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Micro- and Macroparticle enhanced cultivation of filamentous Lentzea aerocolonigenes for increased rebeccamycin production

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1 Introduction and aim of the thesis

The process of natural product formation in filamentous eukaryotic and prokaryotic microorganisms is popular and widely applied for research and industrial purposes. Cultivation of these microorganisms is challenging but also quite worthwhile because of their products. Regarding the pharmaceutical sector actinomycetes are of major importance for production. 60 % of the known secondary metabolites showing biological activity were isolated from actinomycetes in the year 2000 (Kieser et al. 2000). Many actinomycetes exhibit a fungal-like filamentous morphology which is one of the most challenging factors in the cultivation of filamentous microorganisms. These microorganisms are able to grow in different morphological forms ranging from dispersed mycelium to dense pellets (Manteca et al. 2008).

One example for an interesting secondary metabolite is rebeccamycin. Rebeccamycin is an antibiotic and antitumor substance. However, the antitumor effect is of primary interest. Rebeccamycin acts as a topoisomerase inhibitor and thereby interferes with DNA replication. Its analogues, that are easily derived from rebeccamycin, show a similar biological activity (Bush et al. 1987; Long et al. 2002). Becatecarin, an analogue with an increased water solubility, was already part of promising phase I and II clinical trials. In these trials becatecarin was tested for the treatment of e.g. metastatic colorectal cancer, refractory breast cancer or small cell lung cancer (Goel et al. 2003; Burstein et al. 2007; Schwandt et al. 2012). However, for the treatment of cancer patients large amounts of active substance are required. For instance, Schwandt et al. (2012) used a total of 4200 mg m^{-2} becatecarin per patient for the treatment of small cell lung cancer in their study. Regarding rebeccamycin titers described in literature mostly concentrations of under 100 mg L^{-1} are achieved. Moreover, the production is mostly conducted in small scales of around 100 mL (Pommerehne et al. 2019). Hence, research on the provision of larger amounts of rebeccamycin is necessary to allow a sufficient supply for a potential cancer treatment.

Lentzea aerocolonigenes is a filamentous actinobacterium that naturally produces rebeccamycin as one of several secondary metabolites. Due to the molecular structure of rebeccamycin, the biotechnological production potentially entails lower costs for production than a chemical synthesis (Burkart 2003), making the production in the original host *L. aerocolonigenes* more interesting. However, the manifold challenges during the cultivation of filamentous microorganisms have to be considered. One complex aspect is the morphology of these microorganisms. The extreme morphological characteristics are freely dispersed mycelium or dense pellets with various intermediate forms (Manteca et al. 2008). Mycelial structures or hairy pellets lead to an increased viscosity of the cultivation broth

resulting in a reduced mass transfer which should be compensated with an increased power input (Wucherpfennig et al. 2010). However, dense and large pellets can lead to nutrient and oxygen limitations in the pellet core (Hille et al. 2005; Bizukojc and Gonciarz 2015). The most favorable morphology for optimal production yields is depending on the microorganism and the desired product (Whitaker 1992; Walisko et al. 2015).

A wide range of parameters is influencing the cell morphology during cultivation. For example, factors like the medium composition, pH value, osmolality, addition of microparticles, inoculation volume and method of inoculation (e.g., inoculation with spores or vegetative mycelial biomass from a pre-culture) can have a great impact. Moreover, operating conditions of the cultivation process, such as stirrer speed, stirrer geometry and aeration rate or type of aeration can influence growth by inducing (hydro)mechanical stress into the cultivation (Oncu et al. 2007; Papagianni 2004; Wucherpfennig et al. 2010; Walisko et al. 2015).

For the production of rebeccamycin in *L. aerocolonigenes*, Walisko (2017) considered some of these parameters in more detail. Especially the addition of (micro-)particles proved as a successful method for the enhancement of rebeccamycin titers. Walisko et al. (2017) used the addition of talc microparticles (10 g L^{-1}) to cultivations of *L. aerocolonigenes* to increase the rebeccamycin titer to around 120 mg L^{-1} compared to an unsupplemented cultivation with around 40 mg L^{-1}. Microscopic images indicated the entanglement of the talc microparticles in the pellets outer hyphal area. When talc microparticles with different hydrophilic and hydrophobic surface modifications (ø < 30 µm) were applied in cultivations, both increased and decreased rebeccamycin titers compared to an unsupplemented approach or with untreated talc particles were observed. Hence, Walisko et al. (2017) proposed physicochemical surface effects to play an important role in the mechanism for the increased product concentration. The addition of microparticles has also been successfully applied with several other filamentous microorganisms (Kaup et al. 2008; Driouch et al. 2011; Antecka et al. 2016a; Karahalil et al. 2019).

