Development of a shake flask method suitable for effective screening of *Escherichia coli* expression constructs

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Abstract
Screening of expression constructs suitable for protein pharmaceuticals is often done in batch cultivations. But the production of the recombinant protein is made during fed-batch cultivations. The two types of cultivations are different and therefore may good expression constructs that grow poorly in batch cultivations but good in fed-batch cultivations be rejected. Therefore would it be desirable to develop a fed-batch method that can be used in shake flasks. Biosilta has developed a method where starch is broken down into glucose by an enzyme creating fed-batch conditions. This method has been tried out and analyzed during this project. It is shown that the cells grown under these conditions can be glucose limited. However, at a later stage of the cultivation the cells produce a large amount of acetate and pH is not stable. The system builds on a booster tablet which content is unknown. If the booster is not added to the cultivations the cells stop growing, this indicates that there is some other limitation than just glucose. It is also seen that the amount of protein that is produced during this fed-batch mimic cultivation is much lower than that is produced during normal batch cultivations. I would therefore not recommend EnBase as a screening method.

Sammanfattning
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Introduction

1.1 Background
Screening of microbial strains that produces recombinant proteins, to be used as pharmaceuticals, is often done in batch cultivations. But the production of the proteins is often done in fed-batch cultivations. This can be a problem though the cultivation conditions are different in the two different cultivation types. Good drug-candidates that grow poorly in batch cultivations but good in fed-batch cultivations may be rejected [1][2].

This thesis will focus on how to use EnBase to mimic a fed-batch cultivation in shake flasks. EnBase is a product by BioSilta that is available at the market and it contains starch, medium, booster and enzyme. The enzyme will degrade the starch into glucose and in that way create a feed of glucose.

1.2 Aim
The aim with this project is to implement a shake flask procedure using EnBase Flo from Biosilta that mimics the growth of fed-batch cultivation with respect to:

- Growth pattern within 3 standard deviations of a fed-batch in fermentor
- High OD; more than 20
- Low accumulation of acetate; less than 100 mg/L
- Low accumulation of glucose; less than 100 mg/L
- No O\textsubscript{2} limitation
- pH profile within 7.0 ± 1.0 pH units
- High protein production

1.3 Strategy
The strategy is to cultivate genetically modified *Escherichia coli* in bioreactors and in shake-flasks. Shake-flask cultivations will be done both as batch cultivations and with EnBase. Different parameters will then be changed so that the EnBase shake-flasks conditions will mimic the fed-batch conditions. The analysis methods used to control if the goal has been reached are:

- OD; to see how the cells grew using a spectrophotometer.
- Bioprofile; to measure pH, acetate and glucose levels.
- SDS-Page; to analyze product formation.
2. Theory

2.1 Escherichia coli

*E. coli* is a good host for recombinant protein expression though it is easy to genetically modify and inexpensive to culture [3]. *E. coli* can also grow over a wide range of pH, from 4.4 up to 9.2, by adapting its metabolism to the environment. When adapting its metabolism the cells may take up acids or bases from the media, this can slow down growth and reduce the expression of recombinant proteins [4]. *E. coli* can use many different carbon sources such as amino acids, glycerol or glucose. Glucose is the most common raw material used for cultivations of *E. coli*. Glucose is both cheap and it is the preferred energy- and carbon source for *E. coli* [5]. But using glucose can also have some disadvantages; if the glucose concentration is high acetate is formed. This is because the tricarbonic acid cycle and/or the regeneration of NAD$^+$ rate is too slow, which means that all glucose cannot be used and acetate is produced instead [6]. This is overflow metabolism, or as it is also called the Crabtree effect. 10-30 % of the glucose in the media may become acetate, but the amount of acetate produced depends on the strain. The yield of cells per substrate will go down at high acetate concentrations and so will also the protein expression [7][8].

*E. coli* also has some other disadvantages when producing recombinant proteins for pharmaceuticals; it cannot produce post-translational modifications such as glycosylation. This can affect the folding and the activity of the proteins [3]. Some proteins form inclusion bodies which are insoluble aggregates of proteins that often are misfolded. Inclusions bodies are formed under stressful conditions, the stress can depend on that the recombinant proteins are expressed at high rates. To minimize the amount of inclusion bodies can for example the temperature be lowered, the expression rate be lowered, the bacterial host be engineered or the protein be altered. The inclusion bodies are often hard to refold and therefore expensive and time consuming [9].

2.2 Batch cultivations

In a batch cultivation is all components, except the inducer, added at the beginning of the cultivation. The concentration of substrates is therefore high at the beginning. During the cultivation, when the components are consumed, the concentrations go down. *E. coli* grows faster and to higher cell densities if complex medias are used, that is because the nutrient content is high and the buffering better. The cells will in batch cultivation grow unlimited until some nutrition is depleted for example glucose. When preparing a media the concentration of certain substrates cannot be too high because of the toxicity e.g. ammonia. Other components precipitate at high concentrations e.g. magnesium and can therefore not be used in too high concentrations. The oxygen can also become limited, but *E. coli* can grow under anaerobic or oxygen limited circumstances but the production of recombinant proteins will then go down [1][10].

Acetate is often formed as an over-flow metabolite due to the high glucose concentrations at the beginning of the cultivation [6].
2.3 Fed-batch cultivations
A fed-batch cultivation is first a batch phase and after that comes a feed cultivation. The batch phase is started in the same way as a normal batch cultivation, but when the glucose (or other energy source) is depleted starts a feed of glucose and/or other substrates. The growth can be controlled by the substrate feed. Several parameters, that can be a problem in batch cultivations, can be monitored and automatically adjusted in a fermentor, such as pH and oxygen. If this is done correctly there will be no oxygen limitation and the formation of overflow metabolites will be minimized [10]. A schematic picture of a bioreactor and a plot over cultivation parameters can be seen in Figure 1.

