comparison: the average motorist can drive for about 3 years with 100 GJ. The use of food crops for the production of ethanol or biodiesel for transport purposes will therefore occupy a great deal of available agricultural land.

Efficiency

There is another reason why many people are sceptical about the use of corn and grain for the production of bioethanol. It is a fact that a lot of energy is needed to produce energy. Before you can use a fossil fuel as petrol, a lot of energy is lost to obtain the oil, to transport it, and to process it in the oil refineries. The situation is not much different in the case of biofuels.
To start with, the crop has to be grown and harvested. Huge quantities of energy are needed, in particular, for the fertiliser and transport. Thereafter the crop has to be converted into ethanol or biodiesel. This process also requires a lot of energy.
David Pimentel, an ecology and agrarian expert, is of the opinion that the total profitability of a crop like corn is ultimately negative. This means that the energy input is greater than the energy output.
Experts who start with the most up-to-date technology come up with a profitability of 20-30% for corn and 80-90% for sugar cane (there is still a lot of manual processing in Brazil, the biggest producer of ethanol from sugar cane), and 50-60% for biodiesel from rapeseed.

Task 4
Go to the site (►URL 2) and read the interview with Pimentel. Critics of Pimentel said that his calculations used data from 1970 and were thus outdated.
Explain how the use of more recent data would produce better yields.

At first sight biodiesel would seem not to compete with food provision. In Germany rapeseed is grown with the sole purpose of being used in biodiesel production. However, this crop requires agricultural land that could also be used for growing food crops.
There are many more objections to biodiesel production. One of the most productive oil-rich plants is the oil palm. Massive regions of rain forest, particularly in South-east Asia, are being destroyed to make way for oil palm crops. This destruction has a devastating effect on the CO_2 balance. This is because the rain forests are enormous reservoirs of stored CO_2.
The destruction of rain forests therefore results in an immediate and enormous emission of CO_2.

Bioenergy in the Netherlands

Would the Netherlands be able to produce much bioenergy?

Sugar beets are normally used in the Netherlands.
On an annual basis 55 tonnes of sugar beet are produced per hectare. Seventeen percent of this can be converted into sugar. Which can then be converted into ethanol.
The main question is how many hectares of sugar beet must be grown to produce ethanol for all the cars in the Netherlands.

Task 5
In this task you must ultimately make a well-founded estimate of the amount of land needed to produce enough ethanol using sugar from sugar beet to power all the cars in the Netherlands instead of – as now- using fossil fuels.

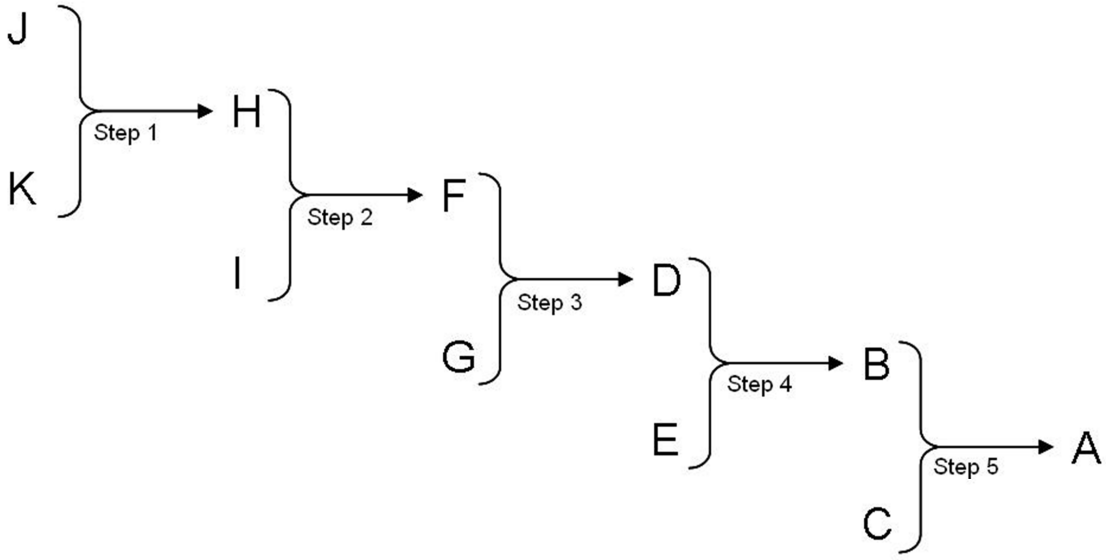


Figure 2 Calculation plan for deducing a variable.

The calculation is done in a number of steps. These steps are indicated in the diagram in Figure 2.
The variables that play a role are denoted by a letter.
Below is a list of the variables in question. The accompanying unit is given afterwards between brackets. But there is no indication (except for A) about which letter belongs to which variable. Given variables are printed in bold.

- The required agricultural acreage: A (ha)
- **Yield crop per ha** (50 tonne/ha)
- **The combustion value of ethanol** (22MJ/litre)
- **The combustion value of petrol** (32MJ/litre)
- The total Dutch requirement in energy for car traffic (MJ)
- **The average petrol use of cars** (12 km/litre)
- **The number of car kilometres driven per year** (100 bn km)
- **Ethanol yield per tonne crop** (108 litre/tonne)
- The total Dutch requirement in ethanol for car traffic (litre)
- Energy use per km (MJ/km)

a) Link every variable to a letter in the calculation diagram.
b) Calculate the required agricultural acreage.

2.4 Second generation bioethanol production

Fortunately the relationship between the energy market and food market is not as critical as the above diagram suggests. The reason for this is that a corn plant, for example, consists of more than just a cob of corn. The majority of the plant consists of non-edible substances, which are perfectly suitable for the production of biofuels. The technologies for converting the non-edible parts of the plant into biofuel are in development. This is what is referred to as second generation biofuels.

The first generation of biofuels focus on three raw materials:

- Sugar (from sugar cane and sugar beet; relatively easy to convert into bioethanol)
- Starch (from grain, corn, barley, potatoes, etc.; a little more difficult to convert into ethanol but there are now tried and tested techniques).
- Oil (from rapeseed, sunflowers, olives, oil palm, etc.; easy to convert into biodiesel).

The rest of the plant contains a lot of cellulose, among other things. The cellulose is also rich in energy but unsuitable for human consumption. Cellulose and related substances can be converted into ethanol. However, the technology for doing this is still in the development stage. This will be looked at in more detail in Chapter 3.
Liquid biofuels from cellulose and related substances are called second generation biofuels.
Second generation biofuels offer great opportunities because they do not compete directly with food provision and can even be made in addition to food.

Waste processing

The extraction of the three raw materials – sugar, starch and oil – from food crops causes major waste flows in agriculture; for example, the waste from grain is a lot of straw. Much of this waste goes to good use, for instance as compost. The result is that normally only a limited amount of the plant's energy is used.
It is perfectly feasible that in the future the grain farmer might deliver his grain to the food market, leave some of the plants in the ground to improve the soil and supply the rest to the ethanol factory.

Growing energy crops on marginal land

Marginal land is land that is not suitable for normal agriculture. The soil has been eroded, become salinated or too rocky.

There are 17 million km^2 or 13% of available marginal land around the world.
The best possibility to produce second generation biofuels is to grow perennial crops that thrive more easily in low quality soil. In comparison with annual (agricultural) crops, perennial crops absorb more carbon dioxide, need less fertiliser and chemicals (which use up a lot of energy!) and the soil is not so easily exhausted. Energy production is cheaper and more efficient. Over time the use of these soils for bioenergy may even improve the soil, making it suitable for food crops.
How much bioenergy can a hectare actually produce if we look at the whole plant? How much energy does a plant actually store? In order to answer this question, we have to go back to the beginning – photosynthesis.

2.5 Photosynthesis for transport

Do biofuels offer a solution to the problem of energy demand? How does the plant actually obtain its energy? Arguably the most important reaction in the biosphere is photosynthesis. This is what the next task is about.

Task 6
Plants grow by means of photosynthesis. Look up the reaction for photosynthesis. The correct reaction diagram for this reaction is:

a. $CO_2 + H_2O \rightarrow C_{12}H_{22}O_{11} + O_2 + \text{energy}$
b. $CO_2 + O_2 + \text{energy} \rightarrow C_6H_{12}O_6$.
c. $6CO_2 + 6H_2O + \text{energy} \rightarrow C_6H_{12}O_6 + 6O_2$
d. $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 + \text{energy}$.

Although crops can differ greatly from one another, photosynthesis seems to give pretty much the same result. When doing calculations it can be assumed that 1 m^2 of crops stores about 50 g CO_2 per day via photosynthesis and that a growth season consists of 100 days.

Task 7
Since the absorbed CO_2 is converted into glucose, the amount of glucose produced can be calculated. The energy content of glucose is known (Binas).
So the amount of solar energy converted by photosynthesis into chemical energy can be calculated.

a) Calculate how much CO_2 is stored per year and per hectare.
b) Calculate how much glucose is made per year per hectare.
c) Calculate how much energy is stored per year and per hectare.

The sun supplies an average of about 100 W/m^2 during the day and night.

d) Calculate the efficiency of photosynthesis.

Task 8
Complete the quiz on the following website ► URL 3.

3. Raw materials

Bioethanol is produced by microorganisms. In this module we will be focusing on ***Saccharomyces cerevisiae***, better known as baker's yeast because it is most commonly used for rising bread dough. Chapter 5, and more particularly section 5.2, takes a closer look at the properties of yeast. Yeast uses glucose as a source of energy. If there is sufficient oxygen the yeast cell can take a lot of energy from the glucose and the cells will quickly multiply. However, if there is too little oxygen, only some of the energy is released. Ethanol therefore contains the energy that the yeast cell was unable to use.

Task 9

These various metabolic processes can be represented as follows:

Aerobic dissimilation:

Glucose+.....→.....+.....+energy

Anaerobic dissimilation:

Glucose→.....+.....+energy

a) Enter the substances in the spaces.
b) Explain which of the above reactions releases the most energy.
c) Both reactions are so-called net reaction diagrams: explain what this means.

Before answering the following questions, look up the formula for glucose and ethanol in Binas or similar source. Use reaction equations for your calculation.

d) If 1 mol glucose is converted during aerobic combustion, how many mol CO_2 are produced? Explain.
e) If 1 mol glucose is converted during anaerobic fermentation, how many mol alcohol are produced? Explain.
f) If 1 kg glucose is converted during anaerobic fermentation, how many grams of alcohol are produced? Explain.

Thus, yeast cells produce alcohol under anaerobic conditions and need glucose to do so. But glucose is not common in free form in nature.
Glucose is often a building block for other, more complex molecules, the most important of which are: sugar, starch and cellulose. These substances, which are produced on a large

scale by plants, function therefore as a raw material for the production of alcohol.
In Figures 3, 4 and 5, you can see how the substances sucrose (saccharose, or normal sugar), starch and cellulose are constructed from glucose.

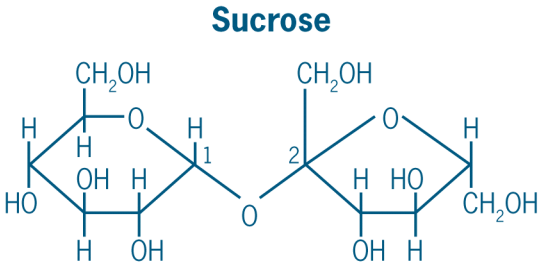


Figure 3 Sucrose consists of two components: glucose and fructose

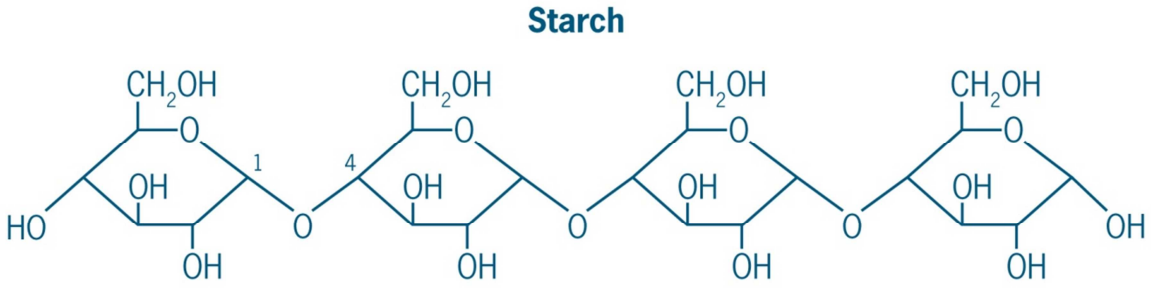


Figure 4 Starch is a polymer of glucose molecules

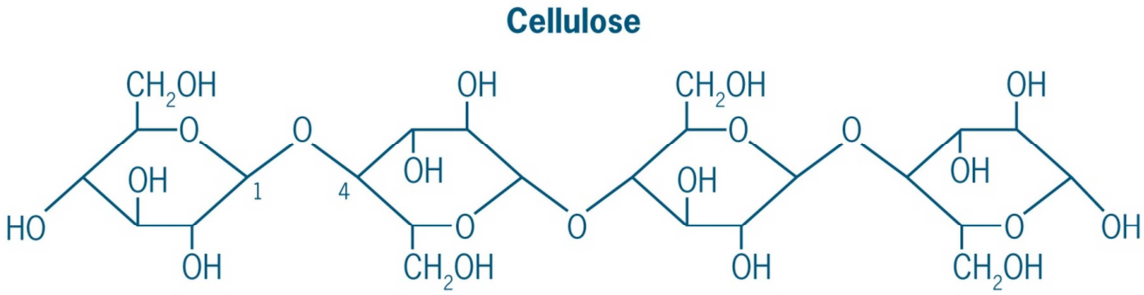


Figure 5 Cellulose is a polymer of glucose molecules. The glucose molecules, in contrast to starch, are attached to each other by another bond.

In order to produce alcohol with the help of yeast cells, a raw material must first be broken down into glucose. This process happens in a similar way in all three substances, sucrose, starch and cellulose. The bonds between the glucose components are broken. Water plays a vital role here (see also section 3.2). This process is called hydrolysis (*ὑδρος* = water, *λύειν* = splitting). Now do the background module for Chapter 3 ►URL 1.

Task 10
Draw a glucose-α and a glucose-β next to each other and show clearly the difference between them. If necessary, consult the internet.

Task 11
Glucose plays a key role in cell metabolism. In bacteria and in animal cells glucose is a source of energy. Why glucose plays this role and not fructose or another simple sugar is a question which is still puzzling molecular evolutionary biologists today.

Explain why glucose is not commonly available in free form in nature.

Task 12
Sugar, starch and cellulose are raw materials in the production of ethanol.

a. Name three plants that each produce sugar, starch and cellulose.
b. Which of these three substances is used in the production of wine?
c. Which of these three substances is used in the production of beer?

3.1 Lignocellulose

Plant cells have a cell wall that consists mostly of cellulose, closely followed by hemicellulose and lignin. Glucose is the sole building block in cellulose, and an important one in hemicellulose.
Almost half of all plant material on earth consists of cellulose. That makes cellulose the most commonly occurring organic substance on earth.

The combination of cellulose, hemicellulose and lignin is called lignocellulose.

Table 2 below shows the percentage of cellulose, hemicellulose and lignin in the dry matter for a number of raw materials:

Table 2 Percentage of cellulose, hemicellulose and lignin in various raw materials

Type of raw material	Cellulose %	Hemicellulose %	Lignin %
Bagasse	41	24	18
Straw	35	35	6
Hard wood	40-55	24-40	18-25
Leaves	15-20	80-85	0
Cotton	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Grass	25-40	35-50	10-30

Cellulose

Plant cells have a recognisable structure with a rigid cell wall. This rigidity is caused partly by the cellulose. The structure of the cell wall is sketched out below (Figure 6).

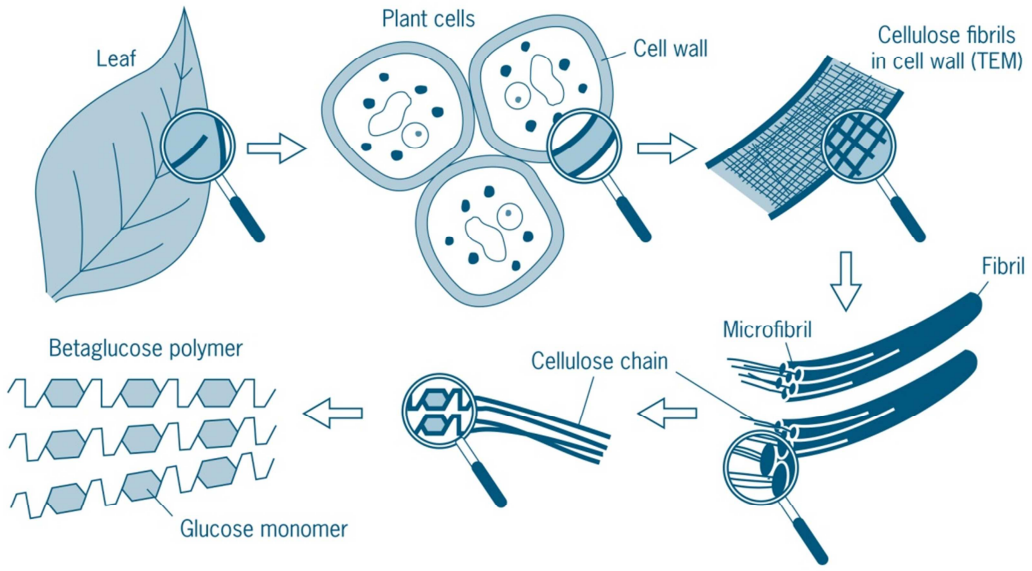


Figure 6 The structure of plant cells. The rigid cell wall consists of fibres made from microfibrils. These comprise about 100 cellulose chains.

