1 Introduction and Objectives

1.1 Introduction

Our industrialized world is driven by using fossil resources like petroleum. Mankind's elevated and thriving demand of these non-renewable resources depletes their availability and can lead to shortages with incalculable consequences for our communities and life. Furthermore, the use of fossil resources in our daily life yields a carbon dioxide fingerprint in every single production process of commercial goods or means of transportation (Becker and Wittmann, 2015). This led to elevated carbon dioxide concentrations in our atmosphere (Crutzen, 2002; Dlugokencky and Tans, 2017). Carbon dioxide, as a greenhouse gas, can influence our climate significantly, leading to deviations from its natural behavior (Crutzen, 2002; UNEP, 2017).

To face these challenges and reduce the dependence on fossil resources as well as greenhouse gas emissions, the recent years have seen a tremendous increase in the sustainable production of chemicals and commercial goods through biotechnology processes (Becker et al., 2015; Werpy et al., 2004). Particularly, the production of amino acids (Becker and Wittmann, 2012b) and organic acids (Becker et al., 2015) plays a key role in the development of a bio-based community.

To broaden this further, the US Department of Energy published a candidate list of 12 top value chemicals, which can be derived through biotechnology processes (Werpy et al., 2004). This list comprises carbon-three to carbon-six chemicals, which are grouped into organic acids, amino acids, (sugar) alcohols and lactones. Organic acids are depicted as the bulk fraction of these platform chemicals (**Table 1.1**).

In detail, 3-hydroxypropionate (C_3), the 1,4-diacids succinate, fumarate, malate (C_4), itaconate, levulinate (C_5), 2,5-furandicarboxylate and glucarate (C_6) are mentioned. In particular, amino acids like aspartate (C_4) and glutamate (C_5) are also identified for application in chemical industry. Furthermore, (sugar) alcohols, like the commonly known glycerol (C_3), a side product of biodiesel industry (Yang et al., 2012), and xylitol (xylose, C_5), arabitol (arabinose, C_5) and sorbitol (sorbit, C_6) are listed (Werpy et al., 2004).

Table 1.1. List of top value chemicals comprising compound details and potential applications. Applications are divided in nine categories e.g. industrial. All categories name specific applications e.g. transportation (fuels, anti-freeze, …). Detailed information is listed in Werpy et al., 2004.

Compound	Compound class	Number of carbon atoms	Derivatives of interest	Application
3-Hydroxypropionate	Organic acid	3	Acrylates, Acrylamides, Esters, 1,3-Propanediol, Malonate	Safe Food Supply, Industrial, Environment, Housing
Glycerol	Sugar alcohol	3	Propylene glycol, Malonate, 1,3-Propanediol, Diacids, Propylalcohol, Dialdehyde, Epoxides	Housing, Industrial, Transportation, Safe Food Supply
Succinate	Organic acid	4	Tetrahydrofuran, 1,4-Butanediol, γ-Butyrolactone, Pyrrolidones, Esters, Diamines, 4,4-Bionelle, Hydroxybutyricacid	Industrial, Textiles, Environment, Transportation, Safe Food Supply, Communication
Aspartate	Amino acid	4	Amino succinate derivatives, Polypyrrolidones	Industrial, Environment, Health and Hygiene
Fumarate	Organic acid	4	Unsaturated succinate derivatives	Transportation, Safe Food Supply, Communication, Housing
Malate	Organic acid	4	Hydroxysuccinate derivatives, Hydroxybutyrolactone	Industrial, Textiles, Environment
Itaconate	Organic acid	5	Methyl succinate derivatives, Unsaturated esters	Transportation, Housing, Textiles, Recreation
Levulinate	Organic acid	5	δ-Aminolevulinate, 2-Methyl Tetrahydrofuran, 1,4-Diols, Esters, Succinate	Industrial, Environment, Health and Hygiene, Textiles, Recreation, Housing
Glutamate	Amino acid	5	Amino diols, Glutarate, Pyrrolidones	Industrial, Textiles, Environment, Safe Food Supply, Health and Hygiene
Xylitol	Sugar alcohol	5	Ethylene glycol, Propylene glycol, Glycerol, Lactate, Hydroxyfurans, Sugar acids	Industrial, Transportation, Safe Food Supply
Arabitol	Sugar alcohol	5		
2,5-Furandicarboxylate	Organic acid	6	Succinate, 2,5-Furan derivatives containing hydroxyl or amino groups	Safe Food Supply, Industrial, Textiles
Glucarate	Organic acid	6	Dilactones, Monolactones, Other products	Industrial, Environment, Health and Hygiene, Housing, Recreation
Sorbitol	Sugar alcohol	6	Ethylene glycol, Propylene glycol, Glycerol, Lactate, Isosorbide	Transportation, Housing, Safe Food Supply, Communication, Recreation,

