CHEMICAL ENGINEERING TRANSACTIONS
VOL. 70, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Timothy G. Walmsley, Petar S. Varbanov, Rongxin Su, Jiří J. Klemeš

ISBN 978-88-95608-67-9; **ISSN** 2283-9216
DOI: 10.3303/CET1870094

Towards the Integration of the Anaerobic Ethyl (S)-3-Hydroxybutyrate Production Process into a Biorefinery Concept

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Ethyl (S)-3-hydroxybutyrate ((S)-E3HB) is naturally found as part of kiwi fruit aroma and is an important precursor for the production of several pharmaceuticals. (S)-E3HB can be produced in a whole cell biotransformation process from Ethylacetoacetate (EAA) using the yeast *Saccharomyces cerevisiae*. One way of improving the sustainability of such processes may be their integration into novel biorefinery concepts, which requires research to characterize the process and the byproduct formation. In this study it could be shown that (S)-E3HB biocatalysis with *S. cerevisiae* can be performed under anaerobic conditions with production rates between 4.0×10^{-3} and 2.5×10^{-2} $g_{(S)\text{-}E3HB}$ $g_{biomass}^{-1}$ h^{-1} which are similar to the rates in conventional aerobic processes. The byproduct spectrum allows for the integration of the new anaerobic biotransformation process into a recently invented biorefinery concept in which the side product CO_2 is further utilized to produce methane.

1. Microbial production of (S)-E3HB

The biotransformation of Ethylacetoacetate (EAA) as a substrate to Ethyl (S)-3 hydroxybutyrate ((S)-E3HB) can be performed as a whole cell biocatalytic process using the yeast *Saccharomyces cerevisiae* (Figure 1). The bio-transformation of EAA to (S)-E3HB is catalysed by a carbonyl reductase, naturally occurring in *S. cerevisiae*. The regeneration of the oxidized Nicotinamide-adenine-dinucleotide-phosphate (NADP+) to NADPH may be powered by the respiratory processes within the cells (Engelking, 2004).
Early investigations on the microbial production of (S)-E3HB using the yeast *Saccharomyces cerevisiae* have been performed by Wipf et al. (1983). They found from batch and fed-batch experiments with suspended cells in an aerated 50 L reactor that substrate concentrations (EAA) for the biocatalysis should be kept below 0.5 gL^{-1}. Product concentrations up to 40 gL^{-1} could be achieved under aerobic conditions. In addition to the substrate EAA Wipf et al. (1983) fed glucose or ethanol as energy yielding carbon sources. They stated, that temperatures below 20 °C and above 40 °C lower the production rate, as found for many other yeast strains as well. Studies by Rohner et al. (1990) showed that the biocatalysis with *S. cerevisiae* yields high optical purities of (S)-E3HB as compared to other yeasts. These authors stated that low pH-values of about 2.2 may have an influence on the optical purity of the product (Rohner et al., 1990). On the other hand Kometani et al. (1991) could not observe any particular influence of the pH-value in the range of 3.5 to 8. A study with glucose and ethanol as energy yielding carbon sources indicated a reduced side product spectrum and a higher optical purity of (S)-E3HB when using ethanol as a carbon source (Kometani et al., 1993). As byproducts acetate, acetone and several butanoates were found (Chin-Joe et al. 2001).
Summing up, the microbial production of (S)-E3HB may be conducted under the following conditions:

- The production of (S)-E3HB is performed aerobically
- Ethanol and glucose may be used as energy sources (C-substrate)
- Less byproducts occur when using ethanol, the optical product purity is higher.
- Ethanol concentration has to be kept below 20 gL^{-1} to avoid inhibition

Please cite this article as: Hirschmann R., Borodkin N., Baganz F., Hass V.C., 2018, Towards the integration of the anaerobic ethyl (s)-3-hydroxybutyrate production process into a biorefinery concept , Chemical Engineering Transactions, 70, 559-564 DOI:10.3303/CET1870094