Cellulose consists of long polymers (chains) of ß-D-glucose molecules (Figure 7). There is a repeating bond between two carbon atoms of the glucose monomers, namely the C1-C4 bond (named after the C atoms involved in the bond)

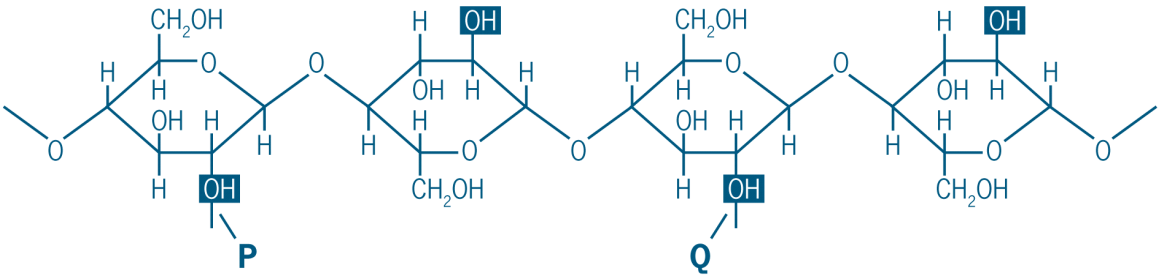


Figure 7 Cellulose chains can bind through hydrogen bonds. That can occur at sites P and Q.

Hydrogen bonds can be created between the chains (see Figure 7). This produces microfibrils, made up of about 100 polymers. The composition of cellulose is arranged in a crystal-like shape. The crystalline structure makes cellulose difficult to break down. As a result wood can stay in a good condition for years if kept dry.

Hemicellulose

Cellulose microfibrils are not tightly packed together but embedded in a less structured mass: an amorphous intermediary substance formed by hemicellulose and lignin. Hemicellulose differs in a number of key areas from cellulose:

- It is a polymer that consists not only of hexoses (i.e. C_6 sugars such as glucose, galactose and mannose), but also of pentoses (C_5 sugars, primarily xylose and arabinose).
- The polymers have a dominant chain that consists of one sort of sugar, usually xylose.
- Since there are several different bonding possibilities, there are side chains on the polymer.
- Sometimes side chains occur with other components such as glucoronic acid.

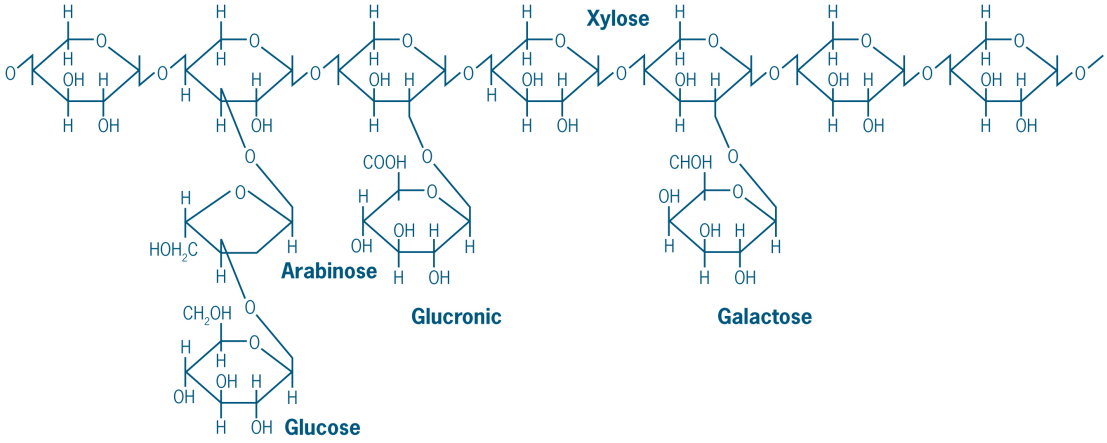


Figure 8 Hemicellulose consists of one polymer with side chains. Here the backbone consists of xylose

The hemicellulose is attached to the microfibrils with hydrogen bonds.

Because of these branches the hemicellulose is not able to build the crystalline structure that is characteristic of cellulose. There is more space for enzymes to break open the bonds. Hemicellulose is consequently more sensitive to humidity and attacks by microorganisms. When wood begins to rot, the hemicellulose disintegrates first. The resulting acids eventually damage the cellulose too.
The total amount of sugars that can be obtained from lignocellulose varies dramatically between plants. Table 3 shows the composition of lignocellulose in a few agricultural waste materials.
The table clearly shows that the monosaccharide xylose as well as glucose is frequently present. This is important for ethanol production because the normal yeast cell cannot ferment

xylose. A technological breakthrough in Delft, however, has changed this. See also H5, New Developments.

Table 3 Mass percentage of sugars in lignocellulose in several agricultural waste materials(After Grohman and Bothast (1994)

Sugar	Corn stalk	Grain straw	Bagasse	Cotton waste	Sugar beet pulp	Crab grass
Glucose	34,6	32,6	39,0	37,1	24,1	31,0
Mannose	0,4	0,3	0,4	1,1	4,6	0,2
Galactose	1,0	0,8	0,5	2,4	0,9	0,9
Xylose	19,3	19,2	22,1	9,4	18,2	0,4
Arabinose	2,5	2,4	2,1	2,3	1,5	2,8

Task 13
Various forms of biomass are named in Table 2. Explain why in nature straw rots more slowly than leaves.

Task 14
Using Table 3, explain which raw material will be most affected by the discovery in Delft: crabgrass or corn straw.

Lignin

Lignin is a large macromolecule with multiple aromatic rings (Figure 9). The molecule does not have a linear structure but together with hemicellulose forms a network in which the microfibrils are embedded. This is what gives the plant cell its rigid structure. The lignin often emerges more slowly and then replaces pectin as an intermediary substance. Grass that is very old contains a lot of lignin and is described as woody. Silage with an excess of this grass can be dangerous for cows. Lignin (also called wood) cannot be digested by cows and other ruminants because there are only a few microorganisms that can break down the molecule.

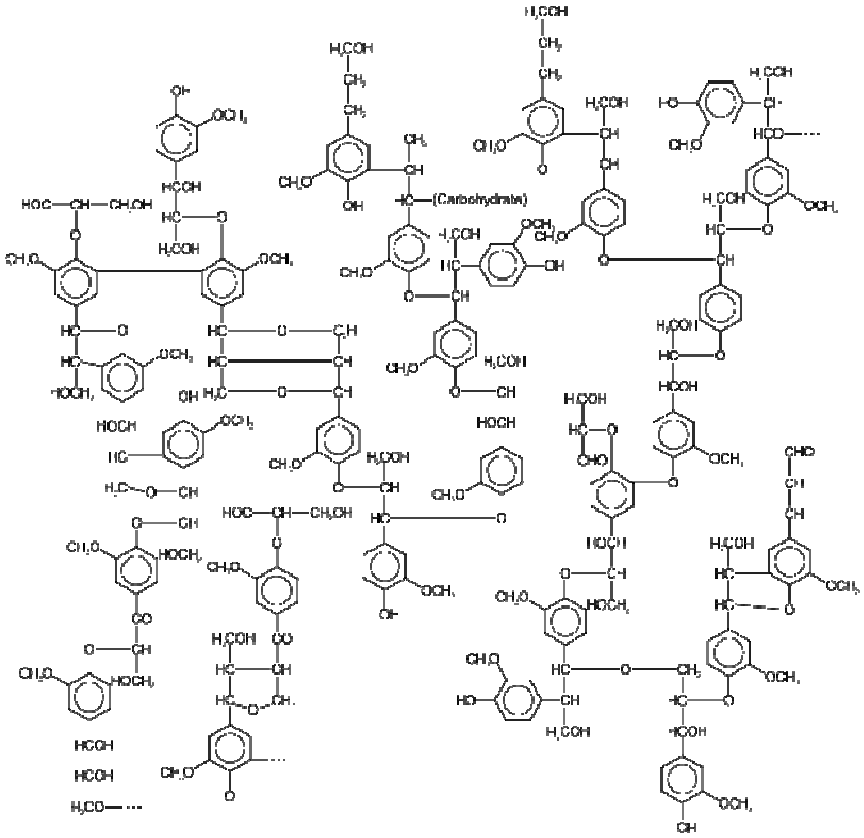


Figure 9 Lignin is a large macromolecule with no fixed structure and with multiple rings and side chains.

3.2 Bioethanol production

Four basic processes are required to make ethanol from plants:

- Pre-processing
- Hydrolysis
- Fermentation
- Re-processing

Pre-processing of the raw materials

The plant or fruit has to be processed. The sugar is usually present in the sap of the plant or fruit. The process of releasing this sap does not usually require a great deal of energy. In starch the pre-processing uses a lot more energy. Starch is usually found in seeds.
These have to be firm in order to serve their purpose. If the seed shell is broken, the starch is accessible.
Cellulose is the least scarce but the most troublesome raw material. Cellulose gives the plant its turgidity. A tree is largely made of cellulose. But in order to be able to break down the cellulose molecules into separate glucose molecules (hydrolysis), the tree has to be pulverised and subjected to a whole range of processes.

Hydrolysis

In hydrolysis, the bonds between the glucose components must be broken. Water is needed for hydrolysis. The bond between the C of a glucose ring and the binding O is broken, an OH group is linked to the C and an H to the O (consuming H_2O), a process creating complete glucose molecules again. Hydrolysis can proceed as a purely chemical process. This process is shown in Figure 10.

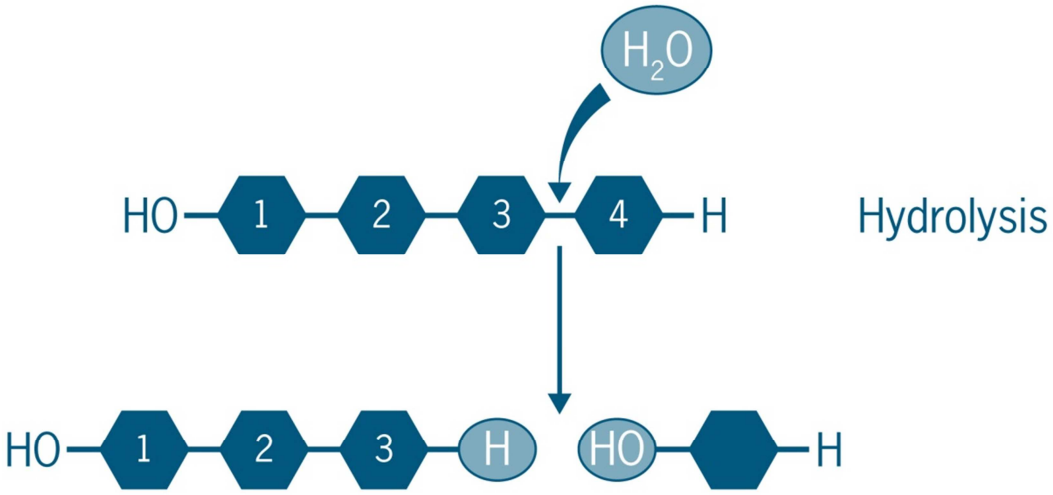


Figure 10 Hydrolysis of a polymer. The bond between glucose molecules 3 and 4 is broken. Then with the help of a water molecule, two new molecules are formed.

Hydrolysis does not take place spontaneously, but can be triggered by, for example, the addition of an acid. The free hydrogen ions weaken the bond between the glucose components.
When hydrolysis is complete, a molecule of water has been used up. The hydrogen ions of the acid have only served as a catalyst.

Task 15
Explain what a catalyst is.

Enzymatic hydrolysis.

Like acids, certain proteins can also act as catalysts. In sucrose (table sugar), invertase acts as a catalyst. Invertase is a protein. Proteins that act as catalysts are called enzymes. Enzymatic hydrolysis is the process that takes place when proteins act as catalysts. In Figure 11 you can see how the enzyme invertase hydrolyses a sugar molecule. The protein can then approach a new molecule.

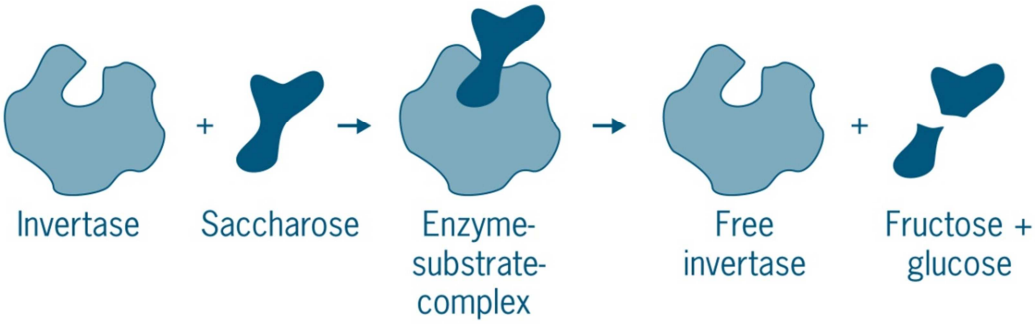


Figure 11 An invertase molecule, an enzyme, binds to sucrose, breaks the bond, releasing the products.

Task 16
Using Google, look for hydrolysis animations. Keywords: animation – hydrolysis.

Task 17
Why does hydrolysis involving an enzyme keep slowing down? What can you do to accelerate the reaction?

Fermentation.

During fermentation yeast cells convert glucose into alcohol. Providing there is no oxygen present. Fermentation can take place on a small scale but also on an industrial scale. The photo below (Figure 12) shows a few huge fermentation tanks.

Figure 12 Fermentation tanks.

Filtration and distillation.

Once the yeast cells have done their work, all that remains is a mix of alcohol, water, yeast and possibly some residue sugars.

To obtain pure alcohol, the mixture must be separated. By filtering the mixture, all the solid components are left behind. The yeast included. The liquid contains alcohol, water and dissolved sugars. This liquid is then distilled.

The whole process is mapped out in the diagram below (Figure 13).

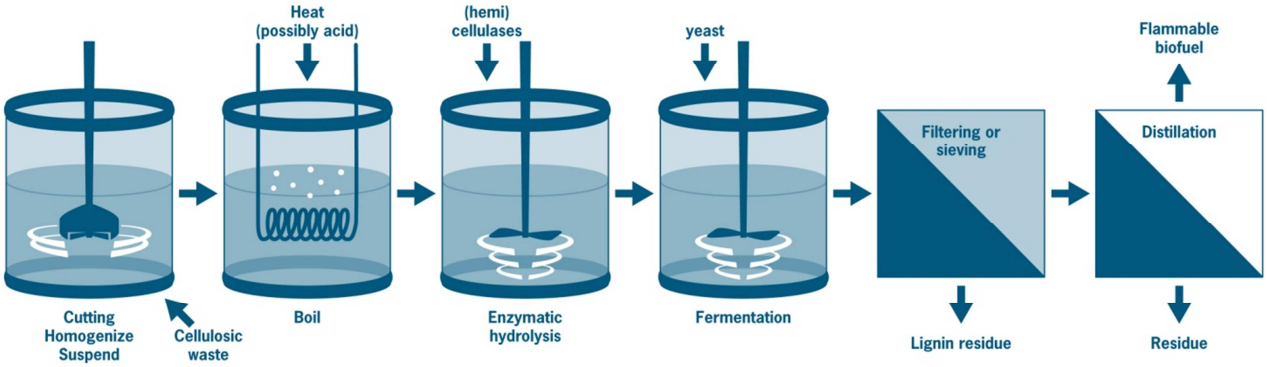


Figure 13 Flow diagram for the production of bioethanol.

In the following chapters, we will take a much closer look at the various production stages.

4. Pre-processing

Chapter 2 explained why ethanol production from lignocellulose is preferable to that from plants containing sugar or starch.
However, there are a number of problems:
The technology already exists for producing alcohol from sugar-containing plants or from corn. Producing alcohol from cellulose is more difficult.
The pre-processing stage for cellulose is much more important. Usually, the cellulose not only has to undergo a mechanical process to unlock the cellulose, but it must also be heated and acid must be added.
The crystalline structure of the cellulose means that there is only a small surface on which hydrolysis can work. Hydrolysis is necessary for obtaining fermentable sugars. Furthermore, cellulose, lignin and hemicellulose together form a matrix, which further reduces the available surface area. Lignin plays no part in ethanol production and has to be removed. First complete the background module that accompanies Chapter 4 (►URL 1).

