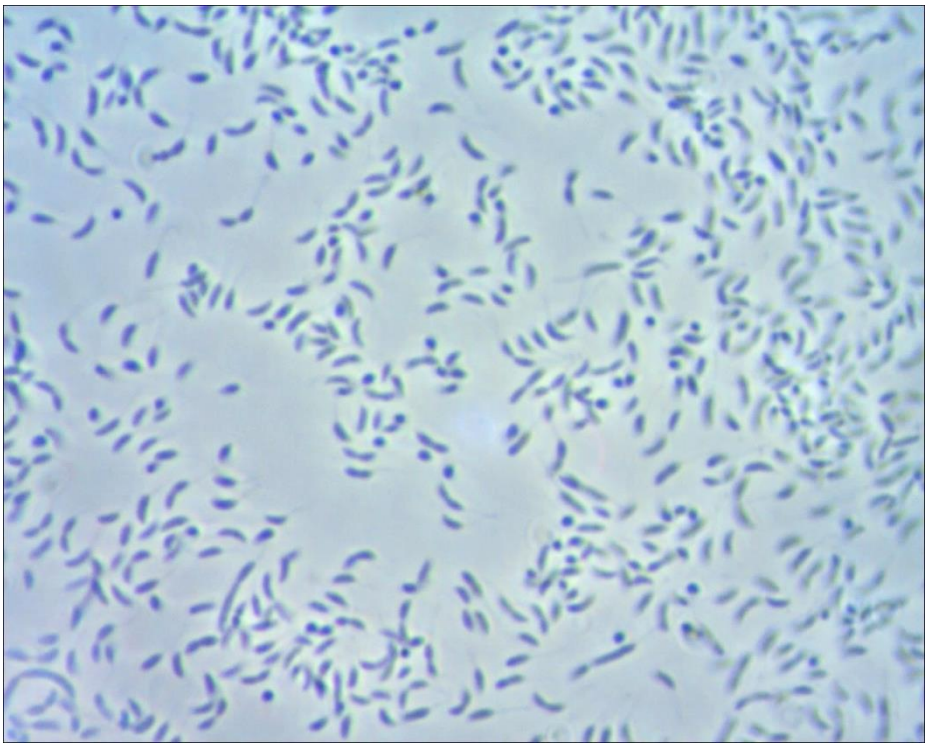
LUND UNIVERSITY

Department of Chemical Engineering

Master's thesis 2016

Physiology of *Caulobacter crescentus* grown on different carbon sources



Sara Lind Jónsdóttir Glaser

Physiology of *Caulobacter crescentus* grown on different carbon sources

Sara Lind Jónsdóttir Glaser

Department of Chemical Engineering

Faculty of Engineering

Lund University

June 2016

Supervisor: Henrik Almqvist

Examiner: Prof. Gunnar Lidén

Picture on front page: *Caulobacter crescentus* taken by Sara Lind Jónsdóttir Glaser

Postal address
P.O. Box 124
SE-221 00 Lund, Sweden
Web address
www.chemeng.lth.se

Visiting address

Naturvetarvägen 16

Preface including Acknowledgments

The degree project was carried out at the Department of Chemical Engineering at Lund University, Faculty of Engineering from November 2015 to June 2016. I would like to thank everyone I worked with at the department. My special thanks to my supervisor, Henrik Almqvist, for guiding me through this project. Your patience, kindness and professional help are greatly appreciated. My examiner, Professor Gunnar Lidèn, for your helpful insights and guidance, humour and positivity. Dr. Lisa Wasserstrom from Division of Applied Microbiology for your kindness and help with the enzyme assay experiments. Also, thanks to Sofia Essén from the Department of Chemistry for the help with the mass spectrometer.

This work is dedicated to my husband, David Glaser, whose love uplifts me every single day, and our son, Markus, whose warm smiles and laughter are more than enough to give me that spark of strength I need to carry on through the challenging days.

Abstract

Freshwater, obligate aerobic, Gram-negative bacterium *Caulobacter crescentus* strain CB2 is able to metabolise various types of carbon sources. In this degree project, growth characterization of *C.crescentus* was made on different substrates including glucose, xylose, and mannose using shake-flask cultivations and bioreactor experiments. Special emphasis was put on the xylose degradation pathway, the Weimberg pathway in *C. crescentus*. This pathway has recently been of interest for introduction into other hosts.

In shake-flask cultivations of *C. crescentus* strain CB2, the highest specific growth rates were found in 10 g/L glucose (0.142 h^{-1}) followed by 10 g/L xylose (0.109 h^{-1}). The organism also grew successfully in 10 g/L mannose but with a lower specific growth rate (0.053 h^{-1}). The main product in cultivations in xylose was α-ketoglutaric acid, the last metabolite in the Weimberg pathway. The strain CB2 showed incomplete substrate consumption whereas another strain tested, CB15, showed complete substrate consumption in shake-flask cultivations using 5 g/L glucose or xylose substrates.

Bioreactor experiments with *Caulobacter crescentus* strain CB2 indicated that the optimal pH for growth was 6.5. The incomplete substrate consumption suggested that growth was limited (or inhibited) by sensitivity to aeration, limiting nutrients (present in yeast extract), or sensivity to some products formed.

Enzyme assays were made for three of the enzymes in the Weimberg pathway (xylose dehydrogenase (*xylB*), xylonate dehydrogenase (*xylD*), and α-ketoglutaric semialdehyde dehydrogenase (*xylA*). Activities of these enzymes were all found, 38.2, 25.4, and 50.9 μmol/(min*mg) protein, respectively, in medium containing only xylose as the carbon source. No activities were found in medium containing glucose as the carbon source.

Table of Contents

Introduction

The world is in great need of a sustainable solution to the exponentially increasing problems associated with oil-based industries. These problems include depletion of natural resources, greenhouse gas emissions, non-sustainable land use, etc. Biorefineries using lignocellulosic feedstock provide a promising answer to this challenge. Unlike its oil counterpart, a biorefinery is based on renewable and sustainable feedstock. Lignocellulosic biomass, which is classified and treated as waste in many commercial and agricultural processes worldwide, is a raw material source. It holds many high-value compounds and can also be converted to a range of compounds, including both high-value and platform chemicals. Ideally, every component of the lignocellulosic feedstock is utilised to produce valuable compounds, and the process is optimised to maximise the market potential (Anastas and Eghbali, 2009; Kamm and Kamm, 2004).

Lignocellulose consists of three major macromolecules: cellulose, hemicellulose and lignin. One of the major hurdles in fully utilising lignocellulose is the conversion of the hemicellulose component. In many plants, hemicellulose is mostly made up of the pentose D-xylose, a sugar which is converted by fewer organisms than glucose. The fungus *Saccharomyces cerevisiae*, known as Baker's yeast, is one of the industrial favourites in fermentation of sugars. It readily ferments hexoses and has high tolerance to heat, pH, and by-products of fermentation such as ethanol (Hahn-Hägerdal et al., 2007). However, Baker's yeast and a few other host organisms used in industry do not convert pentoses. These organisms can be genetically-modified to degrade pentoses by introducing a route leading to its main metabolical pathways (Jeffries, 2006; Chiang et al., 1981; Wang et al., 1980). This is done by introducing genes from organisms that naturally degrade pentoses. In designing metabolic engineering strategies, it is a great advantage to know the natural metabolic behaviour of the organisms where the genes come from. The organism, *Caulobacter crescentus*, holds a pathway for xylose metabolism (Wiebe et al., 2015), i.e. it is a potential source for genes to be used in metabolic engineering.

This thesis work concerns physical characterisation of the organism *Caulobacter crescentus*, which is a freshwater, Gram-negative bacterium able to grow on various carbon sources such as glucose, xylose, and mannose. The aim of the work is in particular to characterise the xylose degradation pathway of *C. crescentus*, which is reported to take place through a metabolic pathway previously identified as the Weimberg pathway (Weimberg, 1961; Stephens, 2007).

For this purpose, shake-flask experiments and bioreactor cultivations of *C. crescentus* were done on various carbon sources, with special emphasis on xylose. Analytical methods such as HPLC, LC-MS and enzymatic assays were used to characterise the metabolites and enzymes in the metabolic pathway.

1 Background

1.1 Lignocellulose

Lignocellulosic biomass, or plant biomass, is a widely untapped renewable resource that can serve as raw material for production of high-value compounds as substitute for the petrochemically-derived chemicals. It is abundant in nature and, importantly, waste from many agricultural and industrial processes is largely comprised of lignocellulose (Hamelinck et al., 2005). Lignocellulosic materials consists of three main components: lignin, cellulose and hemicellulose (Figure 1). Lignin is composed of aromatic polymers.

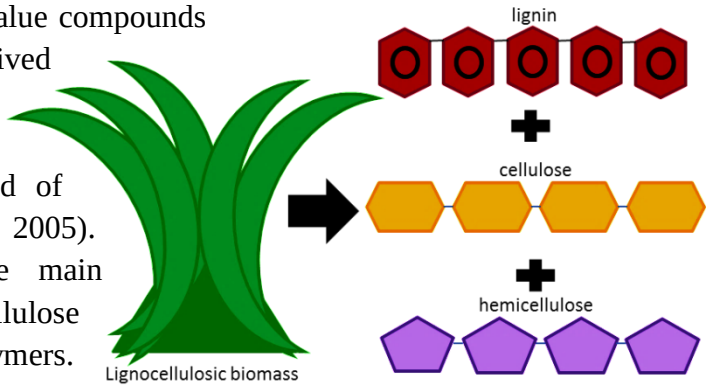


Figure 1. Three main components of lignocellulosic biomass: lignin, cellulose and hemicellulose.

Cellulose is a linear polymer in which glucose monomers are linked by β-(1:4)-glycosidic bonds. Lastly, hemicellulose is mostly composed of xylan and glucomannan polymers. D-Xylose is the most abundant pentose sugar present in hemicellulose (Glazer and Nikaido, 2007).

1.2 Industrial microoganisms used in conversion of lignocellulosic biomass

Industrial processes that utilise lignocellulosic biomass for production of desired compounds require potent microorganisms that are not only efficient producers but can also withstand harsh conditions in the processes. *Saccharomyces cerevisiae* or Baker´s yeast has a long history of being the key organism used in largest fermentation industries in the world. *S. cerevisiae* is not only robust which makes it ideal for industrial processes, it also serves as a key eukaryotic model organism and its cell biology has been extensively studied (Hahn-Hägerdal et al., 2007). Other promising industrial microorganisms include *Pseudomonas putida*, used to produce aromatic compounds from biomass, and *Corynebacterium glutamicum*, used for amino acid production (Meijnen et al., 2009; Gopinath et al., 2011).

Unfortunately, *S.cerevisiae*, *P.putida* and *C.glutamicum* can not degrade xylose. Efforts have been made to genetically engineer these organisms so that they would be able to degrade xylose (Hahn-Hägerdal et al., 2007; Meijnen et al., 2009; Gopinath et al., 2011). Engineering strategies that aim at incorporating a xylose degradation route into the main metabolic pathways of these industrial microorganisms or any desired hosts involve using genes from other organisms that naturally have a xylose conversion pathway. It is of great advantage to characterise the natural behaviour of these source organisms for optimal metabolic engineering designed to maximise the desired product yield with maximum substrate consumption and minimum by-product formation. One example of an organism that holds potential source for genes required in xylose metabolism is *Caulobacter crescentus.*

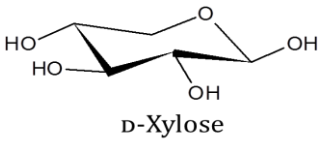


Figure 2. Structure of xylose.

1.3 Caulobacter crescentus

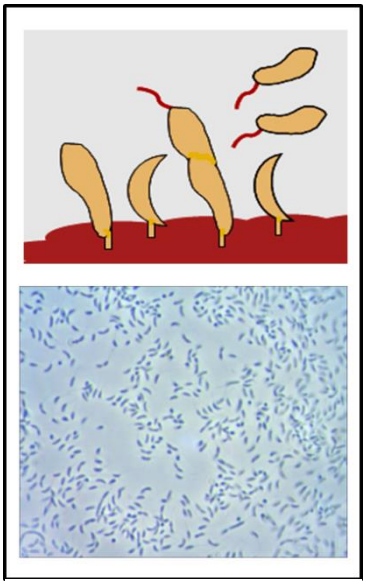


Figure 3. (above) *Caulobacter crescentus* in microscope. (below) Different progenies: swarmer and stalked cells (adapted from Laub et al., 2007).

Caulobacter crescentus is a Gram-negative freshwater aerobic, rod or crescent-shaped bacterium that thrives in very low nutrient conditions. It has an assymetrical cell division mechanism that involves the production of different progenies: stalked and swarmer cells. The stalked cell maximises the uptake of nutrients locally whereas the flagellated swarmer cells move from the site of the „parent" stalked cell to obtain nutrients elsewhere (Figure 3b) (Laub et al., 2007). Stalked cells secrete an adhesive material from its distal end which makes it stick to a surface. Similarly, swarmer cells secrete the same adhesive material from the end of the flagella as they enter their immobile vegetative phase. This adhesive compound is used by *C. crescentus* to stick to any surface including other microorganisms, substrates, or to form a group of clustered cells attached to each other, also called rosettes (Poindexter, 1964)

The natural environment of *C. crescentus* is characterized by extended periods of nutrient starvation. Nutrients and energy source that the organism needs for survival mostly come from the sparsely scattered organic matter from decaying plants (Laub et al., 2007). To cope with these conditions, the organism has evolved a set of cellular mechanisms such as environmental sensors, transporters and metabolic pathways that help facilitate the uptake of the metabolites from plant debris (Blanvillain et al., 2007, Hottes et al., 2004).

Since a significant portion of plant biomass consists of hemicellulose whose primary consituent is D-xylose, it is only logical that *C. crescentus* has a special metabolic pathway that utilises the pentose. Stephens et al. (2007) have identified that the pathway for D-xylose metabolism in *C. crescentus* was comparable to previously studied arabinose-degradation pathway in *Azospirillum brasilense* (Watanabe, 2006). Both of these pathways follow the xylose and arabinose degradation pathways as described by Weimberg (1961) in *Pseudomonas fragi*, hence the name, the Weimberg pathway.

1.4 Weimberg Pathway

The Weimberg pathway in *Caulobacter crescentus* starts with D-xylose as a carbon and energy source. The pathway yields α-ketoglutarate which enters the tricarboxylic acid (TCA) cycle. Figure 4 illustrates the Weimberg pathway on the left-hand side as compared to the glucose metabolism on the right-hand side.

