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Integrating Whole Cell Biotransformation of Aroma Compounds into a Novel Biorefinery Concept

Roland Hirschmann, Waldemar Reule, Thomas Oppenländer, Frank Baganz and Volker C. Hass

Abstract

The synthesis of aroma compounds that are utilized as precursors of multiple synthesis chains in the pharmaceutical industries and as ingredients in food and fragrance industries can be carried out using chemical processes, enzyme biocatalysis and whole cell biotransformation. Whole cell biotransformation has the potential of being more environmentally benign than chemical synthesis and more cost-effective as compared to enzyme catalysis. In a recently published study by the authors, the aroma compound Ethyl(3)hydroxybutyrate was produced by whole cell biotransformation under aerobic and anaerobic conditions. The yield of the anaerobic processes was similar to that of the aerobic processes, but additionally generated CO₂ and ethanol as useful by-products. In this chapter we illustrate how the production process of Ethyl(3)hydroxybutyrate by whole cell biotransformation can be integrated into a novel biorefinery concept, based on the finding that the production of Ethyl(3)hydroxybutyrate under anaerobic conditions is efficient and environmentally friendly. CO₂ may be converted to bio-methane together with H₂ produced from excess regenerative power. A life cycle assessment confirmed that the anaerobic whole cell biotransformation process embedded into a biorefinery concept including bio-methane production has a lower environmental impact as compared to a concept based on the aerobic whole cell biotransformation.

Keywords: biorefinery, ethyl(3)hydroxybutyrate, *Saccharomyces cerevisiae*, whole cell biotransformation, aerobic, anaerobic

1. Introduction

Production of flavors and aroma compounds is a highly complex process with increasing demand. In their review, Carlquist et al. [1] showed that aroma compounds have a reasonable economic impact with a world market of \$24 billion in 2013. Principally, there are three different ways to produce flavors and aroma compounds, namely, chemical synthesis, cell-free biocatalysis, and whole cell biotransformation. Chemical synthesis usually provides high yields; however, they have the disadvantage of resulting in a racemate rather than the preferred enantiomer and

often other unwanted by-products. Additionally, the use of organic solvents renders chemical synthesis environmentally problematic. In comparison to enzymatic processes, efficiency is decreased, and downstream costs may be increased [2]. The third production route utilizes whole cell biocatalysis as the biotransformation step within the aroma compound production. In this way, the natural aroma production capacity of organisms such as the yeast *Saccharomyces cerevisiae* is used. An increasingly important aspect for aromas and flavors is their labeling as bio-, organic, or natural. This kind of labels may be used for products gained by enzymatic reactions or biotransformations as long as the product occurs in nature, too [3]. In particular whole cell biocatalysis processes offer the chance to be beneficially integrated into biorefineries, as will be illustrated in this chapter.

Ethyl(3)hydroxybutyrate (E3HB) is listed in the *Handbook of Flavors* and is specified as fragrance by the International Fragrance Organization [4]. E3HB is characterized as fresh and fruity with grape odor. It is an intrinsic natural part of kiwi aroma and has the second highest concentration of volatile components in fresh kiwi puree [5]. E3HB is a widely used substance in the food and fragrance industry. The annual consumption of E3HB in 2010 has been stated to be 250 kg [6]. Here, the E3HB production process will serve as an example to illustrate the potential of integrating aroma compound production into biorefineries. The chapter concludes with a section describing the results of a life cycle assessment (LCA) for the proposed production of E3HB within a biorefinery concept, in order to investigate the environmental impact of the process.

2. Production of Ethyl(3)hydroxybutyrate

There are three different strategies for the production of Ethyl(3)hydroxybutyrate: (1) chemical synthesis, (2) synthesis by enzymatic biocatalysis, or (3) by whole cell biotransformation with organisms such as the yeast *Saccharomyces cerevisiae*. These strategies will be described briefly in the subsequent sections.

2.1 Chemical synthesis

The enantiomers of E3HB have identical physicochemical properties. However, enantiomers may exhibit different behaviors, e.g., they may trigger different pharmacological effects or different olfactory cognition. (R-) and (S-) configurations of E3HB are obtained depending on the type of catalyst applied to the reaction. The (R-) configuration can be efficiently depolymerized from polyhydroxybutyrate. Seebach and Züger [7] found four possible methods: a reductive depolymerization with LiAlH_4 , a titanium-catalyzed transesterification, an acid-catalyzed depolymerization, and a hydrolysis [7–9]. A broad range of catalysts for the asymmetric reduction of β -ketoesters are listed by Shang et al. [10] including three catalysts for (R)-E3HB but none for (S)-E3HB.

Chemical synthesis of E3HB may also comprise the catalytic hydrogenation of the substrate ethyl acetoacetate (EAA) to yield the target compound Ethyl(3)hydroxybutyrate (**Figure 1**). Seven catalysts for the reduction of EAA to E3HB were listed by Shang et al. [10], five for the (R-) enantiomer and two for the (S-) form. Asymmetric hydrogenation of ethyl acetoacetate (EAA) can, for example, be performed with BINAP-coordinated Ru(II) complexes as catalysts (**Figure 1**) as published by Noyori and coworkers [11, 12]. Jeulin et al. described further modification and optimization of ruthenium(II) complexes with chiral diphosphanes. With these modified catalysts, β -ketoesters have been reduced with an enantiomeric excess close to 100% [13].

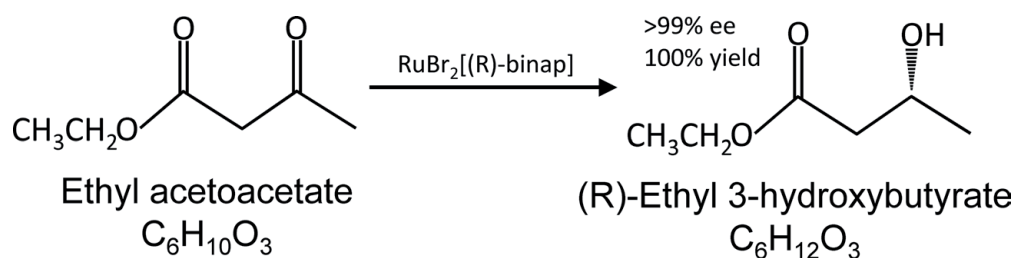


Figure 1.

Ru-BINAP-catalyzed chemical synthesis of (R-)E3HB, according to Kitamura (20–32°C, ethanol, 86 bar, 51 hours) [14].

The reaction temperatures for catalyzed chemical synthesis range from 20 to 70°C, at pressures from 5 to 100 bar. The enantiomeric excess achieved was greater than 97% with a maximum of 99.8%. The reaction solvents used are methanol, ethanol, or toluene. Thus, chemical synthesis necessitates special equipment and harsh conditions to achieve high yields (99%) and enantiomeric excess (99% ee).

2.2 Enzymatic biocatalysis

The advantages of enzymatic biocatalysis are mild reaction conditions like atmospheric pressure and ambient temperature. Often enzymatic reactions are stereoselective. Therefore, problems of isomerization, racemization, epimerization, and rearrangement of molecules are minimized compared to chemical reactions [15]. Enzymes are often used for transforming racemates derived from chemical reactions into one single stereoisomer [16]. Frequently, the cofactors nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) are required. For the enzymatic reduction of EAA to E3HB, the regeneration of the cofactor is necessary (**Figure 2**) because its supply in a stoichiometric amount would be too expensive. The two main methods for cofactor regeneration are the substrate-coupled and the enzyme-coupled regeneration [17]. In principle, regeneration can also be carried out photochemically, electrochemically, or chemically using a catalyst which is regenerating the enzyme [18].