Furthermore, Walisko (2017) added larger glass particles (ø = 0.5 – 5 mm, mostly 80 g L^{-1}) to cultivations of *L. aerocolonigenes*. With 0.5 mm glass beads a rebeccamycin concentration of almost 120 mg L^{-1} compared to an unsupplemented approach with only 6 mg L^{-1} was achieved. With larger glass beads the rebeccamycin concentration was decreased, the pellet size decreased and with 3 and 5 mm glass beads only mycelial structures have been observed. In this case the mechanical stress induced by the glass beads were proposed to be responsible (Walisko 2017). Although the micro- and macroparticle addition proved to be beneficial in regard to rebeccamycin titers, the mechanisms behind these product increasing methods are not yet fully understood.

Due to the rather low rebeccamycin titers described in literature compared to the necessary amounts for cancer treatment, one objective of this thesis was to increase the achievable rebeccamycin concentrations in cultivations of *L. aerocolonigenes*. Different, mostly particle-based, cultivation methods were applied to achieve this aim. Moreover, a better understanding of the mechanisms responsible for the increased rebeccamycin titers with different cultivation methods was intended. In more detail, the aims of this thesis can be described as follows:

- The further investigation of the microparticle addition to cultivations of *L. aerocolonigenes* considering microparticle incorporation and physicochemical effects of surface modified microparticles,
- the characterization of mechanical stress effects of larger macroparticles in cultivations of *L. aerocolonigenes* by the variation of particle size, particle concentration and particle density,
- the development and application of a bubble-free aerated stirred bioreactor for the production of rebeccamycin in a larger scale under defined shear conditions,
- the particle induced rebeccamycin recovery for a facilitated downstream processing of rebeccamycin by addition of adsorbent resins in the shape of particles, and
- the consideration of the interdependency of the cell morphology of *L. aerocolonigenes* and production of rebeccamycin with the aforementioned methods applied.

2 Theoretical background

2.1 *Lentzea aerocolonigenes* and rebeccamycin

Lentzea aerocolonigenes DSM 44217 is an actinomycete originally isolated from a Panamanian soil sample and is obligately aerobic (Bush et al. 1987; Nettleton, Jr. et al. 1985). It is a Gram-positive bacterium (Labeda et al. 2001) with a high guanine-cytosine content (Labeda 1986). A characteristic of actinomycetes like *L. aerocolonigenes* is the complex morphology, in which hyphae can grow to form mycelium or pellets, and the formation of spores. The hyphae appear in a slightly yellowish color during growth (Bush et al. 1987). The microorganism was reclassified several times starting from *Streptomyces* (Shinobu and Kawato 1960) over *Nocardia* (Pridham 1970) and *Saccharothrix* (Labeda 1986) to *Lechevalieria* (Labeda et al. 2001) and finally *Lentzea aerocolonigenes* (Nouioui et al. 2018).

L. aerocolonigenes is a natural producer of various secondary metabolites. However, the most interesting natural product is rebeccamycin (1,11-dichloro-12-(4-O-methyl-beta-Dglucopyranosyl)-12,13-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione). It is a yellow crystalline and hydrophobic substance belonging to the indolocarbazoles with the indolocarbazole framework and two chlorine as well as one methylglucose substituents (**Figure 2.1 a**) (Nettleton, Jr. et al. 1985; Bush et al. 1987; Kaneko et al. 1985).

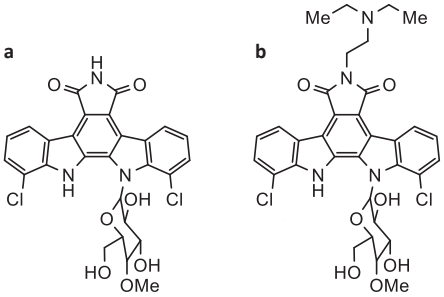

Figure 2.1: Chemical structures of the natural product (a) rebeccamycin and its analogue (b) becatecarin.