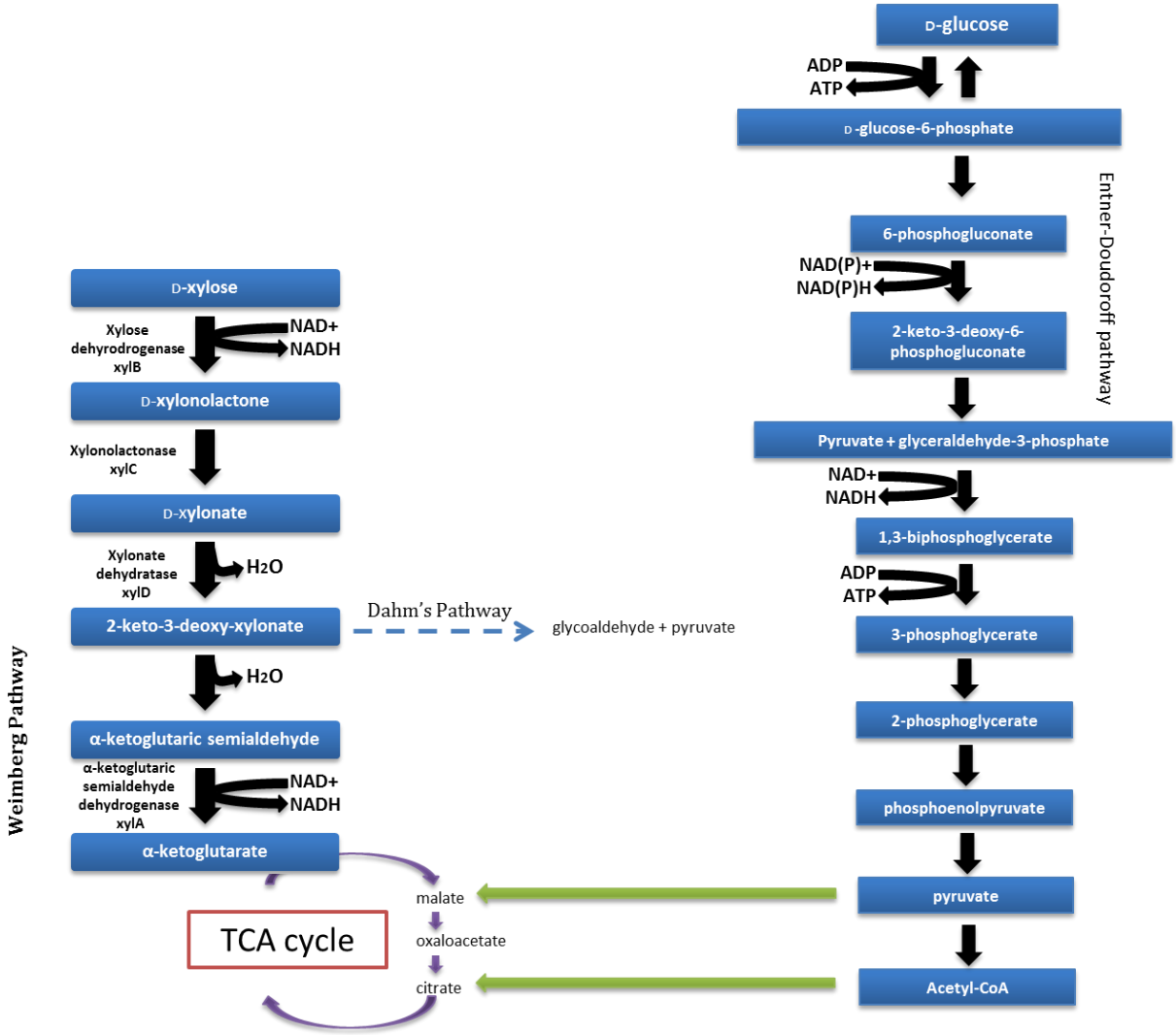


Figure 4. Glucose and xylose metabolic pathways in *Caulobacter crescentus* (adapted from Stephens et al., 2007).

The first part of the Weimberg pathway has similarities with the Entner-Doudoroff pathway – the former produces 2-keto-3-deoxy-xylonate while the latter produces 2-keto-3-deoxy-6-phosphogluconate (Stephens et al., 2007). The similarity is even more apparent in Entner-Doudoroff pathways in archaeic bacteria where there is no phosphorylation of glucose (Romano and Conway, 1996).

Meisenzahl et al. (1996) identified the gene *xylX* on *C. crescentus* that is required for the organism to grow on xylose as a sole carbon source. Subsequently, four other genes (*xylA*, *xylB*, *xylC*, and *xylD*), that code for the enzymes in the Weimberg pathway, were all identified to be necessary for growth on xylose. A deletion of any of these five genes renders no growth on xylose by *Caulobacter crescentus* (Stephens et al., 2007).

The first enzyme in the Weimberg pathway, xylose dehydrogenase (*xylB*), is an NAD+ dependent enzyme. *xylB* catalyses the conversion of xylose into xylonolactone. The second enzyme, xylonolactonase (*xylC*), converts xylonolactone into xylonate, which is then converted into 2-keto-3-deoxyxylonate by the third enzyme in the pathway, xylonate dehydratase (*xylD*). 2-keto-3-deoxyxylonate undergoes a dehydration reaction as catalysed by enzyme *xylX* which leads to α-ketoglutaric semialdehyde. It is then converted into α-ketoglutaric acid by the final Weimberg enzyme, α-ketoglutaric semialdehyde dehydrogenase (*xylA*) using NAD+ as co-factor.

1.5 Recent biotechnological applications of genetic modifications involving *C.crescentus*

The genes encoding for the Weimberg enzymes can be of use for metabolic engineering in various host organisms, e.g. *S. cerevisiae.* The D-xylose dehydrogenase (*xylB*) of *Caulobacter crescentus* has been found to have >30 fold higher activity than D-Xylose dehydrogenase (*xyd1*) of *Trichoderma reesei* (Toivari et al., 2010). When *xylB* was expressed in *S.cerevisiae* for xylonate production from xylose, volumetric and specific production rates of were found to be ten times higher than when *xyd1* from *T. reesei* was expressed in *S. cerevisiae.* Even better xylonate production was observed when *xylC* was also expressed in *S.cerevisiae.* The addition of *xylC* contributed to the increase of metabolic flux towards xylonate instead of xylitol (Toivari, 2015).

In addition to this, genetic modifications done directly on *C. crescentus* have shown to have some promising applications. An engineered strain *C.crescentus* JS40222/p723-6H showed efficiency in removing heavy metal cadmium from contaminated water. The two powerful characteristic of the strain – cell surface display and self-immobilisation – made it possible to completely separate heavy metal ions from water in a single step (Patel et al., 2010).

Research literature shows a huge amount of studies on molecular genetics of *Caulobacter crescentus.* The organism is a model organism for assymetric cell division studies and its genome is well-documented (Jenal and Stephens, 2007; Laub et al., 2000). However, very few studies have been made on the physical characterisation of the organism. It is therefore the primary aim of this Master‘s thesis to investigate the growth characteristics and product formations of *Caulobacter crescentus* on carbon sources including glucose, xylose and mannose both in shake-flask and bioreactor cultivations.

2 Materials and Methods

Bacterial strains

Two different strains of *Caulobacter crescentus* were used in this study; CB2 (DSMZ: strain DSM-4727) and CB15 (NCIMB: strain NCIM89789). Most of the work was done using CB2, and only a few shake-flask experiments were done using CB15.

Media

Rich medium (PYE) consisted of 0.2% peptone, 0.1% yeast extract, 0.8 mM $MgSO_4$, and 0.5 mM $CaCl_2$. 10x stock of minimal medium (M2) consisted of 17.4 g Na_2HPO_4, 10.6 g KH_2PO_4, and 5 g NH_4Cl, per litre. After autoclaving the 10x M2 medium, it was diluted down to 1x. Sugar (200 g/L glucose, xylose, mannose) was aseptically added according to desired concentration (5, 10 or 20 g/L). Mineral medium consisted of 50 mM $MgCl_2$, 50 mM $CaCl_2$, 1 mM $FeSO_4$, and 0.8 mM EDTA, per litre (Ely, 1991).

Shake-flask cultivation

250 mL Erlenmeyer flasks were used with working volume of 50 mL. Duplicate experiments were done on glucose, xylose, arabinose and mannose as substrates. Three different initial sugar concentrations (5, 10 and 15 g/L) were done on glucose and xylose, while 10 g/L was tested mannose.

An overnight culture of CB2 grown in PYE was used to inoculate shake-flasks with M2 medium and the sugars. Inoculation OD_{620} was set at approximately 0.1 Absorbance. The shake-flasks were incubated at 33°C, 200 rpm using Kuhner Shaker and Incubator. Growth in the shake-flasks were observed at different time intervals using optical density measurements and sample taking.

Bioreactor cultivation

1L bioreactors (Sara small bioreactor control serie 2000, Belach AB, Stockholm, Sweden) with working volume of 500 mL were used in batch cultivations. The bioreactors were autoclaved with approximately 400 mL water, 50 mL 10x concentrated M2 medium and 25 mL 1% yeast extract. After autoclaving, 5mL mineral solution (50 mM $MgCl_2$, 50 mM $CaCl_2$, 1 mM $FeSO_4$, and 0.8 mM EDTA), 25 mL glucose or xylose (200 g/L), and an inoculum volume that would correspond to approximately OD_{620} 0.1 in the final volume of the bioreactor. The inoculum was taken from an overnight culture of *Caulobacter crescentus* CB2 in PYE. Figure 5 shows the setup of the batch experiment. 3M NaOH was used for pH control, stirring rate of 300 rpm, 0.1 L/min aeration, and temperature at 33°C.

Figure 5. Bioreactor setup.

Absorbance measurements

1 mL cultivation samples were observed using a spectrophotometer (V-1200, VWR, Radnor, Pennsylvania) at 620 nm throughout the cultivation. The samples were diluted to achieve an optical density (OD) between 0.1-0.4, with DI water.

Dry weight measurements

2mL triplicate samples were taken from the cultivation at late growth phase. The samples were centrifuged for 2 minutes at 12,000 x g u (Mini Scanspeed, Randburg, South Africa). Supernatant was discarded. 1 mL 0.9% NaCl solution was added to the samples for washing. The sample tubes were centrifuged again with the same settings. Supernatant was discarded and 1 mL deionised water was added to each tube, and the cells were mixed until homogenous. The cell solution was transferred to pre-weighed dry weight tubes which had been in 105°C oven (Termaks, Bergen, Norway) for at least 3 hours. The dry weight tubes with the 4 mL sample cell solutions were incubated for approximately 24 hours at 105°C. The tubes were weighed again to determine the weight of the cells without moisture.

Cell dry weight (CDW) was measured to determine the biomass yield as a function of time in the shake-flasks. The correlation factor between OD and biomass was found to be 0.3935 in glucose medium and 0.5642 in xylose. (See Appendix II).

Microscopy

Cell samples were observed using a light microscope (CH40, Olympus, Tokyo, Japan) for general observation and possible contamination checks. The microscopic (Optiphot, Nikon, Tokyo, Japan) photographs were taken using the camera VisiCam®1.3 (VWR, Leuven, Belgium).

YSI Biosensors

A complementary sugar measurement for glucose and xylose were done using a dual channel biosensor (YSI 2700/230V, Yellow Springs, USA). Filtered samples (0.2 µm membrane filter) from cultivation were analysed using the biosensor.

HPLC

600 µL filtered samples from cultivation were transferred into HPLC vials. Two sets of standards were prepared for HPLC (Waters, Millford, MA, USA) analysis: one standard set with glucose, xylose, α-ketoglutaric acid, and glycerol and the other standard set with glucose, xylose and mannose. Two standard sets were necessary to avoid the merging of HPLC peaks for mannose and xylose. The table of dilution is shown in Appendix I. The samples were analysed on Aminex HPX-87H (BIO-RAD Aminex, Hercules, CA, USA) column at 60°C. The mobile phase used was 5 mM H_2SO_4 with a flow rate of 0.60 ml/min. The analytes were detected by both refractive index (Waters 2410) and ultraviolet (Waters 2487) detectors.

LC-MS

Cultivation samples were sent to the Department of Chemistry at Lund University Faculty of Engineering to determine the unknown peaks observed in the HPLC analysis. Liquid Chromatography - Mass spectrometer (UPLC-QTOF/MS, Waters Millford, MA, USA) was used for this purpose.

Cell harvesting

For protein determination and enzyme activity assays, cells were harvested at late exponential phase of growth. The cell sample were centrifuged at 3000 x g, 8°C for 15 minutes using

Hermle centrifuge (Hermle Z513K, Wehingen, Germany). The supernatant was discarded and the cells frozen at -20°C.

Protein extraction

Frozen cells were thawed and centrifuged at 10,000 x g, 4°C for 5 minutes using Heraeus fresco 21 centrifuge (Thermo Fisher Scientific, Langenselbold, Germany). The supernatant was discarded from the cell pellet. 200µL of Yeast Protein Extraction Reagent (Y-PER; Thermo Scientific, Pierce, Rockford, USA) was added for approximately 50 mg of cell pellet. The cells were resuspended in the reagent by pipetting up and down. The cell solution were agitated at 30 rpm, 25°C for 20 min using an incubator shaker (New Brunswick™ Innova 40 Incubator shaker series, Eppendorf, Hamburg, Germany). After agitation, the cell solutions were centrifuged at 14,000 x g, 4°C for 10 minutes. The supernatant containing the extracted protein was transferred to a new Eppendorf tube and kept at -20°C.

Protein determination was done using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) used as the standard curve.

Enzymatic Assays

Enzyme activities were measured using an Ascent Multiscan thermostated plate reader (Thermo Fisher Scientific, Waltham, MA, USA). The enzymes encoded by *xylB* and *xyl*A have a coupled NAD+/NADH conversion hence the enzyme activity is directly proportional to NADH production. The activity of the dehydratase encoded by *xylD* was not directly measured but estimated from the sequential conversion by proteins encoded by x*ylX* and *xylA*, hence the name *xylDXA* in the assays.

The composition of each well in the assays is shown in Table 1.

Table 1. Stock solution composition for the enzyme assays.

xylB	xylDXA	xylA
20 µl 1 M Tris-HCl, pH 8.0	20 µl 1 M Tris-HCl, pH 8.0	20 µl 1 M Tris-HCl, pH 8.0
0.4 µl 1 M MgCl2	2 µl 1 M MgCl2	0.4 µl 1 M MgCl2
20 µl 20 M NAD+	20 µl 20 M NAD+	20 µl 20 M NAD+
139.6 µl MQ-H2O	121 µl MQ-H2O	139.6 µl MQ-H2O
5 µl protein extract	5 µl protein extract	5 µl protein extract
Reactant:	Reactant	Reactant:
15 µl 200 g/L xylose	32 µl 500 mM Ca-xylonate	20 µl 50 mM glutaraldehyde

The reactant was added to each well right before spectrophotometric measurements. Absorbance measurements were done at 340 nm, 30°C for 10 minutes. (Note that glutaraldehyde was used as the reactant for enzyme encoded by *xylA*. Glutaraldehyde has a similar affinity to *xylA* as α-ketoglutaric semialdehyde, the natural substrate of enzyme *xylA*, and is easier to obtain) (Meijnen et al., 2009; Johnsen et al., 2009).

