1 General introduction

1.1 Transfer to a sustainable and environmentally friendly industry

Fossil resources have been used for more than a century to produce platform chemicals for energy production and transportations [1]. The increasing wealth and world population (Table 1) causes an higher demand for energy, food, and transportation fuels [1-4]. However, fossil resources are limited and unequally distributed on earth, which can easily lead to conflicts between countries. The main drawback of a fossil-based industry, however, is the high emission of greenhouse gases, which contribute to global warming [5, 6]. The environmental crisis associated with the exploitive use of fossil resources represents a prominent challenge for humanity. The much-needed shift from the linear fossil-based economy to a circular biobased economy requires renewable feedstocks based on biomass for the production of everyday commodities [7, 8]. In such a scenario, the emitted carbon dioxide would be fixed by plants or dedicated microbes to generate biomass that once again could be used as feedstock for bioproduction processes. Thereby a closed carbon cycle would be established on a short-term basis [9, 10].

Table 1. World population and their development [2].

	Population (millions)			
Region	2017	2030	2050	2100
World	7750	8551	9772	11184
Africa	1256	1704	2528	4468
Asia	4504	4947	5227	4780
Europe	742	739	716	653
Latin America and the Caribbean	646	718	780	712
Northern America	361	395	435	499
Oceania	41	48	57	72

1.2 Organic acids as platform chemicals

Platform chemicals are small molecules that can be utilized as building blocks for the production of chemicals and materials of higher value [11]. In 2004, the U.S. Department of Energy (DoE) tested more than 300 chemicals with great biotechnological potential and identified twelve building block chemicals that could potentially be produced competitively from biomass [12]. The evaluation was organized in several rounds with different characteristics of the compounds. The criteria for selection of the top 12 (Table 2A) was based on the potential markets for the building blocks and their derivatives and the technical complexity of their synthesis pathways. Further, the molecules should have multiple functional groups that possess the potential to be transformed into new families of useful molecules. Among the top 12 building blocks, nine organic acids including itaconate, fumarate, malate, and succinate were listed [12].

Table 2. Building block chemicals. A) Top 12 building block chemicals assigned in 2004 [12] and B) Top 10 building block chemicals assigned in 2010 [13].

A	B
1,4-succinic, fumaric and malic acid	ethanol
2,5-furan dicarboxylic acid	furans
3-hydroxy propionic acid	glycerol and derivatives
aspartic acid	biohydrocarbons
glucaric acid	lactic acid
glutamic acid	succinic acid
itaconic acid	hydroxypropionic acid/aldehyde
levulinic acid	levulinic acid
3-hydroxybutyrolactone	sorbitol
glycerol	xylitol
sorbitol	
xylitol/arabinitol	

In 2010 the list was revisited using following criteria: 1) the compound or technology has received significant attention in literature; 2) the compound illustrated a broad technology applicable to multiple products; 3) the technology provides direct substitutes for existing petrochemicals; 4) the technology is applicable to high-volume products; 5) a compound exhibits strong potential as a platform chemical; 6) scale-up of the product or a technology to pilot, demo, or full scale is underway; 7) The bio-based compound is an existing commercial product, prepared at intermediate or commodity levels; 8) the compound may serve as a primary building block of a biorefinery; 9) commercial production of the compound from renewable carbon is well established [13]. Thus, the new criteria resulted in a new list of the top 10 building blocks (Table 2B). The current market and technology will always influence the request on molecules and thus change the order of the top platform chemicals. Glycerol, for example, has lost attraction, since biodiesel production increased, and crude glycerol was flooding the markets. As a result, companies that produced glycerol chemically lost competitiveness and had to close [14]. But the general desire for low-cost monomers to compete with petroleum-derived molecules will not change [15].

One group of chemicals which is prominently represented in the lists are organic acids. Alone nine of them were among the top 12 building blocks in 2004 [12], while still, five were present in 2010 [13]. This clearly demonstrates the great importance and potential of this group, which is also reflected by its constant appearance in literature [8, 16-25]. The special character of organic acids is that they can be naturally produced by a high number of microorganisms and that their functional groups can be transformed into new families of usable compounds and used as a starting compound for a broad range of applications [8, 20]. However, despite the good basic properties organic acids bring with them, the market size for microbially produced organic acids is small. Only a very limited number of economic processes such as whole-cell biocatalytic citric acid [26], lactic acid [27], D-gluconic acid [28], itaconic acid [29], 2-keto-L-gluonic acid [30, 31] and succinic acid production [32] are established, whereby citric acid is with more than 1 million tons per year the

