Investigating the effects of phosphate limitation in *Escherichia coli* AF1000 for better understanding of 3-hydroxybutyrate production

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Karin Sjöberg Gällnö
gallno@kth.se

Division of Industrial Biotechnology
KTH Royal Institute of Technology
1 Abstract

Phosphate limitation has proven to increase the productivity of 3-hydroxybutyrate (3-HB) in recombinant *Escherichia coli*. The consequences of phosphate limitation on cell physiology and cell metabolism are however not fully known. In this thesis the effects of phosphate limitation on the wild type *E. coli* AF1000 have been investigated in a phosphate limited chemostat. At low dilution rate, D=0.1 h⁻¹, the high affinity phosphate uptake system was activated but no alkaline phosphatase (PhoA) activity was seen. The glucose taken up per cell increased with decreasing growth rate. The maintenance for phosphate was zero whilst the maintenance for glucose was high at 0.4 g glucose/g cells, a consequence of the high carbon dioxide production and acetate formation. In addition to the high production of acetate and carbon dioxide, other organic acids were produced. HPLC analysis indicated that the acids were oxalic acid, pyruvic acid, lactic acid, succinic acid and fumaric acid but it could not be verified. Use of fermentative pathways can be a consequence of redox imbalance caused by inability to produce ATP when phosphate is scarce.

2 Sammanfattning

Fosfatbegränsning har visat sig kunna öka produktiviteten av 3-hydroxybutyrat (3-HB) i rekombinant *Escherichia coli*. Fosfatbegränsnings effekter på cellmetabolism och cellfysiologi är dock inte helt kända. I det här examensarbetet har effekterna av fosfatbegränsning på vildtyp *E. coli* AF1000 undersökts i en fosfatbegränsad kontinuerlig odling (chemostat). Vid låg utspädningshastighet, D=0.1 h⁻¹, aktiverades hög-affinitets upptagssystemet för fosfat, pst, men ingen alkalint fosfatas (PhoA) aktivitet kunde detekteras. Glukosupptaget per cell ökade med minskande tillväxthastighet. "Maintenance-behovet" för fosfat var noll medan det för glukos låg på 0.4 g glukos/g celler, h, vilket är att betrakta som högt. Detta är på grund av en hög koldioxid- och ättikssyraproduktion. Utöver koldioxid och ättikssyra producerades även andra organiska syror. HPLC-analys indikerade att dessa syror kunde vara oxalsyra, pyrodrusyra, mjölsyra, bärnstenssyra och fumarsyra men identiteten kunde inte fastställas. Användet av fermentationsmetabolism kan bero på att cellen lider av redox-obalans orsakad av oförmåga att producera ATP när fosfatnivåerna är låga.
3 Introduction

Plastic is a versatile material that can be used for many different purposes and applications in different fields. There are plenty of different types of plastics, all having in common that they consist of polymers. In many cases the polymers are organic. The main raw material for production of plastics today is petroleum, meaning that these plastics are not renewable and seldom biodegradable. The emissions of greenhouse gases connected to non-renewable products and the fact that oil is a limited resource have given increased interest in finding renewable substitutes for petroleum based products.

There are several different microorganisms that naturally produce organic polymers that can be used for plastic production. One of these organisms is the halophile *Halomonas boliviensis* that produce polyhydroxybutyrate (PHB) as an intracellular storage compound when grown under carbon excess and another substrate is limiting such as P, O, S, N or trace elements; Mg, Ca or Fe. (Lee 1996, Quillaguaman, Hashim et al. 2005). PHB is biodegradable and can be used for many different applications such as packaging materials and because of its biocompatibility it has potential in the medical industry in implants and sutures (Hazer and Steinbuchel 2007, Brigham and Sinskey 2012).

Two enzymes, a thiolase and a reductase, from *H. boliviensis* have been introduced into *Escherichia coli* to produce 3-hydroxybutyrate (3-HB), the monomer of PHB, see Figure 1 (Quillaguaman, Hashim et al. 2005). Firstly two molecules of acetyl-CoA are condensed to acetoacetyl-CoA. This step is followed by a reduction to 3-hydrobutyrate-CoA, it is not clear if this enzyme use NADH or NADPH as reducing agent. The last step is hydrolysis to give the final product 3-HB, this step is either spontaneous or performed by a natural *E. coli* enzyme, a probable candidate is the thioesterase encoded by TesB. *E. coli* and other microorganisms use renewable carbon sources for growth and production, typically glucose, but other sugar or carbohydrates are possible. Earlier studies have shown that phosphate limitation gives the best productivity of 3-HB in *E. coli* in fed-batch cultivations compared to carbon and nitrogen limitation. Phosphate limitation allows control of cell growth at the same time as the carbon source is in excess. This makes phosphate limited fed-batch processes a valuable tool for production of all types of carbon-based products. Limitation of phosphate steers carbon flux towards production of 3-HB instead of cell mass (Schuhmacher, Loffler et al. 2014).
Figure 1. 3-HB production in *Escherichia coli* using recombinantly expressed thiolase (T3) and reductase (Rx) from *Halomonas boliviensis*.

Previous experiments on phosphate limited fed-batches, with and without production of 3-HB, have indicated that acetate is produced throughout the process, even at low growth rates. Acetate production in *E. coli* under aerobic conditions is caused by glycolysis being faster than the capacity of the citric acid cycle (TCA) or oxidative phosphorylation. This results in excess production of acetyl-CoA. *E. coli* deals with this by turning acetyl-CoA into acetate which is secreted and one ATP is gained in the process. For growth rates below 0.3 h⁻¹ this type of acetate production is not typically seen (Enfors 2011, Larsson 2012). The production of acetate withdraws carbon from the glycolysis, carbon that could have been used for production of 3-HB, and thereby reduces the product yield. This is not the only problem connected with acetate production; high acetic acid concentration inhibits cell growth (Luli and Strohl 1990).

Earlier experiments have indicated that the demand for glucose varies with growth rate. In Figure 2 and Figure 3 the growth curves of two phosphate limited fed-batch cultivations, with and without 3-HB production, are shown. Both phosphate and glucose were fed according to the feed-profile but the glucose feed was designed to always be in excess. The glucose concentration was monitored continuously and since glucose levels were dropping it had to be added batchwise to avoid it from becoming the limiting substrate.