- EAA concentration has to be kept below 0.5 gL^{-1} to avoid inhibition
- (S)-E3HB may be produced up to 40 gL^{-1} in the broth
- pH value can be adjusted in a range of 2.2 to 8 and influences optical product purity
- Yield losses by evaporation of EAA, (S)-E3HB and ethanol have to be considered
- Formation of byproducts may have an influence on the product yield

So far, aerobic process conditions were supposed to be essential for the regeneration of the cofactor NADP during the biocatalytic process. However, aerobic processes often show lower carbon efficiency as compared to anaerobic processes. Furthermore, the off gas from anaerobic processes exhibits high carbon dioxide concentrations, and thus may be used as a substrate within novel biorefinery concepts (Reule et. al, 2015). A key property of biorefineries is that the educts are almost completely transformed into products and the amount of non-utilizable products is strictly minimized (Santibañez-Aguilar et al., 2014). Thus, the integration of already existing processes into biorefinery concepts requires research into two directions. First, the process needs to be characterized with respect to its product and byproduct spectrum under different operational conditions to gain the optimal industrial system (Gonela et al., 2014) and second, process strategies need to be found minimizing the amount of non-markeTable or non-utilizable byproducts (Hass et al., 2014).
In this contribution we will focus on the experimental investigation of the (S)-E3HB-process under aerobic and anaerobic conditions. We will compare novel anaerobic processes of biocatalytic (S)-E3HB production to aerobic processes with respect to production rates, yields and major side products.

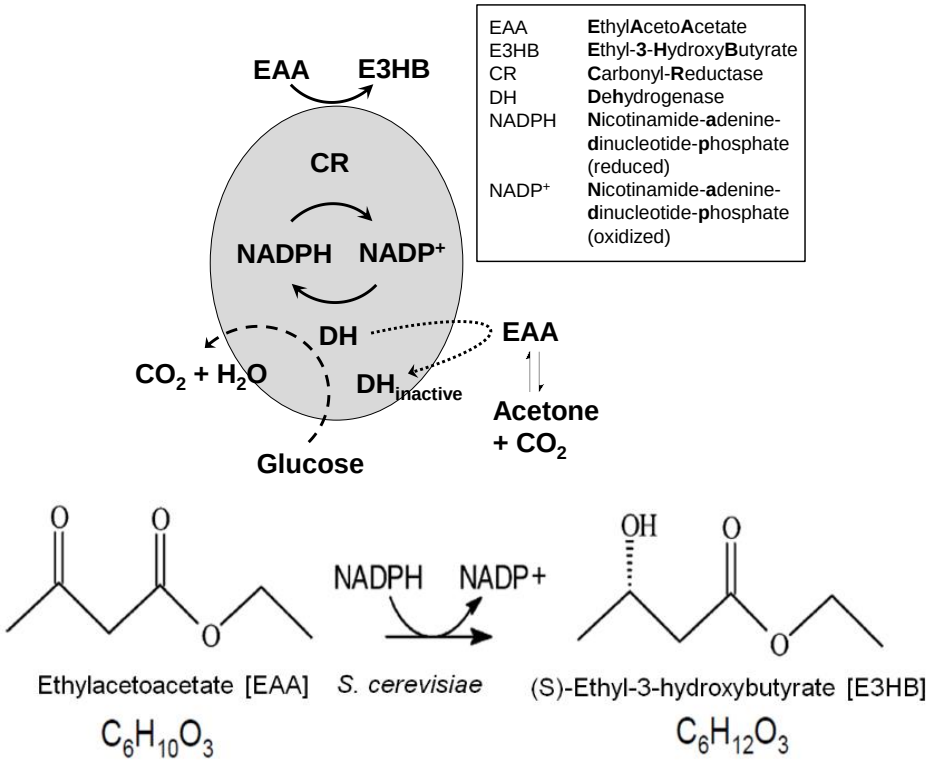

Figure 1: Whole cell biocatalysis of (S)-Ethyl-3-hydroxybutyrate ((S)-E3HB) from Ethyloacetate (EAA) using Saccharomyces cerevisiae as the biocatalyst (adapted from Kometani et al. (1993))

