Aalto-yliopisto
Kemian tekniikan korkeakoulu

Methods lecture

CHEM-E3130
Biolab II

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Aalto-yliopisto
Kemian tekniikan
korkeakoulu

Work 1:
Lab scale recombinant protein production and purification

Contents

- Overview of the laboratory work 1
- *lac* operon
- Antibiotic resistance
- Fermentation
- Purification
- Determination of protein concentration
- SDS-PAGE
- Xylanase activity assay
- Calculations for the work report

Overview of laboratory work 1

- Cell cultivation and enzyme production
  - 1. and 2. work days
  - *Thermopolyspora flexuosa* –bacteria's xylanase gene
  - pJ404 vector (from DNA2.0), protein production induction by IPTG
  - *Escherichia coli* as host
  - Preculture overnight
  - Bioreactor cultivation in controlled conditions

Overview of laboratory work 1

- Purification
  - 3. work day
  - Ammonium sulfate precipitation for concentration
  - Affinity chromatography

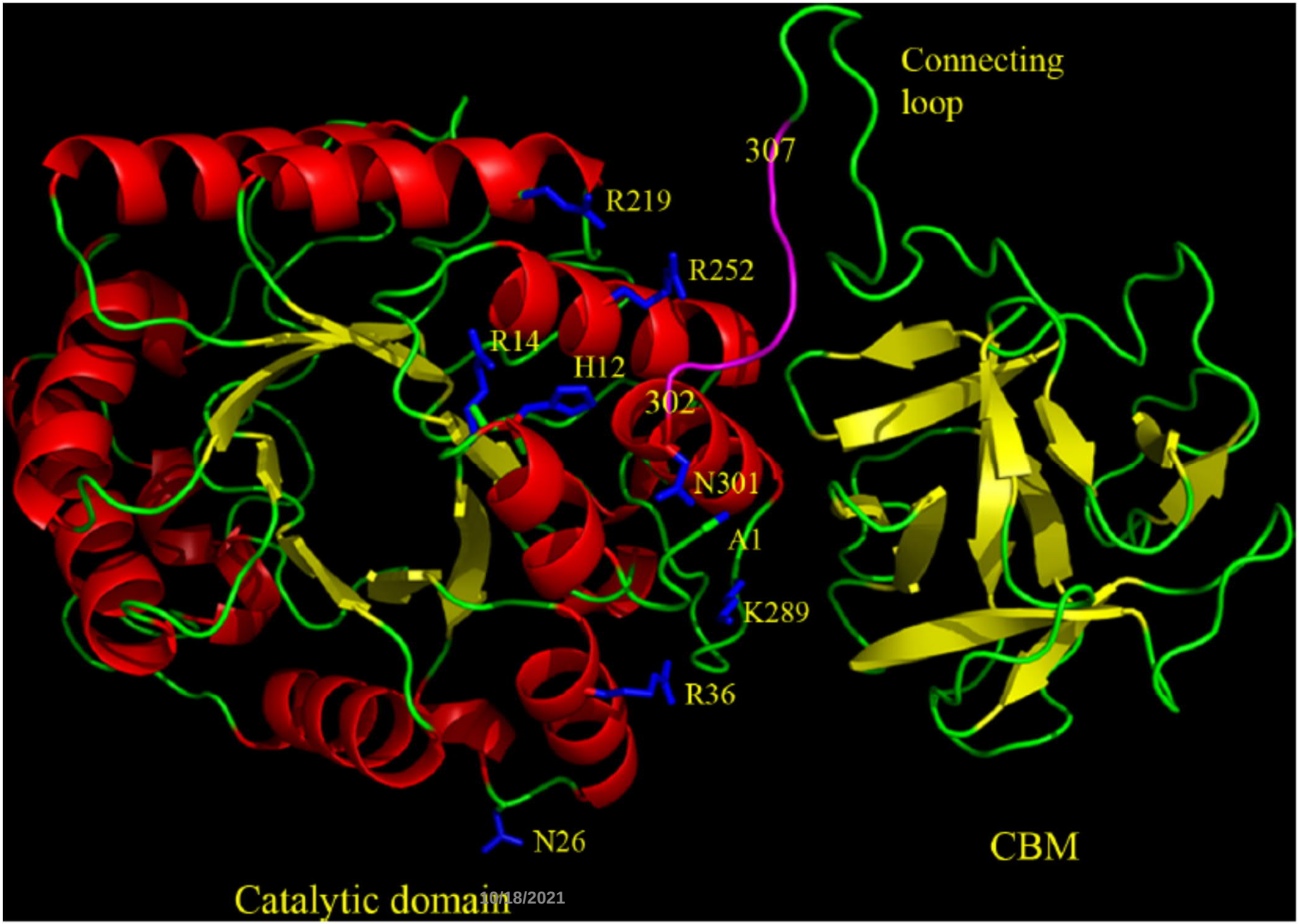
- Analysis
  - 4. and 5. work day
  - Determination of protein concentration with the Bradford method
  - Analysis of purification and molecular weight with SDS-PAGE
  - Determination of xylanase activity with the DNS method

Family GH10 xylanase

MKYLLPTAAAGLLLLAAQPAMA (secretion signal; to periplasmic space in *E. coli*, leaks to medium)

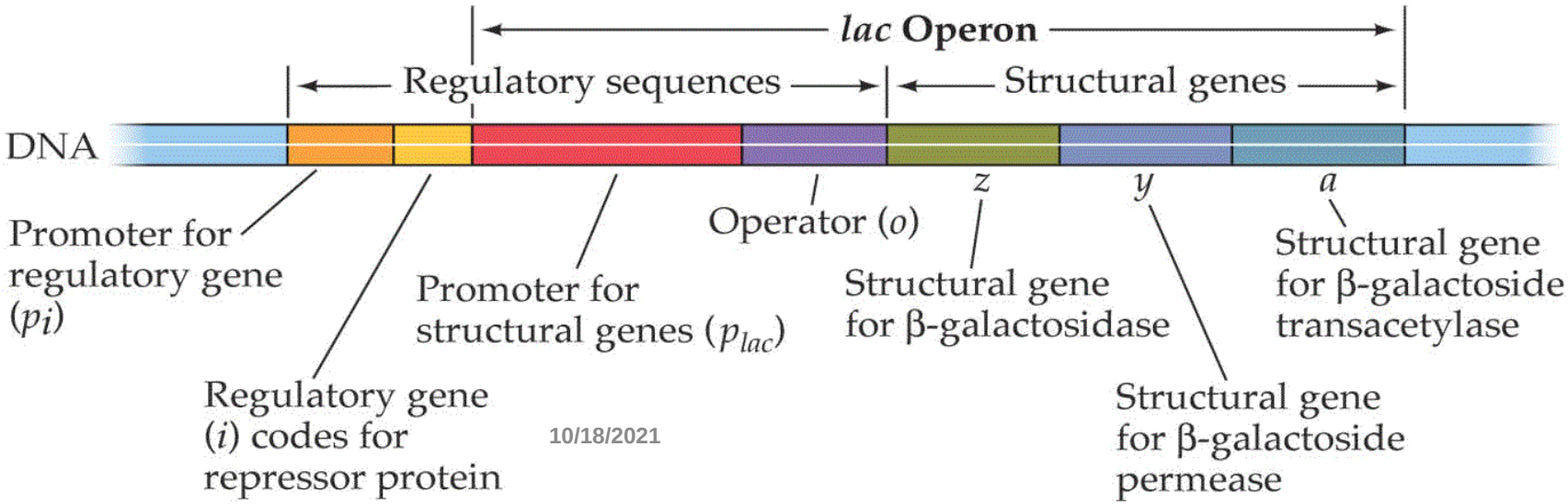
AASTLAEGAAQHNRYFGVAIAANRLNDSVYTNIANREFNSVTAENEMKIDATE
PQQGRFDFTQADRIYNWARQNGKQVRGHTLAWHSQQPQWMQNLSGQAL
RQAMINHIQGVMSYYRGKIPIWDVVNEAFEDGNSGRRRDSNLQRTGNDWIE
VAFRTARQADPSAKLCYNDYNIENWNAAKTQAVYNMVRDFKSRGVPIDCVG
FQSHFNSGNPYNPNFRTTLQQFAALGVDVEVTELDIENAPAQTYASVIRDCLA
VDRCTGITVWGVRDSDSWRSYQNPLLFDNNGNKKQAYYAVLDALN (GH10 catalytic domain)