Huisman et al. [19] described that ketoreductases (KRED) may be used for substrate-coupled regeneration of NADH as well as of NADPH. KREDs can be adapted by enzyme engineering technology and reactor engineering to specific processes and are suitable for large-scale operations from economic and environmental aspects. Moore and his group [20] applied KRED 101 and KRED 107 for EAA conversion to E3HB with excellent yields and high enantiomeric excess of (R-)

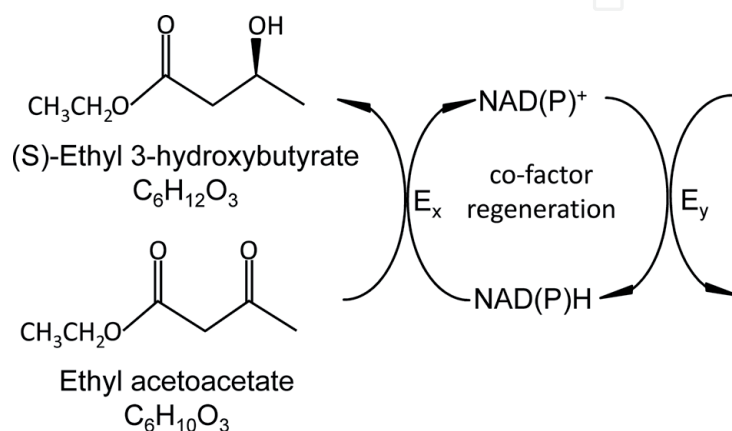


Figure 2.

Enzymatic biocatalysis with cofactor regeneration, according to [17].

E3HB. Kaluzna et al. [21] used KRED 107 for cofactor regeneration during (R-) E3HB production and achieved an ee of >99%. The same group also applied KRED 116–119 supporting the (S-)enantiomer formation and again achieved an ee > 99%.

Besides KREDs, NADPH-dependent alcohol dehydrogenases may be used. An alcohol dehydrogenase from *Kluyveromyces polyspora* DSM 70294 (KpADH) was investigated by Müller [22] achieving an ee > 99.1% of the (S-)enantiomer. Enzymatic reactions mostly are carried out in aqueous solutions.

The enzymatic biocatalysis of EAA to E3HB is performed at ambient temperatures and pressures. The enantiomeric excess achieved was greater than 99.8% with yields of up to 98%. The reaction solvent is an aqueous solution. However, this enzymatic biocatalysis requires cofactor regeneration.

2.3 Whole cell biotransformation

Already in 1931 Friedmann [23] reported the conversion of acetoacetate by yeasts. His work was confirmed by Lemieux and Giguere [24]. In the 1950s Deol et al. [25] improved the process by focusing on clearly defined reaction conditions to obtain a maximum yield. For the reduction of EAA to (S-)E3HB, they achieved a yield of 80%. Also Seuring and Seebach [26] published a detailed laboratory method for the biotransformation of EAA to E3HB with baker's yeast in the late 1970s. In addition to yeasts, also other organisms can be used for whole cell biocatalysis of E3HB. For example, Seebach et al. [9] and Simon et al. [27] reported a method for the production of (S)-E3HB using the bacterium *Clostridium kluyveri* with an enantiomeric excess of 95% [9, 27]. Leuenberger [28] describes whole cell E3HB biotransformations with *Geotrichum candidum* and baker's yeast.

According to Sybesma [29] and his team, *S. cerevisiae* is transforming EAA into (S)-E3HB catalyzed by a carbonyl reductase with NADPH as cofactor (Figure 3). The regeneration of NADP+ is performed via a dehydrogenase with H+ from glycolysis. It has been reported that besides glucose also ethanol may be used as energy source under aerobic conditions [18].

Perles [30] performed an in-depth metabolic study of *S. cerevisiae* under aerobic and anaerobic conditions investigating the E3HB biotransformation including heat and ethanol production and oxygen and glucose consumption in order to find out which compartment of the cell the bioreduction takes place. Under aerobic conditions, the mitochondrial matrix is the location where NADH is regenerated by the

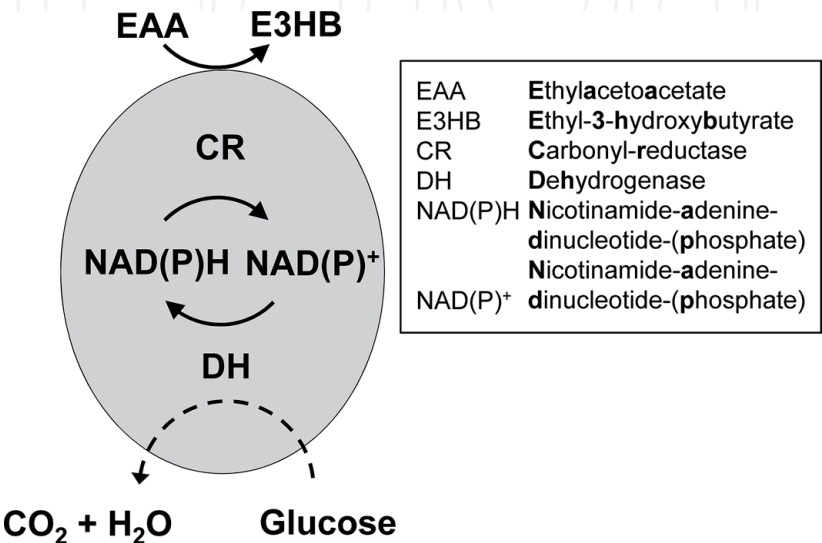


Figure 3. Whole cell biotransformation with regeneration of the cofactor (NAD(P)) under aerobic conditions.

respiratory pathway. Under anaerobic conditions, the reaction is carried out in the cytosol, and NADH is regenerated using the pentose phosphate pathway.

Although some authors just consider the aerobic whole cell transformation, it should be stated that the process can be run either aerobically or anaerobically. In most cases, it is conducted aerobically using fed-batch operation in order to avoid by-product formation and substrate inhibition [18, 29, 31, 32].

2.4 Comparison of the reaction routes

A comparison of all three routes (chemical, enzymatic biocatalysis, and whole cell biotransformation) was published by Borup et al. [33]. Other works compare the chemical synthesis of E3HB with Ru-BINAP to the whole cell biotransformation with baker's yeast [10, 34] or the enzymatic biocatalysis with the chemical synthesis [20]. The most important results of these studies, together with the results of the authors of this contribution, are shown in **Table 1**. In summary, it can be concluded that enzymatic biocatalysis results in the highest product quality

Parameters	Chemical	Biocatalytic	Whole-cell biocatalysis aerobic	Whole-cell biocatalysis anaerobic
Yield	90-95%	98%	<90%	<90%
Space-time Yield	~16 kg m ⁻³ h ⁻¹	~3 kg m ⁻³ h ⁻¹	~1 kg m ⁻³ h ⁻¹	~1 kg m ⁻³ h ⁻¹
Raw materials	EAA Very pure (amine free) Catalyst	EAA Isopropanol Water ~2 kg/kg product NAD or NADP	EAA <i>S. cerevisiae</i> Glucose Water ~100 kg/kg product	EAA <i>S. cerevisiae</i> Glucose Water ~100 kg/kg product
Unit operations	Purification of substrate Chem. reaction Distillation	Enzyme catalyzed reaction Extraction Distillation	Whole cell biocatalysis Extraction Distillation	Whole cell biocatalysis Extraction Distillation H ₂ generation Bio-Methane production
Environment	Organic waste (methanol - toxic) Hydrogen handling Temperature ~100°C Pressure ~5 bars	Ambient conditions	Ambient conditions CO ₂ generation	Ambient conditions
Quality	95-99 % ee	~99.9 % ee	85-98% ee	85-98% ee

Split of whole cell biotransformation into aerobic and anaerobic process.

