Microbial Lactone Synthesis Based on Renewable Resources

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Abstract Lactones are important flavor compounds and find frequent application in the production of food and as building blocks in fine chemical synthesis. The microbial production of optically pure lactones benefits from the high selectivity of biocatalysts and therefore simplifies downstream processing. A summary of different processes applying microorganisms or whole cells for the synthesis of different lactones is presented, starting at the cell metabolism and analyzing the hurdles in view of process development. Prominent examples are the synthesis of γ-decalactone using fungal strains, the Baeyer–Villiger oxidation using recombinant whole cells, and the γ-valerolactone synthesis.

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1 Introduction: Microbial Lactone Synthesis

Lactones are cyclic esters of hydroxy fatty acids. Applications of this versatile class of compounds span from precursors for the synthesis of polymers to high-value, optically pure fragrance compounds. The possibility to produce lactones by microbial fermentation and biotransformation using biogenic carbohydrates and lipids as starting material makes them promising bio-based commodities. Chemical routes for their synthesis often use petrol-based chemicals. Moreover, the chemical synthesis often leads to undesired racemic mixtures, which require further downstream processing. Lactones produced by microbial cultures need to be isolated afterward for use as additives in food manufacture, too. However, these additional isolation and purification steps circumvent the discrimination of two enantiomers and therefore are easier to achieve. Another advantage of the production in microorganisms lies in the possibility to label the resulting product as "natural," which is an important commercial advantage. Examples for lactones as aroma compounds produced by microbial cultures are δ-decalactone, γ-decalactone (γ-DL), δ-dodecalactone, δ-octadecalactone, or (Z)-6-dodecen-δ-lactone (Asunción Longo and Angeles Sanromán 2006).

Biotransformations of precursors for the production of lactones have been established on industrial large scale to produce chiral building blocks. Green and sustainable aspects of such processes are renewable biocatalysts and generally lower toxicity of processes. Several microorganisms are able to convert hydroxy fatty acids to optically pure compounds. The biotransformation is based on chain-shortening reactions using enzymes from the β-oxidation—a reaction cascade which would be extremely difficult to use in a cell-free system. A highly interesting reaction for biotransformation is the Baeyer–Villiger oxidation. This reaction is mostly carried out with whole-cell systems, because of enzyme stability and efficient cofactor recycling. These processes enable access to a multitude of versatile building blocks for asymmetric synthesis (Mihovilovic 2012).

Very recently, lactones have received increasing attention as potential renewable platform chemicals. Perhaps the most prominent bio-based hydroxy fatty acids lactic acid, whose cyclic ester of two lactate molecules serves precursor for the synthesis of bio-based polymers. Fermentative production of hydroxyl–carboxylic acids from agro-industrial waste is an alternative to the synthesis from dwindling fossil resources (Füchtenbusch et al. 2000). The enzymatic machinery for the production of polyhydroxyalkanoates (PHA) in bacteria offers catalytic pathways for the production of these lactone precursors (Efe et al. 2008). Recent examples include the microbial synthesis of γ-butyrolactone and γ-valerolactone. Particularly γ-valerolactone is of importance and ranks among the top key components of the biomass-based economy. Microbial processes thus offer the perspective of a sustainable fermentative production of optically pure renewable lactones.

Already in 2002, Vandamme and Soetaert summarized the hurdles of the application of microbial strains for the synthesis of flavor compounds. Next to low yields of 100 mg L^{-1} a further challenge was the fact that many flavor

Table 1 γ-Decalactone produced by cultured microorganisms (Figueiredo et al. 2010)

Cultured microorganisms	Substrate	γ-Decalactone concentration	Reference
Yarrowia lipolytica	Castor oil or methyl ester of ricinoleic acid	Up to 12.3 g L^{-1}	Rabenhorst and Gatfield (2002)
Candida sp.	Castor oil hydrolysate	10 g L^{-1}	Farbood and Willis (1985)
Yarrowia lipolytica	10 % v/v castor oil or 0.05 % decanoic acid	Up to 6.9 g L^{-1}	Farbood and Willis (1985)
Rhodotorula aurantiaca	Castor oil	6.6 g L^{-1}	Alchihab et al. (2009)
Sporobolomyces odorus	Hydrolyzed castor oil	5.5 g L^{-1}	Lin et al. (1996)
Aspergillus oryzae	Castor oil	0.86 g L^{-1}	Farbood and Willis (1985)
Trichoderma harzianum	Castor oil	0.260 g L^{-1}	Serrano-Carreon et al. (1997)
Geotrichum klebahnii	Castor oil	0.2 g L^{-1}	Farbood and Willis (1985)
Moniliella suaveolens	Castor oil press cake	0.180 g kg^{-1} dry matter	Laufenberg et al. (2004)
Piptoporus soloniensis	Liquid media of yeast extract and glucose	0.008 g L^{-1}	Kenji et al. (2002)
Sporidiobolus salmonicolor	Vinasse and ricinoleic acid methyl ester		Billerbeck et al. (2003)

compounds or their added precursors are inhibitory or even toxic at high levels to the producer strains. Therefore, strategies like fed-batch fermentation and in situ product removal are technologies which could help to circumvent these limitations (Vandamme and Soetaert 2002). The different hurdles as well as recent publications to overcome them will be discussed.

In case of the microbial synthesis of γ-DL, different strains were identified to synthesize this product in high yields. Depending on the substrate and strain, different product concentrations were achieved and are summarized in Tables 1 and 2.

Next to these fatty acids, hydroxy fatty acids can be used as substrates for δ-lactone synthesis via biotransformation in bacterial cells. *Clostridium tyrobutyricum* uses 5-hydroxy-2-decenoic acid or 5-hydroxy-2-dodecenoic acid to produce δ-decalactone (5.95 g L^{-1}) or δ-dodecalactone (13 g L^{-1}) (Pichersky 1998). *Bacillus subtilis* and *Pseudomonas* sp. transform massoia lactone to δ-decalactone (Gocho et al. 1998). Therefore, in case of γ-DL, δ-decalactone, and δ-dodecalactone, the hurdle to achieve product concentrations far above 100 mg L^{-1} can be overcome by screening and choice of appropriate microorganisms.

The conversion of fatty acids into lactones proceeds via an initial hydroxylation of the fatty acyl chain, shortening of the chain by β-oxidation, and lactonization.

Table 2 γ-Decalactone produced by biotransformations of precursors using microorganisms (Figueiredo et al. 2010)

Biotransformations	Substrate	γ-Decalactone concentration	Reference
Candida boidinii	Ricinoleic acid	40.9 g L^{-1}	Mitsuhashi and Limori (2006)
Mucor circinelloides	Ethyl decanoate, ethyl caprinate	10.5 g L^{-1}	Kümin and Münch (1998)
Sporidiobolus ruinenii	Methyl ricinoleate	5.5 g L^{-1}	Dufossé et al. (1999)
Yarrowia lipolytica	Methyl ricinoleate	5.5 g L^{-1}	Wache et al. (2000, 2001)
Sporidiobolus salmonicolor	Ricinoleic acid	0.132 g L^{-1}	Lee et al. (1998)
Sporidiobolus salmonicolor	Ricinoleic acid	0.040 g L^{-1}	Billerbeck et al. (2003)

These reactions may be performed within one cell or in several distinct process steps. The first important step in the conversion of fatty acids to lactones is synthesis of hydroxy fatty acids. The availability of a certain lactone is limited by the availability of the corresponding hydroxy fatty acids. A prominent source is castor oil, which contains a large amount of ricinoleic acid (Biermann et al. 2011). The hydroxy group is inserted by a non-heme hydroxylase, which is closely related to an oleate-12-desaturase (Broun et al. 1998).

These examples illustrate the variety of species for the production of lactones. Within the following subchapters, the focus is set on microbial processes, the involved enzymes and metabolic pathways, the hurdles for the implementation of bioprocesses, as well as strategies to overcome these. Starting with fermentative routes, also whole-cell synthesis and alternative biocatalytic approaches are discussed.