Enzyme Activity calculations

A standardised linear regression of NADH absorbance values at 340 nm as a function of NADH concentration rendered Equation 1.

$$Absorbance\ (340\ nm) = 0.0032\ [NADH] + 0.0566 \qquad \text{Equation 1}$$

See Appendix V for more information on the NADH standard curve (Muñoz de Las Heras, 2016).
The slope (ς) of Eq. 1 , ς = 0.0032, gives the NADH concentration in μmol from the absorbance values.

The unit of enzyme activity is normally characterised as one enzyme unit (**U**) which indicates the conversion of **1 μmol of substrate NAD+ to NADH per minute**. Measured slope (A/min) are converted into μmol NADH using the factor ς = 0.0032 from Eq.1. And finally, the value is multiplied to the amount of protein (mg) in the extract. See Appendix V for protein determination.

Equation 2 shows the calculation for enzyme activity, followed by the calculation for specific enzyme activity per mg protein in the sample.

$$Enzyme\ activity\ \left({}^{\mu mol}/_{\text{min}}\right) = \frac{\left(\frac{Abs_{340}}{min} - blank\right)}{\varsigma} \qquad \text{Equation 2}$$

$$Specific\ enzyme\ activity\ \left({}^{\mu mol}/_{min * mg\ protein}\right) = Enzyme\ activity\ \div (protein\ concentration * extract\ added)$$

An example of calculation of xylose dehydrogenase (*xylB*) activity assay in a protein extract from a bioreactor cultivation with M2 and xylose is as follows.

$$Specific\ enzyme\ activity_{xylB} = \frac{0.006 - 8*10^{-7}}{0.0032} \div \left(\left(4038\ \frac{\mu g\ protein}{mL} * 0.005 mL\right) * \frac{1mg}{1000 \mu g}\right) = 38.18\ {}^{\mu mol}/_{(min * mg)}$$

For flux capacity conversion, specific enzyme activity is converted into specific enzyme activity per gram biomass as shown below.

$$xylB: \frac{specific\ enzyme\ activity}{CDW} * MW_{xylose} * \frac{1h}{60min}$$
$$= \frac{38.18\ \mu molmin^{-1}mg\ protein^{-1}}{2.489\ mg} * 150.13 \frac{g}{mol} * \frac{1\ mol}{1000\ mmol} * \frac{1h}{60min}$$
$$= 0.0382\ g_{xylose} g_{CDW}^{-1} h^{-1}$$

3 Results

3.1 Shake-flask cultivations - growth

Caulobacter crescentus strain CB2 was grown in various substrates (glucose, xylose and mannose) with different concentrations for glucose and xylose. Optical density at 620 nm were measured as a function of time to monitor the growth of the organism. Three concentrations of substrates glucose and xylose (5, 10 and 20 g/L) were used initially, and the measured absorbance values are shown in Figure 6.

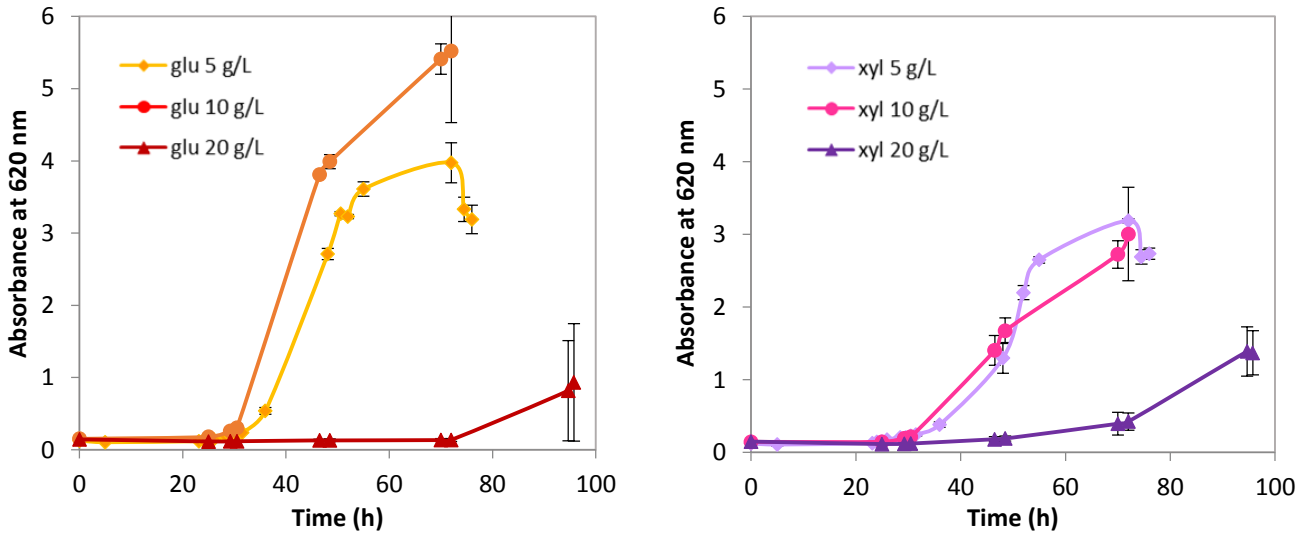


Figure 6. Shake-flask cultivations of CB2 in different concentrations of glucose (left) and xylose (right). Error bars indicate standard deviation of duplicate measurements.

A lag phase of approximately 1 day was observed in both 5 and 10 g/L substrate, whereas in 20 g/L the lag phase was 3 days. Cultivations in glucose generally showed higher growth than in xylose, except for the highest concentration, 20 g/L, where xylose cultivation was slightly higher. To further look into the exponential phase, the specific growth rate (μ) was determined.

$\mu = \frac{ln\frac{x}{x_0}}{t-t_0}$; where x denotes biomass and t denotes time. Equation 3

The specific growth rate was found by plotting the logarithm of the measured optical density as a function of time. The slope of the curve (obtained by linear regression) gives the specific growth rate in the exponential phase of growth of *C.crescentus.*

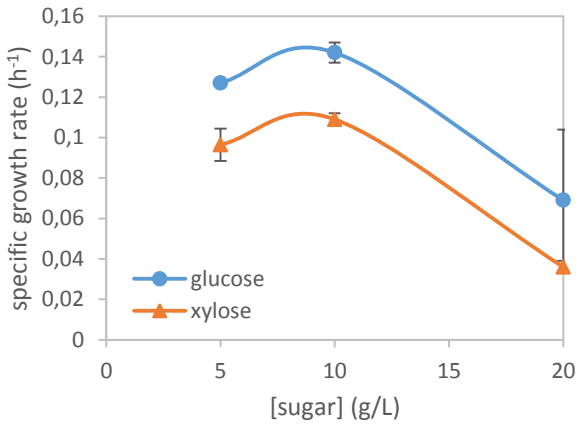


Figure 7. Growth rate as a function of substrate concentration. Error bars indicate standard deviation of duplicate measurements.

Figure 7 illustrates the relationship between the specific growth rates of *Caulobacter crescentus* for various initial concentrations of glucose or xylose. The sharp decrease at 20 g/L indicates substrate inhibition at high substrate concentration.

It can also be seen that the plot deviates from the standard Monod kinetics (Equation 4).

$\mu = \mu_{max} \frac{s}{k_s + s}$; where k_s denotes enzyme saturation constant Equation 4

Instead of reaching maximum growth rate (μ_{max}) at higher substrate concentration as Eq. 4 indicates, *Caulobacter crescentus* cultivation showed growth inhibition at increased substrate concentration.

pH changes

pH values before and after shake-flask cultivations varied as shown in Table 2. Initial pH is usually set at neutral pH 7, however, at the end of fermentation, the pH dropped as low as pH 4.4 in glucose and even more acidic, pH 4.3, in xylose. Similar observations were seen the cultivation on mannose.

Table 2. Initial and final pH of shake-flask cultivations in glucose and xylose.

Substrate	Concentration	pH	
	(g/L)	Initial	Final
glucose	5	7.02	5.03
	10	6.92	4.41
	20	6.94	4.96
xylose	5	7.01	4.49
	10	6.95	4.34
	20	6.96	4.60
mannose	10	7.03	4.83

The pH drop indicates production of acidic compounds during the fermentation. Another possible explanation of the observed drop in pH is the uptake of ammonium ions during growth. The consequent acidic environment in the cultivation may have contributed to the eventual death of the cells, as *Caulobacter crescentus* normally thrives in pH range of 6.1 to 7.8 (Poindexter, 1964).

Product Formation

The products of fermentation of *Caulobacter crescentus* in shake-flask cultivations were analysed using HPLC (Figures 8 and 9).

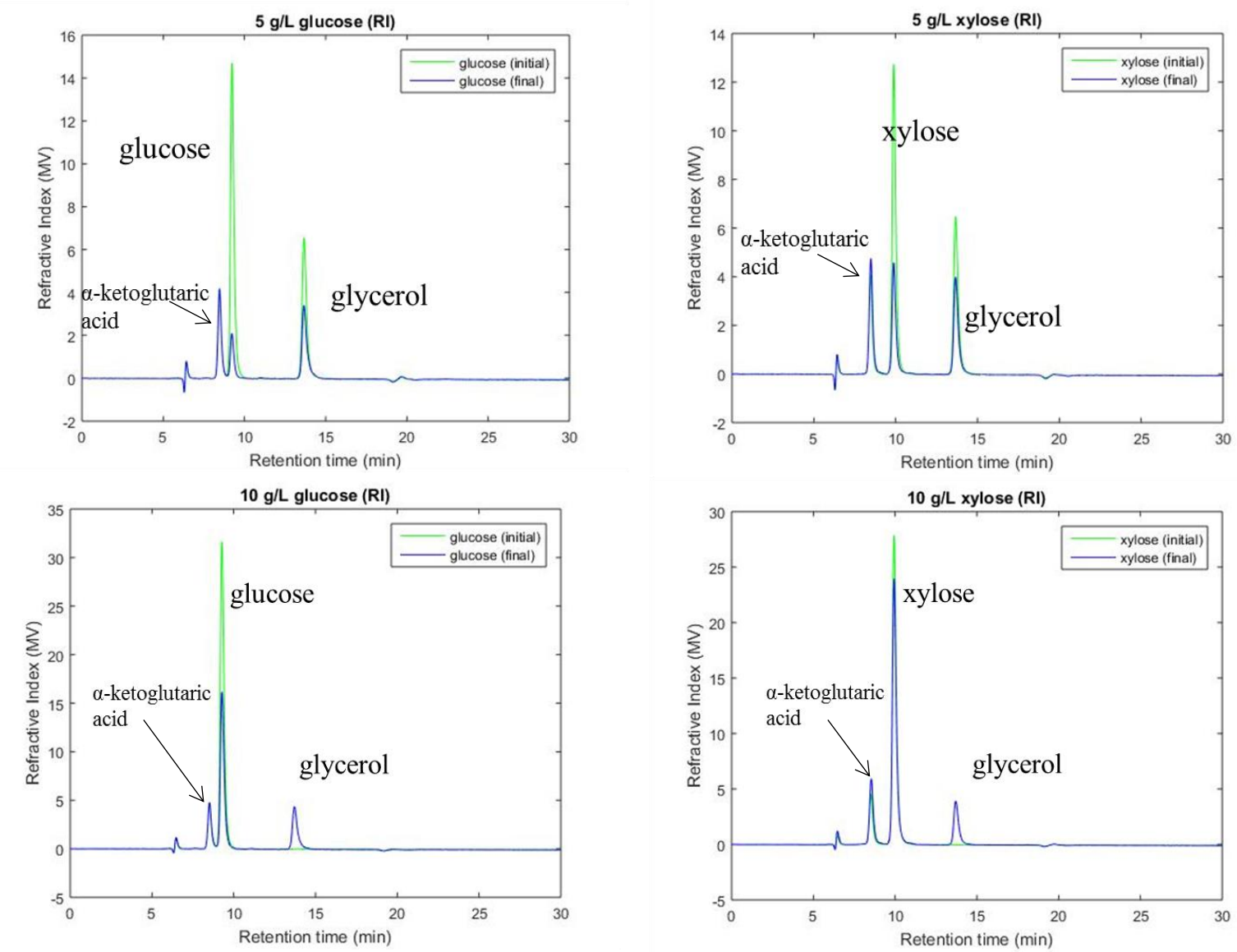


Figure 8. HPLC-RI chromatogram of initial and final samples in shake-flask cultivations with 5 g/L glucose or xylose (above) and 10 g/L glucose or xylose (below).

Figure 8 shows the chromatograms of initial and final samples from shake-flask cultivations in glucose and xylose, 5 and 10 g/L, as detected by RI. Decrease of the substrate concentrations is clearly seen in the chromatograms, at 9.2 and 9.9 minutes retention times, for glucose and xylose, respectively. There was, in all cases, another peak at 13.6 minutes retention time both in 5 g/L and 10 g/L substrate. When a sample was analysed using LC-MS, the mass spectrometer could not measure the mass of the peak indicating that the compound was stable and difficult to ionise. These observations and the measured retention time at 13.6 minutes indicate that the unknown peak is **glycerol.**

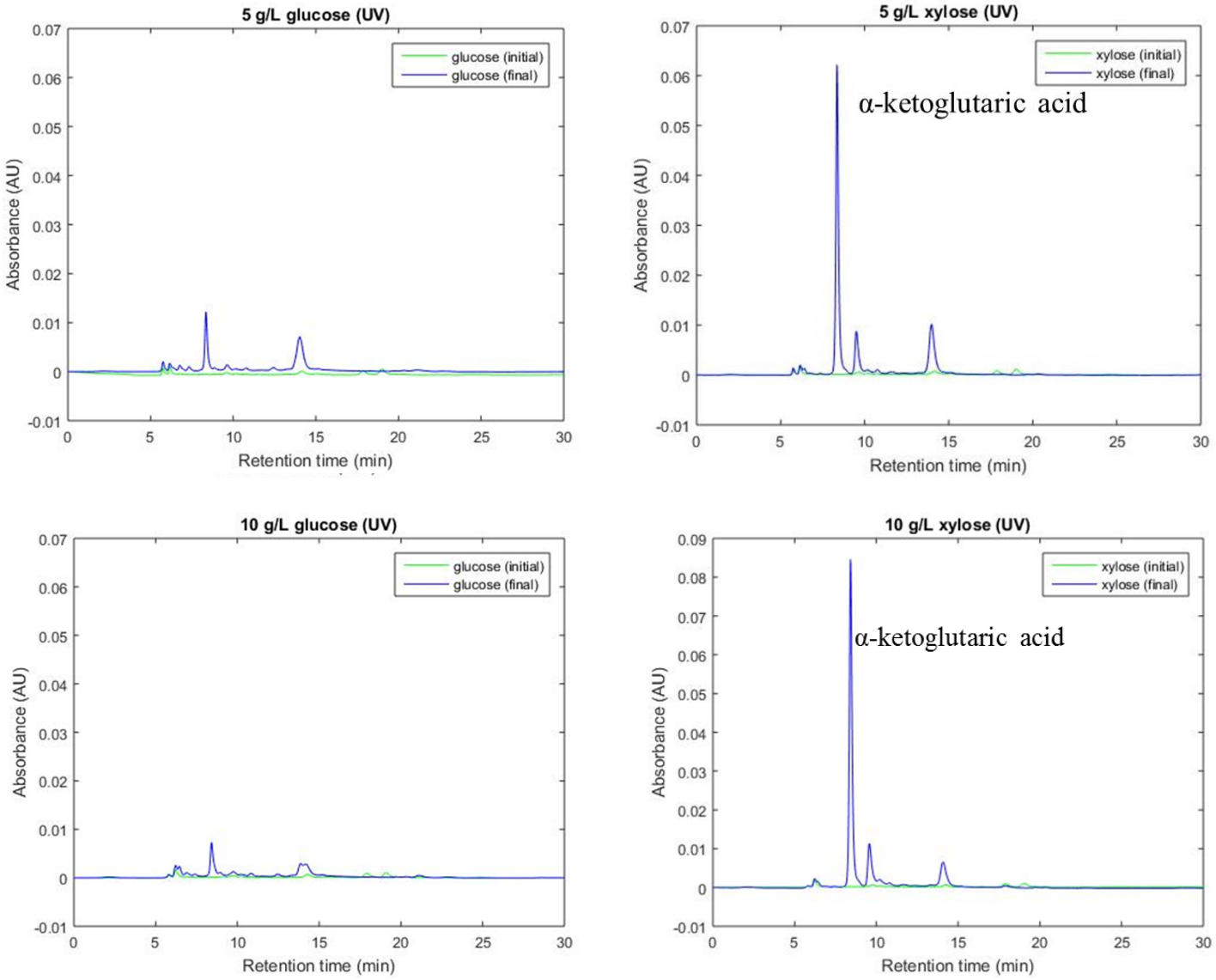


Figure 9. Initial and final HPLC-UV chromatogram of shake-flask cultivation of *Caulobacter crescentus* in 5 g/L substrate (above) and 10 g/L substrate (below).

