Aalto University
School of Chemical Technology

Department of Bioproducts and Biosystems

# CHEM-E3130 Biolab II Instructions

Autumn 2020

## Contents

# Safety instructions

The emergency telephone number is calling 112.

Always clean up after yourself (bench and used equipment).

It is obligatory to use a buttoned laboratory coat and laboratory goggles in the laboratory. Use appropriate protective equipment when handling hazardous chemicals (eg. acids and bases).

Outdoor clothes are not allowed in the laboratory. Jackets are left in the lockers or places shown by the teacher.

Eating and drinking in the laboratory is not allowed.

The fermentation laboratory must be kept tidy and all items must be returned to their places. Table-surfaces are kept aseptic by wiping them with 70% ethanol. Ethanol is kept in orange-capped flasks.

Wastes containing genetically modified microbes are destroyed by autoclaving. With other microbial waste follow the instructions given by the assistants.

Solvents, acids and bases are collected in their own waste flasks.

Needles are collected in their own waste buckets (without paper or plastic). Container for sharp objects can be found in the inoculation room.

Broken glass has its own waste containers. Separate containers for laboratory glass and other glass waste can be found in microbe lab (D436a)

Bioreactors are transferred only with trolleys. Carrying bioreactors is strictly prohibited. Autoclaves, centrifuges or other equipment should not be used without guidance. In all cases of doubt, ask your supervisor.

# Teachers in the course

Responsible teacher
Tero Eerikäinen
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Methods lecture
Tero Eerikäinen

Laboratory teaching
Vijaya Chandgude
Hannamari Haikonen
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Tuukka Levä
Henni Tuomala

# General things of the course

## Content of the course

The objective of the course is to give an overall picture of fermentation in laboratory and pilot scale. The course has two laboratory works

1) Laboratory-scale fermentation of a recombinant protein, protein purification and protein analysis
2) Pilot scale fermentation of a sugar alcohol and downstream processing

The students will learn how to use different laboratory equipment for fermentation, downstream processing and analysis.

## Completing the course

The course includes two different laboratory works, writing laboratory notebook, final reports and entry exam. The laboratory works are also presented in the final seminar presentation.

## Timetable

Timetable is published in MyCourses CHEM-E3130 Biolab II course page
Entry exam is an obligatory part before the laboratory works.

The reports are handed to the responsible teacher. The first version of the report has to be handed as shown in timetable. The final report version has to be returned in two weeks after receiving the report feedback.

## Laboratory works

The course consists of two laboratory works. Both last one week. Works are done in groups of 4-6 students. The groups are formed before the laboratory work. Before each laboratory working day the student will study the instructions for the following day and make all necessary calculations in advance. This applies to the preparation of reagents as well as dilutions of samples. Before each working day, students will ensure that they are familiar with the day's work. A laboratory notebook will be held of all performed laboratory work according to Appendix 1. Each group is responsible for one laboratory notebook. The laboratory notebooks are divided for the groups on the first working day. A final work report of the laboratory works is written as groups (see instructions in Appendix 2). The laboratory works are presented in a seminar as a 20-30 min presentation.

## Grading

The course is graded 1 – 5. There are 100 points divided in the course in the following areas

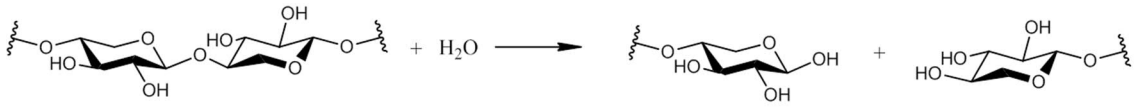
- Activity and attendance in the laboratory 30
- Laboratory work diary 15
- Reports 30
- Seminar presentation 10
- Entry exam 15

Also, the final report is graded on the scale 1 – 5. The grade is given for the first version, but corrections must be done in order to get the report approved. Activity and group dynamics is also measured by group feedback, where group members are confidentially asked to assess their own group member's activity and the ability to cooperate with other members of the group. *If the final report is returned late, 10 points are being reduced! The credits for the course are given only when the final report has been approved and the laboratory notebook has been handed in. In addition, the course feedback found must be completed before passing the course.*

# Theory behind the laboratory work 1

## Introduction

Xylanases are enzymes that break down xylan, which is a component of hemicellulose. In industry xylanases are used in the paper industry for pulp bleaching and in the feed industry to improve silage features. The reaction catalyzed by the xylanase is presented in Figure 1.




1,4-β-xylan xylooligosaccharides

**Figure 1**. The reaction catalyzed by the xylanase.

The objective of the laboratory work 1 is to produce as much pure xylanase as possible. The work is carried out with an *Escherichia coli* XL1-Blue strain, which contains the XYN10A xylanase gene from the bacterium *Thermopolyspora flexuosa* in the pKKtac vector. Anbarasan *et al.* (2010) describe this enzyme (XYN10A-6_His) in their article. Xylanase is produced by cultivating the recombinant strain of *E. coli* in a bioreactor. The xylanase production will be monitored during the cultivation and after the fermentation xylanase is purified from the cultivation broth. The purity, quantity and activity of the product is analyzed at different stages of downstream processing.

## Xylanase production in the bioreactor

The production in industrial bioprocesses is done in a bioreactor. Laboratory-scale experiments are often carried out in flasks, although the adjustment of the conditions and the monitoring of the process are considerably more limited than in the bioreactor. In practice, the bioreactor is a stirred tank, which is instrumented for bioprocess needs and which can withstand sterilization. Typically, the bioreactor has temperature measurement and control, pH measurement and control, aeration connected with the dissolved oxygen measurement and control. Other possible measurements from the bioreactor include the concentration of dissolved carbon dioxide, the composition of the incoming and outgoing gas and the number and viability of biomass. This laboratory work is performed with a laboratory-scale aerated bioreactor.

## Purification of xylanase from the culture broth

A His-tag is a chain with six or more histidine molecules, which is cloned into the N- or C-terminus of the purified protein. His-tag is not expected to significantly affect the properties of the protein. However, the protein's ability to oligomerize might be affected by the His-tag. For the enzyme used in this work the His-tag is has been proven to increase the stability of the enzyme (Anbarasan *et al.*, 2010).

His-tagged proteins can be purified by chromatography with a nickel column. The purification is based on the attachment of the histidine molecules of the protein to the nickel ions, which in turn are immobilized to the carrier material in the column. The proteins that did not attach to the column are washed out of the column with buffer. Imidazole has a higher affinity on the nickel ions than histidine. When the column is eluted with the buffer containing a high concentration of imidazole, the attached His-tag protein detaches from the column and can be collected.

Prior to the chromatographic purification the sample is concentrated by ammonium sulfate precipitation. The ammonium sulfate precipitation is based on the hydrogen bonds that are formed between the soluble proteins and water, which is used as a solvent. The salts compete for the water molecules with the proteins. The more saturated the ammonium sulfate solution, the less water molecules will remain to keep the proteins in water soluble form. As a result, the proteins aggregate with hydrophobic bonds with each other and precipitate. The precipitate can be separated from the solution by centrifugation. Different proteins precipitate in different ammonium sulfate concentrations and therefore the method can be used as a crude fractionation of the protein sample.

## Analysis

### Protein concentration

In this work the Bradford method is used for the determination of the protein concentration. Coomassie dye is red when not bound and its absorption maximum is at the wavelength of 470 nm. Coomassie reacts with arginine, lysine and histidine present in proteins and as a result turns blue with the absorption maximum at the wavelength of 595 nm. The protein concentration of the sample is determined by comparing the absorbance of the samples with a standard curve. The standard curve is determined by measuring absorbance from solutions containing known concentrations of bovine serum albumin (BSA).

### SDS-PAGE

SDS-PAGE stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis. This method is used to analyze the purity of the protein sample and evaluate the molecular weights of the proteins. Proteins bind the negatively charged sodium dodecyl sulfate (SDS) with a constant amount per protein mass. Therefore, the electric charge of the SDS-protein complex per mass is constant. The movement of the SDS-protein complex in the electric field is then proportional to the molecular weight of the protein. The polyacrylamide gel is composed of various sized pores. Small proteins in the gel migrate faster than large ones and this phenomenon is used to separate the proteins of different sizes from each other.

Before the electrophoresis the proteins in the sample are denatured and internal sulfur bridges are broken by reduction with mercaptoethanol. Also proteins composed of subunits are degraded into separate peptides. The separation of the proteins happens between two glass sheets in a casted polyacrylamide gel. In this work pre-casted SDS-PAGE gels are used. Samples containing sample buffer are pipetted into the wells and the gel is placed in an electric field. In Figure 2 the running equipment is shown.