2 γ-DL Production

γ-DL has a distinct peach flavor and is the most important lactone for food production. Biotransformation in fungi has made the commercial production of the "natural"-labeled compound feasible. While *Yarrowia lipolytica* is the mostly used production organism, other microorganisms such as *Fusarium poae*, *Tyromyces sambuceus*, or *Sporidiobolus salmonicolor* have been described (Alchihab et al. 2009). Usually, yields between a few mg up to 10 g L^{-1} can be achieved. Cells from *Candida sorbophila* produced γ-DL with a titer of 49 g L^{-1} (Mitsuhashi and Iimori 2004). Recently, Alchihab and coworkers identified several psychrophilic strains isolated in Antarctica as possible alternative producers. The best results were achieved with the yeast *Rhodotorula aurantiaca* A19, which led to

a production of 5.8 g L^{-1} in shake flasks at 14 °C (Alchihab et al. 2009). This productivity is already similar to those using genetically engineered *Y. lipolytica* strains.

Starting compound is ricinoleic (12-hydroxy-*cis*-9-ene octadecenoic) acid, which is available from castor oil. The yield of the biotransformation, however, is limited by a consumption of the fatty acid by the organism and side reactions leading to hydroxylated and unsaturated lactones. Within the peroxisomes of the yeast cells, β-oxidation leads to a shortening of the C18 chain to C10 and a reduction of the double bond, which is followed by spontaneous lactonization (Fig. 1). β-Oxidation proceeds via four steps: acyl-CoA oxidation, hydration, oxidation of the OH-group, and thiolysis. The regulation of β-oxidation is very complex and depends on the availability of coenzyme A, NADH, and oxygen. *Y. lipolytica* has five acyl-CoA oxidases with different chain length specificity. By inactivation of acyl-CoA oxidases with short-chain specificity, Waché et al. succeeded in increasing the productivity of γ-DL production to 10 g L^{-1} (Romero-Guido et al. 2011; Wache et al. 2001). Yeast strains with deleted acyl-CoA oxidases also showed a slower γ-decalactone degradation and, surprisingly, showed a much improved ratio of γ-DL formation over the undesired formation of hydroxy lactones. Formation of the latter appears to be connected with the availability of NAD^+, which in turn depends much on aeration. This might be an explanation for upscaling problems in the γ-DL production. Recently, Guo and coworkers combined deletion of short-specific acyl-CoA oxidase POX3 with overexpression of endogenous long-chain-specific POX2 (Guo et al. 2012). While the former was supposed to prevent γ-DL degradation, the latter should accelerate lactone formation. Indeed, formation of γ-DL increased from 0.9 g L^{-1} to 2.75 g L^{-1}. The new strain was still able to grow on fatty acids as carbon source.

In addition to the engineering of the cell metabolism, optimization of the fermentation conditions is still going on. When castor oil is used as substrate, the availability of free ricinoleic acid also plays a role in lactone formation. Recently, Braga et al. showed that induction of the endogenous lipase from *Y. lipolytica* leads to a faster formation of γ-DL. After induction with olive oil, maximal production was found after 140 h of culture, compared to 185 h in the non-induced control (Braga et al. 2012). Methyl ricinoleate or castor oil was supplied as oil-in-water emulsions. Gomes et al. investigated the effect of the washing of the cells and droplet size on γ-DL formation. Interestingly, no γ-DL formation was observed

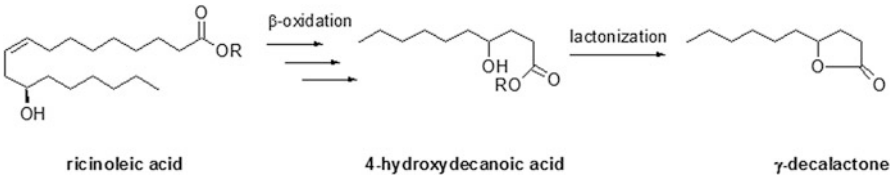

Fig. 1 Biotransformation of ricinoleic acid to γ-decalactone via shortening of the fatty acyl chain by β-oxidation and cyclization (Wache et al. 2001)

with castor oil as substrate and washed cells. Generally, yields where higher using non-washed cells, suggesting that this work-intensive step can be omitted. Larger oil droplets favored aroma production (Gomes et al. 2010, 2011b). Degradation of γ-DL starts when the substrate is consumed. To avoid this, and to minimize inhibitory effects of ricinoleic acid on the cells, Gomes et al. showed that fed-batch cultivation is an interesting alternative. In fed-batch using intermittent feed, they were able to obtain 6.7 g L^{-1}, compared to 1.9 g L^{-1} in batch fermentation. Production of the side product 3-hydroxy-γ-DL increased simultaneously to 10 g L^{-1}. However, due to a longer cultivation time of the fed-batch, the volumetric productivity of the batch (168 mg L^{-1} h^{-1}) was four times higher than that of the fed-batch (43 mg L^{-1} h^{-1}). Accessibility of the substrate is an important issue for the uptake into the cells. Recently, an experimental design study conducted by the same authors optimized the pH values and oxygen concentrations. Response surface methodology analysis showed that these two parameters are indeed critical for the productivity. The best results were achieved with pH 6.17 and 44 % dissolved oxygen concentration (Gomes et al. 2011a).

However, raw material and precursor costs in combination with the removal of unwanted side products and the preparative isolation of the desired products influence the industrial realization of microbial production processes for lactones. Therefore, strategies are asked for keeping the level of side products low. One possibility is the use of a second microorganism metabolizing the side products. In case of the synthesis of γ-DL from ricinoleic acid, the side product 3-hydroxy-γ-decalactone is formed as an intermediate. Because of a dehydration reaction, the 3-decen-4-olide is formed and accumulates during distillation in the distillation residues. The yeast *Saccharomyces cerevisiae* can be used afterward to reduce this compound stereoselectively yielding γ-DL (Gatfield 1995; Vandamme and Soetaert 2002). This strategy allows the conversion of a side product after downstream processing to increase the overall yield of the process (Fig. 2).

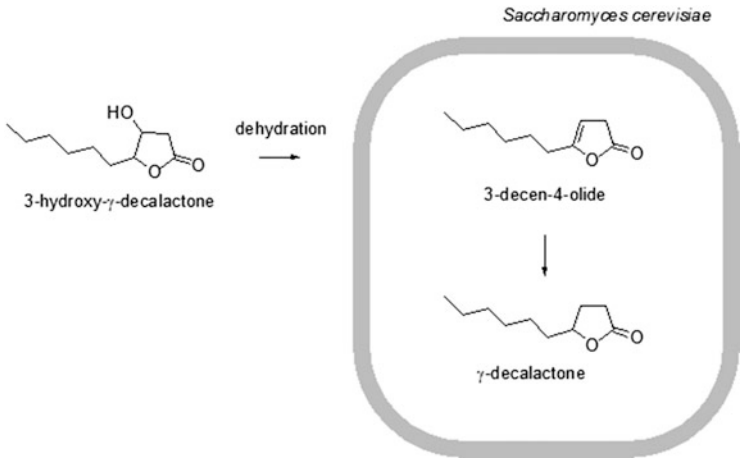

Fig. 2 Conversion of the side product 3-hydroxy-γ-decalactone into γ-decalactone by *Saccharomyces cerevisiae*

A further factor influencing the formation of 3-hydroxy-γ-decalactone is the oxygen transfer rate. The concentration of dissolved oxygen and the oxidative state of the medium influences the pathways of *Yarrowia lipolytica* (Aguedo et al. 2005; Fickers et al. 2005; Kamzolova et al. 2003). In the case of the γ-DL synthesis, the oxygen influence on the β-oxidation is of great importance. From the bioengineer's point of view, the oxygen transfer rate from the gas to the liquid can be improved by increasing the aeration and stirring rates which enhances the k_La value. Alternatively, the air or oxygen pressure of the gas phase directly influences the dissolved oxygen concentration according to Henry's law.