4.1 The first steps

Mechanical processing

The material has to be reduced in size. This can be done by cutting or shredding it into small pieces of a few centimetres, or by grinding or crushing it into fine pieces of 0.2-2mm.
The mechanical process makes the cellulose much more accessible by significantly increasing the effective surface area on which the enzymes for hydrolysis can work.
The disadvantage of the mechanical process is the cost in terms of energy. About one third of the total energy costs associated with the production of ethanol from plant material are required for the mechanical process. And there are no technological developments in the pipeline to drastically reduce these energy costs in the future. Table 4 shows the energy costs of various processes:

\

Table 4 Energy costs of pre-processing (Cadoche and Lopez (1989))

Material	Granule size (mm)	Energy input MJ/tonne Cutting mill	Energy input MJ/tonne Hammer mill
hard wood	1.6	468	468
	2.5	288	432
	3.2	180	414
	6.4	90	342
straw	1.6	27	151
	2.5	23	104
corn plant waste	1.6	-	50
	3.2	72	35

Task 18

Lignocellulose material needs to be pre-treated before enzymatic hydrolysis is possible.

a) Explain what enzymatic pre-treatment is and whether there are any other forms of hydrolysis.

b) Explain why too little ethanol is produced if the lignocellulose is not pre-treated.

Shredding and grinding the cellulose is one form of pre-processing.

c) Explain why this contributes to a bigger yield of ethanol.

Go to ►URL4:

Various pre-processing procedures are mentioned here.

d) Using the internet, look up the meaning of the various terms.

The following site gives an overview of a great many pre-processing methods.

e) Go to the site ►URL 5. Look for the overview of different forms of pre-treatment and the accompanying explanation (page 1627 onwards). Using this description, fill in the table below.

Process	Category (phys-chem-biol)	Description (what happens in this process?)	Disadvantages
grinding	physics	The raw material is divided up into little pieces. The available surface area on which enzymes can work is greatly increased.	Requires lots of energy to pulverise wood.

steam explosion			
Afex			
Ozone (Ozonolysis)			

Acid-catalysed hydrolysis

Concentrated acids such as H_2SO_4 or HCl are also used to treat lignocellulose, for example prior to enzymatic hydrolysis.
In principle strong acids can even hydrolyse the cellulose immediately so that pre-processing and hydrolysis can be carried out in one step. This was done previously in factories where wood was converted into ethanol. Acid hydrolysis is also used nowadays.
There are numerous disadvantages to acid treatment:

- The acid attacks the reactor (unless expensive and strong steel is used).
- The biomass has to be neutralised afterwards for possible subsequent enzymatic hydrolysis and fermentation. This costs money and creates more waste.
- The acid produces toxic residues that are hazardous for later fermentation. These have to be removed.
- A huge waste flow with salt is created.

4.2 Enzymatic hydrolysis

Acid hydrolysis is very energy-intensive. Process temperatures of hundreds of degrees Celsius make it extremely difficult to counter heat loss. There is then the question of whether the final alcohol produced will generate more energy than was required to make it.
A completely different method of hydrolysis uses enzymes. Enzymes that break down cellulose are called cellulases. There are multiple microorganisms that produce these enzymes. These are the same heterotrophic microorganisms that break down wood in nature to obtain their food. Some bacteria and fungi can produce cellulases.

Cellulase occurs in many variants, each with its own function. Three types of enzyme are usually distinguished:

Endo-ß-glucanases

These enzymes break up the long polymers at random locations by hydrolysing the 1-4 bond between two glucose molecules (Figure 14).

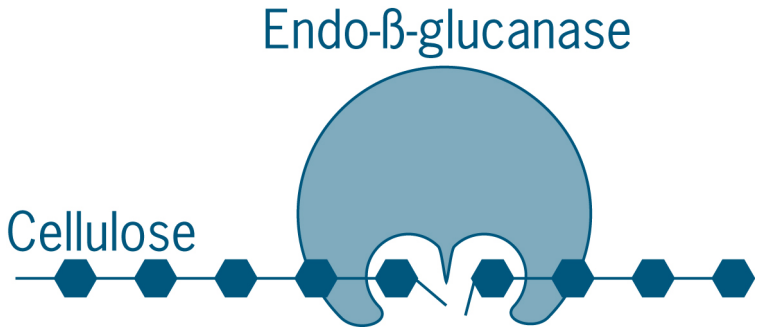


Figure 14 The enzyme endo-ß-glucanase splices a cellulose polymer in two. This results in more extremities to which other enzymes can attach.

Exo-ß-glucanases:

Exo-ß-glucanases attach themselves firmly to the available ends of the polymer chains and splice off short segments (disaccharides and oligosaccharides) from the loose ends (Figure 15). The disaccharides are called cellobioses. Cellobiose is composed of two glucose molecules with a ß-1-4 bond. Cellobiose should not be confused with maltose, which is formed by an α bond between the two glucose molecules. Maltose is easily digested by us and also ferments more easily. Cellobiose is indigestible by us and cannot be fermented by ***S. cerevisiae***.

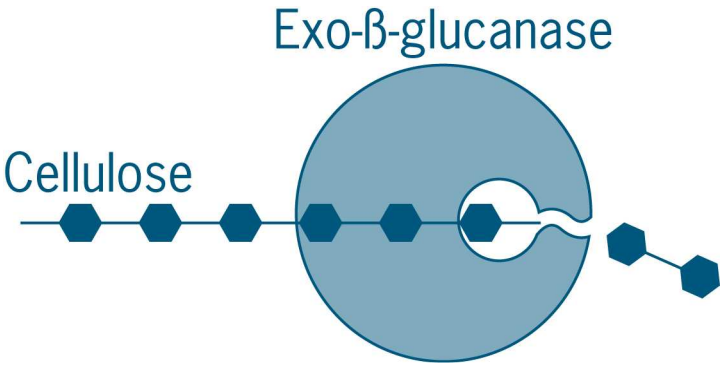


Figure 15 Exo-ß-glucanase splices at the end of a cellulose chain of disaccharides.

Glucosidases

These enzymes splice individual glucose components of oligosaccharides and cleave the cellobioses (Figure 16).

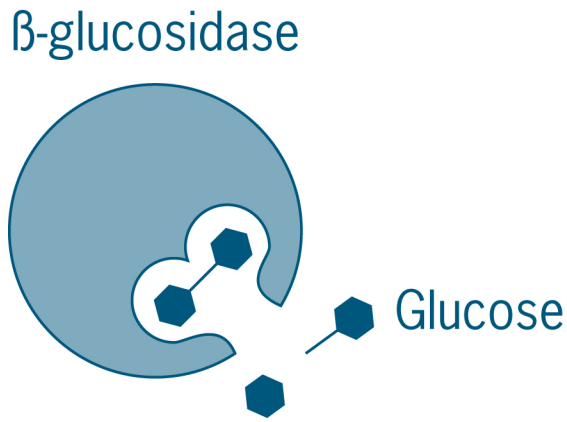


Figure 16 The enzyme ß-glucosidase splices through the disaccharides.

The glucanases are large proteins that consist of two functional units: one in which catalysis takes place, in this case hydrolysis; and another where the protein binds to the hydrocarbon chain. This unit is called the CBM (Carbohydrate Binding Module).

It is clear that the exoglucanases can work faster if more loose ends are created by the endoglucanases.

This is visualised in Figure 17.

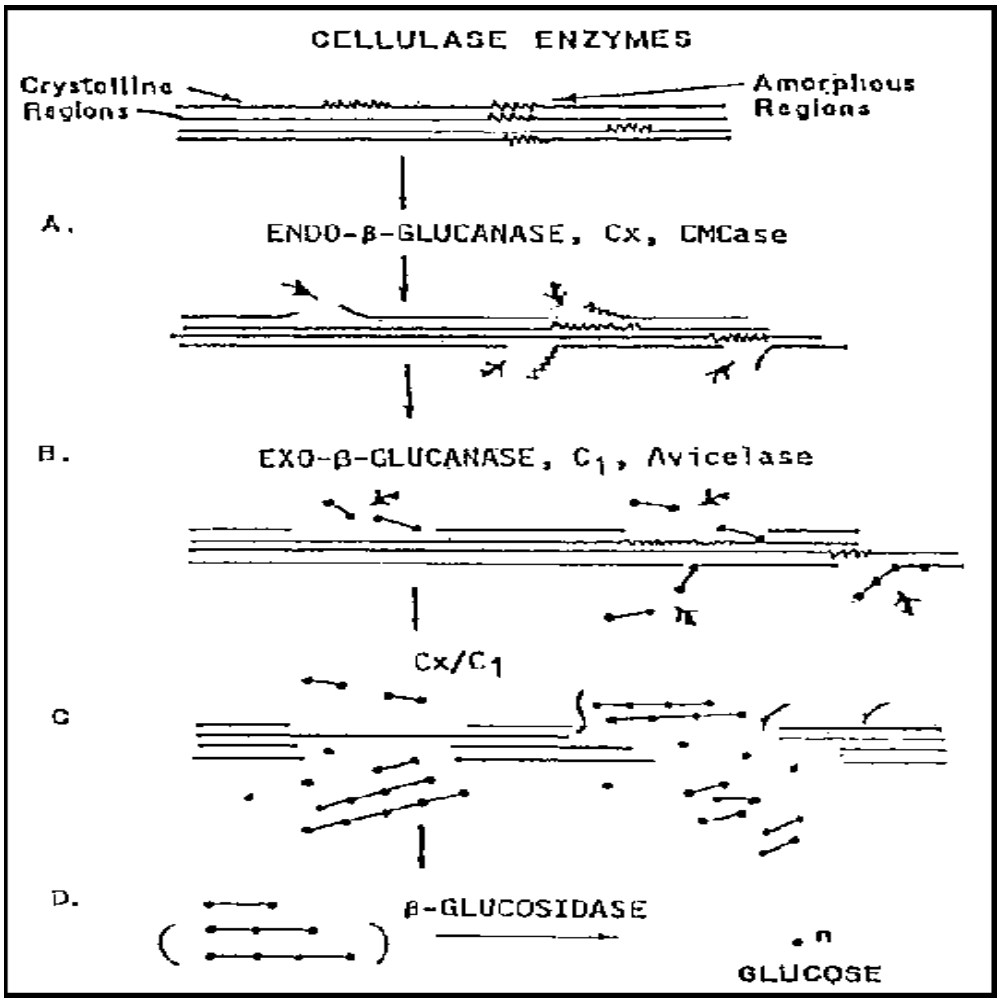


Figure 17 The action of enzymes during the breakdown of cellulose into glucose.

There are also similar enzyme complexes for hemicellulose. However, the issue is complicated by the fact that there are now several sugars that bind to each other in different ways.

Enzymatic hydrolysis often takes place on an industrial scale but not as yet for the production of bioethanol. However, there are an increasing number of trial plants and research in this area is intensifying.

Enzymatic hydrolysis brings with it a number of problems:

- Lignin and hemicellulose surround the cellulose, minimising the available surface area to which the glucanases can bind.
- Enzyme-inhibiting action of the products glucose and cellobiose: too much glucose inhibits the action of the ß-glucosidases. This stagnates the conversion of the cellobioses. Which in turn hinders the exo-ß-glucanases. Therefore the glucose molecules should be removed as soon as they are produced.
- The exo-ß-glucanases must bind to the cellulose chain in order to be able to break off the ends. Sometimes these

proteins bind to the wrong place. They are then unproductive *and* they block the endoglucanases.
- The enzymes can also attach to lignin particles and become unproductive as a result.
- Enzymes can be easily damaged by thermal or chemical activity.

Despite the problems outlined, the expectations surrounding this form of hydrolysis are great. Broad-based research is taking place in an attempt to design efficient forms of enzymatic hydrolysis.
The main attempts focus on:

- The development of biorefineries for EFC (Ethanol from Cellulose) whereby enzymes are produced on site.
- The search for microorganisms via genetic modification which produce enzymes for both cellulose and hemicellulose.
- The development of genetically modified microorganisms which produce the hydrolysis enzymes and take care of the fermentation process. This approach immediately solves the problem of the hydrolysis-inhibiting action of the glucose molecules.

Task 19
Various enzymes are required to hydrolyse cellulose.
a) Name these enzymes.

The following fictitious experiment is performed: three bioreactors with cellulose substrate are consecutively exposed to three enzymes. The other variables are kept constant (why?). At the end of each process the substrate is enzyme-free. The sequence in which the enzymes are added is also varied.
The sequence can be seen below in Table 5:

Table 5 The sequence of adding the enzymes

Bioreactor 1	Bioreactor 2	Bioreactor 3
Exo-β-glucanase	Endo-β-glucanase	Glucosidase
Endo-β-glucanase	Exo-β-glucanase	Endo-β-glucanase
glucosidase	glucosidase	Exo-β-glucanase

b) In one of the reactors, almost no glucose will be produced. Which is the reactor in question?
c) In which reactor will the most glucose be produced? Explain why.

The efficacy of an enzyme depends on the temperature.
This is shown below in Figure 18:

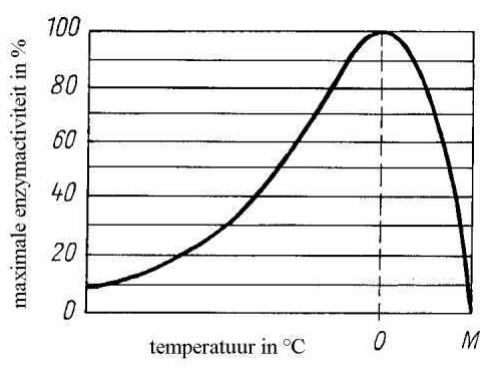


Figure 18 The enzyme activity as a function of temperature.

[maximum enzymatic activity in % - temperature]

d) Explain why enzyme activity initially increases when the temperature rises.
e) Explain why enzyme activity falls off beyond a certain temperature.

Table 6 below contains a few characteristic parameters of a number of enzymes.

Table 6 List of enzymes with their characteristics.

Enzymes	Optimal temperature [°C]	Optimal pH	Inactivation temperature [°C]	Average temperature [°C]
Cellobiase	< 20	4.5-5.0	> 20	37-38
Maltase	35-40	5.3	> 70	
Laminaribiase	37	5.0	> 55	
Lipase	35-40	6.8	> 50	
Exo-beta-glucanase	< 40	4.5	> 40	
Arabinosidase	40	4.5-4.7	> 60	
R-enzyme	40	5.3	> 70	
Endo-ß-glucanase	40-45	4.5-4.8	> 55	45
Aminopeptidase	40-45	7.2	> 50	
Exo-xylanase	45	5.0	> 50	
Endo-xylanase	45	5.0	> 50	
Dipeptidase	40-45	7.8-8.2	> 50	
Saccharase	50	5.5	55	52
Phosphatase	52	4.5-5.0	70	
Endopeptidase	50-60	5.0-5.2	> 80	
Carboxypeptidase	50-60	5.2	> 70	
ß-grensdextrinase	55-60	5.1	> 65	
ß-amylase	60-65	5.4-5.6	70	62
α-amylase	70-75	5.6-5.8	80	72

f) Explain why the addition of a blend of the required enzymes to a cellulose substrate with the characteristics shown in Table 6 will not give an optimal result.
g) Explain how you would advise the manufacturer to increase glucose production.

Enzyme activity

Enzyme activity depends on temperature. If the temperature is too high, the proteins denature. Due to the thermal movement of the molecules in the substrate there is always a little protein damage. In other words, the weak forces that give the protein its specific form are no longer exerted. When the temperature rises, this process is speeded up. Below we investigate this process by means of a dynamic model.

If the number of enzyme molecules is N, then in a period Δt a number of molecules will be lost through this process of denaturation. This reduction is called: ΔN. ΔN is therefore negative.
For the purpose of calculations, it is problematic that N changes in the period Δt. That is why the period taken is small enough that N (and also the other variables that affect the reduction) does not change to any noticeable extent. We call this a reduction dN in a period dt.

The following applies:

dN ~ dt (the greater the time, the more molecules will succumb in that period)
(2 x as many molecules, so 2 x the reduction)

therefore $dN = -k * N * dt$ (1)

Here the proportional factor *k* is a constant that describes to the reduction rate. This factor depends on temperature and other external factors such as pH, etc. But not on N.
To get an idea of the size of k, you can put the question: After what period of time would the population decrease by 10%? And how much time does it take for the number of enzymes to halve? This is called the half-life of the enzymes.

Assume that for a certain cellulase at a certain temperature the half-life is 2 hours. How big is the characteristic variable k?
We can use Excel to work this out.
We will also use a dynamic model.

We start with a fairly random value for the constant k. For example k = 0.001 s^{-1}. This means that:

dN = - k * N * dt = - 0.001 (s^{-1}) * N * 1 (s)

and that would mean that after one second, 1 thousandth of the enzyme has disappeared. What would the half-life of this enzyme be at this temperature?

Starting with a quantity of 100, we will look to see how long it takes for the value to fall to 50. The mathematical process is as follows:

Choose a starting value for t, usually:	t is 0 or t:=0
Choose a starting value for N:	for example N:= 100
Choose a time:	for example dt:= 2 s
Choose a factor k:	e.g. k:= 0.008

Now start the calculation

Step 1:	Now calculate dN	dN:= - 0.008*100*2 = 1.6
Step 2	Calculate the new value of N	N:= N + dN=100-1.6=98.4
Step 3	Calculate the new value of t	t:= t + dt=0+2=2 s
Step 4	Go back to step 1	
Etc.		