2.4 Ways of mimic a fed-batch cultivation
There are several ways to mimic a fed-batch cultivation in a shake flask without manually adding new substrate during the time. One way is by mutate a strain of E. coli so that it reduces its uptake rate of glucose, this has been done by researchers at KTH. The growth rate gets lowered and therefore also the respiration and the formation of acetic acid in a way that mimics a fed-batch cultivation [11].

Another way, developed in Germany, to mimic fed-batch is by using a polymer disc with glucose that slowly releases the glucose. The discs release the glucose depending on the disc height and composition among other things. But it has been shown that the release rate goes down after about 5 hours. With these discs the biomass yield got higher than in normal batch cultures and the amount of overflow metabolites went down similar to fed-batch techniques [1].

A third way to mimic a fed-batch cultivation is by adding a gel containing starch, a storage gel, one regulating gel and then a liquid medium with glucoamylase. The enzyme can break down starch into glucose, this method was initially developed at the University of Oulu (Finland) and is now commercialized by BioSilta. The storage gel contained a high concentration of starch, so high that it was not soluble, through the regulating gel did starch
diffuse into the cultivation. The starch was degraded into glucose by the enzyme and the diffusion made it possible to reach high glucose release without any insoluble starch in the cultivation. A schematic picture of this is showed Figure 2 [6]. When using this method it has been shown that the growth curve is almost linear and the product formation is higher than in normal fed-batch cultivations [12].

![Figure 2. A schematic picture of a cultivation vessel where glucoamylase is used for breaking down starch.][5]

### 2.5 EnBase

The method of a gel with starch and enzyme has been further developed so that the gel is fluid and is available at the market as EnBase Flo. Together with the gel and the enzyme comes a special growth medium and a booster that should be added at induction [13]. By changing the amount of enzyme to the media it will represent different feeds [6].

When buying EnBase EnPresso it includes one bag containing two tablets, media components and starch (polysaccharide) complex, and one bag containing a booster. The booster contains complex additives and buffer [13]. Starch is a polysaccharide that is build up by amylose and amylopectin by green plants as energy storage. A schematic picture of amylose and amylopectin can be seen in Figure 3 [14]. The starch together with the enzyme creates the fed-batch condition. When developing EnBase mineral salt medium was used and it was shown that nitrogen became limiting at high cell densities. If an addition of ammonia was made the cell densities became higher [6]. The exact content of any of the tablets are unknown.
Figure 3. The polymeres that starch is built up by, amylose is on the top and amylopectin in the bottom.

**Glucamylase**

The enzyme that EnBase sells in their kit is called EnZ I’m and is a pre-sterilized enzyme solution. The identity of the enzyme being included is not specified, but according to Panula-Perälä glucoamylase from *Aspergillus niger* was used during the development of the system [6]. Glucoamylase, or 1,4-α-D-glucan glucohydrolase, has the EC number 3.2.1.3. It is an exo-acting enzyme and cleaves of D-glucose units from the non-reducing end of starch. The enzyme can both degrade the α-1,4- and the α-1,6- glucosidic linkages. The glucoamylase can be inhibited in three different ways; competitive inhibition by glucose, substrate inhibition and by competition between substrates [1].

3. Materials and methods

3.1 Bacteria and cultivation conditions

The bacteria strains and plasmids used were *E. coli* BC50 pBV1960, BC50 pBV1976 and RV308 pBV1976. Based the BC50 and RV308 strains are of K-12 origin. The cells were genetically designed to produce a small goal protein that is a possible future drug candidate. Antibiotic was added to all cultivations to maintain the selection pressure.

**Pre-cultures:** 100 µL inoculum was taken from a frozen cell-vial stored at -70 °C and thawed for 15-30 minutes at room temperature before use. The pre-cultures were cultivated for 15 ± 1 hours, in 30 °C and the agitation was 250 rpm (Orbital Shaker from Forma Scientific). A phosphate minimal medium with 5 g, L⁻¹ yeast extract (designated as 2×PO₄+YE) was used as the media for the pre-culture if the cultivation in shake flask or reactor was performed in EnBase-, TB (Terrific Broth)- or 2×PO₄+YE media. If the cultivation was performed in minimal media (designated as 2×PO₄) the pre-culture was cultivated in minimal media.

**Shake flask:** Un-baffled 250 mL or 500 mL shake flasks were used for all cultivations. The cultivation volume was 10 % of the shake flask volume. The temperature was 30 °C and the
agitation was 250 rpm in Orbital Shaker from Forma Scientific. Inoculation volume varied so that OD$_{600}$ started at 0.05 or other wanted inoculation OD. When OD$_{600} = 1 \pm 0.5$ was reached the batch-cultivations were induced by addition of the inducer. To the cultivations of fed-batch characteristic the time of induction varied, and so did also the starting OD. An inducer stock solution, filtered through a 0.2 µm sterile filter (Minisart), was prepared just before use. To some of the cultivations a booster was added. The addition was 10% of the start cultivation volume.

**Bioreactor:** Bioreactor used was Greta from Belach which is a 6×1.0 L reactor, the start volume was 500 mL. Inoculation volume varied so that start OD$_{600} = 0.05$. The glucose feed started when the glucose in the reactor was depleted, a complex nutrient feed started just prior to induction. The glucose profile consisted of three different phases. First, the feed rate was increased linearly to a pre-set maximum value. Second, it was kept constant at this maximum rate and then after induction the feed was lowered to a new constant rate. When a pre-determined OD$_{600}$ was reached the cultivation was induced by adding the inducer. Automatic feed of ammonia kept pH constant and the temperature was held at 30 ºC. The stirrer-speed and supplementation of oxygen changed automatically during the cultivation to maintain the dissolved oxygen (DO) at set point.

See Appendix A for media and booster contents.

### 3.2 Sampling procedures

For induced cultivations samples for further analysis were taken at induction, four hours after induction and twenty hours after induction. OD and Bioprofile measurements were taken during the whole cultivations.