Aguedo et al. investigated the influence of k_La and oxygen pressure on the production of γ-DL (Aguedo et al. 2005). The use of pressure improved the production but increased the concentration of its oxidation compounds. The increase of k_La led to an increase of 3-hydroxy-γ-decalactone and to a smaller extent of decenolides. However, increase of k_La over a certain threshold caused a substantial decrease of all produced compounds, possibly due to the upregulation of the pathway including 3-hydroxy-acyl-CoA dehydrogenase. In summary, the β-oxidation in *Yarrowia lipolytica* is sensitive to the oxidative state of the medium. The dependency on k_La in biphasic medium was also shown by Gomes et al. (2007). Further, the influence of operating conditions like gas flow rate and organic phase composition upon the gas–liquid interfacial area and mass transfer coefficient was analyzed by Gómez-Díaz et al. (2009), illustrating the effect of the reactor setup on the parameter k_La in case of the fermentation of *Yarrowia lipolytica*. A broader study applying the 3^2 level full-factorial design was used to determine the effect of pH in the range of 4.5–6.7 and dissolved oxygen concentration (10–50 %) on the production of γ-DL and 3-hydroxy-γ-decalactone (Gomes et al. 2011a). This study also identified a threshold for dissolved oxygen concentration above which a decrease of 3-hydroxy-γ-decalactone yield was found.

Alternatively, the supply of oxygen can be improved by the use of a second liquid phase of perfluorocarbon, an oxygen-carrying compound, in the culture medium. Here, the availability of oxygen to the microorganisms is increased, and it was found that the specific growth rate of *Y. lipolytica* increases with increasing perfluorocarbon concentration. Most importantly, Amaral et al. (2008) could show that the perfluorocarbon presence lead to an increase of lipase production and not just its secretion to the extracellular medium. Further studies of the same group illustrate that from the bioengineer's point of view the reactor volume, the type of impellers and their position, the organic phase concentration, the aqueous phase composition, and the concentration of inactive biomass are important parameters. In summary, the addition of perfluorodecalin promotes k_La enhancement, while olive oil decreases the overall k_La of the multiphase reactor. Olive oil was used as an inducer for lipase production and also displays very high oxygen solubility (Amaral et al. 2008).

Product recovery is an important step in bioprocesses aiming for products showing a high volatility and low solubility as well as limiting the toxicity of the lactone toward the microorganism. Commonly for the separation from aqueous

media specific membranes or adsorption processes are applied (Edris et al. 2003; Ismadji and Bhatia 2000; Medeiros et al. 2006). The adsorption of γ-DL onto activated carbon and hydrophobic resins by online extraction was reported. However, the presence of these adsorbers in the bioconversion medium also decreased the production of γ-DL (Dufossé et al. 1999; Souchon et al. 1998). Porous organic resins benefit from their hydrophobic nature and their high specific surface area. The adsorption of γ-DL onto Macronet resins (a macroporous polystyrene cross-linked with divinylbenzene) is a method for extracting γ-DL from the culture broth of *R. aurantiaca*, as shown by Alchihab et al. (2010). The studied resins are not chemically reactive with the aroma compounds; they can be easily regenerated and have a low price. Among the tested resins, MN-202 was the most efficient adsorbent for γ-DL.

Fed-batch fermentation is often applied in the production of primary and secondary metabolites, proteins, and biopolymers, because a higher cell density than in batch mode can be achieved (Shiloach and Fass 2005). These high cell densities allow high yields and productivities of desired products. Considering the potential toxicity of the precursors like ricinoleic acid in case of the production of γ-DL, the use of fed-batch fermentation becomes an obvious choice. Different studies were published illustrating the usefulness of fed-batch fermentation for the production of γ-DL (Kapfer et al. 1989; Kümin and Münch 1998; Lee et al. 1995). A recent study of Gomes et al. (2012) illustrates the high potential of fed-batch operation for the *Y. lipolytica* fermentation using methyl ricinoleate as a precursor. The authors compared the constant medium feeding and intermittent fed-batch fermentation with classical fermentation strategies. Although the productivity in view of γ-DL was considerably higher in batch mode (168 mg L^{-1} h^{-1} in comparison to 43 mg mL^{-1} h^{-1}), the substrate conversion to lactone was greater in the intermittent fed-batch yielding 6.8 g L^{-1} instead of 2 g L^{-1} (Gomes et al. 2012).

Another recent study compared batch and fed-batch cultivation for the production of γ-DL using *Yarrowia lipolytica* and castor oil as substrate (Moradi et al. 2013). Also the effect of enhancing oxygen transfer rate by using higher agitation rates or pure oxygen for aeration was investigated. The highest γ-DL concentration (220 mg L^{-1}) was obtained in the fed-batch fermentation using pure oxygen which was three times more compared to the batch cultivation (65–70 mg L^{-1}). Using pure oxygen instead of atmospheric air in the fed-batch fermentation also resulted in 60 % increase of γ-DL production. These studies support the necessity of the control of oxygen as well as precursor concentration in the fermentation medium and the understanding of the involved pathways for the optimized production of lactones.

A highly interesting approach is the use of a lipase to hydrolyze castor oil and yielding ricinoleic acid as a substrate for, e.g., *Yarrowia lipolytica*. Recently Goswami et al. (2013) published a review on the use of lipases for the hydrolysis of castor oil. The activity of lipases of different origins like plants, fungi, and bacteria is compared and clearly illustrates the potential for processes with lower energy consumption, lower temperature, and lower pressure and with higher quality of the final product. Lipases from *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus*

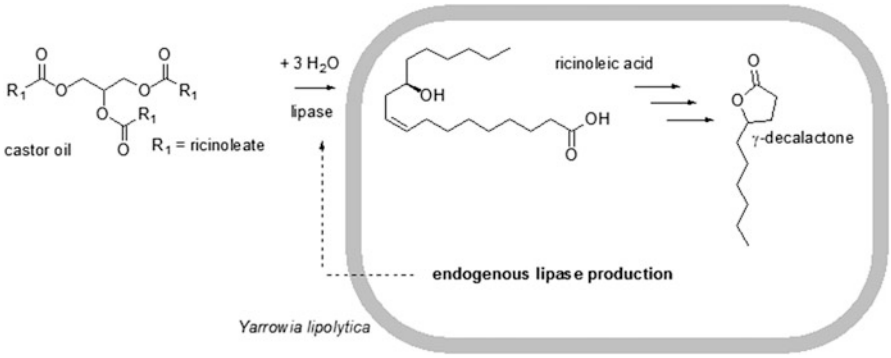

Fig. 3 Conversion of castor oil into ricinoleic acid, the carbon source and precursor for γ-decalactone production by *Yarrowia lipolytica*

delemar, *Humicola lanuginosa*, *Galactomyces geotrichum*, and *Penicillium* sp. were investigated in view of the hydrolysis of castor oil. Different activities were found with reaction times between 1 h and 4 days. Next to these fungal lipases, also bacterial lipases show activity toward this hydrolysis reaction. Neto et al. (2004) showed that the addition of enzymatically hydrolyzed castor oil to the culture medium enhances γ-DL production by *Geotrichum* sp. Braga et al. (2012) published the use of lipase induction in *Yarrowia lipolytica* for castor oil hydrolysis and its effect on γ-DL production (Fig. 3). This approach appears highly interesting, because only one microorganism is involved. The lipase formed by *Yarrowia lipolytica* generates its own carbon source (ricinoleic acid) and the substrate for the bioconversion to yield γ-DL. The goal of this study was to accelerate the hydrolysis of castor oil and in that way to improve the production of γ-DL. The highest values of extracellular lipase activity were obtained with the strain *Y. lipolytica* W29, and with the addition of a lipase preinduction step (using olive oil), it was possible to obtain a gain in the overall process time. Without preinduction of lipase, the lactone production was detected after 120 h, with preinduction after 60 h. However, there might be a limitation because of a possible limitation of the activity of lipase from *Y. lipolytica* toward castor oil.

