RESEARCH ARTICLE

Salt-enhanced cultivation as a morphology engineering tool for filamentous actinomycetes: Increased production of labyrinthopeptin A1 in *Actinomadura namibiensis*

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Abstract

Salt-enhanced cultivation as a morphology engineering tool for the filamentous actinomycete *Actinomadura namibiensis* was evaluated in 500-mL shaking flasks (working volume 100 mL) with the aim of increasing the concentration of the pharmaceutically interesting peptide labyrinthopeptin A1. Among the inorganic salts added to a complex production medium, the addition of $(\text{NH}_4)_2\text{SO}_4$ led to the highest amount of labyrinthopeptin A1 production. By using 50 mM $(\text{NH}_4)_2\text{SO}_4$, the labyrinthopeptin A1 concentration increased up to sevenfold compared to the non-supplemented control, resulting in 325 mg L^{-1} labyrinthopeptin A1 after 10 days of cultivation. The performance of other ammonium- and sulfate-containing salts (e.g., NH_4Cl , K_2SO_4) was much lower than the performance of $(\text{NH}_4)_2\text{SO}_4$. A positive correlation between the uptake of glycerol as one of the main carbon sources and nongrowth-associated labyrinthopeptin productivity was found. The change in the cell morphology of *A. namibiensis* in conjunction with increased osmolality by the addition of 50 mM $(\text{NH}_4)_2\text{SO}_4$ was quantified by image analysis. *A. namibiensis* always developed a heterogeneous morphology with pellets and loose mycelia present simultaneously. In contrast to the non-supplemented control, the morphology of $(\text{NH}_4)_2\text{SO}_4$ -supplemented cultures was characterized by smaller and circular pellets that were more stable against disintegration in the stationary production phase.

KEYWORDS

actinomycete, ammonium sulfate, image analysis, labyrinthopeptin, morphology engineering, salt-enhanced cultivation

1 | INTRODUCTION

Actinobacteria are a rich source for the development of novel anti-infectives. Recently, labyrinthopeptins came into focus as very promising new drug candidates. Labyrinthopeptins belong to the type III lantibiotics, a class of ribosomally

synthesized and posttranslationally modified peptides. Unlike other molecules of this class, they contain the unique carbacyclic triamino triacid labionin (Figure 1A) [1,2]. The only known natural producer strain of labyrinthopeptins is the filamentous actinomycete *Actinomadura namibiensis* DSM 6313, which was isolated from the Namib Desert [3]. Three labyrinthopeptin derivatives, labyrinthopeptin A1 (Figure 1B), A2 and A3, were found in the culture filtrates of *A. namibiensis* [2]. Labyrinthopeptin A1 shows very effective in vitro antiviral activity against human immunodeficiency

Abbreviations: CDW, cell dry weight; NGA, nongrowth-associated; OTR, oxygen transfer rate; RAMOS, respiration activity monitoring system; STY, space-time yield.

virus and herpes simplex virus at submicromolar concentrations without toxic effects on vaginal lactobacilli and without the inflammatory response of peripheral blood mononuclear cells; thus, it is of special interest for the treatment of sexually transmitted viruses [4]. The compound also showed activity against human respiratory syncytial virus subtypes A and B (hRSV) [5] and synergistic effects with other standard antiretroviral drugs [4], making it a candidate for the development of a broad-spectrum antibiotic as well [6].

Generation of class III lantibiotics by genetic engineering is principally conceivable and was investigated by Krawczyk et al. [7] in 250-mL shaking flasks with a 50-mL culture volume. The authors found *A. namibiensis* to be genetically inaccessible; thus, heterologous expression of labyrinthopeptin analogues was performed with *Streptomyces lividans*. However, undesired labyrinthopeptin variants with additional N-terminal amino acids were also produced. This was overcome by the construction of a synthetic labyrinthopeptin A1 gene with additional methionine at the -1 position. Transformation resulted in the production of different labyrinthopeptin A1 variants that were all converted into labyrinthopeptin A1 after longer cultivation times. Finally, a labyrinthopeptin A1 concentration of 86 mg L^{-1} after 16 days [7] and a space-time yield (STY), often also referred to as volumetric productivity, of $5.4 \text{ mg L}^{-1} \text{ d}^{-1}$ was achieved. Since this process was never scaled-up and since the benefit of heterologous expression is uncertain, the production of labyrinthopeptins in the wild-type producer *A. namibiensis* is still important.

An industrial production process for the production of labyrinthopeptins has yet to be established. Rupcic et al. [8] showed the first results of a large-scale production of 580 mg of labyrinthopeptin A1 and 510 mg of labyrinthopeptin A2 isolated from a 7.5-L culture broth of *A. namibiensis* with recovery rates of 72.5 and 42.3%, respectively. However, the cultivation that resulted in maximum yields of over 100 mg L^{-1} of both target molecules after 300 h in a 10-L-scale bioreactor (STY approximately $12 \text{ mg L}^{-1} \text{ d}^{-1}$ per target molecule) still requires further optimization. Until now, no benchmarking study on the most advantageous biotechnological cultivation conditions for the production of labyrinthopeptin has been carried out.

It should be noted that the optimal product yield of filamentous organisms often corresponds with particular morphologic phenotypes [9–14]. The macromorphology determines the microenvironment of hyphae through effects on mixing, mass transfer and broth rheology, which in turn affect product formation [15]. Controlling the morphology of filamentous organisms to enhance the yield of a desired product is still a challenging task.

Despite some obvious differences, the morphological characteristics of filamentous prokaryotes, e.g., actinomycetes, and their impact on cultivation are similar to those of fungi [16]. In submerged cultivations, the morphology of

PRACTICAL APPLICATION

In recent years, the direct interrelation of the morphology and productivity of filamentous organisms has resulted in several attempts to control and regulate the morphology of several economically relevant producer strains of enzymes and secondary metabolites. In this study, salt-enhanced cultivation with $(\text{NH}_4)_2\text{SO}_4$ proved to be a suitable method to tailor cell morphology for increased production of the promising new drug candidate labyrinthopeptin A1 in *Actinomadura namibiensis*. The results will help to design a highly productive biotechnological process for labyrinthopeptin biosynthesis on a large scale. The method of the salt-enhanced cultivation may also be applicable for similar filamentous organisms to produce secondary metabolites in a defined process with an increased salt load.

both varies between freely dispersed mycelia (micromorphology) and dense agglomerated mycelia, the so-called pellets (macromorphology). Therefore, their growth kinetics can be expressed using the same mathematical approaches [17]. To characterize the occurring morphology of filamentous organisms, quantifiable morphological Euclidian parameters (e.g., projected area, perimeter, and maximum Feret diameter) and shape descriptors such as aspect ratio, circularity, roundness, and solidity are applicable. With the introduction of a multifunctional shape parameter termed the nondimensional morphology number, Wucherpennig et al. [18] were able to quantitatively characterize a broad distribution of morphological forms of *Aspergillus niger* SKAn 1015 cells and correlate morphology number with the growth-associated productivity of fructofuranosidase. A relationship between the morphology and productivity of prokaryotic filamentous organisms such as *A. namibiensis* can also be assumed.