Little information on phosphate limited cultivations and how it affects *E. coli* is found in literature. We do not know why the glucose consumption varies with growth rate and how the production of acetic acid is coupled to this.
The cultivation is started with a batch-phase followed by an exponential feed phase (SFR=0.35), a linearly increasing feed-phase (k=0.1) and at the end a phase where no phosphate is fed but the glucose feed is kept constant. Glucose was added batchwise when glucose levels got low.

Figure 2. Phosphate limited fed-batch cultivation of E. coli AF1000 pJBGT3Rx without induction. The cultivation is started with a batch-phase followed by an exponential feed phase (SFR=0.35), a linearly increasing feed-phase (k=0.1) and at the end a phase where no phosphate is fed but the glucose feed is kept constant. Glucose was added batchwise when glucose levels got low.

Figure 3. Phosphate limited fed-batch cultivation of E. coli AF1000 pJBGT3Rx with production of 3-HB. The cultivation is started with a batch-phase followed by an exponential feed phase (SFR=0.35), a linearly increasing feed-phase (k=0.1) and at the end a phase where no phosphate is fed but the glucose feed is kept constant. Glucose was added batchwise when glucose levels got low.
3.1 Phosphate uptake in *E. coli*

Inorganic phosphate (Pi) is part of many important compounds in the cell and its metabolism and uptake is tightly regulated. There are four specific systems for transportation and uptake of phosphate in *E. coli*; The Pi-specific transport system (pst), the phosphate inorganic system (Pit), a Pi-linked antiport system for transport of 3-phosphoglycerate-3-P (GlpT) and a Pi-linked antiport system for transport of glucose-6-P (UhpT) (van Veen 1997). Inorganic phosphate is the preferred form of phosphate in *E. coli* but organophosphates can be used if availability of Pi is scarce. Other organophosphates than 3-phosphoglycerate-3-P and glucose-6-P can be transported into the periplasm via unspecific pore forming proteins, mainly consisting of OmpF and OmpC. Under Pi-limiting conditions a third pore forming protein is produced, PhoE. In the periplasm the organophosphates are hydrolyzed to release Pi by a wide range of enzymes. One such enzyme is the nonspecific alkaline phosphatase (PhoA). For transportation of Pi from the periplasm into the cytoplasm *E. coli* use pst and Pit (van Veen 1997).

The Pit transport system has been called a low affinity-high velocity system with a $V_{\text{max}}$ of 55±1,9 nmol Pi/mg cell dry weight, min and a $K_m$ of 38,2±0,4 µM (Willsky and Malamy 1980). Pit is constitutively expressed and is not affected by Pi deprivation (Rosenberg, Gerdes et al. 1977). The Pit transport is a syport that co-transport phosphate with H⁺. This means that the proton motive force generates the driving force for transport (van Veen 1997).

Phosphate has several different acid and base species. The dominant form is determined by the pH. At physiological pH (5.5-8) $H_2PO_4^-$ and $HPO_4^{2-}$ are the dominating species but in presence of excess $Ca^{2+}$ or $Mg^{2+}$ the neutral, soluble metal chelate MeHPO4 accompanies them. This chemical nature of phosphate has given rise to the idea that the actual substrate for Pit is the metal complex. It has been shown that the Pi uptake via Pit is divalent cation dependent, giving further strength to this statement. The Pit system can also perform homologous exchange of MeHPO4 (van Veen 1997).

GlpT and UhpT are Pi-linked antiport transporters exchanging Pi for their specific substrate, glucose-6-phosphate or 3-phosphoglycerate-3-P. These two systems can also mediate homologous Pi:Pi and organophosphate:organophosphate transport. The transport is driven by downhill transport of Pi (Ambudkar, Larson et al. 1986) (Sonna, Ambudkar et al. 1988). The expression of GlpT and UhpT is induced by extracellular glucose-6-P and 2-deoxyglucose-6-P (van Veen 1997).

The pst system is an ATP-binding cassette (ABC) transporter, with one periplasmic substrate binding protein and three membrane bound components. The substrate binding protein binds to phosphate in the periplasm and redirects it to the transporter for passage into the cytoplasm. Thanks to the substrate binding protein the pst system has high affinity for phosphate with a $K_m$ of 0,43±0,2 µM Pi but its maximum velocity is low compared to that of Pit ($V_{\text{max}} = 15,9±0,3$ nmol Pi/mg dry weight, min) (Willsky and Malamy 1980). The most probable substrate for the *E. coli* pst system is $H_2PO_4^-$ and $HPO_4^{2-}$ (van Veen 1997). Pst’s substrate binding protein is coded by the gene pstS. The pstS
promoter is expressed at low level when Pi levels are high, but when Pi is limiting it shows 100-fold derepression. The *pst* genes forms an operon, *pстSCAB-PhоU*, which expression is tightly regulated. The *pстSCAB-PhоU* operon is part of the phosphate (pho) regulon that contains several more phosphate-starvation-induced (psi) genes including the *PhоE*-porin and *PhоA*. The expression of the Pho regulon is regulated by extracellular Pi-levels. The first step in the signal transduction pathway leading to Pho expression is mediated by pst. Under Pi-limitation Pi is bound and taken up by pst, this mediates a signal to PhoR which autophosphorylates and transmits the signal to PhoB via phosphorylation. PhoB is only active when phosphorylated and acts as a DNA-binding effector protein, binding to the “pho box”, upstream the Pho regulon promoters and activating its transcription. The deactivation, including dephosphorylation of PhoB and PhoR is not well understood, but it is thought to be activated by Pi saturation of PstS. PhoB may also be phosphorylated by CreC (former PhоM) in response to some unknown catabolite and in response to acetyl-phosphate (van Veen 1997).

3.2 How does phosphate limitation affect *E. coli*?
To be able to create and optimize a production process it is essential to know how the process works. In this case the core of the process is phosphate limitation but so far little is known on how it affects *E. coli*. There are however several studies showing that productivity can be increased using phosphate limitation instead of carbon-limitation for production of carbon containing products (Lee, Wong et al. 2000, Johansson, Lindskog et al. 2005, Wu, Hu et al. 2010).

Phosphate have many functions in the cell, it plays a major role in the cell’s energy metabolism in form of nucleotides; ATP, ADP, AMP, GTP, GDP and GMP. It is also an important component in the RNA and DNA backbone and in cell wall structure in the form of phospholipids. Moreover it is a common mediator in many signal transfer pathways. All these compounds have key-functions in the cell, hence limitation of phosphate may affect the cell’s state in many ways (van Veen 1997). In addition to the above-mentioned compounds *E. coli* cells contain a small pool of orthophosphates and polyphosphates, the storage form of phosphate (Egli and Mason 1993, Rao, Liu et al. 1998).