In summary, different approaches were already realized to overcome hurdles of the application of microbial strains for the synthesis of flavor compounds. Yields of much more than 100 mg L^{-1} can be obtained by choosing the appropriate microorganisms. The inhibitory effect of produced lactone can be overcome by fed-batch fermentation, and effects by added precursors can be minimized by adsorption or extraction. Next to these strategies, the influence of the oxygen concentration has to be considered as well as the availability of the substrate. In all cases, for the bioprocess engineering, a detailed understanding of the regulation of different pathways is of great advantage to achieve maximum yields and product concentrations. Also the coupling of steps as shown with the example of lipase catalyzed hydrolysis of castor oil and subsequent conversion to γ-DL illustrates the

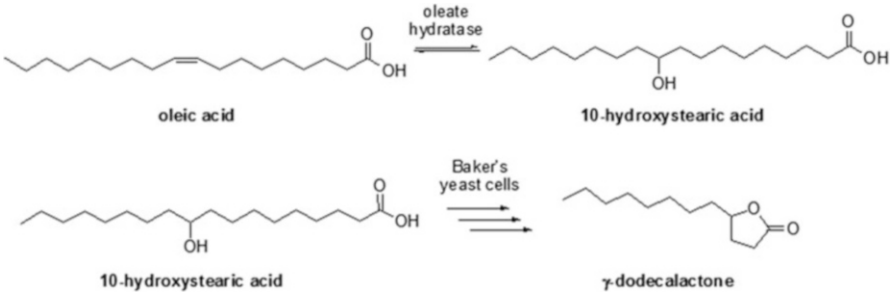

Fig. 4 Enzymatic hydration of unsaturated fatty acids to produce hydroxystearic acid, a precursor of γ-dodecalactone

importance of the mathematical description of enzyme kinetics and growth behavior of microorganisms as well as the microbial transformations. If an accurate adjustment of the kinetics of the different steps is achieved, the long-term stability of the process at maximum productivity can be obtained.

The synthesis of γ-DL is the most prominent example for the microbial production of fragrance lactones. Nevertheless, the biotransformation route via β-oxidation and lactonization (Fig. 1) in yeast can be applied for the production of several lactones provided that a suitable hydroxy fatty acid as starting material is available. For example, ricinoleic acid, the starting compound for the production of γ-DL, can be isolated in large quantities from castor oil. However, other hydroxy fatty acids are more difficult to obtain. For instance, researchers from Takasago International Corporation reported the conversion of 11-hydroxy palmitic acid ethyl ester to δ-decalactone by the yeast *Candida sorbophila* (Mitsuhashi and Iimori 2004). 0.13 g substrate in 30 mL culture medium yielded 0.019 g δ-decalactone in 96 % ee optical purity after 11 days.

γ-Dodecalactone (γ-DoL) is a known whisky flavor. Conversion of 10-hydroxystearic acid (HSA) by Baker's yeast (Fig. 4) formed (*R*)-γ-DoL with a yield of 22 % (Gocho et al. 1995). However, the supply of optically pure (*R*)-10-HSA is difficult. Unlike ricinoleic acid for γ-DL production, HSA does not occur in large quantities in vegetable oils. Very recently, hydratases came into the focus as promising catalysts for a wide range of applications (Jin and Hanefeld 2011). In particular, the hydration of unsaturated fatty acids by enzymatic hydration is a straightforward route to hydroxy fatty acids. Unlike P450-monooxygenases, enzymatic hydration shows excellent product specificity.

Wallen et al. described the hydration of oleic acid to hydroxystearic acid by a *Pseudomonas* strain 3266 already in the 1960s (Davis et al. 1969). Several bacterial strains have been isolated that are able to hydrate oleic acid and in some case catalyze the subsequent oxidation to 10-ketostearic acid (Heo and Kim 2009; Kuo et al. 2000, 2002; Kuo and Levinson 2006). The reaction shows moderate stereospecificity (Gocho et al. 1995). Two groups cloned independently the hydratases from the *Pseudomonas* strain 3266 (Bevers et al. 2009) and *Streptococcus pyogenes*

(Volkov et al. 2010). Since then, several oleate hydratases have been cloned and overexpressed in *E. coli*. A BLAST search of the sequence showed that this enzyme had been previously known as myosin-cross-reactive antigen. The flavin-dependent hydratase may be involved in the production of conjugated linoleic acid. Moreover, there is some evidence that it plays a role in stress tolerance. It could be shown that cells from *Lactococcus* and *Corynebacterium* transformed with the hydratase show an increased viability under heat and solvent stress (Rosberg-Cody et al. 2011). Recently, hydratase was applied in the conversion of ricinoleic acid into a novel dihydroxy fatty acid (Seo et al. 2013). Recombinant oleate hydratases from *Stenotrophomonas maltophilia* and *Lysinibacillus fusiformis* in *E. coli* were successfully applied for the synthesis of 10-hydroxystearic acid with 40 g L^{-1} h^{-1} (Joo et al. 2012; Kim et al. 2012). It can be expected that the efficient supply of 10-HAS by enzymatic hydration will greatly facilitate the microbial production of γ-DoL. Interestingly, δ-DoL was recently isolated from the supernatant of *Lactobacillus* cultures from kimchi, a traditional fermented Korean dish. The authors could show antifungal activity toward *Aspergillus*, *Cladosporium* sp., *Penicillium*, and *Candida*, which might contribute to the conserving properties of the traditional fermentation (Jang and Yang 2011).

3 Baeyer–Villiger Oxidation for Lactone Production

A recently published review summarizes the use of whole-cell biocatalysis for selective and productive C–O functional group introduction and modification (Schrewe et al. 2013). Next to the fermentative routes, such whole-cell processes represent an interesting way to obtain lactones. Within the different host organisms for Baeyer–Villiger monooxygenases (BVMOs) as biocatalyst *E. coli* plays a very prominent role. BVMOs are a group of enzymes that can catalyze the stereoselective oxidation of ketones yielding esters (Kelly et al. 2001). These processes will be discussed in the following. However, one should keep in mind that also various fungal isolates are able to carry out this interesting reaction as a classical microbial biotransformation (Fantin et al. 2006). The authors isolated different microorganisms from environmental samples and found *Fusarium* and *Aspergillus* strains capable to carry out a Baeyer–Villiger oxidation. Also, the discovery of the first Baeyer–Villiger biotransformation was observed in 1948 in fungi (Turfitt 1948). Nevertheless, the bigger part of studies on BVMO processes is using recombinant whole-cell catalysts.

The Baeyer–Villiger oxidation reaction is especially interesting for the oxidation of cyclic ketones into their corresponding lactones. One of the best-characterized BVMO is the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*. The recombinant expression of this enzyme was successful in *Saccharomyces cerevisiae* (Stewart et al. 1998) and *Escherichia coli* (Doig et al. 2001; Mihovilovic et al. 2001). A major limitation of the industrial implementation of BVMOs is the need for efficient recycling of the cofactor NADPH (Baldwin and Woodley 2006). Therefore, the process development has been

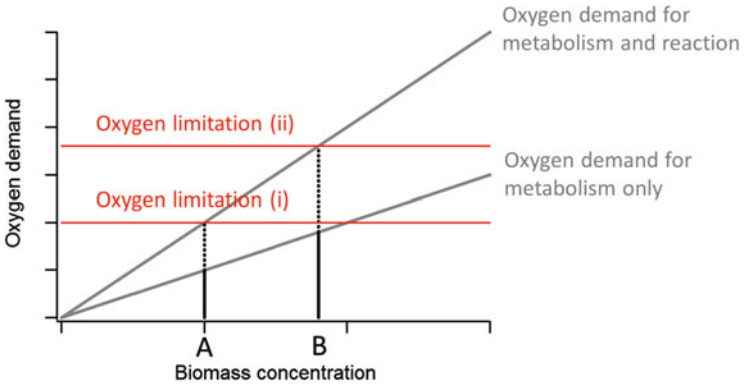

Fig. 5 Conceptual model proposed by Baldwin and Woodley showing the effect of biocatalyst concentration on oxygen demand (Baldwin and Woodley 2006). At a biomass concentration of A, the concentration is optimized for the level of oxygen provided, increasing biomass concentration to B will not increase rate of reaction, unless oxygen supply is increased to a higher value

focused on the use of whole cells, using glycerol or glucose as a reducing equivalent (Walton and Stewart 2002). On the other hand, the lactone production using crude enzyme preparations (CHMO) and applying a second ancillary enzymatic system (glucose-6-phosphate dehydrogenase) was shown to be simple and easy to handle (Ottolina et al. 2005). However, the crude extract contained a dehydrogenase which caused a non-desired reduction. This drawback can be overcome using purified CHMO and illustrates therefore a potential advantage of an enzymatic process.