The potential to convert these platform chemicals into industrial applicable chemicals is huge. A brief overview over the latter comprises: acrylates, acrylamides, 1,3-propanediol, 1,4-butanediol, tetrahydrofuran, γ-butyrolactone (GBL) and polymers like polyhydroxypolyamides. A demonstrative valuable application is the polymerization of acrylate and acrylamides, which can be produced via 3-hydroxypropionate (**Figure 1.1 A**). These compounds can be crosslinked to copolymers which have a very high water absorbency (Liu and Rempel, 1997). This technique is nowadays used in diapers and soft contact lenses. Furthermore, succinate, a metabolite of the tricarboxylic acid cycle in all organisms (Fernie et al., 2004), can be converted to commodity chemicals like tetrahydrofuran or γ-butyrolactone (GBL) (**Figure 1.1 B**). Notably, the latter has a broad application, in industry as plasticizer (McKinlay et al., 2007), as a valuable precursor for pharmaceuticals (Choi et al., 2013) or as a commonly known anaesthetic agent (Lenz et al., 2008).

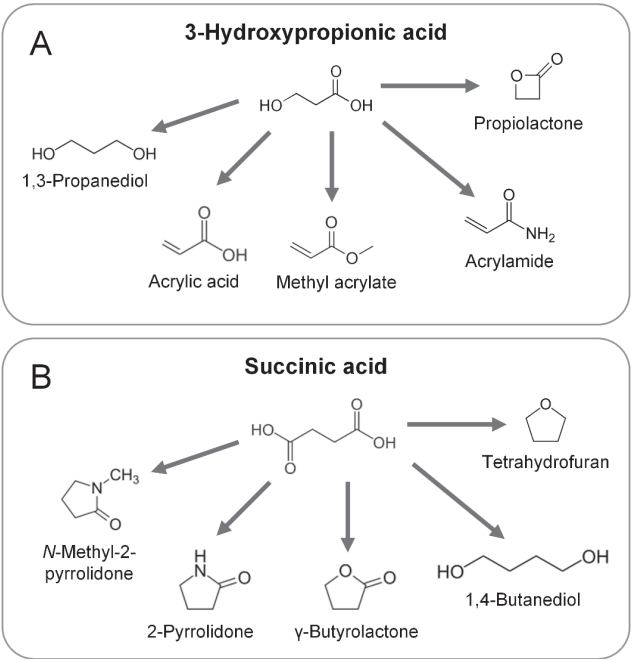

Figure 1.1. Overview of industrial important products derived from platform chemicals. The carbon-three compound 3-hydroxypropionate is used for the production of e.g. acrylates, which are processed to superabsorbent polymers among others (**A**). The carbon-four diacid succinate is processed into solvents, e.g. 1,4-butanediol or tetrahydrofuran (**B**).

1.2 Objectives

The objective of the present work was to broaden the use of *B. succiniciproducens* and derive carbon-three compounds. In particular, the heterologous production of L-alanine, β-alanine, and 3-hydroxypropionate from β-alanine, should be evaluated. Up to date, little is known about the ability of *B. succiniciproducens* to express heterologous genes, in particular suitable promoters, which can be deployed for achieving high expression levels. At first, enhancing the genetic toolbox was targeted. Therefore, set-up of a blue-white screening system in a *lacZ* deficient strain was done. Furthermore, suitable promoters for heterologous expression of various genes should be identified. The second step aimed at the utilization of appropriate genes and gene clusters for heterologous synthesis of the target products. Producing strains should then be developed and improved step-wise, using systems metabolic engineering. Finally, suitable process conditions for overproduction should be identified and applied.

2 Theoretical Background

2.1 *Basfia succiniciproducens* – a novel industrial workhorse

The identification of succinate as a top value added chemical by the US DoE, initialized a rapid and parallelized run for commercialization of bio-based succinate. This led to the discovery of *B. succiniciproducens* DD1 (Kuhnert et al., 2010; Scholten and Dägele, 2008), isolated from the rumen of a Holstein cow (Kuhnert et al., 2010). Subsequent investigation generated succinate overproducers during the last decade (Becker et al., 2013; Lange et al., 2017; Scholten and Dägele, 2008; Scholten et al., 2009; Stellmacher et al., 2010). The microbe can utilize a variety of feed stocks (Kuhnert et al., 2010), including glycerol (Scholten and Dägele, 2008). Glycerol depicts a bulk by-product in biodiesel industry (Yang et al., 2012), make it a cheap and common used substrate.
Alongside, transcriptomics and fluxomics were used to elucidate its unexplored metabolism and identify targets for metabolic engineering (Becker et al., 2013; Scholten et al., 2009; Stellmacher et al., 2010). In particular, gene knockouts (Becker et al., 2013) or homologous gene expression (Lange et al., 2017) were investigated for succinate overproduction. Taken together, this provides a solid start point for further metabolic engineering of *B. succiniciproducens*.