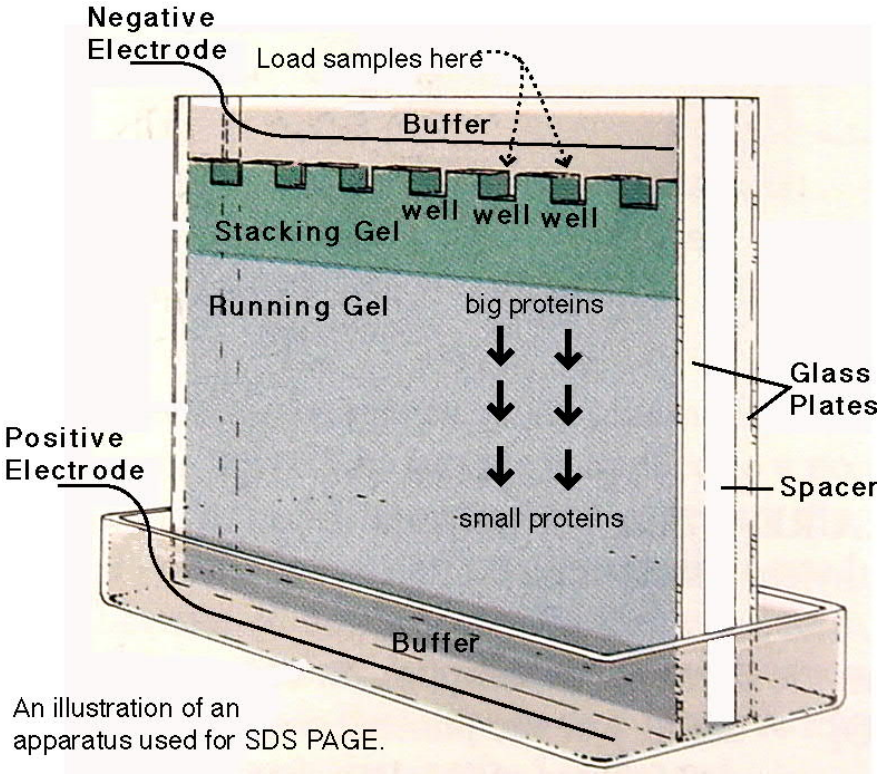



**Figure 2**. The equipment used to run a SDS-PAGE analysis.

After running the gel, it is stained with Coomassie brilliant blue dye. Finally, the color is removed from the gel. The bound proteins keep the color and thus proteins can be displayed in different zones. Figure 3 shows an example of a SDS-PAGE gel.

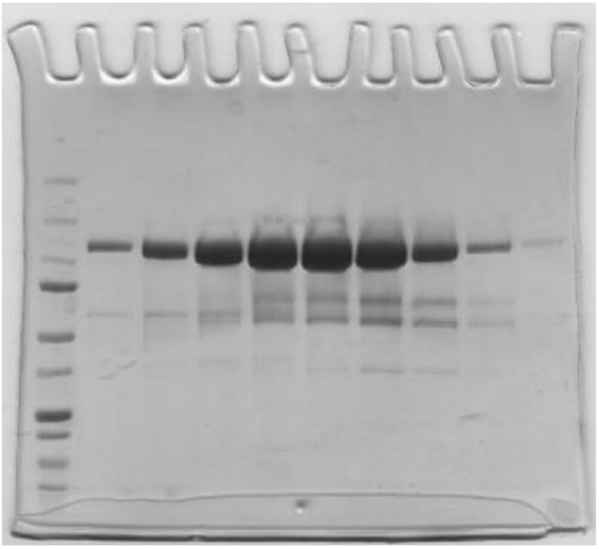


**Figure 3**. An example of a SDS-PAGE gel.

## Xylanase activity

The enzyme activity is the enzyme's ability to accelerate the reaction. This is based on the fact that the enzyme reduces the activation energy of the reaction. In practice, many chemical reactions take place extremely slowly without enzymes, so measuring the activity of a reaction can be assumed to be entirely due to the activity of the enzymes.

For measuring the enzyme activity there must be a way to keep track of the amounts of product or substrate as a function of time. Enzyme activity can be determined either kinetically or with the so called point measurement. In the kinetic measurement the reaction rate is measured continuously from the start of the enzymatic reaction while in the point measurement change in concentration is measured once after an accurately known period of time. In both measurements the aim is to determine the initial rate of the enzyme reaction before the decrease of the substrate concentration or some other factor that is slowing down the reaction. The kinetic measurement is more reliable because it confirms the linearity - or nonlinearity - of the reaction in the function of time.

The xylanase activity is measured in this work by point measurement. This measurement is based on the fact that the enzyme and substrate are incubated under specific conditions for a specific time, after which the reaction is stopped. The concentration of the product or substrate is measured after stopping the reaction. The reaction rate is presumed to be the product formed in the used incubation time. For this measurement type the reaction must be known well, in order to know the reaction to be linear in these conditions throughout the duration of the reaction.

Xylan is the substrate in the xylanase activity assay. The produced xylanase enzyme releases xylo-oligosaccharides from xylan. These are short xylan chains, which have a xylose unit at the end. The reaction catalyzed by the xylanase is presented in Figure 1. Xylose is a reducing sugar and the concentration can be determined by the DNS method.

3,5-dinitrosalicylic acid (DNS) is an aromatic compound, which reacts with reducing sugars and with other reducing compounds. In the reaction 3-amino-5-nitrosalicylic acid is formed, which strongly absorbs light at the wavelength of 540 nm. The reaction between the xylose oligosaccharide and DNS is shown in Figure 4. The DNS method gives the amount of all reducing compounds in the sample and therefore the background needs to be subtracted from the result of the reducing sugars produced by the enzyme.

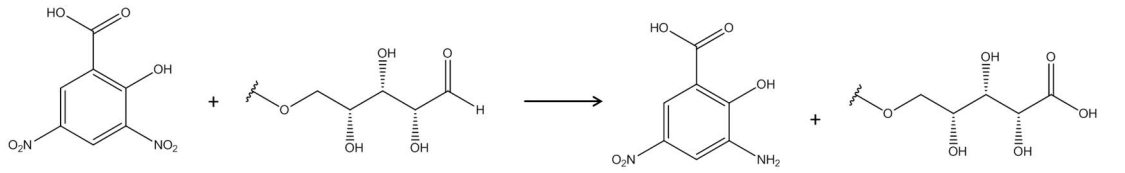



**Figure 4**. The reaction between 3,5-dinitrosalicylic acid and the xylo-oligosaccharide.

# Laboratory work 1

It is recommended that you have a memory stick in the laboratory work in order to collect work data and images used in your laboratory notebook and work report.

## 1. work day

### Reagents

Growth medium: Lysogeny broth (LB, Pronadisa)

Selection antibiotic: Ampicillin, stock solution of 100 mg/ml

### Preculture

A preculture of 50 ml in a 250 ml Erlenmeyer flask is started at 12.00. The strain of *E. coli* XL1Blue is inoculated aseptically in a laminar flow cabinet with an inoculum stick from a plate into liquid LB-medium supplemented with 125 µg/ml ampicillin. The preculture is incubated in an incubator with 37 °C and 250 rpm shaking. Two parallel precultures are prepared, from which one will be selected as the actual inoculum for the bioreactor cultivation.

### Preparing the bioreactor

The bioreactor is prepared for the 1,5 L cultivation. Control of the temperature, dissolved oxygen and pH and the measurement of biomass concentration are needed for the cultivation. Figure 5 shows an example for the assignment of the vessel equipment to the lid ports. The pH-probe is calibrated before autoclaving with standard solutions (pH 7 and pH 4,01). A sampling system is prepared on the lid of the bioreactor. The culture medium used is LB medium. The inoculum volume of 50 ml will be included in the final cultivation volume, which needs to be taken into account when preparing the medium. 2 – 3 drops of anti-foaming agent (Struktol) are added into the medium before sterilization. The reactor is autoclaved with the medium inside in 121 °C for 40 minutes. After autoclaving, the reactor temperature is set to 37 ° C and the stirring is switched on. When the reactor has cooled to 37 °C all connections for probes and aeration are attached. Next, the dissolved oxygen probe is calibrated (zero calibration with nitrogen ($N_2$) and slope calibration with air with the growth conditions of 0,75 lpm and the maximum agitation of 800 rpm). For the night, the stirring is lowered to 100 rpm and the temperature to 15 °C.

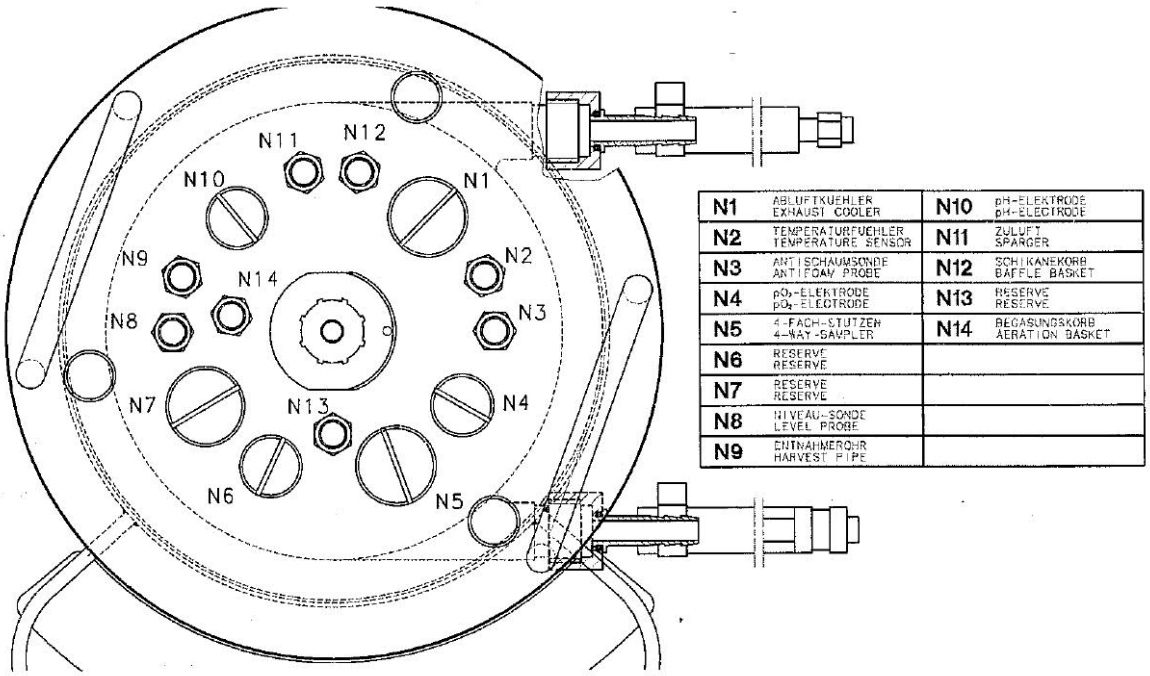



**Figure 5**. Example for assignment of vessel equipment to lid ports.

For the dry weight measurement 8 dry weight tubes are prepared. Clean pre-numbered tubes are placed in an 80 °C oven overnight to dry.