most prominent product.
An optimized fermentation process at lab-scale does not necessarily imply industrial applicability. While certain consumer markets allow a bio-premium in the final price, e.g., Lego, the chemical commodities most often not. Hence, to establish an industrial process, optimizations are needed, which allow production at a lower price compared to the petrochemical route. This is truly challenging as the petrochemical industry is optimized for the last 70 years. One requirement is low-cost substrates like it is common for lactic acid production [8, 33-39]. Even reported repeated fed-batch fermentations or cell retentions systems, enabling high-titer production, do not find application in the industry since they are too complex and too costly [8, 40, 41]. Further, the downstream process plays a significant role in how profitable a process can be. For example, the most produced commodity from lactic acid is polylactide, which is used as low-value packaging material. This automatically means that the production of lactic acid must also be cheap in order for the process to be profitable, especially because the quality of the polymerized product depends on the purity of the lactic acid [42-44]. Moreover, in order to lower production costs, it is required to reduce the use of complex media components such as yeast extract as they lead to high impurities in the fermentation broth and to reduce the production of by-products. In summary, many parameters must be optimized simultaneously to enable a profitable process. In this context, the selection of the microbial host, carbon source, reactor type, process conditions, purification methods, and many other parameters must be carefully considered. However, the market on which the product should be placed is a major determinant, because it specifies the price which ultimately determines allowed production cost and thus the path that can be taken. In the following sections, more detailed information on citric-, succinic-, lactic-, malic-, and itaconic acid are given, whereby the focus is on itaconic acid due to its prevalent significance for this thesis.

1.2.1 Citric acid

Citric acid (Figure 1) (3-carboxy-3-hydroxypentane-1,5-dioic acid, 3-carboxy-3-hydroxypentanedioic acid, 2-hydroxy-1,2,3-propanetricarboxylic acid) (CAS 77-92-9) is a saturated, non-toxic, organic C6-tricarboxylic acid with a molecular mass of 192.12 g mol^{-1}. Its solubility in water is 147.76 g L^{-1} at 20 °C with pK_a-values of 3.13 (pK_{a1}), 4.76 (pK_{a2}), and 6.39 (pK_{a3}). Citric acid was discovered in 1784 by Carl Scheele and the first production was established by extraction from citrus fruits. Historically speaking, the production of citric acid by the filamentous fungus *Aspergillus niger* is the oldest known microbial process for high volume organic acid production [45-51].

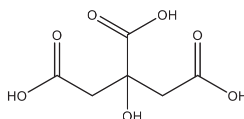

Figure 1. Chemical structure of citric acid.

The main use for citric acid is as a flavoring agent in the food industry. Other applications are as an acidifier and chelating agent in the chemical and pharma industry [17]. Since the demand was constantly increasing, the development was quickly pushed forward. In 1893 first accumulation of citric acid by microbes was observed and 1913 the first patent for citric acid production with *Aspergillus niger* was published [45, 51]. In 1917, a first fermentation process with *A. niger* was

established [46]. The rapid development leads that 90 % of the theoretical yield was reached [47-50]. Automatically, market size increased too. In 1998, the market volume was 879,000 tons [52] and increased further until 2007 to 1.6 million tons. In 2016, the citric acid market increased further and had a global market value of 2.5 billion USD and is estimated to reach 3.83 billion USD by 2025. [53, 54]. Thus, citric acid production is one of the largest biotechnological processes.
On the metabolic level, microbial production starts from pyruvate, which can be generated for example from glucose by glycolysis. Microbial production of citrate starts from pyruvate. In the mitochondrion, the pyruvate dehydrogenase complex converts one molecule pyruvate to one acetyl-CoA, and second pyruvate is carboxylated to oxaloacetate by the pyruvate carboxylase in the cytosol. Afterward, oxaloacetate is converted to malate and transported to the mitochondrion where it is converted by the citrate synthase together with acetyl-CoA to citrate, which is then transported out of the mitochondrion and subsequently out of the cell [55, 56].

1.2.2 Succinic acid

Succinic acid (Figure 2) (butanedioic acid, ethane-1,2-dicarboxylic acid) (CAS 110-15-6) is a saturated, non-toxic, organic, C4-dicarboxylic acid with a molecular mass of 118.09 g mol^{-1} . Its solubility in water is 58 g L^{-1} at 20 °C with pK_a-values of 4.2 (pK_{a1}) and 5.6 (pK_{a2}).

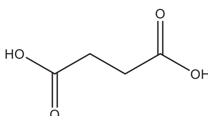

Figure 2. Chemical structure of succinic acid.

