1. Introduction

The Gram-positive soil bacterium *Bacillus megaterium* is used since many decades in industrial biotechnology. This development has been driven by its high natural production capacity towards a rich portfolio of products, meaning that even wild type strains can be directly used for production (Table 1.1).

Table 1.1: Traditional recombinant and wild-type products of *B. megaterium;* [a] n. d. = not determined

Product	Production Strain	Product Yield	Product Titer	Source
Metabolites /vitamins				
L-Lysine	SP14	n.d.	3.56 g L^{-1}	(Ekwealor and Obeta, 2005)
Oxetanocin	NK84-0128 mutant 0161H	n.d.	377.3 ng mL^{-1}	(Morita, et al., 1999)
PHB	BA-019	55.46 $\%_{CDW}$	0.11 g $L^{-1}h^{-1}$	(Kulpreecha, et al., 2009)
Vitamin B_{12}	HBBm1	-	8.51 µg L^{-1}	(Biedendieck, et al., 2010)
Proteins				
α-amylase	n.d.	n.d.	n.d.	(Vihinen and Mäntsälä, 1989)
α -amylase	Aq-2007	n.d.	1015 U mL^{-1}	(Aqeel and Umar, 2010)
β-amylase	n.d.	n.d.	n.d.	(Hebeda R.E., et al., 1988)
β-galactosidase	WH320	4937 Miller U	-	(Rygus and Hillen, 1991)
Glucose dehydrogenase	WH320	101.1 U $mg_{protein}^{-1}$	-	(Rygus and Hillen, 1991)
Keratinase	ATCC14945	n.d.[a]	166.2 U mL^{-1}	(Radha and Gunasekaran, 2007)
Mutarotase	WH320	73.7 U $mg_{protein}^{-1}$		(Rygus and Hillen, 1991)
Penicillin G acylase (natural)	ATCC14945	n.d.	220 U L^{-1}	(Silva, et al., 2006)
Urokinase-like plasminogen activator	WH320	n.d.	400 U mL^{-1} per optical density unit	(Rygus and Hillen, 1991)
Xylanase	B6 ATCC 51946	n.d.	n.d.	(Ray and Nanda, 1997)

As example, vitamins, metabolites and nucleosides can be produced by *B. megaterium* like vitamin B_{12} (Biedendieck, et al., 2010; Wolf and Brey, 1986) pyruvate (Hollmann and Deckwer, 2004), L-amino acids (Ekwealor and Obeta, 2005), polyhydroxybutyrate (Kulpreecha, et al., 2009) and oxetanocin, a viral inhibitor (Morita, et al., 1999; Shimada, et al., 1986; Shiota, et al., 1996; Tseng, et al., 1991). Furthermore, a variety of natural or recombinant proteins expand the

product spectrum like penicillin G acylase, accumulated by the wild type isolate *B. megaterium* ATCC14945 in considerable amounts (Pinotti, et al., 2000), α-amylase (Aqeel and Umar, 2010; Lee, et al., 2001), glucose dehydrogenase (Nagao, et al., 1992), keratinase (Radha and Gunasekaran, 2007) and xylanase (Ray and Nanda, 1997).

Beyond these traditional applications, *B. megaterium* is now gaining particular interest as host for novel recombinant proteins. The organism exhibits different beneficial properties for such products. It has a stable plasmid replication system (Rygus and Hillen, 1991), a high secretion capacity for proteins, lacks alkaline proteases as well as endotoxins (Vary, et al., 2007; Vary, 1992) and shows reasonable production up to temperature as high as 45 °C (Jordan, et al., 2007b). Indeed, a number of recent studies has demonstrated successful application of *B. megaterium* for the production of diverse enzymes (Biedendieck, et al., 2007a; Biedendieck, et al., 2007b) as well as therapeutic proteins (David, et al., 2011b; David, et al., 2011a; Jordan, et al., 2007a; Jordan, et al., 2007b). The product spectrum comprises both, extracellular and intracellular proteins. For the latter, high yields of correctly folded protein in comparison with e.g. *B. subtilis* which facilitates downstream processing and recovery of the active product, can be achieved (Biedendieck, et al., 2007c; Biedendieck, et al., 2007a; Stammen, et al., 2010b).