Table 1.
Evaluation of the E3HB reaction routes, according to [33] and studies by the authors.

and yields, followed by the chemical process. Whole cell biotransformation seems to be less efficient. The volumetric product formation rates reported are $16 \text{ g L}^{-1} \text{ h}^{-1}$ for the chemical synthesis, $3 \text{ g L}^{-1} \text{ h}^{-1}$ for the enzymatic biocatalysis, and $1 \text{ g L}^{-1} \text{ h}^{-1}$ for whole cell biotransformation. The major disadvantages of the whole cell biocatalysis are its low productivity, the low product quality, and the waste generation, which is reported to be 100 L aqueous waste per kg product, compared to 2 L for the enzymatic biocatalysis. The chemical process generates 0.1 L organic waste (methanol and other compounds) per kg product. Nevertheless, whole cell biotransformations have a number of advantages in comparison to cell-free enzymatic biocatalysis and chemical catalytic synthesis. The whole cell biotransformation leads to an enantiomeric excess, which is comparable to the enzymatic biocatalysis and higher as achieved with chemical synthesis [2]. Furthermore, the regeneration of cofactors within the cells enhances the efficiency of the reaction; the cellular environment stabilizes the enzymes. This, together with the non-necessity of enzyme purification, makes whole cell biotransformation economically advantageous as compared to the other two processes [2].

3. Biorefinery concept with E3HB production

A brief but telling definition of biorefineries is given by the International Energy Agency (IEA): “Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy” [35]. Recently, many different kinds of biorefineries were described [36, 37]. Hong and Nielsen [38] gave an overview of biorefinery concepts that could be realized with *S. cerevisiae* in a key transformation process. In the next sections, we will point out the potential of integrating the whole cell biotransformation of EAA to E3HB with *S. cerevisiae* into a biorefinery.

3.1 Potential concepts (aerobic/anaerobic)

The transformation step in the whole cell biotransformation can either be conducted aerobically or anaerobically, as stated earlier in this chapter. The two processes yield a different spectrum of products and, thus, require different biorefinery designs. **Figure 4** illustrates the design of a biorefinery with the aerobic

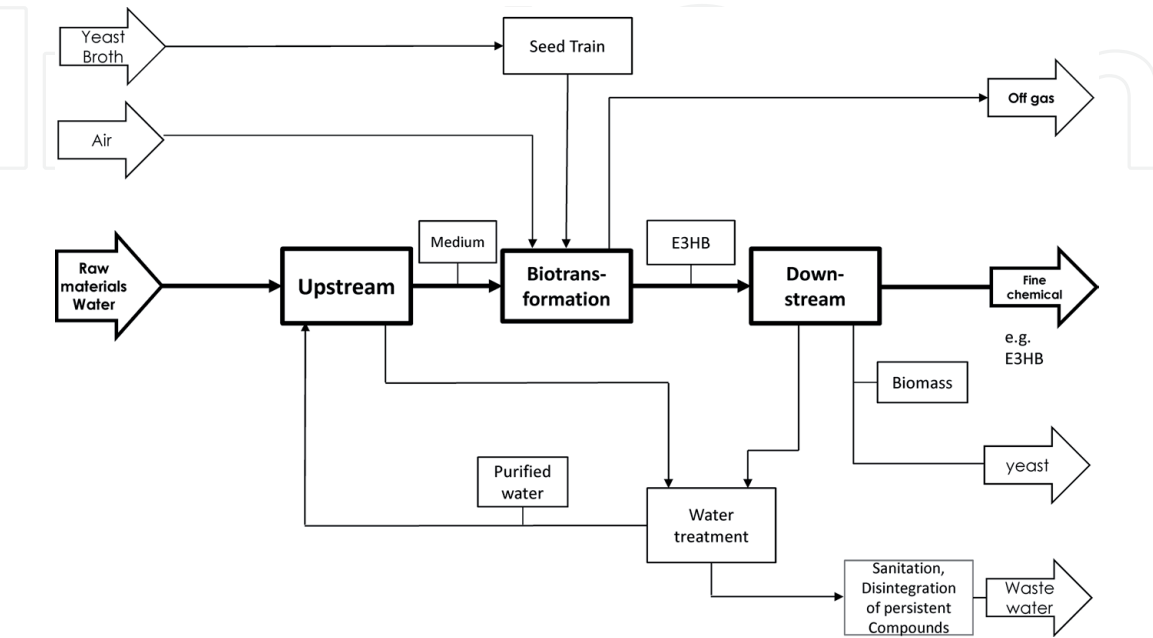


Figure 4. Biorefinery concept with aerobic biotransformation to form E3HB.

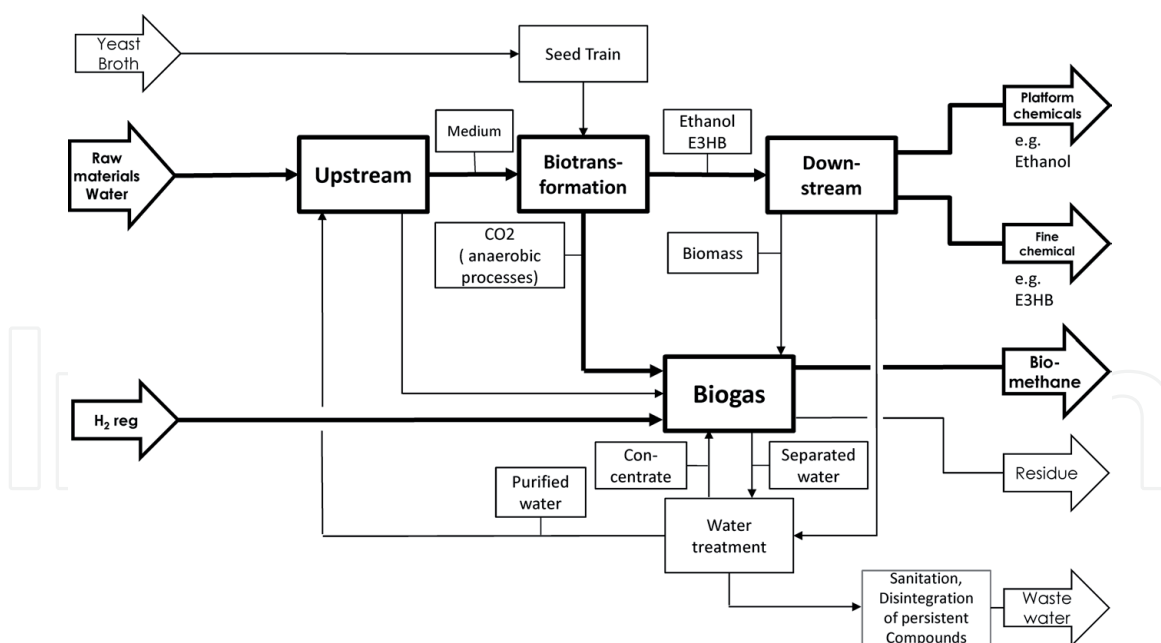


Figure 5.
 Biorefinery concept with the anaerobic biotransformation to form E3HB, combined with a biogas plant [39].

biotransformation of EAA to the target aroma compound E3HB. Raw materials such as starch sources are converted to glucose and used as carbon source in the cultivation medium during upstream processing. The yeast starter culture is produced in a seed train process. Yeast starter culture and cultivation medium are then fed to the main cultivation and biotransformation step. During downstream processing the culture broth is separated into the main products E3HB and yeast biomass plus residuals. CO₂ is released as side product in the off-gas from the biotransformation. Wastewater from downstream processing may be partly recycled and fed back to the medium preparation. The main advantage of the process is the comparably high E3HB productivity during the aerobic biotransformation that will reduce the process cost.

A modified biorefinery concept integrating the anaerobic biotransformation to form E3HB has been patented and published by the authors [39, 40]. This biorefinery concept is shown in **Figure 5**. In principle, it is similar to the concept shown in **Figure 4**. However, during the anaerobic biotransformation, ethanol is formed as an additional product, which can be recovered during downstream processing. Furthermore, the high concentration of CO₂ in the off-gas allows for its subsequent utilization in a newly introduced biogas process to form bio-methane. Excess yeast is also fed into the biogas process. Hydrogen produced with excess regenerative power from wind power plants is fed to the biogas process, in order to transform CO₂ to methane. The wastewater from the downstream processing as well as from the biogas production is recycled and reused in the upstream processing. The main benefit of this new concept is the simultaneous production of the three products E3HB, ethanol, and bio-methane.