Further bottlenecks of the whole-cell biocatalytic process are to be addressed such as substrate and product inhibition, pH and temperature intolerance (Doig et al. 2003), as well as oxygen limitations. In the case of the oxidation of, e.g., bicyclo[3.3.0]-hep-2-en-6-one, the process limitations are the oxygen supply which limits the rate of the reaction, the product inhibition which limits the final yield, and the biocatalyst stability which limits the total reaction time (Baldwin and Woodley 2006). Here, with increasing cell concentrations, the dissolved oxygen tension drops to zero, and the initial specific reaction rate is reduced dramatically.

Figure 5 illustrates one basic problem of the BVMO biotransformation: the role of oxygen in limiting the rate of the reaction (Baldwin and Woodley 2006). The model assumes that oxygen is preferentially used by the microorganism for maintenance (oxidative phosphorylation). As soon as this need has been met, any remaining oxygen can be used in the BVMO reaction. For example, at a biomass concentration of A, there is an oxygen demand for the metabolism and an additional demand for the reaction. This demand can be fulfilled by the oxygen concentration present in the reaction medium [oxygen limitation (i)]. Increasing at constant oxygen concentration, the biomass concentration will reduce the amount of oxygen available for the reaction. Only an increase of the oxygen concentration in the reaction medium [oxygen limitation (ii)] will allow an increase of the reaction rate.

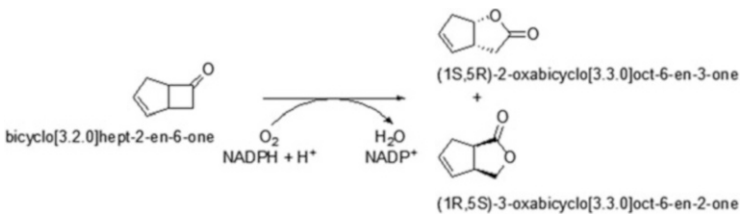

Fig. 6 Cyclohexanone monooxygenase (CHMO)-mediated regiodivergent oxidation of bicyclo [3.2.0]-hept-2-ene-6-one

Hilker et al. (2006) investigated the oxygen transfer rate and its effect on the biotransformation productivity. They could show that an improved mass transfer is beneficial to reach the optimal productivity also at a large scale. Because of the high efficiency of the whole-cell catalyst and the stoichiometric consumption of oxygen in the Baeyer–Villiger oxidation reaction, there is the need for an efficient oxygen supply to the reaction medium. The use of oxygen enriched air is limited because of safety issues. However, Hilker and coworkers achieved good results using a sintered metal sparger, and the oxygen transfer rate increased 40 % in case of the biotransformation of bicyclo[3.2.0]-hept-2-ene-6-one (Fig. 6).

Next to the oxygen supply, the inhibition kinetics of BVMO are of high interest to achieve an economic production of lactones. Substrate inhibition can be addressed using substrate feeding to maintain a low ketone concentration. This attempt aims for a maximal specific activity of the biocatalyst and high final product concentrations. Keeping the feeding rate below the maximum (Doig et al. 2002) could achieve a constant product formation at constant low substrate concentration. However, after 100 min, the product formation rate decreased, and consequently the substrate concentration began to increase. The explanation for the drop in activity was an inhibition by the formed product; because of the similarity of the product and the substrate and the hydrophilic nature of the lactones a selective continuous removal of the product is challenging.

The problem of inhibition, cell toxicity, and substrate solubility as well as substrate and/or product solubility was addressed by (Hilker et al. 2004). They carried out a whole-cell process combined with a “two-in-one” in situ “feeding and product removal” concept (SFPR) using an adsorbent resin (Fig. 7). This approach combines at the same time both in situ substrate feeding and in situ product removal. Thus, substrate and product concentrations in the reaction medium can be controlled to maximize cell productivity and enantioselectivity. The absorbent (macroporous resins) is added to the medium, and desorption of substrates takes place. After diffusion into the cells, the BVMO is oxidizing the substrate. The produced lactones leave the cell and are adsorbed on the resin. Different resins were tested for this reaction system, and Optipore L-493 and Lewatit VPOC 1163 turned out to be the best candidates. They exhibit a high inner surface and small pore diameters. The adjustment of the resin loading in the reactor is of great importance, because the concentrations of substrate and products must be kept under the inhibiting level. Three types of bioreactors were compared (Hilker et al. 2004): a

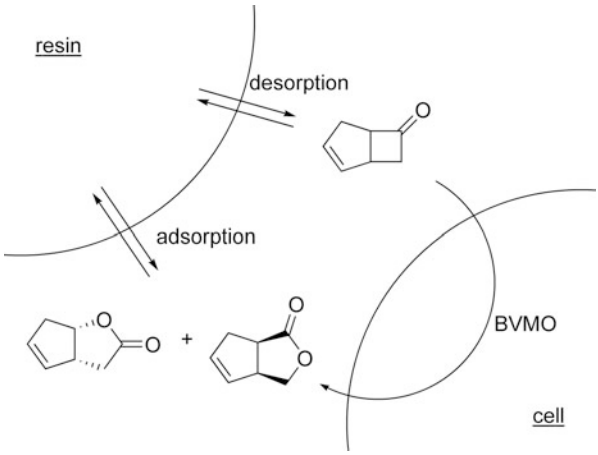

Fig. 7 Resin-based in situ substrate feeding and product removal concept (Hilker et al. 2004)

conventional fermenter, a recycle reactor (the resin is located in a fixed bed in an external loop), and a bubble column reactor. The best one proved to be the bubble column reactor, and both products were obtained in excellent enantiomeric purity (ee $>$ 98 %) and high yield. As an additional benefit, product extraction from the solid adsorbent resin considerably simplified downstream processing (Hilker et al. 2005). Moreover, these authors could show that the adsorbent resin is reusable, which would lower the costs of the entire process.

Another interesting application for adsorbents was shown by Fujii et al. (2007) using the hydrophobic polymer Amberlite XAD-7 to change the reaction selectivity of the microbial conversion of cyclohexyl methyl ketone. The reaction catalyzed by the organism *Dipodascus magnusii* NBRC 4600 in the absence of XAD-7 leads mainly to the reduced product 1-cyclohexylethanol, and the products of the Baeyer–Villiger oxidation reaction are hardly observed (0.2 %). The addition of small amounts of XAD-7 (100 mg XAD-7 per 3 mL) to the reaction system increased the yield of Baeyer–Villiger oxidation products (42 %). Although the cyclohexyl acetate was thought to be the primary Baeyer–Villiger oxidation product, hydrolysis took place and yielded cyclohexanol and acetic acid. Then, cyclohexanol was further oxidized to cyclohexanone (Fig. 8). Possibly, the observed effect is due to the substrate concentration in the aqueous medium. Nevertheless, in comparison to other microorganisms, the reducing power of *Dipodascus magnusii* is higher, and the BVMO reaction can only be observed when adding the resin.