First isolation of succinic acid was reported by Georgius Agricola from amber [57]. In the beginning of industrial production, succinic acid was produced by fermentation but meanwhile, commercial production is via the petrochemical route [57, 58]. Especially because of its high production costs bio-based succinic acid could not assert itself so far [8]. Since it is part of the TCA-cycle, it was investigated in many different organisms like *Fusarium* [59] and *Aspergillus* [60] species, *Penicillium simplicissimum* [61], *Basfia succiniproducens* [62, 63] and yeasts, like *S. cerevisiae* [64] and *C. krusei* [65] and bacteria [57, 58, 66]. The current discussion is on succinate production from CO_2 [67]. Succinic acid is used for the synthesis of surfactant, detergent or foaming agent, as an ion chelator, in the food industry and for pharmaceuticals and antibiotics, and as a precursor for the production of different polymers, resins, and solvents [8, 20, 58]. Although a high number of various substances can be derived from succinic acid, the market size of approximately 131.73 million dollars in 2018 is very small [68]. This is mainly due to the high production costs that are incurred and empede its application although succinic acid would have the chemical potential to replace petroleum-derived maleic anhydride, which has a market size of 213,000 tons per year. Further substances that can be derived from succinic acid are depicted in Figure 3 [8].

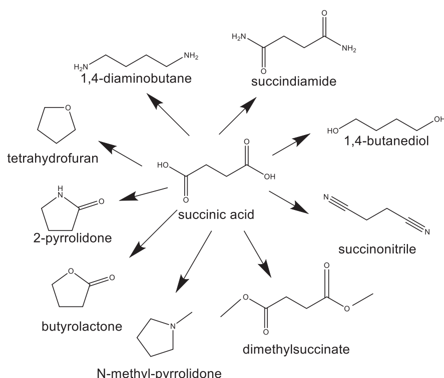

Figure 3. Various substances that can be derived from succinic acid by chemical conversion. The figure was adapted from Sauer *et al.* [8].

Metabolically, three different pathways for microbial production are known: Oxidative and reductive TCA-cycle and the glyoxylate bypass [17]. Advancing genetic tools and the ever-increasing gain of knowledge allow rational modifications within the metabolism to increase product formation in a metabolic engineering approach [69-72]. But also, applying non-rational methods like adaptive laboratory evolution, the production and tolerance could be further increased [73-75].

1.2.3 Lactic acid

Lactic acid (Figure 4) (2-hydroxypropanoic acid) (CAS 10326-41-7) is a non-toxic C3-organic acid with a molecular mass of 90.078 g mol^{-1}. Its solubility is so high that 1 part of lactic acid can dissolve 12 parts of water at 20 °C with pK_a-value of 3.86.

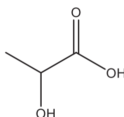

Figure 4. Chemical structure of lactic acid.

Lactic acid was discovered in 1780 by the Swedish chemist, Carl Wilhelm Scheele. He isolated it from sour milk. Up to 1857, it was assumed that lactic acid is a milk component until Pasteur discovered it as a microbial metabolite. Based on this discovery first fermentation and industrial production of lactic acid took place in 1881 in the United States of America [76]. Lactic acid finds many applications in food, pharmaceutical, cosmetic and chemical industry [44, 77]. The most important application is the polymerization of lactic acid to form polylactic acid, which is a polymer that can be produced from renewable resources and thus can replace petroleum-based consumables [78-80]. Lactic acid can be produced by chemical synthesis or by fermentation, whereby fermentation is preferred since it allows pure isomers and the use of renewable feedstocks [81]. In the last decades, lactic acid production increased continuously because more and more

new markets have been opened up. Alone in 2013, global lactic acid was estimated to be 714.2-kilo tons and it is expected to be 1,960-kilo tons by 2020 [82]. Further, global lactic acid production from microbial fermentation accounts for around 90% of total lactic acid production [81]. General lactic acid bacteria (LAB) such as *Lactobacilli* and *Carnobacterium* are used for the production. Further LAB are described in [81]. Depending on the final product, a distinction is made between homofermentative and heterofermentative. While homofermentative LAB convert glucose almost exclusively to lactic acid, heterofermentative LAB additionally form ethanol and CO_2 [81]. Furthermore, heterofermentative LAB can be subdivided in obligatory and facultative [81]. Further LAB have complex nutrient requirements especially because they are limited in synthesizing B vitamins and amino acids. Therefore, nutritionally rich media must be used in LAB fermentations [83]. But in literature also filamentous fungi such as *Rhizopus oryzae* and *R. arrhizus* are described as a natural producer. They utilize glucose aerobically to produce lactic acid and are well discussed in the literature [33, 84-89].

1.2.4 Malic acid

Malic acid (Figure 5) (hydroxybutanedioic acid or 2-hydroxysuccinic acid) (CAS 6915-15-7) has an asymmetric C-atom and thus occurs as L- (CAS 97-67-6) and as D-isomer (CAS 636-61-3). It is a saturated, non-toxic, organic, C4-dicarboxylic acid with a molecular mass of 134.09 g mol^{-1}. Its solubility in water is 558 g L^{-1} at 20 °C for DL-malic acid and 363.5 g L^{-1} for D or L-malic acid; its pK_a-values are 3.46 (pK_{a1}) and 5.10 (pK_{a2}).