Nevertheless, different morphologies seem to be favorable for different nongrowth-associated (NGA) products. Dispersed and agglomerated mycelia (so called clumps) were found to be beneficial for retamycin production in *Streptomyces olindensis* [19], nystatin production in *Streptomyces noursei* [20], and geldamycin production in *Streptomyces hygroscopicus* [21], but productivities were higher in pellets than in dispersive growth forms when producing nikkomycin in *Streptomyces tendae*, avermectin in *Streptomyces avermitilis*, and rebeccamycin in *Lechevalieria aerocolonigenes* [22–24]. In some cases, however, productivity levels for different morphologies were similar [25,26]. Higher productivities in dispersed mycelia and clumps can usually be attributed to an adequate supply of oxygen and other substrates. The overproduction of antibiotics in production systems that favor

FIGURE 1 (A) Structural formula of the triamino triacid labionin that enables the formation of two-ring systems within the peptide chain of labyrinthopeptins. (B) Schematic primary structure of labyrinthopeptin A1 modified from [1] (abbreviations: Ala: alanine, Asn: asparagine, Cys: cysteine, Dhb: dihydrobutyryne, Glu: glutamic acid, Gly: glycine, Lab: labionin, Phe: phenylalanine, Pro: proline, Thr: threonine, Trp: tryptophan, Val: valine)

pelleted growth may be affected by secreted metabolites such as A factor in *Streptomyces griseus* and Factor 1 in *Streptomyces aureofaciens*. These metabolites can accumulate and adhere to the hyphae in pellets and thereby increase productivity [27]. Manteca et al. [35] suggested that the production of secondary metabolites only occurs after differentiation from a young compartmentalized mycelium to a syncytial mycelium after a cycle of programmed cell death. This is also supported by reports of changes in morphology during the course of cultivation, mainly during the transition from the exponential to stationary growth phase [29]. Hence, the correlation between morphology and productivity is until now always strain- and product-dependent.

The development of methods for tailor-made morphologies of filamentous microorganisms is summarized under the term *morphology engineering* [18,22,30–32,62]. Together with empirical modeling of different strain types, this method is used as a tool to increase product concentrations. In addition to the choice of nutrient sources and the generation of physiological stress (e.g., by microparticle-enhanced cultivation (MPEC) [22,30–32]), a sophisticated method to tailor morphology was found to be the manipulation of osmolality by the addition of inorganic salts to the cultivation broth [18]; here—in analogy to the term MPEC—referred for the first time as salt-enhanced cultivation.

Osmolality is generally dependent on the culture medium composition and changes during cultivation through consumption of nutrients, accumulation of metabolic products and *pH* control (addition of acid or base). Polycations enhance pelleted growth, whereas polyanions suppress it, leading to the conclusion that ions influence the agglomeration behavior of naturally charged cell walls [21,27,33]. Only a few studies are devoted to the effect of so-called inert salts such as NaCl or KCl, which are supposed to have no effect on metabolism [34–36]. Increased NaCl concentrations led to stronger mycelial growth and 4.5-fold enhanced growth-associated productivity of glucoamylase and of 18-fold formation of fructofuranosidase in *A. niger* AB1.13 and SKAn1015, respectively [18]. However, the observed changes in productivity might not be due to the change in morphology alone. The parameter of osmolality might have affected the fungal physiology,

which could in turn affect the morphology independently. The increase in the observed productivity was shown to correlate with the active pellet surface area [18].

Similar to the situation of *A. niger*, a study proved that the addition of ions has a distinct impact on the morphology of *Streptomyces azureus* [37]. The authors showed that the addition of excess Mg^{2+} , Ca^{2+} , and Mn^{2+} all induced pelleted growth, although supplementation with Ca^{2+} led to smaller pellets than supplementation with Mg^{2+} . Additionally, Mn^{2+} induced pellet formation at much lower levels than Mg^{2+} and Ca^{2+} . Fuchino et al. [38] recently observed that osmotic upshifts of $1.33 \text{ osmol kg}^{-1}$ with sucrose and $0.97 \text{ osmol kg}^{-1}$ with NaCl both caused a remarkable change in the hyphal growth pattern in *Streptomyces coelicolor*. After a 2 to 3 h period of growth arrest due to the osmotic shock, the existing hyphal tips did not start to regrow, but multiple new branches emerged from the lateral hyphal wall almost simultaneously. Thus, the osmotic shock triggered reprogramming of cell polarity and redistribution of polar growth sites [38].

In this study, the salt-enhanced cultivation was used to increase the production of labyrinthopeptin A1 in wild-type *A. namibiensis* in 500-mL shaking flask cultures with a working volume of 100 mL of culture medium. Therefore, the impact of different salts at various concentrations was tested first. Changes in carbon source utilization and cell morphology induced by the most effective salt, $(NH_4)_2SO_4$, were then investigated in detail and related to labyrinthopeptin productivity.

2 | MATERIALS AND METHODS

2.1 | Strain and maintenance

The filamentous strain *Actinomadura namibiensis* (DSM 6313) used in this study was obtained from the *German Collection of Microorganisms and Cell Cultures*, Braunschweig, Germany. The organism was grown for 7 days on agar plates containing 10 g L^{-1} glucose, 10 g L^{-1} glycerol, 10 g L^{-1} soluble starch (Carl Roth), 2 g L^{-1} yeast extract (Carl Roth), 2 g L^{-1} peptone (Carl Roth), 3 g L^{-1} calcium carbonate

(Sigma-Aldrich), 3 g L⁻¹ corn steep powder (Sigma-Aldrich), and 12 g L⁻¹ agar (Carl Roth). Segments of agar with an area of 1 cm² were stored in 80% glycerol at -80°C.

2.2 | Media and cultivation conditions

For precultures and main cultures of *A. namibiensis*, a modified version of M5294 medium was used, which contained 10 g L⁻¹ glucose, 10 g L⁻¹ glycerol, 10 g L⁻¹ soluble starch, 2 g L⁻¹ yeast extract, 2 g L⁻¹ peptone, 3 g L⁻¹ calcium carbonate, and 3 g L⁻¹ corn steep powder. The pH was adjusted to 7.2 with KOH prior to autoclaving at 121°C for 15 min. Each preculture was inoculated with one thawed agar segment from the cryo culture and cultivated for 3 days at 30°C and 180 min⁻¹ (50 mm orbital shaking diameter) in darkness (Certomat BS-1, Sartorius, Göttingen, Germany) in a 500-mL shaking flask without baffles. Main cultures were inoculated with 5 mL of the preculture of vegetative mycelia and were cultivated in 500-mL shaking flasks without baffles and with 100 mL working volume for 10 days at 30°C and 180 min⁻¹ (50 mm orbital shaking diameter) in darkness. All experiments were carried out in triplicate. Statistical error is given as the mean ± standard deviation.