This is easy to do with Excel. You can then plot N against t in a graph. Below is a sheet from an Excel file. The values shown are calculated using the method outlined above.

t	N	dN		dt =	2
				k =	0,008
0	100	-1,6			
2	98,4	-1,5744			
4	96,8256	-1,54921			
6	95,27639	-1,52442			
8	93,75197	-1,50003			
10	92,25194	-1,47603			
12	90,77591	-1,45241			
14	89,32349	-1,42918			
16	87,89432	-1,40631			
18	86,48801	-1,38381			
20	85,1042	-1,36167			
22	83,74253	-1,33988			
24	82,40265	-1,31844			
26	81,08421	-1,29735			
28	79,78686	-1,27659			
30	78,51027	-1,25616			
32	77,25411	-1,23607			
34	76,01804	-1,21629			
36	74,80175	-1,19683			
38	73,60492	-1,17768			
40	72,42725	-1,15884			
42	71,26841	-1,14029			
44	70,12811	-1,12205			
46	69,00607	-1,1041			
48	67,90197	-1,08643			
50	66,81554	-1,06905			
52	65,74649	-1,05194			
54	64,69454	-1,03511			
56	63,65943	-1,01855			
58	62,64088	-1,00225			
60	61,63863	-0,98622			
62	60,65241	-0,97044			
64	59,68197	-0,95491			
66	58,72706	-0,93963			
68	57,78743	-0,9246			
70	56,86283	-0,90981			
72	55,95302	-0,89525			

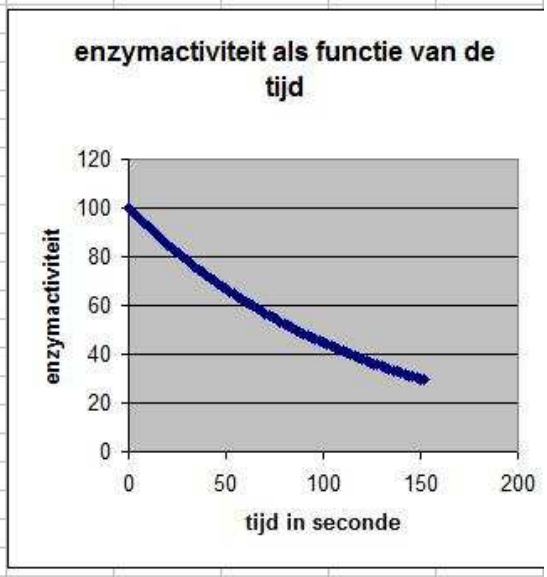


*Figure 19 In Excel the contents of one cell are calculated using the contents of another cell In step 1 in the figure below, the content of cell C2 is calculated using: C2 = F$2*B2*F$1. By copying cell C2 to C3 we get C3=F$2*B3*F$1. The dollar signs prevent reference to another cell when copying.*

[enzyme activity as function of time - enzyme activity - time in seconds]

The advantage of working with this sort of model is that, by 'trial and error', you can find the value of k that goes with a half-life of, for example, 2 hours.
Here we find a half-life of less than 100 s. The temperature is apparently very high.

Task 20
Use Excel to find out which value k should have for a half-life of 2 hours.
Vary the time as well. The smaller that is, the more accurate the results, but it also comes with a downside.

Task 21
Repeat the previous task but use the programme Coach. If you have never worked with this programme before, first do the introductory exercise for Coach.

Enzyme kinetics

In every living cell multiple reactions take place to ensure that the cell can continue to live and multiply. The cell environment is relatively mild. It has a neutral aqueous environment, and a temperature and pressure that is not very different from the surroundings. Under these conditions most reactions inside a cell would not take place. Yet in general they proceed very quickly, because they are nearly all catalysed by a reaction-specific enzyme. Enzymes are proteins and are called the catalysts of life, or biocatalysts. In the absence of these enzymes, most of the reactions in the cell would not take place.

Enzymes can also do their work outside the cell. Some enzymes made by the cell are designed specifically for this purpose and are secreted by the cell, for example to release nutrients from natural polymers. For example, some fungi secrete cellulases that break down cellulose in rotting wood into glucose that can be absorbed by the cells and digested (metabolised). Outside the cell enzymes can also catalyse the formation of a great many commercially interesting substances that are otherwise difficult to produce. This is why there is such interest in enzymes as industrial catalysts. All the more so since the availability of enzymes has been greatly increased by recombinant DNA technology. If the gene that codes for a desired enzyme is not yet available, it can be made or isolated and then cloned in a production organism, for example *E. coli* (a bacterium that also lives in our intestinal flora) or a yeast. The enzyme concerned can subsequently be produced on a large scale, at the desired purity, in a conventional way using the genetically modified organism. This is how enzymes have been produced for years for use in washing powders, for baker's raw ingredients, in the production of soft drinks, wine, antibiotics, etc.

To be as efficient as an industrial biocatalyst, it is best if the enzyme concerned is well characterised, i.e. the action of the enzyme must be known, as well as how fast the enzyme works, i.e. what the enzyme activity is (Exercise 'Conversion'). The enzyme activity is the rate (V) with which the enzyme converts substrate (S) into product (P) or products. For more than a century research has been conducted on enzyme kinetics, i.e. the description of the chemical reaction rates under the influence of enzymes. All chemical reactions are equilibrium reactions, in which the conditions determine whether the equilibrium is more on the side of the reactant(s) or the

product(s). Like all other catalysts enzymes do not change the position of equilibrium, they only increase the rate at which the equilibrium is achieved. Yet enzyme kinetics has its own character, in that the mechanism of the catalysis is different and more complex than that of most conventional chemical catalysts. Enzyme catalysis is characterised by a phenomenon called substrate saturation. If we express the enzyme-catalysed reaction rate V as a function of the substrate concentration [S], then in the simplest case we obtain Figure 20 below, the so-called Michaelis-Menten curve.

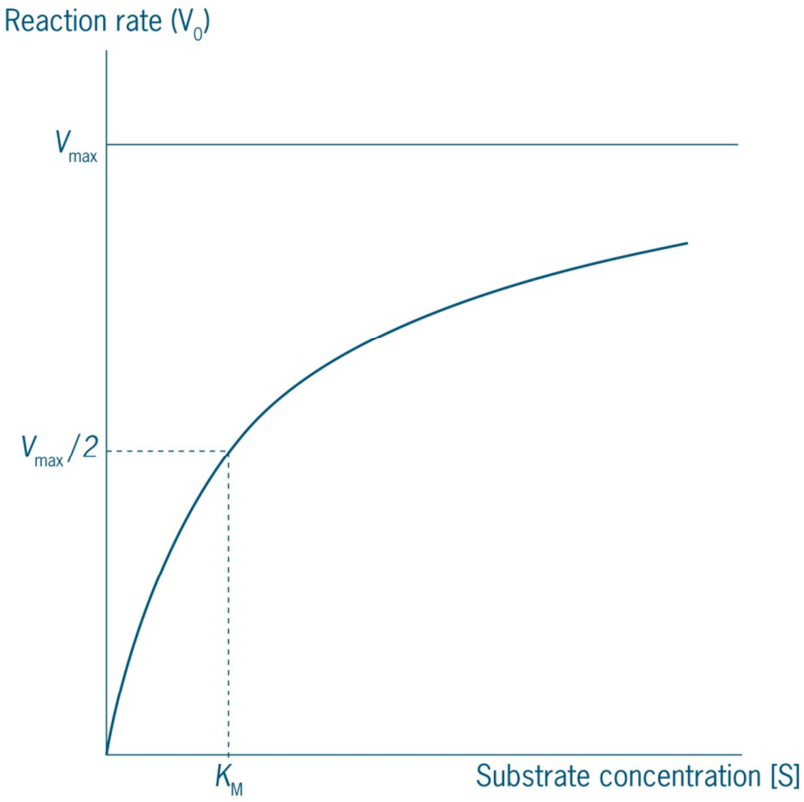


Figure 20: Enzyme kinetics according to Michaelis and Menten.

We see that at a relatively high [S], V hardly increases at all and eventually a maximum reaction rate is reached. This is known as the substrate saturation value, V_{max}. V is then to all intents and purposes zero order in relation to [S], i.e. independent of [S]:

$$V \sim constant = V_{max} \qquad (2)$$

This means that when you have a solution in a flask with a relatively high substrate concentration and you add enzyme, the enzyme essentially works initially at rate V_{max}. However, the more substrate is converted, the smaller V becomes. In Figure 20 we can look at the asymptotic curve from right to left (check this for yourself).

At a relatively low [S] the increase in V is almost directly proportional to [S], i.e. V is essentially first order in relation to [S]:

V=constant*[S] (3)

In the interjacent area the increase in rate continues to level off, giving a reaction order between zero and one. The whole curve can be described using the following equation:

$$V = \frac{V_{\max} * [S]}{K_M + [S]} \quad (4)$$

This is called the Michaelis-Menten (MM) equation and K_M is the Michaelis-Menten constant, named so because two scientists, Leonor Michaelis and Maud Menten theoretically derived this equation. Starting with the characteristic phenomenon of substrate saturation, in 1913 they developed a theory that still forms the basis of quantitative analysis of many aspects of enzyme kinetics. This theory is also the basis for more complex forms of enzyme kinetics too.

According to their theory, in the simplest case, the first step in enzyme catalysis involves the bonding of substrate S to free enzyme E, to form an enzyme substrate complex ES. This is followed by the collapse of this complex into free enzyme and product P. Both steps are equilibrium reactions, but in many cases the equilibrium of the second step is much further to the right, so the reaction equation is simplified to:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P$$

The ks are the specific reaction rate constants and are a measure of the catalytic power of an enzyme. Using the normal principles of chemical reaction kinetics, the Michaelis-Menten equation (4) can be derived, whereby

$$K_M = \frac{k_2 + k_3}{k_1} \quad (5)$$

and

$$V_{\max} = k_3 * [E_T] \quad (6)$$

where [ET] is the total enzyme concentration, or the sum of the free and complex-bound enzyme concentration:

$$[E_T] = [E] + [ES] \quad (7)$$

On closer inspection of the MM equation, we see that K_M units must be equal to those of the substrate concentration [S], thus kg m-3 or mol m-3, if we use the recommended SI units. If the substrate concentration [S] is equal to K_M and we substitute this in equation (4), then it transpires that V = ½ V_{max}! That means that K_M has a numerical value equal to the substrate concentration, whereby the conversion rate is equal to ½ V_{max} (see figure 20).

Task 22

The official SI unit for enzyme activity is the katal. One katal is the amount of enzyme needed to catalyse the conversion of 1 mol substrate per second. The specific activity of an enzyme can therefore be expressed in katal per kg (enzyme)protein, but this unit is not often used. In biochemical research enzyme activity is usually expressed in units. One unit (1 U) enzyme activity is the amount of enzyme needed to convert 1 μmol substrate per minute. The specific activity of an enzyme is usually expressed as μmol min^{-1} mg^{-1} and the enzyme concentration in mg ml^{-1}. Bioprocess technologists prefer using kg, m, s, etc. Calculate V_{max} in these units if it is given that the total enzyme concentration in a bioreactor is 10 mg ml^{-1} and k_3 = 1 μmol $min^{-1}mg^{-1}$.

If [S] >> K_M (as a rule of thumb: [S] > 5 K_M) then we can ignore K_M under the line in the MM equation in relation to [S]. In this way equation (4) can be simplified to equation (2) i.e. V ≈ V_{max}. This fact is often used to make a quick first estimate of reaction rates.

Task 23

In the November-December issue, volume 2007, of the journal Biocatalysis and Biotransformation (25(6): 419-429), Drissen et al describe a model for glucose production from various cellulose substrates (filter paper and wheat straw) using a commercial cellulase complex that contains several enzymes. To summarise, they start from the following simplified reaction mechanism:

cellulose →cellobiose
cellobiose →glucose
cellulose →glucose

Cellulose is therefore converted directly or via cellobiose into glucose by the cellulase complex. We have already seen that cellobiose consists of two interlinked glucose molecules. The enzyme β-glucosidase, also called cellobiase, catalyses the hydrolysis of cellobiose into glucose. Drissen et al measured that the maximum cellobiase activity of the complex is 204 U

ml^{-1}, whereby 1 U per minute, 1 μmol glucose is released from 0.5 μmol cellobiose.
The question is how long it takes before 90% of the cellobiose is converted into a solution containing 34.2 kg cellobiose per m^3. In 1 m^3 there is 1 litre of the enzyme complex and we assume that the substrate concentration after 90% conversion is also at least 5 times K_M.

If [S] << K_M then we can simplify the MM equation to equation (3) whereby

$$C = \frac{V_{\max}}{K_M} \qquad (8)$$

Where C=a constant. The maths therefore becomes simpler, and in practice we can use the fact that [S] < 0.2 K_M to make a fast, less accurate first estimate again. The exercise 'Milk: the white engine!' is an example of this.

In many cases the Michaelis-Menten equation describes enzymatic reaction rates accurately enough for use in the design of bioreactors. In industry most reactions, including enzyme-catalysed reactions, are carried out in batch reactors. An enzyme batch reactor is a tank in which substrate is dissolved in an aqueous medium and to which at time zero enzyme is added to start the reaction. The tank is usually stirred so that the substrate and enzyme are well mixed and to ensure the right pH and temperature. The change in substrate concentration in the tank as a function of the time after addition of the enzyme can be written mathematically as:

$$\frac{d[S]}{dt} = r_s = -v \qquad (9)$$

The left term stands for the change in the substrate concentration over time and is by definition equal to r_S, defined as the volumetric production rate of substrate in kg or mol per volume-unit (m^3) per unit of time (s^{-1}). Here this is equal to –V because substrate is not produced but converted (negative production). The simplest case is seen with substrate saturation (zero order kinetics), whereby $V=V_{max}$ (this is used in the cellobiose hydrolysis exercise). If we substitute this in equation (9), separate the variables ($dS = V_{max}.dt$) and integrate from 0 to t, then we get:

$$\int_{[S(0)]}^{[S(t)]} d[S] = -V_{\max} \int_0^t dt \qquad (10)$$

or

$$[S(0)] - [S(t)] = V_{\max} * t \qquad (11)$$

If the substrate concentration [S] is smaller than 0.2 K_M the whole time, we can substitute equation (9) V = {V_{max}.[S]/K_M}. Separation of variables and integration of 0 to t now gives:

$$\int_{[S(0)]}^{[S(t)]} \frac{d[S]}{[S]} = -\frac{V_{\max}}{K_M} \int_0^t dt \qquad (12)$$

or

$$\ln[S(0)] - \ln[S(t)] = \frac{V_{\max}}{K_M} t \qquad (13)$$

Task 24

Joris Driepinter gets by on three glasses of milk a day, and we want to keep it that way. The price of milk rises dramatically, partly because crops that were previously used for the food and feed industry, are now being used for producing biofuels, as we have seen. In third world countries it is not only the price that makes drinking milk problematic. Many people in Africa and Asia are lactose intolerant. They get sick if they drink milk. That's because their bodies cannot tolerate lactose, milk sugar. Like cellobiose, lactose is also a disaccharide, but in this case there are not 2 glucose molecules attached to each other, but a glucose and a galactose molecule. The enzyme ß-galactosidase, also called lactase, catalyses the hydrolysis of lactose:

Lactose ($C_{12}H_{22}O_{11}$) + H_2O →Glucose ($C_6H_{12}O_6$) + Galactose ($C_6H_{12}O_6$)

Let's say that milk contains 25 g lactose per litre and that we add 1 mg lactase just before the litre packs are sealed in the milk factory. After 1 day a lactose intolerant person buys one of these cartons and immediately drinks a glass of the milk. We know that there can only be a maximum of 1 g per litre, that the maximum conversion rate of lactase is 15 mol lactose per kg enzyme per second (at fridge temperature) and that K_M = 0.4 M. Will the person drinking the milk have a problem?

In many cases we still have to deal with substrate concentrations from around and about the K_M and we cannot get around the MM equation. Substitution of equation (4) by

equation (9), separation of variables and integration of 0 to t now gives:

$$\int_{[S(0)]}^{[S(t)]} \frac{(KM+[S])d[S]}{[S]} = -V_{\max}\int_{0}^{t} dt \qquad (14)$$

or

$$\ln(S(0)] - \ln[S(t)] + \frac{[S(0)]-[S(t)]}{K_M} = \frac{V_{\max}}{K_M}t \quad (15)$$

If we take a good look at this and compare it with equations (11) and (13), then we can see that it is actually a sum of zero and first order reactions.

Task 25

Sucrose is known in layman's terms as sugar and it comes from beet or cane sugar plants. Sucrose consists of a glucose molecule linked to a fructose molecule and is therefore a disaccharide just like cellobiose and lactose. The hydrolysis of sucrose $\{[\ddagger]_D^{20} = +66.5^0\}$ into D-glucose $\{[\ddagger]_D^{20} = +52.5^0\}$ and D-fructose $\{[\ddagger]_D^{20} = -92^0\}$ is often called inversion because it is accompanied by a net change in optical rotation of dextro (right rotating) to levo (left rotating) because glucose and fructose are formed in equimolar quantities. The mixture formed is therefore often called inverted sugar. The hydrolysis of sucrose can be catalysed by acid or by the enzyme invertase and this conversion can therefore be traced in a polarimeter, an apparatus that measures the optical rotation. Fructose (fruit sugar) is sweeter than glucose and sucrose, and inverted sugar is therefore also sweet, albeit less sweet than fructose. It is used in industry and other areas to enhance wine and for champagne and liqueur preparation. Inverted sugar is also used as a honey substitute because honey contains virtually the same proportion of sugar.