## 2. work day

### Reagents

Induction agent: IPTG (isopropyl-β-D-thiogalactopyranoside), stock solution of 500 mM

Salt for used in protein precipitation: Ammoniumsulfate $(NH_4)_2SO_4$

### Cultivation parameters

| | |
|---|---|
| Temperature | + 37 °C |
| pH | no pH control |
| Dissolved oxygen ($pO_2$) | Setpoint 20 % |
| Aeration | 0,5 vvm = 0,75 lpm |
| Stirring | Cascade control with the dissolved oxygen, min. 200 rpm (10 % of the maximum of the control unit), max. 800 rpm (40 % of the maximum of the control unit) |

### Production of xylanase in the bioreactor

The stirring speed is increased to 400 rpm and the temperature to the cultivation temperature of 37 °C and 125 µg/ml ampicillin is added aseptically through the septum on the lid of the bioreactor.

The bioreactor is inoculated in the morning at 8:00 with the overnight grown inoculum of 50 ml. The inoculation is done with a sterile syringe and a needle through the septum on the lid. Before the inoculation, check that all the cultivation parameters are set correctly. The better grown inoculum flask is selected for the main cultivation.

Samples (5 ml + 10 ml) are taken every hour. The samples are named with running numbers e.g. K0, K1, K2 etc. 10 ml samples are collected on ice to wait for the dry weight analysis. From the 5 ml samples 1,5 ml samples are pipetted into two parallel Eppendorf tubes, which are centrifuged in a centrifuge (5 min, 10 000 rpm). After centrifugation the supernatant is transferred with a pipette into two clean Eppendorf tubes, which are placed in the refrigerator for later analysis. The zero sample K0 is taken just after the inoculation.

The optical density of each sample (OD) is measured by spectrophotometer. The sample or a suitable aqueous dilution is measured in the absorbance ($\lambda$ = 600 nm). Water will work as a blank when resetting. Acceptable absorbance readings are between 0,050 and 0,500. The measurement is made in duplicates.

The protein production inducer IPTG is added when the OD of the cultivation is 1,0. The right moment is estimated with the help of OD measurements (and the biomass sensor if available). IPTG is added to a 1 mM concentration in the bioreactor through the septum membrane on the lid with a sterile syringe. The cultivation is continued until the stationary phase. This can be seen when the concentration of dissolved oxygen starts to rise.

The finishing of the fermentation is done in the following order

- Turn the dissolved oxygen control off, but leave the stirring still on.
- Switch off the measurement of the sensors, but do not removed them from the reactor
- Switch off the aeration, stirring and temperature controls.
- Switch the bioreactor off.
- Close the water and air valves.
- Remove the sampling and aeration tubings.
- The reactor is disassembled.
- The culture medium is collected. The cells are separated with centrifugation (15 min, 5 000 rpm, 4 ° C, GS3 rotor) from the culture broth in which the xylanase is secreted.
- All parts of the reactor and the tubes are washed.

## Dry weight measurement

For the determination of dry weight tubes are predried in an oven overnight and weighed the next day after cooling down in a desiccator. Four cultivation samples from the exponential growth are chosen for this analysis. The selected sample is pipetted into two parallel tubes with 5 ml each and the cells are then centrifugated to the bottom (Rotor SS-34, 8 000 rpm, 5 min). The supernatant is carefully removed, so that all cells stay in the tube. The cell pellet is washed twice with 3 ml Milli-Q water. After each wash the cells are centrifuged to the bottom of the tube. The tubes are dried overnight in an oven with 80 °C. The tubes are then cooled in a desiccator and weighed. The difference between the tubes will give the amount of cells and this can be used to calculate the cell density in the bioreactor.

### Protein purification

The xylanase is purified from 300 ml of the supernatant (centrifugated culture medium, from which the rest is put in the refrigerator). A 10 ml sample of the supernatant is taken (P1). Samples are taken at different phases of the purification and they are summarized in Appendix 3.

The first purification step is precipitation with ammonium sulfate. The concentration of ammonium sulfate in solution is raised to 70% (degree of saturation) by adding crystalline ammonium sulfate slowly to the stirred supernatant over 30 minutes in a cold room.

The amount of the ammonium sulfate needed in 0 – 4 °C for can be calculated using the following formula:

$$x = \frac{50{,}6 \times (S_2 - S_1)}{1 - 0{,}3 \times S_2}$$

Where x = needed amount of ammonium sulfate (g) per 100 ml of solution

$S_1$ = degree of saturation of ammonium sulfate in the beginning (here 0)

$S_2$ = required degree of saturation of ammonium sulfate in the end (here 0,7)

## 3. work day

### Reagents

Binding buffer: 20 mM imidazole, 500 mM NaCl, 20 mM sodium phosphate, pH 7,4

Elution buffer: 500 mM imidazole, 500 mM NaCl, 20 mM sodium phosphate, pH 7,4.

His-Trap HP -column (GE Healthcare), V = 1 mL

### Dry weight measurement

The dry weight tubes from the previous day are put from the oven into a desiccator to cool for at least half an hour. The tubes are then weighed, and the weights of empty tubes are used to calculate the dry weight of the samples.

### Protein purification

The precipitated protein formed overnight in the cold room is separated by centrifugation (20 min, 7 000 rpm, 4 ° C, GS3 rotor). A 10 ml sample is taken from the supernatant (P2) and the rest is thrown away. The precipitate is dissolved in 20 ml of binding buffer. The sample is centrifuged (20 min, 13 000 rpm, 4 ° C, SS-34 rotor) and the supernatant is recovered. The supernatant is filtered through a filter of 0,8/0,2 µm pore size and a 1 ml sample is taken (P3).

The purification is carried out with the Äkta Purifier (GE Healthcare) equipment with a His-Trap HP column. The flow rate is 1 ml/min. The column is equilibrated with binding buffer and then the filtered sample is pumped onto the column. The column is then washed with binding buffer until the non-binded sample is eluted. The flow-through and the washing solution are combined

and recovered (P4). The proteins are eluted with a linear imidazole gradient. The concentration of the elution buffer in the flow is increased to 100% over 20 min.

During the elution fractions of 1 ml of the outcoming flow are collected with a fraction collector. The elution of the proteins from the column is detected with an absorbance meter in the outlet of the column at the wavelength of 280 nm. The fractions containing eluted protein are collected and they are numbered F1, F2, etc. The fractions are collected to the cold room waiting for analysis the next day.

## 4. work day

### Reagents

Bradford reagent (5 x concentrate) (Bio-Rad)
Protein standard: BSA (Bovine serum albumin)
4 – 15 % polyacrylamide gel
Running buffer: 25 mM Tris; 0,192 M glycine; 0,1 % SDS; pH 8,3 (Already prepared)
3x sample buffer: 4 % SDS; 1 % mercaptoethanol; 0,002 % bromophenol blue; 40 % glycerol solution in 0,25 M Tris-HCl buffer; pH 6,8
Gel staining solution: 0,25 % Coomassie brilliant blue in R-250 liquid with, 50 % methanol and 10 % acetic acid
Destaining solution: 5 % ethanol and 7,5 % acetic acid
Molecular weight standard: Precision Plus Protein™ Standards, Dual Color, (Bio-Rad 161-0374)

### Determination of protein concentration

The Bradford protein assay is used to measure the concentration of total protein in a sample. The principle of this assay is that the binding of protein molecules to Coomassie dye under acidic conditions results in a color change from brown to blue. This method actually measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to formation of the protein-dye complex.

Microassay Protocol

1. First, a BSA stock solution (100 μg/ml) is prepared with Milli-Q water in a volumetric flask for the protein standards. Seven different dilutions from the BSA stock solution are prepared in glass test tubes of concentrations 1 – 20 μg/ml.

2. The microassay protocol can be performed in two different formats, a 2 ml cuvette assay and a 300 μl microplate assay. We use the microplate assay. The linear range of these assays for BSA is 1.25–10 μg/ml.

3. Remove the 1x dye reagent from the 4°C storage and let it warm to ambient temperature. Invert the 1x dye reagent a few times before use.

4. Depending on the type of standard used, calculate the estimated dilutions needed for each standard, and test the dilutions by running the assay only for the standards if needed. For the diluent, use the same buffer as in the samples. Protein solutions are normally assayed in triplicate.

5.

- Pipet each standard and unknown sample solution into microplate wells.
- Add 1x dye reagent to each tube or cuvette and vortex: for microplates, mix the samples using a microplate mixer.
- Alternatively, use a multichannel pipet to dispense the 1x dye reagent. Depress and release the plunger repeatedly to mix the sample and reagent in the wells. Replace with clean tips and add reagent to the next set of wells.

Assay volume: 300 µL → volume of standard solution = 150 µL; volume of 1x dye reagent = 150 µL.

6. Incubate at room temperature for at least 5 min. Samples should not be incubated longer than 1 hr at room temperature.

