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Development of a NADH-dependent production pathway for anaerobic 3-hydroxybutyric acid production in *E. coli*.

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Development of an anaerobic, NADH dependent production pathway for 3-hydroxybutyric acid in *E. coli*.

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Table of contents

1. Abstract

Anaerobic bioconversion processes are able to achieve higher yields, and have lower energy requirements, than comparable aerobic bioconversion processes. A model bioconversion pathway is the anaerobic production of 3HB by genetically modified *E. coli*. One of the stumbling blocks of this anaerobic bioconversion process is its dependence on NADPH, the flux of which is low under anaerobic conditions. In an attempt to circumvent this NADPH flux bottleneck an NADH dependent 3HB production pathway was made, consisting of the following enzymes: PhaA (acetoacetyl-CoA thiolase), TesB (acyl-CoA thioesterase II) and Hbd ((*S*)-3HB-CoA dehydrogenase). For the purpose of this project an attempt was made to create a plasmid which housed the genes required for NADH dependent 3HB production. The creation of the plasmid, dubbed pHbd, was successful. In order to compare NADH and NADPH dependent synthesis pathways, *E. coli* was transformed with either pHbd or p3HB, with the latter plasmid housing the genes required for NADPH dependent 3HB production. Subsequently, anaerobic fermentations were done using minimal media. No statistically significant difference in 3HB yield on glucose between p3HB and pHbd transformants could be observed, which was 0.09 ±0.01 and 0.08 ±0.00 cmol 3HB/cmol glucose respectively. However, growth analysis showed a higher maximum growth rate for pHbd transformants when compared to p3HB transformants, showing a maximum growth rate of μmax = 0.25 ±0.00 h^{-1} and μmax = 0.22 ±0.00 h^{-1} respectively. The likely cause for the similar yields achieved with p3HB and pHbd transformants, and the observed difference in growth rate, was the activity of PntAB, a membrane bound enzyme which catalyses the transfer of $H^+ + 2e^-$ from NADH to $NADP^+$, thereby creating NADPH. The activity of PntAB is dependent on ATP and effectively couples NADH and NADPH pools. Data from HPLC analysis showed a large degree of variance, and were therefore difficult to interpret. In order to confirm the activity of PntAB and better study anaerobic 3HB production, 3HB titres have to be increased. Titres of 3HB can be improved by increasing biomass concentrations by means of buffered media. Furthermore, the flux towards 3HB production could be increased by knocking out competing metabolic pathways towards lactate and acetate, resulting in a maximum possible yield of 0.67 mol 3HB per mol of glucose.

2. Introduction

Currently, fossil oil is used for the production of various bulk chemicals, which contributes to the formation of greenhouse gasses such as CO_2. Furthermore, fossil oil is a limited resource. Considering the environmental impact of the use of fossil oil, it is interesting to look at more sustainable alternatives [1]. A possible alternative is the use of biomass and microbial bioconversion for the production of bulk chemicals. However, the efficiency of bioconversion processes has to be improved before they can be effectively employed [1]. A potential method for improving the efficiency of such processes is to run these processes anaerobically. The productivity of aerobic bioconversion processes is limited by oxygen transfer, which in turn, is due to the difficulty of dissolving oxygen into water. Thus, the main benefits of anaerobic bioconversion processes are that they don't require sparging and require less mixing when compared to aerobic bioconversion processes. Anaerobic bioconversion processes also generate less heat than aerobic bioconversion processes, and therefore require less cooling [2]. Finally anaerobic bioconversion processes have the potential to have higher yields than their aerobic counterparts because far less substrate is converted into CO_2 and biomass, resulting in a higher carbon flux towards product formation.

A model bioconversion process is the anaerobic production of 3-hydroxybutyric acid (3HB) by genetically modified *Escherichia coli*. During this process, acetyl-CoA is converted into 3HB. In the first step, two molecules of acetyl-CoA are condensed into one molecule of acetoacetyl-CoA by the enzyme PhaA (acetoacetyl-CoA thiolase from *Ralstonia eutropha)*, liberating HS-CoA in the process. During the second step, the newly formed acetoacetyl-CoA is reduced by PhaB ((*R*)-3HB-CoA dehydrogenase from *R. eutropha)* to form 3-hydroxybutyryl-CoA. This step requires NADPH, a redox cofactor which is mainly used in anabolic reactions. The final step, involves the hydrolysis of the thio-ester bond of 3-hydroxybutyryl-CoA by TesB (acyl-CoA thioesterase II from *E. coli* MG1655) forming 3HB, which is excreted into the medium (Figure 1a). [3]