2.3 | Cell dry weight determination

The biomass was determined gravimetrically as cell dry weight (CDW). Culture broth samples of 15 mL were filtered through dried and preweighted membrane filters (filter 389, Sartorius, Göttingen, Germany) and washed with 15 mL high-quality water (MilliQ gradient A10 system, Millipore, Bedford, USA). The filters were dried at 105°C for 24 h and cooled for at least 48 h in a desiccator. The error caused by solid media components (< 0.3%) was negligible. The specific growth rate μ [d⁻¹] was calculated upon the differentiation of an asymptotic approximation curve over time that fitted best to the experimental biomass data. The volumetric product formation rate r_p [mg_p L⁻¹ d⁻¹] was calculated by linear regression of the product concentration values from cultivation day 4 to 10. The specific productivity q_p at sample time t_i was calculated as follows:

$$q_{P,i} = \frac{c_{Product}(t_i) - c_{Product}(t_{i-1})}{[t_i - t_{i-1}] \cdot [c_{Biomass}(t_i) - c_{Biomass}(t_{i-1})]} \quad (1)$$

where, the product and biomass concentrations were taken from the linear regression and the nonlinear approximation curve, respectively.

2.4 | Glucose and glycerol determination

Glucose and glycerol were analyzed by HPLC (Hitachi LaChrom Elite, Hitachi, Tokyo, Japan). Water was used as the eluent with a flow rate of 0.6 mL min⁻¹. For separation, a

MetaCarb 87C column (300 × 7.8 mm, Agilent Technologies, Santa Clara, USA) and a MetaCarb 87C Guard precolumn (50 × 4.6 mm, Agilent Technologies) at 85°C were used. Glucose and glycerol were detected by a Hitachi L-2490 RI detector with retention times of approximately 11 and 16.4 min, respectively.

2.5 | Starch determination

To determine the amount of residual starch in the cultures, a sample (0.2 mL) of the culture broth supernatant was treated with an aqueous solution (1 mL) of iodine (0.22 g L⁻¹) and KI (0.44 g L⁻¹). The mixture was diluted 1:20 with deionized water, and the absorbance at 546 nm was related to a calibration line given by known concentrations of starch (0 to 10 g L⁻¹; $R^2 = 0.9996$).

2.6 | Labyrinthopeptin A1 determination

For preparation of a crude extract of labyrinthopeptin A1, 1.5 mL of XAD16N adsorber resin (Sigma-Aldrich, Munich, Germany) was added to each culture broth sample of 15 mL and shaken for 60 min in an overhead shaker (Intelli-Mixer RM-2 M, LTF Labortechnik, Wasserburg, Germany). The mixture was then centrifuged for 10 min at 2300 × g (Heraeus Varifuge 3.0R, Thermo Fisher Scientific, Waltham, USA), and the supernatant was removed. The residual material was shaken with 30 mL acetone (Sigma-Aldrich) for 60 min in the overhead shaker. The suspension was centrifuged under the same conditions as before, and the supernatant was air-dried. For HPLC analysis, the residue was dissolved in 2 mL of a methanol solution (90% v/v) in high-quality water (MilliQ gradient A10 system, Millipore).

Labyrinthopeptin A1 was analyzed by HPLC (Hitachi LaChrom Elite). An XBridge Shield RP18 column (5 μm, 250 × 4.6 mm, Waters, Milford, USA) and a Hypersil ODS precolumn (5 μm, 50 × 4.6 mm, Thermo Fisher Scientific) at 40°C were used with an injection volume of 20 μL. Labyrinthopeptin A1 was analyzed at a flow rate of 1.5 mL min⁻¹. The eluents were 90% acetonitrile (eluent A), 12.5 g L⁻¹ (NH₄)₂SO₄ pH = 9 (eluent B) and high-quality water (MilliQ gradient A10 system, Millipore) (eluent C). A gradient starting with 5% eluent A, which was raised to 60% over 10 min, held constant for 0.5 min and then raised again to 90% over 1 min, was used. The detection was carried out by a diode array detector (Hitachi L-7450) at a wavelength of 280 nm with a retention time of approximately 9 min for labyrinthopeptin A1.

2.7 | Osmolality determination

In this work, the unit osmolality (osmol kg⁻¹) instead of the more common osmolarity (osmol L⁻¹) was used since

osmolality is independent of temperature and pressure. Osmolality of media and culture supernatants were measured with a freezing point depression osmometer (Osmomat 030, Gonotec, Berlin, Germany). For determination of osmolality, 50 μL of bulk sample was analyzed. The osmolality of the standard medium was approximately $0.2 \text{ osmol kg}^{-1}$.

2.8 | Cultivation and online analysis using the respiration activity monitoring system

For respiration activity monitoring, main cultures were inoculated as described before and cultivated in modified non-baffled 500-mL shaking flasks with 100 mL filling volume using an in-house manufactured respiration activity monitoring system (RAMOS) device [59,60]. Commercial versions of the RAMOS device can be obtained from Kühner AG (Biersfelden/Switzerland) or HiTec Zang GmbH (Herzogenrath, Germany). These cultivations were performed in darkness at 30°C using an orbital shaker (Climo-Shaker ISF1-X, Kühner AG, Switzerland) with a shaking diameter of 50 mm and a shaking frequency of 180 min^{-1} .

2.9 | Microscopy and image analysis

Cell morphology was monitored offline by acquisition of a minimum of 500 pellets per sample with a digital inverse microscope (EVOS xl, AMG, Bothell, USA). A sample of 0.3 mL cultivation broth was diluted with 1 mL 0.9% NaCl and spread on a 90-mm petri dish. The brightness of the microscope was set to 1% below overexposure. Image capturing was performed at $2\times$ magnification ($4.5 \mu\text{m}$ per pixel). For image analysis, the public domain software Fiji/ImageJ v1.52 (National Institute of Health, Bethesda, USA) was used. Each image was converted into an 8-bit grayscale image. A pixel intensity threshold of 111 was used to distinguish between dense (pellets or clumps) and dispersed mycelial biomass. Pixel intensity values above 223 were caused by some mycelia lying outside the focus area and therefore were regarded as background noise. The relative area covered by pellets was calculated as the quotient of the sum P of pixels with intensity $I \leq 111$ to the sum of pixels with intensity $I \leq 223$ for each image:

$$A_{\text{Pellet,rel}} = \frac{\sum_{I=0}^{111} P(I)}{\sum_{I=0}^{223} P(I)} \quad (2)$$

Since a proportional correlation between the fraction of pellets present in the culture and the relative pellet area on the microscopy images is assumed, the relative pellet area was taken as value for the pellet fraction. Euclidian morphology parameters (projected area, perimeter, minimum, and maximum Feret diameter) and shape descriptors (aspect ratio, circularity, roundness, solidity) were determined using ImageJ's built-in particle analysis function. Pellets intersecting the edge

FIGURE 2 Kinetics of carbon source consumption (starch metabolism similar to glucose, see supporting information Figure S1), cell growth and labyrinthopeptin A1 formation in *A. namibiensis*

of an image were excluded. Furthermore, only pellets with a minimum projected area of $10\,000 \mu\text{m}^2$ were counted and included in the calculation of morphology parameters.