Rebeccamycin is poorly soluble in water (< 1 µg mL^{-1}) but is highly soluble in organic solvents (e.g. tetrahydrofuran and dimethylsulfoxide) (Bush et al. 1987; Nettleton, Jr. et al. 1985; Kaneko et al. 1990). It gained more interest than other natural products of *L. aerocolonigenes* because of its antibacterial effects against certain Gram-positive bacteria and additional antitumor effects, which makes it pharmaceutically relevant (Bush et al. 1987; Nettleton, Jr. et al. 1985).

The poor water solubility of rebeccamycin is critical for the use as a drug in the human body, which is why analogues with increased solubility in water were developed. Becatecarin (**Figure 2.1 b**) (1,11-Dichloro-6-[2-(diethylamino)ethyl]-12,13-dihydro-12-(4-O-methyl-beta-D-glucopyranosyl)-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione) is a promising analogue with increased water solubility. It can be derived from rebeccamycin by adding an aminoalkyl group. The antibacterial and antitumor activity is maintained during the structural modification (Kaneko et al. 1990).

2.1.1 Medical applications

Rebeccamycin acts antibacterially against the Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus faecalis*. Although this might already be interesting for a medical application, the antitumor properties of rebeccamycin are paramount. It is intercalating in the DNA and causes double-strand breaks as well as inhibits topoisomerase I (Bush et al. 1987; Facompré et al. 2002). The analogue becatecarin may even additionally inhibit topoisomerase II (Long et al. 2002). However, due to the low water solubility of rebeccamycin, mostly analogues were further investigated in clinical trials. Several phase I and II clinical trials with becatecarin with regard to small cell lung cancer, breast cancer, metastatic colorectal cancer and metastatic renal cell cancer were conducted (Nock et al. 2011; Burstein et al. 2007; Hussain et al. 2003; Schwandt et al. 2012; Goel et al. 2003). Furthermore, the effects on solid tumors in children was studied in clinical trials (Lagmay et al. 2016; Sánchez et al. 2006a; Langevin et al. 2008). The applied doses of becatecarin to children with solid tumors in the study of Langevin et al. (2008) was 650 mg m^{-2} every 21 days for maximal 16 cycles. However, in certain cases an increased dose of 780 mg m^{-2} per cycle was given to the patients. For the treatment of small cell lung cancer in adults 140 mg m^{-2} becatecarin each day for five days with a repetition every three weeks for maximal 6 cycles was applied in the study of Schwandt et al. (2012).

The applied doses in these clinical trials are rather high compared to rebeccamycin titers from biotechnological production processes published in literature (see **Table 2.1** in **chapter 2.1.2**). Therefore, the relevance of further investigations of the production process and possibilities for increased production becomes apparent.

2.1.2 Production of rebeccamycin

Rebeccamycin can be produced in three different ways: Chemical synthesis, heterologous production in other microorganisms or production in the natural producer *L. aerocolonigenes* (Kaneko et al. 1985; Gallant et al. 1993; Faul et al. 1999; Hyun et al. 2003; Casini et al. 2018; Onaka et al. 2015; Walisko et al. 2017). **Table 2.1** gives an overview of the currently achieved rebeccamycin titers and space-time-yields (STY) under various conditions presented in literature and will be considered in more detail below.

Table 2.1: Overview of rebeccamycin concentrations and space-time-yields (STY) achieved with different process conditions from literature. Selected examples based on highest concentration and space-time-yield are presented.