HHHHHH (His-tag for purification)


Connecting
loop
307
R219
R252
R14
H12
302
N301
A1
K289
R36
N26
Catalytic domain
CBM

lac operon: Definition

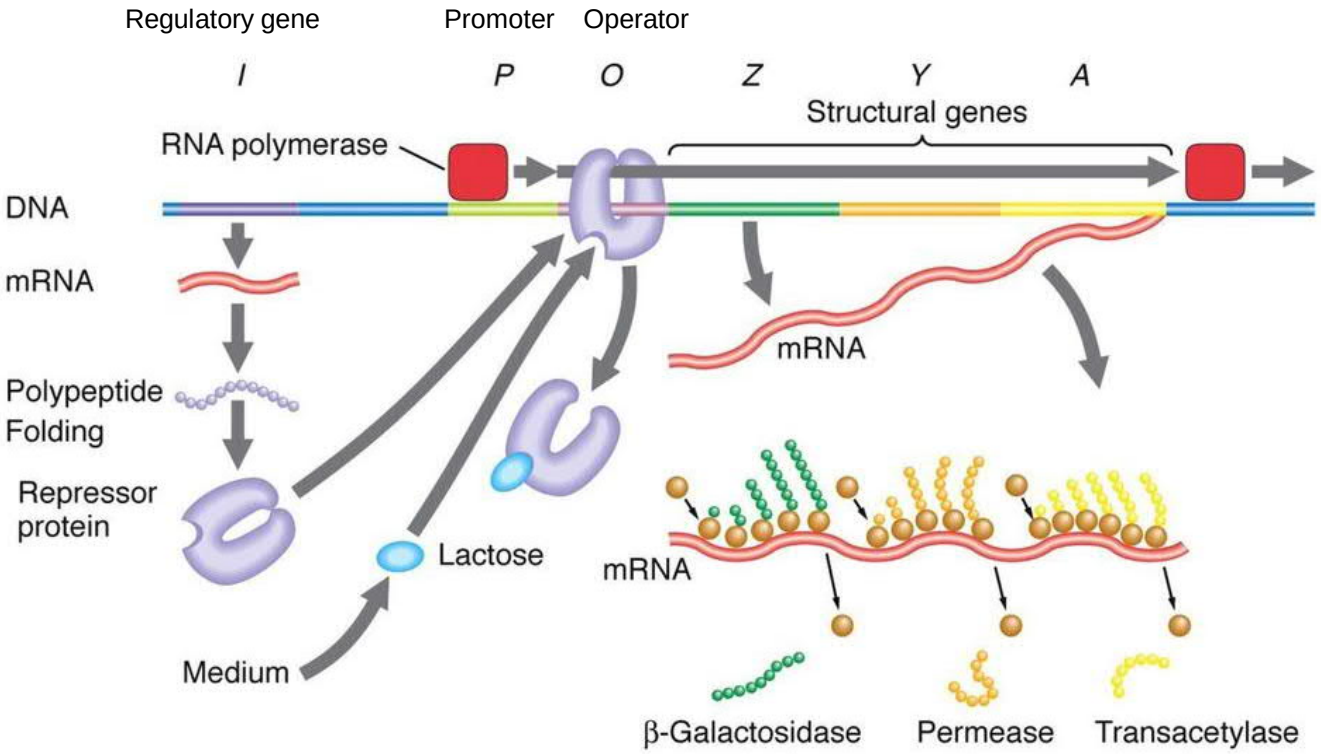
- *Operon* : the DNA sequence in which the enzyme-producing genes are closely together
- *Promoter* : the point to which RNA polymerase binds
- *Terminator* : the end point of the transcription
- *Regulator* : encodes the repression protein
- *Operator* : the point where the repression protein binds



lac-operon

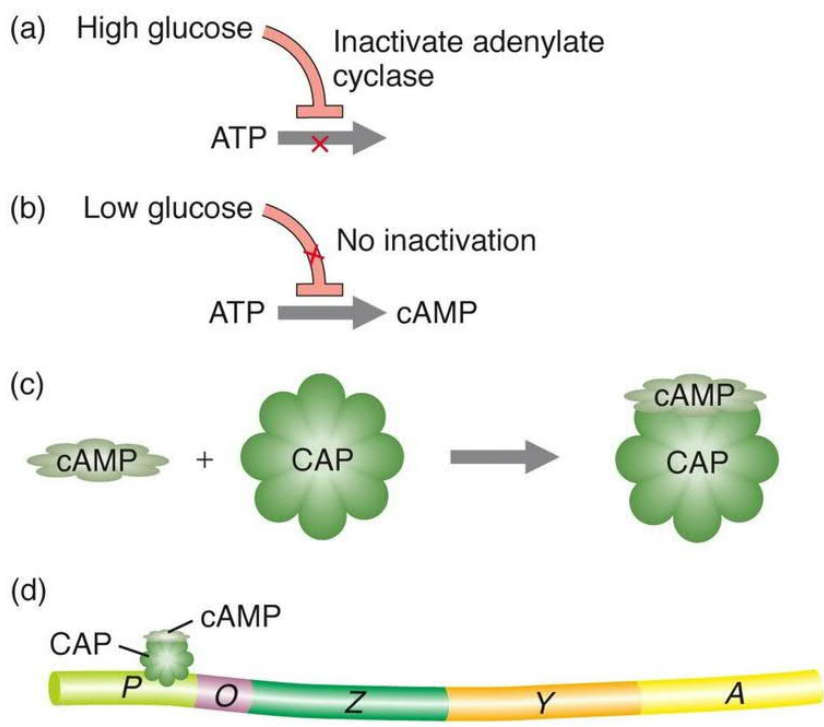
- Lac-operon affects for the transport and metabolism of lactose in bacteria like *Escherichia coli*
- Enables efficient digestion of lactose
- Enzymes should not be generated if
  - glucose is available
  - lactose is not available
- Two-step control mechanism

lac-operon: Control mechanism



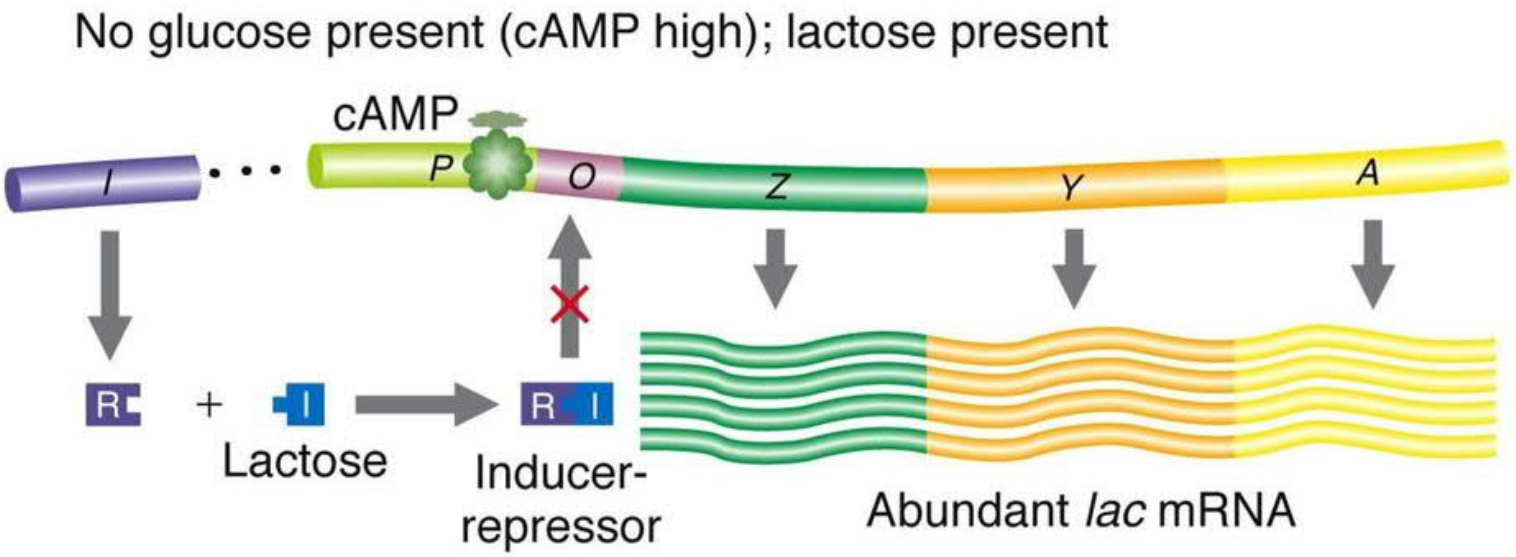
- **When no lactose is available**
  - The regulator expresses repressor protein
  - Repressor binds to the operator and inhibits the transcription
- **When lactose is available**
  - Lactose binds to repressor and prevents it from binding to the operator
  - RNA polymerase can work and mRNA transcription can begin