7. Set the spectrophotometer to 595 nm. Measure the absorbance of the standards, blanks, and unknown samples.

Protein concentration is determined from all the purification step samples (P1 – P4) and selected purification fractions (F1, F2 etc.). Sample dilutions of 1:10 and 1:50 are used first (and later diluted more if needed).

The standard curve is obtained by representing the absorbances of the standards as a function of BSA concentration. Protein concentrations in the samples can then be calculated with the standard curve equation, which shows the correlation between absorbance and protein concentration.

The absorbances of the samples have to be within the standard curve region and between 0.15 – 1.5 to be reliable. If the used dilutions do not give acceptable results for all samples, the estimated results are used for making suitable dilutions for repeating the protein analysis. In addition, if there is a large difference between the sample triplicates, the determination must be carried out again.

## SDS-PAGE

Gel electrophoresis is performed on samples P1, P3, P4, and five fractions obtained from the chromatographic purification step. 3 µl of 4x sample buffer is added to 9 µl of sample and mixed. Samples are kept lids closed in a heat block for 5 min at temperature of 95 °C (in the fume hood). After heating, the samples are centrifuged to the bottom of the tubes and they are ready to be pipetted on the gel. If samples are high in protein, less sample can be pipetted on the gel. From samples P1 and P3 2,5 – 5 µg protein should be aimed to get into the well. An appropriate amount of the purified protein on the gel is 0,25 – 2,5 µg. However, the same volumetric amount of all fractions is pipetted on the gel.

When ready to run a SDS-PAGE gel, the plastic tab at the bottom of is removed from the gel (in ready-to-use gels). The gel storage solution contains small amounts of toxic sodium azide, so it is necessary to use protective gloves. Two gels can be put into the chamber, but if there is only one gel, a buffer dam is used instead of the second gel. The shorter of the two glass plates of the gel is put towards the inside of the gel chamber. The gel and the buffer dam are placed

vertically, and the gel chamber is locked. A sealed intermediate space is formed. The gel chamber is transferred into the running chamber. The SDS equipment is shown in Figure 6.

**Figure 6.** Electrophoresis equipment.

When the gel running equipment is assembled, the running buffer is poured into the outer chamber's buffer reservoir so that about 2 cm the lower edge of the gel is covered. The space between the two gels is completely filled with running buffer and the comb covering the wells is carefully removed from the gel.

Next, 2 µl of the molecular weight standard and previously given amounts of denatured protein samples are pipetted into to the wells. The sample buffer contains glycerol, which causes the sample to settle to the bottom of the well. The equipment's lid is put in place and the electrode cables are connected to the power source. It is important to check the correct connection of the cables. The samples are run with 160 V voltage until the blue sample buffer front reaches the bottom level of the gel (approx. 45 – 60 min).

After the run the glass plates on the gel are separated from each other and the gel is transferred to the staining solution. The gel is stained for 60 minutes on a shaking platform in a fume hood. Then the staining solution is poured out and replaced with the destaining solution. The destaining solution can be changed after 10 – 15 minutes. The gel is left in the destaining solution overnight in the fume hood. As a result of the treatment the color that does not stick to protein comes off and the stained proteins can be detected as blue bands on the gel. Sample bands are compared with the standard bands (Figures 3 and 7) and the size of the examined protein can be defined. A picture of the gel is taken.


**Figure 7.** Molecular weight standard sizes and corresponding bands on the gel.

The xylanase assay is done on day 4 (Thu) or day 5 (Fri), depending on how quickly the Bradford assay and the gel are done.

## Reagents

Citrate-phosphate buffer (0,05 M, pH 4.8)

Substrate solution (1,0 g birch xylan in 100 ml citrate-phosphate buffer (pH 4.8)

DNS reagent

Xylose (for standards → calibration curve)

## Xylanase activity assay

Preparation and measurement of standards: In room temperature 180 µl of substrate solution and 20 µl of standard dilution is pipetted into each microplate well. In addition, the reagent blank is prepared with 180 µl of substrate solution and 20 µl of buffer. The solutions are mixed by vortexing and all tubes are boiled for exactly 5 minutes. The solutions are analyzed by DNS assay together with the samples. Finally, the standard curve is plotted with the absorbance (λ = 540) as a function of xylose concentration, as described in the assay protocol below.

Preparation and measurement of samples: 180 µl of substrate solution is pipetted into each microplate well. The plate is heated at 70 °C for 5 min. Add 20 µl enzyme sample of enzyme sample and mixed by pipetting up and down. The reaction is continued in a water bath of 70 °C for exactly 5 minutes. The reaction is stopped 60 µl into each PCR plate well containing the DNS reagent (step 2. in the DNS assay protocol). Next, the samples are analyzed following the DNS assay protocol below.

DNS assay protocol:

Preparations:

Weigh in 2 g Dinitrosalicylic acid (DNS), 0.4 g Phenol, 0.1 g sodium sulfite ($Na_2SO_3$) and 40 g Potassium sodium tartrate tetrahydrate. Dissolve in 100 ml 2% NaOH and fill up with H2O to 200 ml. Store at room temperature in a bottle covered with aluminum foil (reagent is toxic!!) *(Technicians prepare this!)*

$V_{assay}$= 180 µl

1. Add 120 µl DNS reagent to each microplate well.

2. Pipette 60 µl of reaction sample or standard into 96 well PCR plate (dilute strong samples with 50 mM NaAc buffer pH 4.8 if necessary, i.e. if they are outside the st curve).

3. Cover plate with sealing tape (best- Sealing Tape Aluminium non Steril; Nunc)

4. Incubate for 5 min at 95°C in PCR machine.

5. Let plate cool to room temperature (5 min on ice).

6. Pipette 160 µl H2O into a 96 well microtiter plate, add 36 µl from the DNS reaction of the PCR plate. Mix thoroughly with pipette.

7. Measure absorbance at 540 nm (“kinetic cycle”, 10 reads) with a plate reader.

8. Collect DNS waste and dispose it in the appropriate container (Cupboard in fume hood, Gene lab), dispose used PCR and microtiter plate as well as tips in separate waste container (DNS solid waste - Chemical room).

Calibration curve:

1 – 20 mg/ml glucose in 50 mM NaAc or citrate-phosphate buffer (pH 4.8). Pipette 120 µl DNS reagent to each PCR plate well. Pipette 20 µl standard into 96 well PCR plate, add 40 µl of buffer (50 mM; pH 4.8). Proceed as described above.

Reaction blank: 60 µl 50 mM NaAc buffer (pH 4.8)

Enzyme blank (when measuring sugar release from enzymatic hydrolysis): 20 µl culture media (for cultivation samples) plus 40 µl NaAc buffer.

Example calibration curve:

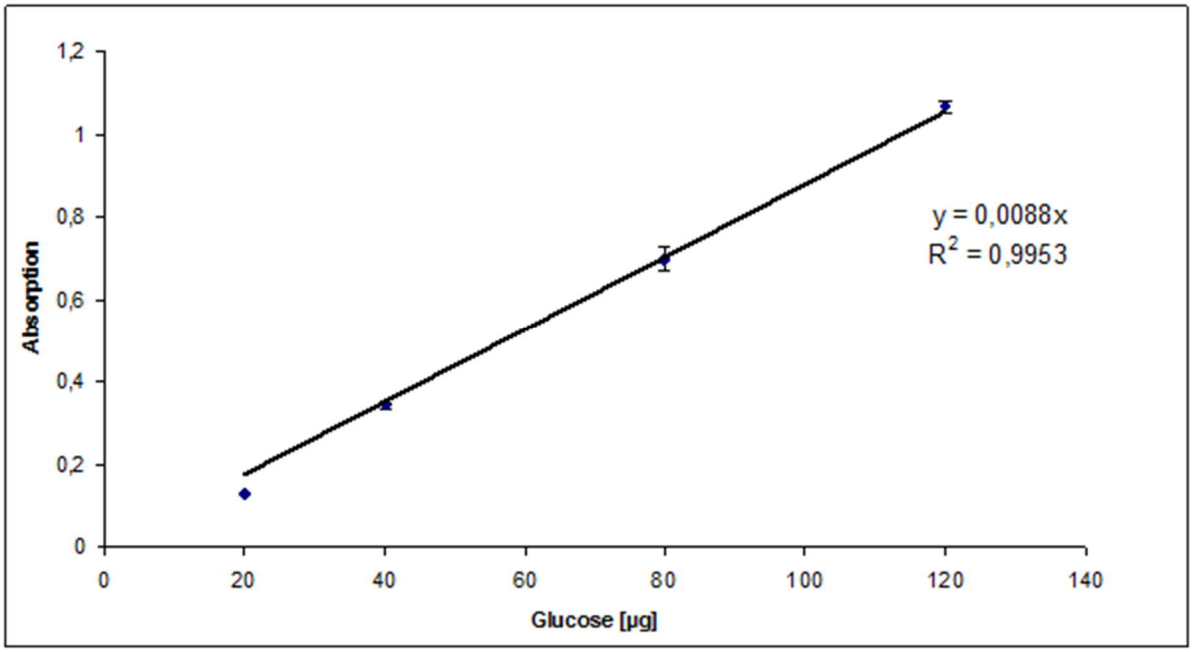



**Figure 8.** Calibration curve for DNS assay

1-20 mg/mL glucose standards are prepared for the calibration curve (by sequential dilution). The stock solution (20 mg/mL) is made in NaAc or citrate-phosphate buffer (50 mM M, pH 4.8). Next, six standard dilutions with a glucose concentrations between 1 and 20 mg/mL are prepared.

The sensitivity of the assay is in the range 1-20 mg/mL glucose.