Egli and coworkers have made extensive investigations on how bacteria react to exhaustion of different nutrients. Although these results are not directly applicable to the nutrient-limited conditions that occur in fed-batch and continuous cultivations their results can give a hint on what happens in the case of phosphate limitation. They have shown that when cells of *Klebsiella pneumoniae* are exhausted on phosphate their growth continues but ceases, implying that the cell can assimilate phosphate from intracellular deposits. For cells exhausted in glucose a degradation of rRNA can be seen immediately after the onset of exhaustion to provide energy and precursors for synthesis (Egli and Mason 1993). Since rRNA is a large carrier of phosphate the same response is seen for phosphate starved cells after consumption of intracellular orthophosphates (Egli and Mason 1993). As DNA is vital for both production of new cells and cell survival scavenging on DNA is not likely. It has been shown
that RNA is degraded to give building blocks for DNA, suggesting that DNA is of major priority of the cell (Egli and Mason 1993).

As for the case of phosphate starvation, it is probable that the cell will give priority to the phosphate-containing compounds essential for cell growth, i.e. DNA, phospholipids and to some extent ATP, when phosphate is limiting. When no more phosphate can be taken up from the surrounding these compounds are expected to be produced at expense of other phosphate containing compounds. Firstly the cell’s small storage of ortho- and polyphosphates and after that RNA mainly in the form of rRNA is degraded. rRNA constitutes the main part of degradable RNA in the cell and therefore it is the preferred RNA fraction (Egli and Mason 1993). To some extent, phosphate might also be taken from cell wall phospholipids, leading to a change in cell morphology. Phospholipids and cell wall material are however vital for production of new cells and their decrease is expected to be minor. No change in protein content is expected since both carbon and nitrogen are in excess. On the other hand induction of PhoA is likely to occur in accordance with induction of pst since they are expressed from the same regulon.

ATP is vital for many different cell functions and it is therefore likely that its production is prioritized. There are however investigations showing that energy charge is lowered when cells are subjected to phosphate limitation (Schuhmacher, Loffler et al. 2014). This indicates that what actually limits growth of the cells is an incapability of producing enough ATP.

Phosphate is not consumed in the metabolism, it is rather shuffled between different compounds (e.g. the reaction from ADP to ATP or phosphoenolpyruvate to glucose-6-phosphate), meaning that phosphate is only used in the production of new cells and not for supporting cell survival. This gives that the maintenance requirement ($q_{\text{mPO43-}}$) should be zero.

Phosphate limitation can also be seen as a case of glucose excess. When glucose is in excess the normal cell response is production of overflow metabolites, acetate in the case of *E. coli* (Enfors 2011). Therefore a non-growth coupled production of acetate is anticipated. The consequence of a high production of acetate is a large maintenance requirement for glucose ($q_{\text{mglu}}$).

All these different consequences of scarce phosphate are believed to be seen in a stepwise fashion with increased phosphate limitation (lower phosphate feed) in a continuous cultivation.

### 3.3 Present investigation

For development and optimization of a process, knowledge on how the production host reacts to the production conditions is vital. From a production process development point of view there are questions that need to be answered. For design of feed-profiles knowledge on how the glucose uptake varies with growth rate is needed. To be able to minimize the production of byproducts such as acetic acid we must first know how it is related to phosphate limitation and cell growth. The more we know about how *E. coli* is affected by
phosphate limitation the better possibilities we get to develop an efficient production process of 3-HB based on phosphate limitation.

In this thesis the aim is to investigate what happens in the wild type *E. coli* AF1000 when phosphate is limiting. Based on available literature different factors that are thought to be affected by phosphate limitation have been chosen for investigation. These factors are; intracellular content of phosphate, amount of rRNA, PhoA activity, energy charge, acetic acid and byproduct formation, glucose and phosphate uptake. The factors will be investigated in a phosphate limited chemostat cultivation to see how they are affected by growth rate.
4 Materials and methods

4.1 Bacterial strain and cultivation conditions
The bacterial strain used was *Escherichia coli* AF1000 (Sanden, Prytz *et al.* 2003). Continuous cultivation (chemostat) was carried out in a 3-liter fermenter (The ant, Belach Bioteknik AB) with a working volume of 2 l. The temperature was kept constant at 37°C and the pH at 7, using automatic titration of 25%NH₄OH. The stirring was kept constant at 1500 rpm and the air inflow at 2.5 l/min, this gave a DOT of approximately 60%. Two different feed solutions were used to control the growth of cells. Feed 1 was based on a minimal medium but the amount of phosphate was reduced to 0.4 g/l and it did not contain glucose. See medium recipe in appendix. Glucose (500 g/kg) was fed separately, named feed 2. This double-feed was set-up to be able to control the feed rate of phosphate and glucose independently. The continuous (chemostat) culture was accomplished by adjusting the speed of the inflow and outflow pumps, where the inflow pumps were used to set the dilution rate (D). The outflow pump was turned on in intervals at a set speed to maintain a constant weight of the reactor, this was automatically regulated by the WebAnt® control software (Belach Bioteknik AB).

7 different dilution rates were tested and each dilution rate was tested at least two times to get cultivation duplicates. 2 dilution rates were also tested with a lower feed rate of glucose (D=0.1 h⁻¹ and D=0.3 h⁻¹). For exact feed rates see Table 1. Samples were taken after 5 residence times.

Table 1. Feed rates at different dilution rates.

<table>
<thead>
<tr>
<th>“D”</th>
<th>D̄real</th>
<th>FₚO₄₃-</th>
<th>Fglu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[h⁻¹]</td>
<td>[l/h]</td>
<td>[l/h]</td>
</tr>
<tr>
<td>0.1</td>
<td>0.106</td>
<td>0.2</td>
<td>0.012</td>
</tr>
<tr>
<td>0.2</td>
<td>0.212</td>
<td>0.4</td>
<td>0.024</td>
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<tr>
<td>0.3</td>
<td>0.318</td>
<td>0.6</td>
<td>0.036</td>
</tr>
<tr>
<td>0.4</td>
<td>0.424</td>
<td>0.8</td>
<td>0.048</td>
</tr>
<tr>
<td>0.5</td>
<td>0.530</td>
<td>1.0</td>
<td>0.061</td>
</tr>
<tr>
<td>0.6</td>
<td>0.636</td>
<td>1.2</td>
<td>0.073</td>
</tr>
<tr>
<td>0.7</td>
<td>0.742</td>
<td>1.4</td>
<td>0.085</td>
</tr>
<tr>
<td>0.1lowGlu</td>
<td>0.103</td>
<td>0.2</td>
<td>0.005</td>
</tr>
<tr>
<td>0.3lowGlu</td>
<td>0.308</td>
<td>0.6</td>
<td>0.017</td>
</tr>
</tbody>
</table>

4.2 Sampling procedure
The bioreactor used has a sampling port with a rubber membrane on top. From the port samples are withdrawn using a syringe.