Figure 9 shows a distinct peak at approximately 8.2 minutes retention time that is primarily present in xylose, and at much lower levels in glucose. Since the final pH of cultivation is lower, it is assumed to be an organic acid. One potential compound originating from the Weimberg pathway (Figure 4) α-ketoglutaric acid – formed in the final step in the Weimberg pathway.

From LC-MS analyses it was confirmed that the peak was **α-ketoglutaric acid.**

There were two other peaks (retention times 9.9 and 14 minutes) observed in the UV detectors that were not present in the initial samples but were produced towards the end of the cultivations (Figure 9). However, due to the relatively low concentrations of these peaks, they were not investigated any further.

The time profiles of the thus identified compounds present in the cultivations samples are shown in Figure 10.

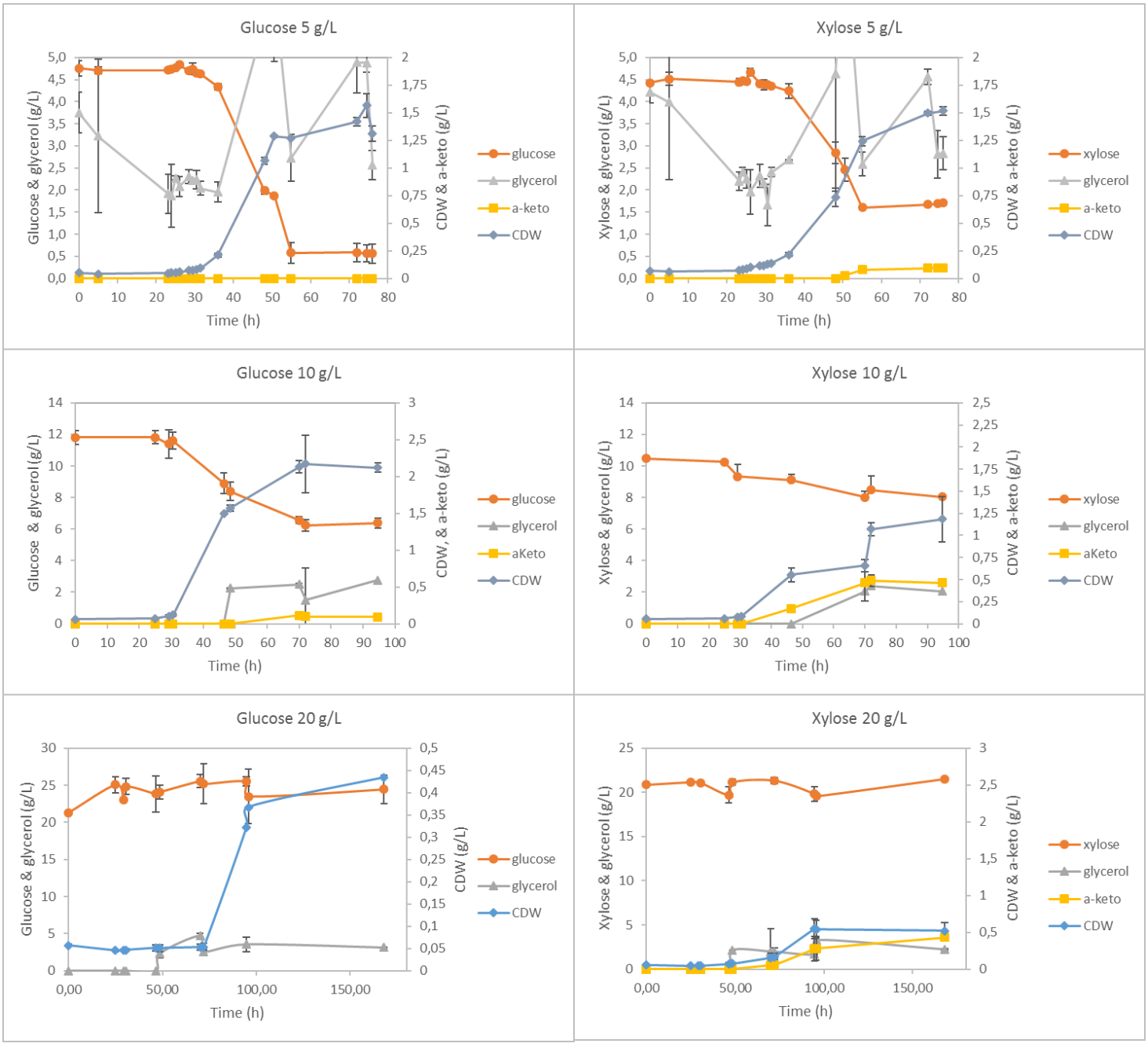


Figure 10. Shake-flask cultivations of *Caulobacter crescentus* CB2 in various concentrations of glucose and xylose. All experiments done in duplcates. Error bars indicate standard deviation. Glycerol levels in all plots except for glucose 20 g/L were calculated from a previously used glycerol standard curve (See Appendix I).

The substrates were not fully consumed in any of the cultivations. 5 g/L glucose cultivation showed the highest percentage of substrate consumption, 88%, followed by 61% consumption in xylose 5 g/L . Substrate consumption in 10 g/L cultivations throughout the cultivations were 46% and 23% respectively for glucose and xylose. The lowest substrate consumption was observed in 20 g/L cultivations with only 6.2% and 6.1% substrate consumption for glucose and xylose, respectively.

Biomass formation follows the substrate consumption, as not much biomass was formed in the cultivations where the substrate levels remained virtually unchanged, such as in the 20 g/L cultivations. Interestingly, towards the end og 20 g/L cultivation on glucose, there was a sudden increase of biomass formation while almost only noise was observed in the glucose measurements.

α-ketoglutaric acid was mostly present in xylose cultivations, with the highest observed concentration on 10 g/L xylose, at 0.5 g/L at the end of the cultivation. There was a slight α-ketoglutaric acid production in 10 g/L glucose. Since α-ketoglutaric acid is present in the TCA-cycle in cell metabolism, it is not unusual to see the compound in a glucose medium.

There were prominent levels of glycerol in all of the cultivations especially in 5 g/L substrate. Around 3.5 – 4 g/L glycerol was present at the start of the cultivation in both 5 g/L glucose and xylose as the level highly fluctuated throughout the cultivation. This indicated that glycerol was present in the pre-culture with PYE. In comparison, there were no glycerol present at the start of the cultivation in both 10 and 20 g/L substrates but the concentration of glycerol increased before and during the exponential growth phase of the cells..

Average volumetric substrate consumption rates were nearly identical for concentrations of 5 and 10 g/L: 0.06 g/Lh in glucose and 0.04 in xylose (Table 3). As evident from the plots in Figure 10, the substrate consumption rate were much lower in 20 g/L substrate. Yields of biomass over substrate were fairly consistent with the aforementioned values. For instance, 20 g/L glucose exhibited the lowest yield at 0.17 g/g ompared with the lower glucose concentrations. In contrast, 20 g/L xylose exhibited a higher biomass yield than 10 g/L, but lower than 5 g/L xylose concentration.

Specific α-ketoglutaric production rate was the highest in 20 g/L xylose at 0.006 g/gh and interestingly enough, it was higher than in 10 g/L xylose at 0.004 g/gh.

Table 3. Summary of the results from the shake-flask cultivations. All shake-flasks experiments were done in duplicates. The uncertainty values indicate standard deviation of the duplicates.

substrate	Concen-tration (gL^{-1})	growth rate (h^{-1})	Initial substrate concentration (g/L)	Final substrate concentration (g/L)	Substrate consumption rate ($gL^{-1}h^{-1}$)	α-ketoglutaric acid production rate ($gL^{-1}h^{-1}$)	Specific α-keto production rate ($g\,g^{-1}\,h^{-1}$)	Yield biomass /sugar (gg^{-1})	Yield α-keto /sugar ($g\,g^{-1}$)
glucose	5	0.127 ± 0.001	4.8 ± 0.2	0.6 ± 0.2	0.055 ± 0.001	0	0	0.301 ± 0.003	0
	10	0.142 ± 0.005	11.8 ± 0.5	6.6 ± 0.2	0.057 ± 0.002	0.001 ± $3*10^{-4}$	$4.9*10^{-4}$ ± $2*10^{-4}$	0.38 ± 0.01	0.018 ± 0.006
	20	0.07 ± 0.04	26 ± 1	23 ± 3	0.03 ± 0.03		0	0.2 ± 0.1	0
Xylose	5	0.096 ± 0.008	4.42 ± 0.01	1.61 ± 0.04	0.036 ± 0.001	0.001 ± $1*10^{-4}$	$8*10^{-4}$ ± $1*10^{-4}$	0.531 ± 0.004	0.035 ± 0.004
	10	0.109 ± 0.003	10.474 ± 0.003	8.0 ± 0.4	0.037 ± 0.001	0.005 ± $2*10^{-5}$	0.004 ±$2*10^{-5}$	0.2 ± 0.2	0.17 ± 0.01
	20	0.036 ± 0.003	20.86 ± 0.06	19.58 ± 0.02	0.010 ± 0.007	0.003 ± $1*10^{-4}$	0.006 ± $1*10^{-4}$	0.32 ± 0.04	0.19 ± 0.1
mannose	10	0.053 ± 0.002	11.6 ± 0.6	4.8 ± 0.2	0.065 ± 0.005	0	0	0.3 ± 0.1	0

Shake-flask cultivations on mannose

The growth of *Caulobacter crescentus* on another carbon source, mannose, was also investigated in a series of shake-flask experiments. The specific growth rate in mannose (0.053 h^{-1}) was found to be lower than on glucose and xylose with the same concentration of 10 g/L. The organism managed to grow fairly well in mannose with overall substrate consumption of 56.6% . Interestingly, mannose cultivations exhibited a higher substrate consumption rate than in glucose and xylose as shown in Table 3. The yield of biomass over substrate for mannose was comparable to glucose 5 g/L at 0.30 g/g. There were no by-products formed in the cultivations as observed in the HPLC chromatograms of the cultivation samples.

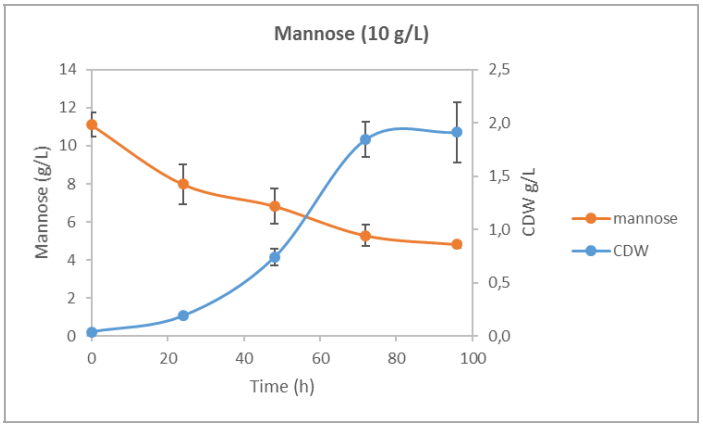


Figure 11. Growth and substrate consumption profile of *Caulobacter crescentus* CB2 grown in shake-flasks with mannose as carbon source. Error bars in mannose indicate standard deviation of the duplicate meaasurements.

Comparison between strains of Caulobacter crescentus

Growth *Caulobacter crescentus* strain CB15 was investigated in shake-flask experiments with glucose and xylose as the carbon source in order to make comparisons to the default strain CB2 which was primarily used in the project.

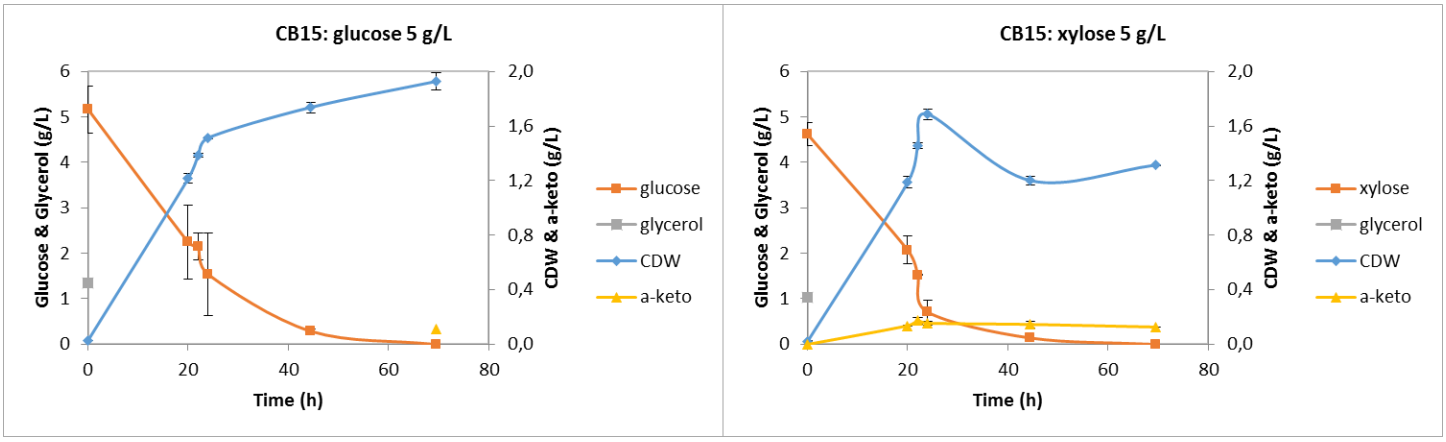


Figure 12. Growth and product profiles of *Caulobacter crescentus* strain CB15 in shake-flask cultivations in glucose and xylose. Error bars indicate standard deviation of duplicate measurements.