Catabolite repression: Control mechanism



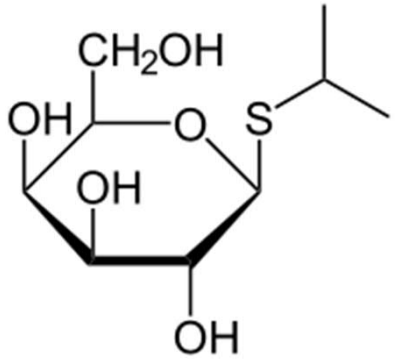
When glucose is running out:
→ cAMP (*cyclic adenosine monophosphate*) is synthesized from ATP
→ cAMP binds to CAP-protein (*catabolic activator protein*)
→ cAMP-CAP binds to DNA near the promoter and helps the RNA polymerase to bind on the promoter
→ Transcription is stimulated. Enzymes for the metabolism of other carbon sources are synthesized

lac-operon: Control mechanism



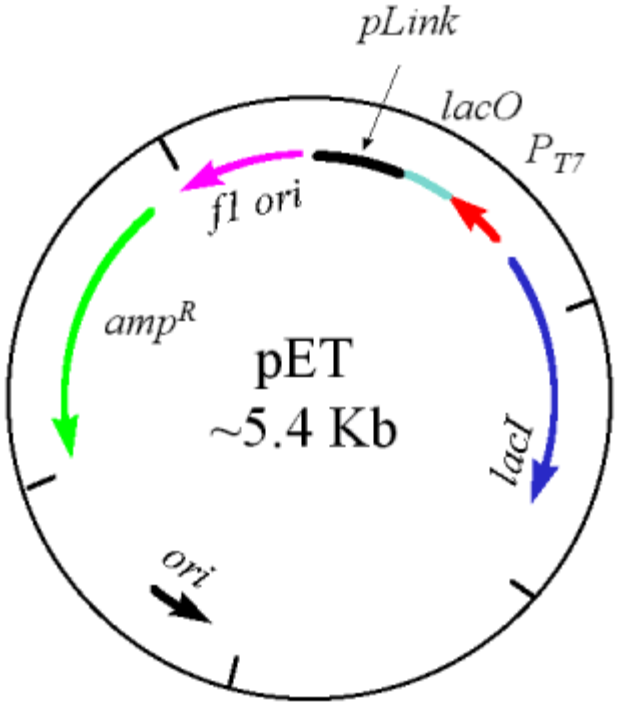
lac-operon: IPTG

- Inducer of *lac*-operon
- **I**so**p**ropyl β-D-1-**t**hio**g**alactopyranoside
- Mimics lactose
- Does not metabolize
  - Amount remains constant
- For the recombinant enzyme
  - Put in the vector *lac* operon
  - Instead of the operon's own gene sequence is the sequence of the desired enzyme
  - The desired enzyme expression is induced by addition of IPTG

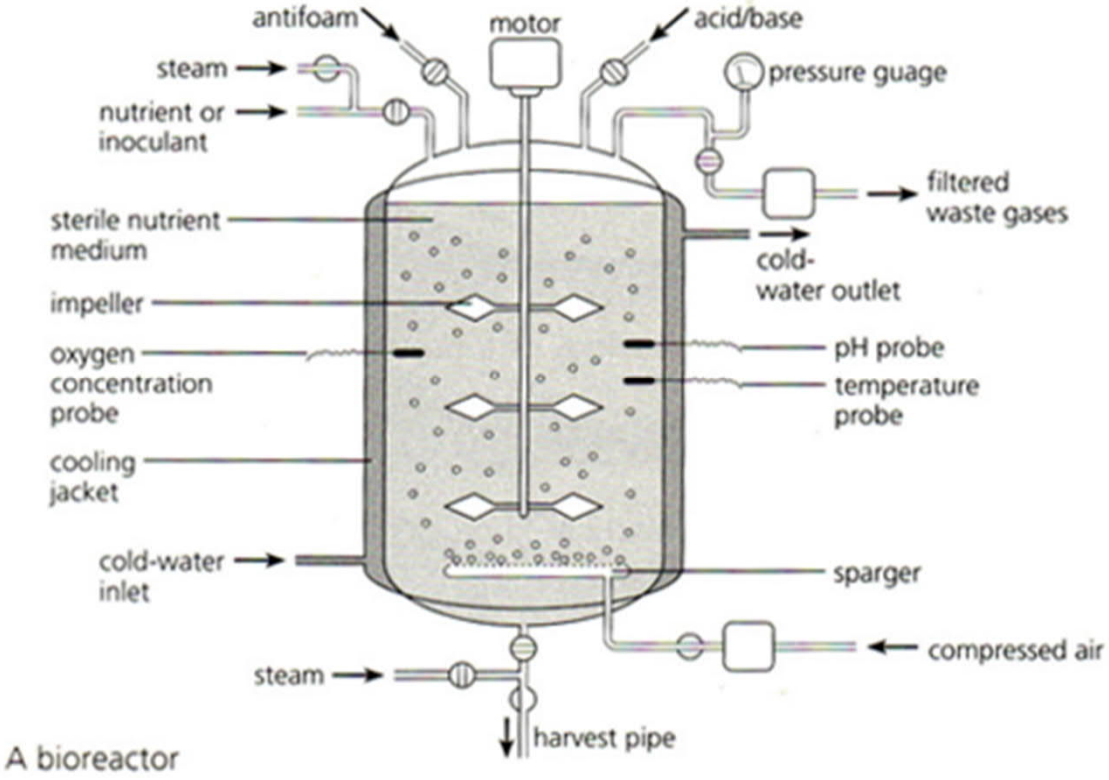


Antibiotic resistence

- Plasmid may include an antibiotics resistance gene
- In cloning
  - Successfully transformed cells are selected
  - Success of ligation examined separately
- In the production phase
  - Prevents contamination
  - The plasmid remains within the cell
- Wanted gene is cloned into plasmid at the polylinker site (pLink)



Bioreactor



A bioreactor

Bioreactor – different sizes

Purification

- Ammonium sulphate precipitation
  - Between proteins and water are hydrogen bonds
  - The salts compete for water molecules
  - The proteins aggregate with hydrophobic bonds with each other → precipitate
  - Solution to fractionation or concentration next