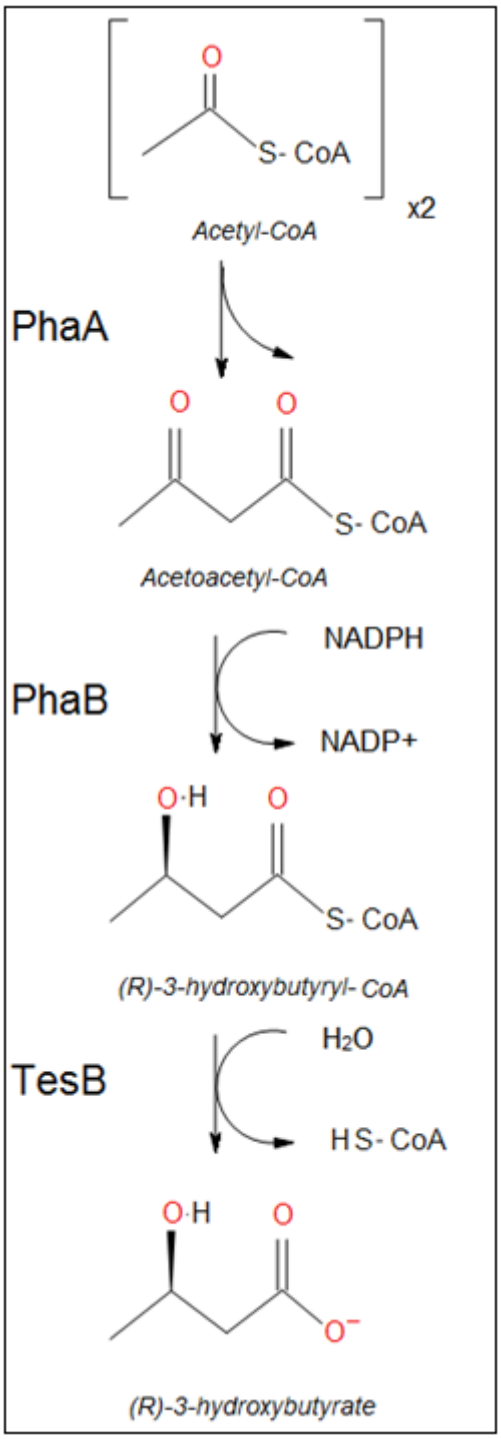

Figure 1a: *NADPH dependent synthesis pathway of R-3-hydroxybutyric acid.*

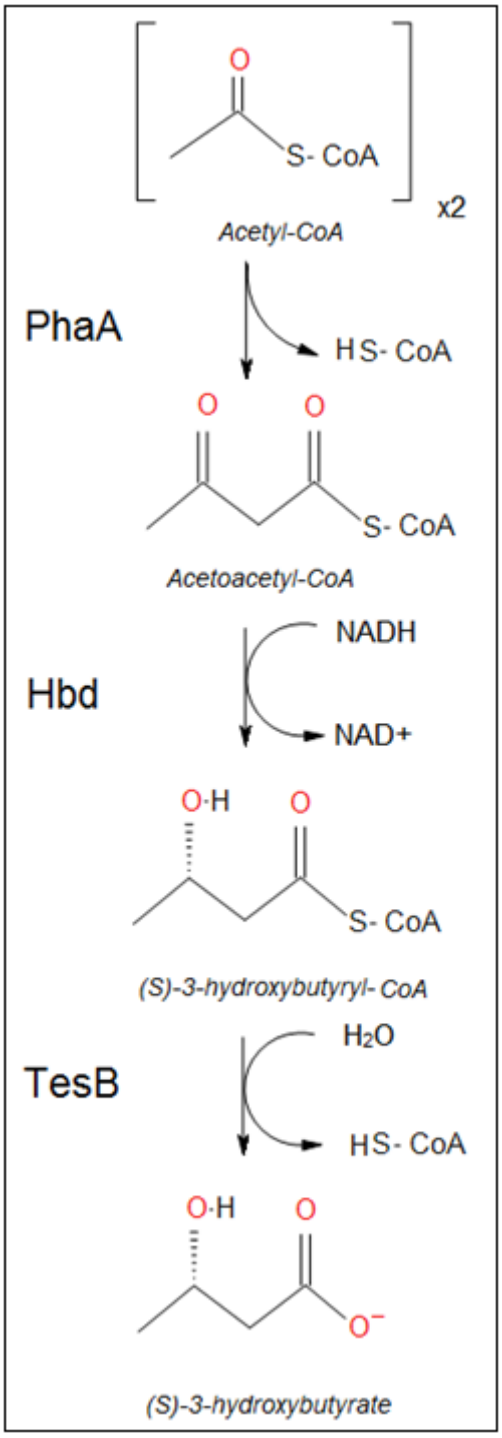

Figure 1b: *NADH dependent synthesis pathway of S-3-hydroxybuteric acid. This pathway is almost identical to the NADPH dependent pathway however, the reduction of acetoacetyl-CoA is mediated by Hbd instead of PhaB.*

The production of 3HB by genetically modified *E. coli* serves as a good model for anaerobic bioconversion processes for several reasons. First the synthesis pathway uses acetyl-CoA, linking it directly to the central metabolism of *E. coli,* thereby easing flux calculations. Furthermore, *E. coli* is a well-studied organism and many techniques exist to genetically modify it. Lastly, 3HB itself is interesting as it can be used by the chemical industry, for example for the synthesis of vitamins, antibiotics, and flavour compounds. [4]

One of the stumbling blocks for using anaerobic bioconversion is the regeneration of redox cofactors such as NADPH. *E. coli* grows slowly under anaerobic conditions and the flux of NADPH, which is required for 3HB production, is directly coupled to the production of biomass. Thus anaerobic growth results in a bottleneck in 3HB production. On the other hand the flux of NADH is high under anaerobic conditions due to a comparatively high glycolytic flux, which occurs during anaerobic growth [5]. Thus a solution for the low NADPH flux could be to switch 3HB production from an NADPH dependent process to an NADH dependent process. (Figure 2).

The aims of this study were to establish a pathway for the NADH dependent synthesis of 3HB and to compare NADH and NADPH dependent 3HB synthesis pathways.

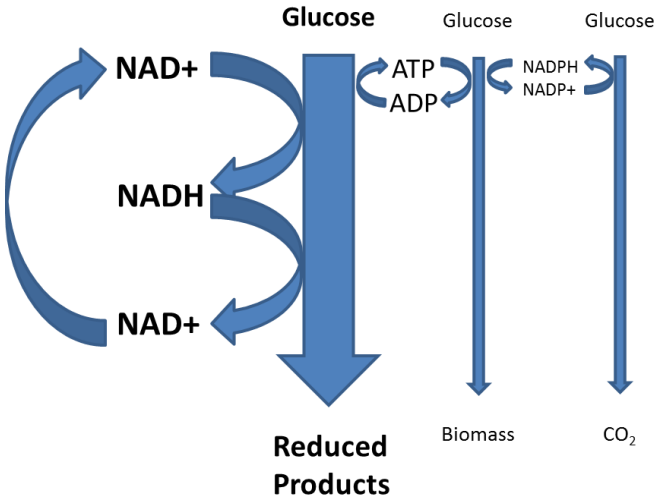

Figure 2: *NADH flux vs. NADPH flux under anaerobic conditions. The flux from glucose to reduced products (e.g. lactate, ethanol and succinate) is high under anaerobic conditions which in turn results in a high flux through NADH. Conversely, the flux from glucose to biomass is low under anaerobic conditions which results in a low NADPH flux.*

An NADH dependent 3HB production pathway was created by replacing the enzyme PhaB with Hbd ((*S*)-3HB-CoA dehydrogenase) which was originally derived from *Clostridium acetobutylicum*. The end product of the NADH dependent production pathway used in this study is *S*-3HB compared to *R*-3HB which is the end product of the NADPH dependent 3HB production process (Figure 1b). It was not possible to create an NADH dependent *R*-3HB production pathway as a study of existing literature showed that no bacterial NADH dependent *R*-3HB-CoA dehydrogenase is currently known to exist [6]. In order to compare 3HB yields between NADPH and NADH dependent 3HB production, *E. coli* was transformed with two plasmids, p3HB and pHbd, and subsequently anaerobic fermentations were performed. p3HB contained the genes *phaA*, *phaB* and *tesB* which were required for NADPH dependent *R*-3HB production and pHbd contained *phaA*, *hbd* and *tesB* which were required for NADH dependent *S*-3HB production.

3. Material and Methods

2.1 ***Microorganism***

The working strain for cloning was *E. coli* XL1-Blue. For fermentations *E. coli* BW25113 was used.

2.2 ***Plasmids used and plasmid (pHbd) construction***

An NADPH-dependent 3HB production plasmid, named p3HB, was obtained from I. Lamot. p3HB was based on pUC19 and contained a chloramphenicol resistance marker gene (*cmR*) furthermore p3HB housed the following genes in an operon, under the constitutive promotor *phbCab*: *phaA*, *phaB* derived from *R. euthropha* and *tesB* derived from *E. coli MG1655*. For plasmid construction, *phaB* was removed from p3HB using the restriction enzymes MaubI and AflII. A gene fragment, *hbd,* housed on pUC57-Hbd*,* was also excised with MaubI and AflII and ligated into de remaining p3HB fragment. *hbd* was originally derived from *Clostridium acetobutylicum.* This gene was provided by GenScript inc. and codon optimized for *E. coli*. (Figure 3)