Rebeccamycin concentration [mg L^{-1}]	Organism	Medium	Cultivation volume [L]	T [°C]	Cultivation time [d]	STY [mg L^{-1} d^{-1}]	Comments	Reference
27.1	*Lechevalieria aerocolonigenes*	H34	0.1	27	7	3.87		Bush et al. (1987)
71.3	*L. aerocolonigenes*	H95	0.1	27	7	10.19		Bush et al. (1987)
160.8	*L. aerocolonigenes*	H96	0.1	27	7	22.97		Bush et al. (1987)
183	*L. aerocolonigenes*	H96	0.1	27	7	26.14		Nettleton, Jr. et al. (1985)
0.3-0.5	*Escherichia coli* (recombinant)	LB/ amp	1	26	2	ca. 0.2		Hyun et al. (2003)
0.7	*E. coli* (recombinant)	LB Miller	0.05	26	2	0.35	Rebeccamycin aglycone	Casini et al. (2018)
ca. 8	*Streptomyces lividans (recombinant)*	A-3M	0.1	30	12	0.67	Pure culture	Onaka et al. (2015)
multiplied	*Streptomyces albus* (recombinant)	R5A	0.4	30	5			Sánchez et al. (2002)
663	*L. aerocolonigenes*	G134	30	27	8.5	78.00	Bioreactor scale	Bush et al. (1987)
52.5	*L. aerocolonigenes*	G134			9	5.83		Onaka et al. (2003a)
18.4	*L. aerocolonigenes*	MM + starch	0.1	28	6	3.07		Lam et al. (1989)
44.5	*S. lividans (recombinant)*	A-3M	0.1	30	12	3.71	Co-culture with *T. pulmonis*	Onaka et al. (2015)
ca. 30	*S. lividans (recombinant)*	A-3M	0.1	30	12	2.50	Co-culture with *R. erythropolis*	Onaka et al. (2015)
ca. 15	*S. lividans (recombinant)*	A-3M	0.1	30	12	1.25	Co-culture with *C. glutamicum*	Onaka et al. (2015)
9.6	*L. aerocolonigenes*	GYM	0.05	28	8	1.20	No talc supplement	Walisko et al. (2017)
ca. 18	*L. aerocolonigenes*	GYM	0.05	28	8	2.25	Talc 10 g L^{-1}	Walisko et al. (2017)
94.3	*L. aerocolonigenes*	GYM	0.05	28	8	11.79	Talc $(PDA/Carr)_3$ 10 g L^{-1}	Walisko et al. (2017)
6	*L. aerocolonigenes*	GYM	0.05	28	8	0.75	No glass beads	Walisko et al. (2017)
116	*L. aerocolonigenes*	GYM	0.05	28	8	14.50	Glass beads 80 g L^{-1}, Ø = 0.5 mm	Walisko et al. (2017)

Chemical syntheses

Different chemical syntheses for the rebeccamycin production can be found in literature (Kaneko et al. 1985; Gallant et al. 1993; Faul et al. 1999). The first two approaches of a chemical rebeccamycin production were presented by Kaneko et al. (1985). One approach was a Diels-Alder reaction between N-benzyloxymethylmaleimide and 7,7′-dichloroindigo. The other approach was based on the indole Grignard method, which was later used for further developments. Gallant et al. (1993) improved this total synthesis by a facilitated glycosylation. Faul et al. (1999) introduced a versatile method that allowed the production of either rebeccamycin or its analogues i.e., 11-dechlororebeccamycin. The overall yield for rebeccamycin in this approach was 12 %. Further approaches for a total chemical synthesis of rebeccamycin were scarce, rather a total synthesis for derivatives (Marminon et al. 2003) or chemical transformations of rebeccamycin (Anizon et al. 2009) were presented. A more detailed overview of the chemical syntheses presented in literature is given in Pommerehne et al. (2019).

Heterologous production

The heterologous production of rebeccamycin has various advantages over the natural production in *L. aerocolonigenes*. Challenges by the complex morphology (see also **chapter 2.2**) and the relatively unknown microorganism regarding metabolic pathways and cultivation conditions can be circumvented by the transfer of the rebeccamycin gene cluster into a well-studied model microorganism. Hyun et al. (2003) transferred the gene cluster into *Escherichia coli* and successfully produced rebeccamycin, however, only in small concentrations between 0.3 - 0.5 mg L^{-1} (see **Table 2.1**). Casini et al. (2018) were able to produce the precursor called rebeccamycin aglycon in *E. coli*. A concentration of 0.7 mg L^{-1} was estimated, assuming a similar UV absorbance-to-titer conversion factor for both rebeccamycin and rebeccamycin aglycon. The transformation of a cosmid with the rebeccamycin gene cluster into *Stretompyces lividans* was conducted by Onaka et al. (2003b). A successful rebeccamycin production was confirmed by HPLC measurements but was not quantified. Later on, Onaka et al. (2015) quantified the rebeccamycin production in *S. lividans*. After 12 days of cultivation approximately 8 mg L^{-1} of rebeccamycin were produced. Sánchez et al. (2002) transformed genomic DNA of *L. aerocolonigenes* into *Streptomyces albus* and stated that the production levels were several fold higher than in *L. aerocolonigenes* under the same conditions. However, no numbers were given in this particular case.