2.1.1 The distinct metabolism and physiology of *Basfia succiniciproducens*

The first isolate of *B. succiniciproducens*, named DD1, was enriched from the bovine rumen of a Holstein cow, while screening for native succinate producers (Scholten and Dägele, 2008). Seven strains were isolated (Kuhnert et al., 2010) and taxonomically assigned to the family *Pasteurellaceae* by assessing the 16S rRNA, *rpoB*, *infB* and the *recN* gene sequences. Distinct phenotypical aspects of the *Pasteurellaceae* family include a capnophilic, facultative anaerobic, non-spore-forming, and non-motile lifestyle. The cells are gram-negative and coccoid to rod-shaped (Kuhnert et al., 2010). The *Pasteurellaceae* family comprises primary or opportunistic pathogens of the respiratory and genital tract of vertebrates (Dousse et al., 2008; Guettler et al., 1999). However, the isolated strain DD1 is neither toxic nor pathogenic against bovine, human or fish cell lines (Kuhnert et al., 2010). This is beneficial for development of industrial relevant processes, by reducing costs and handling risks. Additionally, only a few

vitamins are essential for the strain (Hong et al., 2004), allowing minimal medium cultivation and consequently exact investigations of phenotypic aspects.

The genome sequence of *B. succiniciproducens* DD1 comprises 2.34 Mbp with 2363 open reading frames (ORFs) (Kuhnert et al., 2010). It is related (95 % on DNA and amino acid sequence level) to *Mannheimia succiniciproducens* MBEL55E, another prominent succinate producer (Lee et al., 2002). Of the 2380 ORFs found in the *M. succiniciproducens* genome, 2006 ORFs are homologous to *B. succiniciproducens* and might be regarded as a core genome (Kuhnert et al., 2010).
The central carbon metabolism of *B. succiniciproducens* DD1 (**Figure 2.1**) is composed by the Embden-Meyerhof-Parnas (EMP) pathway, and fueling the pentose phosphate (PP) pathway. Furthermore, the gluconeogenesis is found, which uses malic enzyme (*sfcA*), malate dehydrogenase (*mdh*), phosphoenolpyruvate carboxykinase (*pckA*) and fructose-1,6-bisphosphatase (*fbp*, *glpX*) to bypass the irreversible reactions of EMP pathway. The Entner-Doudoroff (ED) pathway, connected to gluconate metabolism, is found inactive (Becker et al., 2013). Several (carbohydrate) transporters, comprising phosphotransferase systems and ABC transporters, are annotated, which obviously mediated succinate production from different substrates (glycerol, sucrose, glucose, fructose, xylose, arabinose, galactose and mannose) (Scholten and Dägele, 2008). Due to its niche lifestyle in the bovine rumen, the observed efficient succinate formation from different feedstocks evolved by natural selection. The role of *B. succiniciproducens* in the bovine rumen is specialized as a part of the hosts microbial digestion, in a symbiotic manner. Its broad substrate spectrum enables conversion of plant carbohydrates from the bovine feed into succinate. Succinate is subsequently decarboxylated into propionate by the microbial rumen flora. The latter is then utilized by the host, underlining the importance of *B. succiniciproducens* as a member of the rumen flora (Guettler et al., 1999). Due to the specialized function of the rumen, the phenotypical succinate production is furthermore found in other rumen bacteria, e.g. *M. succiniciproducens* (Lee et al., 2002) and *Actinobacillus succinogenes* (Guettler et al., 1999). Additionally, acetate, formate and lactate and ethanol are the major and minor by-products of the metabolism, respectively (Becker et al., 2013).

Recently, metabolic flux analysis (Becker et al., 2013) allowed first insights into the specialized metabolism of *B. succiniciproducens*. High yield succinate production is mainly achieved through the reductive branch of the TCA cycle, whereas the contribution of the oxidative branch is negligible (**Figure 2.1**). The latter is a consequence of the beneficial incorporation of CO_2, while ATP is formed. This step is conducted by the anaplerotic ATP-dependent phosphoenolpyruvate carboxykinase (**Figure 2.1**), fueling the reductive branch of the TCA cycle (Becker et al., 2013). The enzyme links CO_2 incorporation to energy generation and cell growth. This was demonstrated by *pckA* knockout studies in *M. succiniciproducens* (Lee et al., 2006). As suggested for *M. succiniciproducens*, it appears likely that fumarate functions as an electron acceptor (Hong et al., 2004).