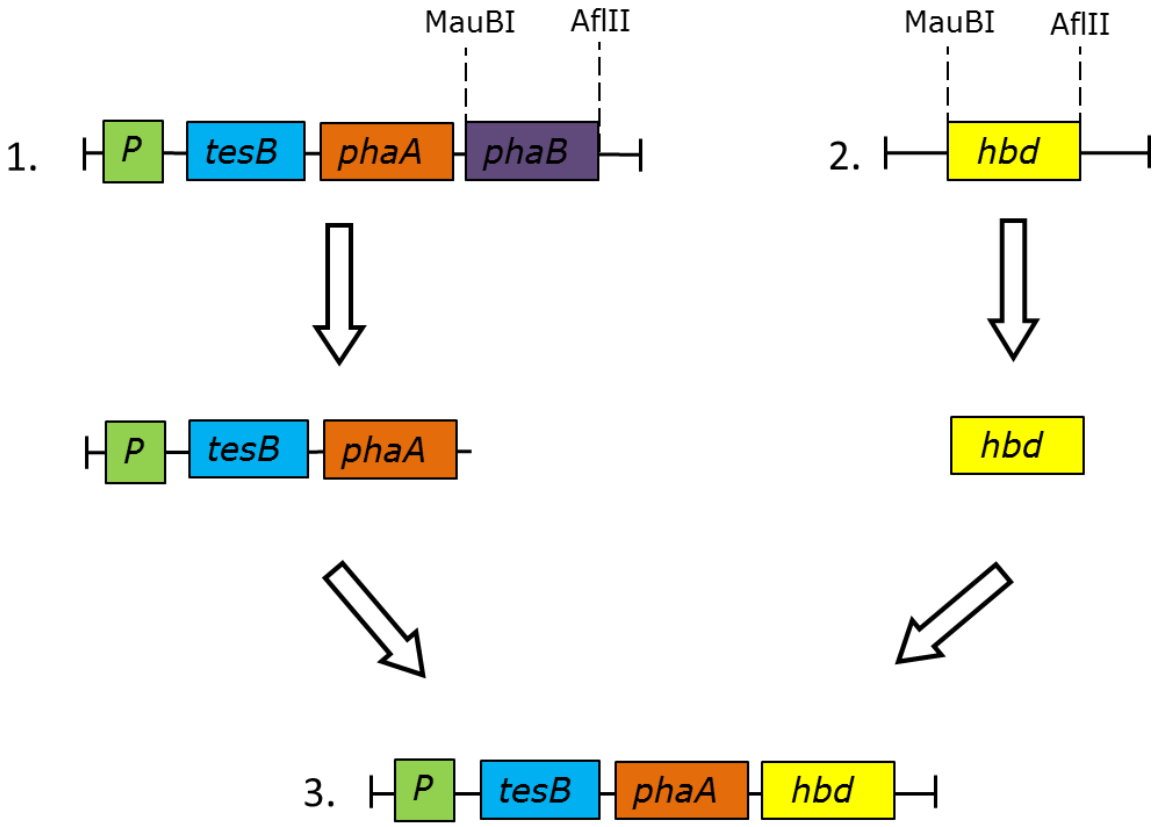

Figure 3*: Construction of pHbd. hbd originally from Clostridium acetobutylicum ATCC 824 was synthesised and codon optimized for E. coli by GenScript USA (Inc.) and provided on pUC57. 1) operon present on p3HB consisting of tesB, phaA and phaB under a constitutive promotor [P] 2) hbd gene 3) operon present on pHbd consisting of tesB, phaA and hbd*

For anaerobic fermentations a control plasmid was used named pBlank. This plasmid was based on pUC19 into which *cmR* was inserted. pBlank lacked *tesA*, *tesB*, *hbd*/*phaB* and was provided by I. Lamot. Plasmid isolation, restriction enzyme digestion, agarose gel electrophoresis, transformations and DNA ligation were performed following standard procedures.

2.3 ***Pre culture conditions***

Fresh transformants were transferred to 3 ml high salt Lysogeny Broth (LB)-medium (Difco) and were grown aerobically overnight at 37 ˚C, on a rotary shaker (Innova 4335 New Brunswick Scientific), at 250 rpm. Overnight LB cultures were transferred to fresh LB medium at a ratio of 1 ml old medium and 4 ml fresh medium. LB cultures were allowed to grow for another 5 hours to ensure exponential growth. Subsequently, 50 µl of these exponentially growing LB cultures was used to inoculate 40 ml Evans medium and grown aerobically, overnight at 37 ˚C, on a rotary shaker (Innova 44 incubator series shaker, New Brunswick) in 250 ml Erlenmeyer flasks, at 250 rpm. Cultures were kept under antibiotic stress using chloramphenicol (concentration 50 µg/L). For the composition of Evans medium see Index I.

2.4 ***Anaerobic fermentations***

Anaerobic fermentations were performed in 100 ml nitrogen gas saturated serum bottles with butyl rubber stoppers, at a temperature of 37 ˚C on a rotary shaker (Innova 44 incubator series shaker, New Brunswick), at 200 rpm. Samples for HPLC analysis, pH measurements, and optical density (OD) measurements were taken every hour. OD measurements were performed on a Ultrospec 2000, Pharmacia Biotech at a wavelength of 600nm. For HPLC analysis, samples were centrifuged, and supernatants were frozen for further use.

2.5 ***HPLC analysis***

In order to determine metabolite formation during anaerobic fermentations, HPLC analysis was performed. For the detection of glucose and ethanol a refractive index (RI) detector was used. For the detection of succinate, lactate, formate, acetate and 3HB, an UV detector was used. In order to lyse any remaining cells and to insolubilize proteins, samples were mixed 1:1 with 1M H_2SO_4. Different HPLC setups were used for fermentation run 1 and 2.

2.6 ***Determination of 3HB stereoisomers***

In order to differentiate between the different stereoisomers of 3HB an enzymatic assay was used in conjunction with HPLC analysis. The presence of *R*-3HB could be directly assayed by use of the D-3-Hydroxybutyric acid assay kit acquired from Megazyme international (Ireland, Wicklow). The D-3-Hydroxybutyric acid assay could not detect *S*-3HB. In order to detect the presence of *S*-3HB, HPLC analysis was used in conjunction with the D-3-Hydroxybutyric acid assay. For this assay samples were taken from anaerobic cultures of *E. coli* BW25113, transformed with either pBlank, p3HB or pHbd. Cultures were grown on Evans medium and samples were taken after 24 hours. The D-3-Hydroxybutyric acid assay was performed according to the manufacturers protocol.

4. Results

3.1 ***Proof of plasmid construction***

In order to create a pathway for the NADH dependent production of 3HB an attempt was made to construct a plasmid which housed the genes *phaA*, *hbd* and *tesB*. This plasmid was named pHbd. In order to determine whether construction of pHbd was succesful, a restriction analysis was performed. The two restriction enzymes which were used for this essay were EcoRI and EcoRV. The expected restriction fragment lengths can be seen in Table 2. The digestion patterns of a pHbd transformed colony can be seen in Figure 4, together with a p3HB control. The digestion patterns seen matched the predicted fragment lengts. However, the 186 bp fragment, expected from the EcoRV and EcoRI + EcorRV double digest of pHbd, was not visible on the gel in Figure 4. Given that all other bands seen correspond to expected fragment lengts, and the decreasing visibility of fragments on gel in occordance with decreasing fragment length, it is likely that this small 186 bp fragment band is not visible due to it's small size.

Table 2*: Restriction digest fragment list of EcoRI, EcorV and EcorI and EcorV double digests on pHbd and p3HB plasmids.*

Plasmid	Restriction Enzyme(s)	Fragment lengths (bp)
pHbd	EcoRI	4691, 3236
pHbd	EcoRV	6873, 868, 186
pHbd	EcoRI + EcoRV	3236, 2856, 868, 781, 186
p3HB	EcoRI	4579, 3236
p3HB	EcoRV	7815
p3HB	EcorI + EcoRV	3802, 3236, 781

3.2 ***Determination of enzyme expression***

In order to check if pHbd was expressed in *E. coli*, an enzymatic assay for the detection of *R*-3HB was performed in combination with an HPLC assay. pHbd transformants were negative for the enzymatic assay and positive for the HPLC assay showing an *S*-3HB concentration of 0.42 ±0.01 g/L. p3HB transformants were used as a postive control and were positive for the enzyme assay showing a *R*-3HB concentration of 0.40 ±0.07 g/L. p3HB transformants were also positive for the HPLC assay showing a *R*-3HB concentration of 0.55 ±0.07 g/L. As a negative control *E. coli* transformed with pBlank, a plasmid which did not house the genes required for the production of 3HB, was also assayed. pBlank transformants were negative for both the enzyme assay as well as the HPLC assay.