The substrate was fully depleted in both glucose and xylose cultivation indicating a much better growth by strain CB15 in 5 g/L glucose and xylose than with CB15. More and steady biomass formation of CB15 was observed in glucose than in xylose, even though the growth rate at exponential phase was higher in xylose. Biomass formation in xylose halted suddenly shortly after the exponential phase as the substrate was consumed completely.

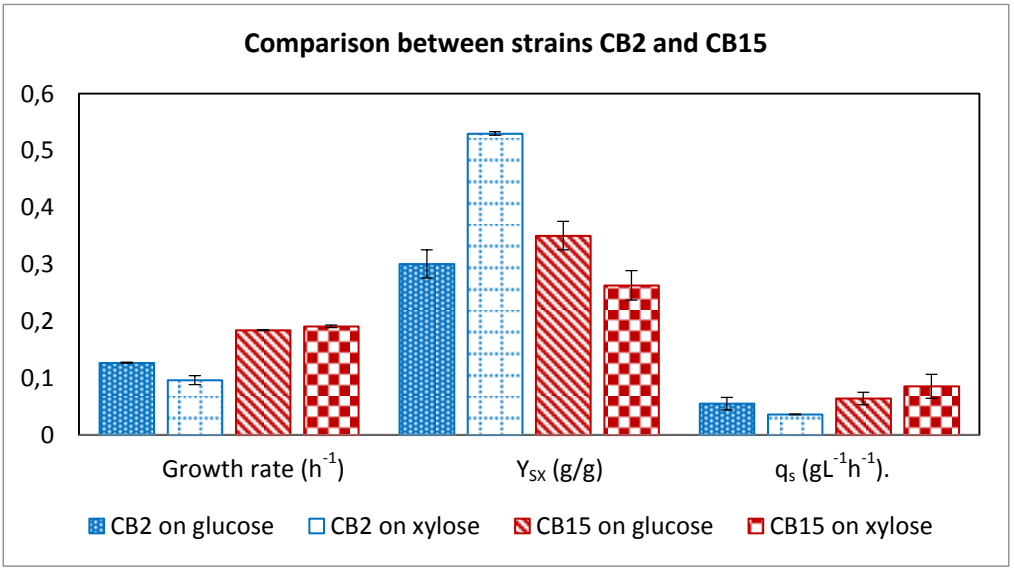


Figure 13. Comparison of growth rates, biomass yields over substrate (Ysx), and substrate consumption rates (qs)between *Caulobacter crescentus* strain CB2 and strain CB15. Error bars indicate standard deviation of the duplicates.

Figure 13 shows the comparison of characteristic values obtained in shake-flask cultivations of strains CB2 and CB15 in 5 g/L concentration of glucose and xylose. The growth rate may be higher in CB15 but the biomass yield over substrate was notably higher in CB2 cultivation in xylose. The yield of CB2 cultivation in glucose was, however, slightly lower than in CB15. The substrate consumption rates were slightly higher in CB15.

3.2 Bioreactor experiments

Initially, bioreactor experiments were inoculated from a 2-day pre-culture of *Caulobacter crescentus* in M2 medium in order to avoid the 1-day lag phase as observed in 10 g/L shake-flask cultivation. The pH was set to 7 controlled by 3 M NaOH, 300 rpm aeration, and 0.5 L/min aeration. This experiment design rendered very low and inconsistent growth rates in glucose and xylose duplicate batch cultivations.

Table 4. Growth rates in initial batch cultivations.

	Growth rate (h^{-1})	
	glucose	xylose
Batch 1	0.004	0.011
Batch 2	0.010	0.005

Several factors that could have inhibited the growth of the organism in the bioreactor cultivations were investigated. These included pH, aeration, and the medium of pre-cultures. Experimental investigations in both shake-flask and bioreactor cultivations were done. Finally, growth had been achieved using lower aeration rate of 0.1 L/min aeration, inoculation using an overnight culture in PYE, skipping the 2-day pre-culture in M2 medium, addition of 0.5% yeast extract and different pH were tested.

Figure 14 shows the state of the working volume of the bioreactor at a daily basis during the cultivation period. High cell density was observed with naked eye after only 24 hours, and foam formation had been observed in the bioreactors with pH control using 3M NaOH. For comparison, no anti-foam chemicals were used in any of the experiments and duplicates performed.

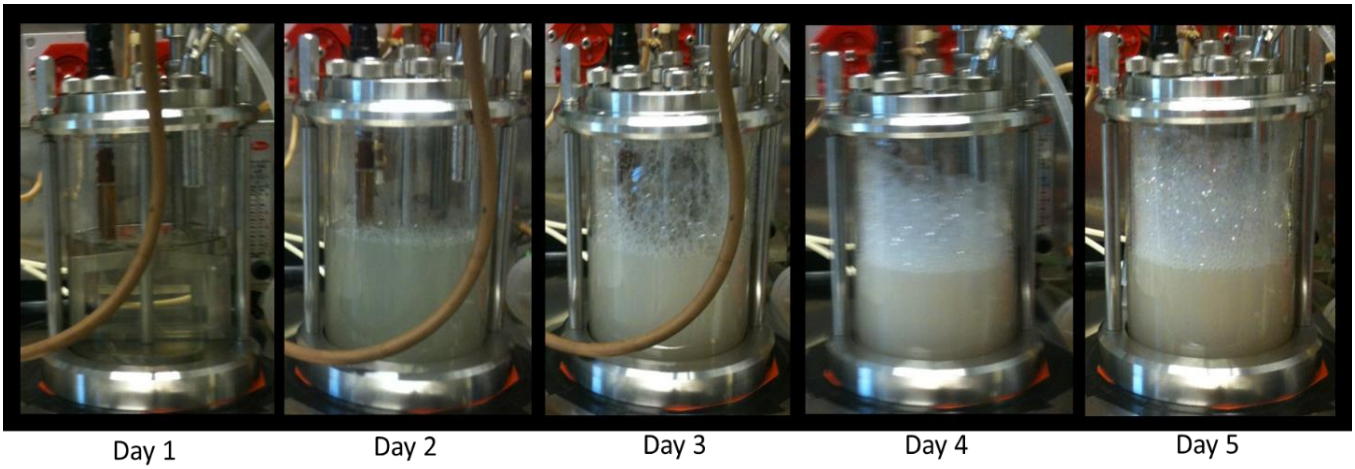


Figure 14. Bioreactor experiment in 10 g/L xylose at pH 7 throughout the whole cultivation period.

Duplicate bioreactor experiments were done on 10 g/L glucose with pH control at 7, 10 g/L xylose with pH control at 7, and 10 g/L xylose with no pH control. Other bioreactor experiments without duplicates were also done on 10 g/L glucose with no pH control, 10 g/L xylose with pH control at 5.9.

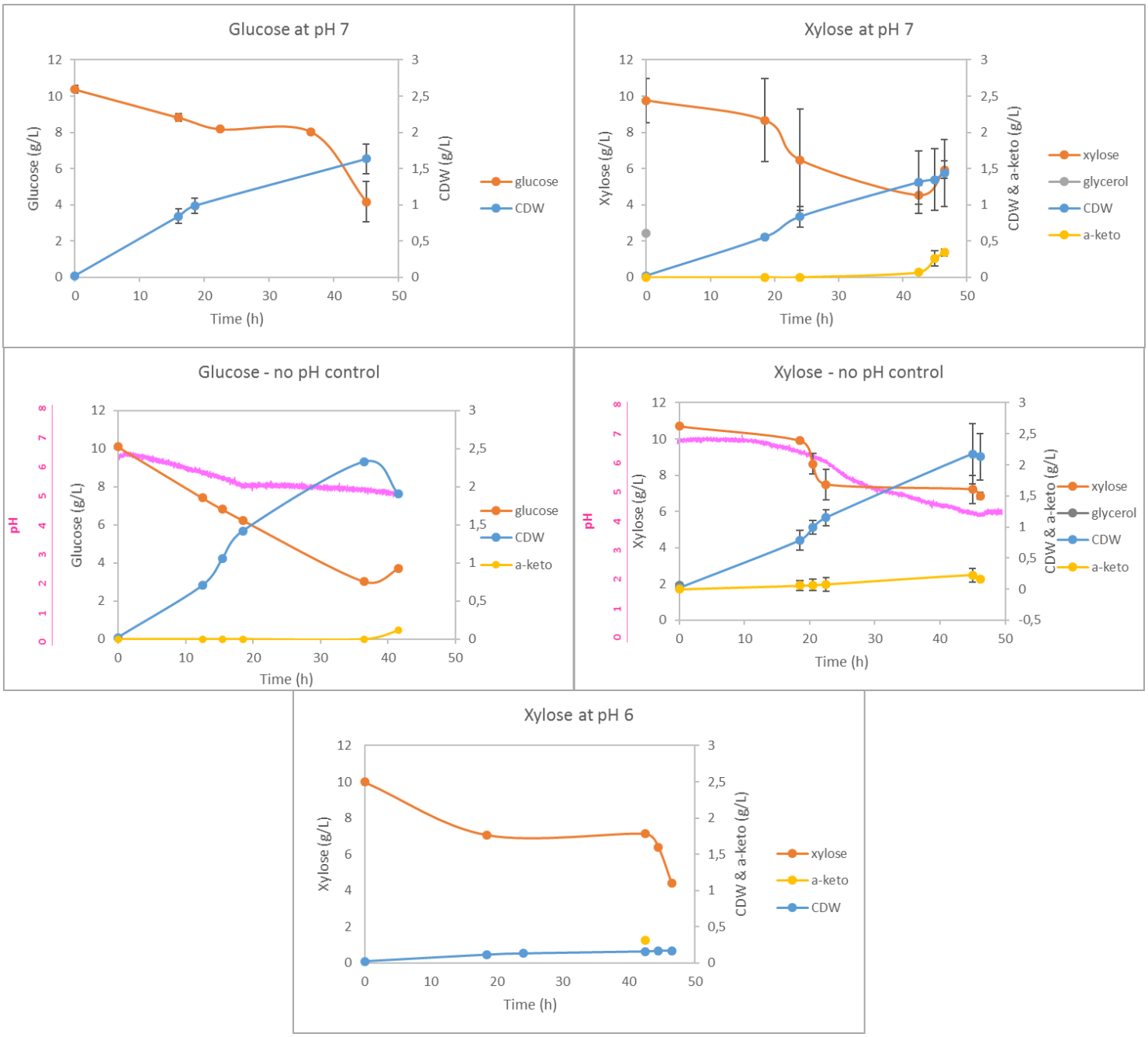


Figure 15. Batch cultivations of *Caulobacter crescentus* CB2 in 10 g/L glucose or xylose, with variation in pH setup. All experiments done in duplicates except for glucose without pH control and xylose at pH 6. Error bars indicate standard deviation.

It is evident that higher cell densities (Figure 15) and growth rates (Table 5) were observed in bioreactor experiments without any pH control. This indicates an optimal pH lower than 6.7. (the pH control at 7 had a lower pH limit set at 6.7). Another batch experiment in xylose (no duplicate) controlled at pH 6 yielded a very low growth rate of 0.08 h^{-1}. From the growth profiles in Figure 15, the optimal pH for the growth of *Caulobacter crescentus* seems to lie approximately at pH 6.5. Again, incomplete substrate consumption was observed during the whole cultivation period in the bioreactor experiments: 60% susbtrate consumption for glucose with control at pH 7, 39% and 36 % for xylose with control at pH 7 and no pH control respectively.

Sugar consumption rate was higher in glucose medium than in xylose medium. Variations in pH appears to affect α-ketoglutaric acid production. Even though there was minimal growth in pH 6, the yield of α-ketoglutaric acid over substrate was higher than in cultivations at pH 7 and no pH control.

Table 5. Summary of the results from batch cultivations. The batch experiments were done in duplicates , but due to time constraints and the length of time it took to run bioreactors, only one bioreactor experiment was done on those marked with (*).

Medium	pH	Growth rate (h^{-1})	Initial substrate concentration (g/L)	Final substrate concentration (g/L)	sugar consumption rate ($gL^{-1}h^{-1}$)	α-ketoglutaric acid production rate ($g\ L^{-1}h^{-1}$)	Specific α-keto production rate ($g\ g^{-1}\ h^{-1}$)	Yield biomass/sugar ($g\ g^{-1}$)	Yield α-keto/sugar ($g\ g^{-1}$)
M2 + glucose	pH 7	0.23 ± 0.04	10.4 ± 0.2	4 ± 1	0.14 ± 0.03	0	0	0.27 ± 0.09	0
M2 + glucose*	No control	0.26	10.1	3.7	0.15	0.003	0.002	0.30	0.02
M2 +xylose	pH 7	0.157 ± 0.007	10 ± 1	5.9 ± 0.5	0.08 ± 0.04	0.007 ± 0.001	0.005 ± 0.003	0.37 ± 0.05	0.09 ± 0.06
M2 +xylose	No control	0.193 ± 0.009	10.70 ± 0.05	6.9 ± 0.2	0.083 ± 0.005	0.005 ± 0.001	0.002 ± 0.001	0.5 ± 0.1	0.06 ± 0.05
M2 + xylose*	pH 6	0.077	9.99	4.42	0.12	0.0073	0.047	0.026	0.11

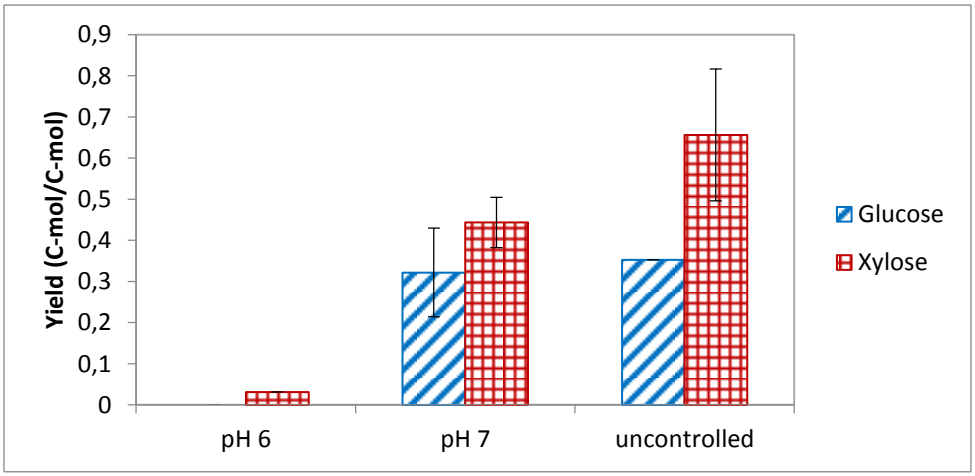


Figure 16. Comparison of yields (C-mol biomass /C-mol substrate) in bioreactor cultivations with varied pH. Error bars indicate standard deviation of the duplicates.