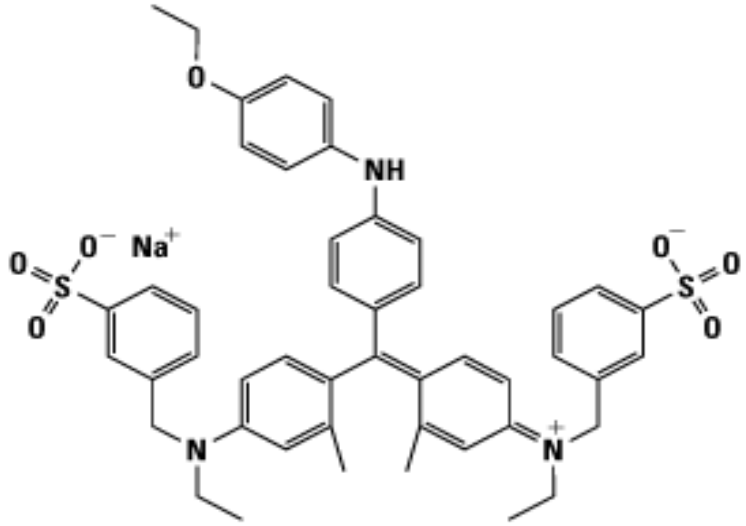
- Affinity chromatography
  - In column Ni^{2+} bound on carrier material
  - His-tag on the protein binds to nickel
  - Elution with imidazole

Centrifugation

- Relative centrifugal force RCF (marked 20000 x g)
- Revolutions per minute RPM (marked 13000 rpm)
- In the instructions RPM values are given, but for the work report RCF values are needed
  - Convertible with the radius of the rotor
  - RCF is independent of the size of the rotor

Determination of the protein concentration

Determination of the protein concentration



Coomassie Brilliant Blue G-250 Dye
$C_{47}H_{48}N_3NaO_7S_2$
MW 854.02

- Bradford method
- Based on the Coomassie Brilliant Blue G250 dye
  - Acidic compound
  - Unbound red colored
  - Bound blue colored with absorption maximum at 595 nm

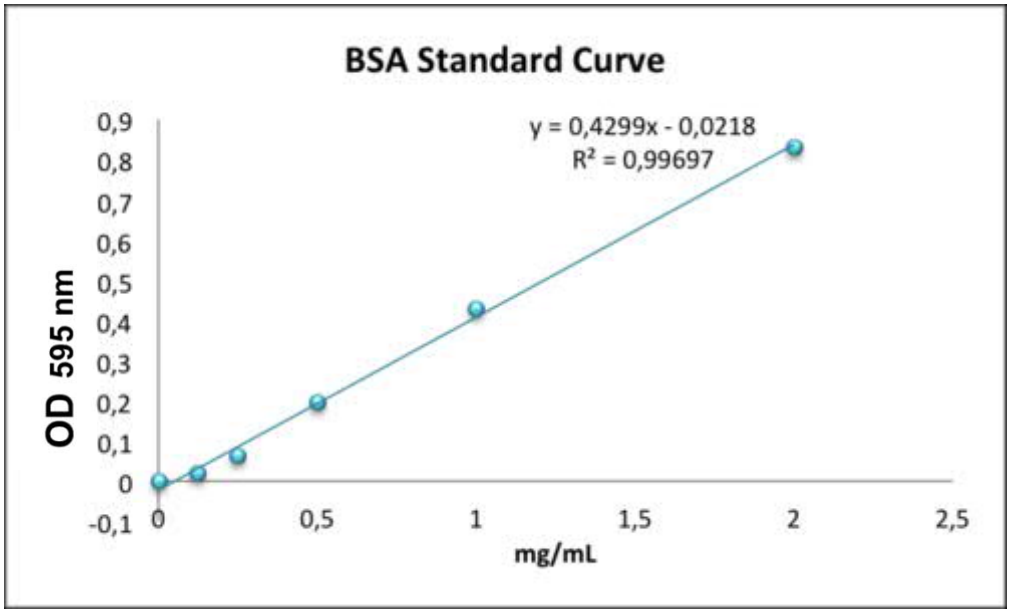
Determination of the protein concentration: Coomassie

- Reacts with the amino acids in protein
- Van der Waals forces and hydrophobic interactions affect also dye binding
- Valid for protein size of more than 3000 Da

- Bio-Rad concentrate is toxic!

Determination of the protein concentration: Standard

- BSA
  - Bovine Serum Albumin
  - Cheap
  - It doesn't affect the reactions
  - Provides the average absorbance
- Standard curve
- Protein concentrations of the samples

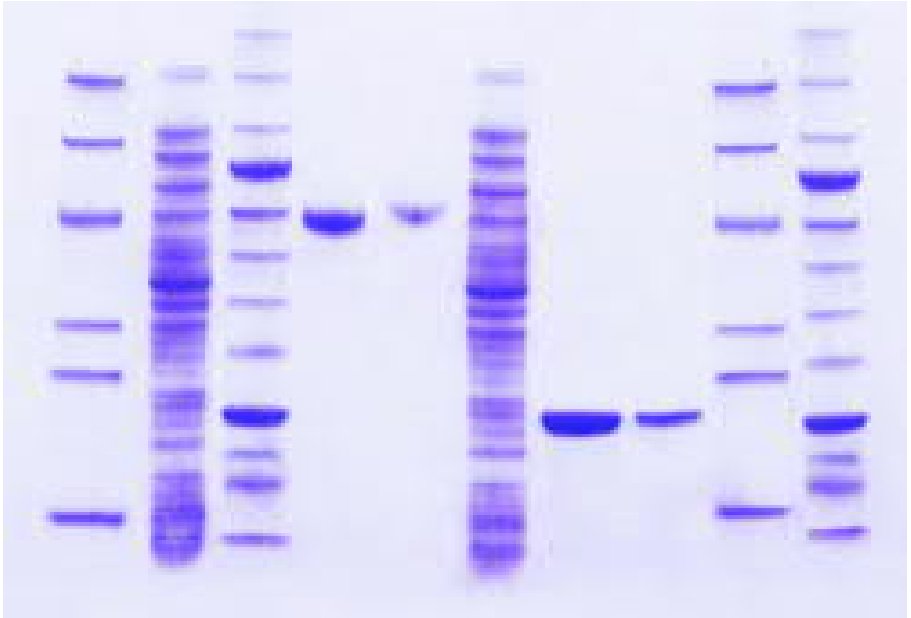


Determination of the protein concentration: Analysis procedure

- The absorbances of the samples in the area of the standard curve
- Parallel samples
- Sample dilutions of 1:10 and 1:50
- Planning before the work day
  - 100 µg/mL stock solution of BSA
  - NOTE! Minimum accurately weighed mass is 50 mg
  - Dilution series for standards 1 – 20 µg/ml
  - Sample dilutions decided on product phase (see instructions)

SDS-PAGE

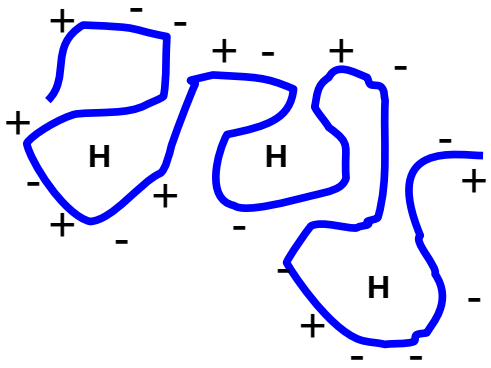
- **S**odium **D**odecyl **S**ulphate **P**oly**a**crylamide **G**el **E**lectrophoresis
- Separation and analysis of proteins
- The proteins are denatured and separated by size



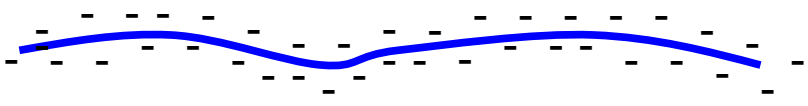
SDS-PAGE: Denaturation

- Denaturation with anionic detergent, sodium dodecyl sulfate (SDS)
- Breaks the secondary and non-disulfide tertiary structure
- Protein becomes negatively charged in relation to the mass
- The sample buffer's mercaptoethanol cuts the sulfur bridges