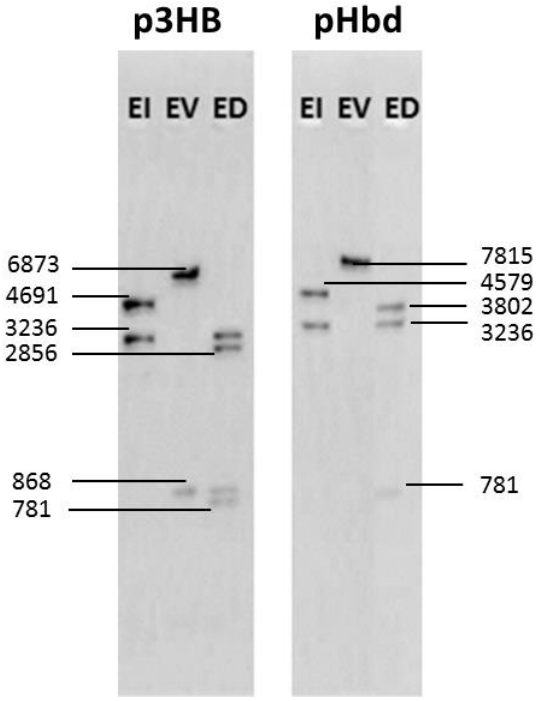

***Figure 4**: Restriction analysis gel where: **EI** = EcoRI digest, **EV** = EcoRV digest, **ED** = EcoRI + EcoRV double digest. Numbers indicate fragment sizes in bp.*

3.3 ***Growth experiments***

NADPH is a anabolic redox cofactor used mainly for growth. It was expected that an NADPH dependent 3HB production pathway could draw NADPH away from anabolic processes present in the cell, thereby slowing growth. In order to see a difference in growth, three different types of *E. coli* BW25113 transformants were compared. These transformants housed three different plasmids, namely: p3HB, pHbd and pBlank. Growth was measured over a period of 24 hours. Samples were taken every hour for the first 7 - 9 hours and after 24 hours a final sample was taken. Exponential growth occured between 1 to 5 hours. This was determined using curve fitting. Fermentations were performed in two seperate runs called fermentation 1 and 2. During the first run, starting optical density (OD, wave length = 600nm) measurements between duplicates varied. For the second run, starting OD's were identical between the three transformants. The maximum OD's observed during experiments were 1.29 - 1.88 for p3HB, between 1.05 - 1.15 for pHbd and between 1.17 - 1.29, for pBlank. (Figure 5a and Figure 6a). Maximum growth rates were also determined and can be seen in Table 3. For both fermentation runs pHbd and pBlank transformants show significantly higher growth rates when compared to p3HB transformants.

As Figures 5b and 6b show, over the course of anaerobic fermentations, pH decreased in concurence with bacterial growth. HPLC analysis showed that glucose was still present at the end of all fermentations, the concentrations of which can be seen in Table 4.

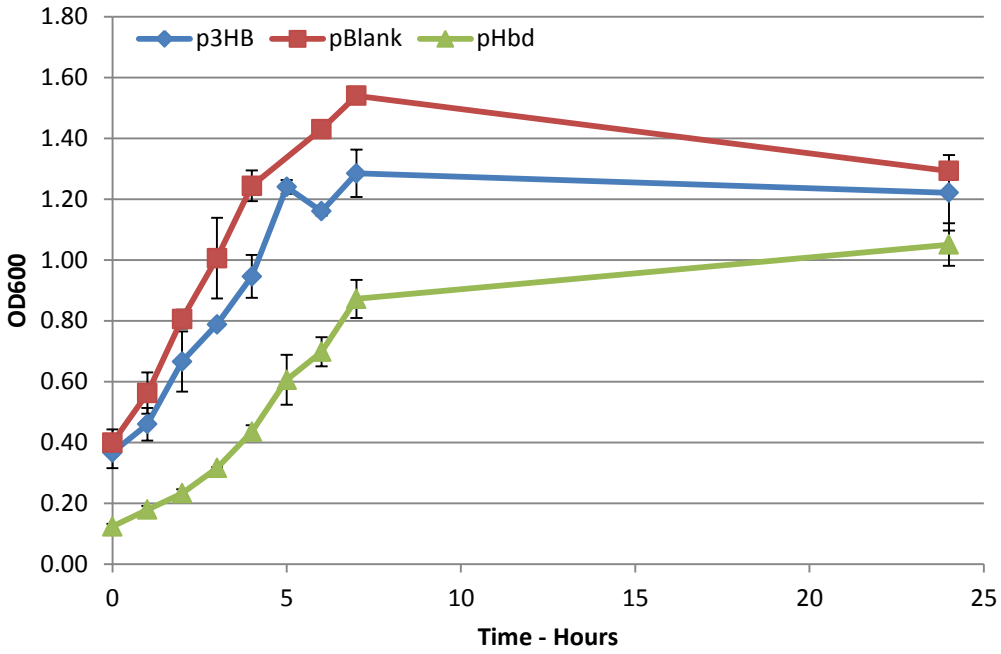

Figure 5a: *Growth curves for fermentation run 1 using E. coli BW25113 transformed with p3HB (diamonds), pBlank (squares) and pHbd (triangles). Fermentations were anaerobic and the medium used was Evans mineral medium supplemented with 10 g/L glucose. Measurements were done in duplicate. Error bars represent standard deviation*

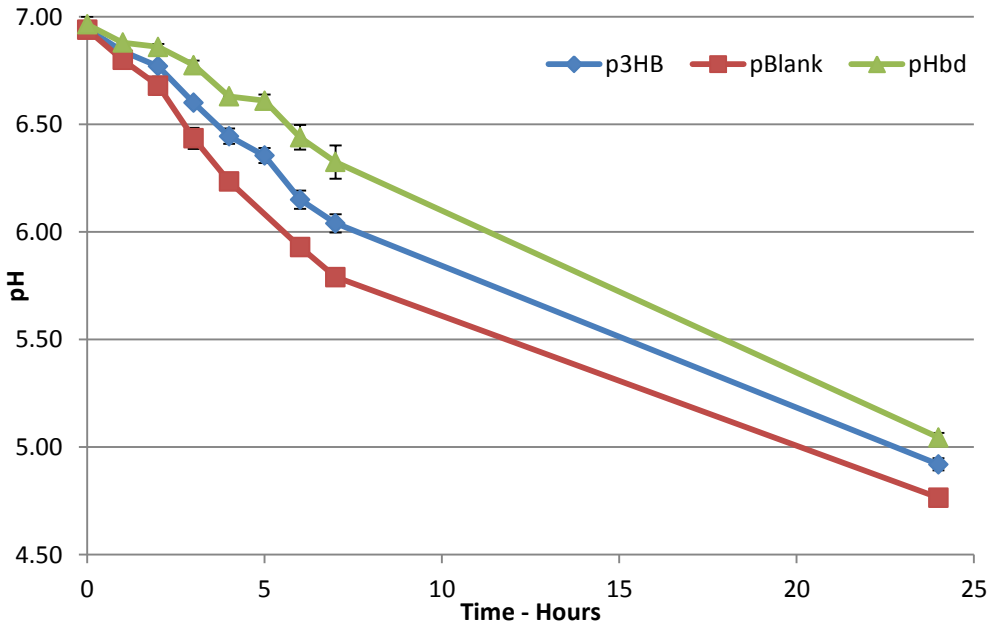

Figure 5b: *pH curves for fermentation run 1 using E. coli BW25113 transformed with p3HB (diamonds), pBlank (squares) and pHbd (triangles). Fermentations were anaerobic and the medium used was Evans mineral medium supplemented with 10 g/L glucose. Measurements were done in duplicate. Error bars represent standard deviation*