2. Whole cell biocatalysis of (S)-E3HB

In the course of our research eleven biotransformation processes have been carried out in either 5 L or 20 L stirred tank bioreactors, equipped with online probes for temperature, pH and dissolved oxygen tension. The exhaust gas concentrations of O_2 und CO_2 (aerobic conditions) and the volumetric flow rate of CO_2 (anaerobic conditions) were also measured online. Samples were taken frequently to determine concentrations of glucose, biomass, ethanol, EAA and (S)-E3HB as well as the side products lactic, acetic and formic acid. Dry biomass

concentrations were determined using a Sartorius moisture analyser. Glucose and ethanol concentrations were determined using commercial enzymatic testkits (r-biopharm). EAA and (S)-E3HB concentrations were determined by high pressure liquid chromatography (HPLC) (Dionex Ultimate 300, PDA 100) with Rezex ROA organic acid column, 0.005 N H_2SO_4, flow rate 0.3 ml min^{-1}, UV detector at 210 nm, 25 °C, (S)-E3HB retention time: 47.84 min, EAA retention time: 58.56 min). HPLC was also used to determine the concentrations of lactic, acetic and formic acid. The eleven biotransformation runs were performed using the operational conditions given in Table 1. Besides aerobic and anaerobic fed-batch experiments also continuous trials with a perfusion system were carried out under aerobic and anaerobic conditions.

Figures 2(a) and 2(b) illustrate typical courses of aerobic and anaerobic fermentation processes. Prior to the biocatalytic phase yeast was cultivated in aerobic batch or fed-batch mode until approximately 20 to 25 gL^{-1} of dry biomass were achieved. Thereafter the feed of glucose solution was reduced and the biotransformation of EAA to (S)-E3HB was started by feeding EAA solution. In case of the anaerobic biocatalysis aeration was stopped at this point. After starting the biocatalysis by feeding EAA biomass concentrations still slightly increased (Figure 2(a)) under aerobic conditions, whereas growth stops and the biomass concentration decreases under anaerobic conditions (Figure 2(b)), which is partly due to the dilution with media. After starting to feed EAA the (S)-E3HB concentrations increased under aerobic as well as under anaerobic conditions. A maximum (S)-E3HB concentrations of 12 gL^{-1} was achieved under aerobic conditions. Under anaerobic conditions (S)-E3HB concentrations of more than 20 gL^{-1} could be reached (run 9, Table 1).

Our experiments indicate that the feeding strategies of the energy yielding carbon source (glucose) and the substrate EAA for biocatalysis strongly influence the productivity of the process. Sufficient glucose feed is required to ensure energy metabolism within the cells, while overfeeding may lead to product dilution and reduced carbon efficiency due to side product formation (for example ethanol production under aerobic conditions). The EAA feed should ensure EAA concentrations at about 0.5 gL^{-1}. Lower concentrations limit the (S)-E3HB formation, while concentrations higher than 2.0 gL^{-1} may cause substrate inhibition of the cell energy metabolism by EAA, also lowering the (S)-E3HB production (Rohner et al., 1988). This effect can be observed under aerobic as well as anaerobic conditions (Figure 2).

Table 1: (S)-E3HB production under aerobic and anaerobic conditions in fed-batch and continuous mode

run	mode	runtime h	feed rate h^{-1}	yield $g_{E3BH}\ g_{EAA}^{-1}$	final conc. $g_{(S)\text{-}E3HB}\ L^{-1}$	production rate $g_{(S)\text{-}E3HB}\ g_{biomass}^{-1}\ h^{-1}$
1	aerobic fed-batch	52	0.0267	0.64	12	0.0080
2	anaerobic fed-batch	72	0.0267	0.19	2	0.0041
3	aerobic fed-batch	52	0.1000	0.31	7	0.0120
4	aerobic fed-batch	52	0.0200	0.80	4	0.0061
5	aerobic continuous/perfusion	72	0.014 - 0.18 stepwise increasing	0.64	12	0.0060
6	anaerobic continuous/perfusion	72	0.014 – 0.18 stepwise increasing	0.46	5	0.0068
7	Aerobic fed-batch	52	0.01	0.41	6	0.004
8	Anaerobic fed-batch	52	0.008	0.47	3	0.004
9	Anaerobic fed-batch	52	0.028	0.91	21	0.025
10	Aerobic fed-batch	52	0.012	0.87	11	0.01
11	Anaerobic fed-batch	52	0.031	0.50	12	0.013