**Shake flask:** The samples were taken with a sterile pipette in a fume cupboard.

**Bioreactor:** The samples were taken with a sterile syringe through a membrane. The membrane was sprayed with ethanol before the sample was taken.

### 3.3 OD measurements

For optical density (OD) measurements a Novaspec II from Pharmacia Biotech and 4 ml PS-cuvettes from Plastibrand were used and the wavelength was 600 nm. As a zero-sample was 0.9 % NaCl solution used, the same solution was used for dilution of the samples. The OD was diluted so that OD$_{600}$ was between 0.4 and 0.8. According to earlier experience at Sobi, the ratio between OD$_{600}$ and cell dry weight is approximately 2.7 for this spectrophotometer.

### 3.4 SDS-Page

Osmotic shock and medium samples from some of the induced cultivations were analyzed with SDS-Page. Three parts of sample was mixed with one part redx4-buffer and incubated for 10 minutes in 80 ºC. The volume added to the gel depended on estimated protein concentration and OD value at sample taking. NuPAGE 10 % Bis-Tris gels from Invitrogen were used, the run times were 38 minutes at 200 V. After the run the gels were stained with instant blue from Expedeon for approximately one hour, de-stained in distilled water over night and finally scanned.
Osmotic shock

1 mL (5 mL if the sample was from a fermentor) cell broth was centrifuged, the supernatant discarded and the cell pellet saved on ice until the start of the procedure. 0.5 mL (1 mL if the sample was from a fermentor) periplasmatic extraction buffer was added, see Appendix B, and the pellet was re-suspended. The solution was incubated on ice for 20 minutes and then centrifuged for 35 minutes at 4 °C, 3000 × g. The supernatant was then discarded and the pellet re-suspend in 1 mL (5 mL if the sample is from a fermentor) MgCl-buffer and incubated on ice for 20 minutes. The solution was then centrifuged for 35 minutes at 4 °C, 3000 × g. The supernatant was saved in freezer at -70 °C until further analysis.

Medium

1 mL of cell broth was centrifuged. The supernatant was saved in freezer at -70 °C for further analysis.

3.5 Metabolite measurements and pH measurements

Glucose, Acetate, Ammonia and Phosphate measurements

Metabolite levels in the media were measured with a Bioprofile 300A instrument (Nova Biomedical). Before using the Bioprofile the samples were centrifuged and the cell pellet discarded. If the samples were not analyzed directly they were stored in freezer at -70 °C and re-thawed for 15-30 minutes before analysis.

Note 1: The Bioprofile does not measure all kind of phosphate, only ortho-phosphate.

Note 2: The glucose measurements are not exact at low concentrations of glucose. A sample that according to the Bioprofile contained 0.68 g, L^{-1} glucose was also analyzed with an enzymatic kit from r-biopharm (kit number E0176251). According to the enzymatic-kit measurements was the glucose concentration too low to measure i.e. lower than 0.08 g, L^{-1}.

pH measurements

pH was measured with Bioprofile if the sample could be analyzed directly. Before using the Bioprofile the samples were centrifuged and the cell pellets discarded.

If the samples could not be analyzed directly by Bioprofile the samples were analyzed with a pH prob. The samples were then directly put in a water bath of 30 °C and pH measured.

3.6 Calculations

Formulas used for calculations can be seen in Table 1. Most experiments are made in duplicate; the values in the calculations and graphs are mean values of these.
Table 1. Formulas and values used for calculations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Unit</th>
<th>Formula or value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific growth rate</td>
<td>h⁻¹</td>
<td>( \mu = \frac{\ln (\frac{OD_{t2}}{OD_{t1}})}{t_2 - t_1} )</td>
</tr>
<tr>
<td>Specific consumption rate</td>
<td>g₆Gl, g₆cell⁻¹ h⁻¹</td>
<td>( q_s = \frac{\mu}{Y_{X/Glc}} )</td>
</tr>
<tr>
<td>Yield coefficient</td>
<td>g₆Glc, g₆cell⁻¹</td>
<td>( Y_{X/Glc} = 0.4 ) [16]</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>-</td>
<td>( \sigma = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} )</td>
</tr>
</tbody>
</table>

3.7 Chemicals
Chemical used were from Merck, Sigma, BD and Riedel de Haën. For a list see Appendix C.

3.8 Experimental designs
A table of all the cultivations can be seen in Appendix D. In the table can inoculation OD, type of strain and plasmid, media, booster addition time, induction time and concentration of EnZ I’m be seen.

4. Results

4.1 Fed-Batch in fermentor
Experiments with BC50 pBV1960 constructs were cultivated in triplicate in fermentor in a way that is standard at Sobi AB. The results of these cultivations were used as a goal when developing the shake-flask procedure with EnBase. The cultivations ended at a final OD in the range of 100 after 40 hours. In Figure 4 the growth and the specific growth rate can be seen. In the graph three standard deviations was marked out. The specific growth rate, \( \mu \), at induction was 0.23 ± 0.02 h⁻¹ and three standard deviations was therefore 0.06. During the linear phase \( \mu \) was 0.14 h⁻¹ and the specific consumption rate of glucose, \( q_s \), was 0.35 g₆Gl, g₆cell⁻¹ h⁻¹.

The glucose concentration starts at almost 15 g, L⁻¹ and after a couple of hours it has decreased to zero and the feed starts, during the feed the glucose concentration in the media is kept at zero. The acetate levels started at 0.2 g, L⁻¹ and when the glucose feed started it had increased to 0.5 g, L⁻¹.
4.2 Batch cultivations in shake flasks

Batch cultivations of BC50 pBV1960 were made in different media to see the differences between them. The media used were EnBase with booster-, EnBase without booster-, TB-, 2×PO₄⁻ and 2×PO₄ + YE media. In Figure 5 OD and the specific growth rate can be seen for the different cultivations. The values were mean values from duplicate cultivations and all cultivations were induced. The final OD varied with the media, highest OD was reached in TB media (about 17 after 24 hours), the cultivations in 2×PO₄ and 2×PO₄ + YE ends at the lowest OD (about 5 after 24 hours).