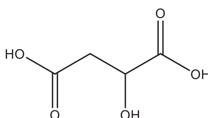

Figure 5. Chemical structure of malic acid.

In 1785, malic acid was isolated from apples, a source that was used for several decades [90]. It is mainly used in the beverage and food industry, but also in smaller amounts in metal cleaning, electroless plating, pharmaceuticals, infusions in hospitals, and in paints [18]. Due to high production costs, malic acid has a small market compared to other chemicals [8]. Approximately 40,000 tons malic acid were produced per year [18]. Yet until today, malic acid is mainly produced by hydration of maleic or fumaric acid resulting in a racemic mixture. Another option is the biotransformation of fumaric acid with immobilized cells expressing the enzyme fumarase. Depending on which organism is used yields for L-malic acid between 70 and nearly 100 % are possible [18, 91-94]. Since L-malic acid is an intermediate of the TCA-cycle, it is produced by all organisms. As is the case for many other organic acids, fungi are in focus of malic acid production, since they can naturally produce high titers [18, 91, 95-98]. Next to fungal production, also for some bacteria and *S. cerevisiae* strains malic acid production is reported [99-101]. Altogether there are four reported malic acid pathways: 1) TCA-cycle, 2) cytosolic rTCA-cycle, 3) cyclic glyoxylate pathway, and 4) non-cyclic glyoxylate pathway [99]. The biggest hurdle in microbial malic acid production is attaining sufficient product yields as yields are mostly far below theoretical, since always byproducts exist and the cumbersome purification from water, due to the high solubility [102].

1.2.5 Itaconic acid

Itaconic acid (Figure 6) (2-methylidenebutanedioic acid, 1-propene-2,3-dicarboxylic acid, methylenesuccinic acid) (CAS 97-65-4) is an unsaturated, non-toxic, organic, C5-dicarboxylic acid with a molecular mass of 130.1 g mol^{-1}. Its solubility in water is 83 g L^{-1} at 20 °C with pK_a-values of 3.84 (pK_{a1}) and 5.55 (pK_{a2}).

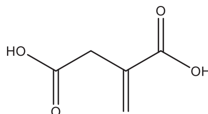

Figure 6. Chemical structure of itaconic acid.

First documentation about itaconate and its production was in the year 1837 [103]. The first production methods were based on chemical synthesis procedures [104-106]. Due to the high production costs involved in chemical synthesis, it was not possible to establish a foothold in the market [12, 107]. The first report about microbial production was published in 1931 with *Aspergillus itaconicus* [108] following by the first patent by Charles Pfizer Co. for the production with *A. terreus*. Over time, the fermentation process with *A. terreus* could be optimized, finally outcompeting the chemical process [109]. Thus since 1950 *A. terreus* is used for industrial itaconate production [110]. Due to its two functional groups, itaconate can be used versatile building block in the polymer industry. Radical polymerization and/or esterification with a wide range of co-monomers allow a rapidly expanding application range [25, 110]. Itaconate is mostly used as a co-monomer in the production of styrene-butadiene rubber and acrylate latexes, which are used in the paper and architectural industry [111]. Further polymerizations with acrylamide or methyl-methacrylate results in superabsorbent hydrogels for pharmaceutical applications [112-115]. Itaconate can be also polymerized with itself to obtain a polymer with high cation binding capacity, whereby the derivatives can be used as detergent additives, scale inhibitors, and dispersing agents [116]. Furthermore, itaconate is produced by mammalian macrophages where it plays a key role in the human immune response [117-119] against microbial pathogens, with possible applications as therapeutic agents for autoimmune diseases [120]. Further application can be found in Okabe *et al.* [110] or in Figure 7. Even though itaconate has so many potential applications, in 2011 it´s market size and value was only 41,400 t and $74.5 million, respectively [23, 111]. This is caused by the high price with around two dollars per kilogram and is an exclusive criterion for further market penetration. To be competitive against petrol-based products, costs need to reduce to around $0.5 per kg [121]. Assuming, that the price would decrease, itaconate has the possibility to replace polyacrylic acid whose production is petroleum-based and has a market worth of $11 billion [23, 107, 110].

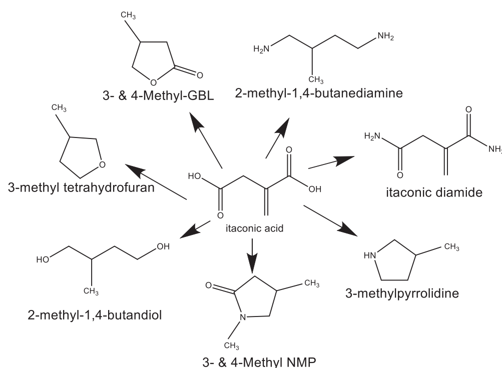

Figure 7. Derivatives of itaconate. The figure was adapted from Werpy [12].