Samples for OD, cell dry weight, alkaline phosphatase activity, total protein and rRNA were taken directly from the reactor. Samples for OD, CDW and alkaline phosphatase were analyzed directly.
Samples for medium glucose, medium phosphate and acetate analysis were taken into a syringe containing 2 ml pre-cooled (8°C) perchloric acid (0,13 M). The sample size was 2 ml. The syringe was weighed before and after addition of acid and sample for calculation of the dilution factor. Directly after sample taking the sample was centrifuged for 10 min at 4500 rpm. 3,5 ml of the supernatant was neutralized with 0,075 ml of pre-cooled potassium carbonate (8°C, 500 g/l). After 15 minutes on ice the sample was centrifuged (5 min, 4500 rpm) and the supernatant was saved for analysis (Larsson and Törnkvist 1996). The samples were kept in fridge (-20°C) until time of analysis.

Since the turnover of intracellular metabolites is fast the samples for intracellular metabolite analysis had to be inactivated efficiently. For this a sampling method adapted to this type of reactor based on the one developed by Meyer et al. was established (Meyer, Noisommit-Rizzi et al. 1999). Samples were taken into a syringe containing approximately 8,5 grams of glass beads (diameter 0,5-0,75 mm) and 1 ml of inactivation medium. The type of inactivation medium depended on the following analysis. The syringe with beads and inactivation medium was kept in a freezer (-20°C) until sample taking. 3 ml of sample was taken into the syringe and the syringe was weighed after addition of beads and after addition of acid and sample to calculate the dilution factor. 2 different inactivation media were used depending on the following analysis; HClO4 (35% w/v) or 4M HCl (Theobald, Mailinger et al. 1997, Meyer, Noisommit-Rizzi et al. 1999) The samples where kept in fridge (-80°C) until day of analysis.

4.3 OD
OD was measured at 600 nm (Novaspec II, visible spectrophotometer). Samples were diluted to absorption of approximately 0.1.

4.4 Cell dry weight
5 ml of cell suspension was centrifuged for 10 minutes at 4500 rpm in pre-weighed glass tubes. The supernatant was discarded and the cell pellet was resuspended in 5 ml of saline solution (0,9 % w/v). Centrifugation was repeated and the supernatant was discarded. Cell pellets were dried in oven (105°C) over night and weighed.

4.5 Alkaline phosphatase activity
Cells for alkaline phosphatase analysis were disrupted using a french press (SLM instruments inc.). 100 µl of pressed sample was added to 900 µl of Alkaline Phosphatase Yellow (pNPP) Liquid substrate System for ELISA (Sigma-Aldrich, P7998). The absorbance was monitored at 405 nm.

4.6 Total protein
The protein content was analyzed in three different fractions; extracellular, intracellular and a total fraction, where no separation of cells and medium had been carried out, containing both intracellular and extracellular proteins. The extracellular sample was taken from the supernatant of the cell dry weight, after centrifugation, this sample was used to estimate the cell lysis. The intracellular sample was taken according to the procedure of cell dry weight but as a last step the pellet was resuspended in 5 ml of saline (0,9% NaCl).
The protein concentration was analyzed using the Bradford protein assay (Bradford 1976). 1 ml of Bradford reagent (Coomassie Brilliant blue G250,100 mg/l, 95% ethanol 50 ml/l, 85% (w/v) phosphoric acid 100 ml/l) was mixed with 20 µl of sample and incubated for 5 minutes. The absorbance was measured at 595 nm. A protein standard prepared from bovine serum albumin was used for quantification.

4.7 Acetic acid, glucose and byproducts
Acetic acid and glucose was analyzed on HPLC (Waters alliance Separation module 2695, Waters 2410 Refractive index detector, Waters 2996 photodiode array detector). The stationary phase was a Bio-Rad Aminex HPX-87H column (300*7,8 mm). 0,004 M sulphuric acid was used as running buffer in an isocratic run, the flow rate was kept at 0,5 ml/min and the sample time was 40 minutes. The column and detector was kept at room temperature, approximately 25°C. Samples were heated to 80°C for 15 minutes and centrifuged at 13000 rpm for 10 minutes prior to analysis. Standard solutions containing acetic acid or glucose were used for quantification. For byproduct analysis no quantification was done and the retention times and absorbance spectra was compared to standards of succinic acid, malic acid, formic acid, lactic acid, pyruvic acid, oxalic acid, fumaric acid, ethanol and methylacetoacetate and methylacetoacetate (KOH 1M).

4.8 rRNA
RNA was extracted using Qiagen’s RNeasy Mini Kit and treated according to the supplementary protocol “Purification of total RNA from bacteria using the RNeasy® Mini Kit” also provided by Qiagen. Samples were diluted to OD 1, 1 ml of diluted sample was centrifuged (5300g, 5min) to get a cell pellet containing approximately 10⁹ cells. Cell pellets were frozen at -80°C until day of analysis. For lysis of the cells 40-80 mg acid washed and autoclaved glass beads (diameter 0,5-0,75 mm) were put in a safe-lock tube, together with the sample, according to the protocol, the lysis was performed by vortexing of the cells for 30 seconds followed by cooling on ice for 30 seconds. This cycle was repeated 8 times. 350 µl of the supernatant was taken in step 5 and 350 µl of ethanol was added. The protocol was followed for the rest of the procedure.

The total RNA concentration was measured on Nanodrop. Agarose gel (1%) electrophoresis was used to separate the ribosomal RNA. TBE was used as running buffer and GelRed 10000x (Biotium) for visualization of the bands. The ladder was Generuler 1kb. Bands were quantified using the ImageJ software.

4.9 Medium phosphate
The phosphate content in the medium was determined using Sigma-Aldrich’s Phosphate Colorimetric Kit (MAK030). Samples were heated to 80°C for 15 minutes and centrifuged at 13000 rpm for 10 minutes prior to analysis.