Protein structure before the SDS addition



Protein's primary structure after the SDS addition

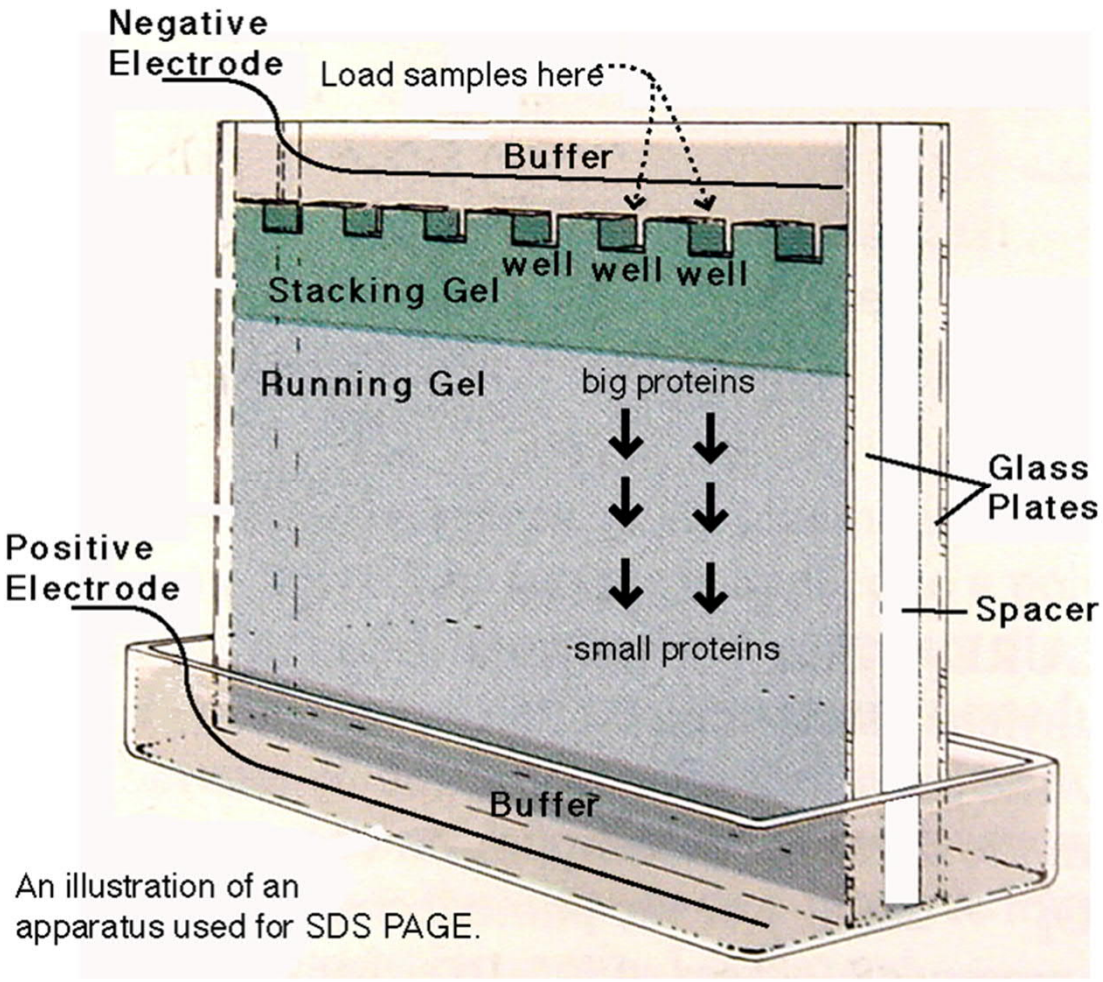


H= hydrophobic areas

+/- = positively and negatively charged side groups

SDS-PAGE: Gel

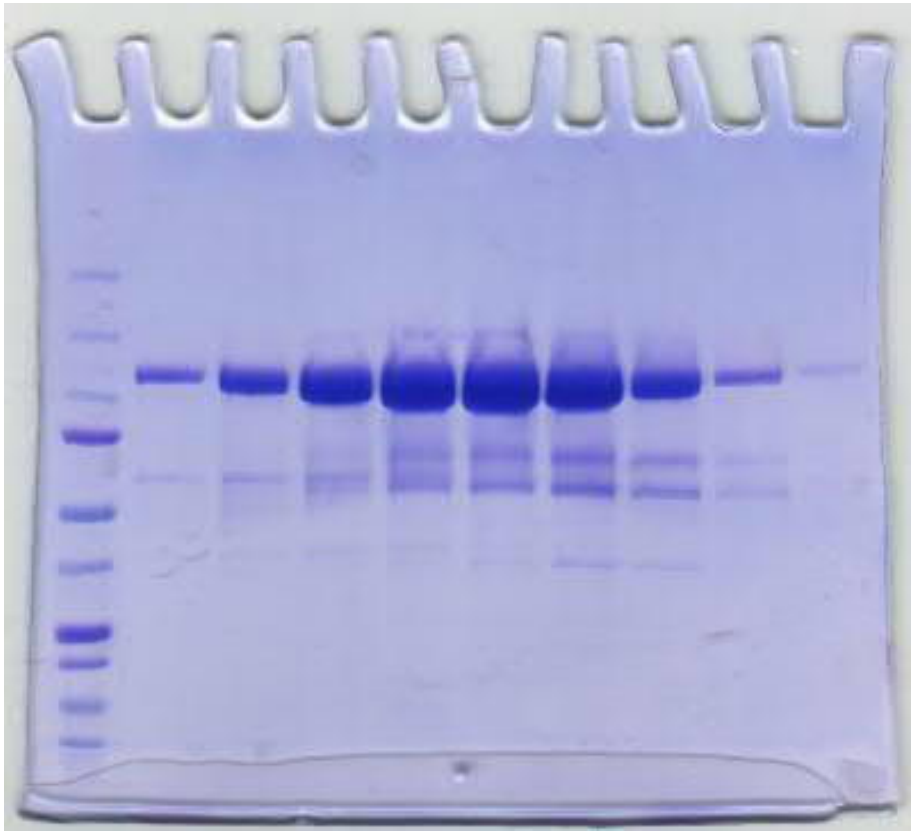
- Gel is composed of various sized pores
  - Changing the acrylamide gel concentration for different pore size
- The movement of proteins in the gel is mainly influenced by
  - The pore size of the gel
  - The voltage difference between the anode and the cathode
  - The size of the protein



An illustration of an apparatus used for SDS PAGE.

- Acrylamide polymerized with APS and TEMED
  - Ammonium persulfate (APS) is a source of free radicals and it's frequently used as an initiator for gel formation
  - Tetramethylethylenediamine (TEMED) stabilizes free radicals and improves polymerization

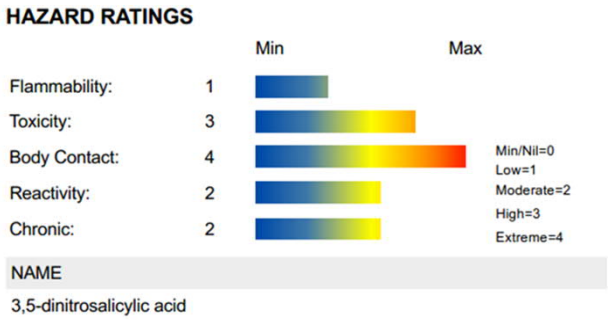
SDS-PAGE: Staining of the gel and detection



- Stained in Coomassie Brilliant Blue R-250 solution
  - 0,25 % Coomassie
  - 50 % methanol and 10 % acetic acid
- Coomassie binds to proteins
- Excess dye is removed
- On the gel stained protein bands appear
- Detection