As shown in Table 1, the final product concentrations and yields depend on the operational conditions used. In the aerobic experiments a specific (S)-E3HB production rate of 4.0×10^{-3} to 2.5×10^{-2} $g_{(S)\text{-}E3HB}\ g_{biomass}^{-1}\ h^{-1}$ was observed, which is well within the range of the reported (S)-E3HB production rate of 2×10^{-3} to 5.4×10^{-2} $g_{(S)\text{-}E3HB}\ g_{biomass}^{-1}\ h^{-1}$ (Sybesma et al., 1998). In contrast to the results published of Sybesma et al. (1998) and and also by Rohner et al. (1988) who did not expect (S)-E3HB to be produced under anaerobic conditions, we found a specific (S)-E3HB-production rate of up to 2.5×10^{-2} $g_{(S)\text{-}E3HB}\ g_{biomass}^{-1}\ h^{-1}$ under anaerobic conditions.

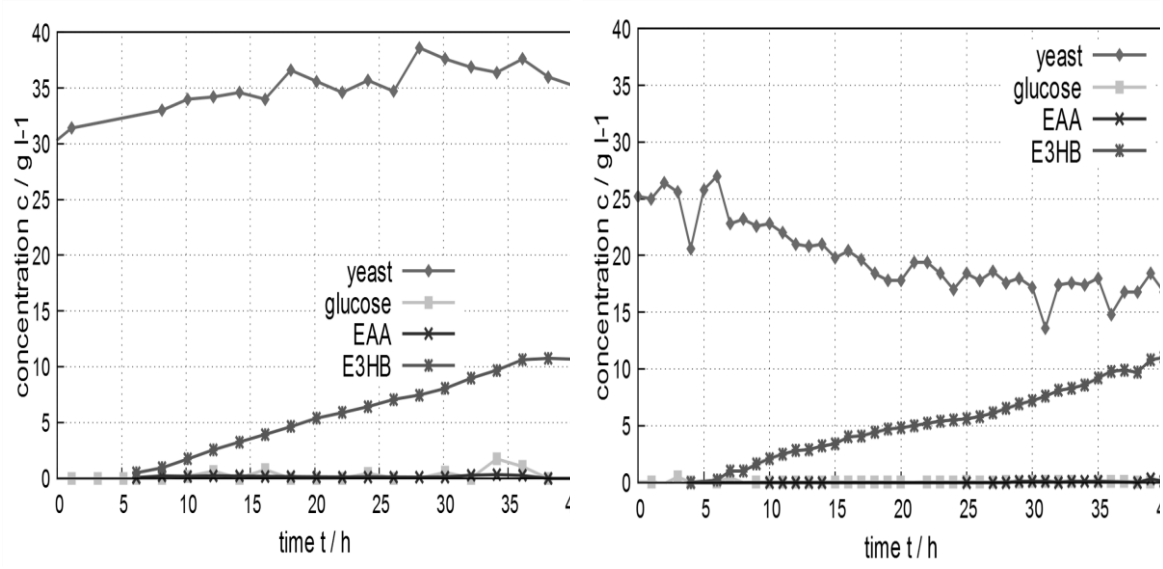

Figure 2: Production of Ethyl (S)-3-hydroxybutyrate in a whole cell biotransformation process. (a) aerobic process (run 1), (b) anaerobic process (run 11) profiles.