The high industrial relevance has stimulated more recently systems-wide analyses of the metabolic and regulatory networks of *B. megaterium* (Figure 1.1). Today, a full range of experimental and computational systems biology tools is available to unravel the various cellular components on the level of transcriptome (Borgmeier, et al., 2011a), proteome (Wang, et al., 2006a; Wang, et al., 2006b), metabolome (Korneli, et al., 2012a) and even their functional interaction, i.e. the fluxome (Fürch, et al., 2007a; Fürch, et al., 2007b; Biedendieck, et al., 2011).

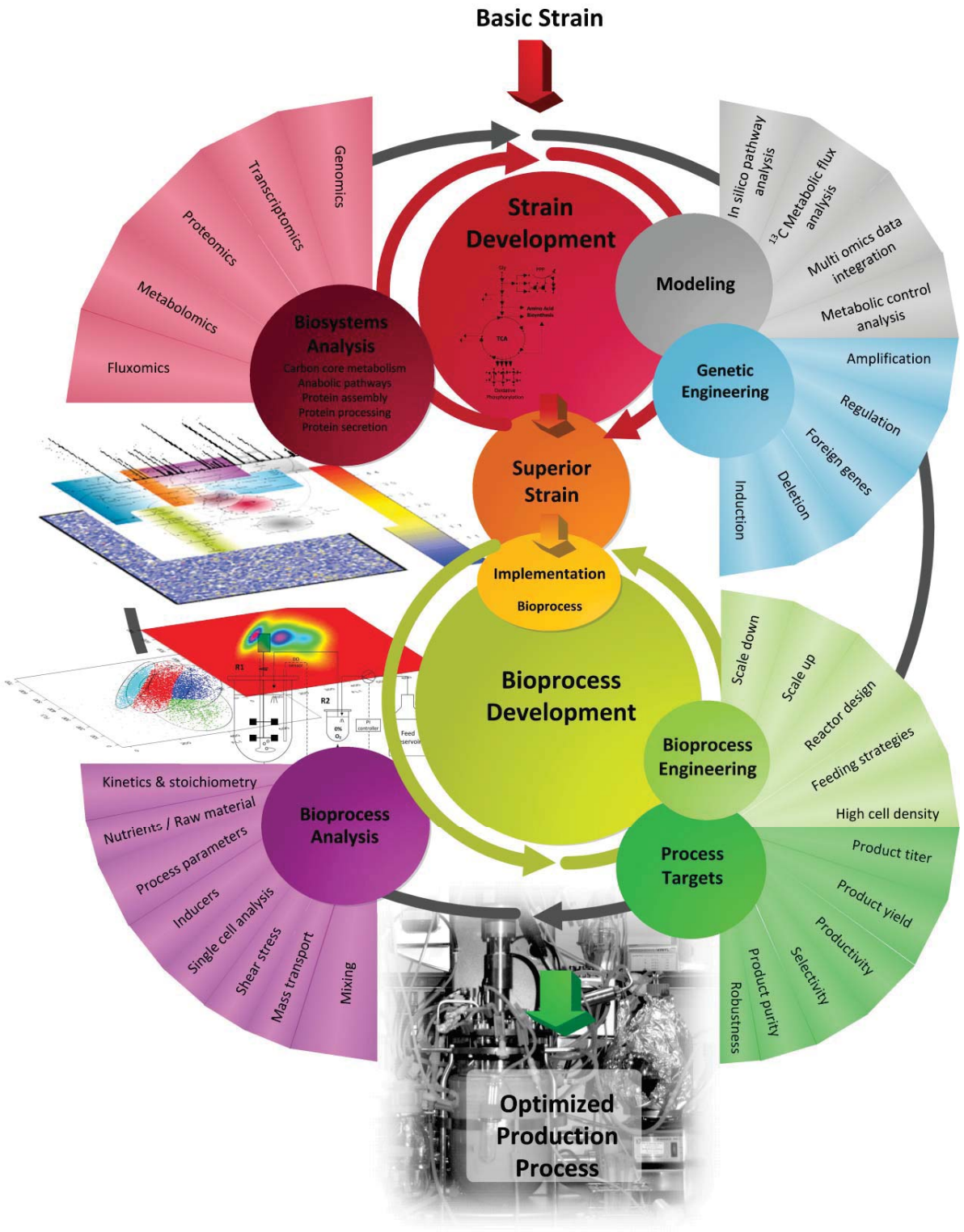

Figure 1.1: Systems biotechnology of *B. megaterium* involving strain engineering via polyomics based target identification, genetic engineering for strain evaluation as well as bioprocess engineering for the developed producer strains (Korneli, et al., 2012b).