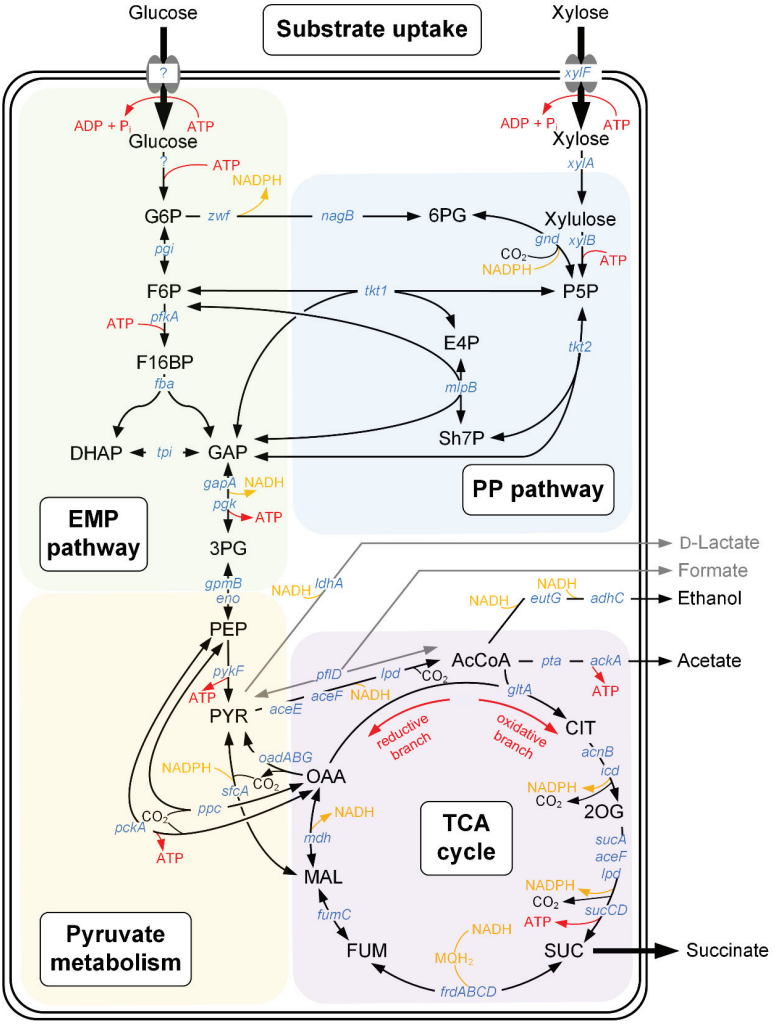

Figure 2.1. Central carbon metabolism of *B. succiniciproducens* DD1. Substrate carbon is taken up and channeled through the core metabolism by the EMP (glycolysis) and pentose phosphate (PP) pathway. Specific reactions at the pyruvate node and in the TCA cycle act reversible. The genes, encoding enzymes which catalyze the interconversions, are given in *italics*. Reactions corresponding to energy conversion are depicted in red, while redox reactions are indicated in orange. Gray arrows indicate reactions, which are eliminated in the used strain of the present study: *B. succiniciproducens* Δ*ldhA* Δ*pflD* (DD3). Question marks (?) denote unidentified genes. Abbreviations are: G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; P5P, pentose 5-phosphate; Sh7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; F16BP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; 3PG, 3-phospho glycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; AcCoA, acetyl-CoA; CIT, citrate; 2OG; 2-oxoglutarate; SUC, succinate; FUM, fumarate; MAL, malate; MQH_2, menaquinol; ATP, adenosine triphosphate; NADH, nicotineamide adenine dinucleotide, reduced; NADPH, nicotineamide adenine dinucleotide phosphate, reduced.

2.2 The C_4 platform chemical succinate – an industrial production accessible using *Basfia succiniciproducens*

Since biotechnological production of acetate and citrate, back in 1823 (Raspor and Goranovič, 2008) and 1913 (Zahorski, 1913), organic acids belong to the veterans of bio-based goods (Alonso et al., 2014; Becker et al., 2015). Beyond traditional uses in feed and food, particularly the renaissance of bio-plastics has pushed bio-production of organic acids as bi-functional monomers. Acids with additional keto- or hydroxyl-groups are desirable building blocks for polyesters, and di-carboxylic acids are used for the production of polyamides with advanced material properties (Becker and Wittmann, 2015). Recently, succinate, an intermediate of the tricarboxylic acid (TCA) cycle, evoked a strong research interest for its bio-based production. This carbon-four 1,4-diacid provides a basic chemistry, similar to the petrochemically derived maleic acid/anhydride, which is beneficial for manufacturing biopolymers or solvents like butanediol, maleic anhydride and nylon-type polymers (Beauprez et al., 2010; Becker et al., 2015; Werpy et al., 2004).