In Section 4 of this chapter, we will show the results of a life cycle assessment of the two competing biorefinery concepts illustrated in **Figures 4** and **5**. In subsequent Section 3.2, we will briefly outline the process operation strategy of the anaerobic biotransformation that has been derived from the aerobic process.

3.2 Development of an anaerobic process

So far no industrial process for the whole cell biotransformation of E3HB has been reported. However, there is a proposal for an aerobic process as shown in **Figure 4**. In the work by Hirschmann et al. [41], the anaerobic process is

investigated in more detail, because it was supposed to be beneficial to simultaneously produce E3HB and the by-products carbon dioxide and ethanol.

The reduction of aliphatic β -keto esters with baker's yeast was extensively reviewed by Csuk and Glaenger [42]. Kometani et al. [31] investigated the influence of various by-substrates on the reaction rates. In their experiments the reduction did not proceed under anaerobic conditions. Some investigations showed that stereoselectivity may be influenced by feeding strategies and oxygen limitation. Best results for the production of (S)-E3HB with an enantiomeric excess (ee) of 99% were obtained under aeration and slowly added gluconolactone within a fed-batch process [43]. In 2002 Seebach et al. [44] published a lab scale process to produce (S)-E3HB from EAA with *S. cerevisiae* with an optical purity of 93%. Engelking [45] published a very detailed report about the reaction technology for the asymmetric reduction of β -ketoesters with recombinant yeasts and evaluated the influence of reaction conditions like pH, nutrients, and temperature. The work was carried out using wild-type strains of *S. cerevisiae* and *Pichia pastoris* as well as the recombinant strains *S. cerevisiae* FasB His6 and *P. pastoris* GC909. Also Matsuda et al. [46] gave a broad overview of reaction mechanisms and process conditions for reductive whole cell biotransformations with many organisms and substances.

3.2.1 Selection of process conditions and operational strategy

In order to carry out the biotransformation to form E3HB, EAA is needed as a substrate being converted to E3HB. Additionally, a carbon and energy source is needed to meet the cells' energy demand and enables cofactor regeneration.

It has been shown previously that EAA has an inhibitory or toxic impact on *S. cerevisiae*. Wipf et al. [32] found a limit of 0.5 g L^{-1} for inhibition of the E3HB biotransformation and 15 g L^{-1} to be toxic for the yeast. Ebert et al. [47] reported that high substrate concentrations (EAA) may have an effect on yield and ee. These results have to be considered with caution. They performed small-scale experiments (1000 mL bottom flask, 200 mL liquid) with baker's yeast suspended in distilled water and added EAA in concentrations of 4 and 8.5 g L^{-1} at the beginning of their experiments. The obtained ee for 4 g L^{-1} was $>98\%$ and for 8.5 g L^{-1} 95% .

Within a range between 0 and 15 g L^{-1} , glucose seems to have no effect on the biotransformation [48, 49]. In addition, due to the Crabtree effect, ethanol might be formed at glucose concentrations above 0.1 g L^{-1} , even if the process is operated aerobically.

Katz et al. [49] found that ethanol concentrations above $19\text{--}30 \text{ g L}^{-1}$ lead to strong inhibition of the reduction of EAA to E3HB. They assumed that ethanol leads to intracellular accumulation of acetaldehyde, which then inhibits the biotransformation. Ethanol may also influence membrane functions.

Hence, fed-batch is the most preferred operating concept. With this strategy up to 40 g L^{-1} E3HB could be produced [32] in aerobic operation. Both glucose and ethanol can be used as carbon and energy source under aerobic conditions for maintenance purposes during the biotransformation. Katz et al. [49] found that ethanol consumption is 10 times less than glucose while yielding the same amount of E3HB. Typical feeding rates are $0.05 \text{ mol glucose kg}_{\text{yeast}}^{-1} \text{ h}^{-1}$ and $0.15 \text{ mol ethanol kg}_{\text{yeast}}^{-1} \text{ h}^{-1}$. The ethanol concentration has to be kept below 19 g L^{-1} under aerobic conditions. Higher feeding rates of the carbon source cause an increase in production of biomass and a lower ee value. Alternatively to continuous glucose feeding, it has been suggested to use raffinose as an energy yielding co-substrate [49]. This strategy avoids ethanol formation due to the Crabtree effect, occurring at glucose concentrations above $0.1\text{--}0.5 \text{ g L}^{-1}$.

An important factor, influencing the productivity of the whole cell biotransformation is the balancing of the cofactor regeneration and the biotransformation of

the ester. Katz et al. [49] optimized the reduction of a bicyclic diketone by balancing the reactions of reduction and cofactor recycling, using a genetically engineered strain. The best yield was 250 mg ketone per gram co-substrate (glucose) compared to 25 mg ketone per gram glucose for the lowest yield.

The pH may be adjusted to the normal range for standard *S. cerevisiae* cultivations. Dahl et al. [50] could shift the ee from 68.7% (S-) enantiomer to 87.3% (R-) enantiomer by changing pH from 5 to 8 and the addition of allyl alcohol. Engelking observed an optimum for ethyl (S)-4-chloro-3-hydroxybutanoate of 94% ee at pH 8 but strongly decreased yield [45]. From their findings, it can be concluded that for practical reasons a pH around 5 is favorable due to low inhibition effects, low foam formation, and a certain barrier for other organisms to grow.

S. cerevisiae is frequently cultivated at temperatures around 30°C. A temperature increase during biotransformation seems to favor ee and yield. In experiments performed by Hirschmann et al. [41], the maximum temperature was 34°C. However, the temperature should be kept lower than 35°C in order to avoid cell inactivation [47]. The yield of the process may also be influenced by various other factors. Under aerated conditions the evaporation of the educt EAA with 6% of the fed quantity may not be neglected. If there is an ethanol formation due to high glucose concentrations (>0.1%), the evaporation of ethanol can be 25% [51]. Therefore, a good off-gas cooler is required to minimize this effect. Another factor that might decrease yields is the absorption of E3HB by the yeast cells. Highly concentrated yeast (50 g L⁻¹ yeast dry matter) may absorb up to 20–30%. Furthermore, hydrolysis of EAA may not be negligible [51].

In a recently published study by Hirschmann et al. [41], the anaerobic biotransformation of EAA to E3HB could be demonstrated and was directly compared to the aerobic biotransformation. Their processes were performed in 0.2–15 L stirred tank bioreactors in fed-batch mode using *S. cerevisiae* as organism for the biotransformation of EAA to E3HB (**Figure 6**).

All experiments comprised an aerobic yeast cell propagation phase of approx. 25 hours (phase I) which was followed by whole cell biotransformation (approx. 55 hours). Whole cell biotransformation was performed either aerobically or anaerobically. In **Figures 7** and **8**, two typical time courses of the process, one aerobic and one anaerobic, are shown. Subsequently, the phases of the process will be described to some detail.

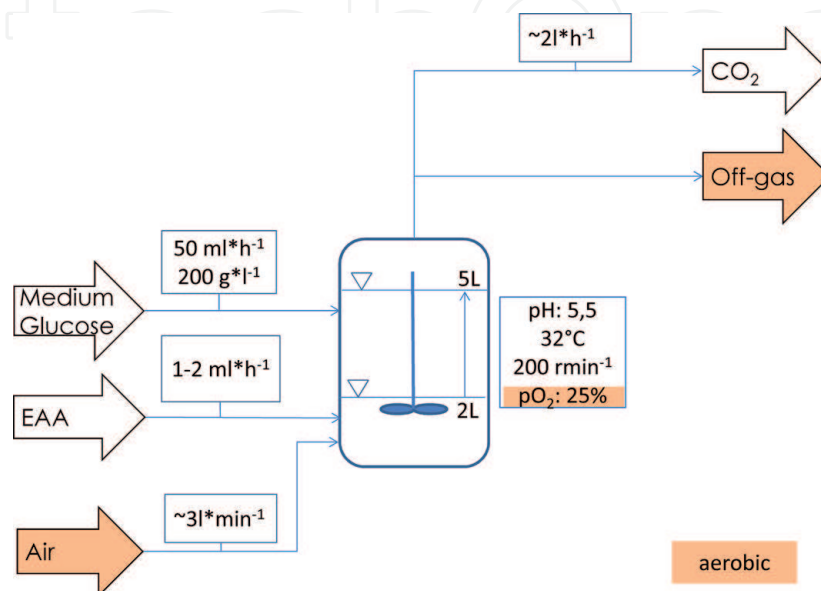


Figure 6.
 Aerobic and anaerobic fed-batch conditions.