Natural production in L. aerocolonigenes

The natural production of rebeccamycin in the original microorganism *L. aerocolonigenes* is described several times in literature. Cultivations after the first isolation resulted in rebeccamycin concentrations between 27.1 and 183 mg L^{-1} in shaking flasks by a variation of the media components (Bush et al. 1987; Nettleton, Jr. et al. 1985). With the H96 medium a rebeccamycin concentration of 160.8 mg L^{-1} after 7 days was achieved in shake flasks. An improved medium with 15 g L^{-1} additional starch (G134 medium) led to 663 mg L^{-1} rebeccamycin after 8.5 days of cultivation in a 30 L bioreactor (Bush et al. 1987), which until now is the highest ever achieved rebeccamycin concentration according to literature (see **Table 2.1**). However, later Onaka et al. (2003a) were not as successful using the same G134 medium. In shake flasks only 52.5 mg L^{-1} were achieved. The different rebeccamycin titers resulting from various media compositions and cultivation parameters already indicate that cultivations of filamentous microorganisms, and thereby their productivity, can be influenced by various parameters. Further insight into this topic is given in **chapter 2.2**.

2.1.3 Purification of rebeccamycin

Rebeccamycin produced during cultivations of *L. aerocolonigenes* or other microorganisms needs to be purified in order to use it for medical purposes. Therefore, the first step is usually the extraction of rebeccamycin from the cultivation broth using tetrahydrofuran (Pearce et al. 1988), acetone (Lam et al. 1989) or ethyl acetate (Walisko et al. 2017). Walisko (2017) added the fatty alcohol ethoxylate Genapol C 200 at the end of the cultivation to facilitate the extraction by avoiding organic solvents. An aqueous extraction of hydrophobic substances like rebeccamycin was enabled using this substance. In literature the description of further purification steps is scarce. Mostly quantitative HPLC analyses are conducted to measure product titers (Walisko et al. 2017; Onaka et al. 2015). However, an HPLC based purification of rebeccamycin with an C18 column and 0.1 M ammonium acetate-MeOH-MeCN (4:3:3) was presented by Pearce et al. (1988). A UV detector was used to detect rebeccamycin at 313 nm. Unfortunately, no further details on the purification process were given. Another purification method is the crystallization of rebeccamycin described by Nettleton, Jr. et al. (1985). Several steps of extraction were done and the final crystallization was conducted with hot methanol.

2.2 Morphology and productivity of filamentous microorganisms

Cell morphology of filamentous microorganisms is more complex than that of unicellular microorganisms. The formation of hyphae that are intertwining to loose mycelia or dense pellets leads to challenges in the cultivation process (Walisko et al. 2015; Wucherpfennig et al. 2010) as is exemplarily shown in **Figure 2.2**. However, these challenges are accepted since many filamentous bacteria and fungi produce substances that are interesting for food

or pharmaceutical industry (Walisko et al. 2015). The bacterial filamentous *Actinomycetes*, including *L. aerocolonigenes*, are especially interesting because of the production of mainly pharmaceutically relevant secondary metabolites (Nielsen 1996; Kieser et al. 2000). In the year 2000 60 % of the known biologically active secondary metabolites were isolated from *Actinomycetes* (Kieser et al. 2000). Although there are clear differences between filamentous bacteria and fungi, their morphology and its influence during a cultivation process are similar (Olmos et al. 2013; Nielsen 1996).