You can increase sensitivity if needed by preparing a more concentrated DNS reagent, and increasing sample volume. Also preparing a standard curve in lower concentration range and pipetting a higher volume (for standards and samples) into the microwell plate used for measurement (steps 6. and 7.) can work.

The samples that are measured:

Each cultivation sample gets an enzyme blank. With the enzyme blank the reducing agents in the culture medium are measured that can be subtracted from the result of the sample activity. In this way, the result is the xylanase activity formed in the culture medium where the background effect is eliminated. In the enzyme blank the 50 µl of sample is pipetted into the tube after the DNS addition.

The xylanase activity is measured from all cultivation and purification samples as well from selected purification fractions. Two parallel determinations are made of all samples and standards.

The cultivation samples K0, K1 etc. are used undiluted. However, from the last cultivation sample also a dilution of 1:2 is prepared. From the purification sample P1 a dilution of 1:5 is done. The purification samples P2 and P4 are used undiluted and from sample P3 dilutions 1:10 and 1:100 are made.

From the best purification fractions dilutions of 1:50 and 1:500 are prepared. The purification samples with less protein can be used with a dilution of 1:10 or undiluted depending on the amount of the protein. The assistant help you choose the correct dilutions.

If the absorbance of the measured sample is outside the determination range, the measurement need to be repeated with new dilutions. The measurement also needs to be repeated, if the results of the parallel samples differ strongly from each other.

## Calculations needed for the report

When plotting the standard curve the exact standard concentrations should be used, which are calculated using the amount of substance weighed and the volume used to solubilize it. For the final product the best fraction is chosen with as much pure xylanase as possible. In the calculations the dilution factor has be taken into consideration.

### Calculated dry weight

The calculated dry weight is determined for all cultivation samples using a standard curve. The determined dry weight values are plotted as a function of the measured OD values, which will give a standard curve. This standard curve is used to calculate the dry weight for all other samples.

### Protein concentration

The protein concentrations for all samples can be calculated with the standard curve from the Bradford analysis.

### Amount of total protein

The total amount of protein in each sample can be calculated by multiplying the protein concentration of the sample with the total volume in that step. The total protein amount needs to decrease in the purification process, because the amount of impurities decreases with each purification step.

### Activity per volume

With the standards the correlation between the concentration and the absorbance of xylose can be determined. With this xylose standard curve the concentration of xylose in the assay can be

calculated, which was formed during the incubation. The activity of the sample can be calculated from xylose and the reaction time using equation 1.

$$\text{activity} = \frac{\text{xylose concentration } \frac{\text{mol}}{\text{l}}}{\text{reaction time s}} \quad (1)$$

Since the unit of the activity will be $\frac{mol}{s \times l}$ and the definition of Katal is $kat = \frac{mol}{s}$, we get the unit $\frac{kat}{l}$. If the sample is diluted, the correction is made with the dilution rate to give the enzyme activity of the original sample.

## Total activity

The total activity decreases in the purification process, because some enzyme is lost within the various stages of the process. The total activity is calculated for all the purification and fraction samples. The total activity can be calculated using the equation 2.

$$\text{total activity kat} = \text{activity } \frac{\text{kat}}{\text{l}} \times \text{volume l} \quad (2)$$

## Specific activity

The specific activity should increase during the purification process, because the xylanase concentration increases in the total protein concentration while unwanted proteins disappear. The specific activity is calculated for those samples, where the protein concentration is measured. Specific activity is calculated using the equation 3.

$$\text{specific activity } \frac{\text{kat}}{\text{g}} = \frac{\text{activity } \frac{\text{kat}}{\text{l}}}{\text{protein concentration } \frac{\text{g}}{\text{l}}} \quad (3)$$

## Purification factor

The purification factor shows the increase of the specific activity of the sample in the purification process. The purification factor is calculated for the sample P3 and the final product. The purification factor is calculated by dividing the specific activity of the purification step with the specific activity of the cultivation sample P1.

## Yield

The yield represents the decrease of the total activity during the purification process. The yield is calculated for the same samples as the purification factor. The yield is calculated by dividing the total activity of the purification with the total activity of the cultivation sample P1. The yield can never be greater than one.

## Molecular weight

From the SDS-PAGE gel the molecular weight of the produced xylanase is determined. For the calculation of the molecular weight first the distance traveled by proteins in the gel is

determined ($R_f$). This is obtained by dividing the distance traveled by the protein with the distance of the dye (Figure 9). In addition, the $R_f$ value is calculated for each protein of the molecular weight standard.

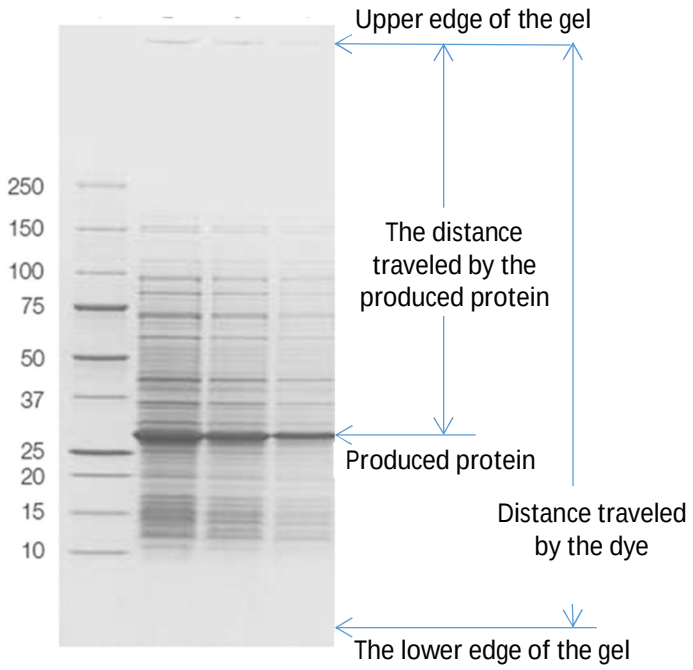



**Figure 9**. Determining the distances traveled by the proteins.

The logarithms of the molecular weights log (MW) are plotted as the function of the $R_f$ values, which gives a standard curve. With the standard curve and the $R_f$ values of the desired proteins the molecular weights of these proteins can be calculated. If some of the standard bands of the molecular weight do not fall within the linear area, they are not taken into account when drawing the standard line.

# Laboratory work 2

## Introduction

The laboratory work 2 is a scale-up of a bioprocess. The workgroups will consist 5-6 students and subgroups of 2-3 students. The students will find out the details for the work from a given dissertation work "Process development for mannitol production by lactic acid bacteria" (von Weymarn, 2002; http://lib.tkk.fi/Diss/2002/isbn9512258854/isbn9512258854.pdf ). Read pages 9-16; 21-27; 39-45; 54-61; 80-84 and 97-99 from the dissertation. This laboratory work will last for one whole week. The work includes the biotechnical production of a sugar alcohol in pilot scale and its downstream processes including cell separation, product concentration and crystallization. Every group will write their own notes of this laboratory work and a report of the main observations. Results will be presented in the seminar presentation.

Mannitol is traditionally manufactured by a chemical process, i.e. by hydrogenation of glucose-fructose syrup. In such case, however, the main product is sorbitol. It is therefore worthwhile to come up with a new way to produce only mannitol. Many microbes are known to secrete mannitol to growth medium. In particular, lactic acid bacteria are known to efficiently produce mannitol from fructose. The aim in this work is to utilize fermentation to produce 3 kg of pure crystallized mannitol during one week. In a profitable process this amount should be able to be produced of 10 kg of fructose. The purity of the produced crystals should be analyzed. In this work biotechnical production of mannitol is carried out using *Leuconostoc mesenteroides* ATCC-9135 strain.

## Main apparatus

Following apparatus are used during the work 2 (separate user instructions for these devices exist in the laboratory file collection):

- Marubishi 200-liter bioreactor (recommended for cultivating capacity up to 100 l)
- Sartorius 15-liter bioreactor (culture volume 8 L)
- Pellicon tangential flow filtration holder and Sine pump
- Millipore filter cartridge for the previous filter holder (cut-off 1000 kD, suitable for bacteria separation, filtration surface area of 2 $m^2$)
- Alfa-Laval Centri-Therm centrifugal vacuum evaporator
- Cooling crystallizer
- Crystal separator

**Figure 10.** Marubishi bioreactor and Pellicon filter holder

## Workflow

Biolab II –Scale up: Mannitol production by *L. mesenteroides.* The aim is to produce 3 kg of mannitol.

Monday: Cultivation media preparation for each cultivation steps. Inoculation of the first pre-culture step.

1. Prepare the inoculation flask and adjust the incubation cabinet conditions
2. Prepare the MRS solution according to the instructions in the jar, 300 ml (16.5 g MRS) of the prepared solution (do not put in a bottle as it is difficult to measure the pH, preferably in a beaker)
3. Check the pH of the MRS solution and adjust it to 6.2 with HCl if necessary [even if initially pH is about 7.2, 1 mL of HCl is too much at a time!].
    a. Divide the second stage pre-culture into pre-5 * 50 mL Erlenmayer vials for autoclaving, the remainder can be autoclaved in the source vial
4. Sterilize the MRS solution in an autoclave (121 ° C, 20 min, while sterilizing the inoculation bottle + tube (thin tube!) and parts of the 15 L reactor, at least the inoculation tube and the Marubishi inoculation tube).
5. Prepare also 200L reactor glucose and fructose solutions (50g / L and 100g / L) Add 20 L cold water into Diessel tank and warm it to 50-60 C. Weigh glucose and fructose, and pour into Diessel-tank with stirring.
6. Sterilize the sugar solutions in Diessel-tank (121 ° C, 1 h)
7. During sterilization, weigh the nutrients needed for 15 L and 200 L growth in decanters, including glucose for 15 L growth.
8. Distribute 10 mL of sterilized MRS solution into three Falcon tubes (do this in a laminar flow cabinet!)
9. Transfer the test tubes from the glycerol stock (still in the laminar flow cabinet) (*Leuconostoc mesenteroides*, which we use in this work is in a narrow tower nr.4, quite near the right corner of the 2nd floor deep freezer, top box).
10. Grow in incubator at T = 30 ° C for 10 h, no mixing, caps slightly open (timed growth overnight)
11. Bring 2 ml Eppendorf tubes (about 16) to dry in the oven for the following day's cdw assays.