The interest for enantiomerically pure compounds and high product yields led to the development of dynamic kinetic resolution processes. This approach combines an in situ racemization with a kinetic resolution step. Thus, a theoretical product yield of 100 % is possible. Enzymes and whole cells can contribute either to racemization or to kinetic resolution. BVMO were highly efficient to achieve asymmetric Baeyer–Villiger oxidation reactions with high enantioselectivity (Mihovilovic et al. 2002). Gutiérrez et al. (2005) investigated a dynamic kinetic resolution process using an anionic exchange resin to carry out the racemization

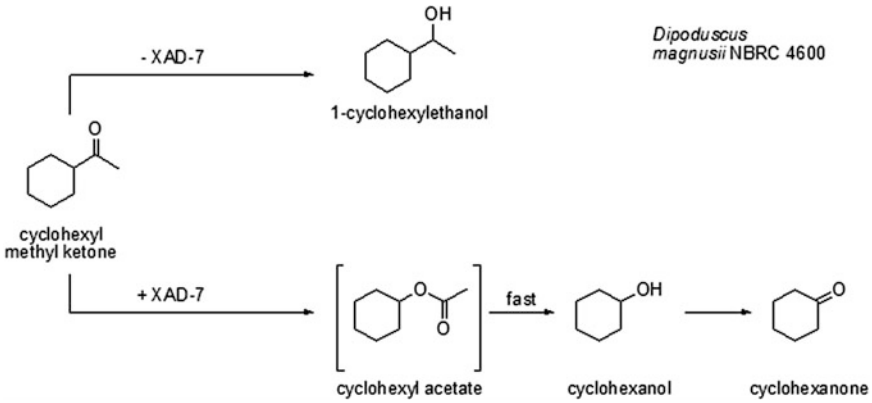

Fig. 8 Reaction of cyclohexyl methyl ketone by *D. magnusii* and effect caused by addition of the adsorbent XAD-7

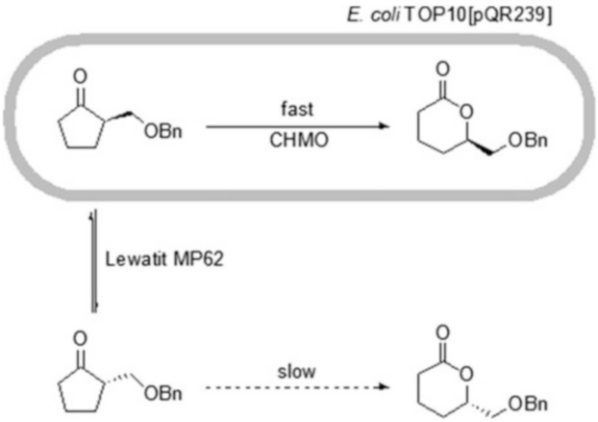

Fig. 9 Dynamic kinetic resolution of benzyloxycyclopentanone catalyzed by whole cells harboring CHMO from *Acinetobacter calcoaceticus* NCIMB 9871 and Lewatit MP62 for racemization

step (Fig. 9). The selective racemization of the substrate is based on the relatively high acidity of the proton on the Cα of the carbonyl group. Insertion of an oxygen atom stabilizes the product against racemization. In comparison to the base-catalyzed variant investigated beforehand, the loss of activity observed at pH 8.5 was reduced when using the resin (Gutiérrez et al. 2005). The authors could show that a satisfactory dynamic kinetic resolution process could be set up combining whole-cell-catalyzed Baeyer–Villiger oxidation and in situ racemization catalyzed by the weakly basic anion exchanger Lewatit MP62. Starting from racemic benzyloxycyclopentanone, nearly enantiopure lactone was obtained (97 % ee) with a yield of 84 %.

4 Lactones from Renewable Feedstocks as Future Platform Chemicals

Lactone production from biomass feedstocks would be a sustainable source of platform chemicals (Efe et al. 2008). Lactic acid is a well-known bifunctional molecule with a hydroxyl and a carboxyl moiety. Therefore, cyclic esters can be synthesized easily from this substance. The esters formed by two molecules of lactic acid are called lactides and can further be converted to polylactide which is a very interesting, biodegradable polymer (Doi and Steinbüchel 2002). The green biorefinery allows the production of lactic acid from green biomass via silage fermentation applying bacteria, e.g., *Lactobacillus plantarum*, *Lactobacillus casei*, *Pediococcus acidilactici*, and *Enterococcus faecium* (Langeveld et al. 2012). This intensively studied example will be used to shortly illustrate benefits and challenges of fermentation to produce small molecules like acids or lactones.

By anaerobic degradation of sugars, lactic acid is formed from silage. Starting from the vegetable carbohydrates, the glycolysis can produce pyruvate which is converted to lactic acid. Next to the carbohydrates, proteins can be degraded via deamination yielding oxoacids, which are converted to lactic acid, too. Using NAD^+ as redox equivalent, ethanol, propionate, butyrate, and lactic acid are formed from pyruvate (Kongo 2013).

The fermentative routes starting at the carbohydrates fructose or glucose can be subdivided into homofermentative and heterofermentative routes. Within the homofermentative process, sugars are exclusively converted to lactic acid. Heterofermentative bacteria produce significant amounts of other metabolites like acetic acid, ethanol, and glycol via a different pathway. Therefore, the industrial processes are desired which are based on the homofermentative route (Chang et al. 1999) to obtain high yields of the desired product from the substrate. Figure 10 illustrates the biochemistry of these two different routes in the case of lactobacilli.

The homofermentative route produces pure lactate from sugar applying the glycolysis pathway. To reduce pyruvate, the formed NADH is directly used yielding lactate, and the energy yield of this route is two molecules of ATP per molecule of glucose. Very small amounts of acetate, diacetyl, and acetoin are formed as side products leading to high yields. However, many homofermentative bacteria can be induced when applying pentoses as an additional substrate resulting in a heterofermentative process.

In comparison to the homofermentative processes, within the heterofermentative route, glucose is not converted by the glycolysis pathway but by the pentose phosphate pathway. Fructose-1,6-bisphosphate-aldolase and triosephosphate isomerase are not involved. The microorganisms are adapted to the utilization of pentoses as carbon source and in addition convert hexoses like glucose via this route. These pentoses are found when degrading plant material and are mainly xylose, ribose, or arabinose. They are converted via xylulose-5-phosphate which is converted to acetyl-phosphate and glycerinaldehyde-3-phosphate. Afterward,

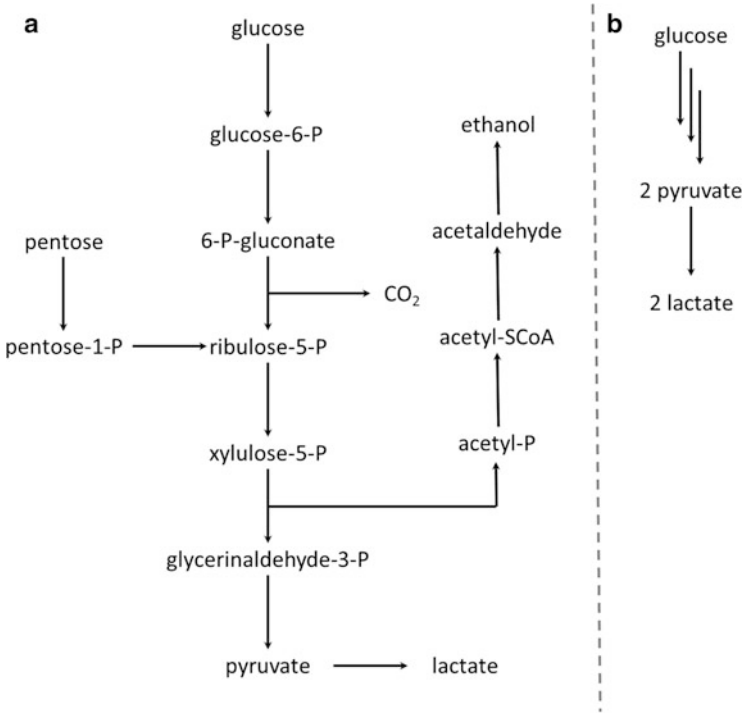

Fig. 10 (**a**) Heterofermentative and (**b**) homofermentative fermentation. The heterofermentative route yields lactate, ethanol, and CO_2 with an energy yield of 2 mol ATP per mole pentose. The homofermentative route produces pyruvate via glycolysis which is reduced to lactate with an energy yield of 2 ATP per mole hexose. For reasons of clarity, cofactors like ATP, NAD^+, and $NADP^+$ are not shown

glycerinaldehyde-3-phosphate is oxidized in the glycolysis yielding pyruvate and lactate.

These very prominent and well-described examples show the versatility of microbial conversions to yield small acids—in this case hydroxy acids. Central challenges of microbial conversions can be derived from this example, which are the carbon efficiency and the formation of side products. Therefore, there is a high interest in the productivity and yield of microbial processes and a detailed understanding of the underlying metabolic pathways. Tools of molecular biology and genetic engineering are used to optimize these pathways next to classical screening approaches identifying promising strains and species to produce small hydroxy acids as well as lactones.