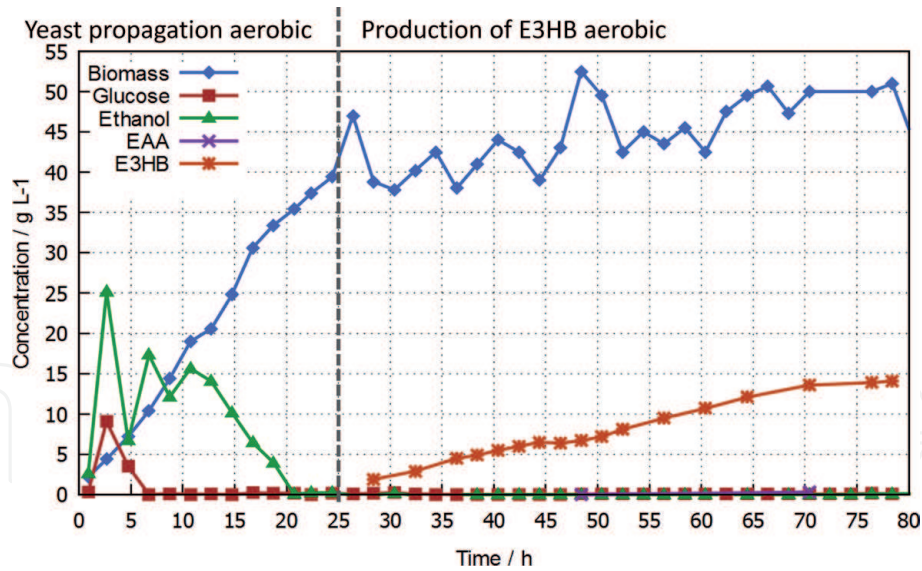


Figure 7.
Time course of fed-batch culture with aerobic biotransformation.

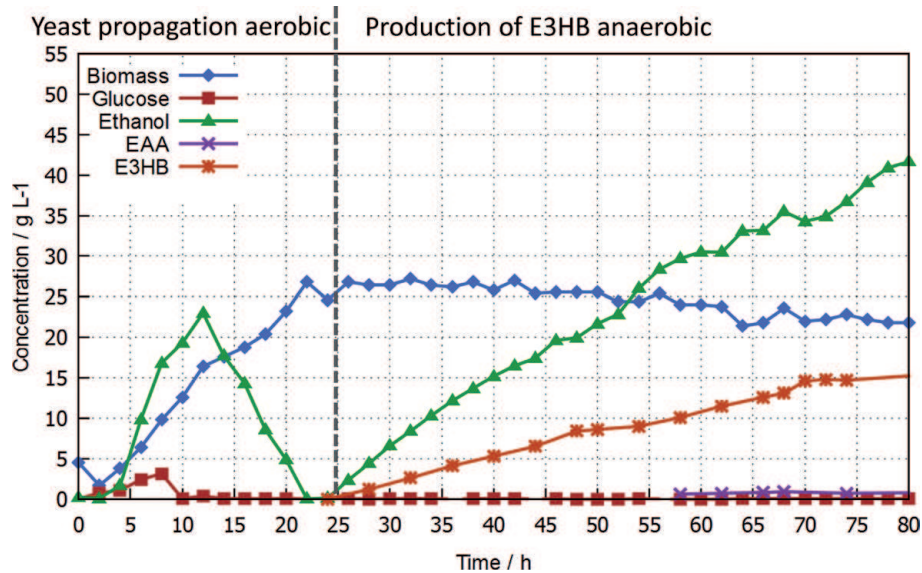


Figure 8.
Time course of fed-batch culture with anaerobic biotransformation.

3.2.2 Phase I: aerobic propagation of the yeast cells

In order to provide sufficient biomass for the whole cell biotransformation, it is necessary to start with the propagation of *S. cerevisiae* (WB 06 Fermentis). A fed-batch process is suitable for this purpose. The aim was to achieve a *S. cerevisiae* concentration of at least 25 g L^{-1} . The starting medium was water with 12 g L^{-1} yeast extract, peptones, and ammonium sulfate as well as 6 g L^{-1} potassium dihydrogen phosphates. After addition of the biomass, a fed-batch cultivation was carried out with a feed medium containing 300 g L^{-1} glucose in addition to the substances contained in the starting medium. The propagation lasted 25 hours with an aeration rate of 2 L min^{-1} . The pO_2 value was kept at approx. 25%. The stirrer speed was used as the control variable for pO_2 . The feed was manually adjusted to keep the respiratory quotient (RQ) at values of approx. 1.1. The mole fractions of O_2 and CO_2 in the exhaust gas were measured. From these values the RQ could be calculated using also the aeration rate. The medium temperature was controlled at 30°C . The pH value was adjusted and controlled to 5 by addition of 10% sodium hydroxide and 10% phosphoric acid

solutions. The ethanol and glucose concentrations were determined with an enzyme kit photometrically. A gravimetric method with prior filtration (0.2 μm) was used to determine the biomass content. For both, the subsequent aerobic and the anaerobic whole cell biotransformation, the same yeast cell propagation process was used.

3.2.3 Phase II: aerobic and anaerobic biotransformation

For both, the aerobic and the anaerobic biotransformation, the same carbon source feed medium was used for the yeast propagation. The feeding strategy for the carbon source was 0.8 g glucose per g yeast and hour. EAA was dosed at a ratio of 0.15 w/w to the added glucose. The duration of the biotransformation was about 55 hours.

In the aerobic case, the mole fractions of O_2 and CO_2 in the off-gas were measured, while in the anaerobic case, the resulting volumetric flow of the CO_2 off-gas was measured.

EAA and E3HB concentrations were determined off-line by HPLC.

A resulting course of aerobic yeast propagation and a subsequent aerobic biotransformation process are shown in **Figure 7**. The concentration of biomass could be increased from approx. 2 g L^{-1} to approx. 40 g L^{-1} during propagation. At the beginning of the yeast propagation phase, overdosing of the glucose solution with associated ethanol formation (25 g L^{-1} and an increase of the glucose concentration to approx. 9 g L^{-1}) may occur. By reducing the feed rate, the glucose concentration could be reduced to <0.1 g L^{-1} after 7 hours and the ethanol concentration to <0.5 g L^{-1} after 20 hours. During the dosing of EAA from 25 hours, there was no accumulation of EAA in the culture solution (concentration of EAA < 0.2 g L^{-1}). The E3HB concentration increased continuously to approx. 14.8 g L^{-1} . The yeast concentration also increased gradually to approx. 50 g L^{-1} .

A resulting time course of aerobic yeast propagation with subsequent anaerobic biotransformation is shown in **Figure 8**. The concentration of biomass increased from approx. 2 g L^{-1} to approx. 26 g L^{-1} during propagation. At the beginning of the process, overdosing the glucose solution was associated with ethanol formation (23 g L^{-1} and an increase of the glucose concentration to approx. 3 g L^{-1}). By reducing the glucose feed, the glucose concentration was reduced to <0.1 g L^{-1} after 10 hours and the ethanol concentration to <0.5 g L^{-1} after 22 hours. During the dosage of EAA from 25 hours, there was a slight accumulation of EAA in the culture solution (concentration of EAA max. 1.0 g L^{-1}). The EAA concentration increased continuously to approx. 15.0 g L^{-1} , while the yeast concentration decreased to approx. 22 g L^{-1} . Due to the increase in volume in the reactor, however, an increase in the total amount of biomass during anaerobic biotransformation from approx. 72 to 110 g could be observed. The ethanol concentration increased to 41.6 g L^{-1} during the biotransformation phase.