Tuesday: Inoculation of the second pre-culture. Finalizing cultivation media for reactor cultivations. Preparation and sterilization of reactors for 8 L and 100 L growth volumes. Inoculation of the third pre-culture.

12. Take the Eppendorf tubes to cool in a desiccator. At some point during the day, remember to weigh the tubes for Wednesday's sample processing.
13. NOTE! This should be started so that the next breeding step begins at about 10 am. Measure the OD600 of the parallel first-stage precultures and select the best breeding for the next preconditioning step (sample sterile in a laminar cabinet!) (1:10 dilution).
14. Inoculate 1.3 mL (1.25 mL = 2.5%) of the best grown tube to 50 mL Erlenmayers.
15. Incubate 50 mL of MRS and inoculum vials again at T = 30 ° C for 10 h (8-12h), no mixing.
16. Start preparing the 15 L reactor.
17. Calibrate the 15 L reactor pH sensor (with pH 4.01 and 7.0 solutions) and secure it to the reactor connector [screw the pH sensor wirelessly!].

18. Calibrate the NaOH pump [6 M NaOH, graduated glass]. Rotate the feed line to the "closed" position during sterilization.
19. Close all 15 L reactor taps and check that all openings are blocked (see Reactor Reference, Be sure to insert a septum for inoculation)
20. Prepare a growth glucose solution for 15 L reactor (Dissolve 8L*30g/L=240 g glucose in about 1.5 L milli-Q water) and also a yeast extract mineral solution (according to SCP2) (approximately 1.5 L water). The solutions are sterilized in the reactor.
21. Add media to the 15 L reactor
    a. a. Add 1-2 drops of Struktol to prevent foaming [3 drops were sufficient when sterilized with glucose]
    b. b. Add tap water to give a total volume of about 9 L (up to 1-2L can be lost during sterilization, so that the total volume of liquid before sterilization could be about 9 L)
22. The 15 L reactor is sterilized at 121 ° C for 20 min. Switch on the program from the control unit
    c. Monitor reactor until it reaches 121 ° C and proceeds to sterilization step. In COOL2 step manually open exhaust filter coolant valve.
23. The sample valve and the bottom valve are sterilized before use with steam produced by the Veit Steam Generator
24. Prepare a 100 L pilot culture medium excluding sugars and feed into the reactor. But only when the next steps d) and e) are done!
25. Sterilize the Marubishi reactor
    d. Calibrate the pH sensor on the external IWaki pump unit. After calibration, attach the sensor to the reactor.
    e. Close all 200 L reactor valves and side door
    f. Pour the medium into the reactor and add 3-4 drops of Struktol to prevent foaming
    g. Fill the reactor with 60 L of tap water (during / after sterilization, the fluid volume may increase / decrease depending on whether the safety valve opens. Check the situation after sterilization and, if necessary, autoclave additional water)
    h. Close the Cover Input Door and check that all connections are blocked.
    i. Begin sterilization according to Marubish instructions
26. The reactor is left with an excess pressure of about 0.2 bar and a temperature of 25 ° C until it is inoculated with 15 L of broth. The second stage pre-cultures are combined with an autoclaved inoculum flask after about 10 h of growth and inoculated into a sterilized 15 L reactor ). Take an inoculate sample (12 mL) for OD and dry weight for assays prior to inoculation with the reactor.
27. 15 L reactor conditions during cultivation: pH 5.5, T = 30 ° C, stirring 50 rpm. The pH is adjusted with 6 M NaOH.

Wednesday: Inoculation of 100 L production reactor. Taking manual samples from the reactor for 8 hours (see analysis), HPCL- standard preparation. Tangential flow filter assembly and test.

28. 15 L reactor growth takes 8-10 h (until late exponential growth phase, growth progress is monitored by base consumption -> when consumption stabilizes may stop.).
29. At the end of the 15 L cultivation, take a sample of approximately 12 mL for the sugar assay and the OD assay, also cdw. Veit steam generator is used for this, the sample line is steamed for 10 min before sampling.

30. After inoculation, wash the reactor. (Instructions in the reactor manual. After emptying the reactor, fill with water and heat to 60 degrees for 30 minutes. After cooling down, rinse and disassemble).

Pilot-scale cultivation

Set 200 L reactor at T = 30 ° C, pH 5.0 and stirring at 50 rpm

31. Add the sugar solutions aseptically from Diessel-tank to a 200 L reactor
32. Inoculation from 15 L reactor pre-growth through a sterile hose to a 200 L reactor
33. After inoculation, take the first sample from Marubishi for OD and HPLC assays
34. When the pH of the culture has dropped to 5.0, start the pH adjustment with NaOH (with Iwaki-pump)
35. Take samples first at two hours intervals then after 5 hours, more frequently according to assistant instructions. After sampling, take the HPLC samples in the freezer to wait for analysis, the ODs are done immediately to see the progress of growth. Put the CDW-Eppendorf tubes in the oven for the night.
36. If necessary, add Struktol during growth to prevent foaming, or if the pH threatens to drop too much NaOH, too ((watch with reactor lid and NaOH tube)
37. Bioreactor cultivation is continued overnight
38. During the day, also assembling the tangential flow filter and measuring the water permeability should be done.

Thursday: End of production cultivation. Start of downstream processing. Weighting CDW samples.

39. The previous day's Eppendorf tubes in a desiccator to cool.
40. Growth is stopped when NaOH consumption equilibrates and OD values stabilize (should be at about 7-10h but continue overnight)
41. Immediately before completion, take final samples for OD and HPLC assays
42. Filter the culture medium with a tangential flow filter, concentrate the filtrate on a vacuum evaporator and transfer the concentrate to a crystallizer.
43. Wash the Marubishi reactor
44. Weighing of Marubishi cdw samples

Friday: End of downstream processing. Cleaning up.

45. Recovery of mannitol crystals with a centrifuge filter
46. Dry weight of mannitol crystals
47. Preparation of HPLC standards and HPLC runs
48. Sugar standard: mannitol, glucose, fructose. Starting at 4 g / L, dilute the series at 3, 2, 1 and 0.5 g /L
49. Acid standard, e.g.: 100% acetic acid 95.2 µL and 90% lactic acid 92.6 µL are dissolved in 25 mL H2O. Dilution series similar to sugars.
50. Cleaning of down-stream equipment, measurement of the water permeability of the tangential flow filter.

## Pre-culture fermentations:

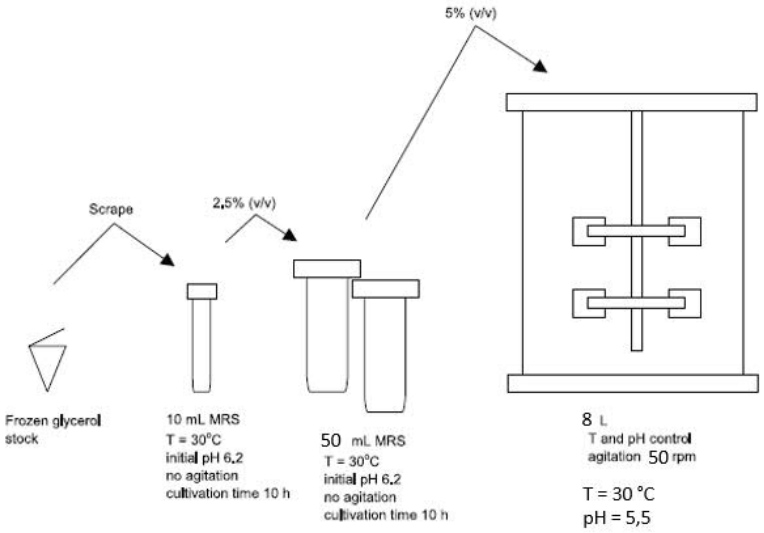



**Figure 11.** Mannitol cultivation up to 8L

First, MRS growth medium is prepared according the introduction on the MRS can. The pH is adjusted to 6.2 with HCl before sterilization. 5 x 10 mL of standard MRS growth medium is inoculated from a frozen glycerol stock and is grown in a test tube at 30°C for about 10 hours without mixing. Next, from the best grown 10 mL test tube, broth is added to fresh MRS medium in five 50 mL Erlenmeyer flasks and is grown at 30°C for about 10 hours without mixing. After that, the broth of this pre-culture is used to inoculate the 8 L of SCP2 medium in bioreactor.

| *Composition of SCP2 medium* | *(g/L)* |
|---|---|
| Yeast extract (LAB M, International Diagnostics Group, England) | 15 |
| Glucose | 30 (50) |
| (Fructose | 100) |
| K2HPO4 | 2 |
| MgSO4 | 0.2 |
| MnSO4 | 0.05 |

All dry matters are diluted into cold tap water.