To reduce the price of itaconate, more improvements are necessary. Certainly, fermentation conditions and especially the downstream process influence the price of the product [8]. But also used microorganism and C-sources influence the whole process, which in turn has an impact on the price. Unquestionably, many other factors such as location and energy costs play a role, in the following sections, only the influence of microorganism, fermentation, and downstream process is discussed.

1.2.5.1 Microbial hosts and metabolism for itaconate

Currently, *A. terreus* is used for industrial itaconic acid production. First, *A. terreus* strains producing itaconate were isolated in 1935 [122]. A screen of more than 300 strains in 1945 resulted in *A. terreus* NRRL 1960 as the best-producing strain, which was used for industrial itaconate production by Pfizer Co. in Brooklyn, NY, USA [106, 123]. Further factories were founded in England, Japan, and France. To reduce production costs facilities are nowadays located in the Asia-Pacific region [110, 111]. Next to *A. terreus*, also Ustilaginaceae such as *Pseudozyma* and *Ustilago* species, [95, 124-127] *Rhodotorula,* [128] *Candida* [127, 129], and *Helicobasidium* [130] species are known to produce itaconate. An overview of itaconate producing wildtype strains including titer, yield, and productivity is given in Table 3. Although the production parameters of the other strains such as *Ustilago* are so far apart, the search for new production organisms continues. Despite the long history and experience, itaconate production in *A. terreus* remains challenging and will be discussed in the next sections. Next, to the filamentous *A. terreus*, the yeast-like growing *U. maydis* is well studied and is a naturally itaconate producing organism.

Table 3. Itaconate production of wildtype strains generated by random mutagenesis [21].

Microorganism	Substrate	Itaconate (g L^{-1})	Yield (g_{ITA} g_{GLC}^{-1})	Productivity (g L^{-1} h^{-1})	Reference
A. terreus DSM 23081	glucose	129	0.58	1.15	[131]
A. terreus NRRL 1960	glucose	130	-	-	[22]
A. terreus DSM 23081	glucose	160	0.46	1	[132]
A. terreus SKR10 N45[a]	hydrolyzed corn starch	50	0.42	0.35	[133]
A. terreus R104[a]	glucose	52.7	0.72	0.55	[134]
A. terreus IFO-6365 TN-484[a]	glucose	82.3	0.54	0.57	[135]
Candidia sp.strain B-1[a]	glucose	35	-	0.29	[129]
Helicobasidium sp.	-	-	-	-	[130]
P. antarctica NRRL Y-7808	glucose	30	0.38	-	[124]
P. tsukubaensis H488	glucose	74.7	0.49	0.36	[125]
U. cynodontis K470	glucose	28.4	-	-	[136]
U. maydis DSM 17144	glucose	44.5	0.24	0.31	[137]
U. rabenhorstina IFO 8995	glucose	15.7	-	-	[136]

[a] Strains generated by random mutagenesis

In both organisms, the genes enabling itaconate biosynthesis are clustered and co-regulated [138, 139]. The biosynthetic pathway starts with the transport of *cis*-aconitate from the mitochondria to the cytosol by a mitochondrial tricarboxylate transporter, encoded by *Um_mtt1* or *At_mttA* [25, 139, 140]. Both transporters are assumed to do so by antiport exchange with cytosolic malate [141]. In *U. maydis*, this transport poses the rate-limiting step in itaconate production, since overexpression of *mtt1* leads to a strong increase in itaconate production [139]. In *A. terreus*, the cytoplasmic *cis*-aconitate is converted directly to itaconate by a cytosolic *cis*-aconitate decarboxylase (*cadA*) [142-144]. In contrast, *U. maydis* first isomerizes *cis*-aconitate to *trans*-aconitate by a cytosolic aconitate-δ-isomerase (Adi1). This *trans*-aconitate is subsequently decarboxylated to itaconate by a *trans*-aconitate decarboxylase (Tad1) [139]. *U. maydis* can further convert itaconate to (*S*)-2-hydroxyparaconate by an itaconate P450 monooxygenase (Cyp3). The latter is the lactone of L-itatartarate, which is also found in the supernatants of *U. maydis* [126, 136, 140]. This was also reported for some *A. terreus* strains [126, 145], likely through a similar P450 enzyme encoded by the *cypC* gene directly adjacent to the itaconate gene cluster [140, 146]. Interestingly (*S*)-2-hydroxyparaconate formation was not reported for *A. terreus* production strains [132, 147], maybe because these strains were selected by screening for high itaconate production leading to the selection of a defect *cyp*C expression. Further in both organisms itaconate is transported out via a major facilitator superfamily transporter, encoded by *Um_itp1* and *At_ mfsA*. Because both products (itaconate and (*S*)-2-hydroxyparaconate) are produced *via* the same pathway, and the gene clusters of both organisms only contain one gene encoding a cytosolic exporter, it is reasonable to assume that the same protein secretes both products. Metabolism and

cluster structure are depicted in Figure 8. This knowledge about metabolism allowed optimization by strain engineering in *Ustilago* and *Aspergillus* but also in other strains such as *E. coli*. Some examples are given in Table 4.