4.10 Intracellular phosphates
For determination of intracellular levels of different phosphate compounds two different pretreatment steps were applied. For acid soluble phosphates (e.g. polyphosphates and some nucleic aids) sample were taken into cool HCl (4 M) as described in the sampling procedure section, section 4.2. The samples were
subjected to 1 freeze-thaw cycle and put on heating block (95°C) for 60 minutes (Ohtomo, Sekiguchi et al. 2004, Torres-Dorante, Claassen et al. 2005).

For determination of the more persistent phosphate containing compounds (e.g. phospholipids and other organophosphates) samples were taken in HClO₄ (35% w/v) as described in section 4.2. To 800 µl of sample 160 µl of KOH (6 M) and 200 µl of potassium persulfate (50 mg/ml) was added. Samples were put on heating block (90°C) for 16 hours as described by (Huang and Zhang 2009).

Quantification of hydrolyzed Pi for both acid soluble and total phosphate was done identically. Samples were centrifuged at 13000 rpm for 10 minutes. The phosphate content in the samples after heat treatment was determined using Sigma-Aldrich’s Phosphate Colorimetric Kit (MAK030). The acid soluble fraction was calculated by subtracting the medium concentration from the measured value. The persistent phosphate fraction was calculated by subtracting the acid soluble and medium concentration.

4.11 Energy charge

Samples were taken according to sampling procedure (section 4.2) in cool HClO₄ 35% (w/v). Samples were put on ice for 5 minutes and neutralized with a solution consisting of 2 M KOH and 0.5 M imidazole. Samples were centrifuged (5 min 5300g) to remove salts and the supernatant was frozen (-80°C). Samples were subjected to 2 freeze-thaw cycles and centrifuged before determination of nucleotide content using RP-HPLC. Quantification was done using the method described by Folley (Folley, Power et al. 1983). The column used was a C-18-RP column (15 cm*4,6cm, 3µm) (Supelcosil LC-18-T, Supelco), no guard column was used. The HPLC configuration and run was done according to the protocol described by Meyer et al. with the only modification that the flow rate was lowered to 0,5 ml/min (Meyer, Noisommit-Rizzi et al. 1999). Energy charge was calculated using the equation below:

\[
\text{Energy charge} = \frac{[ATP] + \frac{1}{2}[AMP]}{[ATP] + [ADP] + [AMP]}
\]
5 Results

5.1 Cell growth and nutrient uptake – Rates and yields

5.1.1 Cell mass and substrate uptake
When cells are grown in a chemostat the cell mass is determined by the concentration of the limiting substrate in the inlet flow. When glucose is the limiting substrate the cell mass will be constant if the feed composition is constant. The results from this phosphate limited chemostat are however different, the cell mass decreases with increased flow rate, see Figure 4.

At D=0,1 h⁻¹ the concentration of phosphate in the medium is significantly lower than for the other dilution rates, we also see an increased cell mass in this point. The significant increase of phosphate uptake at dilution rate 0,1 h⁻¹ is due to the induction of the pst uptake system. The yield of cells over phosphate (Yₓₚ) is constant during the whole cultivation, Figure 5, i.e. the increase in cell mass is only due to the increased assimilation of phosphate and not due to a change in Yₓₚ. The higher cell mass causes an increase in the volumetric glucose uptake. The increase in glucose uptake is however not only due to an increased amount of cells. At dilution rates below 0,5 h⁻¹ more glucose is taken up per cell and the uptake is increasing with decreasing growth rate, seen in Figure 5 as a decrease in yield of cells over glucose (Yₓ₉₉ₗ). At growth rates above 0,5 h⁻¹ Yₓ₉₉ₗ reaches a maximum value of 0,36 g cells/g glucose h⁻¹. The point at D=0,5 h⁻¹ showing a Yₓ₉₉ₗ far above the maximum theoretical Yₓ₉₉ₗ of 0,5 g cells/g glucose is considered an outlier (Larsson 2012).
At $D=0,1 \text{ h}^{-1}$, $D=0,1 \text{ h}^{-1}$ lowGlu and $D=0,3 \text{ h}^{-1}$ lowGlu both the glucose and phosphate concentrations in the reactor are low, Figure 4, meaning that the cells in this point are limited in both glucose and phosphate. It is legitimate to believe that if more glucose had been available at $D=0,1 \text{ h}^{-1}$ the bacteria would have had a higher glucose uptake i.e. the glucose uptake is not saturated in this point.

![Graph](image_url)

**Figure 5.** The yield of cells over glucose and phosphate.

Both the specific glucose uptake rate ($q_{glu}$) and the specific phosphate uptake rate ($q_{PO43}$) vary linearly with growth rate, Figure 6. The maintenance coefficient for phosphate ($q_{mPO43}$) is zero, showing that phosphate is only needed for biomass formation and not for life-supporting activities. The maintenance coefficient for glucose ($q_{mglu}$) is 0,4 g glucose/g cells, h. For *E. coli* grown under glucose limiting conditions a $q_{mglu}$ in the range of 0,04 g glucose/g cells, h is normally seen (Larsson 2012) giving that the $q_{mglu}$ observed here is extremely high. This high $q_{mglu}$ indicates either an increased need for ATP or reducing equivalents or that more glucose is needed for ATP production.
5.1.2 Acetic acid production

Acetic acid is produced at all dilution rates, except for $D=0.1 \text{ h}^{-1}\text{lowGlu}$, Figure 7. Between $D=0.2 \text{ h}^{-1}$ and $D=0.6 \text{ h}^{-1}$ $q_{HAc}$ increases linearly with growth rate. Above $D=0.6 \text{ h}^{-1}$ $q_{HAc}$ shows a decreasing trend when growth rate increases.

For $0.1 \text{ h}^{-1}<D<0.6 \text{ h}^{-1}$ the acetate yield over glucose is constant at its highest value while it is lower for $D=0.1 \text{ h}^{-1}$ and $D>0.6 \text{ h}^{-1}$, Figure 7. At $D=0.1 \text{ h}^{-1}$ both glucose and phosphate have been limiting which could explain the lower yield ($Y_{HAcGlu}$) seen in this point. The dilution rates with the highest yield of acetic acid correlates with the increased uptake of glucose per cell seen in Figure 5.