We buy a commercial invertase powder and add 1 g to 0.1 m^3 sugar solution (100 kg sugar per m^3). The K_M of invertase is 0.1 M. After one day, the concentration of sugar is 10 kg m^{-3}. Calculate the maximum specific enzyme activity in kg substrate, per kg enzyme, per second.

5. Fermentation

Since the beginning of time man has used the fermentation process to prepare food and drink. You may have learned about this already on your biology course. Check if your knowledge is up-to-date by looking at the background module (►URL 1).

5.1 Classical biotechnology

The use of fermentation is a typical example of classical biotechnology. It was not until the 19th century that scientists uncovered the existence of yeast cells. Louis Pasteur is the most famous scientist who studied microorganisms. Nowadays there are entire industries cultivating yeast cells using biotechnology. Every day tonnes and tonnes of yeast are produced for bakers and breweries.

Task 26

a) Look up the meaning of biotechnology.
b) Give three examples of classical biotechnology.
c) Indicate what distinguishes modern biotechnology from classical biotechnology and give an example.
d) Search the internet for a Dutch company that produces yeast.
e) Search the internet for companies, with the exclusion of bakeries and breweries, that need yeast for their production process.

Task 27 experiment

How many yeast cells are there in 1 kg dry baker's yeast?
Since a yeast cell is extremely small, there will be a great many of them.
You will need a microscope to count them.
Mix 1 g yeast with 100 ml water. Take a drop of this mixture and look at it under a microscope. Draw what you see.
Think of a method for estimating the number of yeast cells in one gram of yeast.
Describe the method in a number of steps and explain which calculations are required for each step.
Put your method into practice.
(you may use the fact that there are 20 droplets in 1 ml for your calculations).

The first application of yeast cells

Wine-making is probably the oldest form of biotechnology. Wine is easy to make because alcohol often occurs

spontaneously in fruit juice. Yeast cells are present everywhere and there are always a few on unwashed fruit. The discovery that the drink produced by fermentation kept longer than fruit juice may have been entirely accidental. It must have been a wonderful revelation for hunter-gatherers. These days the wine industry is a scientifically advanced sector which does not rely on wild yeast cells. Lots of research is conducted on yeast.

Task 28 Experiment:
The following experiment can be conducted in order to check whether wild yeast cells also occur in nature.

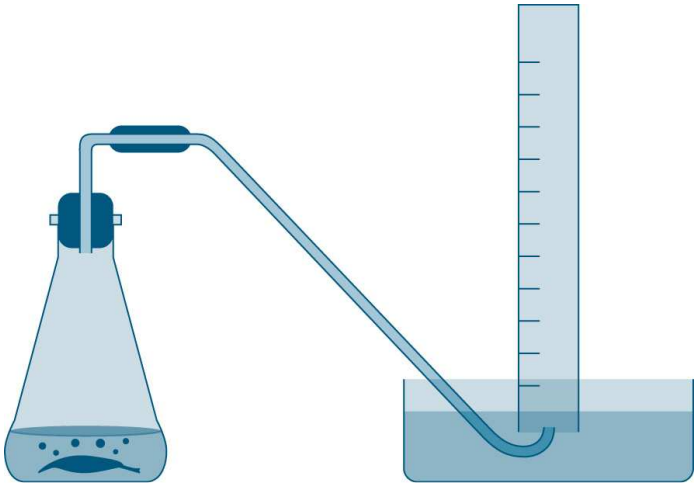


Figure 21 Set-up for experiment 28

Two of these set-ups are arranged. In one Conical flask, grape juice from a carton is used. In the second, grape juice is also used but in addition a piece of unwashed fruit, a sprout or some leaves are added. The set-up should be left standing for several days. One of three possible things should now happen.

- There is no fermentation in either flask.
- Fermentation occurs in both flasks.
- Fermentation occurs in one flask.

Before you begin the experiment, check which conclusions you should draw from each of the results stated above.

a) Explain how, using this experiment, you can check whether fermentation has taken place.
b) For each possibility, explain which conclusions can be drawn.
c) Explain why all the equipment you use must be clean.

Conduct the experiment and make a note of your observations. Examine a droplet from both flasks and draw what you see.

5.2 The yeast cell

The most well-known microorganism able to convert glucose into ethanol is available in the supermarket. It is probably the only living organism on sale in regular packaging, sitting on the shelf between the sugar and flour. The official name is ***Saccharomyces cerevisiae***, but this single cell organism is better known as baker's yeast. It is one of the most researched living organisms and is produced on an industrial scale.
The yeast cell is a heterotrophic organism. This means that the cell is reliant on other living organisms for its existence. This is in contrast to autotrophic organisms. These are able to make the complex molecules of life from non-organic substances. Plants are autotrophic. Plants use water, CO_2 and minerals with the help of sunlight to create organic substances. One of the substances produced in this way is glucose, which is then used to make more substances. The yeast cell needs glucose as nutrition.

Cells

Life on earth is always organised in cells. All cells are composed in a very similar way. Yeast cells are shown in Figure 22.

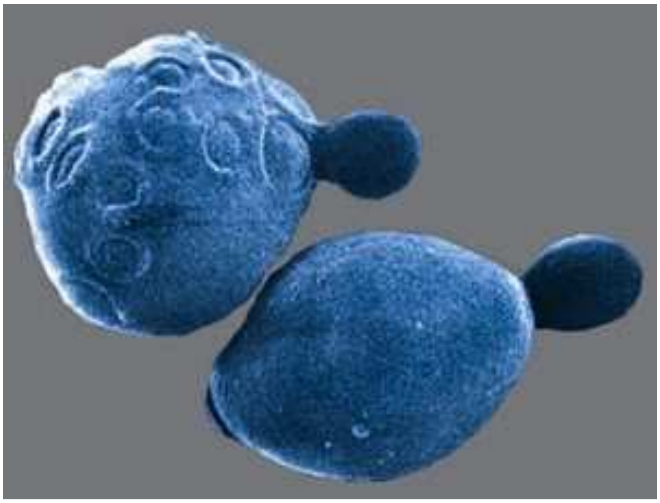


Figure 22 Saccharomyces cerevisiae

After the discovery of the cell by Robert Hooke in 1665, our understanding that all life, at least all life on earth, is organised in cells, slowly evolved. A cell membrane protects the cell against chemical activities in the outside world and allows special chemical processes to take place inside the cell. The similarities at the cell level are amazing. Horses have more in common with a dandelion than was first thought. This is with respect to the composition of their cells, and the chemical processes that take place inside of them. The same is also true for yeast cells. Yeast cells are showed in figure 22.

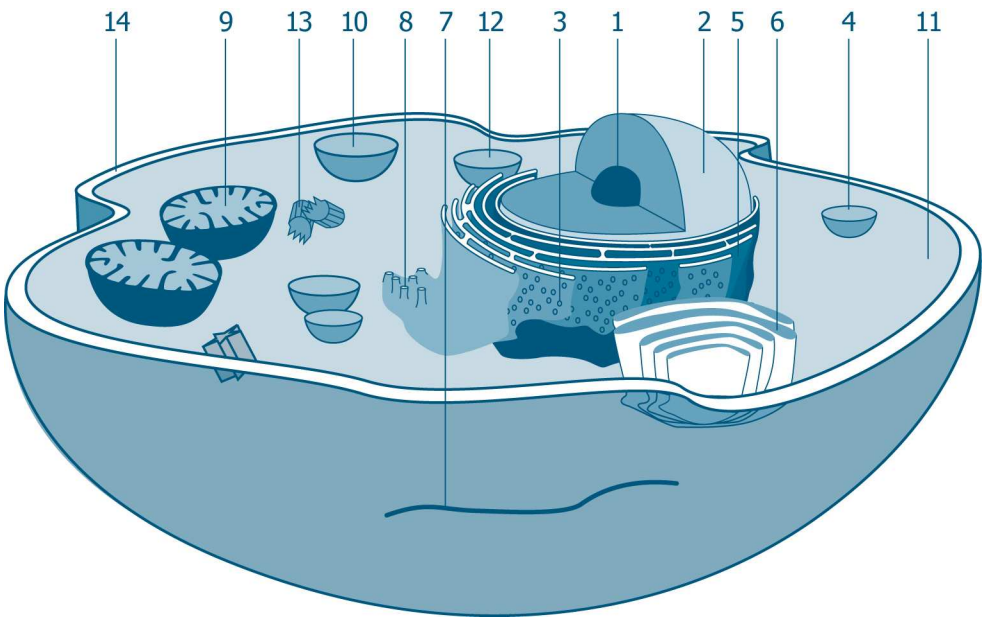


Figure 23 The composition of a cell.

The nucleus contains the DNA, which contains the genetic code with which the yeast cell passes on its characteristics to daughter cells.
The cell is surrounded by a cell wall, which protects the complicated chemical processes that take place within the cell from chemically active substances outside the cell.
The cytosol is the fluid which fills the cell. Key processes also take place here. The most important is surely glycolysis: the process whereby glucose is converted into alcohol. There are several organelles or cell components floating in the cytosol.

Task 29
If a cell is compared to a factory, the organelles are the parts of the factory with a specific function:
- There is the head office where the most important decisions are made;
- There are the production sites where the proteins are produced: these are the substances necessary for all important functions of the cell;
- There are energy plants which have to supply the cell with energy.
- There is an emergency power-unit in case the energy plant does not work.
- There is an import/export department which takes care of the delivery of the goods;
- There is a waste processing department.
- There is a transport system to take waste outside.

a) Look up the action of a cell.
b) Describe the role of the various organelles.

c) Compare the functions of the organelles with the statements listed above.

5.3 Biochemistry

Cell chemistry is the chemistry of substances dissolved in water where the carbon atom (C) is the key building component. The carbon atom plays an important role because it can make a maximum of four single, double and even triple bonds with other atoms. This gives rise to molecular structures with a carbon skeleton of chains, rings and combinations thereof. Outside living organisms, there are even cylindrical and spherical structures (nanotubes and buckyballs).
Carbon chemistry facilitates the complexity that characterises life.
Despite the practically unlimited possibilities that carbon chemistry offers, cell chemistry only uses a limited number of molecular building blocks and structures. Half a dozen substances or families of substances play a key role in the chemistry of life, biochemistry:

Lipids

Lipids are molecules with long chains. There are two important subsections. One group is formed by fats, whose main function is to store energy for the cell. The other group consists of phospholipids. These are significant because they spontaneously form membranes which enable cell activity to be separated from the outside world and from each other.

Carbohydrates

Cellulose, starch and glycogen are biopolymers built up of glucose molecules. The carbohydrates are built up of only one kind of building block and so have only a few features for specificity. These substances often only have one function. Sugars and starch serve as a fuel. Cellulose occurs in plants and gives the plant its turgidity.

Proteins

Proteins are also polymers. Proteins are chains of amino acids. Proteins are the ‘work horses’ in the cell and carry out a multitude of tasks. There are proteins that function as building materials, such as keratin, which is key for nails; there are proteins that have a transport function, such as haemoglobin, which is responsible for transporting oxygen in the blood; and there are proteins that function as chemical catalysts which facilitate or prevent chemical reactions.

Nucleic acids (DNA and RNA)

DNA is the carrier of the genetic code. Proteins are put together with the help of this code. It is the only molecule that can divide and copy itself with the help of proteins. RNA plays a role in the coding process of the proteins.

Adenosine phosphates (AMP-ADP-ATP)

These molecules function as 'small change', energetically financing chemical reactions.
Sugars and fats are the fuels for the cellular activity. The direct breakdown of these fuels would, however, throw up two problems. Firstly, the amount of energy released would be too big. The cell would become overheated. Secondly, the energy has to be available much quicker than the breakdown of sugars or fats would allow.
So energetic 'small change' is present in nature in the form of ATP (adenosine triphosphate) The ATP molecule can be broken down into an ADP molecule. In addition, a phosphate group is split up; under normal circumstances 50 KJ/mol is released in the process.
This process is shown in figure 24.

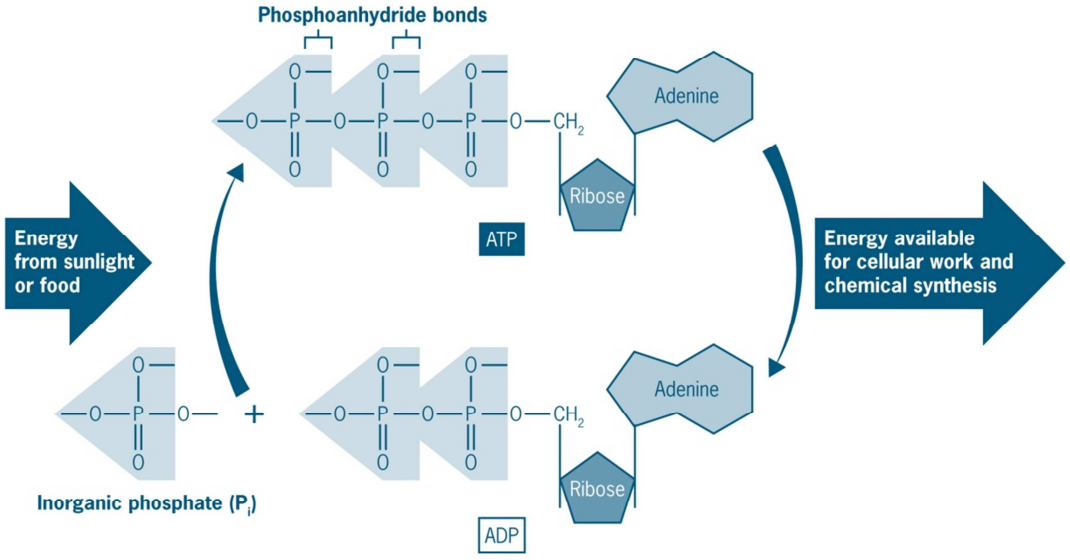


Figure 24 ADP can be converted into ATP by absorption of energy. ATP then functions as an energy carrier. When the energy is used, ATP reverts to ADP.

Metabolism

Metabolism comprises all the chemical activities that enable living organisms to create information and build up complex structures with the help of substances and energy from outside. Metabolism also has anabolic pathways, where substances are synthesised as proteins. In addition, there are also catabolic pathways, which supply the energy required for the synthesis by, for example, the breakdown of glucose.

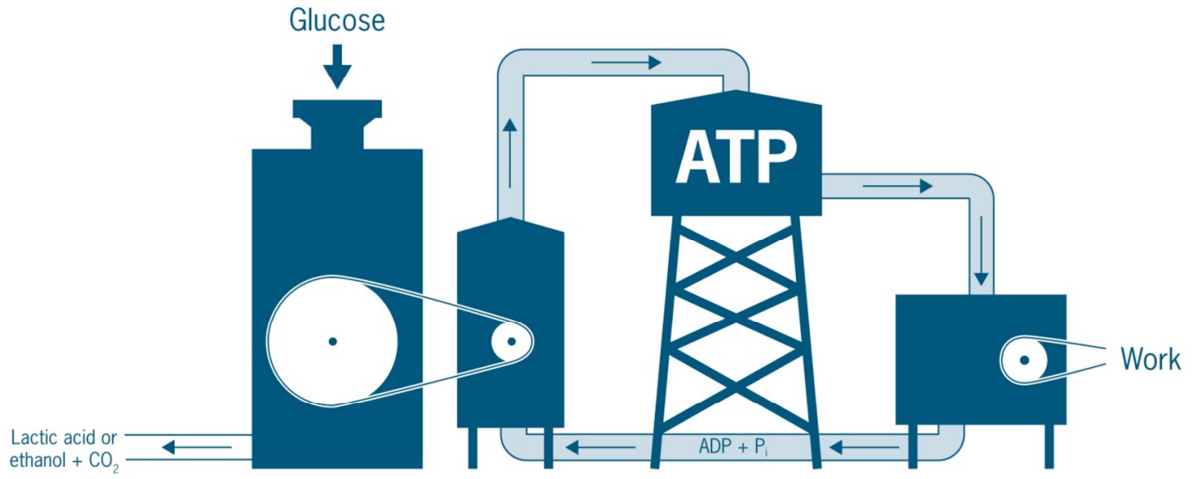


Figure 25 By burning glucose, energy is released to upgrade ADP to ATP. When energy is required, for work for example, ATP can release its energy by changing into ADP.