Table 4. Itaconate production of genetically modified organism. [21].

Microorganism	**Substrate**	**Itaconate ($g\ L^{-1}$)**	**Yield ($g_{ITA}\ g_{GLC}^{-1}$)**	**Productivity ($g\ L^{-1}\ h^{-1}$)**	**Reference**
A. niger AB1.13 CitB#99	glucose	26.2	0.37[b]	-	[148]
A. terreus A729	glucose	45.5	-	-	[149]
C. glutamicum AO-2/pEKEx2-malEcadopt	glucose	7.8	0.29	0.16	[150]
E. coli ita23	glucose	32	0.49[b]	0.27	[151]
S. cerevisiae	glucose	0.168	-	-	[152]
Synechocystis sp. PCC6803	CO_2	0.0145	-	-	[153]
U. maydis MB215 Δ*cyp3* P_{etef}*ria1*	glucose	63.2	0.23[b]	0.38	[140]
Y. lipolytica	glucose	4.6	0.058	0.045[a]	[152]

[a] Maximum productivity
[b] Itaconate per consumed glucose

Despite the possibilities given by metabolic engineering, still *A. terreus* is a highly efficient filamentous fungus achieving nearly theoretical yields and titers over 100 g L^{-1} at low pH values at suitable conditions [21, 131, 132].