Figure 4. Growth curve for BC50 pBV1960 in fermentor with 3 standard deviations marked out by lines. (●) OD and the line represents 3 standard deviations, (○) specific growth rate (h⁻¹) and the dashed line represents 3 standard deviations. The arrow represents the time of induction.

Figure 5. Growth curves. (●) OD, (○) specific growth rate (h⁻¹) and the colors represent the media the cells have been grown in; (purple) 2×PO₄, (red) TB, (blue) 2×PO₄ + YE, (green) EnBase with booster, (orange) EnBase without booster.
In Figure 6 the glucose- and acetate concentrations during the cultivations in the different media can be seen. TB medium contained glycerol instead of glucose and therefore the glucose concentration was close to zero during the cultivation.

![Figure 6. Metabolite concentrations in the media. (●) glucose (g, L$^{-1}$), (○) Acetate (g, L$^{-1}$) and the colors represent the media the cells have been grown in; (purple) 2×PO$_4$, (red) TB, (blue) 2×PO$_4$ + YE, (green) EnBase with booster, (orange) EnBase without booster.](image)

Specific growth rate and glucose consumption rates during the exponential phase and lowest and highest pH during the experiments can be seen in Table 2. Most acetate (5.47 g, L$^{-1}$) was formed by the cells grown in EnBase media with booster and second most (4.95 g, L$^{-1}$) of cells grown in 2×PO$_4$ + YE media. Least amount of acetate was produced by the cells grown in 2×PO$_4$ media (0.53 g, L$^{-1}$). The specific growth rates at induction were between 0.39-0.48 h$^{-1}$, the highest in EnBase media with booster and the lowest in 2×PO$_4$ and EnBase media.

The pH was most stable in TB media, it differed the most by 0.48 pH units. The biggest pH differences was measured in 2×PO$_4$+YE media where the difference was as big as 2.17 pH units.

<table>
<thead>
<tr>
<th>Media</th>
<th>$\mu$ (h$^{-1}$)</th>
<th>$q_s$ (g$<em>{Glc}$/g$</em>{cell}$ h$^{-1}$)</th>
<th>Acetate concentration (g, L$^{-1}$)</th>
<th>Highest pH</th>
<th>Lowest pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2×PO$_4$</td>
<td>0.39</td>
<td>0.93</td>
<td>0.53</td>
<td>7.05</td>
<td>5.82</td>
</tr>
<tr>
<td>2×PO$_4$ + YE</td>
<td>0.44</td>
<td>0.81</td>
<td>4.95</td>
<td>7.10</td>
<td>4.93</td>
</tr>
<tr>
<td>TB</td>
<td>0.44</td>
<td>-</td>
<td>1.69</td>
<td>7.24</td>
<td>6.76</td>
</tr>
<tr>
<td>EnBase</td>
<td>0.39</td>
<td>0.86</td>
<td>0.68</td>
<td>6.86</td>
<td>5.97</td>
</tr>
<tr>
<td>EnBase and booster</td>
<td>0.48</td>
<td>1.03</td>
<td>5.47</td>
<td>6.83</td>
<td>5.18</td>
</tr>
</tbody>
</table>

At induction (OD ≈ 1) was $\mu$=0.53 h$^{-1}$ in 2xPO$_4$ media, $\mu$=0.83 h$^{-1}$ in TB media, $\mu$=0.69 h$^{-1}$ in 2xPO$_4$+YE media $\mu$=0.57 h$^{-1}$ in EnBase media and $\mu$=0.74 h$^{-1}$ in EnBase with Booster media.
4.3 EnBase
Induced and non-induced experiments were compared in some experiments, AI6712-SF1-4 according to Appendix D. Non-induced cultivations of BC50 pBV1960 ended at OD = 18.3 ± 0.8 while induced BC50 pBV1960 ended at OD = 18.7. The cells grew very much alike and therefore could induced and non-induced cultivations be compared to each other.

EnBase without cells
To be able to mimic the feed in the fermentor it is crucial to know how much glucose that was released at different enzyme concentrations. Therefore, experiments without any cells were done. The glucose concentrations were measured at different concentrations of EnZ I’m and thereafter were estimated OD values calculated based on the glucose released. The estimated OD and the glucose concentrations can be seen in Figure 7 (negative values have been deleted, and the values are mean values of duplicates). The glucose release rate was on average 0.03 g, L⁻¹h⁻¹ when 0.3 U/L EnZ I’m was used, 0.08 g, L⁻¹h⁻¹ when 0.3 + 0.6 U, L⁻¹ EnZ I’m was used (0.6 U, L⁻¹ was added after 18 hours before that was the glucose release rate 0.03 g, L⁻¹h⁻¹ and after the addition 0.09), 0.11 g, L⁻¹h⁻¹ when 1 U, L⁻¹ EnZ I’m was used and 0.22 g, L⁻¹h⁻¹ when 5 U, L⁻¹ was used.

EnBase without booster addition
Biosilta recommends that a booster was added to the cultivations, but in some early experiments the booster was not added. When no booster was added to the EnBase media the cells stop growing. A graph over OD and specific growth rate can be seen in Figure 8, the experiment was made in duplicate and the values are mean values. The cultivations were not induced. The EnZ I’m concentration was 0.3 U, L⁻¹. It is seen that after 10 hours the OD was 6.38 and after 30 hours 6.55.