Task 30

Explain whether the following reactions are anabolic or catabolic:

a) the combustion of glucose.
b) Hydrolysis
c) The formation of proteins from amino acids.
d) Photosynthesis
e) Alcohol fermentation

5.4 Fermentation reaction

Alcohol fermentation

When glucose is burned it forms the catabolic mechanism whereby the yeast cell generates energy for its living processes. The yeast cell contains mitochondria which can burn the glucose if oxygen is present. During the breakdown of 1 glucose molecule, approximately 16 ATP molecules are formed in yeast. But in nearly all circumstances, the yeast cell prefers to use alcohol fermentation. The net result of this is only 2 ATP. The yeast cell will have to be satisfied with this under most circumstances. The remaining energy stored in the glucose molecule is now partly situated in the ethanol molecule. If the alcohol concentration is too high, the yeast cells will die. That happens in about 14% of cases; some yeast strains can stand somewhat higher concentrations. Figure 26 shows the alcohol fermentation.

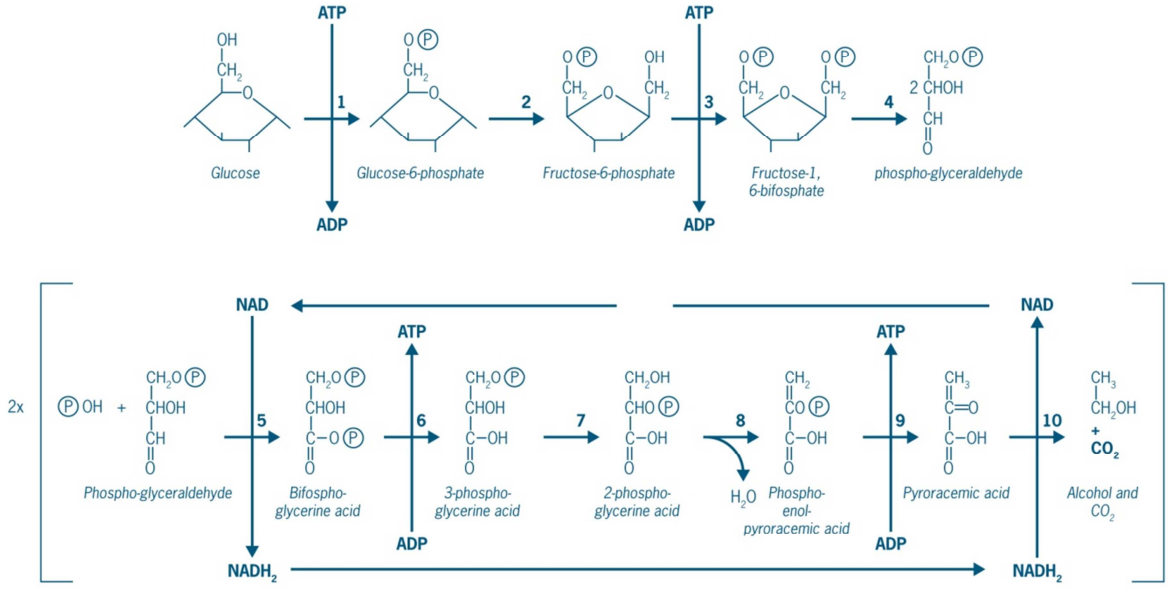


Figure 26 Glycolysis in the yeast cell Glucose is converted into CO_2 and ethanol in 10 steps.

The first nine steps of glycolysis are the same in all cells. In the tenth step there is a choice. In many other microorganisms (but also in human muscle cells, for example) lactic acid is produced in place of alcohol and CO_2. In 1856 this turned out to be the cause of a personal tragedy suffered by Louis Dominique Bigo, a distiller from Lille ,who suddenly found himself on the brink of bankruptcy when it appeared that all his yeast tanks had been acidified by sugar beet juice. In the end he called on a young chemist who was working at the university of Lille and whose research was said to be brilliant. The man agreed to help Bigo and saved his business. But the discovery needed to bring about this rescue would change the world. He discovered that fermentation was the work of microorganisms. And his name was Louis Pasteur.

Task 31

a) Explain what happened to Bigo's yeast tanks.
b) Explain how Bigo would probably be able to prevent the same disaster from happening in the future.

Task 32

Look at the film clip about glycolysis in ►URL 6.

Answer the following questions:

a. Is lactic acid or ethanol produced via this process?
b. Is CO_2 released?
c. Sourdough can be made with flour and water. The sourness comes from lactic acid that is produced by bacteria. Can the dough rise purely as a result of the activity of lactic acid bacteria?

d. Does glycolysis generate energy or use energy, and how much? (expressed your answer in ATP units)

Task 33

a. Look up the formula for lactic acid
b. Check whether the production of lactic acid is also possible in step 10 of glycolysis.

Task 25a, Demonstration experiment

Fermentation rate is an important variable for a producer of alcohol.
Indicate the unit in which fermentation rate can be expressed.

a. Explain why fermentation rate is important for the producer.
b. Name a few factors that are key to fermentation rate.

Using the set-up from chapter 5, task 20, the fermentation rate can be investigated. It is also possible to use a sensor for this purpose. A test-tube is filled with a sugar-yeast solution, covered with a thin layer of oil. The test-tube is then sealed with a pressure sensor.
See figure 27.

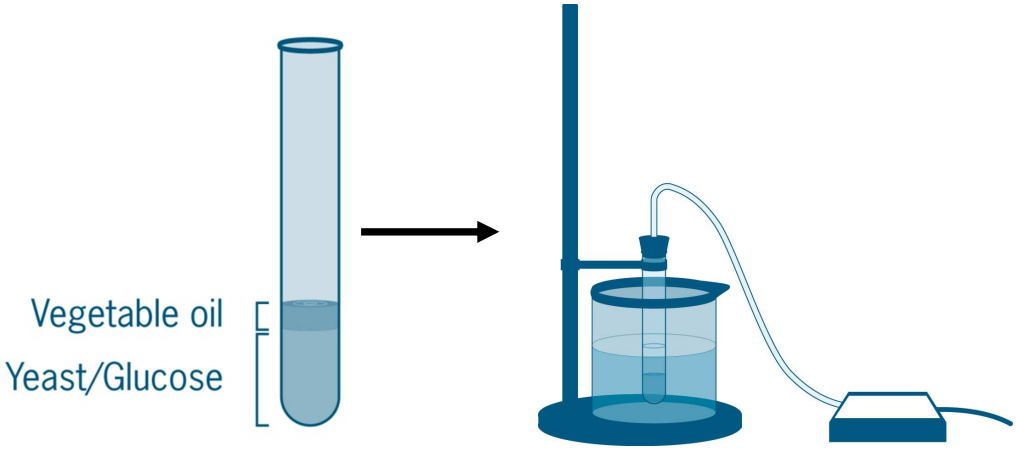


Figure 27 A set-up for measuring fermentation rate. The CO_2 tension is measured with a pressure sensor.

c. With the set-up shown above, how can you investigate the effect of the different factors?

5.5 New developments

Around the world attempts are being made to use ***Saccharomyces cerevisiae*** for the production of ethanol from plant material. The yeast cell is specialised in converting glucose. As already mentioned in Chapter 2, a transition is taking place from the use of plant components with sugar and starch to the use of lignocellulose. But lignocellulose does not consist purely of the easily fermentable C_6 sugars, such as glucose. Hemicellulose, in particular, also contains many so-

called C_5 sugars, such as xylose. In some plants the amount of xylose can be significant (see Chapter 3, table 3). The yeast cell, however, can do little with a sugar like xylose. Baker's yeast lacks an essential enzyme for fermenting xylose. Which is why the yield of ethanol production remains low. If xylose could be used as well, ethanol production would rise by 20%.

Elephant excrement

A discovery by a group of microbiologists from Nijmegen offers new possibilities. They came across the fungus ***Piromyces*** in elephant excrement. This fungus *can* convert xylose thanks to the enzyme xylose-isomerase. This discovery gave microbiologist Marko Kuyper of the Technical University of Delft (one of the partners in the Kluyver Centre for Genomics of Industrial Fermentation) the idea to incorporate the gene from this fungus coding for the enzyme into the genome of baker's yeast, so that it could also convert xylose.
The modified baker's yeast seemed to grow only slowly in an oxygen-free environment. For alcohol production an oxygen-free environment is vital. Kuyper finally succeeded in making a yeast that could grow faster. This yeast can therefore also make biofuel more quickly.

5.6 The bioreactor

A set-up in which a tank of microorganisms is kept for production purposes is called a bioreactor.
A good example of a business that uses bioreactors is a yeast manufacturer. A yeast manufacturer is interested in the fastest possible increase in the number of yeast cells. A yeast manufacturer must therefore have access to a research department. There, mathematical models are always used to mirror life in the reactor.
There are various types of bioreactor using various different processes:

- Batch process:
  In the batch process a quantity of yeast cells are put together with nutrients in a bioreactor. The quantity of yeast cells in the reactor increases and over time is harvested thereafter the reactor is refilled.
- Continuous process:
  In a continuous process there is a continuous influx of nutrients accompanied by a continuous outflow of product. The process within the reactor is stationary, i.e. the quantity of yeast cells and the quantity of nutrients within the reactor are constant.

Task 34

a. If we want to produce yeast cells in a batch process, is it an aerobic or anaerobic process?
b. Explain whether substances must be added to a batch process.
c. Explain whether substances must be removed in a batch process.

It can be assumed that the quantity of yeast cells in the tank during a batch process is at its maximum at a given moment.

d. Explain whether this moment occurs when there are no more nutrients or whether it occurs earlier.
e. Make a sketch of how, over time, the quantity of yeast cells in the reactor tank changes in a batch process.

In a batch process the time for harvesting the yeast has to be chosen. One possibility would be to wait until the quantity of yeast cells stops increasing. This has an upside and a downside.

f. Name the upside and downside.
g. Explain why the bioreactor has to be thoroughly cleaned after each use.

A stationary process takes place in a continuous reactor.

h. Explain what the consequence of this is for the supply and drainage flows.
i. Carbon, C, is added to and removed from the reactor: in which form?

5.7 Cell growth

The concentration of yeast cells is very important for the production of bioethanol. However, the number of yeast cells may vary. Cells grow and cells die. Mathematical models are required for making predictions.

The growth of yeast cells in a closed tank can be described in mathematical terms. There are various growth models depending on the circumstances.

Exponential growth

At the start of the growing process enough nutrients are available for the yeast cells present. The conditions are optimal. The growth of the yeast cells is entirely determined by the rate with which yeast cells can multiply.

In a short period dt in which the conditions change negligibly, the number of yeast cells N will increase by a small amount dN.

This means that dN is proportional to N because the more yeast cells there are, the more yeast cells there will be created. In addition, it figures that dN is proportional to the length of the period dt. The longer this period, the more additions there are.
Therefore

$$dN = \text{constant} * N * dt$$

A process to which this applies is called exponential growth. If the volume of the tank is V, the concentration c of the yeast cells can also be investigated. If we divide the left and right term by the volume of the tank, then:

$$dc = \mu c * dt$$

or

$$dc/dt = \mu c$$

Task 35
The above equation tells us that a quantity c per time unit always grows by the same factor. Assume that this factor (μ) is 0.04.
Take a starting value of 100 for N and opt for time points dt=1

a. Now fill in the following table:

t	dN	N
0	0	100
1	0.04*100=4	104
2	0.04*104=4.01	108.1
3		
4		
5		
6		

b. Check how many divisions are needed to double N. We call this the doubling time.
c. Explain how with a growth of 4% per time unit, less than 25 time units are needed for doubling.
d. Yeast cells have a doubling time of 1.5 hours. Calculate what the growth factor, μ, per hour should be.

Dynamic models

Dynamic models are models in which the development of variables in time can be calculated. There are computer programmes that can calculate models really quickly. A graphic model environment is normally used. A model environment can also be of great use when studying the growth of yeast cells. We are interested in a condition variable: for example, the concentration of yeast cells, c in g/litre. How does c change

during a growth process in a closed bioreactor? The information known concerns the flow variable dN/dt.
The calculation using the model now proceeds as follows:
The chosen time point dt is so small that the conditions (quantity of food, oxygen, etc.) in this period barely change. A starting value is chosen for N. For time t the starting value 0 is chosen.

Then the following steps are calculated:

dN = k*dt (k = a constant)
N = N + dN (N is the old value of N, raised by dN)
t = t + dt (t becomes t+dt)

This is then repeated many times until a certain previously defined stop condition is fulfilled, for example t = 100 units.

Task 36

a. Make a model with exponential growth. Choose the time point dt = 1 and choose the growth factor = 0.04. Check the results of the previous task.
b. Use your model to determine the doubling time.
c. Change the time point and see how this affects the doubling time.

In optimal conditions yeast cells multiply an average of once every 90 minutes.

d. Choose a time point of 1 second and use your model to check how big the specific growth rate μ should be.

Logistical growth

The previous model was concerned with unlimited growth. Of course, in reality, there are always limits. As these limits occur, growth is limited. One limiting factor is, for example, the available oxygen. This is usually permanently fed into the reactor tank, but if there are more yeast cells, consumption increases. With a constant oxygen supply, the availability of oxygen per yeast cell decreases.

Task 37

In an experiment the oxygen supply to the bioreactor is stopped at a certain moment. Indicate how this will affect

a. the number of yeast cells N
b. the growth rate dN/dt

At a certain moment there is also no more nutrients. Indicate how this will affect

c. the number of yeast cells N
d. the growth rate dN/dt

In mathematical terms, this could be shown as follows:

As the concentration of yeast cells increases, problems A, B, etc., arise for the yeast cells. (lack of oxygen, etc.). A number of these factors only inhibit growth. Others are fatal.

$dc/dt = \mu c - kA - mB - \ldots..$

Factors A, B, etc., increase as c increases. Then there is a concentration c whereby problems A, B, etc., are so great that growth is 0. The concentration has reached its maximum value. We call this maximum concentration E, where

$dc/dt = \mu c (1 - c/E)$

If concentration c is much smaller than E, the second term in parentheses is practically zero and there is unlimited growth. If c = E then growth is zero.

Task 38
Extend your model. Add a term to the definition of the flow variable dc/dt. Choose your own starting values. See what effect this term has on the progress of c.

6. Re-processing

As soon as the yeast cells have converted sufficient substrate, it is time to start harvesting the ethanol. In order to use ethanol as a fuel, it must be (relatively) pure or mixed with other fuels. It should obviously contain no water or solid components. But the microbiological origin of the bioethanol means that this fuel results from a complex mixture of water, dissolved solids (sugars, waste from yeast cells, substrate residues), other dissolved substances (ethanol, carbon dioxide) and undissolved solids (mainly dead and living yeast cells). First do the background module ►URL 1. This module will refresh your knowledge about re-processing and separation methods.

6.1 Filtration

It is still easiest to separate the solids from the mixture. Separation methods such as sedimentation and filtration can be used for this purpose. However, it is difficult to do this efficiently and cheaply. If you make ethanol using a ***continuous process***, it is also practical to let the filtration step occur continuously. One possibility is the continuous filtration shown in figure 28 and 29.

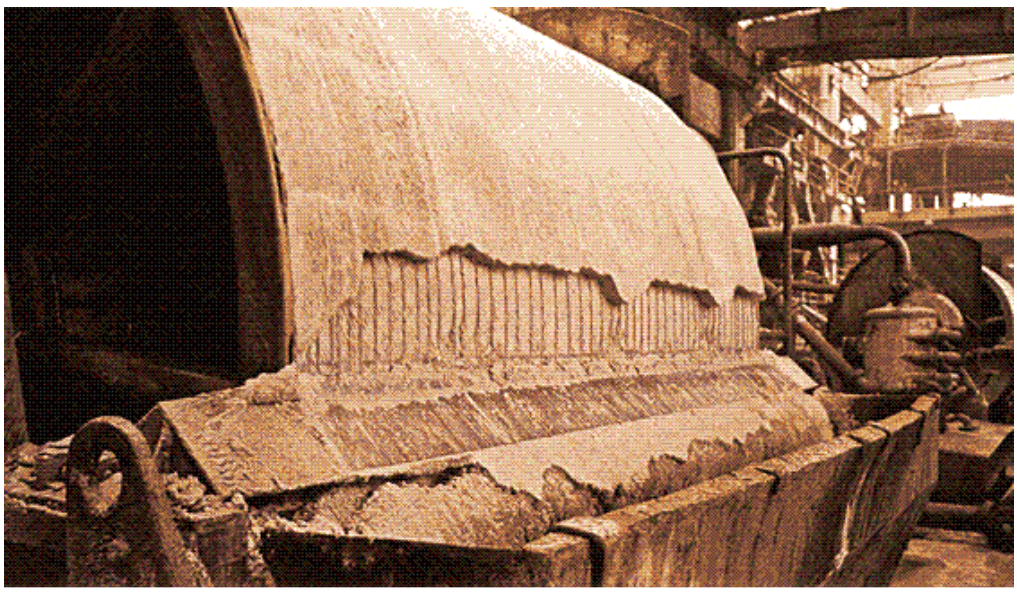


Figure 28 Continuous filtration with vacuum drum filter

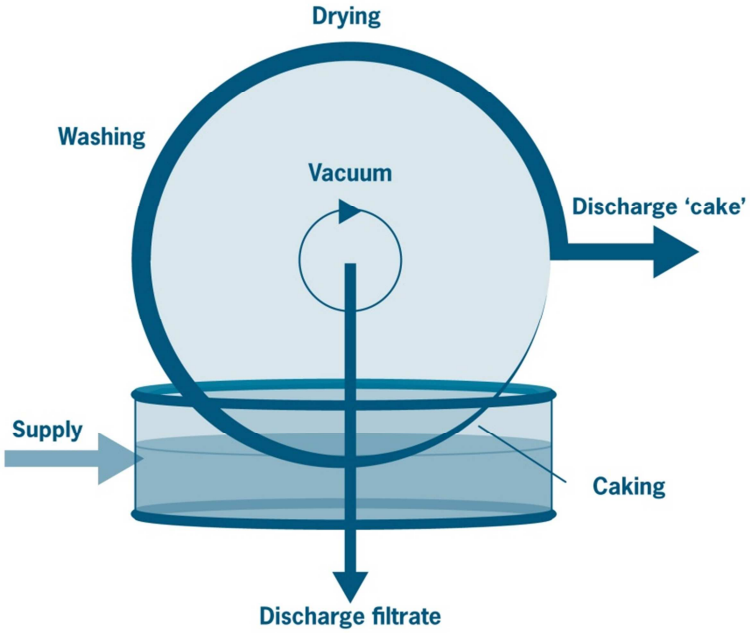


Figure 29 Continuous filtration with vacuum drum filter - schematic

Task 39
In your own words explain how a continuous filtration with 'vacuum drum filter' works. Make sure you include the following aspects in your discussion:
- vacuum,
- the Dutch term for 'drum',
- continuous,
- the construction of the filter,
- the path along which the filtrate is drained off.