From preliminary experiments it was concluded that byproducts may be formed during the process. From our data we calculated elemental carbon balances on a mol basis, in order to check if the C-balances are closed. The balances are based on the assumption that *S. cerevisiae* has an average elemental composition $CH_{1.67}O_{0.5}N_{0.16}$. The balances take into account the concentrations of glucose, ethanol, EAA, yeast dry matter, (S)-E3HB, acetic acid, lactic acid, formic acid and carbon dioxide measured at the beginning and at the end of the process. Figure 3 shows Sankey diagrams resulting from elemental C-balances for the aerobic and the anaerobic processes displayed in Figure 2.

It is shown, that the C-balances could be closed with an error of less than 17 % for both experiments. It also can be seen, that the byproduct spectrum clearly depends on the operational mode. During the aerobic process mainly carbon dioxide (51 %), biomass (30 %) and (S)-E3HB (18 %) are formed (Figure 3(a)). In the anaerobic process the products are mainly ethanol (39 %), carbon dioxide (22 %), (S)-E3HB (12 %) and biomass (7 %) (Figure 3(b)). To a smaller extend lactic acid (4 %), acetic acid (< 1 %) and formic acid (< 1 %) are formed mainly under anaerobic conditions. The formation of organic acids to higher concentrations is an indicator for suboptimal process conditions.

Our experimental results clearly show that the anaerobic biocatalysis of EAA to (S)-E3HB using *S. cerevisiae* is possible. Additionally, the carbon dioxide produced has a higher purity as compared to the aerobic process and may be further utilized in subsequent process steps. Finally, the ethanol produced anaerobically may be separated in subsequent downstream processing, which could further contribute to an increase of the overall carbon efficiency of the process.

These findings support the integration the (S)-E3HB biotransformation into a biorefinery concept, where the anaerobically produced CO_2 is further utilized (Reule et al. 2015) as shown in Figure 4.

The proposed biorefinery concept mainly consists of two key elements. An anaerobic biotransformation unit is used to produce fine chemicals like (S)-E3HB in parallel to platform chemicals like ethanol. The CO_2 produced in this step is of high purity and may then further be utilized for methane production using H_2, produced by electrolysis with excess green electricity. Further studies will be performed in order to increase the process performance of the (S)-E3HB production under anaerobic conditions. In parallel, life cycle assessment methods will be used to evaluate the overall process on the basis of experimentally obtained data (Santibañez-Aguilar et al., 2014).

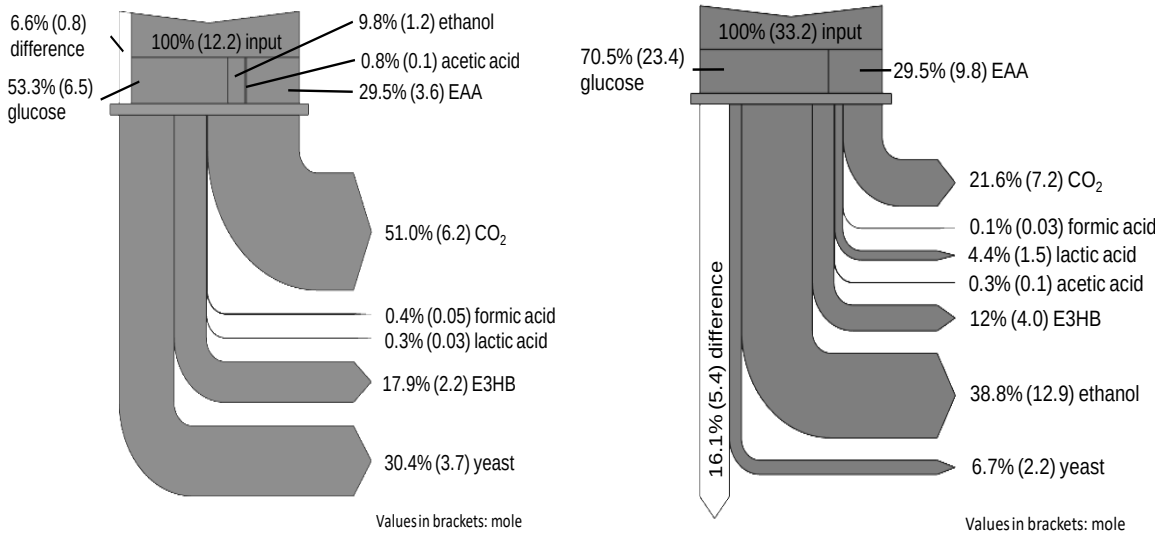

Figure 3: Integral process balances of the (a) aerobic and (b) anaerobic biocatalysis