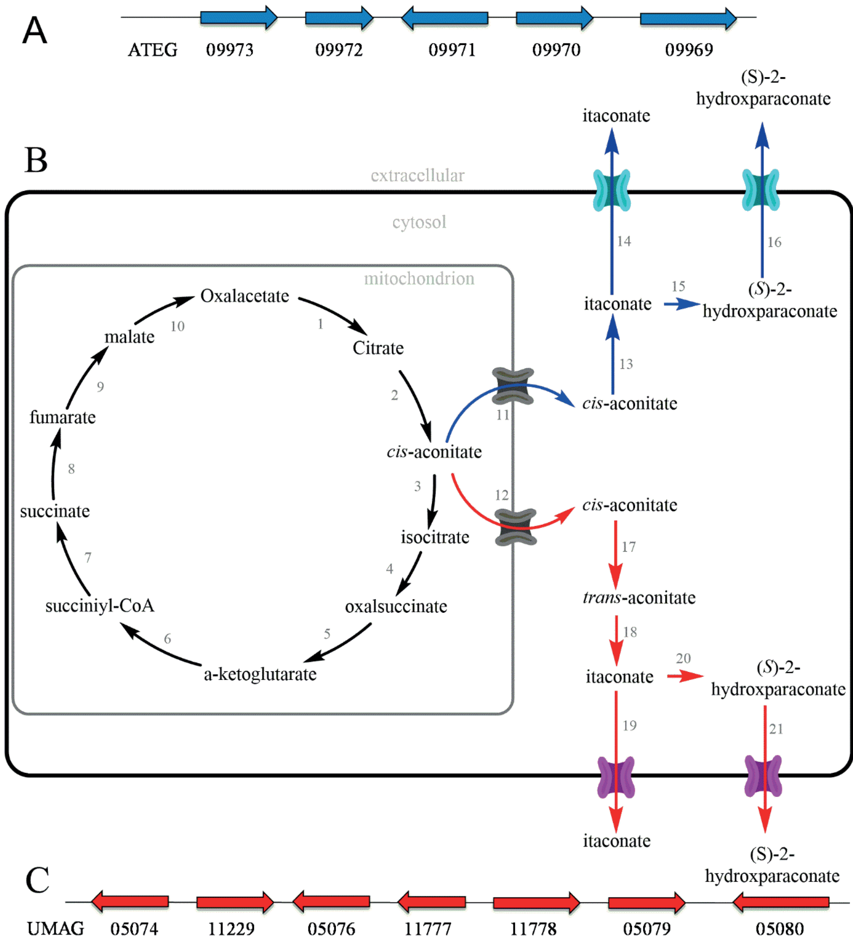

Figure 8. Itaconate metabolism in *U. maydis* and *A. terreus*. Itaconate cluster of *A. terreus* (blue) (A): ATEG_09973 (*cypC*), ATEG_09972 (*mfsA*), ATEG_09971 (*cadA*), ATEG_09970 (*mttA*) and ATEG_09969 (*iaR*); TCA-cycle including metabolic steps for itaconate in *A. terreus* (blue) and *U. maydis* (red) (B): citrate synthase (1), aconitase(2, 3), isocitrate dehydrogenase (4, 5), a-ketoglutarate dehydrogenase (6), succinyl-CoA synthetase (7), succinate dehydrogenase (8), fumarase (9), malate dehydrogenase (10), citrate malate antiporter (11,12), *cis*-aconitate decarboxylase cad (13), major facilitator superfamily protein *mfsA* (14, 16), P450 monoxygenase *cypC* (15), aconitate- δ -isomerase *adi1* (17), *trans*-aconitate decarboxylase *tad1* (18), P450 monooxygenase *cyp3* (20) and itaconate transport protein *itp1* (19, 20); Itaconate cluster of *U. maydis* (red) (C): UMAG_05074 (*cyp3*), UMAG_11229 (*rdo1*), UMAG_05076 (*tad1*), UMAG_11777 (*itp1*), UMAG_11778 (*adi1*), UMAG_05079 (*mtt1*) and UMAG_05080 (*ria1*). Pathway of *A. terreus* was adapted from Steiger *et al.* [25].

1.2.5.2 Itaconate production

To be competitive, the productivity of itaconate production should be 2.5 g L^{-1} h^{-1} [12], this is far from best published values, since *A. terreus* has a long fermentation time of up to 45 days [21, 110]. The morphology of *A. terreus* ranges from branched to densely mycelium masses [154] and efficient itaconate production is only achieved in a pellet form with a diameter smaller < 0.5 mm [23, 155, 156]. Thus, itaconate production is morphology-dependent in *A. terreus*. Hence, the control of morphology plays a major role in *A. terreus*; especially it is influenced by many factors. In contrast, most Ustilaginaceae such as *U. maydis* produces itaconate with a stable yeast-like growth behavior [95, 107, 137]. For many medium components like iron, zinc, calcium, cobalt, nickel and especially manganese ions it is reported that they influence the morphology of *A. terreus* above a specific concentration or even inhibit key enzymes involved in itaconate production [22, 147, 156-160]. This represents a hurdle to decrease production costs with second generation feedstock, such as (hemi)cellulosic fraction of wood, which contains a mixture of these elements. This causes additional costs since the substrate has to be pretreated and if necessary, to purified from the fermentation medium [157, 161]. Further, low pH values are necessary for mycelium growth, otherwise, no itaconate is produced. It is assumed that low pH values induce relevant enzymes for itaconate production [162]. Meanwhile, different pH strategies are established, resulting in different titers, rates, and yields [131, 147]. Currently, highest titer with 160 g L^{-1} is achieved under pH control at 3.4 in the production phase [132]. For purification usually, itaconate is crystallized by evaporation-crystallization systems. Different strategies were established to achieve high quality and purity of itaconate [161, 163-169]. To reduce costs and purification steps coming from by-product formations and impurities from substrates, reactive extraction can be used. Since *A. terreus* react sensitively against solvents, this method is in their infancy [107, 170]. Despite the drawbacks associated to the morphology of *A. terreus*, *A. terreus* has been asserting itself for 60 years as itaconate producing strain. Especially the fact that morphology is a burden to improve itaconate production, new organisms should be established featuring a robust non-filamentous phenotype that is insensitive towards feedstock impurities and thus allows itaconate production in an efficient way. In the past, members of the Ustilaginaceae have particularly proven themselves to be good organic acid producers [95]. Mainly because of their versatile range of value-added chemicals, they became interesting for the biotechnology industry [126, 171, 172]. Since most of the members of Ustilaginaceae are plant pathogens, they were mostly investigated in terms of pathogenicity. They mainly infect crops, such as maize, barley, corn, wheat, oats, sorghum, and sugar cane [173]. They are responsible for high crop losses in the agricultural industry and cause a great deal of economic damage. The most prominent member in terms of itaconate production is *U. maydis* [171]. *U. maydis* is a well-established model organism for studies of biotrophic plant-pathogen interaction [171, 174-176], cell biology, DNA repair, mRNA transport and molecular methods and techniques [177-180]. In 2006, the genome sequence was fully annotated which was the basis for further developments applying genetic engineering [181, 182] such as, FLP/FRT system [183], Golden Gate Cloning [184] and CRISPR/Cas9 system [185]. Further, strong and constitutive promoters (P*otef*, P*oma*) [186-188] and inducible promoters (*crg1, nar1, mig, prf1*) [189-191] were established and antibiotics (carboxin, hygromycin, nourseothricin, phleomycin, geneticin) with their corresponding resistance cassettes are available [182, 192-196]. This development also made it possible to establish *U. maydis* as an itaconate producer. The highest published titer for itaconate of 63 g L^{-1} so far was published by Geiser *et al.* [140]. However, especially its yeast-like growth behavior, resistance against osmotic pressure,