3 | RESULTS AND DISCUSSION

3.1 | Growth and product kinetics

In preliminary tests, a complex medium based on medium 5294 [39] containing glucose, glycerol, and soluble starch as carbon sources and a mixture of yeast extract, peptone and corn steep as source of nitrogen, vitamins, and trace elements was the best production medium for *A. namibiensis*. With the omission of one of these carbon sources or the complex components, either growth or product formation was substantially lower. Corn steep was found to be an essential complex component for sufficient growth and used with a concentration of 3 g L^{-1} in the cultures. Figure 2 shows the profile of growth and labyrinthopeptin A1 production by *A. namibiensis* in a batch experiment of 500-mL shaking flask cultures (working volume 100 mL). Glucose and starch (not shown in Figure 2) are consumed simultaneously (see Supporting Information Figure S1) within the first four days of cultivation and are mainly used for biomass production. Subsequently, the exhaustion of glucose and starch causes a diauxic shift to glycerol, which is metabolized slower than glucose, while the biomass remains stationary in the range of 10 to 12 g L^{-1} . NGA labyrinthopeptin A1 production starts when glucose is almost depleted. The highest labyrinthopeptin A1 concentration is reached in the late stationary phase. After 14 days of cultivation, degradation of labyrinthopeptin A1 is frequently observed. Thus, under the given culture conditions, the best time for product harvesting is in the range of 9–11 days of cultivation. Several studies show, that a cultivation time of

FIGURE 3 Comparison of maximum labyrinthopeptin A1 concentrations in 500-mL shaking flask cultures for 10 days: (A) Supplemented with ammonium- and sulfate-containing salts ((NH₄)₂SO₄, NH₄Cl, and K₂SO₄ between 0 and 100 mM). (B) After 4, 6, 8 and 10 days of cultivation and (NH₄)₂SO₄ supplementation (between 0 and 200 mM) in the medium

approximately one week and more is not uncommon for filamentous organisms [40–44].

3.2 | Increase in productivity by salt-enhanced cultivation

To determine the effect of salt-induced osmotic stress, sodium chloride (NaCl), ammonium sulfate ((NH₄)₂SO₄), ammonium chloride (NH₄Cl), and potassium sulfate (K₂SO₄) were added to the cultivation medium. The four examined salts cover the physiologically most important ions, except for phosphate, that may be examined in further studies. Phosphate salts were not investigated, because they buffer the *pH* at undesired values which may affect growth, morphology, and productivity. When the cultivation medium was supplemented with (NH₄)₂SO₄, K₂SO₄ or NH₄Cl in concentrations between 25 and 100 mM, the produced amount of labyrinthopeptin A1 within the first 10 days of cultivation was significantly increased compared to the non-supplemented control (Figure 3A).

In the case of (NH₄)₂SO₄ supplementation, the product concentration increased steadily over cultivation time (Figure 3B). The highest labyrinthopeptin A1 concentration of 325 mg L⁻¹ was achieved by the use of 50 mM (NH₄)₂SO₄, which is seven times more than that in the non-supplemented medium. At higher concentrations of (NH₄)₂SO₄, labyrinthopeptin A1 concentrations increased to a lesser extent as growth slowed with increased (NH₄)₂SO₄ concentration, resulting in decreased final biomass concentration (Figure 4). Long error bars were obtained, because heterogeneous, vegetative biomass was used for inoculation instead of spores. This method was chosen since the organism

FIGURE 4 Biomass growth in medium without (NH₄)₂SO₄ supplementation (0 mM) and with 50 mM (NH₄)₂SO₄ supplementation and final biomass concentrations of all tested (NH₄)₂SO₄ concentrations

does not sporulate well. Additionally, it takes a very long time for the spores to germinate. Nevertheless, the changes evoked by different salts are greater than the variability within the triplicates.

The biomass curves show, that *A. namibiensis* grows nearly linear in the first four days of cultivation, before growth decelerates. A linear instead of exponential growth is typical for many filamentous organisms. As the biomass concentration and the viscosity of the culture broth rises, mass transfer limitation may occur. On the one hand, mycelial aggregates eventually reach a critical size, so that the inner

FIGURE 5 Comparison of *A. namibiensis* respiration activity monitored via the (A) oxygen transfer rate and the (B) integrated oxygen transfer rate without any salt supplementation and supplementation of either 100 mM $(\text{NH}_4)_2\text{SO}_4$ or 100 mM NH_4Cl . For clarity, only every seventh data point is shown

core is not supplied with oxygen and nutrients anymore. On the other hand, the gas/liquid mass transfer may also be limited. The nutrient which is most likely to be limited first is oxygen [45]. It was therefore examined whether enough oxygen was available to the cells during cultivation.

A commonly applied method to monitor the respiration activity of microorganisms in shaking flasks is the cultivation using a RAMOS device [60]. Oxygen limited cultures are characterized by a plateau of the oxygen transfer rate (OTR) at the maximum oxygen transfer capacity of given culture conditions [61].

Shaking flask cultivations of *A. namibiensis* using either the non-supplemented medium 5294 or one of two salt supplements did not show any plateauing of the OTR at high values (Figure 5A). Additionally, none of the cultures reached OTR values close to the theoretical maximum oxygen transfer capacity of $9.3 \text{ mmol L}^{-1} \text{ h}^{-1}$ (according to Meier et al. [61] at an osmolality of $0.48 \text{ osmol kg}^{-1}$, see Supporting Information Figure S2). Therefore, an oxygen limitation caused by the process conditions can be ruled out. This conclusion was supported by further experiments where the same OTR trend was observed for culture conditions with higher maximum oxygen transfer capacities set by using lower culture filling volumes (50 and 25 mL, data not shown).

The OTR of the control cultures during the first four to five days correspond to biomass growth, whereas the production phase of labyrinthopeptin A1 is indicated by a slightly decreasing OTR plateau from $1.0 \text{ mmol L}^{-1} \text{ h}^{-1}$ at a cultivation time of six days to $0.7 \text{ mmol L}^{-1} \text{ h}^{-1}$ at the end of the cultivation (Figure 5A). Compared to the non-supplemented cultures, the integrated OTR of the $(\text{NH}_4)_2\text{SO}_4$ -supplemented cultures was slightly lower during the first four to six days (Figure 5B) which is consistent with lower biomass growth

(Figure 4), but showed a more elevated OTR level during days four to ten when labyrinthopeptin A1 production takes place (Figure 2).

The deviation of the OTR progression for duplicates of the same culture condition reflects the before mentioned heterogeneity of the preculture. However, the integrated OTR for supplemented and non-supplemented media were clearly following separate trends (Figure 5B). In the cultures supplemented with $(\text{NH}_4)_2\text{SO}_4$, this deviation might have been amplified due to the occurrence of foam in the shaking flasks. Peculiarly, the non-supplemented as well as the NH_4Cl supplemented shaking flask cultures did not show any foaming.