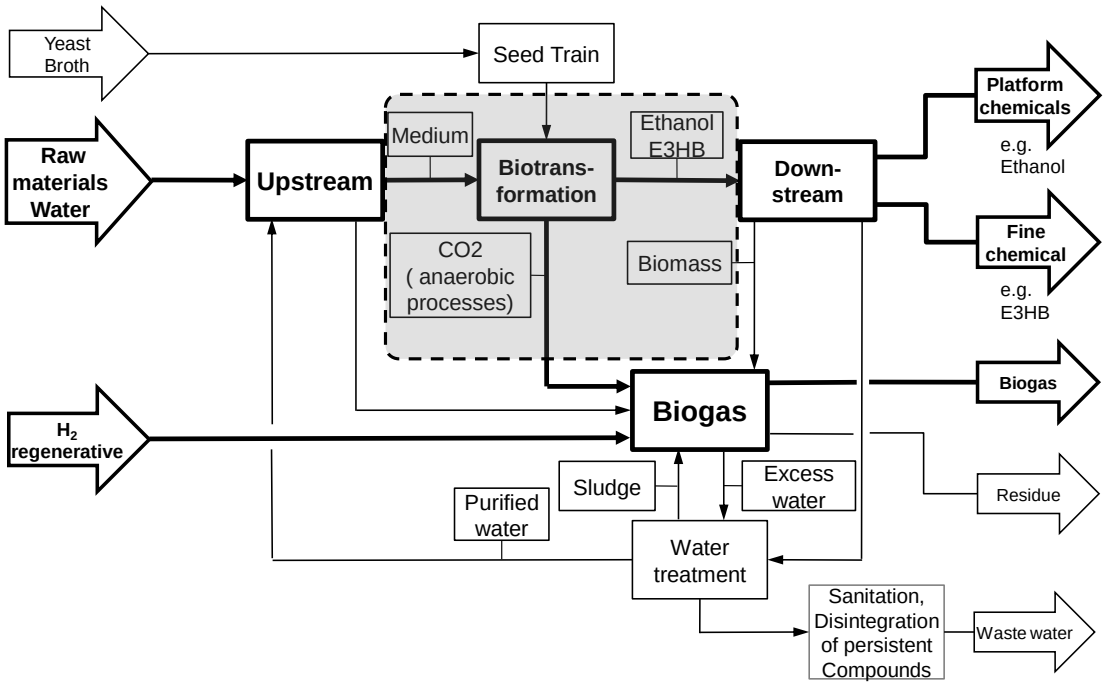

Figure 4: Biorefinery concept (Reule et al. 2015) with integrated (S)-E3HB biocatalysis

3. Conclusions

As revealed, research on the biocatalytical production of (S)-E3HB from EAA focused on the aerobic whole cell biotransformation with *S. cerevisiae* or other yeasts. In this contribution it could be shown experimentally that also anaerobic whole cell biotransformation of (S)-E3HB is possible at production rates of more than 0.01 $g_{(S)\text{-}E3HB}\ g_{biomass}^{-1}\ h^{-1}$, which is in the same order of magnitude as compared to the aerobic process. The byproduct CO_2 produced in the anaerobic process is of high purity and may, thus, be further utilized within a new biorefinery

concept as proposed by Reule et al. (2015). Anaerobically performed whole cell biotransformation processes may be integrated into biorefineries leading to improved overall carbon efficiency and further investigations are made to increase the yield of the anaerobic process. Thereafter a comparison of anaerobic and aerobic production of (S)-E3HB with life cycle analysis will be conducted to get information about the environmental burden of both production methods.

Acknowledgments

We gratefully acknowledge the support given by the Hochschule Furtwangen University and the University College London.

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