6.2 Distillation

Once the solids are removed from the mixture, distillation is practically the only way to get ethanol out of what remains. You already know that distillation is one of the key separation methods (figure 30). This method is based on the principle of a difference in boiling point of the various components, of which water and ethanol are the most important here. Water has a boiling point of 100 °C, ethanol about 78.5 °C.

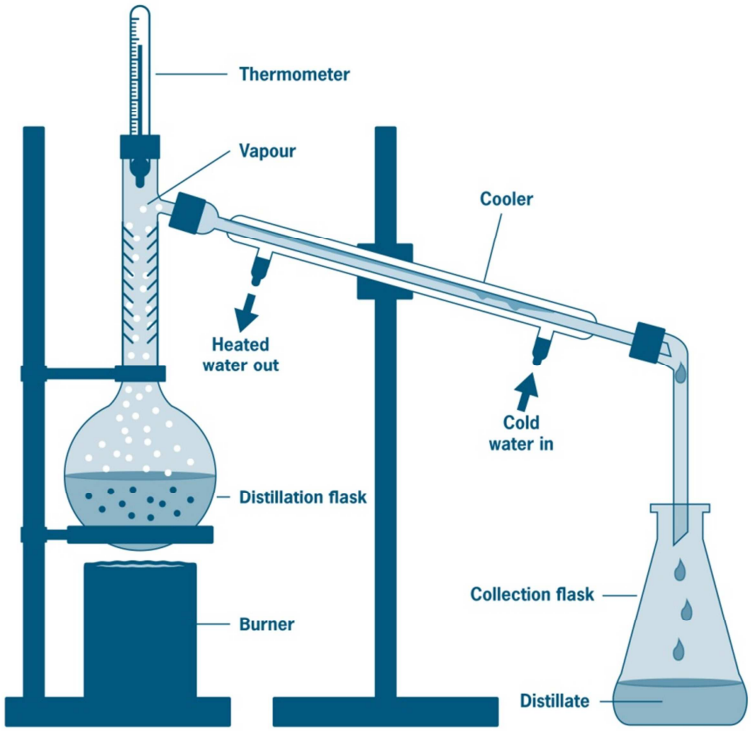


Figure 30 A simple distillation apparatus

We used this in the third year to explain how distillation works: ethanol has the lowest boiling point and therefore evaporates first. The ethanol vapour condenses in the cooler and is collected at the end of this process. Water is left behind. This also relates nicely to something else that you learned in the third year, namely that a mixture of ethanol and water does not have a boiling point, but a ***boiling trajectory***. If a mixture has a boiling trajectory, the temperature of the mixture rises while boiling. This means that the composition of the mixture changes while boiling: The component with the lowest boiling point disappears first, leaving behind the component with the highest boiling point. The ratio between the components in the fluid is therefore continuously changing. That was what we learned from the third year of chemistry. In reality the process is much more complicated.

We are now going to take a much closer look at the phenomena that play a role here. Boiling is in itself a special evaporation process. What exactly happens during boiling? In order to know that, you must first know something about vapour pressure.

Vapour pressure

Imagine a closed system with a liquid, for example water. There is water vapour above the liquid surface. Molecules are constantly escaping from the liquid. We call this the outgoing flow.

At the same time, molecules are returning to the liquid. This is the incoming flow.
At a certain moment, there will be a dynamic equilibrium whereby incoming flow and outgoing flow are equal. The vapour is then saturated. At this point a certain vapour pressure is generated above the liquid.
As the temperature rises, so does the vapour pressure (figure 31).

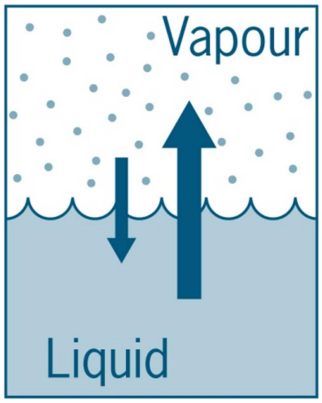


Figure 31 A closed system with vapour and liquid. The outgoing flow depends on temperature and here it is bigger than the incoming flow, therefore the vapour density rises. Vapour equilibrium occurs when the incoming flow becomes bigger with increasing vapour density.

Task 40

The following factors will affect this process of incoming and outgoing flow: temperature, vapour pressure, size of the liquid surface, type of liquid.

a. Which factors does the outgoing flow depend on?
b. Which factors does the incoming flow depend on?
c. If the temperature rises, one of the flows increases: which one?
d. Why does the other flow eventually have to adapt to the first?
e. Why is it that after a rise in temperature, a new equilibrium is created at a higher vapour pressure?
f. Explain why the saturation pressure – or the vapour pressure – is not dependent on the size of the liquid surface but on the type of liquid and the temperature.
g. Explain how the vapour pressure rises more than proportionally to the temperature.
h. Use Binas to look up the temperature at which water has a vapour tension of 1.0 bar
i. Do the same for ethanol.
j. Use Binas to look up what the vapour pressure of water is at 300°C.
k. Why is it dangerous to heat up liquid in a closed tank?
l. Why is it dangerous to let a droplet of water fall on hot oil?

Boiling

During the boiling process vapour bubbles appear in the liquid. Where do these bubbles come from? Why does water have a boiling point of 100°C?
Vapour bubbles are constantly appearing in water. However, they disappear because the vapour pressure is lower than the outside pressure. Look at the drawing below (figure 32). As the temperature increases, so does the vapour pressure. If the vapour pressure is equal to the outside pressure or even greater, the vapour bubble swells. The bubbles then rise to the surface of the liquid.

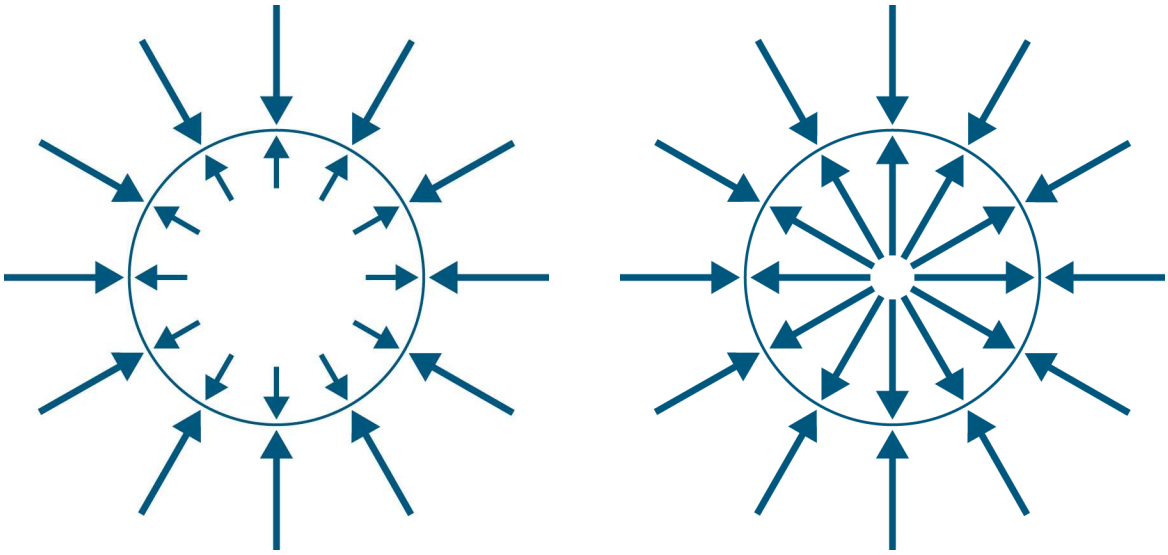


Figure 32 If the vapour pressure is not big enough, a vapour bubble will disappear. If the vapour bubble is big enough, a boiling bubble is created.

Task 41

The vapour pressure of liquids depends on the temperature.

a. Why does water boil at 100°C?
b. Why does water boil at a lower temperature in the mountains?

Distillation

In distillation a ***mixture*** of water and ethanol are brought to the boil.
In the boiling liquid, vapour bubbles appear again because the vapour pressure is equal to the outside pressure. However, this time there is not one but two liquids involved.

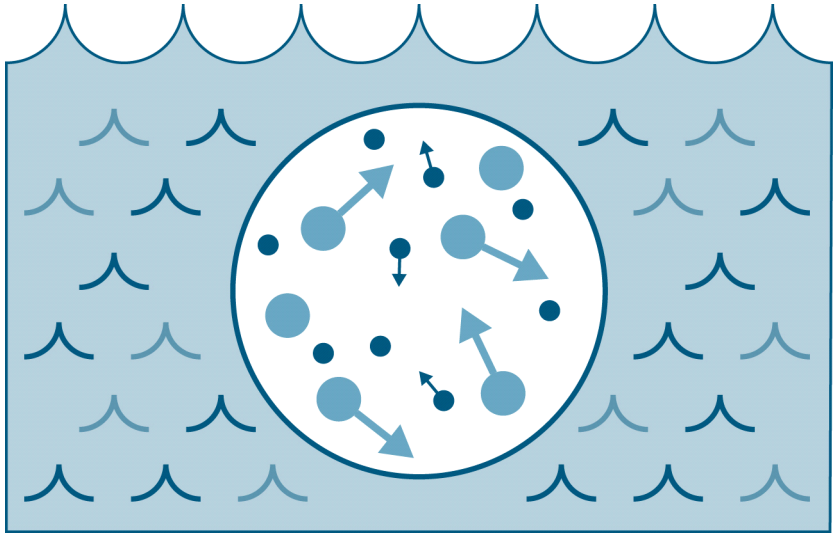


Figure 33 A vapour bubble with ethanol and water molecules.

Within the vapour bubble there are both ethanol and water molecules (Figure 30). The total number of molecules, N in the bubble consists partly of water molecules, N_w and partly of ethanol molecules, N_a.
Both sorts of molecule contribute to the total vapour pressure in the bubble. This is in turn equal to the outside pressure, i.e. 1 atmosphere.
The boiling point of water is 100°C. The boiling point of ethanol is 78.5°C. Using Raoult's law, the boiling point of a mixture can be calculated.

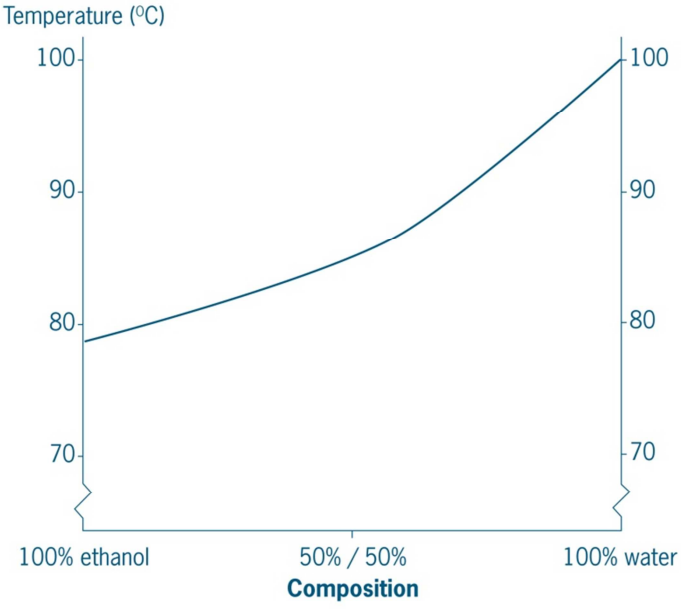


Figure 34 The boiling point of a mixture depends on its composition (theoretical values). [composition]

In above you can see how the boiling point of a water/ethanol mixture depends (theoretically) on the composition. This is the background to the boiling trajectory of the mixture.

If we now look at the composition of the vapour, we see that it is different from that of the liquid. Is that what we expected? Absolutely, because we expected the ethanol to evaporate and it did, but ***water also*** evaporated. In percentage terms the vapour contains less water than the liquid. At every boiling temperature there seems to be a certain ethanol/water ratio necessary for the vapour to exist. These data can be shown in a so-called T,x diagram (see figure 35). You will recognise the liquid line from figure 34. The T refers to the temperature and the x refers to the x-axis, which represents the composition of the mixture (here too the values are theoretical).

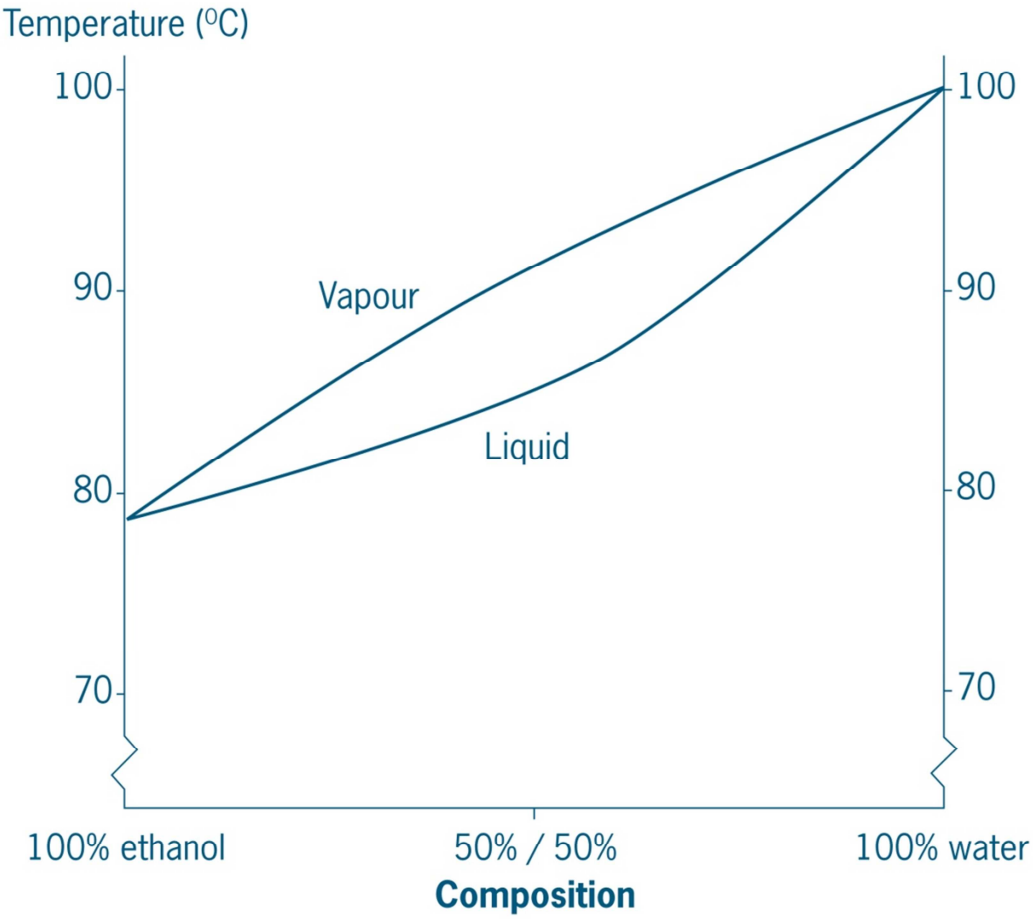


Figure 35 A T,x diagram of a water-ethanol mixture.

How should you interpret such a diagram? Choose a certain water:ethanol ratio, for example that in wine, where there is about 12% volume ethanol. That becomes point A in figure 36. Then you can read the boiling point at point B. Point C gives the ratio of the components in the vapour phase at equilibrium.