The biomass-related product yield coefficients $Y_{P/X}$ were estimated after 10 days of cultivation at different $(\text{NH}_4)_2\text{SO}_4$ concentrations between 0 and 200 mM. $Y_{P/X}$ is increased by a factor of 12.5 when comparing the non-supplemented control with the addition of 50 mM $(\text{NH}_4)_2\text{SO}_4$ (Table 1). Then, with an increased salt amount, $Y_{P/X}$ decreased to the initial value of approximately $2.6 \text{ mg}_P \text{ g}_{CDW}^{-1}$ at 200 mM $(\text{NH}_4)_2\text{SO}_4$. The STY values show a similar progression, with 32.1 and $29.5 \text{ mg}_P \text{ L}^{-1} \text{ d}^{-1}$ being the highest values at 50 and 100 mM $(\text{NH}_4)_2\text{SO}_4$ supplementation, respectively. The maximum specific productivity q_P was 4.2 times higher with $(\text{NH}_4)_2\text{SO}_4$ supplementation than in the non-supplemented cultivation. Taken together, these parameters show that the positive impact of $(\text{NH}_4)_2\text{SO}_4$ on product formation is greater than the negative impact on biomass growth.

While glucose metabolism is slightly affected by $(\text{NH}_4)_2\text{SO}_4$ supplementation (Figure 6A), the uptake of glycerol is significantly accelerated by $(\text{NH}_4)_2\text{SO}_4$ supplementation of 50 and 100 mM. A higher $(\text{NH}_4)_2\text{SO}_4$ supplementation resulted in decreased metabolism of glycerol (Figure 6B).

TABLE 1 Values of growth and product formation of *A. namibiensis* calculated from cultures of 10 days with $(\text{NH}_4)_2\text{SO}_4$ supplementation between 0 and 200 mM

$(\text{NH}_4)_2\text{SO}_4$ addition [mM]	Biomass concentration [g L ⁻¹]	Space-time- yield STY [mg _P L ⁻¹ d ⁻¹]	Max. specific productivity $q_{P, \max}$ [mg _P g _{CDW} ⁻¹ d ⁻¹]	Yield coefficient $Y_{P/X}$ [mg _P g _{CDW} ⁻¹]
0 ^a	12.0 ± 0.3	3.2 ± 0.4	13.1	2.7 ± 0.4
50 ^a	9.4 ± 0.2	32.1 ± 1.5	54.3	33.9 ± 5.1
100	9.3 ± 0.2	29.5 ± 4.3	— ^b	31.7 ± 5.3
150	7.9 ± 0.8	20.9 ± 10.1	— ^b	26.5 ± 14.9
200	6.6 ± 1.0	1.73 ± 1.3	— ^b	2.6 ± 2.4

^aDetailed profile of biomass growth is given in Figure 4.

^bNot determined.

FIGURE 6 (A) Glucose concentration and (B) glycerol concentration over cultivation time in cultures with $(\text{NH}_4)_2\text{SO}_4$ supplementation between 0 and 200 mM. (C) Correlation between glycerol consumption rate and labyrinthopeptin A1 production rate with $(\text{NH}_4)_2\text{SO}_4$ supplementation between 0 and 200 mM. (D) Specific production rate q_P for labyrinthopeptin A1 as a function of specific growth rate μ without (0 mM) and with (50 mM) $(\text{NH}_4)_2\text{SO}_4$ supplementation (determination with Equation 1)

The amount of labyrinthopeptin A1 production correlates well with the glycerol consumption rate (compare Table 1, Figure 6C). Although supplementation of the cultivation medium with 50 to 100 mM $(\text{NH}_4)_2\text{SO}_4$ results in faster glycerol consumption; biomass concentration is higher in the

non-supplemented control. This suggests that glycerol is mainly used as a carbon source for the biosynthesis of secondary metabolites and is scarcely used to accumulate biomass. Moreover, many organisms accumulate or release electrolytes and small organic solutes such as glycerol in

the case of an osmotic upshift. In *S. coelicolor*, glycerol is taken up by facilitated diffusion [46]. Hence, the increased production of labyrinthopeptin A1 in *A. namibiensis* could also be related to facilitated glycerol uptake due to the osmotic pressure induced by $(\text{NH}_4)_2\text{SO}_4$. However, with increasing $(\text{NH}_4)_2\text{SO}_4$ concentrations over 100 mM, glycerol consumption and labyrinthopeptin A1 production decreased, since higher osmolality inhibits growth and substrate metabolization.

In Figure 6D, the specific production rate q_P of standard cultivation and the salt-enhanced cultivation with 50 mM $(\text{NH}_4)_2\text{SO}_4$ is plotted as a function of the specific growth rate μ to show that the product formation kinetics of labyrinthopeptin A1 are nearly independent of the biomass concentration at sample time, which differs between the investigated cultivation conditions. When μ trends to 0, q_P increases significantly for the non-supplemented and the 50 mM $(\text{NH}_4)_2\text{SO}_4$ case, which is expected for NGA product formation. At $\mu < 0.2 \text{ d}^{-1}$, q_P increases slightly in the case of the non-supplemented control, whereas with the salt-enhanced cultivation, the increase in q_P intensifies strongly.

The overall osmolality of the cultivation medium with 50 mM $(\text{NH}_4)_2\text{SO}_4$ decreased by approximately $0.25 \text{ osmol kg}^{-1}$ over the cultivation time, whereas the osmolality of the non-supplemented control and cultures with higher amounts of $(\text{NH}_4)_2\text{SO}_4$ decreased by no more than $0.1 \text{ osmol kg}^{-1}$ (Supporting Information Figure S2). Since sodium chloride (NaCl) did not lead to increased labyrinthopeptin A1 production in concentrations up to 100 mM, but equiosmolal concentrations of NH_4Cl increased the labyrinthopeptin A1 concentration by 50 to 100%, it is likely that ammonium and sulfate ions in particular have a positive impact on labyrinthopeptin A1 formation. The concentration of ammonium ions decreased by 70 and 33% in cultivations with 50 mM $(\text{NH}_4)_2\text{SO}_4$ and 100 mM NH_4Cl supplementation, respectively (Supporting Information Figure S3), suggesting that the supplemented ammonium was partly used as an additional nitrogen source. Thus, the increased productivity of labyrinthopeptin A1 could not only be a result of higher osmolality, but also related to the excess of nitrogen source. However, nitrogen-free K_2SO_4 performed similar to NH_4Cl , and productivity was much higher with 50 mM $(\text{NH}_4)_2\text{SO}_4$ than with 100 mM NH_4Cl containing the same amount of nitrogen. Therefore, the type and concentration of ammonium salt plays an important role, but the total amount of ammonium is a minor factor.