It seems as the production of acetic acid is not directly coupled to growth rate instead it is fair to believe that it is coupled to the glucose uptake. There may however be additional influencing factors such as the metabolic state of the cell.
5.1.3 Carbon dioxide production

At $D=0.1\ h^{-1}$ the carbon dioxide production is high, dropping to its lowest measured value at $D=0.2\ h^{-1}$, Figure 8. From $D=0.2\ h^{-1}$ the specific carbon dioxide production rate increases with growth rate and it reaches a plateau at $D=0.6\ h^{-1}$.

The yield for carbon dioxide per glucose is the same for the high and low glucose cases but the production rate is affected. At the $D=0.1\ h^{-1}\ lowGlu$ the production rate is significantly lower while it is higher at $D=0.3\ h^{-1}\ lowGlu$. 

Figure 7. Specific acetic acid production rate and acetate yield over glucose.
5.1.4 Carbon recovery and redox balance

To see if all carbon containing products had been accounted for a carbon and redox balance was calculated. The balances were based on glucose and oxygen as ingoing substrates and the theoretical cell mass $\text{CH}_{1.8}O_{0.5}N_{0.2}$, acetic acid and carbon dioxide as products. The carbon recovery is well below 100% at all dilution rates and the degree of reduction is negative for all points except one, see Figure 9. This indicates that there are products that have not been accounted for. The carbon and the redox balance follows the same pattern as $q_{\text{HAc}}$ indicating that the decrease in acetic acid production at growth rates above 0.5 h$^{-1}$ is a consequence of production of some other product. One of the points at $D=0.5$ h$^{-1}$ is considered an outlier since its carbon recovery is above the theoretical maximum of 100%.

Figure 8. Specific carbon dioxide production rate and the yield of carbon dioxide per glucose.

$q_{\text{CO}_2}$ [g CO$_2$/g cells, h], $Y_{\text{CO}_2}$/glu [g CO$_2$/g glucose]

Dilution rate [h$^{-1}$]

- $q_{\text{CO}_2}$
- $q_{\text{CO}_2}$ lowGlu
- $Y_{\text{CO}_2}$/glu
- $Y_{\text{CO}_2}$/glu lowGlu
5.1.5 Possible byproducts

Because of the inadequate closure of the carbon and redox balance the HPLC-spectra from glucose and acetate analysis were more thoroughly analyzed. Several peaks showing the same type of alteration in peak area as acetic acid were identified. For the DAD-detector the interesting peaks had retention times of 9.5, 11.6, 12.5, 15.9, 16.8 and 22 minutes. The spectrum from the RID-detector showed interesting peaks at retention time 23.5 minutes and it also showed the same peak as the DAD-detector at retention time 9.5 minutes. This shows that there are several different byproducts that have not been accounted for.

The peak spectrum and retention time for each peak were compared with standards for succinic acid, malic acid, formic acid, lactic acid, pyruvic acid, oxalic acid, fumaric acid, ethanol and methylacetoacetate and methylacetoacetate (KOH 1M). The peak at 9.5 minutes showed the same spectrum and retention time as oxalic acid, 11.6 as pyruvic acid, 15.9 as lactic acid, 16.8 as succinic acid and 22 as fumaric acid. Their identities cannot however be verified but this serves as an indication on that these substances might be present. Some peaks were not possible to identify at all.

Lactate and succinate together with acetate and carbon dioxide are typical fermentation products in *E. coli* (Gottschalk 1986) indicating that fermentation pathways are active.
5.2 The metabolic state of the cell

5.2.1 Intracellular phosphates

Two types of intracellular phosphates were measured; acid soluble phosphates and more persistent phosphate compounds, degraded by oxidation. Some points showed a negative value after subtraction of medium or acid soluble phosphates, these points were considered outliers and were not included in the final results. The inconsistent data complicates the interpretation of the result.

In Figure 10 it can be seen that the levels of both types of phosphates vary equally with growth rate. The phosphate content is constant or perhaps slightly increasing with growth rate from D=0,1 h⁻¹ to D=0,6 h⁻¹. At D=0,7 h⁻¹ the levels reach its lowest value for both persistent and acid soluble phosphates.

The highest content of persistent phosphates is seen in the low glucose point for D=0,1 h⁻¹. The high content of persistent phosphates is difficult to explain. It was expected that this fraction would remain more or less constant or perhaps show a slight decrease with decreasing growth rate, since the phosphates in this fraction are resistant.

![Figure 10. Content of phosphate tied to different types of intracellular compounds. Acid soluble phosphates mainly come from Pi, polyphosphates and some nucleic acids while the persistent compounds mainly consist of phospholipids and other organophosphates.](image)

5.2.2 Protein content and PhoA expression

The intracellular protein content increases with growth rate reaching a plateau at D=0,6 h⁻¹, Figure 11. A minimum protein content is found at D=0,2 h⁻¹. At D=0,1 h⁻¹ lowGlu the protein content reaches its highest value. As stated earlier the protein levels were expected to be constant since both carbon and ammonium are in excess, thus this result is surprising.
There are studies showing that protein expression changes and so called “stress proteins” are produced when bacteria are stressed (Hemm, Paul et al. 2010), but the studies are not showing if the proteins actually accumulate or if they are produced at expense of other proteins. Thus it cannot serve to explain the high protein content seen at D=0,1 lowGlu. The studies of Egli et al. indicate that the protein levels in the cell are constant even at very low medium phosphate concentrations. Consequently no good explanation to the increase in protein content can be stated.

No PhoA activity could be detected at any dilution rate. This shows that there is no direct relationship between pst expression (seen at D=0,1 h\(^{-1}\)) and PhoA expression.

![Figure 11. Protein content at different dilution rates.](image)

**5.2.3 Lysis**

The concentration of proteins in the supernatant was below detection limit at all dilution rates except for the points at low glucose and low growth rate (0,1 h\(^{-1}\) lowGlu). At these points a protein concentration of 0,15 and 0,13 mg/ml in the supernatant was detected indicating that cell lysis occurred.

**5.2.4 Energy charge**

The energy charge measurement using HPLC was not successful. No peak corresponding to neither ATP nor ADP or AMP could be identified in any of the samples. Therefore no conclusions can be drawn from this analysis.
5.2.5 RNA

The RNA content of the cells was measured using two methods; Nanodrop, where the total RNA was measured, and agarose gel, where the RNA fractions were separated and the band for the large ribosomal unit was quantified (see gel in appendix). In Figure 12 results from both methods can be seen. It is difficult, to not say impossible, to draw any reliable conclusions from these results. The results from both methods vary to the same extent implying that it is either the sampling method or the purification that have caused this variance in the data.