To conclude this section, some key process parameters and results have been summarized in **Table 2**. A constant cultivation temperature of 32°C and a pH of 5.5 were used in the aerobic as well as the anaerobic biotransformation process. The aeration with 3 L min^{-1} during the aerobic process ensured aerobic conditions in the medium. After a start volume at the beginning of the propagation (2.6 L), the medium was fed for the propagation as well as for the biotransformation (3.1 L aerobic, 2.7 L anaerobic). During the biotransformation EAA was added in parallel (88.2 g aerobic, 87.0 g anaerobic). At the end of the processes (at 80 hours process time), the substrate glucose was almost consumed. Small residual concentrations of EAA were measured. During the aerobic biotransformation, 15.0 g L^{-1} of E3HB could be produced. In the anaerobic process, 14.8 g L^{-1} E3HB and 41.6 g L^{-1} ethanol were produced. The yield $Y_{\text{E3HB/EAA}}$ for the anaerobic process was 0.78 as compared to 0.92 for the aerobic process. These results show that the biotransformation may be conducted aerobically as well as anaerobically, although up to now it was usually conducted aerobically.

		Aerobe	Anaerobe
Temperature	°C	32.0	32.0
pH	-	5.5	5.5
Aeration	L min ⁻¹	3.0	0.0
Start volume propagation	L	2.6	2.6
Start volume biotransformation	L	4.1	3.5
End volume	L	5.4	4.6
Feed glucose medium	L	3.1	2.7
Feed glucose	g	921.0	816.0
Feed EAA	g	88.2	87.0
Glucose 80 h	g L ⁻¹	0.0	0.0
EAA 80 h	g L ⁻¹	0.2	0.4
Ethanol 80 h	g L ⁻¹	0.1	46.1
E3HB 80 h	g L ⁻¹	15.0	14.8
EAA 80 h	g	1.1	1.8
Ethanol 80 h	g	0.7	212.5
E3HB 80 h	g	81.0	68.2
Y _{E3HB/EAA}	-	0.92	0.78
Y _{E3HB/Glucose}	-	0.088	0.084
Y _{Ethanol/Glucose}	-	0.001	0.260

Table 2.
Comparison of the aerobic and the anaerobic biotransformation.

4. Life cycle assessment

In order to assess and compare the environmental impacts of biorefinery concepts, life cycle assessments (LCA) are appropriate tools. According to ISO 14.040 [52], the framework conditions for the LCA have to be defined at the beginning of the life cycle assessment process. Usually, a product-specific life cycle assessment comprises the entire life cycle of a product from “cradle to grave” and gives an estimate of its environmental impact. A full “cradle to grave” analysis would comprise the raw material production, the production of aroma and other products, their usage, as well as the disposal of all end products. However, in the case of the aerobic/anaerobic E3HB biotransformation, the final usage and disposal of the products were not known. Therefore, in this case a “cradle to gate” analysis was carried out by Hirschmann et al. [41], ending at the gate of the production site. The method interpreting the results of the LCA was a comparative analysis, which is systematically comparing production alternatives (aerobic and anaerobic). This method is used to find the environmentally better process for a product. Absolute values of the results are less important, and uncertainties of used data from databases are less critical as many of these data are applied in both processes [53].

4.1 LCA model

For the biorefinery concepts embedding the aforementioned biotransformation processes, the LCAs were conducted using the software Umberto NXT LCA from ifu, Hamburg. This software facilitates graphical modeling of all energy and material flows and offers access to a variable inventory of databases. It comprises performance analysis and evaluation modules. The evaluation results can be visualized and transferred to other evaluation programs [54]. The Umberto NXT LCA software uses the LCA databases GaBi and ecoinvent (version 3.3) [53, 55], which offer a high number of data sets of processes and materials. Local data records from Europe and

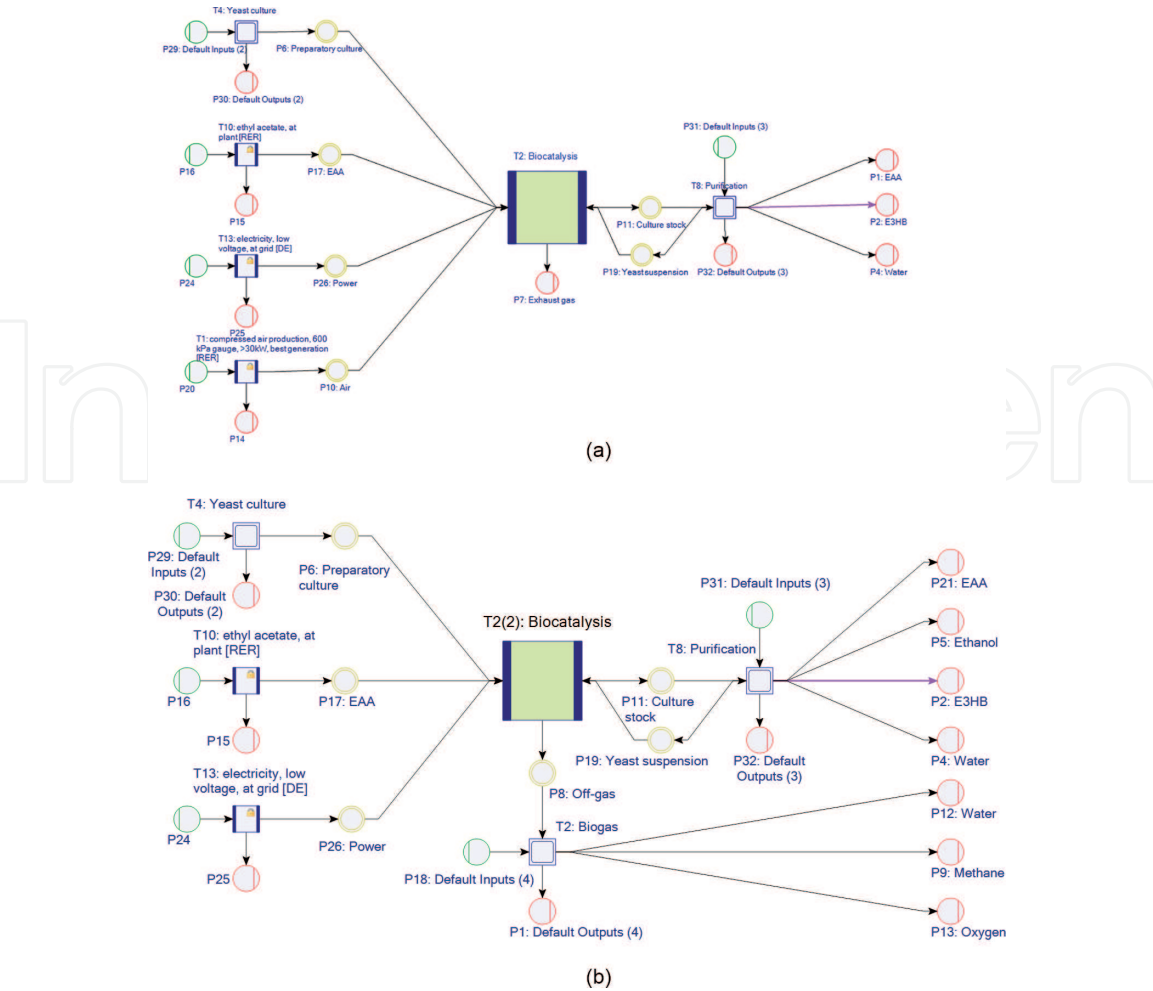


Figure 9. LCA models of the aerobic (a) and anaerobic (b) production of E3HB modeled with Umberto NXT LCA software (basic model).