The intramolecular esters of larger hydroxy fatty acids are receiving increasing attention due to their interesting chemical properties and their usefulness for a range of applications, including “green” solvents such as methyl tetrahydrofuran and new biofuels. Recently, the catalytic conversion of microbially produced polyhydroxybutyrates to tetrahydrofuran was suggested (Van et al. 2012). The catalytic process

produces γ-butyrolactone as intermediate, which is further hydrogenated to tetrahydrofuran. Van et al. used a genetically modified strain that increased the content of 4-hydroxybutyrate in the PHA over that of 3-hydroxybutyrate. 4-Hydroxybutyrate is formed via succinic acid semialdehyde (SSA) from the intermediates from the tricarboxylic acid cycle: α-ketoglutarate and/or succinyl-CoA. For the best strain, Van et al. reported a titer of poly-4-hydroxybutanoate of 46 % dry cell weight. Interestingly, overexpression of succinate semialdehyde dehydrogenase, which catalyzes conversion of succinyl-CoA to SSA, was crucial for the success, whereas overexpression of α-ketoglutarate decarboxylase did not lead to increased 4-hydroxybutyrate formation. Fermentative production of γ-butyrolactone would be an alternative to the production from petrol-based chemicals and the catalytic dehydration of pentoses from lignocelluloses, which requires the use of strong acids and high temperature. In conclusion, the investigation of fermentative PHA production as a future synthesis route for sustainable chemicals remains a thrilling field, which leads to expect further findings of this fascinating microbial machinery for polymer synthesis.

γ-Valerolactone (γ-VL) is under consideration as one of the main bases for a future bio-based economy as it is available in large quantities from biomass-derived feedstocks (Bozell 2010; Horvath et al. 2008). Potential applications range from a precursor of valeric biofuels (Palkovits 2010) to green solvents such as 2-methyl tetrahydrofuran (Geilen et al. 2010). It can be produced by chemical reduction from levulinic acid. Levulinic acid is a dehydration product of simple sugars like glucose, and the cellulosic fraction of lignocellulosic biomass can be converted into levulinic acid (Fig. 11). This makes levulinic acid an inexpensive starting material for the production of platform chemicals, green solvents, and even biofuels (Lange et al. 2010).

The first step for a utilization is the reduction of levulinic acid to 4-hydroxyvalerate (4-VL) followed by subsequent cyclization to γ-VL. Several groups have investigated the bioreduction of levulinic acid to 4-hydroxyvaleric acid and subsequent cyclization to γ-VL. A major obstacle lies in the difficulty to find suitable dehydrogenases with high activity for the reduction of levulinic acid, as no wild-type dehydrogenase for this substrate has been reported. Martin et al. used the observation that cells from *Pseudomonas putida* contain polyhydroxyalkanoic acid polymers with 4-HV monomers when grown on media supplemented with levulinic acid.

To redirect hydroxyvalerate production from PHA synthesis, they investigated the use of a *Pseudomonas* strain with systems for a removal of the CoA carriers from the hydroxy acids. Wild-type *Pseudomonas* overexpressing the gene of a recombinant thioesterase from *E. coli* yielded 14 g L^{-1} 4-hydroxyvalerate and 5 g L^{-1} 3-hydroxyvalerate as by-products (Fig. 12). They used minimal medium which was supplemented with glucose and levulinic acid (Martin and Prather 2009). Regarding the production of γ-VL, they faced the problem that the intramolecular cyclization of 4-hydroxyvaleric acid is pH dependent and that the equilibrium at pH 7.5 in the cytosol is unfavorable for lactonization. By using a variant of human paraoxonase I, they achieved a substantial increase in γ-VL production.

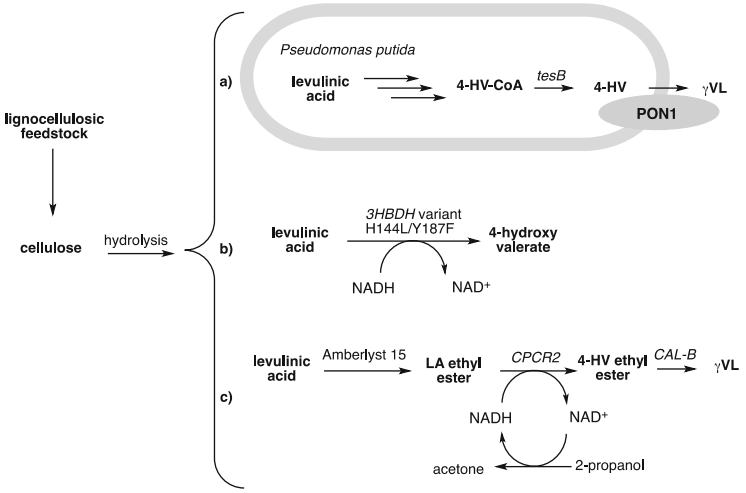

Fig. 11 Biotechnological approaches for the production of γ-valerolactone (γ-VL) from levulinic acid. The key step is the bioreduction of levulinic acid 4-hydroxyvalerate (4-VL), which subsequently can lactonize to γ-VL. (**a**) Biotransformation using *Pseudomonas putida* whole-cell overexpression of thioesterase *tesB* and paraoxonase I *PON1* (Martin et al. 2010). (**b**) A new variant of 3-hydroxybutyrate dehydrogenase *3HBDH* from *Alcaligenes faecalis* catalyzes the bioreduction of levulinic acid to 4-hydroxyvalerate (4-HV) (Yeon et al. 2013). (**c**) Chemoenzymatic route using carbonyl reductase from *Candida parapsilosis CPCR2* and lipase B *CAL-B* from *Candida antarctica* (Götz et al. 2013)

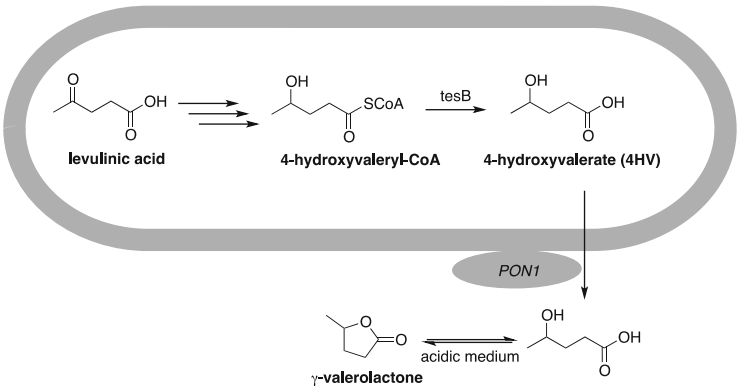

Fig. 12 Biotransformation of levulinic acid to γ-VL using whole cells from *Pseudomonas putida*, a thioesterase from *E. coli* (tesB) and extracytosolically expressed PON1. The cyclization at a low pH value is mediated by the human paraoxonase PON1. From Martin et al. (2010)

The important step was the extracytosolic expression of the paraoxonase I, which allowed to catalyze the pH-dependent lactonization in the acidic medium. Biotransformation and extracytosolic cyclization yielded 27 g L^{-1} 4-hydroxyvalerate and 8 g L^{-1} γ-VL, respectively (Martin et al. 2010). The enzymes involved in the

reduction of 4-VL remain unknown. The increase of 4-HV formation by the expression of a thioesterase suggests, however, that the substrate of the bioreduction is a CoA-ester of levulinic acid.