Nevertheless, the secondary metabolism of *A. namibiensis* may be nitrogen-controlled as described for many other *Actinobacteria*. However, some kind of ammonium repression of antibiotic production is observed in many cultivations when ammonium salts are supplied in excess [47–49]. A comprehensive review on preferred nitrogen sources and the influence of ammonium on *Actinobacteria* was given

by Wohlleben et al. [50]. Usually, the onset of secondary metabolite production is repressed by ammonium, and antibiotic production is stimulated with ammonium exhaustion, but some studies also reported a stimulation of antibiotic production by ammonium salts such as $(\text{NH}_4)_2\text{SO}_4$ [50]. The optimal $(\text{NH}_4)_2\text{SO}_4$ concentration was 0.6 mM for the biosynthesis of clavulanic acid in *S. clavuligerus* [51], 1.5 mM for gilvocarcin in *S. arenae* 2064 [52], 10 mM for avilamycin in *S. viridochromogenes* [53], 11 mM for avermectin in *S. avermitilis* [54], 40 mM for rapamycin in *S. hygroscopicus* [55], and 113 mM for the immunosuppressant L-683,590 in *S. hygroscopicus* MA7040 [56]. In most of the mentioned cases, $(\text{NH}_4)_2\text{SO}_4$ concentrations higher than the optimum for antibiotic production further promoted growth but drastically decreased product concentrations. By contrast, the generated data from this study show a growth-inhibiting effect with increased amounts of $(\text{NH}_4)_2\text{SO}_4$ (Table 1, Figure 4). Presumably, this effect can be attributed to the induced osmotic stress caused by the relatively high salt concentration applied in this study.

3.3 | Cellular morphology

To quantify the impact of $(\text{NH}_4)_2\text{SO}_4$ supplementation on cellular morphology, the shape and size of pellets were measured. Since ammonium and sulfate ions are able to bind a large number of water molecules, they may compete with water molecules in the hydrate shell of the outer filaments of the pellets and change the physicochemical properties next to the cell wall. Thus, pellet formation can be influenced, resulting in a change in the macromorphology. The distributions of Euclidian morphology parameters are depicted for the Feret diameter (Figure 7A), projected area (Figure 7B), aspect ratio (Figure 7C) and circularity (Figure 7D) of the pellets after 10 days of cultivation.

Compared to the non-supplemented control, the use of 50 mM $(\text{NH}_4)_2\text{SO}_4$ resulted in pellets with smaller Feret diameters (A), but the distribution of the projected area (B) differed only slightly between both cultivation conditions. Thus, the aspect ratio (C) of these pellets is clearly shifted towards more spherical morphologies with values close to one, whereas the non-supplemented control contains a larger proportion of elongated pellets (aspect ratio > 2). This also coincides with the values of circularity (D). Here, the distribution of the values of the salt-enhanced cultivation is shifted to more circular morphologies, because the corresponding pellets have smaller perimeters. In Figure 8, this finding is illustrated by microscopic images. When comparing the images of day four, it becomes clear that pellets grown with $(\text{NH}_4)_2\text{SO}_4$ exhibit a circular and less irregular shape. While the highest average pellet diameter in the non-supplemented control is observed after two days of cultivation, the pellets grown with salt-enhanced cultivation reach their maximum diameter after

FIGURE 7 Distributions of Euclidian morphology parameters for (A) Feret diameter, (B) Projected area, (C) Aspect ratio, and (D) Circularity of *A. namibiensis* pellets grown for 10 days without (0 mM) and with $(\text{NH}_4)_2\text{SO}_4$ (50 mM) supplementation of the medium

FIGURE 8 Development of cell morphology of *A. namibiensis* grown in complex medium: (A) without $(\text{NH}_4)_2\text{SO}_4$ addition (0 mM) and (B) with 50 mM $(\text{NH}_4)_2\text{SO}_4$ supplementation in 500-mL shaking flasks. The number in each picture indicates the cultivation time in days. The edge length of each picture corresponds to 5 mm

approximately four days of cultivation due to slower growth and are smaller on average.

However, *A. namibiensis* always develops a highly heterogeneous mycelial morphology, in which pellets with a broad

size range and free mycelia coexist. Figure 9 shows the fraction of pellets estimated from the relative pellet area in the microscopic images as determined by Equation (2) over cultivation time. It is noticeable that pellets grow predominate

FIGURE 9 Pellet fraction estimated from the relative pellet area of microscopic images ($n = 10$) according to equation (2) (bars) and fraction of individual pellets with a projected area $> 40\,000\ \mu\text{m}^2$ (boxes) over cultivation time

in the first three days of cultivation as long as glucose is still available. In the subsequent stationary phase, the pellet fraction gradually decreases. Instead, more and more mycelia are freely dispersed or loosely aggregated. After 10 days of cultivation, over 50% of the captured area is covered by loose mycelia for both cultivation conditions.

Furthermore, it was found that the space between the pellets was gradually filled by loose mycelia and that a reduction of pellet number occurred during the production phase on glycerol. This is generally applied to pellets of all sizes, both in the control and the salt-enhanced cultivation. A difference between both cultivation conditions regarding pellet reduction was only found in the largest pellets with an area $> 40\,000\ \mu\text{m}^2$. In the growth phase on glucose, large pellets more frequently formed in the non-supplemented control and decreased rapidly after 4 days of cultivation, while their reduction was slower in the salt-enhanced cultivation. Thus, the addition of $(\text{NH}_4)_2\text{SO}_4$ not only changed the morphology of the pellets but also affected the speed of pellet formation and disintegration of the pellet fraction in the stationary growth phase, leading to different time-dependent compositions of pellet fragments, loose clumps and free mycelia in the cultivation broth.

A transition from pellet to dispersed morphology, i.e., the disintegration from pellets into looser fragments after a certain period of cultivation time, was also reported for bioreactor cultivations of *Penicillium chrysogenum* under diverse process conditions [17,57,58]. Posch and Herwig [58] found that this effect depends on the specific growth rate normalized with respect to the spore inoculum concentration used and, to a lesser degree, on the fraction of the culture of *P. chrysogenum* present in pellet morphology. Increased pellet growth in the exponential growth phase later caused

increased pellet erosion and breakage, resulting in an accelerated transition to a dispersed morphology. This is in accordance with the present observation on the morphology of *A. namibiensis* under the conditions of the salt-enhanced cultivation. In the non-supplemented culture, pellets grew faster and reached larger sizes. Correspondingly, they also disintegrated faster than the smaller pellets of the cultures with 50 mM $(\text{NH}_4)_2\text{SO}_4$ supplementation.

By having smaller diameters on average, the pellet cores of biomass from cultures with $(\text{NH}_4)_2\text{SO}_4$ are less substrate-limited. On the other hand, high amounts of mycelium can lead to a higher broth viscosity and a poorer transport of oxygen and nutrients within the broth. A mixed morphology of small pellets and a specific amount of dispersed mycelia may provide a good balance between the advantages and disadvantages of both morphological growth forms. However, the composition of pellets and dispersive mycelium changes considerably over cultivation time. Since glycerol consumption is affected by $(\text{NH}_4)_2\text{SO}_4$, it is likely that this inorganic salt affects the morphology indirectly through changes in bacterial physiology. An additional direct external influence such as changed physicochemical properties near the cell walls is also possible.