European processes and infrastructure were used as a basis for the LCAs performed (exception: potassium carbonate data were only available globally in the database).

Figure 9 shows (a) the aerobic and (b) the anaerobic graphical LCA model, developed by the authors. Rectangles symbolize processes, whereby circles with arrows pointing to processes represent inputs and circles which are pointed at from process rectangles represent outputs. Double-edged circles serve as connecting points between processes. For a better overview, some processes are grouped into subnets, which are represented by double-edged rectangles. A lock in the rectangles indicates the usage of predefined process frames from the database ecoinvent. The functional unit of the model is 1 kg E3HB (indicated by the purple arrow).

As shown in **Figure 10a** and **b**, the biotransformation (biocatalysis) process formed the center of the LCA models. Pre-cultivation of the yeast, represented by a subnet, delivered its main product to the biotransformation. The performance of the biotransformation process required substrate EAA and energy as well as oxygen for the aerobic process. Main outputs from the aerobic and the anaerobic biotransformation including purification were E3HB, water, and exhaust gas. The anaerobic process also yielded ethanol and methane. As all materials may have an impact on the LCA, they are outputs in the LCA model.

Figure 9a shows the model for the aerobic process. The focus was on whole cell biotransformation (T2, biocatalysis). Biotransformation requires the production of sufficient biomass (*S. cerevisiae*). This process was described using the subnet yeast culture (T4) representing the aerobic propagation of yeast as shown in the experimental results previously. The subnet, which is not shown here in detail, contained all necessary steps

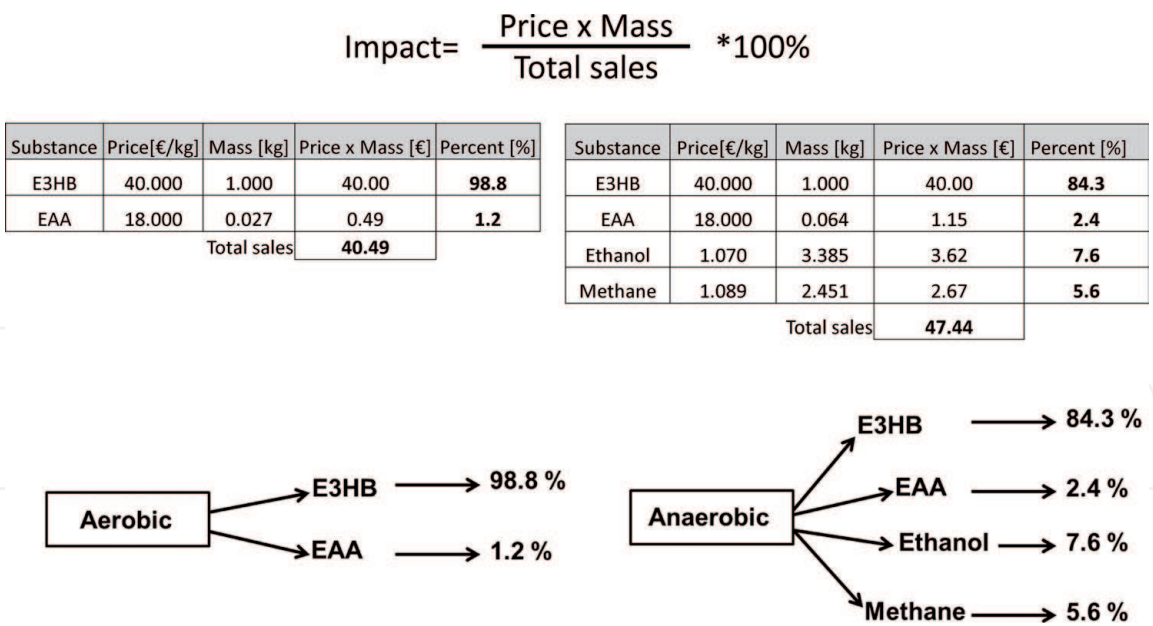


Figure 10.
Economic allocation for the biorefinery processes with aerobic (left) and anaerobic (right) biotransformation.

for the production of yeast, for example, the production of the culture medium, the sterilization of the medium, and of course all raw materials used and energy required. In addition to the formed yeast, the inputs EAA (T10), electrical energy (T13), and compressed air (T1) for aeration are also shown. Biotransformation (T2, biocatalysis) was followed by processing the culture broth in the LCA models. The data for this downstream part of the process were estimated from theoretical process design. The downstream process, which is also not shown in detail, comprised the centrifugation of the yeast cells, from which some of the yeast containing sludge was recycled to biocatalysis (P11).

The culture broth separated from the yeast was then assumed to enter an extraction process. In this way E3HB may be extracted from the culture broth using Methyl-tert-butyl-ether (MTBE). The extraction step was followed by purification in a rectification process, from which the residual MTBE was recycled to the extraction process. Here too, electrical energy was accounted for, to run the unit operations. EAA (P1), E3HB (P2), and water (P4) as well as exhaust gas (P7) were seen as output from the overall process. EAA as output represents the remaining EAA from biotransformation. All other inputs and outputs have been taken from the data sets used in the ecoinvent and GaBi databases.

Figure 9b shows the anaerobic process. Compressed air for the gassing as input to the biotransformation step was not required. The subnet purification (T8) additionally contained a second rectification unit between centrifugation and extraction for the separation of the ethanol produced here. Otherwise, the process model was identical to the one for the aerobic process. An important modification in the anaerobic process model as compared to the aerobic model was the additional subnet biogas (T2). The CO₂ produced during biotransformation was assumed to be fed into a biogas process to which also hydrogen was fed simultaneously, according to Reule [40]. The hydrogen was assumed to be obtained from an electrolysis process for which regenerative electricity could be used, as also suggested by Reule [40]. The gained bio-methane may be fed into the natural gas grid. This could be a contribution to the power to gas concept and at the same time an opportunity to improve the efficiency of biogas plants. Eventually, in the LCA model describing the biorefinery concept embedding the anaerobic biotransformation, ethanol (P5) and methane (P9) become additional outputs. Oxygen (P13) from electrolysis was not evaluated

because it was not considered as a marketable product or to have a significant impact on the LCA results.

4.2 Allocation

A major challenge in life cycle assessment is the solution of allocation problems, when multiple products are formed. The various environmental impacts of processes and products need to be allocated to the products in a sensible manner. At present there is no general method available, which could be applied to all allocation problems [53, 55]. On the other hand, the assignment of environmental impacts to the respective products is the most critical part of a LCA and may significantly influence its results. In order to ensure an equitable distribution of the environmental impacts on the products, guidelines and different methods for carrying out an allocation were defined internationally in the ISO Standard 14,044 [56]. In ISO Standard 14,044, allocations by mass, by physical laws (stoichiometry), or based on economic values of the products and by-products are proposed.

Both, in the aerobic and the anaerobic processes for E3HB production, by-products such as water and off-gas are formed in addition to the main product. In this work the allocation is based on economic values of the products and by-products, since only those products having an economic value can at least theoretically create an economic benefit to the producer.

The impact factor based on economic values for each product was calculated by multiplying the product price with the product mass (**Figure 10**). The result was then divided by the total potential sales. The total potential sales are the sum of the specific costs of all products [52, 53, 55] times the product mass. All masses were related to the functional unit of 1 kg E3HB. **Figure 10** shows the percentages of environmental impacts for both processes.

In the anaerobic process, the two by-products ethanol and methane are produced. As a result, the environmental impacts were weighted differently and were allocated only with 84.3% to the main product E3HB for the anaerobic process as compared to 98.8% for the aerobic process. Nevertheless, E3HB has the major environmental impact in the anaerobic process, too.