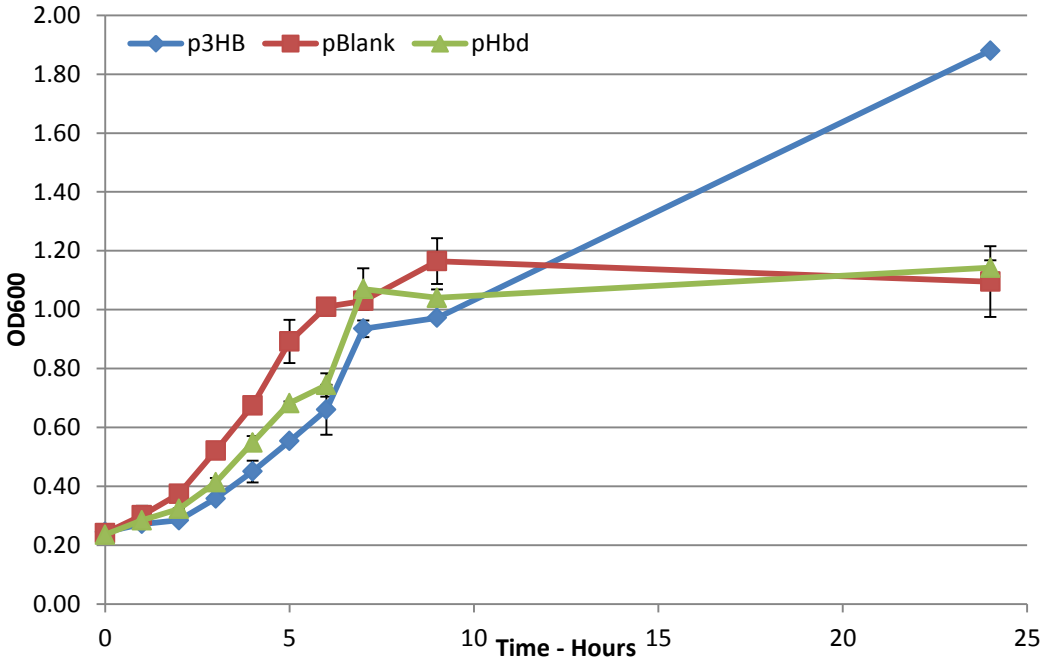

Figure 6a: *Growth curves for fermentation run 2 using E. coli BW25113 transformed with p3HB (diamonds), pBlank (squares) and pHbd (triangles). Fermentations were anaerobic and the medium used was Evans mineral medium supplemented with 10 g/L glucose. Measurements were done in duplicate. Error bars represent standard deviation.*

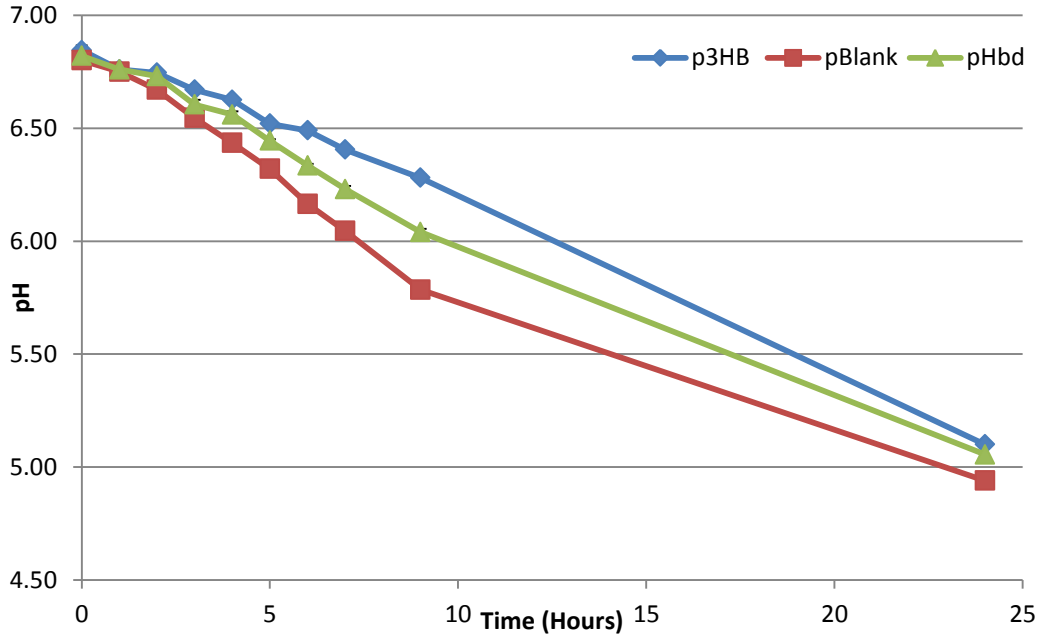

Figure 6b: *pH curves for fermentation run 1 using E. coli BW25113 transformed with p3HB (diamonds), pBlank (squares) and pHbd (triangles). Fermentations were anaerobic and the medium used was Evans mineral medium supplemented with 10 g/L glucose. Measurements were done in duplicate. Error bars represent standard deviation*

Table 3: *Maximum growth rates (µmax) for pHbd, p3HB and pBlank E. coli BW2511 transformants during fermentation run 1 and 2. Measurements were done in duplicate. Error ranges represent standard deviation*

Fermentation	µmax pHbd	µmax p3HB	µmax pBlank
1	0.30 ±0.02	0.23 ±0.03	0.26 ±0.01
2	0.25 ±0.00	0.22 ±0.00	0.29 ±0.04

Table 4: *glucose concentrations in g/l of p3HB, pBlank and pHbd transformants at the end of fermentations 1 and 2 . Error ranges represent standard deviation*

	Fermentation 1	Fermentation 2
p3HB	3.95 ±0.34	3.28 ±0.43
pBlank	4.99 ±0.32	2.57 ±0.21
pHbd	3.13 ±0.07	5.36 ±0.79

3.4 ***Metabolite yields***

In order to determine the difference in 3HB yield between NADH and NADPH dependent 3HB production pathways, HPLC analysis was performed on *E. coli* BW25113 transformants housing either pHbd, p3HB or pBlank. Due to low reproducibility of HPLC measurements at low metabolite concentrations, only 24 hour measurements were used to calculate yields, as it was at this time point that product concentrations were high enough to see significant differences in metabolite concentrations between samples. Yields are expressed as cmol product formed per cmol glucose consumed. The production of CO_2 was not measured directly, rather, yields were calculated with the formula shown in Figure 7.

$$\mathbf{[acetate] + [ethanol] + 2\,[3HB] - [succinate] - [formate] = [CO_2]}$$

Figure 7*: formula for the calculation of yield of CO_2, in cmol/cmol, on glucose*

As can be seen in Figures 8 and 9, fermentations show a relatively high production of lactic acid for p3HB, pHbd, and pBlank transformants, when compared to all other mixed acid fermentation products. Between the three transformants, the only mixed acid fermentation product that differed significantly in yield was lactic acid. There was no significant difference in 3HB production between p3HB and pHbd. Note that for fermentation 1 the sum of all yields was lower than 1. For fermentation 2 the sum of the yields was higher than 1. This indicated that there were possible innacuracies in the HPLC measurements.