Figure 7. Glucose concentration in the media and calculated OD depending on the glucose concentration. (purple) 5 U/L, (red) 1 U/L, (orange) 0.3 U/L + 0.6 U/L after 18 hours and (blue) 0.3 U/L. (●) glucose concentration (g, L⁻¹) and (○) estimated OD.
8 hours after inoculation the highest acetate concentration was measured, 0.56 g, L⁻¹, and the pH was at its lowest, 6.34 (start pH was 6.72). The initial glucose concentration was 0.85 g, L⁻¹ and after some hours it had decreased to a concentration around 0.45 g, L⁻¹. The 0.45 g/L level measured by the Bioprofile was verified by the glucose kit assay to be < 0.08 g/L.

One theory was that the cells stop growing due to low pH and another was that the cultivation run out of some complex substrate. Therefore two different pH buffers was added to the cultivations; alone and in combination with yeast extract. The pH buffers where TRIS with pH = 7.5 or 2×PO₄-buffer with pH = 7.2. In the experiments where only TRIS was added the cells grow from OD = 5.1 to OD = 7.2 after 20 hours, the pH went from 6.6 to 7.1. When TRIS and yeast extract was added the cells grow from OD = 5.1 to OD = 5.2 after 20 hours, the pH went from 6.6 to 7.2. When only 2×PO₄ was added to the cultivation the OD was 5.0
and 20 hours later it was 3.9, pH went from 6.7 to 6.9. When 2×PO₄ and yeast extract was added the OD went from 5.2 to 5.1 20 hours later, the pH went from 6.7 to 6.8.

**EnBase with booster addition**

In some first experiments was the amount of EnZ I’m used evaluated. According to the EnBase protocol a booster- and a second EnZ I’m addition and induction should be done after one night of cultivation. In Figure 10 OD and µ can be seen for cells grown in EnBase with a booster and EnZ I’m addition after 16.5 hours, the EnZ I’m concentration is 0.3 + 0.6 U, L⁻¹ and 5 U, L⁻¹. Neither of the experiments were induced. The values seen in the graph was mean values from duplicate experiments. In Figure 11 the acetate concentration and pH can be seen for the same experiments. OD ended around 20 for all experiments but the acetate concentrations differ, in the experiments with the higher amount of EnZ I’m the acetate concentration is over 2 g. L⁻¹ after the booster addition while for the cultivation with 0.3 + 0.6 U, L⁻¹ about 0.9 g, L⁻¹ acetate was formed.

![Figure 10. OD and μ for BC50 cells grown according to the EnBase protocol. (●) OD, (○) specific growth rate. (blue) 0.3 + 0.6 U, L⁻¹ and booster after 16.5 hours, (red) 5 U, L⁻¹ booster after 16.5 hours.](image-url)
Figure 11. Acetate concentration and glucose concentration in the media during cultivation of BC50 grown according to the EnBase protocol. (●) Acetate, (○) Glucose concentration (blue) 0.3 + 0.6 U, L\(^{-1}\) and booster after 16.5 hours, (red) 5 U, L\(^{-1}\) booster after 16.5 hours.

In one experiment the second addition of EnZ I’m was evaluated. OD can be seen in Figure 12. The OD ended at almost the same value. The acetate concentration peaked at 1.2 g, L\(^{-1}\) and 0.9 g, L\(^{-1}\) for the cultivation without EnZ I’m addition and the cultivation with EnZ I’m addition. But the cultivation without EnZ I’m addition had almost 0.3 g, L\(^{-1}\) acetate at start. If this amount is subtracted the acetate peak is almost the same.

Figure 12. OD and acetate comparison between 0.3 U, L\(^{-1}\) and 0.3 + 0.6 U, L\(^{-1}\). (●) OD, (○) Acetate concentration in the media (red) 0.3 + 0.6 U, L\(^{-1}\) and booster after 10.7 hours, (blue) 0.3 U, L\(^{-1}\) booster after 10.8 hours.

All cultivations grown in EnBase reached a plateau when OD was about 6-7. After the booster addition the cells started growing again and the acetate concentration in the media got higher. This can be seen in Figure 13 were OD and acetate concentrations can be seen for BC50 pBV1960 were the inoculation OD and booster addition time was varied. It was seen that all cultivations has a lag-phase except one, the one that has an inoculation OD of 0.01 and an
addition of booster after 6 hours, this cultivation did not reach the plateau before the booster addition. In the graph it also seen that the concentration of acetate in the media gets higher after booster addition but the concentration goes down again after some hours. The experiments were not induced.

Figure 13. OD and acetate concentrations for some typical cultivation in EnBase with booster addition. (●) OD, (○) Acetate concentration in the media (red) inoculation OD was 0.01 and booster addition was made after 18 hours, (orange) inoculation OD was 0.5 and booster additions were made after 18 hours, (green) inoculation OD was 0.07 and the booster addition was made after 12 hours (purple) inoculation OD was 0.5 and booster addition was made after 6 hours (blue) inoculation OD was 0.01 and the booster addition was made after 6 hours.

In Table 3 OD approximately 20 hours after induction or booster addition, time of booster addition, strain, plasmid and the highest amount of acetate during the cultivations can be seen for cultivations that had inoculation OD of 0.05. It was seen that the highest concentrations of acetate was achieved when the booster addition was made early of the cultivation for BC50 while it for RV308 at all times form much acetate.
Table 3. Shake flask performance parameters from cultivations with inoculation OD 0.05 in EnBase media.