For the final pre-cultivation step (8 L) glucose is sterilized in the reactor with other medium components, but for the production step (100 L) sugar solutions are autoclaved separately, other medium components are sterilized in the reactor. Glucose: 30 g/L for the last pre-culture step and 50 g/L for the production step. Fructose is used only in production step.

## Pilot scale fermentation and downstream processing:

Production reactor (100 L) is inoculated with 8 L fermentation broth from the last pre-culture step. Production conditions: pH: 5,0;T = 30°C; agitation: 50 rpm

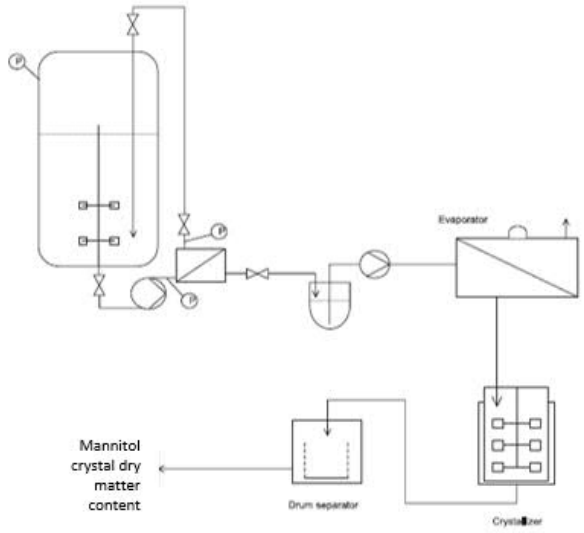



**Figure 12.** Downstream Processes: cell separation, product concentration and crystallization

Table 1. Mannitol work sampling suggestions

| Growth phase | Sample | | | |
|---|---|---|---|---|
| | OD | cdw | HPLC | |
| MRS-broth | X | | | |
| 10 mL precultivation end | X | | | OD: dilution 1:10 |
| 50 mL precultivation end | X | | | OD: dilution 1:10 |
| 8 L precultivation beginning | | | X | |
| 8 L precultivation end | X | X | X | |
| Pilot-cultivation start, 0h | X | X | X | HPLC: dilution 1:20<br>take first cdw-sample supernatant and make the dilution from it |
| Pilot-cultivation every hour | X | X | X | |
| Pilot-cultivation end | X | X | X | |

## Assay methods

Optical density The optical density of the fermentation broth is measured at 600 nm against distilled water. The samples are diluted in such a manner that the absorbance values are in the range of 0.1 to 0.6.

The cell dry weight (CDW) is measured as follows: a sample of bioconversion broth (2 mL) is pipetted into a pre-weighted centrifuge tube followed by centrifugation at 6000 g for 5 min. The cell pellet is washed with deionized water, the centrifugation is repeated. Washing is repeated after which the centrifuge tube is dried at 80 °C overnight.

HPLC (High Performance Liquid Chromatography) Concentrations of fructose, glucose and mannitol as well as concentrations of acetic acid and lactic acid are assayed with HPLC. HPLC samples are prepared from the supernatant of the first centrifugation cycle. Supernatant is diluted with deionized water so that concentrations of the analyzed components are between 0 and 4 g/L. Prepare two HPLC vials from each sample; one is used for sugar analysis and the other one for acid analysis. Samples are transferred into vials through 0.45 µm filter with a syringe. Do not over fill vials because they might break during freezing,~1 mL is enough.

Prepare HPLC standards:

Glucose, Fructose, Mannitol, 0.5 g/L; 1 g/L; 2 g/L; 4 g/L. Start by preparing 4 g/L solution, which contains each sugar. Prepare sample vials as fermentation samples.

Acetic acid and Lactic acid: similar dilution series as for sugars.

# References

Anbarasan, S., Jänis, J., Paloheimo, M., Laitaoja, M., Vuolanto, M., Karimäki, J., Vainiotalo, P., Leisola, M. ja Turunen, O., Effect of Glycosylation and Additional Domains on the Thermostability of a Family 10 Xylanase Produced by *Thermopolyspora flexuosa*, *Applied and Environmental Microbiology* 76:1 (2010) 356-360.

von Weymarn N., Process development for mannitol production by lactic acid bacteria. Doctoral dissertation, Helsinki University of Technology, Department of Chemical Technology, Espoo 2002, 110 p.

# Instructions for laboratory notebook

All the laboratory work is constantly kept up-to-date in the laboratory notebook. Each member of a group writes in turn in the notebook and takes the main responsibility for recording and reporting the results from the fermentation, xylanase purification, protein analysis and xylanase activity. Learning lab journaling is particularly important for possible future tasks. In the industry and business life the laboratory notebook can be used as evidence in for example patent litigations. Therefore, right from the beginning the laboratory notebook is done in the way, what employers are using in the field. It is not wise to write notes on loose papers, because they have a tendency to disappear. At worst, the disappearance of important weighing or measurement results might lead to a renewal of the whole work, because calculations will be impossible without the primary results. The lab work stages are described using either passive or imperfect. You may refer to work instructions, but all dilutions should be written down. The lab notebook should include the following among other things:

- Start the notebook with two empty leafs for the Table of contents!!
- The date, the name of the writing person and group number, course code and topic, as well as the page number
- The principle and purpose of the work in brief (what is done and why)
- Methods used (how something was done), remember to use titles
- Used equipment (information about the equipment, the manufacturer and manufacturing country) and special reagents (manufacturer, manufacturing country)
- Experimental observations as they are identified (primary notes)
- Other work-related remarks (e.g. method and equipment problems)
- Primary weighing and measuring results in tables

The lab notebook is a book, which is all the time with you in the lab and in which work is written down while being performed. Figure 1/1 shows an example of a page from a lab notebook.

| 27.10. 2016 | Group 2<br>Isaac Einstein | CHEM-E3130 Biolab II<br>Labwork 1 | 5 |
|---|---|---|---|

## Analysis of protein concentration

First was a stock solution prepared with BSA-concentration 100 μg/ml. 50,8 mg of BSA (Albumin, Bovine, Sigma USA lot 1090221) was weighted in a measuring bottle and filled to 500 ml with milli-Q water.

Standard series was prepared from stock solution according to the Table 5.

Table 5. Standard dilutions of the protein standards

| Standard (μg/ml) | Stock solution (μl) | Water (μl) | Total volume (μl) |
|---|---|---|---|
| 1 | 30 | 2970 | 3000 |
| 2,5 | 75 | 2925 | 3000 |
| 5 | 150 | 2850 | 3000 |
| 7,5 | 225 | 2775 | 3000 |
| 10 | 300 | 2700 | 3000 |
| 15 | 450 | 2550 | 3000 |
| 20 | 600 | 2400 | 3000 |

Dilutions 1:20 and 1:100 were prepared from fractions F1-F6.
Dilutions 1:20 and 1:50 were prepared from samples P1-P4.
Dilutions were prepared according to Table 6.

Table 6. Pipeting table for the protein concentration analysis

| Dilution | Sample (μl) | Water (μl) | Total volume (μl) |
|---|---|---|---|
| 1:10 | 250 | 2250 | 2500 |
| 1:20 | 125 | 2375 | 2500 |
| 1:50 | 400 dil. 1:10 | 1600 | 2000 |
| 1:100 | 400 dil. 1:20 | 1600 | 2000 |

Figure 1. Example page of a lab notebook.

# Instructions for the work report

Each group will write a work report of a laboratory work. In the report the work carried out, the main results and discussion of the results are described. The layout of the work report is done according to the Chemical Engineering study program's guide (Guideline for written reports). Figures 2 and 3 provide examples of suitable graph presentation. The work instructions should not be copied! The recommended length of the work report is 20 – 30 pages.

Examples are given in relation to work1 but they hold for work 2 as well. The work report is divided into the following sections:

*Summary*

In the summary the objectives of the work and the main results are presented. The maximum length of the summary is one page and it does not include images or tables. Summary is written in a separate page before the list of contents and it is not included into the list.

*Introduction*

The purpose of the introduction is to introduce the reader to the background of the work. In the end of the introduction it is good to describe the purpose and objective of the work in a couple of sentences.

*Materials and Methods*

In this section the materials and methods of the work are briefly and objectively explained so that the reader is able to repeat the work if wanted. All essential information on work reproducibility must be found, but any excess information will be left out. This is not supposed to be a copy of the instructions of the laboratory work, but only a short version. In the work report f. ex. pipetting or the weighing amounts are not reported, but only the final concentrations of solutions. Furthermore, the centrifugation is expressed with RCF-value (relative centrifugal force), so that the reader can repeat the steps with a different rotor. The models and manufacturers of the equipment and manufacturers of the materials are also given to ensure the reproducibility of the work.

*Results*

The results include the primary results (in the appendices) as well as the calculated actual results. Graphs and tables are recommended. Duplications should be avoided and most of the primary results are placed in the appendices instead of the actual text. Long uninformative tables do not belong to the report's actual text. Instead well designed graphs should be used. The results are rounded, as long as they stay rational and reliable. The result should not contain more significant figures than the output values. When calculating the results, they should be considered whether they are possible, f. ex. the yield cannot be more than 100%. In case of impossible results, you should check the calculations, and if you still get such results, the issue should definitely be considered in the error discussion.