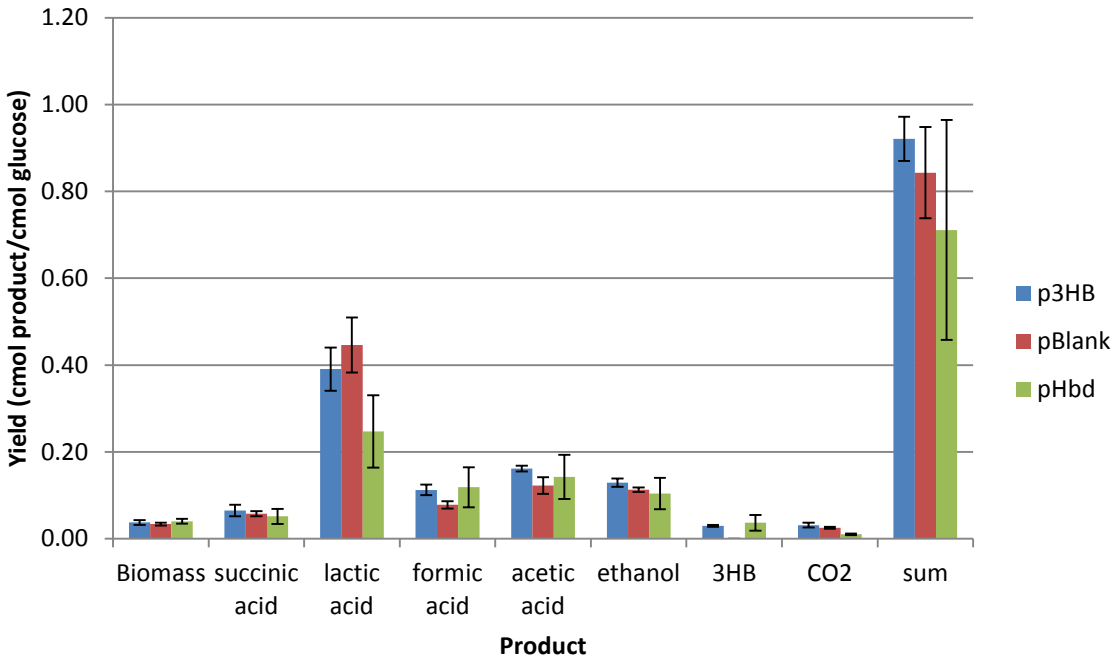

Figure 8: *Product yields (cmol product/cmol glucose) at t= 24 h for fermentation 1, using E. coli BW25113 transformed with p3HB (Blue), pBlank (Red) and pHbd (Green). Fermentations were anaerobic and the medium used was Evans mineral medium supplemented with 10 g/L glucose. Measurements were done in duplicate. Error bars represent standard deviations*

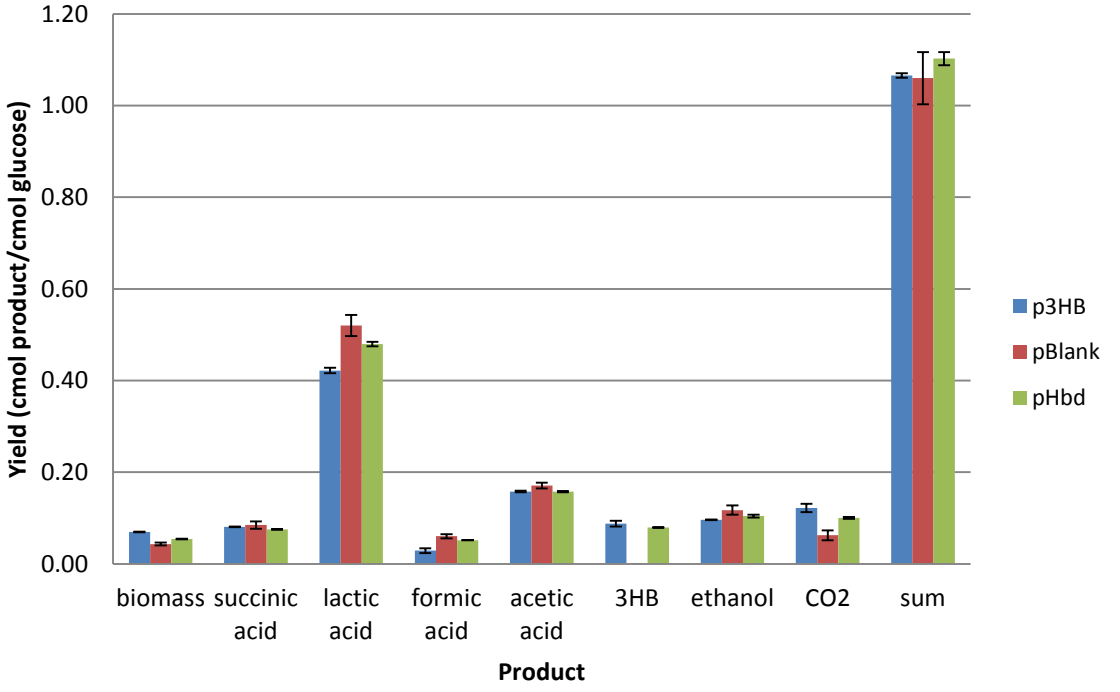

Figure 9: *Product yields (cmol product/cmol glucose) at t= 24 h for fermentation 2, using E. coli BW25113 transformed with p3HB (Blue), pBlank (Red) and pHbd (Green). Fermentations were anaerobic and the medium used was Evans mineral medium supplemented with 10 g/L glucose. Measurements were done in duplicate. Error bars represent standard deviations*

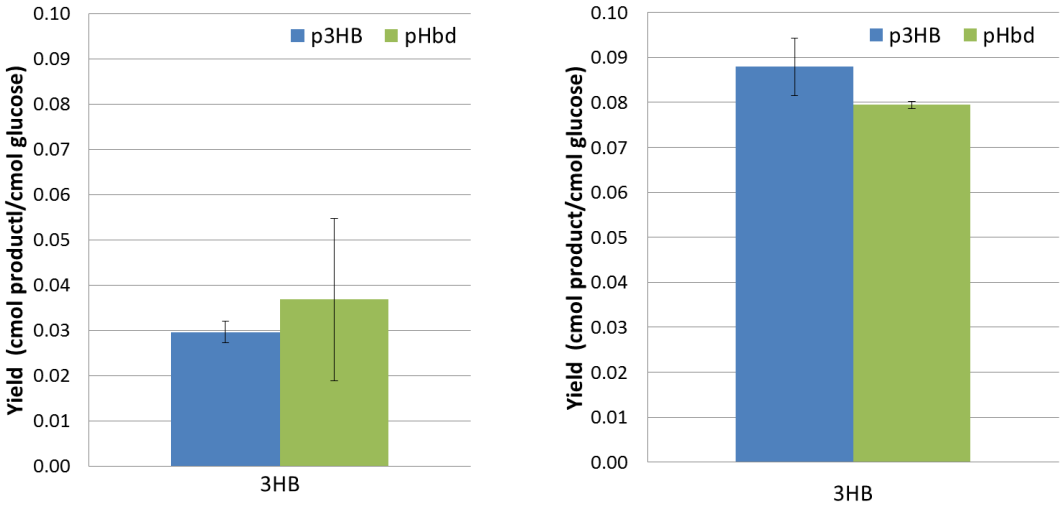

Figure 10: *3HB yields (cmol product/cmol glucose) at t= 24 h for fermentation 1 (left) and 2 (right), using E. coli BW25113 transformed with p3HB (Blue) and pHbd (Green). Fermentations were anaerobic and the medium used was Evans mineral medium supplemented with 10 g/L glucose. Measurements were done in duplicate. Error bars represent standard deviations*