<table>
<thead>
<tr>
<th>Production system</th>
<th>EnZ I´m (U, L⁻¹)</th>
<th>time of booster addition</th>
<th>Highest acetate concentration</th>
<th>Final OD</th>
<th>highest pH</th>
<th>lowest pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC50, pBV1960</td>
<td>0.3 + 0.6</td>
<td>10.8</td>
<td>0.93</td>
<td>21.9</td>
<td>6.85</td>
<td>6.30</td>
</tr>
<tr>
<td>BC50, pBV1960</td>
<td>0.3 + 0.6</td>
<td>16.4</td>
<td>0.73</td>
<td>19.8</td>
<td>6.9</td>
<td>6.52</td>
</tr>
<tr>
<td>BC50, pBV1960</td>
<td>5</td>
<td>16.7</td>
<td>2.08</td>
<td>21.2</td>
<td>6.75</td>
<td>5.74</td>
</tr>
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<td>BC50, pBV1960</td>
<td>0.3</td>
<td>7.0</td>
<td>3.68</td>
<td>18.3</td>
<td>7.18</td>
<td>6.42</td>
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<td>0.3</td>
<td>7.2</td>
<td>2.71</td>
<td>18.7</td>
<td>7.11</td>
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</tr>
<tr>
<td>BC50, pBV1976</td>
<td>0.3</td>
<td>10.7</td>
<td>1.20</td>
<td>21.5</td>
<td>7.12</td>
<td>6.29</td>
</tr>
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<td>BC50, pBV1976</td>
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<td>12</td>
<td>1.42</td>
<td>17.8</td>
<td>7.07</td>
<td>6.11</td>
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<tr>
<td>BC50, pBV1976</td>
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<td>15</td>
<td>0.99</td>
<td>17.3</td>
<td>7.06</td>
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<td>17</td>
<td>1.31</td>
<td>17.6</td>
<td>7.23</td>
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<td>RV308, pBV1976</td>
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<td>12</td>
<td>2.33</td>
<td>20.9</td>
<td>6.84</td>
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<td>RV308, pBV1976</td>
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<td>19.9</td>
<td>6.94</td>
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<td>2.33</td>
<td>20.2</td>
<td>6.88</td>
<td>5.73</td>
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In Table 4 the highest measured acetate concentration, OD 20 hours after induction or booster addition, time of booster, En Z I´m concentration and cell can be seen for experiments where the inoculation OD varied. Also here it can be seen that the acetate concentration was highest when the booster addition was made early, but it was also seen that the most amount of acetate was produced when inoculation OD was low in a combination with early booster addition. The OD after 20 hours was not affected much by either the inoculation OD or the time of booster addition.

Table 4. Shake flask performance parameters from cultivations with varied inoculation OD in EnBase media

<table>
<thead>
<tr>
<th>Production system</th>
<th>inoculation OD</th>
<th>EnZ I´m (U, L⁻¹)</th>
<th>time of booster addition</th>
<th>Highest acetate concentration (g, L⁻¹)</th>
<th>Final OD</th>
<th>highest pH</th>
<th>lowest pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC50, pBV1960</td>
<td>0.01</td>
<td>0.3</td>
<td>6</td>
<td>2.64</td>
<td>18.3</td>
<td>7.18</td>
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<td>0.3</td>
<td>12</td>
<td>1.31</td>
<td>19.7</td>
<td>7.02</td>
<td>6.00</td>
</tr>
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<td>0.3</td>
<td>12</td>
<td>0.6</td>
<td>17.4</td>
<td>7.09</td>
<td>6.32</td>
</tr>
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<td>0.3</td>
<td>6</td>
<td>1.81</td>
<td>17.2</td>
<td>7.00</td>
<td>6.09</td>
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<td>22.7</td>
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<td>0.3</td>
<td>18</td>
<td>0.61</td>
<td>15.0</td>
<td>6.99</td>
<td>6.30</td>
</tr>
</tbody>
</table>

The lowest amount of acetate was produced when the inoculation OD was 0.07 and the booster addition was made after 12 hours. The two highest amounts of acetate were formed when the time of booster addition was low and the inoculation OD either was 0.5 or 0.01.
4.4 SDS-Page results
The product levels were roughly estimated from the SDS-Page gel by comparing the intensity obtained from the cultivation sample with a reference sample of known concentration. All the SDS-Page gels can be seen in Appendix E.

In the fermentor at induction, there were no, or a too small amount to be measured, product. After 4 hours the product had accumulated to gram levels in the periplasm and after 20 hours it was still at the same level, but the cell specific production was lower than at 4 hours since the amount of product per OD is lower. After 20 hours some of the product were in the media, but the sample moved a bit strangely and it was hard to make an estimation of how much.

In batch cultivations made in 2×PO₄ + YE and TB media the amount of product in the media was higher after 20 hours than after 4 hours, while the concentration of product was lower after 20 hours than after 4 hours for cultivations in 2×PO₄ media. The osmotic shock samples from the cultivation in 2×PO₄ showed more product after 20 hours than after 4 hours while the other cultivations did not. In neither of the samples the concentration was higher than 0.02 g, L⁻¹. The SDS-Page with samples from batch culture in EnBase media showed that the product formation was very low. This was both 4 hours after induction and 20 hours after induction, both in the media and in the periplasm.

When EnBase was used as a fed-batch and the booster was added after 8 hours it was seen that there were almost no product. In the osmotic shocked sample 4 hours after induction there was a small band of product, and after 20 hours it was even smaller. An almost invisible shadow could also be seen in the media sample 20 hours after induction. If the booster was added 15 hours after induction almost no product was formed either. The only visible band at the gel was in the media sample 4 hours after induction. It was a very light band.

5. Discussion
To use EnBase to create glucose limitation may not fully work, several problems have occurred during the project. One problem was that we cannot be sure that there is a glucose limitation during the cultivation. The bioprofile that was used for measurements of glucose was not sensitive enough. One test was done with another, enzyme based, glucose measurement method and it showed a lower glucose concentration than the bioprofile. This confirms that there was glucose limitation when it according to the bioprofile was a low concentration of glucose. It would be interesting to see if there is a small glucose accumulation during the lag phase and after the booster is added, and this cannot be done by the Bioprofile. One goal was to have a low, under 0.1 g, L⁻¹, glucose accumulation but due to the poor accuracy of the bioprofile it is not sure if the goal been reached or not. According to the bioprofile the goal was not reached, but when doing the other glucose test the sample had very low concentration so it is considered to have been reached.