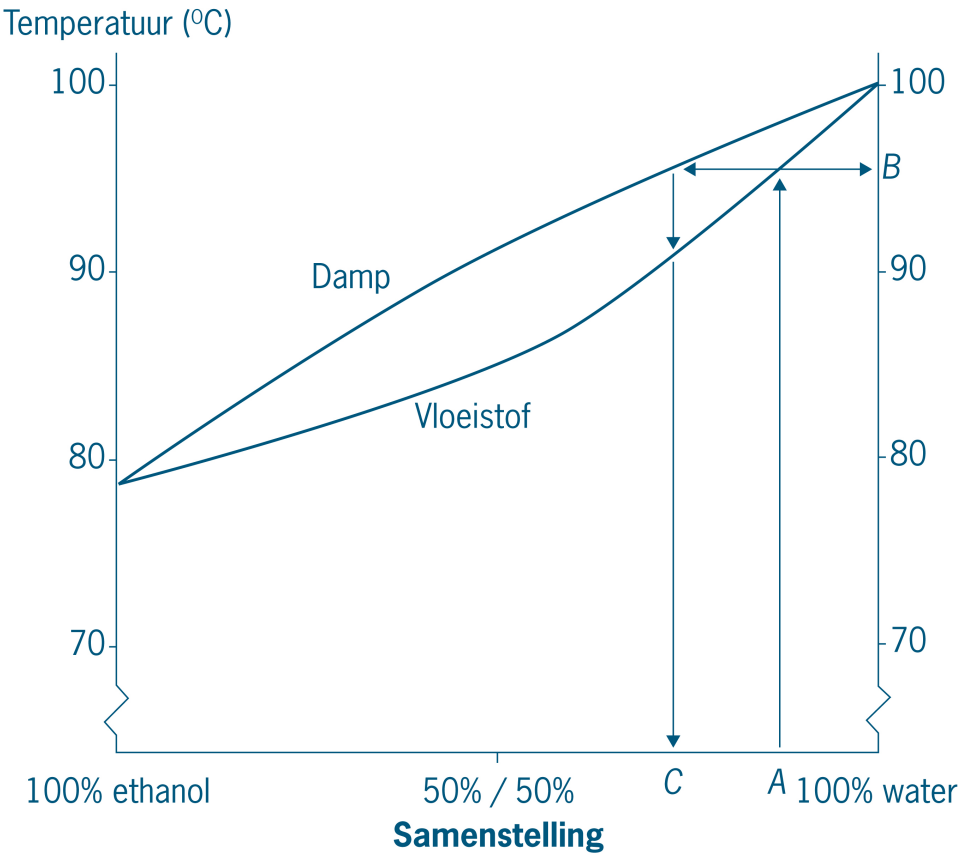


Figure 36 A T,x diagram of a water-ethanol mixture.

And now we can see how distillation works. The vapour has a different composition from the original liquid. According to the diagram we now have 30% ethanol. If you let this vapour condense, you obtain a new liquid mixture that you can distil a second time. This gives an even higher ethanol content. After several repeated distillations you would arrive in the region of 100% ethanol.

Box text Filtration and distillation

In the fictitious experiment we were waiting for the creation of an equilibrium between the liquid and the vapour. In practice that can take a long time, which is why distillation apparatus is usually equipped with devices to intensify the contact between the liquid and the vapour.

In a glass distillation set-up like the ones at school, a so-called *vigreux* condenser is normally attached. This has a massive internal surface, along which the condensed liquid can descend towards the rising vapour.

In a huge industrial distillation column, usually called a *rectification column*, there are horizontal steel plates situated at various intervals to enable the contact between vapour and liquid via a system of pipes and domes. In an ideal scenario, you will achieve a liquid/vapour equilibrium at each platform. Because a rectification column contains a whole series of plates, with an increasingly lower temperature towards the top, more purification takes place at the top of the component with the lowest boiling point. So with one apparatus you can achieve high purity, because it carries out several distillation steps in succession.

Task 42
Search the internet for clear pictures of a vigreux condenser and a plate in a rectification column with domes. In both cases try and find a photo as well as a schematic diagram.

Task 43
What happens to any carbon dioxide present during distillation?

Task 44
a Using a pencil, draw in Figure 33 what happens during a second and third distillation.
b Estimate how many distillation steps are needed – according to this diagram – to reach ethanol levels of 90%.

In the legend to figure 34 you will see the term 'theoretical values' in parenthesis.
This means that there is something about the water/ethanol mixture that does not completely comply with the theory. The molecules of these two substances display so much interaction that the vapour composition is different from that given in the diagrams above for the 'ideal case'.
The real T, x diagram of water/ethanol is shown in figure 37.

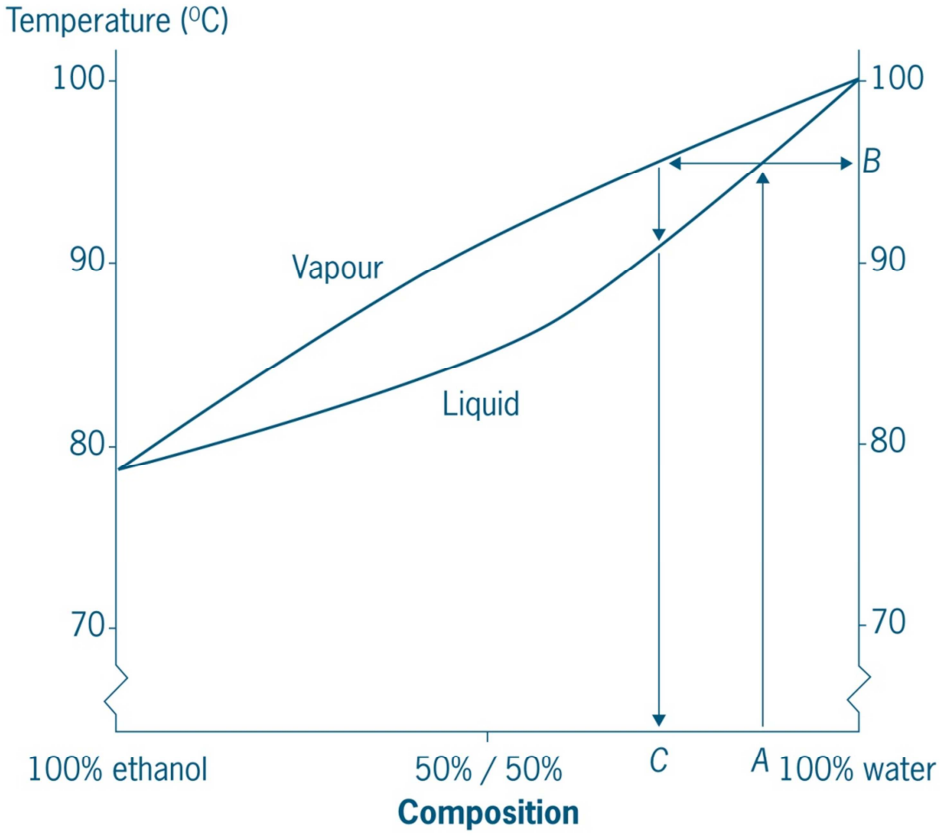


Figure 37 The real T, x diagram for a water/ethanol mixture.

What you will notice immediately is that the left extremities of the curve no longer represent the minimum. There is a new low point at about 78 ºC and 96 (volume)% ethanol. The curves also meet at that low point. If you use this diagram to simulate another series of distillations, you will notice that you get stuck in that low point. You cannot get further than 96% ethanol with distillation.

You can also view it like this: Conduct a distillation with an ethanol/water mixture that contains 96% ethanol. The diagram shows the composition of the vapour. It is ***the same*** as that of the liquid you are dealing with. If you let that vapour condense again in the cooler, you simply get your original liquid again. The distillation process has therefore not increased the ethanol content. If you want to remove the last remaining water to get 100% purity (absolute alcohol), you will need to look for another method.

A liquid that has the same composition as the vapour is called an ***azeotrope***. Like many scientific terms, this word has its origins in Greek. The overall meaning of azeotrope is 'no change on boiling'.

Task 45
What sort of interaction takes place between the molecules of water and ethanol?

Task 46
Using a pencil, draw in figure 37 what happens during a series of 10 distillations, beginning again with a mixture of 12% ethanol.

7. Practical

The theoretical part dealt more with the production of bioethanol. Bioethanol is reasonably easy to make and it would be great if this fuel could be produced from material for which there is no further use, i.e. waste. About 70% of our household waste consists of material that contains cellulose, such as wood and paper. That also goes for tissues: the waste product that will be used in this practical. Household waste products, such as tissues, are normally incinerated, but there is a far more useful purpose for it: biofuel production. That is the aim of this practical. We are going to convert cellulose from tissues into ethanol in a number of steps. In figure 38 you can see how this happens in industry.

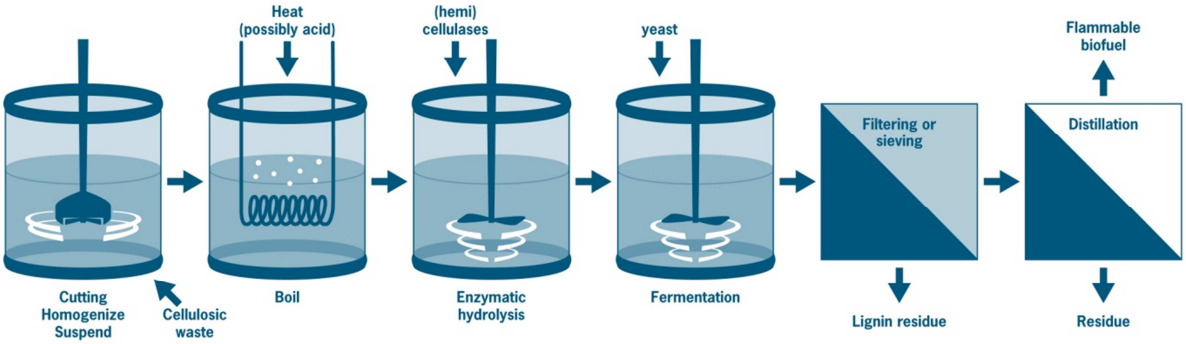


Figure 38: The global process for the industrial production of bioethanol.

During this practical, however, we will only conduct three steps from this diagram, namely:
Step A hydrolysis → Step B fermentation → Step C distillation.

First look up the reactions that take place in steps A and B. Only then begin with the practical.

7.1 Hydrolysis

Equipment:

0.1 M acetate buffer pH 4.5 (made by lecturer or TOA)
Enzyme solution (made by lecturer)
Tissues
Sealable container
Oven or water bath at 50°C
Glucose strips
Weighing scales
Measuring cylinder (250 ml)

- Weigh about 30 g tissues and put them in the container.
- Measure 200 ml buffer in a measuring cylinder and add 5 ml enzyme solution.
- Pour the buffer with enzyme solution over the tissues, so that they are thoroughly soaked.
- Close the container tightly and put it in the oven or water bath at 50°C.

Measuring the glucose content

We use an indicator to measure the glucose content. These are also used by doctors to find out if there is sugar in a patient's urine. Unfortunately the strips are not very accurate for high sugar concentrations, which is why the sample has to be diluted for time periods 24, 48 and 72 hours. The colour change from yellow to green is the most reliable, so we will look for this colour change in a dilution series.

The dilution series
Let the container cool down a little and take out a sample of 5 ml using a pipette. Use this 5 ml to make up four little pots with different dilutions:
1:10 1:50 1:100 1:150
1:10 means 1 part sample (e.g. 1 ml) to 9 parts water. To make a 1:100 dilution, you can take 1 ml of your 1:10 dilution and add another 9 ml water.

Using the glucose strip
Put a glucose strip into each pot. Hold it in the liquid for 2 seconds and then take it out. After 15 seconds look at the colour and make an estimate as to how much sugar the dilution contains, referring to the glucose strip packaging. Write up this value immediately.

Calculate the sugar concentration for each pot. This should be the same for all four.
Which of the four measurements will you use and why?

- After 1 hour measure the glucose content with the glucose strips (see 'Measuring the glucose content') and repeat this after approximately 24 hours, 48 hours and 72 hours.

What is the glucose concentration that you have measured?

Time	glucose content

1 hour	
24 hours	
48 hours	
72 hours	

7.2 Fermentation

Equipment:

The solution from part A
500 ml reaction tank
Water seal with accompanying rubber cap, to attach to the reaction tank
Yeast (10 g)
Magnetic stirrer
Stirrer
Water bath or oven at 33°C
Weighing scales

- Put the suspension from part A, including the remaining tissues, into the reaction tank and add 10 g yeast and a stirrer.
- Close the reaction tank using the water seal.
- Put the tank in an oven or water bath at 33 degrees, so that the stirrer can be set in motion by the magnetic stirrer.
- Programme the stirrer at 500 revolutions/min and note the time.
- During the next half hour, check the water seal regularly.

Note your observations and give an explanation.

- Leave the fermentation set-up to stand for about one day. There's no harm in leaving it longer.
- Measure the glucose content again with a glucose strip and note the glucose concentration.
- Smell the solution.

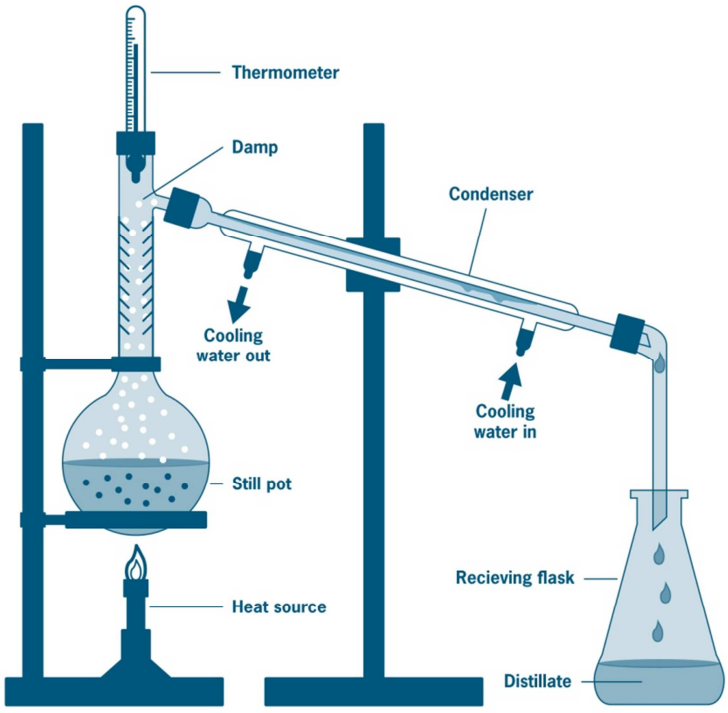
Explain your observations.

7.3 Distillation

Equipment

Solution from part B
Set-up for distillation with vigreux column with a 250 ml flask (set up by lecturer)
Various 10 ml cylinders to collect the distillate.
Heat source (preferably a heating muff)
Cooking stones
Small dish
Matches
Safety glasses
Spoon

- Below is a diagram of a distillation set-up.



- The set-up for distillation will be arranged by the lecturer.
- Make sure that the flask is clean and dry
- Make sure that the thermometer reservoir is at the same height as the point where the vapour enters the cooler.
- Using the measuring cylinder, measure how much solution you have left from part B and put this in the distillation flask. Add a couple of cooking stones to it.
- Put on your safety glasses and start heating it up. Put the heat at maximum until the liquid begins to boil. Then put the heating element at half capacity and keep an eye on the thermometer. You will see that the temperature

suddenly starts to rise quickly. At 80 degrees the temperature remains stable for a short while. You can see the first droplets dripping into the collection flask. Change the collection flask as soon as the temperature reaches 93 degrees. You will then have collected about 5 ml liquid. Keep on distilling and collect the distillate in fractions of 5 ml each time. Stop when you have four fractions (including the first).

- You now have four fractions with different ethanol percentages.

Which fraction do you think has the highest ethanol percentage?

- We are now going to determine the combustibility of the fractions. Put on your safety glasses! Put half of fraction 1 in a small dish. Try to set fire to the liquid using a match.

Does this fraction burn?
You will see that not all of the liquid in the dish burns. Why not?

- Repeat the previous step for the other fractions.
- Not all fractions will burn. Use the last fraction that does not burn in the following step:
- *Warning: do not carry out this step with a fraction that does burn well!*
  Pour a little liquid onto a spoon, hold it carefully for a few seconds over a Bunsen burner and try to light it again. Be very careful: an ethanol flame is not always very visible. Test whether the liquid on the spoon is already burning by holding a fresh match above the spoon. The match will ignite if the ethanol is burning.

If you start with the assumption that a solution containing an ethanol content of 40% and upwards will burn, which fractions contain more than 40% ethanol?

How efficient do you think this experiment is?

8. URL list

URL1 Digital background module: Create an account and click on B-basic.
http://pkedu.fbt.wur.nl/moodle/

URL2 Cornell ecologist's study finds that producing ethanol and biodiesel from corn and other crops is not worth the energy
http://www.news.cornell.edu/stories/july05/ethanol.too costly.ssl.html

URL3 Bioenergy quiz ANW-active
http://www.vijfvannijgh.nl/anwactief/jun2007/vraag1.php

URL4 Unlocking lignocellulose
http://www.senternovem.nl/EET/projecten/Ontsluiting_van_lignocellulose_met_biologische_zuurrecycling.asp

URL5 Pre-treatment of Lignocellulosic Wastes
http://www.mdpi.com/1422-0067/9/9/1621/pdf

URL6 Glycolysis:
http://instruct1.cit.cornell.edu/Courses/biomi290/MOVIES/GLYCOLYSIS.HTML