5. Discussion and Conclusion

One of the stumbling blocks for the anaerobic production of 3HB by *E. coli* is the dependency of the process on the redox cofactor NADPH. In order to circumvent this NADPH bottleneck, an attempt was made to create an NADH dependent 3HB production pathway. This pathway would be based on an NADPH dependent pathway consisting of the enzymes: PhaA, TesB and PhaB. The genes required for expression of the NADPH dependent pathway were situated on a vector, named p3HB. In order to create an NADH dependent pathway *phaA* was exchanged for *hbd*. The resulting plasmid was dubbed pHbd. In order to check if plasmid construction was successful, a restriction analysis was performed, the results of which can be seen in Figure 4. The observed band pattern was in accordance with the expected fragment sizes. The desired fragment sizes can be seen in Table 2. As the observed band pattern matched the expected band pattern we could conclude that plasmid construction was successful. Following plasmid construction, the expression of Hbd was determined by detecting the production of *S*-3HB in anaerobic cultures of *E. coli* transformed with pHbd. In order to confirm the expression of Hbd, the production of *S*-3HB was assayed. In order to detect the production of *S*-3HB, HPLC analysis was performed in combination with a *R*-3HB specific enzyme assay. The enzyme assay did not show the formation of *R*-3HB in pHbd transformed cultures. This was to be expected, as the assay should not show a positive for *S*-3HB. As a control for the enzyme assay, p3HB and pBlank cultures were also tested for the production of *R*-3HB. As expected, the enzyme essay showed the presence of *R*-3HB for p3HB transformed cultures, and was negative for pBlank transformed cultures. HPLC analysis showed the presence of 3HB for pHbd and p3HB transformants, but not for the pBlank transformants. This was as expected, as the HPLC analysis method used for this study did not discriminate between *R*- and *S*-3HB. The negative result from the *R*-3HB enzyme essay, combined with the positive result from the HPLC analysis, indicated that *S*-3HB was produced by the pHbd transformants. In short, the aforementioned results showed that we were successful in creating an NADH dependent 3HB production pathway.

In order to compare the yields of 3HB on glucose between NADPH and NADH dependent 3HB production, *E. coli* was transformed with two plasmids, p3HB and pHbd. Subsequently, anaerobic fermentations were carried out. HPLC analysis was performed on fermentation samples to determine metabolite production and glucose consumption. Furthermore, OD measurements and pH measurements were performed to determine growth and medium acidification.

HPLC analysis (Figure 8 and 9) showed no significant differences in 3HB yield between pHbd and p3HB transformants. The highest 3HB yields achieved during this study were 0.09 ±0.01 and 0.08 ±0.00 cmol 3HB/cmol glucose for p3HB and pHbd transformants respectively. This result was unexpected, as it was thought that the comparatively high flux through NADH, when compared to NADPH dependent 3HB production, would result in a higher 3HB yield. However, a difference in growth rate was observed between p3HB and pHbd transformants. During anaerobic fermentations pHbd transformants appeared to grow faster (μmax = 0.25 ±0.00 h^{-1}) than p3HB transformants (μmax = 0.22 ±0.00 h^{-1}). The difference in growth rate could be explained by the fact that Hbd requires NADH and PhaB requires NADPH. NADPH is an anabolic redox cofactor mainly used in biomass synthesis. Over the course of fermentation 1 the total NADPH requirement for biomass formation, assuming that 16 mmol NADPH is required per gram of dry matter [7], was 4.72 ±0.34 and 4.24 ±0.34 mmol NADPH for p3HB and pHbd cultures respectively . The amount of NADPH or NADH required for the production of 3HB was 2.28± 0.18 and 2.04 ±0.62 mmol for p3HB and pHbd cultures respectively. For p3HB cultures a large amount of NADPH was required for the production of 3HB, with roughly 1/3 of all NADPH formed, used for the production of 3HB, and the other 2/3 for biomass formation. When NADPH is used for the production of 3HB, less NADPH is available for the production of biomass. In order to maintain NADPH levels $NADP^+$ is actively reduced to NADPH by pyridine nucleotide transhydrogenase (*pntAB*). This membrane bound enzyme transfers H^+ + 2

electrons from NADH to $NADP^+$. This reaction is ultimately ATP dependent and would thereby slow growth rate [7]. The activity of PntAB could also be the cause for the similar 3HB yields achieved with p3HB and pHbd transformants, because pntAB effectively couples NADH and NADPH pools. However, differences in yield might also be due to differences in enzyme activity of Hbd compared to PhaB. It was assumed that by using a constitutive promotor, and a high copy vector, cellular enzyme concentrations would be sufficiently high to mitigate the difference in enzyme activity between Hbd and PhaB. In order to ensure that differences in enzyme activity had no influence on the experiments the enzymatic activity of Hbd and PhaB should be determined. Furthermore, it is also possible that the absence of a significant difference in 3HB production between p3Hb and pHbd transformants could be and artefact of the experimental methods used. There were large standard errors for all compounds measured and, compared to other groups, comparatively low 3HB titres and biomass concentrations were achieved (Table 5). The highest biomass concentration and yield on glucose achieved during fermentations were 0.55 g/l and 0.09 cmol/cmol glucose respectively . Low titres of 3HB were likely caused by the low biomass levels and product yields achieved during this study. Low biomass concentrations were likely caused by acidification of media, the use of minimal media, and anaerobic culture conditions. Low 3HB yields were caused by the co-formation of mixed acid fermentation products such as lactic acid, in turn caused by the anaerobic culture conditions used. Difficulties were also encountered with the HPLC method itself, specifically the separation of 3HB and formic acid peaks was difficult, lowering the accuracy of the method.

Table 5: *Comparison of yields (cmol/cmol glucose), (S/R)-3HB titres (g/L) and biomass concentrations (g/L) achieved under aerobic and anaerobic conditions by various other research groups. Error ranges represent standard deviation.*

Author	Medium	(An)aerobic	3HB (g/L)	Yield (cmol/cmol glucose)	Biomass (g/L)
Tseng [2]	LB	aerobic	2.92	0.35	0.80
Liu [9]	LB	aerobic	4.03 ±0.13	0.28	2.23 ±0.07
Gao [8]	LB	aerobic	2.23	0.38	2.38
Liu [9]	LB	anaerobic	2.37 ±0.12	0.41	0.33 ±0.02
Lamot	LB	aerobic	3.09 ±0.00	0.50	2.05 ±0.02
Lamot	LB	anaerobic	0.22 ±0.01	0.07	0.40 ±0.03
Lamot	MM	aerobic	2.12 ±0.03	0.17	1.14 ±0.02
Lamot	MM	anaerobic	0.22 ±0.01	0.04	0.23 ±0.01
This study	MM	anaerobic	0.55 ±0.07	0.09	0.26 ±0.01

To ensure that the lack of an observed difference in NADPH and NADH on anaerobic 3HB production was not due to inaccuracies in the methods used, biomass concentrations and 3HB yields have to be improved. HPLC analysis indicated that not all glucose was consumed at the end of fermentations (Table 4). This would imply that growth was not limited by the availability of glucose, but rather by the drop in pH which occurred during growth experiments. Culture media were buffered using a phosphate buffer. However, in order to increase the amount of biomass the buffering capacity of the media needs to be increased. Interesting buffers to use would be the Good's buffers MOPS ((3-(N-morpholino) propanesulfonic acid)) or PIPES (piperazine-N,N'-bis (2-ethanesulfonic acid)), because these buffers have strong buffering capacity around pH 6.9, the starting pH for the anaerobic flask experiments. [10] Due to the overlap between formate and the 3HB peaks, the accuracy of the HPLC method used would benefit from the removal of formate from the medium. Over time formate is converted by *E. coli* to CO_2 and H_2 by means of the membrane bound formate hydrogenlyase complex. Considering that formate was still detected in culture media after 24 hours, it is likely that the time span for formate removal is longer than 24 hours. Therefore it is suggested to sample at later time points during anaerobic fermentations.