The feed of glucose was controlled by the amount of EnZ I´m added. But when measurements were done to see how much glucose that actually was released at different enzyme concentrations the amount of released glucose was very low. When predicting the cell densities it seemed very low, but when adding cells to EnBase the OD was much higher than
predicted. Exactly how much glucose that was released during cultivations was hard to know
due to that inhibition of the enzyme might have occurred in the flask experiments without
cells. It therefore also was hard to predict exactly how much EnZ I´m that should be used to
mimic a fed-batch feed. It also was strange that BioSilta recommended a second addition of
EnZ I´m after about 15 hours. This addition did not affect the growth pattern of the
cultivations and I therefore could not see a direct reason why to do it. However, the protein
expression was not evaluated in this study with and without second EnZ I´m addition and the
expression may be affected by the second EnZ I´m addition.

One of the specific goals with this project was to reach a high final OD. In bioreactor the cell
density ended at OD in the range of 100, in a batch phase with 2×PO₄ + YE did the OD end
just above OD 5. When using EnBase and booster the OD ended around 20. So if the booster
was used the goal was reached, but if the booster addition was not used the OD ended
between 6-7. If a high OD would be the only goal another way of reaching it would be by
changing the media to a complex media, when TB media was used OD ended at almost 18.

The booster tablet was a bit of a mystery. Something in the booster made the cells start
growing again after the cells went into the lag phase. This may be a sign that it was not the
glucose that was limiting for the growth. When the booster was added the cells also started to
produce acetate. In batch-cultivations with EnBase and booster it was also seen that high
amounts of acetate was formed, about 5 g, L⁻¹. If the batch cultivation was made in only
EnBase media the acetate concentration on the other hand was very low, about 0.4 g, L⁻¹. The
amount of acetate that was produced depends on the time of the addition and strain. If the
booster addition was made early much acetate was formed, if the booster was added late the
amount of acetate formed was lower. So to minimize the acetate production the booster
needed to be added late, but if the growth pattern of a fed-batch cultivation should be mimic
the addition should be made early. In a fed-batch cultivation there was no lag phase, and it
would therefore be good if the mimic did not have that either. Neither the acetate production
or the lag phase have been mentioned in the literature [6] [10][12]. One goal with this project
was to have a low, under 0.1 g, L⁻¹, acetate accumulation. This goal was not reached due to
the acetate production after the booster addition. In some cases was the glucose accumulation
quite low, but still higher than 0.1 g, L⁻¹, and in other cases was the acetate accumulation very
high, over 4 g, L⁻¹. Worth noticing is that the acetate that was produced in a couple of hours
was gone, taken up by the cell. So if only a comparison between inoculation time, when the
acetate concentration was zero, and the end of a cultivation, 20 hours after induction or later,
would be done it would look like the acetate accumulation would be very low or none. It was
also seen that during the first phase, before the addition of booster was made, the acetate
concentrations were lower than in a normal fed-batch. In the first phase it were unusual to see
acetate concentrations higher than 0.5 g, L⁻¹ glucose, except when the acetate concentration
were high at start but the glucose concentration did not at these times increase more than 0.5
g, L⁻¹.

Another goal was that the pH should be stable at pH 7 ± 1. This was not completely achieved.
In some experiments the pH was within the goal but many times it got lower. One cause of
why the pH went down as much as it did was because of the acetate produced. Comparing
start pH with the pH at the end of cultivation the pH has not decreased, since that is because the booster contains some buffer with a higher pH.

When evaluating the product formation with SDS-Page it was seen that the amount of goal protein produced was very low when EnBase was used. Lower than the production in normal batch-phases. In some cases it was so low, or none, that it could not be noticed. The literature [10] says that 10 times more, compared to batch cultivations, soluble recombinant proteins has been formed when EnBase has been used this was, consequently, not the case in our project. The low amount of formed product may be because of the high amount of acetate that was formed. Worth noticing is also that the samples from “fed-batch” with EnBase do not show almost any proteins on the gel. This could be a sign that something went wrong during the extraction procedure or in the dying of the proteins or the run of the gel. I do not think that the gels were reliable, and to be sure a new gel and staining would be recommended.

Another thing that was worth noticing was that if a method should be used it is good if it is robust. A small error such as too much or too little inoculation, a late or early addition of something or some kind of miscalculation should not affect the outcome of the cultivation much. EnBase did not seem to be as robust as wanted. If the booster was added one hour early it could change the acetate levels much. To make the system as robust as possible the booster and induction should not been done until hours after the cultivation gone into the lag-phase. If this is done the inoculum is not as important either, if the inoculum is big the cultivation will go into the lag-phase earlier.

**Conclusions and future perspectives**

I would not recommend Sobi AB to use EnBase instead of normal batch screening method. I have not been able to develop a method that creates a fed-batch process similar to the ones in bioreactor processes. The booster that was added is a mystery and it is not sure that the cells are growing under glucose limitation the whole time. The booster may contain some kind of energy source that makes the cells start growing, and to use EnBase without booster does not either seems like a good option. If booster is not added the cells stop growing after a couple of hours just as in a batch cultivation.

Further experiments would be to try to find out what it is in the booster that makes the cells grow again. Experiments should also be done to try to find out why the cells stop growing, is there some kind of limitation? If so is the case what substrate is depleted, an amino acid analysis would be a good start to clear out some of the question marks.

Several glucose measurements should also be done so it can be sure that the growth is during glucose depletion. It would also be good to make cultivations with the EnBase system in minimal media. Also, it may be useful to perform experiments to verify that there is no oxygen limitation during any stage of the cultivation.

It should also be explored if it is good to make the induction at the same time as the booster is added or before or after. Is there a big difference in product formation depending on when the induction is made?
References


Appendix A
All media were sterilized by heating to 121 °C for 30 minutes. The post sterilization additions were sterilized separately by heating to 121 °C for 30 minutes except for the trace element solution that is sterile filtered through a 0.2 µm sterile filter (Avisart).