4.3 Impact assessment

The life cycle impact assessment (LCIA) is part of the life cycle assessment and is used to identify and assess the magnitude and significance of potential environmental impacts of a process (for cradle to gate: used raw materials, energy, the production process, the emissions, and the product(s)) during its lifetime. LCIA supports the assessment of the LCA balance sheet in order to better understand the environmental relevance of the investigated process by grouping the environmental impacts of a process or product(s) into impact categories. Each impact category is related to an environmental issue, such as climate change, eutrophication, or land occupation, which is influenced by the process under consideration. The environmental impacts of the products are quantified by an impact indicator. As an example, carbon dioxide equivalents may be used to quantify the category climate change. In the work described in this section, the eco-indicator 99 (E,E) (**Table 3**) was used for the LCIA of the comparative life cycle assessment of E3HB. (E,E) means egalitarian and egalitarian weighing and includes long-term effects to the environment. Eco-indicator 99 (E,E) reviews the environmental damage in Europe and accounts for the impact categories' ecosystem quality, human health, and resources, each consisting of several subcategories (**Table 3**). This method is used for the damage-oriented estimation of environmental effects and is already

Ecosystem quality	Human health	Resources
Land occupation	Climate change	Fossil fuels
Ecotoxicity	Ozone layer depletion	Mineral extraction
Acidification & eutrophication	Carcinogenic	
	Ionising radiation	
	Respiratory effects	

Table 3.
Impact categories within eco-indicator 99.

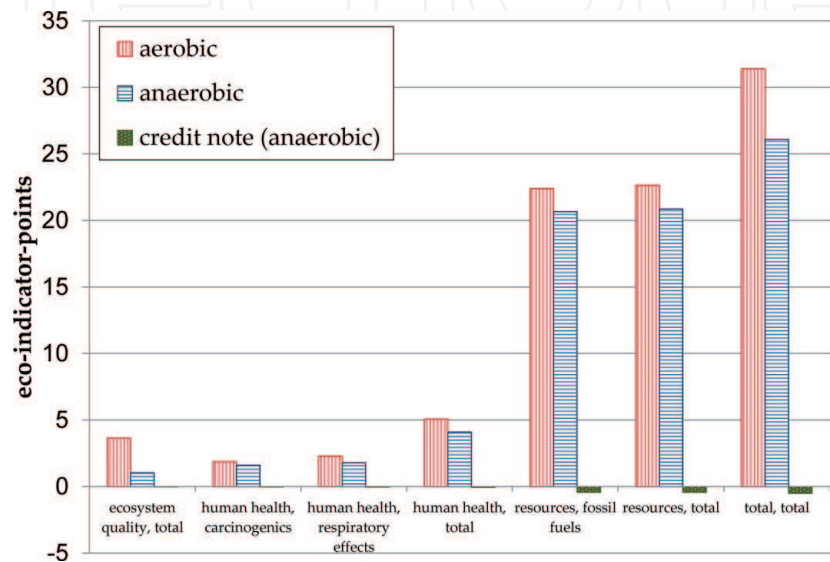


Figure 11.
Comparative life cycle assessment for aerobic and anaerobic production of Ethyl 3-hydroxybutyrate with eco-indicator 99.

integrated in the *Umberto NXT LCA* software [54, 57–59]. The individual damage categories have different physical units. For example, climate change is calculated in kg CO₂ equivalents and acidification in SO₂ equivalents. In order to enable a comparison between the aforementioned categories, the respective balance results are converted into a comparable, aggregated, numerical, and unitless impact indicator (eco-indicator points) for each environmental area. This approach allows to compare the different categories directly [59].

Results of the performed LCA are shown in **Figure 11**. Here, a comparative LCA was conducted, emphasizing the differences of the environmental impacts between the aerobic and the anaerobic process. The absolute eco-indicator point values were not taken as basis for evaluation in this study. On the ordinate in **Figure 11**, the results are plotted as eco-indicator points, which are dimensionless (see above). The higher the number of eco-indicator points, the stronger is the effect to the environment, human health, or resource consumption. On the abscissa, only those impact categories are shown which are characterized by eco-indicator points larger than 1. The last pair of bars (total/total) shows the environmental effects of all impact categories in total [54].

Most eco-indicator points are gained from resources, subgroup fossil fuels. Fossil fuels are needed for the production of the raw materials like sugar and for the production of the used electricity, which is a mix from German electricity production. Only for the biogas process, electricity from the wind and sun was used. The environmental impacts of human health and ecosystem quality result from the production of the agricultural raw materials which are necessary for the processing of the culture media.

Both, the aerobic and the anaerobic whole cell biotransformation of E3HB, produce CO₂ as output. This CO₂ is renewable; its origin is the carbon source (glucose). However, within the anaerobic production of E3HB, the biogas process leads to a saving of environmental impacts, since CO₂ is converted to bio-methane, which replaces natural gas of fossil origin. In the LCA this leads to eco-indicator point credits lowering the environmental impact of the anaerobic process. In order to model the replacement of fossil resources, a separate process was used in which the same amount of natural gas was produced which could be replaced by bio-methane. The natural gas was then converted into CO₂ in a simplified combustion step. The results are the negative values shown in **Figure 11**. As can be seen from **Figure 11**, this effect is small (about 2%) as compared to the total eco-indicator points (total, total) for the whole process.

All indicated impact indicators show that the anaerobic process has lower eco-indicator points than the aerobic process. For total, total, the difference is about 17%. This is within the range of the allocation of the additional products of the anaerobic process which is 15% less as compared to the aerobic process.

4.4 Interpretation of the LCA

The application of the eco-indicator 99 (E,E) method within a LCIA provides the basis for the comparison of the two biorefinery concepts. A credit for the substitution of natural gas from fossil resources by bio-methane was accounted for in the evaluation of the anaerobic process. The comparative LCA shows that the anaerobic production of E3HB has about 17% less of the environmental impact than the aerobic process.

The exhaust gas from the anaerobic process, which mainly consists of CO₂, is processed in a biogas process to form bio-methane substituting natural gas. In the aerobic process, the formed CO₂ is directly released into the atmosphere. The main contribution to the advantage of the anaerobic process in comparison to the aerobic biotransformation results from the by-product formation. Due to the formation of ethanol and bio-methane in the anaerobic process, the total eco-indicator points were reduced by 15%. Only further 2% eco-indicator point reduction resulted from the minimized CO₂ release of the anaerobic process.

All material and energy flows, except for the biogas process, the downstream process, and the natural gas combustion (for crediting the bio-methane production), were fully recorded in previous laboratory tests. Therefore, the data used were considered to be reliable. With regard to the biogas process, the downstream process, and the credit note (natural gas combustion), the data for procedures, implemented in the LCA software package, should be experimentally cross checked.

Various methods have been used for the sensitivity analysis of the aerobic and anaerobic LCA models by the authors. Different weightings were made in the economic allocation, and different allocation methods were applied. Various data sets from ecoinvent were used and compared for the production of E3HB. In the case of the credit, bio-methane was also burned instead of natural gas in the separate process. However, the analysis did only show slight fluctuations in LCIA results.

5. Conclusions

The presented data from the fed-batch biotransformation processes show that anaerobic production of the aroma compound E3HB from EAA is possible. The production efficiency of the anaerobic process is similar as compared to the aerobic production. This makes it possible to integrate both, the aerobic and the anaerobic processes, into two process-specific biorefinery concepts.

A LCA showed that the integration of the anaerobic process into a biorefinery results in a production process with lower environmental impact than a biorefinery with the aerobic process. In both alternatives environmentally friendly ambient conditions are applied. In particular, the anaerobic process produces ethanol as an additional by-product, replaces natural gas by the production of bio-methane, and does not produce CO₂ as a waste product. The knowledge gained during this work can probably be transferred to similar bioreductions, which can be carried out with the yeast *S. cerevisiae*.

Taken together the goal of integrating the whole cell biotransformation of the aroma compound E3HB into an anaerobic biorefinery process was achieved. The present study shows that anaerobic biotransformation can be used as a production process for E3HB, resulting in less environmental impact compared to the aerobic process as part of a biorefinery.

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