In order to increase the 3HB yields of anaerobic fermentation, competing synthesis pathways have to be knocked out. For instance, knocking out lactate and acetate production, by means of deletion of *ldhA* (D-lactate dehydrogenase) and *pta* (phosphate acetyl transferase), would increase the availability of acetyl-CoA and NADH respectively (Figure 11). Due to the activity of *pntAB* the availability of NADPH would also increase. From this point it is not possible to further increase the yield of 3HB on glucose due to the fact that 3HB production from glucose is not redox balanced. During the production of 3HB from glucose there is a net surplus of one NADH molecule per molecule of 3HB produced (Figure 12). The only way to achieve redox balance is to co-produce 3HB with ethanol and succinate, as each have a net consumption of one NADH molecule per molecule of ethanol or succinate formed (Figure 13a, Figure 13b).

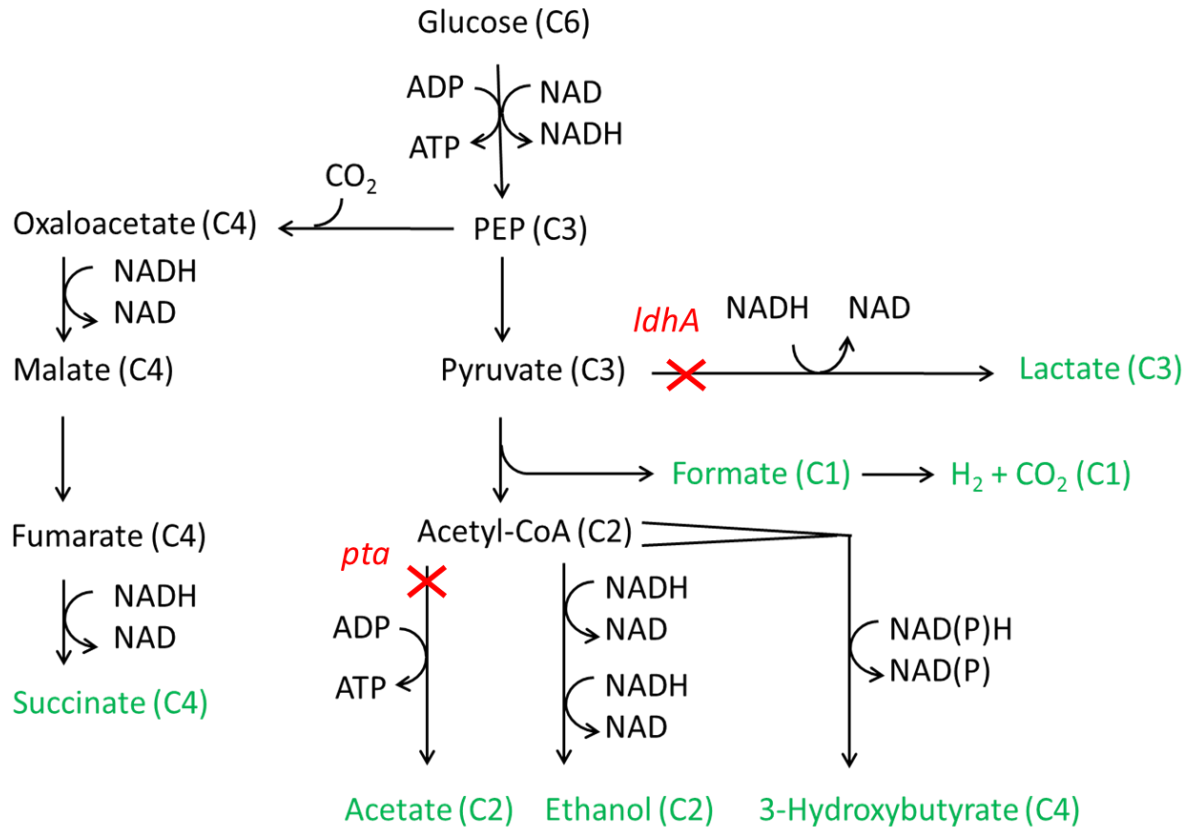

***Figure 11**: Simplified metabolic flow sheet of mixed acid fermentation in E. coli. Carbon chain length of compounds shown in parentheses. Compounds that are excreted are green. Potential deletions denoted with red crosses for pta and ldhA.*

Figure 12: *Reaction scheme for the production of 3HB from glucose.*

The maximum possible yield of 3HB on glucose, assuming no biomass accumulation or maintenance, would be 0.67 mol of 3HB per mol of glucose. Assuming that succinate and ethanol are produced in 1:1 ratio, 0.33 mol succinate and 0.33 mol ethanol per mol of glucose would also be produced.

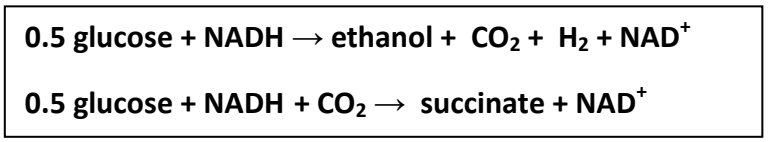

Figure 13a: *Reaction scheme for the production of ethanol and succinate*

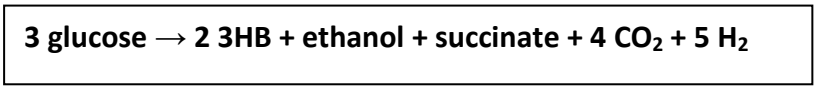

Figure 13b: *Reaction scheme for the co-production of 3HB with ethanol and succinate*

In conclusion, this study succeeded in expressing an NADH dependent 3HB production pathway in *E. coli* and subsequently, succeeded in the anaerobic production of 3HB using the aforementioned NADH dependent 3HB production pathway. No difference was seen in 3HB production between NADH and NADPH dependent pathways. The likely cause for a lack of a significant difference in 3HB production was the activity of pntAB. However, In order to confirm the effect of pntAB and to better study the production of 3HB in general, 3HB yields have to be further increased by knocking out competing pathways and higher 3HB titres have to be achieved by increasing biomass concentrations.

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Index I

Composition of Evans medium

The composition of 1 liter Evans medium, prior to sterilization, can be seen in Table 6. The composition of the trace elements mix required for Evans medium can be seen in table 7. Components from Table 6 and 7 were sterilized by autoclaving. Subsequently, selenite and thiamine were added through a sterile 0.2 µM filter to a final concentration of 30 µg/l and 15 mg/l respectively.

***Table 6**: composition of 1 liter Evans medium prior to sterilization. [1]glucose concentration for aerobic pre cultures. [2]glucose concentrations for anaerobic cultures.*

compound	concentration (g/L)
NaH_2PO_4 .2 H_2O	15.6
KCl	0.75
$MgCl_2$ .6 H_2O	0.25
NH_4Cl	5.35
Na_2SO_4	0.28
$CaCl_2$ .2 H_2O	0.003
Glucose[1]	20
Glucose[2]	10
Titriplex	0.39
Trace elements mix	100 ml

***Table 7**: composition of trace element mix.*

compound	concentration (g/L)
ZnO	0.412
$FeCl_3$ .6 H_2O	5.4
$MnCl_2$.4 H_2O	2
$CuCl_2$. 2 H_2O	0.172
$CoCl_2$. 6 H_2O	0.476
H_3BO_3	6.4E-02
Na_2MoO_4. 2 H_2O	4.0E-03
HCl (37%)	20 ml/L