Beside the biotechnology workhorses *Escherichia coli* and *Corynebacterium glutamicum*, members of the *Pasteurellaceae* family appeared promising for succinate production (Guettler et al., 1999; Lee et al., 2002; Scholten and Dägele, 2008). *A. succinogenes* (Pateraki et al., 2016), *M. succiniciproducens* (Kim et al., 2017) and *B. succiniciproducens* (Becker et al., 2013; Cimini et al., 2016) revealed their potential in bio-based succinate production. These bacteria are to date well-established industrial bio-succinate producers with a broader substrate spectrum (Dousse et al., 2008; Guettler et al., 1999; Kim et al., 2004; Scholten and Dägele, 2008).

The intensive research in this field contributed to our current understanding of microbial succinate fermentation and its optimization (Becker et al., 2015). In brief, reviewed rational strategies exploited the elimination of by-product formation (Becker et al., 2013; Cheng et al., 2013; Litsanov et al., 2012) and amplification of the anaplerotic flux toward the reductive TCA cycle branch (Cheng et al., 2013; Litsanov et al., 2012). Additionally, renewable substrates like lignocellulosic derived xylose (Salvachúa et al., 2016) and sucrose (Lange et al., 2017) were considered, recently.

As example, Lange et al. (2017) demonstrated the powerful use of ^{13}C metabolic flux analysis for metabolic engineering of *B. succiniciproducens* for efficient sucrose utilization. The overexpression of a newly discovered fructokinase (*rbsK*), the deletion of the competing fructose PTS and the combination of both strategies led to synergistic improvements in succinate production from sucrose. It was demonstrated, that *B. succiniciproducens* DD1 Δ*fruA*, lacking the fructose PTS, produced 71 g L^{-1} succinate at a yield of 2.5 mol mol^{-1}. Apparently, a 12 % improvement of the succinate titer was achieved, compared to the parent strain *B. succiniciproducens* DD1. An overview of the most relevant native and recombinant microbial succinate production hosts is given in **Table 2.1**.

Table 2.1. Succinate production performance of native and recombinant microbial production hosts.

Organism	Substrate	Process operation	Max. titer [g L^{-1}]	Max. yield [g g^{-1}]	Max. STY[a] [g L^{-1} h^{-1}]	Reference
Corynebacterium glutamicum Δ*ldhA*-pCRA717	Glucose	Dual phase fed-batch	146.0	0.92	3.17	(Okino et al., 2008)
Corynebacterium glutamicum BOL-3/pAN6-*gap*	Glucose and formate	Dual phase fed-batch	134.0	1.09	2.53	(Litsanov et al., 2012)
Escherichia coli NZN111 Δ*pflB*Δ*ldhA*	Cassava starch	Dual phase fed-batch	127.0	0.86	3.23	(Chen et al., 2014)
Actinobacillus succinogenes 130Z^T	Glucose	Batch	106.0	0.80	1.34	(Guettler et al., 1996)
Escherichia coli AFP111-*pyc*	Glucose	Dual phase batch	99.2	1.10	1.30	(Vemuri et al., 2002)
Basfia succiniciproducens Δ*fruA*	Sucrose	Fed-batch	71.0	0.84	3.13[b]	(Lange et al., 2017)
Mannheimia succiniciproducens Δ*ldh*Δ*pfl*Δ*pta*Δ*ack*	Glucose	Fed-batch	52.4	0.76	1.80	(Cheng et al., 2013)
Basfia succiniciproducens DD1 Δ*ldhA*Δ*pflD*	Glucose	Batch	31.7	0.71	1.50	(Becker et al., 2013; Scholten and Dägele, 2008; Stellmacher et al., 2010)
Basfia succiniciproducens CCUG 57335	DDAPH[c]	Batch	30.6	0.69	1.05[b]	(Salvachúa et al., 2016)
Basfia succiniciproducens DD1	Glycerol	Batch	8.4	1.20	0.90	(Scholten and Dägele, 2008)

[a] STY = Space Time Yield
[b] estimated from reference
[c] DDAPH is defined as high xylose-content hydrolysate from corn stover (see reference)