4 | CONCLUDING REMARKS

The salt-enhanced cultivation was applied with the aim of increasing the production of labyrinthopeptin A1 by *A. namibiensis*. The highest labyrinthopeptin A1 concentrations within 10 days of cultivation in a complex medium was obtained with ammonium salts (particularly 50 mM $(\text{NH}_4)_2\text{SO}_4$). Thus, the process kinetic values for STY , q_P , and $Y_{P/X}$ are also significantly increased compared to those of the non-supplemented control. It was shown that the conducted shaking flask cultures were sufficiently supplied with oxygen during the whole cultivation time. Since the measured OTRs of the replicates differed due to culture heterogeneities and foam formation, the influence of such homogeneities on the main culture and the origin of foam formation in non-baffled flasks should be further investigated. The inorganic salt also enhanced the metabolization of glycerol, one of the main carbon sources used, and affected the strain's pellet macro-morphology, which was characterized in detail for the first time. Whether additional salt was used in cultivation or not, *A. namibiensis* developed a heterogeneous morphology in which both pellets and mycelia were present simultaneously. Pellets only grew as long as glucose was available in the medium and showed disintegration in the stationary production phase. In contrast to the pellets of the non-supplemented cultivation, the pellets produced with 50 mM $(\text{NH}_4)_2\text{SO}_4$ were considerably smaller, circular and less frayed. During

the production of labyrinthopeptin A1, more and more pellets disintegrated. Accordingly, the percentage of loosened mycelium increased in the stationary growth phase. The larger pellets of the non-supplemented cultivation disintegrated fastest. Hence, $(\text{NH}_4)_2\text{SO}_4$ prevented the pellets from total disintegration by limiting their maximum size, suggesting that compact, small pellets are beneficial for labyrinthopeptin production in *A. namibiensis*. Most likely, the morphological differences between the salt-enhanced cultivation and the non-supplemented control result from metabolic changes that were induced by the higher supply of nitrogen in the form of ammonium and sulfate ions, respectively. The exact mode of action of these ions with regard to the physiology, morphological alteration and product formation of filamentous organisms still has to be investigated. For studies on the involved metabolic pathways and their genetic regulation in *A. namibiensis*, a suitable minimal medium for labyrinthopeptin production should be developed. Investigating the metabolism of *A. namibiensis* and other filamentously growing bacteria will help to provide a better understanding of the complex relationship between morphology, labyrinthopeptin formation and biomass production. This knowledge is important for developing a robust and economically feasible biotechnological process for labyrinthopeptin production.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

REFERENCES

- Meindl, K., Schmiederer, T., Schneider, K., Reicke, A. et al., Labyrinthopeptins: A new class of carbacyclic lantibiotics. *Angew. Chemie Int. Ed.* 2010, 49, 1151–1154.
- Seibert, G., L., V., Wink, J., Winkler, I. et al., Antibacterial and antiviral peptides from *Actinomadura namibiensis*. International Application No. PCT/EP2007/008294, 2008.
- Wink, J., Kroppenstedt, R. M., Seibert, G., Stackebrandt, E., *Actinomadura namibiensis* sp. nov. *Int. J. Syst. Evol. Microbiol.* 2003, 53, 721–724.
- Férir, G., Petrova, M. I., Andrei, G., Huskens, D. et al., The lantibiotic peptide labyrinthopeptin A1 demonstrates broad anti-HIV and anti-HSV activity with potential for microbicidal applications. *PLoS One* 2013, 8, e64010.
- Haid, S., Blockus, S., Wiechert, S. M., Wetzke, M. et al., Labyrinthopeptin A1 and A2 efficiently inhibit cell entry of hRSV isolates. *Eur. Respir. J.* 2017, 50, 4124.
- Martinez, J. P., Sasse, F., Brönstrup, M., Diez, J. et al., Antiviral drug discovery: broad-spectrum drugs from nature. *Nat. Prod. Rep.* 2015, 32, 29–48.
- Krawczyk, J. M., Völler, G. H., Krawczyk, B., Kretz, J. et al., Heterologous expression and engineering studies of labyrinthopeptins, class III lantibiotics from *Actinomadura namibiensis*. *Chem. Biol.* 2013, 20, 111–122.
- Rupcic, Z., Hüttel, S., Bernecker, S., Kanaki, S. et al., Large scale production and downstream processing of labyrinthopeptins from the actinobacterium *Actinomadura namibiensis*. *Bioengineering* 2018, 5, E42.
- Driouch, H., Hänsch, R., Wucherpfennig, T., Krull, R. et al., Improved enzyme production by bio-pellets of *Aspergillus niger*: targeted morphology engineering using titanate microparticles. *Biotechnol. Bioeng.* 2012, 109, 462–471.
- Papagianni, M., Matthey, M., Morphological development of *Aspergillus niger* in submerged citric acid fermentation as a function of the spore inoculum level. Application of neural network and cluster analysis for characterization of mycelial morphology. *Microb. Cell Fact.* 2006, 5, 1–12.
- Ahamed, A., Vermette, P., Effect of culture medium composition on *Trichoderma reesei*'s morphology and cellulase production. *Biore-sour. Technol.* 2009, 100, 5979–5987.
- Driouch, H., Roth, A., Dersch, P., Wittmann, C., Optimized bioprocess for production of fructofuranosidase by recombinant *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 2010, 87, 2011–2024.
- Babič, J., Pavko, A., Enhanced enzyme production with the pelleted form of *D. squalens* in laboratory bioreactors using added natural lignin inducer. *J. Ind. Microbiol. Biotechnol.* 2012, 39, 449–457.
- Wucherpfennig, T., Lakowitz, A., Driouch, H., Krull, R. et al., Customization of *Aspergillus niger* morphology through addition of talc micro particles. *J. Vis. Exp.* 2012, 1–6.
- Wucherpfennig, T., Kiep, K. A., Driouch, H., Wittmann, C. et al., *Morphology and Rheology in filamentous Cultivations*, 1st ed., Elsevier Inc. 2010.
- Olmos, E., Mehmood, N., Haj Husein, L., Goergen, J. L. et al., Effects of bioreactor hydrodynamics on the physiology of *Streptomyces*. *Bioprocess Biosyst. Eng.* 2013, 36, 259–272.
- Nielsen, J., Modelling the morphology of filamentous microorganisms. *Trends Biotechnol.* 1996, 14, 438–443.
- Wucherpfennig, T., Hestler, T., Krull, R., Morphology engineering - Osmolality and its effect on *Aspergillus niger* morphology and productivity. *Microb. Cell Fact.* 2011, 10, 1–15.
- Pamboukian, C. R. D., Facciotti, M. C. R., Production of the antitumoral retamycin during continuous fermentations of *Streptomyces olindensis*. *Process Biochem.* 2004, 39, 2249–2255.
- Jonsbu, E., McIntyre, M., Nielsen, J., The influence of carbon sources and morphology on nystatin production by *Streptomyces noursei*. *J. Biotechnol.* 2002, 95, 133–144.
- Dobson, L. F., O'Cleirigh, C. C., O'Shea, D. G., The influence of morphology on geldanamycin production in submerged