Xylanase activity assay with the DNS method

Xylanase activity assay: DNS



- 3,5-Dinitrosalicylic acid
- Reacts with reducing compounds
- Harmful substance
  - Inhaled, on skin and if swallowed
  - Irritating to eyes, respiratory system and skin
  - If contact with eyes, rinse immediately and seek medical advice
- Material Safety Data Sheet

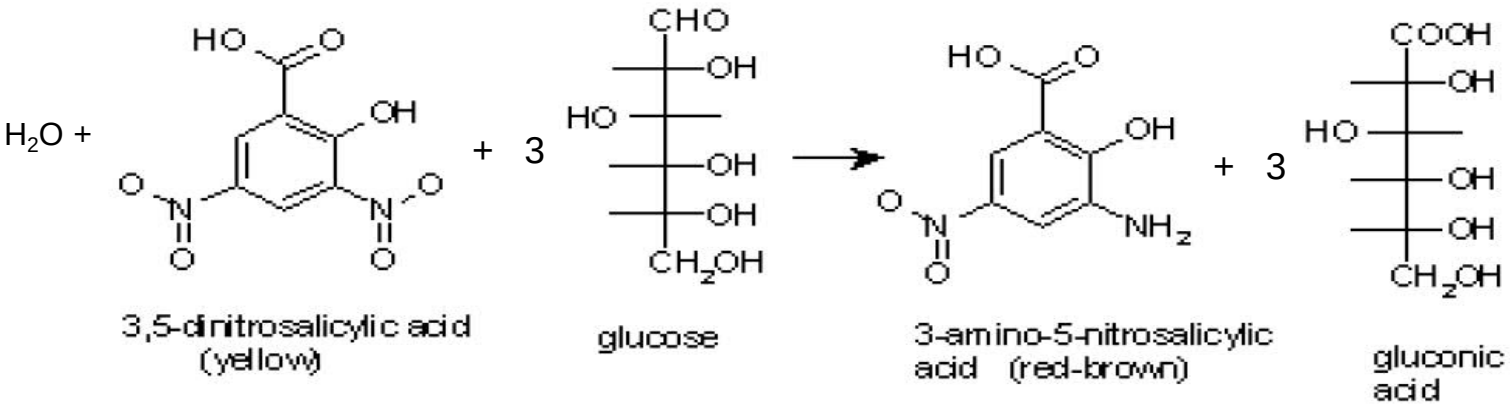
http://datasheets.scbt.com/sc-214181.pdf

Xylanase activity assay: Enzyme reaction

- Substrate + enzyme → product
- Xylan + xylanase → xylo-oligosaccharides
- Reducing ends of sugar chains are released
- Reaction with reducing ends
- Stop the reaction by addition of DNS

Xylanase activity assay: Color reaction

- The formed amount of reducing sugars is measured
- Color reaction from yellow to reddish-brown
- Measured at 540 nm
  - 1 mol of reacted DNS is equivalent to 3 mol of oxidized sugar
- If xylose (reducing sugar) is standard → product is xylonic acid (oxidized product)
- Here same with glucose and gluconic acid:



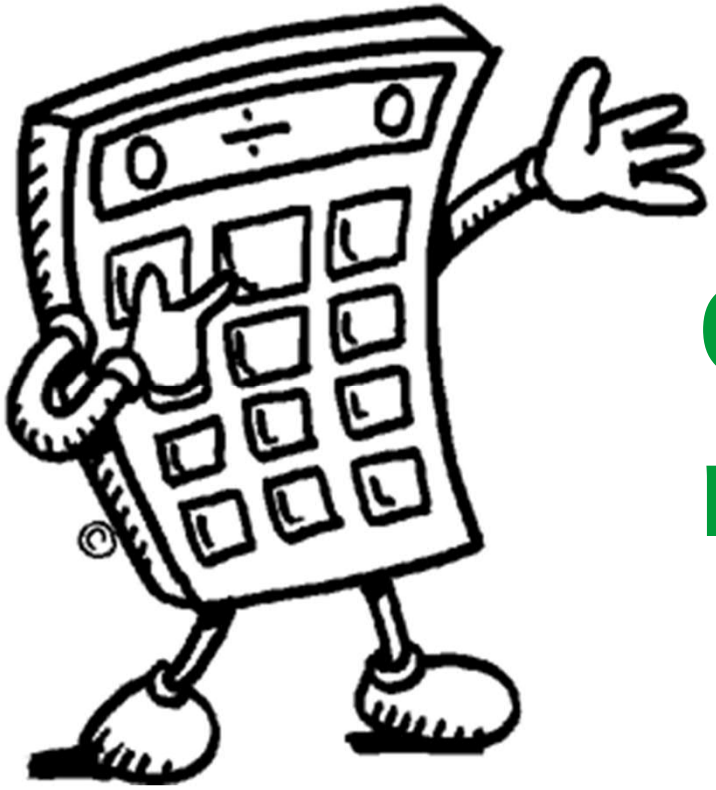
Xylanase activity assay: Procedure

- Preparation of the xylose standard
  - 0.02 M stock solution
  - Dilution series 1 – 20 µmol/ml
- Sample dilutions
  - Given in the work instructions
  - Pipetting chart
- Enzyme blanks
- Approved absorbance values 0.3 – 1.0

Xylanase activity assay: Procedure

- Accuracy with the time measurements!
- The sample measurements have to go on the standard curve
- DNS method has to be done in a fume hood
- Careful handling of DNS
- DNS-waste into a waste container

- In xylanase and Bradford assay → microplate automation will be utilized.



Calculations needed

Activity

- Notice the volumes and dilutions
- Per volume (nkat/mL)
- The xylose standard's absorbances as a function of the concentration of the reducing sugars
  - In purified samples no interfering compounds
  - Cultivation samples need enzyme blank
    - Deleting the background
- With the absorbances of the samples → the amount of reducing sugars generated mol/ml
- The activity is obtained by dividing with the reaction time, mol/s = kat
- Linear reaction is assumed

Total activity

- *Total activity (nkat) =*

  *activity per volume (nkat/mL) * V_{total} (ml)*
- Decreases in the purification process
- When taking only a part of the product to purification calculate final values as if whole product was used

Specific activity

- *Specific activity (nkat/mg) =*

$$\frac{\textit{Activity of sample per volume (nkat/ml)}}{\textit{total protein concentration (mg/ml)}}$$

- Increases in the purification process, when impurities disappear

Purification factor

- Specific activity increases in the purification process

- *Purification factor =*

$$\frac{\textit{Specific activity of sample}}{\textit{specific activity of cultivation sample P1}}$$

- Can be calculated for each step separately or compared to the beginning
- Here the purification factor is calculated compared to the first purification sample P1

Yield

- Total activity decreases in the purification process
- *Yield* =

$$\frac{\Sigma \text{ total activity (nkat)}}{\text{total activity of cultivation sample P1 (nkat)}}$$

- Can be calculated for each step or compared to the first sample
- Here we compare to the first sample

Calculations in report

- These calculations go into the report
- For every calculation there must be an example calculation
  - The report must be readable for someone who has not done the work

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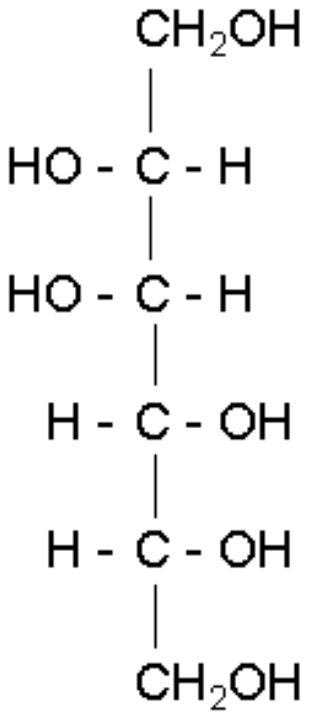
Work 2:
Mannitol production using lactic acid bacteria

Contents

- Mannitol properties
- Applications
- Traditional manufacturing
- Production with lactic acid bacteria

D-mannitol

- Sugar alcohol (polyole)
- White, crystalline
- In nature not in L-form
- Manna, mannite,
  manna sugar
- In plants and animals
  (manna ash
  =mannasaarni)