hydromechanical stress, impurities, and its tolerance against high product concentrations, give *Ustilago* a few advantages over *Aspergillus* [107, 171, 197]. Further, it can utilize several carbon sources such as, glucose, glycerol, xylose, xylan, CM-cellulose, and homogenized plant tissue [137, 171, 197-200]. So far *U. maydis* has not yet been able to assert itself against *Aspergillus*. Although *U. maydis* has so many advantages, reached titers were still low compared to *Aspergillus* and production was limited to pH values above 5. Lower pH values enable reduced base consumption, easier downstream processing, and autosterility, which help to reduce the costs [8, 201]. But also members of Ustilaginaceae such as *U. cynodontis* are pH-tolerant organisms and able to produce itaconate under low-pH conditions. [95]. Thus, *U. cynodontis* can be used as a pH-tolerant production strain. It is also resistance against osmotic pressure, impurities, and is tolerance against high product concentrations. Additionally, experience from *U. maydis* such as medium composition and fermentation condition can be transferred from *U. maydis* to *U. cynodontis*. However also, further improvement in *U. maydis* is possible, concerning the yield and the fermentation process, especially its yeast-like growth makes it favorable in large-scale fermentations [24, 140].

1.3 Scope and outline of this thesis

The overall aim of this thesis was to establish efficient itaconate production hosts by strain engineering and optimization of the fermentation process in the yeast-like *Ustilago maydis* and pH-tolerant *Ustilago cynodontis* to enable a sustainable and bio-ecological process. This chapter 1 is a general introduction and provides the necessary information for a bio-based industry and the importance of building block chemicals, especially of the organic acids in terms of market potential and applications. This includes advantages and disadvantages of itaconate production in *A. terreus*, *U. maydis* and *U. cynodontis* and their importance as microbial catalysts.

To enhance itaconate production in other Ustilaginaceae such as *U. cynodontis*, *U. xerochloe*, and *U. vertiveriae* responsible gene cluster for itaconate biosynthesis were investigated in 13 strains from seven species in chapter 3.1. The sequences of the gene cluster for itaconate synthesis were analyzed and compared to the cluster of *U. maydis*, and the phylogenetic relationship of the itaconate cluster transcription factor of Ria1was investigated. The itaconate gene cluster of *U. cynodontis* was further investigated in chapter 3.3.
Chapter 3.2 investigates the roles of the extracellular and mitochondrial transporters which are involved in itaconate production in *U. maydis* and *A. terreus*. In complementation studies and systematic cultivation, it could be shown that involved transporters had different product affinities. This knowledge enabled us to boost itaconate production remarkably in production strains generated in chapter 3.3 and 3.5.
In chapter 3.3, the strong filamentously growing and pH-tolerant *U. cynodontis* was established as itaconate production strain. Since no molecular tools were available, they were established based on *U. maydis* plasmids and methods. After that morphological engineering was applied to modify the strong filamentous growth into stable yeast-like growth. Responsible genes for filamentous growth were determined by BLAST and compared to *U. maydis*. The deletion of these genes resulted in stable yeast-like growth under process-relevant conditions. For further enhancement of itaconate production in *U. cynodontis* by metabolic engineering, the clarification of the genes belonging to the itaconate cluster, which was annotated in chapter 3.1 was necessary. These findings allowed to increase *U. cynodontis* itaconate production up to 6.5-fold compared to the wildtype. Furthermore, first fermentation experiments confirmed stable yeast-like behavior in bioreactor systems.
In chapter 3.4, production strains that were generated in chapter 3.3 were further investigated in bioreactors. Itaconate production with *U. cynodontis* could be further improved by determination of the optimal pH value combined with process optimizations and different feeding strategies. Thereby titer, rate, and yield could be increased drastically for itaconate.
In chapter 3.5, we could combine recently published metabolic engineering strategies with discovered morphological improvements from chapter 3.2 and 3.3 in *U. maydis*. This powerful combination led to the highest produced itaconate titer achieved in a biotechnological process.
This thesis clearly demonstrates the potential of the pH-tolerant *U. cynodontis* and yeast-like *U. maydis* as itaconate production organisms. Building on previous works, significant increases were achieved, and it was confirmed that *Ustilago* is on the right track to make the itaconate production process more sustainable and cost-effective. Based on the results, further optimizations should be continued in the future in order to achieve the primary goal of an environmentally friendly and sustainable process.