Yields of biomass over substrate (in C-moles) in the bioreactors on either glucose or xylose with varied pH are shown in Figure 15. It is worth noting that the yields were higher in xylose cultivations than in glucose cultivations, and especially higher in uncontrolled pH. Higher yields in cultivations with uncontrolled pH coincide with the respective growth rates but yields in xylose were unexpectedly higher than in glucose.

The organism‘s better efficiency in glucose was reflected on how much substrate was consumed during the cultivations. Glucose cultivation controlled at pH 7 consumed 60% of the initial substrate, while the cultivation with no pH control consumed 63%. Xylose cultivation controlled at pH 7 consumed only 39% of the initial substrate, the other experiment with no pH control consumed an even lower percentage of 36%, and surprisingly, the cultivation controlled at pH 6 consumed 56% of the initial substrate.

Cell Morphology

During the experiments, the cells were occasionally checked under a light microscope for possible contaminations. *Caulobacter crescentus* cells were easily recognisable under a microscope because of its characteristic crescent shape. The organism adapts to different medium therefore some changes in morphology and behaviour were observed in various media used in the experiments.

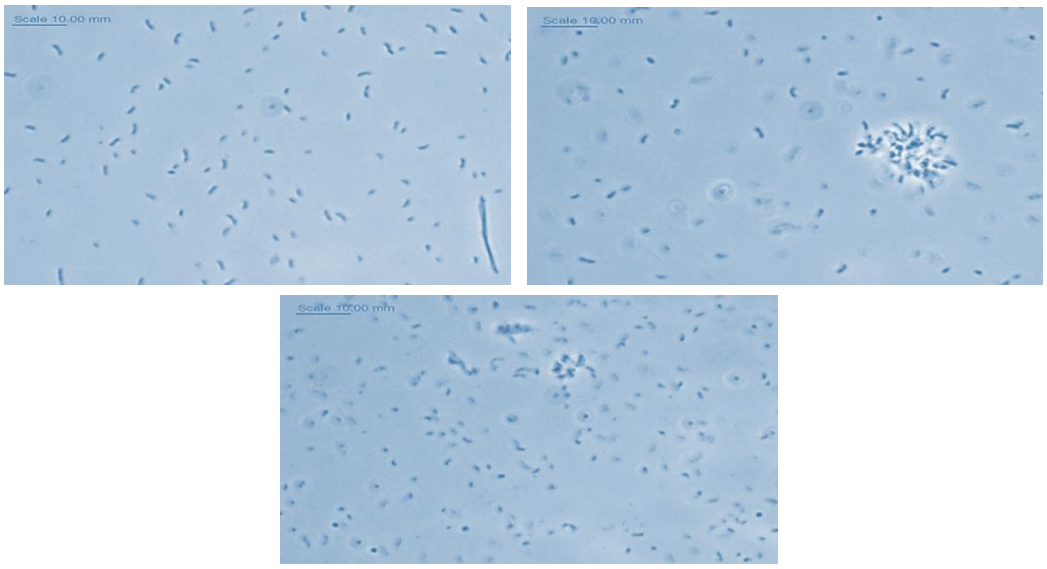


Figure 17 (upper left) CB2 in M2+glucose (upper right) CB2 in M2+xylose (below) CB2 in PYE

Figure 17 shows *Caulobacter crescentus* in PYE and M2 media, with glucose or xylose. The cells appear to be slightly longer in M2 glucose and xylose than in PYE. Also, there appears to be a really long strand of cell in M2 glucose, as well as a cluster of cells in M2 xylose. It was also observed that there were more cellular movements in M2 xylose and glucose than in PYE.

3.3 Enzyme activities

Activities of three enzymes in the Weimberg pathway (encoded by *xylB*, *xylD* and *xylA*) were analysed in crude extracts from *C.crescentus*. Two of the enzymes catalyse dehydrogenase reactions (*xylB* and *xylA*), in which NADH is formed from NAD+ (Fig 18), which makes it possible to assess activity by the formation of NADH by absorbance at 340 nm. The activity of the third enzyme, xylonate dehydratase (*xylD*) was not possible to measure alone, but only through the sequential conversion by the enzymes encoded by *xylX* and *xylA* as described in M&M. The reactions catalysed by the Weimberg enzymes were indirectly measured by increasing absorbance at 340 nm as NAD+ gets converted into NADH (Figure 18).

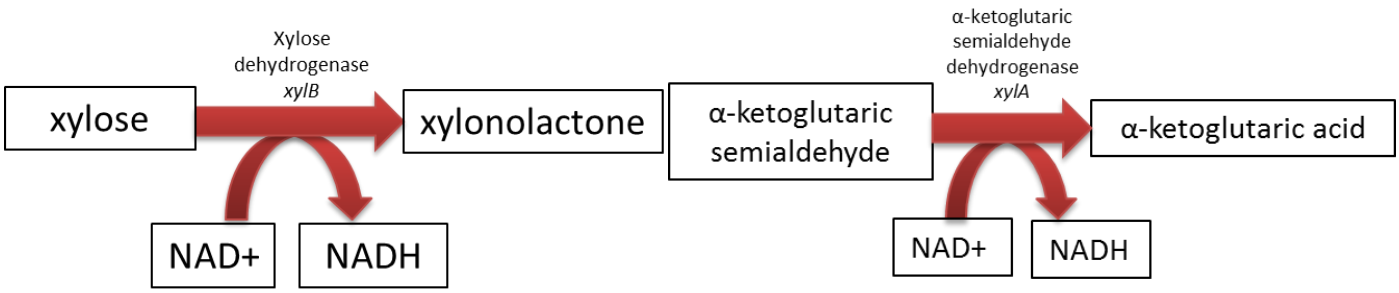


Figure 18. Schematic diagrams of the chemical reactions as catalysed by xylose dehydrogenase (*xylB*) and α-ketoglutaric semialdehyde dehydrogenase (xylA) coupled with NADH conversion from NAD+.

The summary of the activities of the three Weimberg enzymes – both in PYE medium and M2 medium, glucose and xylose as substrates – are shown in Figure 19.

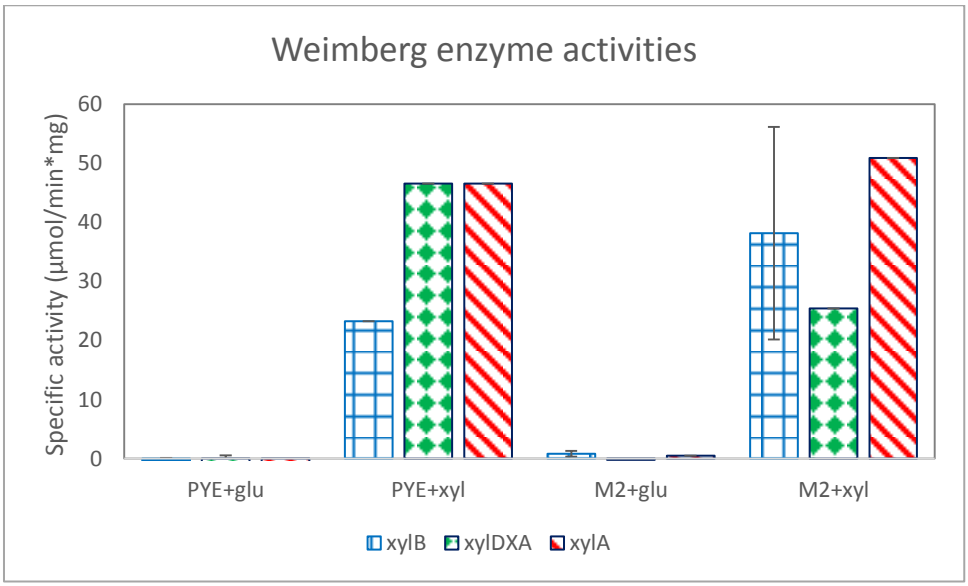


Figure 19. Enzyme activities for xylB, xylDXA and xylA. All measurements were done in duplicates. Error bars indicate standard deviation. Error calculations were based on the uncertainties of both protein and absorbance measurements.

Enzyme activity measurements showed the activity of the enzymes encoded by *xylB*, *xylD* and *xylA* mainly when xylose was used as substrate in the cultivation, both in PYE and M2 media. High uncertainty was observed in xylose dehydrogenase (*xylB*) activity in M2+xylose, however the rest of the measured activities had almost identical measurements for the duplicates. Estimated activities for xylonate dehydratase (*xylD*) marked *xylDXA* and α-ketoglutaric semialdehyde dehydrogenase (*xylA*) were measured at 56 µmol/(min*mg) protein

in PYE+xylose while xylose dehydrogenase (*xylB*) had an activity of only half of the former two, at 23 μmol/(min*mg). On the other hand, xylose dehydrogenase (*xylB*) activity in M2+xylose was higher at 38 μmol/(min*mg), but with more uncertainty. Estimated activity for the enzyme encoded by xylonate dehydratase (*xylD*) in M2+xylose was lower than in PYE+xyl, whereas the enzyme encoded by *xylA* in M2+xylose showed the highest activity, at 51 μmol/(min*mg).

No enzyme activities were observed in both PYE and M2 media with glucose as the carbon source, except for minute activities in M2 + glucose. These values, however, are error-prone, as the linear regression of absorbance vs time in the respective samples had R2 values below 0.5.

The measured activities were converted to a flux capacity, which could be compared to the specific substrate consumption rate in the same cultivation. Specific activities per g biomass of *xylB, xylDXA* and *xylA* were 0.038, 0.026 and 0.051 $g_{xylose}g_{CDW}^{-1}h^{-1}$. These estimates could be compared to the specific xylose consumption rate, which was calculated at 0.057 g_{xylose} g_{CDW}^{-1} h^{-1}.

4 Discussion

4.1 Shake-flask cultivations

Glucose and xylose

Growth profiles of *Caulobacter crescentus* strain CB2 in 5, 10 and 20 g/L glucose and xylose and 10 g/L arabinose and mannose showed various growth characteristics of the organisms. The clearly lower growth rates of the organism at the highest initial substrate concentrations (Fig 7) indicated a strong substrate inhibition.Product inhibition could also be a case since α-ketoglutaric acid is produced during the fermentation, but the product is not initially present, which points more towards substrate inhibition. The natural habitat of the organism is characterised by very low nutrient condition. Thus, it is not a surprise to see that it did not grow well in a high concentration of either glucose or xylose. Since only three concentrations were investigated in glucose and xylose, and therewas no attempt made to quantitatively model the growth of the organism. *Caulobacter crescentus* showed the best growth rate at 10 g/L substrate concentration and hence this concentration was chosen for further experiments.

Another observation was that substrates were not fully utilised in any of the cultivations done on glucose or xylose, in three concentration values investigated. The highest substrate consumption observed was in 5 g/L glucose and xylose, 88.2% and 61.4%, respectively. The seizure of growth could be caused by several different factors, including nutrient limitation, pH change or accumulation of an inhibiting compound in the medium. With respect to decrease in pH, cultivations in xylose exhibited lower final pH than cultivations in glucose (Table 2) . This could be one explanation for lower substrate consumption and hence lower biomass yields in xylose cultivations

Production of α-ketoglutaric acid was mainly observed in shake-flask cultivations in xylose, with only minute amounts observed in glucose. This can be connected to the lower final pH in xylose cultivations than in glucose. This further supports the enzyme activity measurements showing that the Weimberg pathway was indeed activated in xylose cultivations as α-ketoglutaric acid is the end product of the pathway.

Glycerol levels were particularly evident at the start of 5 g/L cultivations on glucose and xylose and highly fluctuated throughout the cultivation. This indicates that a siginificant amount of glycerol was present in the pre-culture in PYE. Even though the pre-culture was inoculated with a loop from frozen cell stock in glycerol, the amount of glycerol in the pre-culture should not reach the levels measured at the start of the cultivation on M2. It is possible that there were errors in glycerol values measured by HPLC since the glycerol standard curve used was obtained from another analysis. Even so, the glycerol peaks were highly evident in the chromatograms, therefore a repetition of this part of the experiment may be necessary to determine accurate glycerol measurements on cultivations on 5 g/L glucose and xylose.

In comparison, there was no glycerol present at the start of the cultivations on 10 g/L and 20 g/L glucose and xylose. However, glycerol was produced during the cultivations reaching a final glycerol concentration ranging from 2-3 g/L. This is particularly interesting, especially in 20 g/L cultivations where there was not much biomass formation. This shows the organism‘s preference of utilising the carbon present in its environment. The main product of cultivation was glycerol, not biomass formation in both 10 g/L and 20 g/L cultivations.

Mannose

Caulobacter crescentus showed growth on mannose as the carbon source, albeit with lower growth rate than in glucose and xylose. Mannose utilisation in *Caulobacter crescentus* occurs in pathways other than Entner-Doudoroff and Weimberg pathways. There were no by-product formation observed in HPLC analysis of the cultivation samples. Biomass yields over substrate was 0.25 and 0.30 g/g for arabinose and mannose, respectively. Biomass yield over mannose was higher than xylose (0.24 g/g) but lower than glucose (0.38 g/g) at the same concentration. Since mannose is also a 6-carbon compound like glucose, this suggests that *Caulobacter crescentus* utilises 6-carbon compounds more efficiently than 5-carbon compounds.

Comparisons with Caulobacter crescentus strain CB2 and strain CB15

Shake-flask cultivation of the wild-type strain CB15 were done on 5g/L glucose and xylose. Much higher growth rates were observed in CB15 than in CB2, and the substrates were fully utilised at the end of cultivation. This is a stark contrast to CB2 cultivations in which no complete substrate consumption was observed. It was previously suggested that cell death of CB2 in shake-flask cultivations were due to decrease in pH, but final pH in CB15 cultivations were identical to CB2 cultivations – also lower in xylose. This opens up other possible factors that contributed to the cell death in CB2 leading to incomplete substrate consumption.

It was established in previous studies that some strains of *Caulobacter crescentus* are more tolerant to phosphate concentrations than others (Ely, 1991). Since phosphate buffers were used in the minimal medium, CB2 may have not been completely tolerant to phosphate ions, even though it could grow in the presence of the compound. In contrast, strain CB15 showed complete tolerance to the medium.

Despite lower growth rates and substrate consumption rates in CB2 than CB15, biomass yield over substrate was much higher in xylose cultivation of CB2 than both glucose and xylose cultivation of CB15. Glucose cultivation of CB2, however, was lower than the glucose cultivation of CB15, although not completely significant since the differences were within the uncertainties of the duplicates. A possible explanation to these discrepancies may be that the subtle differences between the wild-type strains CB2 and CB15 may have been within the xylose degradation pathway. Translation of Weimberg enzymes may have been enhanced in CB2.

4.2 Bioreactor experiments

The fact that *C. crescentus* did not grow at the initial attempts with inoculation using pre-culture in M2 indicated that the organism needed some growth precursors only obtained from the yeast extract. Yeast extract was therefore added at 0.5 g/L in the biroeactor experiments published in the project. However, other parameters were also changed when attempting to grow the organism in a bioreactor. Aeration was lowered from 0.5 L/min to 0.1 L/min, for instance. This might indicate that *C. crescentus* has a limit for aeration to be able to grow.