These provide a rational platform for systems metabolic engineering of *B. megaterium*. In line, a number of interesting studies have particularly focused on studying recombinant *B. megaterium* in its industrial bioprocess environment thus integrating systems metabolic engineering with systems biotechnology and providing the full picture towards an optimal process. Relevant investigations cover the level of single cells by fluorescence labeling and flow cytometry (David, et al., 2011a) as well as large scale production which can be assessed by specific scale-down reactor configurations (Korneli, et al., 2012a; Korneli, et al., 2011). The future perspectives of protein producing *B. megaterium* are promising as the market for high-value therapeutics like antibodies or insulin as well as enzymes for the food or feed sector is rapidly growing and expanding (Ahlawat, et al., 2009; Hensing, et al., 1995; Shojaosadati, et al., 2008). As example, biopharmaceuticals already contribute to about 20 % of the total pharmaceutical market with further increase expected (von Holleben M von, 2012).

2. Objectives

Aim of this work was to unravel metabolic and process dependent bottlenecks during recombinant protein production with *B. megaterium* and the development of a high yield production process for this microbial host. The formation of both, intracellular as well as extracellular proteins should be examined. Favorable environmental conditions should be assessed and the metabolic state as well as cellular processes should be studied in detail using state-of-the-art methods of systems biotechnology. The focus was set on investigation of basic culture parameters of influence as well as of metabolic burdens under large scale conditions. Finally, this knowledge should be applied to develop a high cell density process for the production of extracellular levansucrase – an enzyme of great industrial interest. As economic point of view the usability of alternative industrial waste substrates should display a further target.

3. State of Knowledge – *Bacillus megaterium* as Recombinant Host

3.1. Basic physiology and metabolic pathway repertoire of *B. megaterium*

B. megaterium has been discovered as Gram-positive soil bacterium more than hundred years ago (De Bary, 1884). The rod shaped bacterium has a length up to 4 µm with a volume 100 times higher than that of *E. coli (*Figure 3.1).

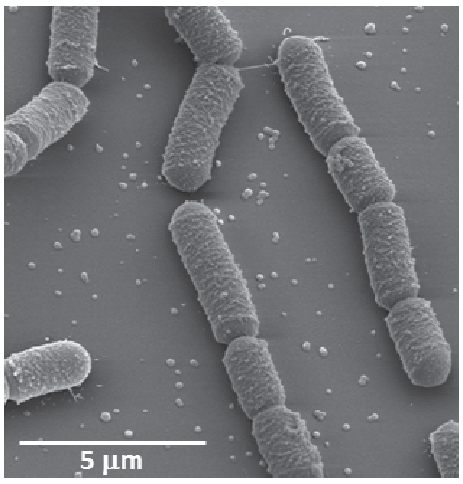

Figure 3.1: Scanning electron microscopic image of *B. megaterium* cells. *B. megaterium* strain DSM319 was cultivated in complex medium and grown for 14 h at 37 °C under aerobic conditions. Magnification × 6,000. (M. Rhode, HZI, 2008).

Due to its enormous cell size, *B. megaterium* is a giant within the bacterial world, expressed by its name megat(h)erium, which is Greek for "big animal". Different *B. megaterium* isolates strongly differ in their content of plasmids. Strains with up to ten plasmids, besides their chromosome, have been observed (Carlton and Helinski, 1969; Kim, 2003), but also plasmid-less strains like DSM319 have been described (Eppinger, et al., 2011). Recently, the genomes of two different *B. megaterium* strains, DSM319 and QM B1551, were sequenced and annotated (Eppinger, et al., 2011). Both strains harbor a chromosome of around 5.1 Mbp with about 5,300 open reading frames. In addition, the strain QM B1551 possesses seven plasmids with additionally 523 genes (Eppinger, et al., 2011). As further shown, 300 chromosomal genes are strain-specific and account for differences in experimentally confirmed phenotypes. With