Properties

- Relative sweetness to sucrose 40-50%
- Low solubility to water
- High positive enthalpy of solution in water
- Very low hygroscopicity
- Chemically inert
- Used in tablets and granulated powders

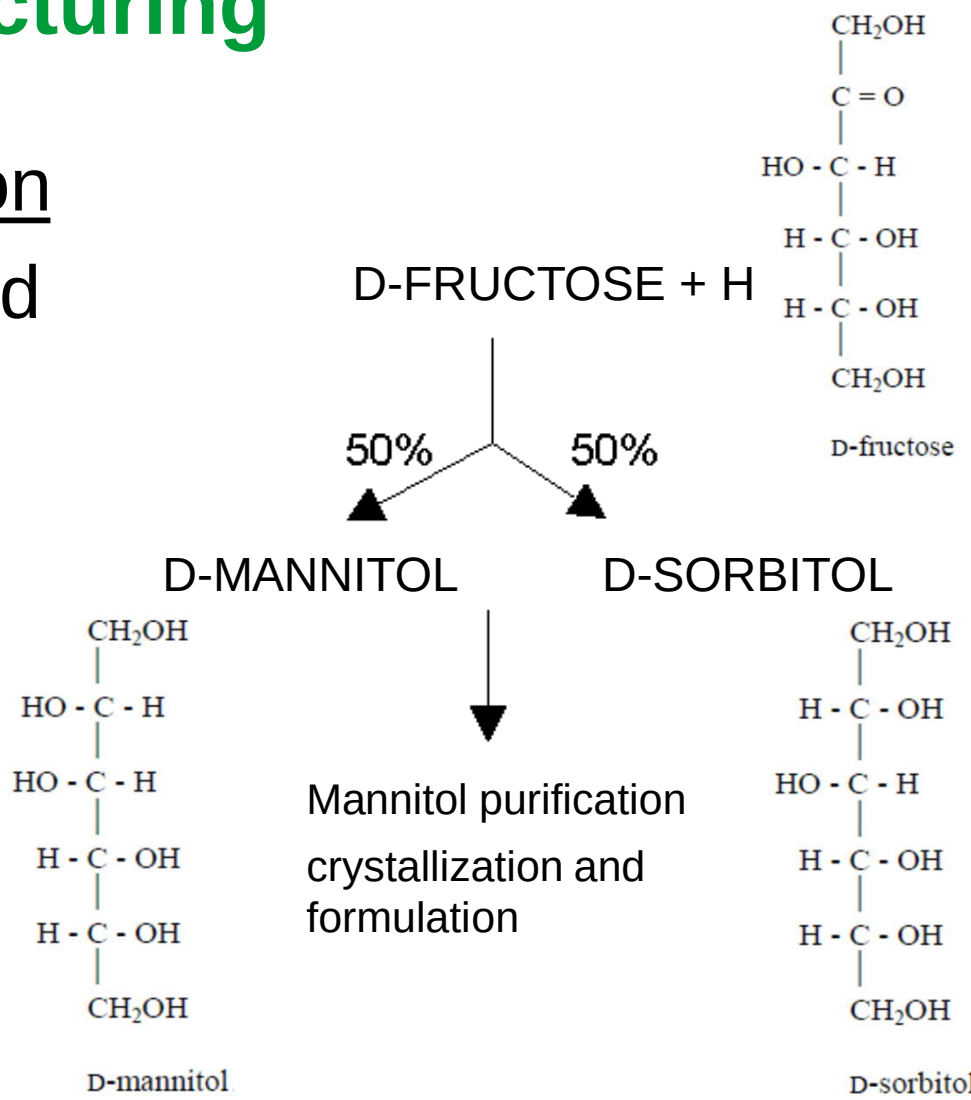
Applications

1. Food products
   - cooling sensation, a sweetener e.g. in sugar-free chewing gums
2. Pharmaceutical product formulation
   - low chemical reactivity, excellent mechanical compressing properties and low hygroscopicity
3. Pharmaceutical product
   - drugs referred to as osmotic diuretics
4. Chemical industries
   - artificial resins and plasticizers

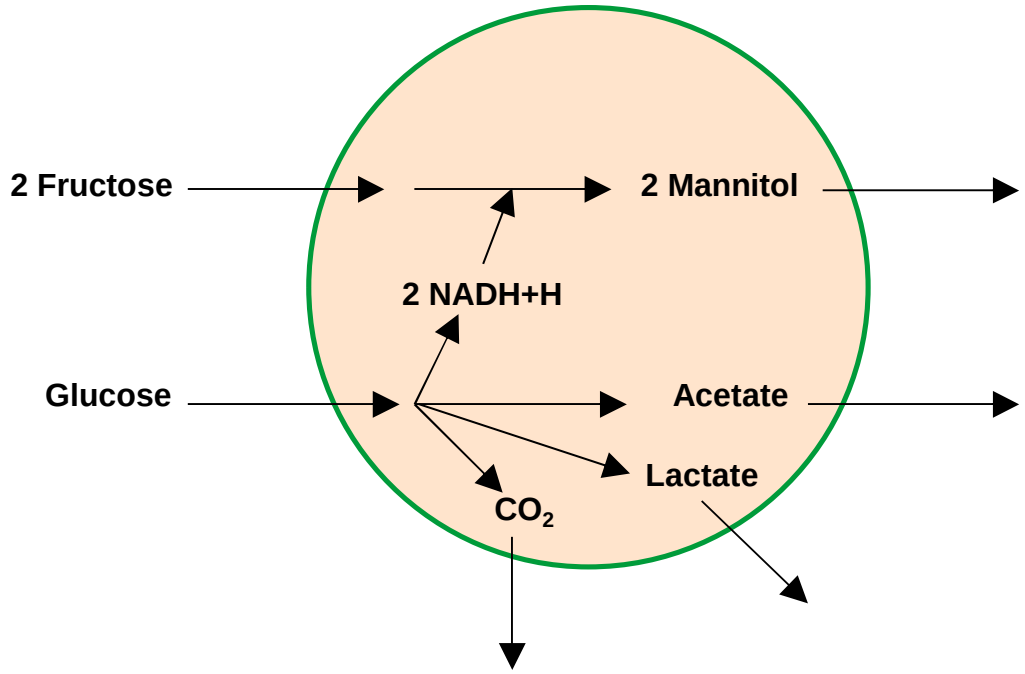
Traditional manufacturing

Chemical hydrogenation

- High temperature and pressure
- Pure fructose
- Pure hydrogen
- Metal catalyst
- Demanding down-stream processing



Mannitol production with lactic acid bacteria

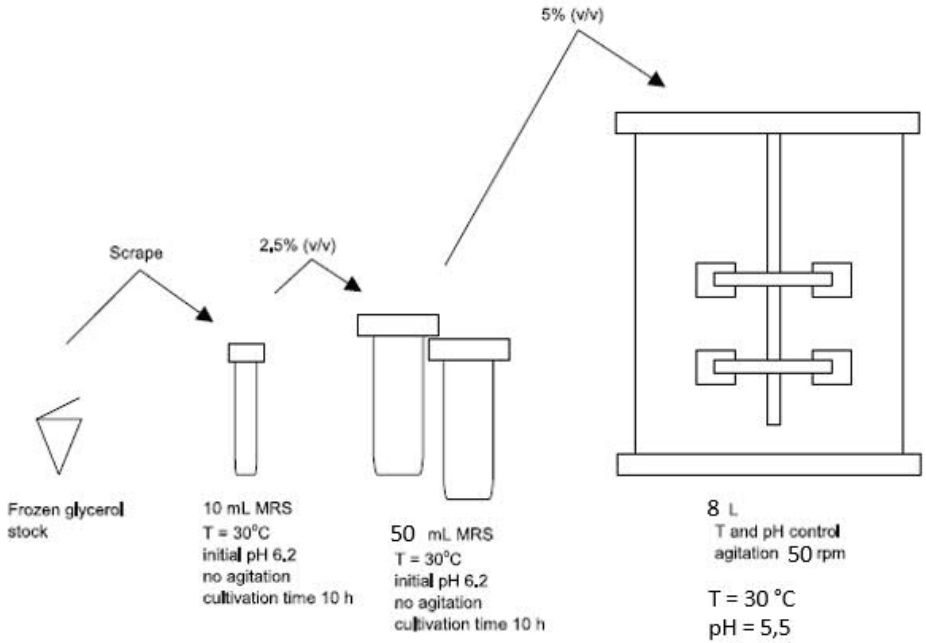


- In homofermentative species, both glucose and fructose are used to produce lactate
- On the other hand, in most heterofermentative species, fructose can act as an external electron acceptor in a reaction involving mannitol dehydrogenase (MDH).