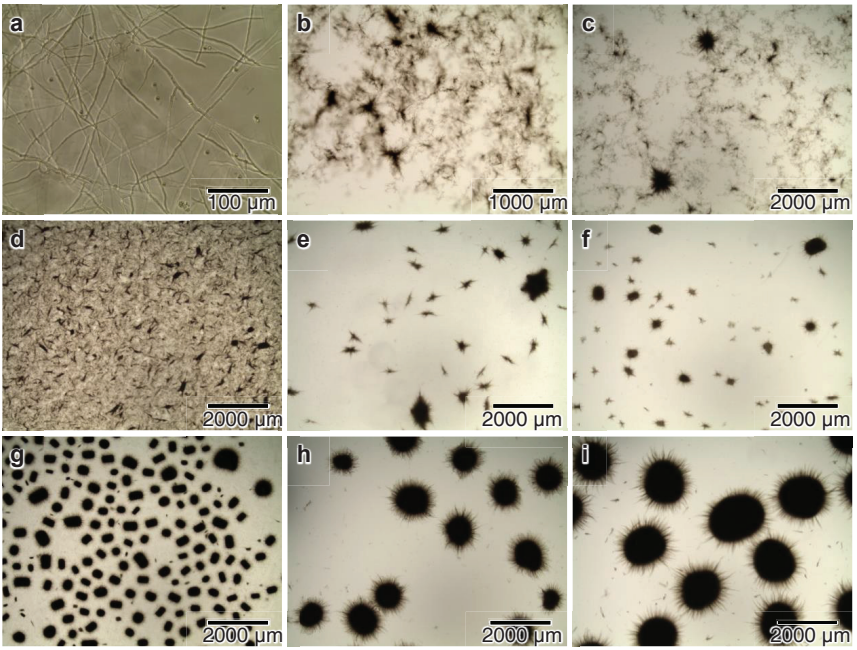

Figure 2.2: Morphologic diversity of the filamentous actinomycete *Lentzea aerocolonigenes* in different cultivations ranging from (a) single hyphae, over mostly mycelial structures (b – d) up to dense and defined pellets in various sizes (e – i).

The different morphological forms of filamentous microorganisms can affect the cultivation process by influencing different parameters such as the viscosity of the cultivation broth and thus the mixing performance as well as the costs for downstream processing (Wucherpfennig et al. 2010). At high biomass concentrations mycelial structures or pellets with many freely exposed hyphae in their periphery can lead to a high viscosity with a non-Newtonian flow behavior (Wucherpfennig et al. 2010; Bliatsiou et al. 2020). In a highly viscous cultivation broth, the mass transfer and mixing performance are impeded. In order to ensure a sufficient nutrient and oxygen supply of the microorganism an increased power input is necessary (Oncu et al. 2007). A large power input in turn causes high mechanical stress and therefore

leads to morphological changes (El-Enshasy et al. 2006) as well as variations in biomass growth and product titers (Rosa et al. 2005). Furthermore, dense pellets with a large diameter often face oxygen and nutrient limitations in the pellet core (Hille et al. 2005; Bizukojc and Gonciarz 2015). The diffusion of oxygen in the pellet core is impeded when a critical diameter is exceeded (Hille et al. 2009). This also affects the microorganisms metabolism and the product formation (Phillips 1966; Elmayergi et al. 1973).

For many filamentous microorganisms a correlation between morphology and productivity has been observed (Antecka et al. 2016a). Which morphology is beneficial in regard to productivity, however, highly depends on the microorganism and the desired product. Mycelial structures are advantageous for the production of geldamycin in *S. hygroscopicus* and tylosin in *S. fridae* (Dobson et al. 2008; Tamura et al. 1997). Other microorganisms like *S. tendae* and *S. avermitilis* show an improved production of nikkomycin and avermectin, respectively, in pellets (Vecht-Lifshitz et al. 1992; Yin et al. 2008). However, there are also microorganisms that exhibit nearly equal productivity in different morphological forms like *S. clavuligerus* and *S. virginiae* when producing clavulanic acid or virginiamycin (Belmar-Beiny and Thomas 1991; Yang et al. 1996). For the rebeccamycin production with *L. aerocolonigenes* Walisko et al. (2017) stated the pelleted morphology to be beneficial.

The cell morphology of filamentous microorganisms can be influenced by various process conditions (Oncu et al. 2007; Papagianni 2004; Wucherpfennig et al. 2010; Walisko et al. 2015). By adjusting the cell morphology according to the preferences of the microorganism a targeted optimization of the product concentration is desired. Thus, different aspects need to be considered. For several actinomycetes a high energy dissipation and high dissolved oxygen levels lead to pelleted growth whereas a lower energy dissipation generates fluffier pellets and low dissolved oxygen levels promote mycelial growth (Vecht-Lifshitz et al. 1990; Tough and Prosser 1996; Bellgardt 1998; Yin et al. 2008). Moreover the inoculum size and type, the pH value, the composition of the cultivation medium or the presence of certain ions were observed to influence the morphology and productivity of various filamentous fungi and bacteria (Walisko et al. 2015). The addition of inorganic salts, the so called *salt-enhanced cultivation*, has already been investigated by Wucherpfennig et al. (2011) for cultivations of *A. niger*. The supplementation of sodium chloride led to an increased productivity and a decreased pellet size. Similar observations were made for the addition of ammonium sulfate to *Actinomadura namibiensis* (Tesche et al. 2019). Furthermore, the addition of various other additives to the cultivation can influence the cell morphology. The addition of a silicone antifoam, Tween 80 and Triton X-100 to cultivations of *Streptomyces hygroscopicus* var. *geldanus* resulted in decreasing pellet diameters with increasing concentrations of the corresponding surfactant (Dobson et al. 2008). O'Cleirigh et al. (2005) added xanthan gum to