regard to recombinant protein production, *B. megaterium* comprises a rich set of pathways from catabolic breakdown of the carbon source to the final product synthesis and secretion (Eppinger, et al., 2011). For growth on sugars, it uses the Embden-Meyerhof-Parnas pathway and the pentose phosphate pathway (PPP), followed by a classical tricarboxylic acid cycle (TCA-cycle). In contrast to other bacilli, *B. megaterium* contains an intact glyoxylate pathway which enables re-utilization of acetate, a typical by-product in production processes. Derived from its carbon core network, *B. megaterium* seems to have the anabolic pathways for the synthesis of all twenty proteinogenic amino acids, all nucleotides and cofactors and a full spectrum of fatty acids. In addition, the genes necessary for transport of a wide variety of carbon sources have been found. The ability to cope with a wide diversity of carbon sources broads the spectrum of suitable growth media to cheap industrial resources like glycerol or molasses (Vary, et al., 2007). It is interesting to note that the carbon source has a strong influence on the growth and production performance (Fürch, et al., 2007b). Among various substrates tested, fructose and glycerol have shown to be optimal for antibody fragment production and secretion (David, et al., 2011b). Following the nutritional requirements of *B. megaterium*, a well suited defined minimal medium could be successfully developed with stepwise reduction of complex ingredients (Yang, et al., 2006). Also the composition of trace elements was tailored towards recombinant protein production. Model-based medium optimization by a genetic algorithm identified calcium, manganese, cobalt and especially magnesium as key elements. This allowed adjusting for optimal concentrations regarding antibody fragment secretion and biomass formation (David, et al., 2010). In summary, this has enabled high growth rates, high cell densities and biomass yield, favorable characteristics with regard to volumetric productivity and product (Table 3.1).

Table 3.1: Growth and production properties of *B. megaterium* wild type DSM319 and producing strains

Strain	μ_{max} [h^{-1}]	q_{Sub} [mmol g^{-1} h^{-1}]	$Y_{X/S}$ [mmol g^{-1}]	x_{max} [g L^{-1}]	Comment
DSM319	1.3	15.3	85	2.1	Unpublished data; cultivation at 37 °C, 5 g L^{-1} glucose, in 50 mL M9 medium
YYBm1					
pEJBmD1.3scFv	0.6	5.36	99	25.4 (fed-batch)	(David, et al., 2011b)
pSSBm85	0.42	2.6	93.6	20 (fed-batch)	Unpublished data

B. megaterium is capable to directly secrete proteins into the surrounding medium. However, the titers are typically low (Yang, et al., 2006; Bunk, et al., 2010a). Further, undesired formation of inclusion bodies can thus be avoided, favoring efficient downstream procedures (Biedendieck, et al., 2007a; Biedendieck, et al., 2007c; Rygus and Hillen, 1991; Rygus, et al., 1991; Stammen, et al., 2010b). In line, optimized protein maturing and secretion by co-expression of cleavage proteins or chaperones seems to be important to improve production performance (Borgmeier, et al., 2011a). Generally, the microorganism is genetically accessible. For transfer of genetic material protoplast transformation is most established (Biedendieck, et al., 2011; von Tersch and Robbins, 1990) while trans-conjugation (Biedendieck, et al., 2011; Richhardt, et al., 2010), electroporation (Moro, et al., 1995) or even biolistic transformation (Shark, et al., 1991) were described.

3.2. Genetic engineering

3.2.1. Selection markers and knock-out mutants

Initially, the plasmid-less *B. megaterium* DSM319 appeared most straightforward as host for heterologous, i.e. plasmid encoded, protein production (Meinhardt, et al., 1989). Already in 1991, strain development started from this parent isolate. As first step, the β-galactosidase negative *B. megaterium* mutant WH320 was selected after random chemical mutagenesis (Rygus, et al., 1991) enabling the use of the encoding *lacZ* for subsequent gene deletion in the genetic background of WH320. As example the *lacZ* marker was used for the construction of the mutant WH323 which cannot metabolize xylose as carbon source (Δ*xylA*) (Rygus and Hillen, 1992). This later enabled optimized use of xylose inducible expression systems without metabolization of the inducer. In addition a collection of deletion strains was constructed from DSM319 aiming at biological containment (Borgmeier, et al., 2011b; Wittchen, et al., 1998; Vary, et al., 2007; Biedendieck, et al., 2011). Interesting mutants having lost the ability to form viable endospores could be derived (Nahrstedt, et al., 2005b; Wittchen, et al., 1998) whereas other approaches towards increased UV-sensitivity have only be partly successful (Nahrstedt, et al., 2005a). Interesting studies focused on deletion of extracellular proteases, potentially