Workflow

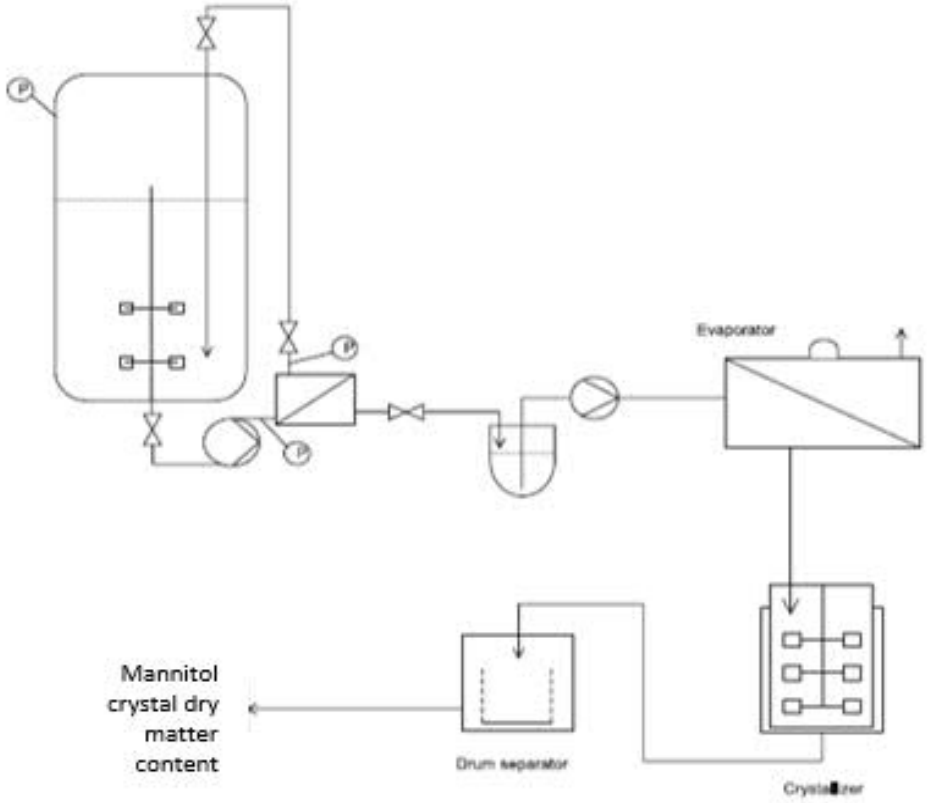
- The aim is to produce 3 kg of mannitol.
- **Monday**: Cultivation media preparation for each cultivation steps. Inoculation of the first pre-culture step.
- **Tuesday**: Inoculation of the second pre-culture. Finalizing cultivation media for reactor cultivations. Preparation and sterilization of 8 L and 100 L reactors. Inoculation of the third pre-culture.
- **Wednesday**: Inoculation of 100 L production reactor. Taking manual samples from the reactor for 8 hours (see analysis), HPCL- standard preparation.
- **Thursday**: End of production cultivation. Start of downstream processing. Weighting CDW samples.
- **Friday**: End of downstream processing. Cleaning up.

Cultivation up to 8L



- First, MRS growth medium is prepared
- 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 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.

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
- **Downstream Processes:** cell separation, product concentration and crystallization

Assay methods

- **Optical density** The optical density of the fermentation broth is measured at 600 nm against distilled water. The samples diluted in the absorbance 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 ^{o}C overnight.
- **HPLC** (High Performance Liquid Cromatography) 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 0.5 and 4 g/L.
- **Prepare HPLC standards:**
  - Glucose, Fructose, Mannitol, 0.5 g/L; 1 g/L; 2 g/L; 4 g/L.
  - Acetic acid and Lactic acid: similar dilution series as for sugars.

Cell production and bioconversion

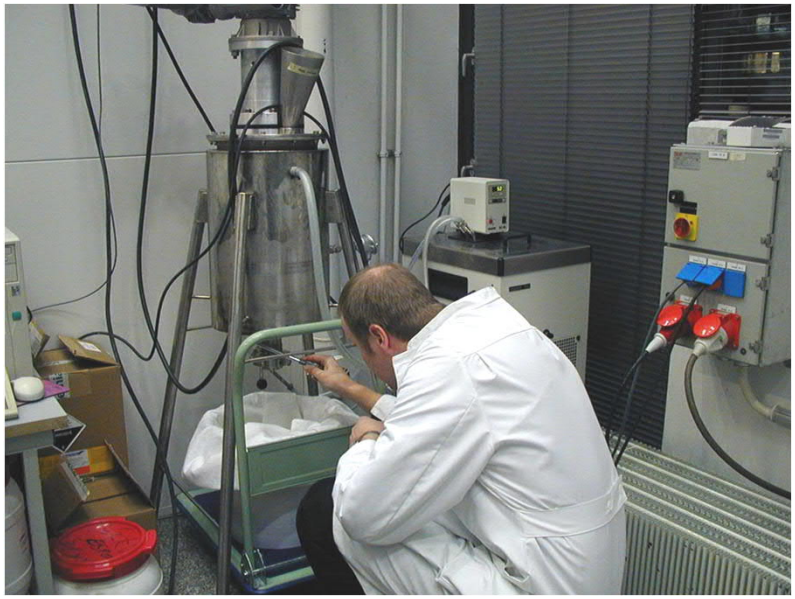
Mixing tank

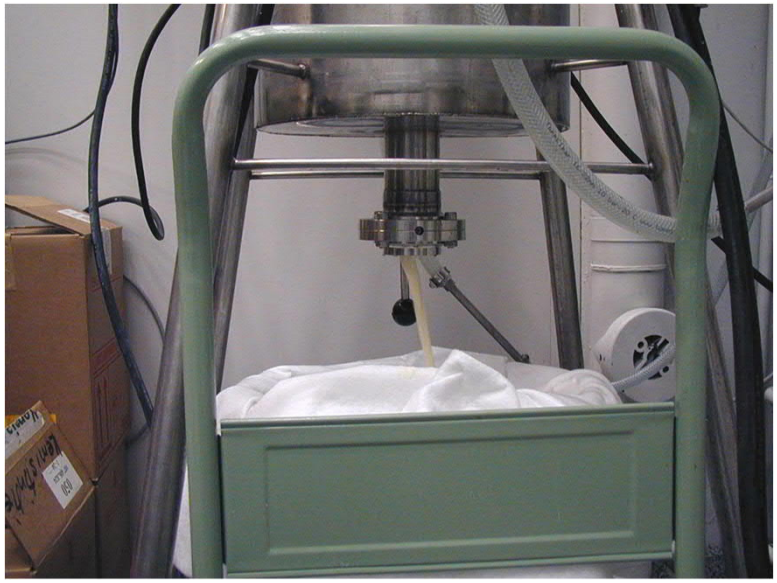
200 L bioreactor

Crossflow filter

Concentration with evaporation

Crystallization





Crystal separation

Important

- Laboratory coat, goggles and masks
- When needed, gloves
- Careful and precise working
- Handling of cuvettes
- Cleaning up after the work