*Figure 12. RNA levels in the cells at different dilution rates. The nanodrop value corresponds to total RNA whilst the gel value only presents the 16s rRNA fraction.*
6 Discussion

6.1 Metabolism when phosphate is limiting

We have seen that cell growth and metabolism of *E. coli* grown under phosphate limitation differs from bacteria grown under glucose limitation or nutrient excess. The most striking results are mentioned below; Production of lactic acid, fumaric acid and succinic acid are normally only seen in the mixed acid fermentation of *E. coli* (Lengeler, Drews et al. 1999) i.e. their presence indicate that fermentation pathways are active. These products have been produced although the reactor has been well aerated, with a DOT constantly above 50%. The high carbon dioxide production indicates that TCA is active. An increased glucose uptake per cell is seen when going to lower growth rates. These discoveries have altogether lead on to a new theory on the cell metabolism when phosphate is limiting.

Although the energy charge measurement was not successful the hypothesis that production of ATP is hampered when phosphate is limiting remains. ATP is produced by two main mechanisms; substrate level phosphorylation and oxidative phosphorylation. Substrate level phosphorylation occurs in the glycolysis and the citric acid cycle. From the glycolysis and TCA reducing equivalents, such as NADH, are also produced. NADH donates its electrons to the respiratory chain and is thereby reoxidized to NAD⁺. In the respiratory chain the electrons are transferred to an electron acceptor, oxygen for aerobic growth. This transfer of electrons is coupled to pumping of protons and build-up of an electrochemical potential over the membrane, the proton motive force. The proton motive force is used to fuel the production of ATP from the enzyme ATP synthase. This process is called oxidative phosphorylation. The production of ATP in the respiratory chain is far more efficient than the production in the glycolysis and TCA. The glycolysis and the TCA generates 2 mols of ATP per mol of glucose each, giving in total 4 mols ATP whilst the respiratory chain generates approximately 34 mols of ATP per mol of glucose (Lengeler, Drews et al. 1999).

The $K_m$ of ATP synthase for Pi is 4,2 mM, this is considerably high compared to the enzymes responsible for phosphate uptake in substrate level phosphorylation. Phosphate acyltransferase has a $K_m$ of 2,1 mM and glyceraldehyde 3-phosphate dehydrogenase has a $K_m$ of 0,53 mM (Eyschen, Vitoux et al. 1999, Iino, Hasegawa et al. 2009, Campos-Bermudez, Bologna et al. 2010). This means that of the enzymes responsible for ATP production, ATP synthase is the one that is first affected by low phosphate levels. Since the cell still has the same energy requirement as before it must take up more glucose to be able to produce the energy needed when using substrate level phosphorylation. This serves as an explanation to the increased glucose uptake per cell seen at growth rates below 0,6 h⁻¹, Figure 5. The increased uptake of glucose also correlates with the increased yield of acetate ($Y_{\text{AcGlu}}$) seen for dilution rates between 0,2 h⁻¹ and 0,5 h⁻¹, Figure 7. Production of acetate yields one ATP but no reducing equivalents are produced and thereby this pathway contributes to the energy state of the cell without affecting the redox balance. The lower yield of acetate seen at $D=0,1$ h⁻¹ is a cause of both phosphate and glucose being limiting in this point.
If the limitation of phosphate causes ATP synthase to be incapable or at least inefficient in producing ATP, what happens with the rest of the respiratory chain? Schuhmacher et al. have proposed a decoupling scenario of ATP synthase meaning that it can decouple the production of ATP from pumping of protons. This would mean that the cell still could keep an active respiratory chain for regeneration of NAD⁺ even though no or little ATP is produced (D’Alessandro, Turina et al. 2011, Schuhmacher, Löffler et al. 2014). Their theory does not however explain why fermentation products are produced therefore another theory on the metabolism is suggested below.

6.2 Phosphate limitation causes inactivation of the respiratory chain

The increased uptake of glucose and the high carbon dioxide production suggests that the flux in the glycolysis and TCA is still high even though the respiratory chain is hampered. This increased flux is a cause of ATP synthase being inefficient, as explained above. If ATP synthase is inactivated by the low phosphate levels rather than being decoupled as stated by Schuhmacher et al. a build up of the proton motive force occurs due to NADH still being produced further up in the metabolism. To keep the redox state over the membrane and avoid the excessive build up of proton motive force the whole respiratory chain is more or less inactivated. This on the other hand leads to accumulation of NADH since the respiratory chain no longer is capable of electron transport, Figure 13.
Figure 13. Accumulation of reducing equivalents occurs due to respiratory chain inactivation caused by low phosphate levels.

This scenario where reducing equivalents are in excess and the respiratory chain is inactivated or inefficient is actually quite similar to the state of the cell at anaerobic growth. Therefore it is plausible that the bacteria use fermentation pathways to regenerate NAD⁺, Figure 14. The common fermentation pathways in *E. coli*, called the mixed acid fermentation, results in formation of succinate, lactate, acetate, ethanol, carbon dioxide and hydrogen gas (Lengeler, Drews et al. 1999). Presence of lactic acid and succinic acid has been indicated in addition to acetate. The formation of lactic acid regenerates one NAD⁺ whilst formation of succinic acid regenerates 2 NAD⁺. Although we have not been able to determine the exact nature of the byproducts other researchers have qualitatively verified presence of fermentation products when *E. coli* are cultivated at phosphate limitation. Johansson *et al.* have detected formic acid, succinic acid and ethanol in phosphate limited fed-batch cultivations of wildtype W3110 (Johansson, Lindskog *et al.* 2005).
Figure 14. To be able to reoxidize NADH to NAD+ the cell use fermentation pathways leading to accumulation of the fermentation products. The green circles show the products whose presence has been indicated.

In short the theory means that when *E. coli* is subjected to phosphate limitation it enters a “pseudo-anaerobic state” caused by inefficiency in the respiratory chain. The inefficiency is caused by ATP synthase not being able to produce ATP when phosphate is low because of poor affinity for phosphate. To avoid accumulation of reducing equivalents the cell turns to fermentation pathways for regeneration of NAD⁺.

This theory is also supported by the fact that cell lysis is observed for D=0,1 h⁻¹ lowGlu. For this point the availability of glucose is too little for the cell to produce enough energy via substrate level phosphorylation, leading to cell stress and lysis.