Since the drop of pH in shake-flask cultivations was observed which may have contributed to the death of the cells, it was deemed necessary to investigate the variations in pH in the bioreactor experiments. Bioreactor cultivations were done in glucose and xylose 10 g/L controlled at pH 7, no pH control and at pH 6. Again, the substrates were not fully utilised in any of the bioreactor cultivations. Better growth of the organism was observed in bioreactors with no pH control than at pH 7, whereas worst growth was observed at pH 6. The drop of pH

was monitored throughout the cultivation without any pH control. At exponential growth, pH lied around 6.5. This is consistent with the published value for the optimal pH of *Caulobacters* which is at pH 6.5 (Poindexter, 1964).

Higher yields (C-mol biomass over C-mol substrate) observed in xylose bioreactor cultivation compared to glucose cultivation, despite lower growth rates in xylose than in glucose, shows difference in the utilisation of the metabolites in the respective degradation pathway *C. crescentus* chooses according to which carbon source is present. Xylose goes through the Weimberg pathway whereas glucose goes through the Entner-Doudoroff pathway. This discrepancy was further illustrated at the percentage values at which the carbon balances closed. Cultivations on xylose at pH 7 and no pH control closed at 74% and 93%, respectively, whereas cultivations on glucose at pH 7 and no pH control closed at 45% and 51%, respectively. These values did not take into account the carbon dioxide produced in the reaction hence the remaining percentage lost can be attributed to carbon dioxide. Entner-doudoroff pathway involves more carbon dioxide production such as the conversion of pyruvate to acetyl-coA. Weimberg pathway, in contrast, maintains the carbon molecule throughout the pathway before it enters the TCA cycle (Figure 4).

In this project, we were not able to fully understand the reason for incomplete substrate consumption in the biroeactor cultivations It would be interesting to see if the bioreactor cultivation is controlled at optimal pH 6.5 but from the results of all the executed experiments, it appears to be that other factors are crucial to proper growth of *Caulobacter crescentus.* For instance, wild-type strain CB15 underwent full substrate consumption but not CB2. Cultivating strain CB15 in a bioreactor seems to be the obvious next step, as it would be interesting to see its behaviour in a more controlled environment. In addition to this, changes in aeration might give us more insight on growth characteristics of *Caulobacter crescentus* since it was one of the parameters changed when attempting to grow the organism in the bioreactor. Its effect was not particularly understood within the constraints of the project. Although an obligate aerobe, there is a possibility that *Caulobacter crescentus* is sensitive to excessive aeration as this leads to more oxidative stress which may be detrimental to the growth of the organism. Also, another possible explanation would be the particular nutrient the organism only gets from the yeast extract. 0.5 g/L yeast extract was used in the bioreactor experiments. Determination of that limiting nutrient only found in the yeast extract and which metabolic pathway it is needed may shed light on growth mechanics of *Caulobacter crescentus.*

Cell morphology

Caulobacter crescentus cells showed differences in morphology with respect to the medium they grow in. The cells appeared to be slightly longer in the minimal medium with either glucose or xylose only as the carbon source compared to the cells in the rich medium (Figure 17). The organism is known to adapt to different levels of nutrients available and therefore these changes in morphology were expected. Stalk length had been shown to increase when nutrient levels are low in order to enhance acquisition of nutrients. This increases the effective membrane areas available for nutrient transport systems within the cell without significantly increasing the volume of the cytoplasm (Hirsch, 1986). In addition to changes in physical features, it was observed that there were more moving cells in the samples from the minimal

medium than in the rich medium. This can be explained by previous studies showing that more swarmer cells are produced at very low nutrient availability. This gives the cells the ability to find other sources of nutrients using their chemotactic sensors (Hirsch, 1986).

4.3 Enzymatic assays

The Weimberg enzymes encoded by *xylB, xylDXA* and *xylA* were shown to be primarily active in cultivations run on xylose as a carbon source. This is as expected, since with xylose as the only carbon source in the medium the Weimberg pathway must be activated in *C. crescentus* for survival. Xylose dehydrogenase (*xylB*) activity was higher, albeit with higher uncertainty, in M2 and xylose (38 μmol/(min*mg)), than in PYE and xylose (23 μmol/(min*mg)). Two factors can possibly explain this behaviour. Firstly, PYE medium contains other sources of carbon for the cells, such as amino acids from the yeast extracts, rendering other metabolic pathways active for cell survival and metabolism whereas in cultivations with only M2, the Weimberg pathway was practically the only known option for cell survival. Secondly, the cultivation with PYE was done in shake-flask while the other was done in a bioreactor, hence the higher percentage of other potential carbon sources in the total volume of medium culture (50 mL) compared to a batch cultivation in M2, though inoculated with pre-culture in PYE. The measured activities for xylose dehydrogenase (*xylB*) was comparable to the published enzyme activity (27.5 μmol/(min*mg)) for the same enzyme obtained from a cultivation in PYE and xylose (Stephens et al., 2007).

The estimated activity of xylonate dehydratase (*xylD*) was higher in PYE than in M2. A potential explanation of this would be a difference in water activities in PYE and M2 media since a product of the reaction that xylonate dehydratase (*xylD*) catalyses is water.

Last but not of least interest, the enzyme activity of α-ketoglutaric semialdehyde dehydrogenase (*xylA*) was slightly higher in M2 than in PYE. This is an interesting measurement since the two previous enzymes had lower activities in M2 medium. This indicates a stronger metabolic flux in the lower part of the Weimberg pathway which is noteworthy of further investigations.

Since xylose dehydrogenase (*xylB*) catalyses the first step in the xylose degradation pathway, the specific activity measured in this enzyme was attempted to be compared to specific substrate consumption rate of the bioreactor cultivation where the cells were harvested from. The specific xylose consumption rate was found to be 0.06 $g_{xylose}\ g_{CDW}^{-1} h^{-1}$ while the specific enzyme activity of xylose dehydrogenase (*xylB*) was 0.04 $g_{xylose}\ g_{CDW}^{-1}\ h^{-1}$, showing a comparable flux of xylose in the pathway. Specific enzyme activities of *xylD* and *xylA* were 0.03 and 0.05 $g_{xylose}\ g_{CDW}^{-1}\ h^{-}1$, respectively, were also within the range of the flux. It should be noted though that comparisons of these measurements are typically prone to errors since uncertainties in dilutions, HPLC analysis, cell harvesting, weighing and dry weight measurements add up.

The most important finding in the enzyme activity experiments was the observation that there was virtually no Weimberg enzyme activities measured in *Caulobacter crescentus* cells grown in glucose medium, both in PYE and M2. This indicates that only in the presence of xylose are the key Weimberg enzymes activated. It was shown by Stephens et al. (2007b) that

a promoter (P_{xylX}) is responsible for controlling the expression of *xyl* operon which holds the encoding genes for the Weimberg enzymes. P_{xylX} is regulated by a LacI-type repressor (*xylR*). Xylose was found to deactivate the repressor *xylR* and hence xylose metabolism promoter P_{xylX} gets activated (Stephens et al., 2007b). This important finding by Stephens et al. (2007b) was successfully demonstrated in the results of the enzyme activity experiments.

4.4 Future work

The next possible experiments after this thesis work could be to examine growth and product profiles of *Caulobacter crescentus* on other carbon sources such as α-ketoglutaric acid, glycerol, and combinations of sugars that had previously been explored. The organism‘s preference of carbon source would also be interesting to investigate. This can be done by cultivating the bacterium in minimal medium with more than one carbon source and then analysing the consumption rates of the carbon sources. Enzymatic assays can be performed from cell extracts obtained from different carbon sources to determine enzymatic activities when these carbon sources are available for the organism. Also, it would be interesting to characterise the metabolic flux in the Weimberg pathway.

Another interesting experiment with bioreactor cultivation of *Caulobacter crescentus* would be to vary the aeration rates.

5 Conclusion

In this project, growth of *Caulobacter crescentus* strain CB2 on various carbon sources including glucose, xylose and mannose was characterised using shake-flasks and bioreactors. α-ketoglutaric acid was found to be the main by-product formed in xylose cultivations, with some traces of glycerol present. Bioreactor experiments showed an optimal pH 6.5 for cultivating the organism. Special emphasis was given to the xylose degradation pathway, the Weimberg pathway. Three of the Weimberg enzymes (encoded by *xylB, xylD and xylA*) were shown to be active when the organism was grown with only xylose as the carbon source, but not when the organism was grown on glucose. This hallmarks the versatile capability of *Caulobacter crescentus* as it efficiently controls enzyme activations with respect to which carbon source is available in the environment.

Incomplete substrate consumption and non-optimal overall growth of strain CB2 led to discussion on possible reasons for the inhibition of growth in the organism. Possible explanations could be: variations in aeration, limiting nutrient only found in yeast extract, or product inhibition. This could be strain dependent, since another wild-type strain CB15, showed a more complete substrate utilisation in shake-flask experiments.

Bibliography

1. Anastas, P., and Eghbali, N. (2010). Green chemistry : principles and practice. *Chemical society reviews.* 39, pp. 301-312.
2. Blanvillain, S., Meyer, D., Boulanger, A., Lautier, M., Guynet, C., et al. (2007). Plant carbohydrate scavenging through TonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. *PLoS One.* 2, p. 224.
3. Bradford, M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 7(72), pp. 248-254.
4. Chiang, L.C., Gong, C.S., Chen, L.F., Tsao, G.T. (1981). D-Xylulose fermentation to ethanol by *Saccharomyces cerevisiae. Applied Environmental Microbiology.* 42, p. 284.
5. Glazer, A. and Nikaido, H. (2007). *Microbial Biotechnology: fundamentals of applied microbiology*, 2nd edition. W.H. Freeman and Company. United States of America.
6. Gopinath, V., Murali, A., Dhar, K.S., Madhava Nampoothiri, K. (2012). *Corynebacterium glutamicum* as a potent biocatalyst for the bioconversion of pentose sugars to value-added products. *Applied Microbiology and Biotechnology*. 93, pp. 95-106.
7. Hahn-Hägerdal, Karhumaa, K., Jeppsson, M., Gorwas-Grauslund, M.F. (2007). Metabolic engineerng for pentose utilization in *Saccharomyces cerevisiae. Advances in Biochemical Engineering and Biotechnology*. 108, pp. 147-177.
8. Hamelinck, C.N., van Hooijdonk, G., Faaij, A.P. (2005). Ethanol from lignocellulosic biomass: technoeconomic performance in short-, middle- and longterm. *Biomass and Bioenergy*. 28, pp. 384-410.
9. Hirsch, P. 1986. Microbial life at extremely low nutrient levels. *Advances in Space Research*. 6, pp. 287–298.
10. Hottes, A.K., Meewan, M., Yang, D., Arana, N., Romero, P., et al. (2004). Transcriptional profiling of *Caulobacter crescentus* during growth on complex and minimal media. *Journal of Bacteriology.* 186, pp. 1448–1461.
11. Jeffries, T. (2006). Engineering yeasts for xylose metabolism. *Current Opinion in Biotechnology*. 17, pp. 320-326.
12. Jenal, U., and C. Stephens. 2002. The *Caulobacter* cell cycle: timing, spatial organization and checkpoints. *Current Opinion in Microbiology.* 5, pp. 558–563.
13. Johnsen, U., Dambeck, M., Zaiss, H., Fuhrer, T., Soppa, J., Sauer, U., Schönheit, P. (2009). D-xylose degradation pathway in the halophilic archaeon *Haloferax volcanii*. *The Journal of Biological Chemistry*. 284(40), pp. 27290-27303.
14. Kamm, B., and Kamm, M. (2004). Principle of biorefineries. *Applied Microbiology and Biotechnology*. 64, pp.137-145.
15. Laub, M, Shapiro, L, McAdams, H. (2007). Systems Biology of Caulobacter. *Annual Review of genetics.* 41, pp. 429-41.
16. Laub, M. T., H. H. McAdams, T. Feldblyum, C. M. Fraser, and L. Shapiro. (2000). Global analysis of the genetic network controlling a bacterial cell cycle. *Science.* 290, pp. 2144–2148.
17. Meijnen, J.P., de Winde, J.H., Ruijssenaars, H.J. (2009). Establishment of oxidative D-xylose metabolism in *Pseudomonas putida* S12. *Applied and Environmental Microbiology*. 75(9), pp. 2784-2791.
18. Muñoz de las Heras, A. (2016). Experimental Data. Division of Applied Microbiology. Faculty of Engineering Lund University. Sweden.
19. Patel, J., Wilson, G., Michael, R, McKay, L., Vincent, R., Xu, Z. (2010). Self-immobilization of recombinant Caulobacter crescentus, and its application in removal

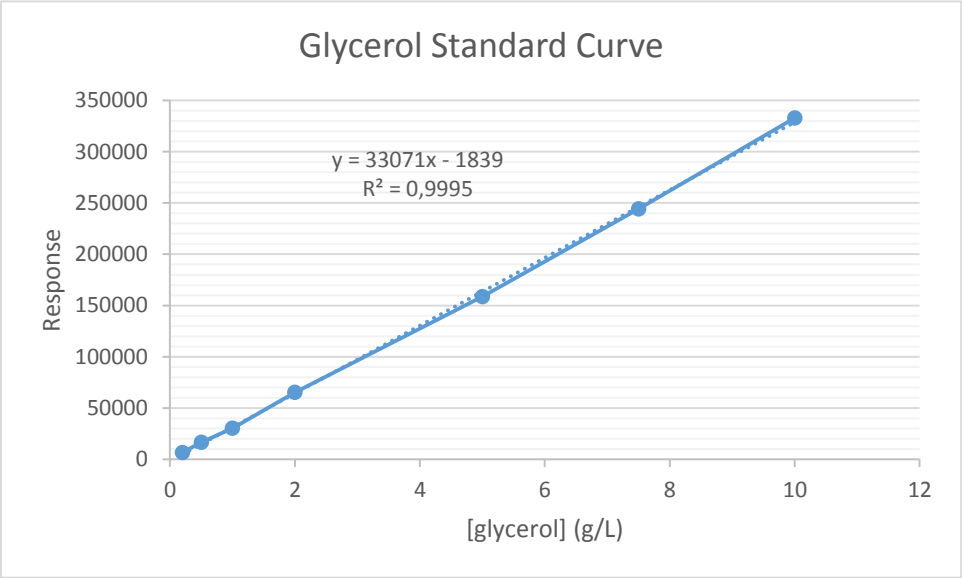
of cadmium from water. *Applied Biochemistry and Biotechnology*. 162(4), pp. 1160-1173.
20. Poindexter, J. S. (1964). Biological properties and classification of the Caulobacter group. *Bacteriological Reviews*. 28, pp. 231–295.
21. Romano, A. H., and T. Conway. (1996). Evolution of carbohydrate metabolic pathways. *Research in Microbiology*. 147, pp. 448–455.
22. Stephens, C., Christen, B., Fuchs, T., Sundaram, V., Watanabe, K., Jenal, U. (2007). Genetic analysis of a novel pathway for D-xylose metabolism in Caulobacter crescentus. *Journal of Bacteriology*. 189(5), pp. 2181-2185.
23. Stephens, C., Christen, B., Watanabe, K., Fuchs, T., Jenal, U. (2007b). Regulation of D-xylose metabolism in Caulobacter crescentus by a LacI-type repressor. *Journal of Bacteriology. 189*(24): 8828-8834.
24. Toivari, M., Nygård, Y., Kumpula, E. et al. (2012). Metabolic engineering of *S.cerevisiae* for bioconversion of D-xylose to D -xylonate. *Metabolic Engineering*. 14, pp. 427-436.
25. Toivari, M., Ruohonen,L.,Richard,P., Penttila, M.,Wiebe, M.G. (2010). *Saccharomyces cerevisiae* engineered toproduce D-xylonate. *Applied Microbiology and Biotechnology*. 88, pp. 751–760.
26. U.S. DOE. (2014). U.S. Department of Energy Bioenergy Research Centers, DOE/SC-0162. Office of Biological and Environmental Research within the DOE Office of Science. Available at http://genomicscience.energy.gov/centers/brcbrochure/
27. Wang, P.Y., Shopsis, C., Schneider, H. (1980). Fermentation of pentose by yeasts. *Biochemical and Biophysical Research Communications.* 94, p. 248.
28. Wasserström, L. (2016). Experimental Data. Division of Applied Microbiology. Faculty of Engineering Lund University. Sweden.
29. Watanabe, S., T. Kodaki, and K. Makino. (2006). A novel alpha-ketoglutaric semialdehyde dehydrogenase: evolutionary insight into an alternative pathway of bacterial L-arabinose metabolism. *Journal of Biological Chemistry*. 281, pp. 28876–28888.
30. Weimberg, R. (1961). Pentose oxidation by *Pseudomonas fragi*. *Journal of Biological Chemistry*. 236, pp. 629–635.
31. Wiebe, M.G., Nygård, Y., Oja, M., Andberg, M., Ruohonen, L., Koivula, A., Pentiilä, M., Toivari, M. (2015). A novel aldose-aldose oxidoreductase for co-production of D-xylonate and xyliton from D-xylose with *Saccharomyces cerevisiae. Applied Microbiology and Biotechnology*. 99, pp. 9439-9447.