The main results from the cultivation phase:

- The measurements from the bioreactor (pH, pO2, stirring speed and the OD measurements)
- The dry weight results
- The calculated dry weight
- The xylanase activity during growth and the effect of induction

In the downstream the preservation of the xylanase activity in the various stages of the purification and the purification of xylanase are discussed. The following results are presented in the work report:

- The graph of the chromatographic purification
- The imidazole concentration, when the protein eluates
- The SDS-PAGE gel and a calculated molecular weight of the xylanase
- The protein concentration and total protein amount
- The xylanase activity (total activity and specific activity)
- The purification factor and the yield for the sample P3 and for the end product
- A purification table (total protein amount, protein concentration, total activity, specific activity, yield and purification factor)

*Discussion of the results and errors*

In this section the obtained results and the reasons for them are discussed. In the error discussion f. ex. the reliability of the parallel measurements, the reliability of the standard curves and the preservation of the total amount of protein in of the various stages are discussed. Naturally, human and technical errors should be considered in this section. In addition, own results are compared with the corresponding results found in the literature and the reasons for potential differences are discussed.

*Conclusions*

In this section possible follow-up measures are presented and how the work could proceed afterwards. Proposals for improving the work can also be included, f. ex. what would you do differently, if you would do the work again.

*Appendices*

All the primary results as well as standard curves are put in the appendices.

The introduction for the work report could be written to before starting with the laboratory work. This would make you more familiar with the topic prior to the start of the work. The first version of the work report will be returned within less than two weeks following the last laboratory work day. The report should be sent responsible teacher as soon as possible at an agreed time. Responsible teacher will ask assistant to make a first look at the report. The final approved version must be returned within three weeks after the receiving the correction suggestions. Please note that the report may have to be revised several times. All possible questions relating to the work report should be addressed to the responsible teacher of the course (Tero Eerikäinen) who also reads and grades all final reports.

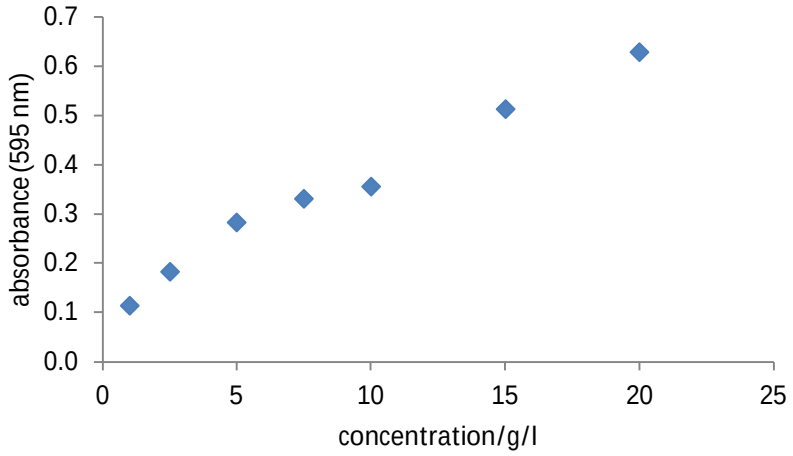



Figure 2. An example of the presentation of a graph in the work report.

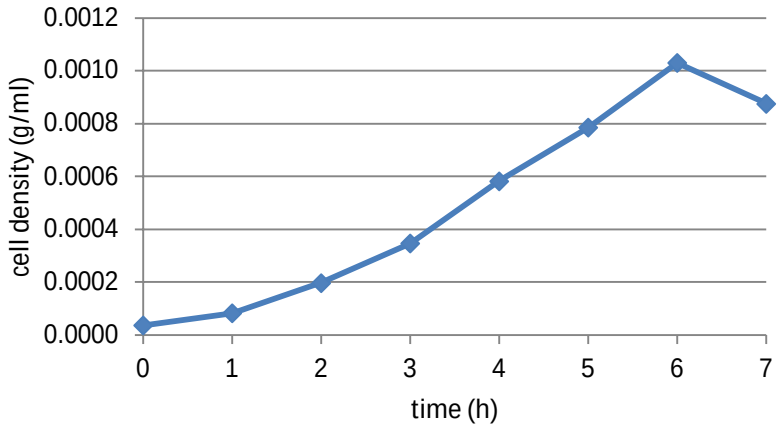
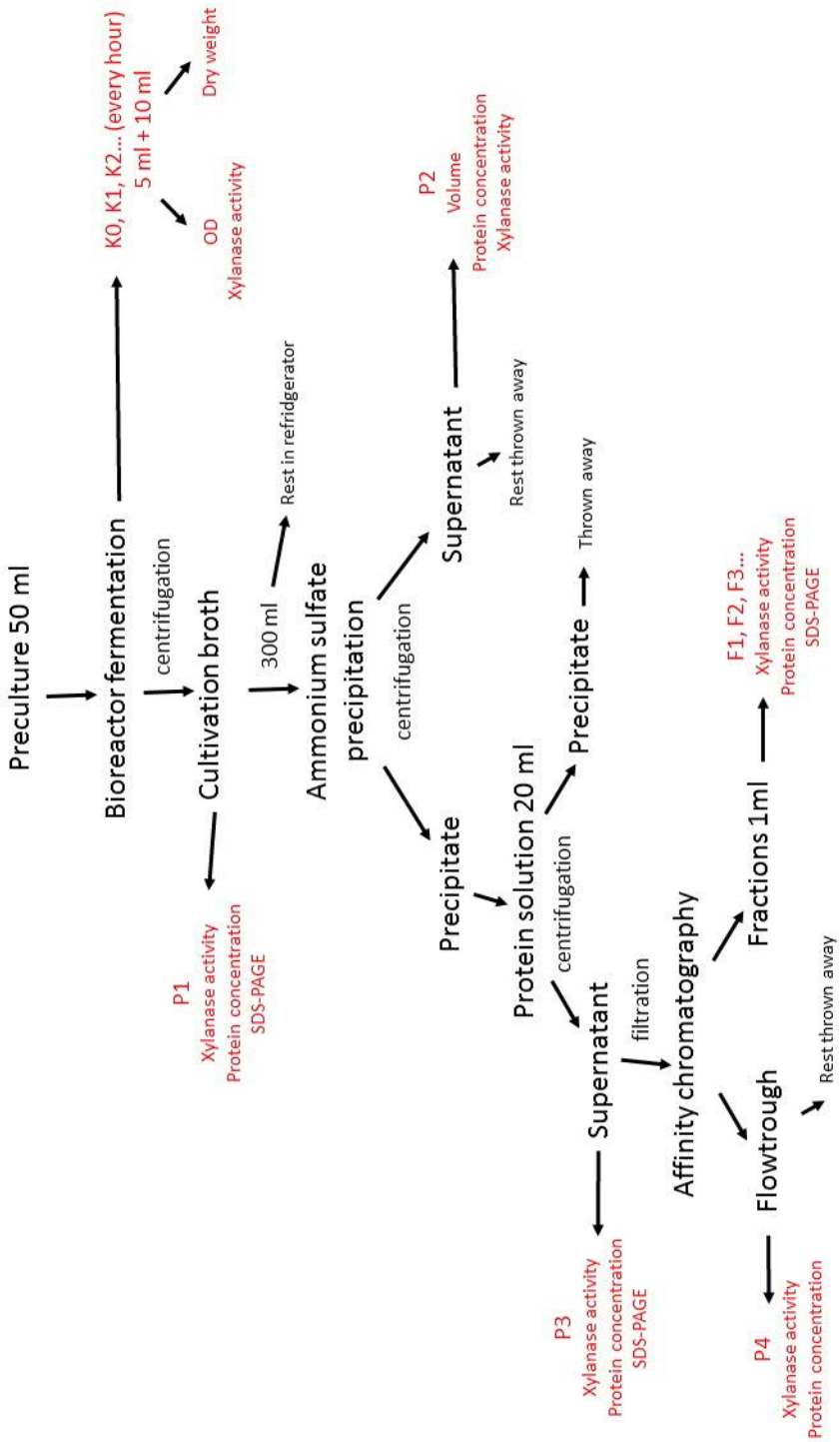



Figure 3. An example of the presentation of a graph in the work report.

# Sample chart



## Preparation of Citric Acid – Na2HPO4 Buffer Solutions, pH approx. 2.6–7.61

Citric acid monohydrate, C6H8O7 • H2O, M. wt. 210.14; 0.1M-solution contains 21.01 g/l. Na2HPO4, M. wt. 141.98; 0.2M-solution contains 28.40 g/l, or Na2HPO4 • 2H2O, M. wt. 178.05; 0.2M-solution contains 35.61 g/l.

| pH | x ml 0.1M-citric acid | y ml 0.2M-Na2HPO4 |
|---|---|---|
| 2.6 | 89.10 | 10.90 |
| 2.8 | 84.15 | 15.85 |
| 3.0 | 79.45 | 20.55 |
| 3.2 | 75.30 | 24.70 |
| 3.4 | 71.50 | 28.50 |
| 3.6 | 67.80 | 32.20 |
| 3.8 | 64.50 | 35.50 |
| 4.0 | 61.45 | 38.55 |
| 4.2 | 58.60 | 41.40 |
| 4.4 | 55.90 | 44.10 |
| 4.6 | 53.25 | 46.75 |
| 4.8 | 50.70 | 49.30 |
| 5.0 | 48.50 | 51.50 |
| 5.2 | 46.40 | 53.60 |
| 5.4 | 44.25 | 55.75 |
| 5.6 | 42.00 | 58.00 |
| 5.8 | 39.55 | 60.45 |
| 6.0 | 36.85 | 63.15 |
| 6.2 | 33.90 | 66.10 |
| 6.4 | 30.75 | 69.25 |
| 6.6 | 27.25 | 72.75 |
| 6.8 | 22.75 | 77.25 |
| **7.0** | **17.65** | **82.35** |
| 7.2 | 13.05 | 86.95 |
| 7.4 | 9.15 | 90.85 |
| 7.6 | 6.35 | 93.65 |