The high carbon dioxide production can be explained by the increased flux in the citric acid cycle, but carbon dioxide can also be produced as a result of the fermentative pathway that converts formate to carbon dioxide and hydrogen gas, Figure 14. Formate is produced from pyruvate by pyruvate formate lyase and this reaction also yields one acetyl-CoA. Formate is cleaved into carbon dioxide and hydrogen gas by formate-hydrogen lyase. This reaction results in neither ATP nor regeneration of NAD⁺ and probably serve as a way for the cell to get rid of toxic formate (Murarka, Clomburg *et al.* 2010). The electrons from formate are inserted into H₂. However, if fumarate is present the electrons from formate are...
instead used by fumarate reductase to form succinate (Gottschalk 1986). Pyruvate formate lyase is said to be inactive at aerobic conditions but studies measuring hydrogen gas development have shown on its activity under microaerobic conditions (Cleland 1988, Sawers 2005). This way of forming acetyl-CoA is redox neutral i.e. no NADH is formed as opposed to the common aerobic pathway where pyruvate is directly converted to acetyl-CoA by pyruvate dehydrogenase forming one NADH in the process. Since the cells are thought to have excess reducing equivalents the formate-pathway is beneficial from a redox point of view and it is possible that this pathway is active. However, for certainty presence of hydrogen gas and formate must be investigated.

6.3 Production of 3-hydroxybutyrate
An optimal production process is when the productivity is high while byproducts are minimized. 3-HB production competes with carbon dioxide, cell mass, acetate and possibly other organic acids for the ingoing carbon. To achieve a high productivity of 3-HB the key is to keep a high flux in the glycolysis and being able to direct it towards product formation. We have seen that phosphate limitation can be used to limit cell growth while maintaining a high carbon flux but on the other hand unwanted byproducts in form of organic acids are produced.

In theory, for the sole production of 3-HB the TCA and respiratory chain need not to be active. These pathways are actually contributing to the loss of carbon to byproducts. In the TCA carbon is lost to carbon dioxide. TCA is also the major pathway leading to production of cell mass, which in this sense also is a byproduct that withdraws carbon. This means that by limiting the flow of carbon into TCA two carbon withdrawing components are minimized and the precursor of 3-HB, acetyl-CoA is accumulated. Limitation of TCA can to some extent, be achieved by process design using nutrient limitation but the actual mechanism behind is not fully known. According to the theory presented in this thesis phosphate-limitation might actually cause the opposite, an increased flux in TCA. However, studies so far show that phosphate limitation gives the best productivity of 3-HB compared to ammonium- and glucose-limitation.

Limitation of TCA is not as easy as it may seem, as one of the cell’s most central pathways it is affected by and affects a number of other pathways. It is also a key player for the redox state of the cell. For each acetyl-CoA produced in glycolysis two reducing equivalents (NADH) are produced. For each 3-HB-molecule produced two molecules of acetyl-CoA is needed and one reducing equivalent (NADH or NADPH) is consumed. This means that if the TCA and fermentative pathways were to be completely shut down, to increase flux of carbon to 3-HB, redox imbalance occurs caused by accumulation of reducing equivalents.

To avoid the above-mentioned scenario another strategy is to allow competition between byproducts and 3-HB for carbon, letting product and some byproduct being formed simultaneously. Focus would then be to minimize the relative flux of carbon to byproducts. This study shows that acetic acid and carbon dioxide production is diminished at high growth rates. The question is how the production of 3-HB relates to this, is it lowered or is it constant or perhaps
increasing? Another strategy is to co-produce 3-HB with one or several valuable products that reoxidize NADH to NAD⁺ when formed.

A strategy that has shown to increase productivity of PHB and decrease productivity of lactate when ammonium is limiting is use of gluconate as substrate (Sekar and Tyo 2015). Since gluconate is more oxidized than glucose its metabolism requires NADH, diminishing accumulation of reducing equivalents.

Whatever type of production strategy that is chosen feed-back regulation on the glucose feed is desirable. This is due to the variations in glucose uptake (Q_{glu}, g glucose/l, h) at different growth rates. The glucose uptake is affected by both varying cell mass depending on activation of pst and a q_{glu} (g glucose/g cells, h) that varies with growth rate making the glucose uptake difficult to predict. To maintain a high productivity excess glucose is important.
7 Future work

There are two main paths that can be followed for further investigation. Firstly, there are still questions to answer about how the cell metabolism is affected by phosphate limitation and secondly the information gained in this investigation should be used for improvement of 3-HB productivity at phosphate limitation.

The byproducts that we have indicated presence of need to be verified and quantified to further strengthen the proposed theory. Different ratios of glucose to phosphate feed can be tested to investigate the effects of limitation of both glucose and phosphate limitation, which also is an interesting metabolic scenario. The two dilution rates with lower glucose feed tested only serves to give a hint on what happens in the dual substrate limitation case.

To benefit from the results presented here further investigations must be done on 3-HB producing cells and how phosphate limitation can be used to improve its productivity. A repetition of the cultivation performed in this thesis with the only difference that a 3-HB producing strain e.g. AF1000 pJBG73Rx is used can give a lot of information on the actual production process and how to optimize it. That cultivation can show on the influence of growth rate on 3-HB production relative to byproduct formation.

To sum up, there are still questions to be answered regarding phosphate limitation and production of 3-HB but I hope that this work will serve to give information and inspiration for further investigations.
8 References


9 Appendix

9.1 Medium recipe

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<th>Concentration</th>
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<td>5 g/l</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0,14 g/l</td>
</tr>
<tr>
<td>Na₂HPO₄ x 2 H₂O</td>
<td>0,57 g/l</td>
</tr>
<tr>
<td>(NH₄)₂-H-citrate</td>
<td>0,5 g/l</td>
</tr>
</tbody>
</table>

*Added separately after sterilization*

- 1 M MgSO₄ (1000X) 1 ml/l
- Trace element (1000X) 1 ml/l
- Antifoam 50 μl/l

Trace element stock solution (1000X)

<table>
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<th>Component</th>
<th>Concentration:</th>
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<tr>
<td>FeCl₃ x 6 H₂O</td>
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<td>ZnSO₄ x 7 H₂O</td>
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<td>CuSO₄ x 5 H₂O</td>
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<td>MnSO₄ x 1 H₂O</td>
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<td>CoCl₂ x 6 H₂O</td>
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</tr>
<tr>
<td>Na-EDTA:</td>
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</tr>
</tbody>
</table>
9.2 RNA agarose gel

Figure 15. RNA agarose gel. Ladder is Generuler 1kb. Dilution rate $[h^{-1}]$ marked on top of lane.