Appendix

Appendix I – HPLC standards

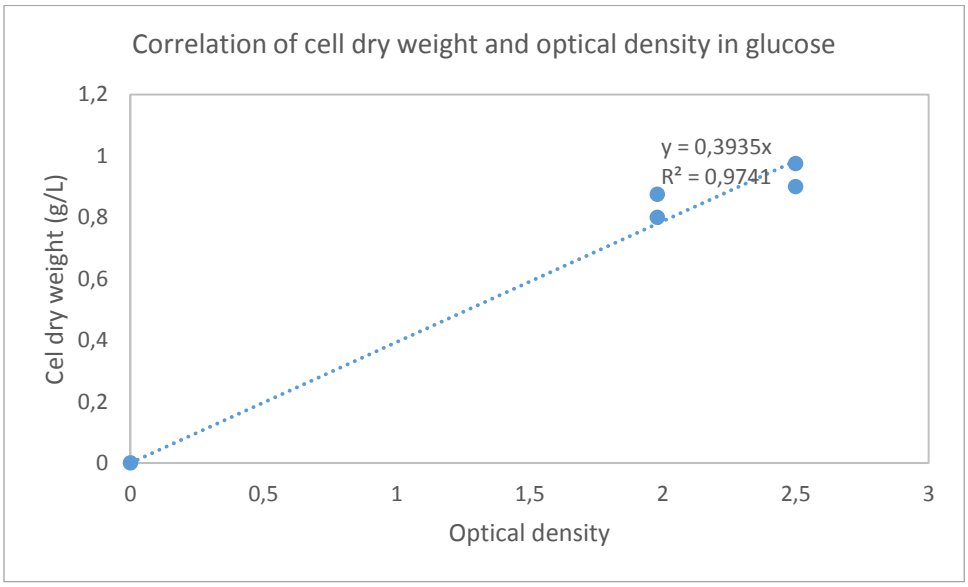
Level	Glucose (g/L)	Xylose (g/L)	Glycerol (g/L)	α-ketoglutaric acid (g/L)	arabinose (g/L)	mannose (g/L)
1	10	10	10	5	10	10
2	7,5	7,5	7,5	3,75	7,5	7,5
3	5	5	5	2,5	5	5
4	2	2	2	1	2	2
5	1	1	1	0,5	1	1
6	0,5	0,5	0,5	0,25	0,5	0,5
7	0,2	0,2	0,2	0,1	0,2	0,2

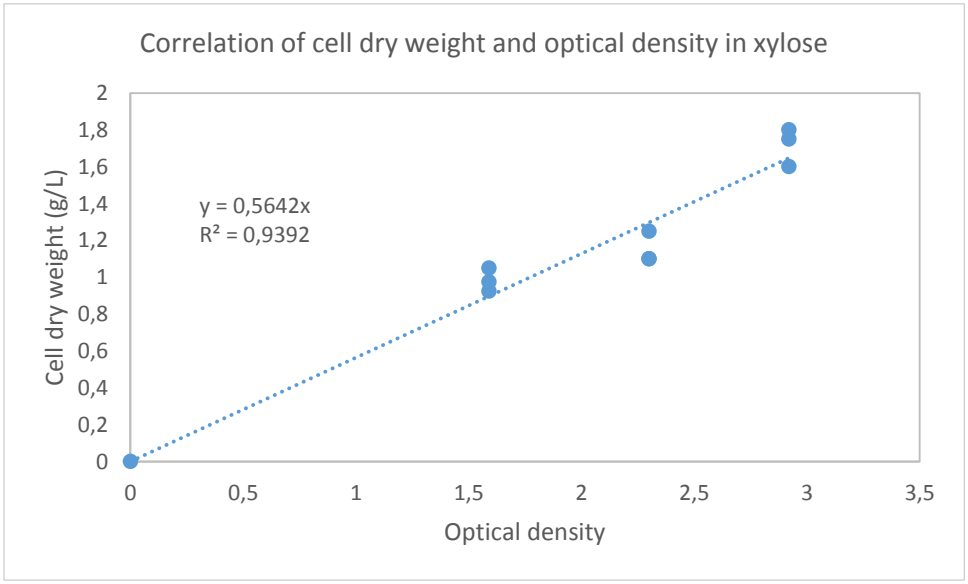
Glycerol Standard curve



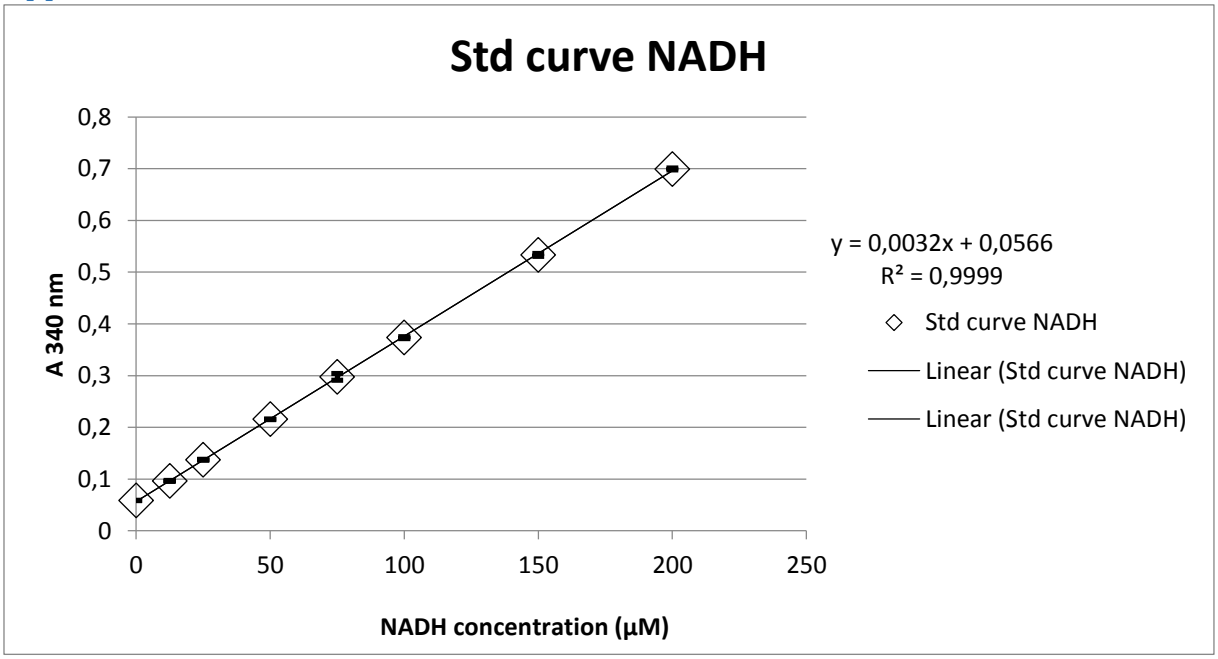
Appendix II – Dry Weight measurements

Several dry weight measurements were taken in different cultivations. The ones with the high OD values were chosen for the correlation graphs. The average of two measurements with different OD values was calculated for both glucose and xylose in M2 medium.



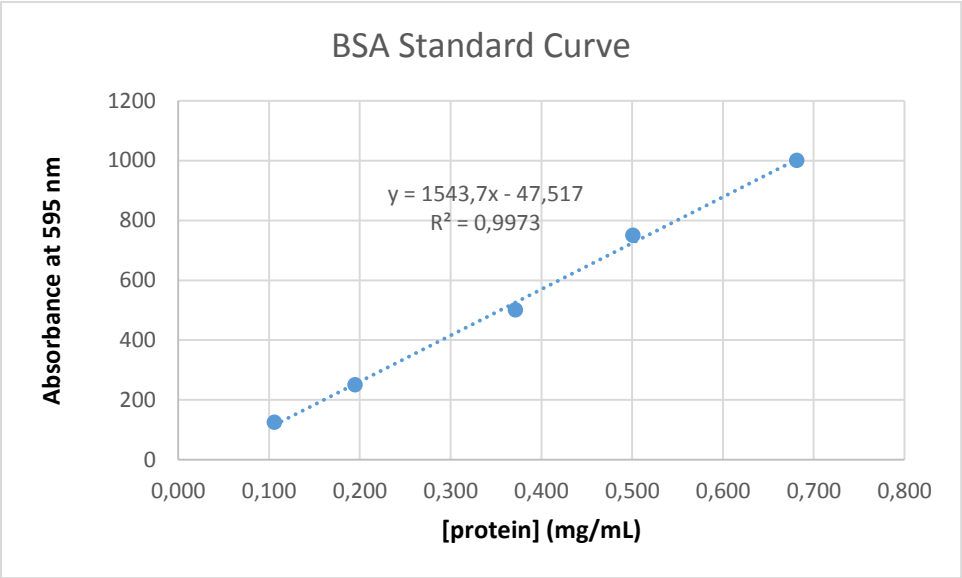


Appendix III– Standard Curve of NADH



Source: Muñoz de las Heras, 2016

Appendix IV – Protein determination for the enzymatic assays



The slope is ς=1543.7 with y-intercept value at -47.52 (Source: Wasserström, 2016).

	Absorbance $_{595nm}$		**[protein] in sample**	
	Sample 1	Sample 2	mg/mL	standard deviation
PYE + glu	0.542	0.566	4.038	0.128
PYE + xyl	0.551	0.554	4.028	0.020
M2 + glu	0.553	0.537	3.971	0.088
M2 + xyl	0.503	0.513	3.683	0.055

Example of calculation for protein determination:

$$[protein] in\ sample = (Abs_{595} * \varsigma - blank) * \frac{1mg}{1000\mu g} * dilution\ factor$$

$$= (0.542 * 1543.7 - 47.517) * \frac{1}{1000} * 5 = 3.946 mg/mL$$

Appendix V - Popular Science Summary

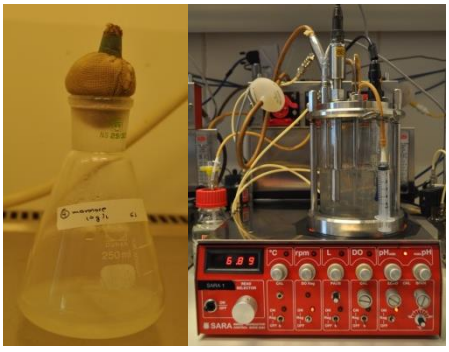
The world desperately needs a solution to an ever increasing environmental problems connected to oil-based industries. Today, unfortunately, almost every material we come in contact with was produced directly or indirectly using petrochemicals. Teams of scientists all over the world have worked with the idea to produce necessary chemicals from other more environmental-friendly sources such as plant biomass – more specifically, the part of the plant which is disposed of by many agricultural and industrial processes. Since this is waste, it would not compete with food production because competition with food would be the last thing the society needs right now.

In order to convert this waste part of the plants into something useful for us, microorganisms such as certain harmless bacteria and yeasts are used. There are millions to choose from but one of the most ideal ones for industrial use is the fungus *Saccharomyces cerevisiae*, also known as Baker's yeast. Yes, the one you use for baking! The problem is, Baker's yeast can only work with around 40% of the waste part of the plant, the part composed of sugar with 6 carbon atoms. So teams of specialized scientists have been working on changing the genetic make-up of the yeast so that it could convert almost 100% of the waste part of the plant.

They do this by taking some important genes from other microorganisms that do convert the remaining component of the waste part of plants, the part composed of sugars with 5 carbon atoms. In this case, an organism called *Caulobacter crescentus* which naturally degrades xylose, a sugar with 5 carbon atoms. This organism is normally found in freshwater and thrives on decaying plant material. The perfect target!

Caulobacter crescentus was the main focus of the degree project. Getting to know its growth behaviour in different environments was investigated with the aim of shedding light on how this organism can be used to improve Baker's yeast. *Caulobacter crescentus* was grown in shake-flasks and bioreactors (see figures below). It was found that this organism grows the best in glucose (sugar with 6 carbon atoms) followed by xylose. Also, it was found that when xylose is the only food for *Caulobacter crescentus*, an acid called α-ketoglutaric acid is formed, which is not the case when glucose is the only food. This organism was also shown to be quite smart. In fact, it changes its mechanism of survival depending on which sugar to eat. *Caulobacter crescentus* uses different sets of tools, in this case, enzymes, as a response to which type of food is available. For instance, the enzymes used for xylose do not appear when only glucose is available. For an organism that is about 170 million times smaller than yourself, that is indeed very intelligent!

The degree project has opened many doors for further research. There were some discoveries, but more questions were left unanswered such as why *Caulobacter crescentus* died before finishing all its food. The logic of the question may not be applicable to human beings but for a bacteria grown in a bioreactor, or shake-flasks for that matter, it does.



(left) a shake-flask (right) a bioreactor

All in all, it was an interesting project and hopefully to some degree this would contribute to developing a more sustainable solution to some of the major problems the world is facing today.