TB media
Solution 1:
Tryptone: 12 g, L⁻¹
Yeast extract: 24 g, L⁻¹
Glycerol (87%): 11.5 g, L⁻¹

Solution 2:
KH₂PO₄: 2.3 g, L⁻¹
K₂HPO₄: 12.5 g, L⁻¹

Post sterilization:
Mix nine parts of Solution 1 with one part of solution 2.

Minimal media; 2 x phosphate media
(NH₄)₂SO₄
K₂HPO₄
KH₂PO₄
Na₃-citrat

Post sterilization additions:
MgSO₄×7 H₂O: 3.35 mL, L⁻¹
Trace elements solution: 1 mL, L⁻¹
Glucose monohydrate: 11 g, L⁻¹

2 x phosphate + 5 g/L yeast extract media
(NH₄)₂SO₄
K₂HPO₄
KH₂PO₄
Na₃-citrat
Yeast extract

Post sterilization additions:
MgSO₄×7 H₂O: 3.35 mL, L⁻¹
Trace elements solution: 1 mL, L⁻¹
Glucose monohydrate 11 g, L⁻¹

EnBase media
EnBase Medium tablets, from Biosilta, are dissolved in 50 ml sterile water (sterilized by heating to 121 °C for 30 min).
EnBase Booster Tablets, from Biosilta, are dissolved in 5 ml sterile water.

EnZ I´m 600 U, L⁻¹, from Biosilta, volume varied after wanted concentration.
Trace element solution
The trace element stock solution was consisting of the metal ions Fe, Zn, Co, Cu, Mn and Ca.

MgSO4 solution
MgSO4×2H₂O
Appendix B

Periplasmatic extraction buffer

*Tris*: 30 mM  
*Sucrose*: 20 %  
*EDTA*: 2 mM

MgCl buffer

*MgCl*: 1 mM
Appendix C

\((NH_4)_2SO_4\): article number 1.01216.5000, Merck

\(K_2HPO_4\): article number 1.05101.5000, Merck

\(KH_2PO_4\): article number 1.04871.5000, Merck

\(C_6H_5Na_3O_7 \times 2H_2O\) (tri-sodium citrate): article number 1.06448.1000, Merck

*Bacto Tryptone*: article number 211705, BD (Becton, Dickinson and Company)

*Bacto yeast extract*: article number 212750, BD (Becton, Dickinson and Company)

*Glycerol 87%*: article number 33224, Riedel de Haën

*NaCl*: article number: 1.05404.1000, Merck

*dH_2O*: Purified Water at Sobi AB
## Appendix D

### Table 5. Experimental design

<table>
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<tr>
<th>experiment number</th>
<th>Strain</th>
<th>Plasmid</th>
<th>Medium</th>
<th>Inoculation OD</th>
<th>time of booster addition (h)</th>
<th>induction time (h)</th>
<th>EnZ I’m (U, L⁻¹)</th>
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Appendix E

Figure 14. SDS-Page from fed-batch cultivation in fermentor. Samples from left to right; reference; osmotic shock sample at induction, osmotic shock sample 4 hours after induction, osmotic shock sample 20 hours after induction and last one medium sample 20 hours after induction.

Figure 15. SDS-Page media samples from batch cultivation in different media, pBV1960. Samples left to right; marker, standard 0.6 µg, sample from other experiments, 25 µL sample from cultivation in 2xPO4 + YE media at induction, 25 µL sample from cultivation in 2xPO4 media 4 hours after induction, 25 µL sample from cultivation in 2xPO4 media 20 hours after induction, 10 µL sample from cultivation in TB media 4 hours after induction, 10 µL sample from cultivation in TB media 20 hours after induction, 25 µL sample from cultivation in 2xPO4 + YE media 4 hours after induction, 25 µL sample from cultivation in 2xPO4 + YE media 20 hours after induction
Figure 16. SDS-Page osmotic shocked samples from batch cultivations in different media, BC50 pBV1960. Samples left to right: marker, sample from other experiments, standard 0.6 µg, 25 µL sample from cultivation in 2xPO4 + YE media at induction, 25 µL sample from cultivation in 2xPO4 media 4 hours after induction, 25 µL sample from cultivation in 2xPO4 media 20 hours after induction, 10 µL sample from cultivation in TB media 4 hours after induction, 10 µL sample from cultivation in TB media 20 hours after induction, 25 µL sample from cultivation in 2xPO4 + YE media 4 hours after induction, 25 µL sample from cultivation in 2xPO4 + YE media 20 hours after induction. The intensive band positioned above the product band is Lysozyme used in this extraction.

Figure 17. SDS-Page of a fed-batch cultivation in EnBase medium, 0.3 U/L with booster and induction after about 8 hours. Samples from left to right: marker, 0.5 µg reference, 1 µg reference, 20 µL medium at induction, 10 µL medium 4 hours after induction, 5 µL medium 20 hours after induction, 20 µL osmotic shocked sample at induction, 10 µL osmotic shocked sample 4 hours after induction and 5 µL osmotic shocked sample 20 hours after induction.
Figure 18. SDS-Page of a batch cultivation in EnBase + booster media, BC50 pBV1960, and EnBase fed batch cultivation with a booster addition after 15 hours, BC50 pBV1976. Samples from left to right: 1 µg reference, 20 µL osmotic shocked sample from the batch cultivation 4 hours after induction, 20 µL media sample from the batch cultivation 4 hours after induction, 20 µL osmotic shocked sample from the batch cultivation 20 hours after induction, 20 µL media sample from the batch cultivation 20 hours after induction, 10 µL osmotic shocked sample from the fed-batch cultivation 4 hours after induction, 10 µL media sample from the fed-batch cultivation 4 hours after induction, 7 µL osmotic shocked sample from the fed-batch cultivation 20 hours after induction, 7 µL osmotic shocked sample from the fed-batch cultivation 20 hours after induction.