Use of isolated enzymes would avoid side reactions and thus lead to much higher molar yields. Very recently, Yeon et al. redesigned the active site of 3-hydroxybutyrate dehydrogenase (3HBDH) from *Alcaligenes faecalis* (Yeon et al. 2013). While the wild-type enzyme does not convert levulinic acid, a new variant produced 4-hydroxyvaleric acid with an activity of $k_{cat}/K_M = 578\ min^{-1}\ M^{-1}$, leading to 57 % conversion within 24 h. Based on a docking study of the substrate in the active site of the dehydrogenase, Yeon et al. investigated the potential interaction of levulinic acid with residues in the active site. They focused on the distance between the C4 atom of NADH and the carbonyl group and the distance between the oxygen atom of the active site residue Tyr155 and the carbonyl oxygen atom. 6 Residues with potential impact on the substrate specificity were identified. In an in vitro assay, only mutant H144L showed some activity. This was confirmed by a docking study of the six mutants, in which H144L mutant was the only one where suitable distances were observed. This mutant was used as initial variant for further mutagenesis studies. Double mutant H144L/Y187F showed a further 11-fold increased activity compared to the initial single mutant H144L. This study is an interesting example how protein engineering can create novel biocatalysts for practical applications. Alternatively, Hilterhaus et al. circumvented the lack of suitable dehydrogenases for levulinic acid conversion by using levulinic esters instead of the free acid as substrate. Carbonyl reductase from *Candida parapsilosis* showed excellent activity toward levulinic acid ethyl ester. They developed a chemoenzymatic process for the bioreduction of levulinic acid by using recombinant dehydrogenases and cofactor recycling (Götz et al. 2013)

All the abovementioned approaches depend on the supply of levulinic acid from cellulose. Two recent patents show a perspective for the production of levulinic acid and γ-VL from other feedstocks which would allow utilization of a much wider range of carbohydrates as renewable feedstocks. Kohei et al. have patented the fermentative production of levulinic acid from vanillic acid (Mase et al. 2012). Figure 13 depicts the reaction cascade, which starts with the ring opening by using the dioxygenase PcaHG. 3-Carboxy-cis,cis-muconate cycloisomerase PcaB and 4-carboxymuconolactone decarboxylase PcaC form muconolactone. β-Ketoadipate enol-lactone hydrolase PCaD forms 2-ketoadipic acid, which is subsequently decarboxylated to yield levulinic acid. This fascinating approach is a potential utilization of lignin-derived compounds such a ferulic acid.

Recently, the US company Arzeda has suggested a novel enzymatic pathway for the fermentive production of γ-VL (Fig. 14). The pathway starts with pyruvate, which is derived from the primary metabolism. Pyruvate decarboxylase converts pyruvate into acetaldehyde, which is added by an aldolase to another molecule of pyruvate to 4-hydroxy-2-oxo-pentanoic acid. A specific dehydrogenase reduces this intermediate to 2,4-dihydroxypentanoic acid, and a second catalyzes a selective oxidation of the hydroxyl group on C4 to give 2-hydroxy-4-oxo-pentanoic acid (2-hydroxy-levulinic acid). A hydratase eliminates water and produces 4-oxo-2-

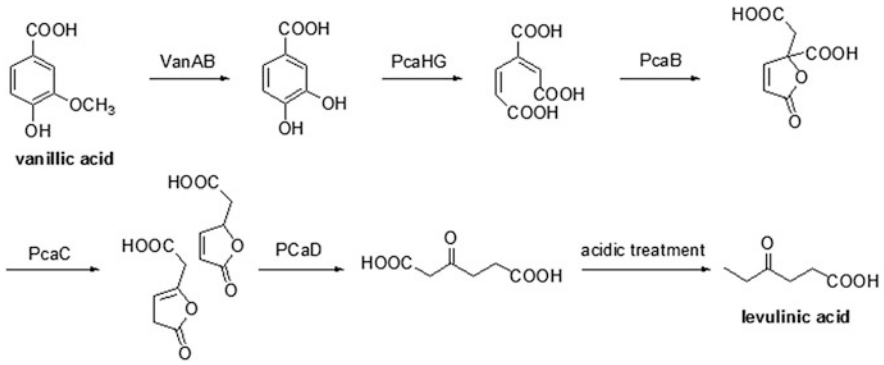

Fig. 13 Reaction sequence for the conversion of vanillic acid in recombinant cells from *Pseudomonas putida*. Abbreviations: *VanAB* vanillate O-demethylase, *PcaHG* dioxygenase, *PcaB* 3-carboxy-cis,cis-muconate cycloisomerase, *PcaC* 4-carboxymuconolactone decarboxylase, *PcaD* β-ketoadipate enol-lactone hydrolase

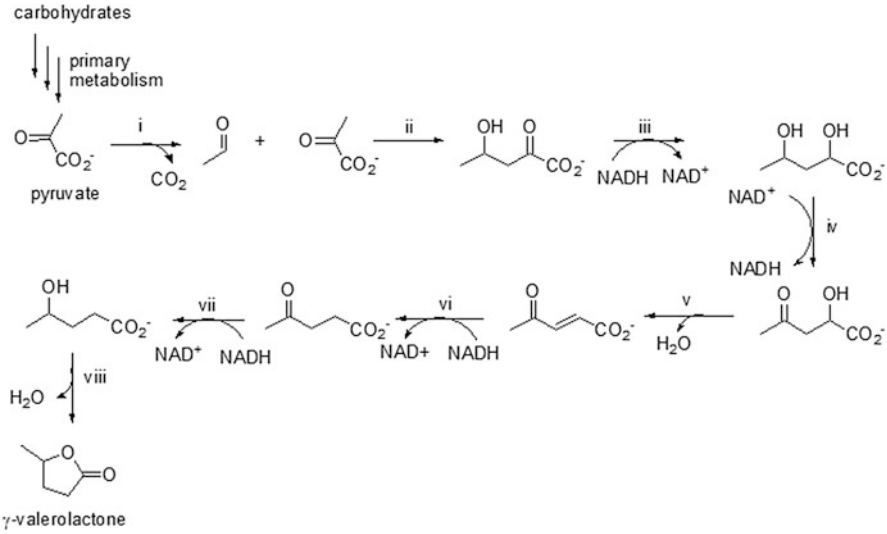

Fig. 14 The US company Arzeda suggested a biosynthetic pathway for the conversion of carbohydrates to γ-valerolactone. i: pyruvate decarboxylase; ii: aldolase; iii, iv, vii: specific dehydrogenases; v: dehydratase; viii: spontaneous lactonization

pentenoic acid, which is again reduced by an enoate reductase to levulinic acid. Levulinic is then reduced by a third dehydrogenase to 4-hydroxypentanoic acid and undergoes spontaneous cyclization to γ-VL. This impressive concept combines enzymes from different sources and pathways to a new metabolic pathway (Zanghellini 2012). It will be thrilling to get to know how all the practical

challenges of the approach will be met, particularly in view of the specificity of dehydrogenases in the presence of several keto acids and acetaldehyde.

Conclusion

Due to the foreseeable depletion of fossil resources, biomass represents a renewable feedstock for the production of different chemicals. Cellulose and hemicellulose can be degraded from vegetable waste by using different thermal processes as well as efficient enzymes to small sugar molecules. These carbohydrates can be used for the production of bioethanol or for fine chemicals in biorefineries. Lignocellulosic biomass typically contains more than 50 % sugar by weight, and valuable platform molecules such as levulinic acid and γ-valerolactone can be obtained therefrom. Next to the potential as platform chemicals to produce different derivatives, lactones represent important flavor compounds and find frequent application in the production of food. They can be produced from fatty acids, which are itself renewable resources. In the last decade, different hurdles of the application of microbial strains for the synthesis of lactones were found and overcome. Yields of more than 10 g L^{-1} were achieved, enabling economically feasible processes. Challenges regarding inhibitory or toxic effects of precursors or products within in microbial conversion were overcome by bioprocess engineering. In future, this story of success must be extended in view of the substrates applied. Here, new origins for precursors must be found, and downstream processing needs to be established. Next to the wild-type microorganisms, the exemplified BVMO processes illustrate the potential of recombinant microorganisms to achieve new products. However, most of these products are formed from substrates which are not originating directly from renewable resources. Here, further research and development needs to be carried out to broaden the range of biomass-derived substrates for lactone synthesis. In conclusion, the high selectivity and the synthesis of optically pure lactones is the outstanding property of microbial production processes. Applications range from potential bulk chemicals to high-value products that have the potential to complement other production processes in the biorefinery. They thus increase the added value and make an important contribution for the transformation of our industry into a truly sustainable, bio-based economy.

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