- fermentations of *Streptomyces hygroscopicus* var. *geldanus*. *Appl. Microbiol. Biotechnol.* 2008, 79, 859–866.
22. Walisko, J., Vernen, F., Pommerehne, K., Richter, G. et al., Particle-based production of antibiotic rebeccamycin with *Lechevalieria aerocolonigenes*. *Process Biochem.* 2017, 53, 1–9.
 23. Vecht-Lifshitz, S. E., Sasson, Y., Braun, S., Nikkomycin production in pellets of *Streptomyces tendae*. *J. Appl. Bacteriol.* 1992, 72, 195–200.
 24. Yin, P., Wang, Y. H., Zhang, S. L., Chu, J. et al., Effect of mycelial morphology on bioreactor performance and avermectin production of *Streptomyces avermitilis* in submerged cultivations. *J. Chinese Inst. Chem. Eng.* 2008, 39, 609–615.
 25. Yang, Y. K., Morikawa, M., Shimizu, H., Shioya, S. et al., Image analysis of mycelial morphology in virginiamycin production by batch culture of *Streptomyces virginiae*. *J. Ferment. Bioeng.* 1996, 81, 7–12.
 26. Belmar-Beiny, M. T., Thomas, C. R., Morphology and clavulanic acid production of *Streptomyces clavuligerus*: effect of stirrer speed in batch fermentations. *Biotechnol. Bioeng.* 1991, 37, 456–462.
 27. Braun, S., Vecht-Lifshitz, S. E., Mycelial morphology and metabolite production. *Trends Biotechnol.* 1991, 9, 63–68.
 28. Manteca, A., Alvarez, R., Salazar, N., Yagüe, P. et al., Mycelium differentiation and antibiotic production in submerged cultures of *Streptomyces coelicolor*. *Appl. Environ. Microbiol.* 2008, 74, 3877–3886.
 29. Treskatis, S. K., Orgeldinger, V., Wolf, H., Gilles, E. D., Morphological characterization of filamentous microorganisms in submerged cultures by on-line digital image analysis and pattern recognition. *Biotechnol. Bioeng.* 1997, 53, 191–201.
 30. Antecka, A., Bizukojc, M., Ledakowicz, S., Modern morphological engineering techniques for improving productivity of filamentous fungi in submerged cultures. *World J. Microbiol. Biotechnol.* 2016, 32, 193.
 31. Gonciarz, J., Bizukojc, M., Adding talc microparticles to *Aspergillus terreus* ATCC 20542 preculture decreases fungal pellet size and improves lovastatin production. *Eng. Life Sci.* 2014, 14, 190–200.
 32. Kaup, B.-A., Ehrich, K., Pescheck, M., Schrader, J., Microparticle-enhanced cultivation of filamentous microorganisms: increased chloroperoxidase formation by *Caldariomyces fumago* as an example. *Biotechnol. Bioeng.* 2008, 99, 491–498.
 33. Elmayergi, H., Moo-Young, M., Scharer, M., Effects of polymer additives on fermentation parameters in a culture of *A. niger*. *Biotechnol. Bioeng.* 1973, 15, 845–859.
 34. Allaway, A. E., Jennings, D. H., The effect of cations on glucose utilization by, and on the growth of, the fungus *Dendryphiella salina*. *New Phytol.* 1971, 70, 511–518.
 35. Bobowicz-Lassociska, T., Grajek, W., Changes in protein secretion of *Aspergillus niger* caused by the reduction of the water activity by potassium chloride. *Acta Biotechnol.* 1995, 15, 277–287.
 36. Fiedurek, J., Effect of osmotic stress on glucose oxidase production and secretion by *Aspergillus niger*. *J. Basic Microbiol.* 1998, 38, 107–112.
 37. Okba, A. K., Ogata, T., Matsubara, H., Matsuo, S. et al., Effects of bacitracin and excess Mg^{2+} on submerged mycelial growth of *Streptomyces azureus*. *J. Ferment. Bioeng.* 1998, 86, 28–33.
 38. Fuchino, K., Flärdh, K., Dyson, P., Ausmees, N., Cell-biological studies of osmotic shock response in *Streptomyces* spp. *J. Bacteriol.* 2017, 199, e00465-16.
 39. Wink, J., Methods for the taxonomic description of the Actinobacteria. *Compendium of Actinobacteria*. 2009.
 40. Nevalainen, K. M. H., Te'o, V. S. J., Bergquist, P. L., Heterologous protein expression in filamentous fungi. *Trends Biotechnol.* 2005, 23, 468–474.
 41. Manzoni, M., Bergomi, S., Rollini, M., Cavazzoni, V., Production of statins by filamentous fungi. *Biotechnol. Lett.* 1999, 21, 253–257.
 42. Litthauer, D., van Vuuren, M. J., van Tonder, A., Wolfaardt, F. W., Purification and kinetics of a thermostable laccase from *Pycnoporus sanguineus* (SCC 108). *Enzyme Microb. Technol.* 2007, 40, 563–568.
 43. Rau, U., Nguyen, L. A., Schulz, S., Wray, V. et al., Formation and analysis of mannosylerythritol lipids secreted by *Pseudozyma aphidis*. *Appl. Microbiol. Biotechnol.* 2005, 66, 551–559.
 44. Lin, Z. J., Zhu, T. J., Chen, L., Gu, Q. Q., Three new aspothalasin derivatives from the marine-derived fungus *Spicaria elegans*. *Chinese Chem. Lett.* 2010, 21, 824–826.
 45. Prosser, J. I., Mathematical modelling of fungal growth. *Grow. Fungus* 2007, 319–335.
 46. Bertram, R., Schlicht, M., Mahr, K., Nothhaft, H. et al., In silico and transcriptional analysis of carbohydrate uptake systems of *Streptomyces coelicolor* A3(2). *Society* 2004, 186, 1362–1373.
 47. Aharonowitz, Y., Nitrogen metabolite regulation of antibiotic biosynthesis. *Annu. Rev. Microbiol.* 1980, 34, 209–233.
 48. Martin, J. F., Demain, A. L., Control of antibiotic biosynthesis. *Microbiol. Rev.* 1980, 44, 230–251.
 49. Sanchez, S., Demain, A. L., Metabolic regulation of fermentation processes. *Enzyme Microb. Technol.* 2002, 31, 895–906.
 50. Wohlleben, W., Bera, A., Mast, Y., Stegmann, E., Regulation of secondary metabolites of Actinobacteria, in: Wink, J., Mohammadipani, F., Hamedi, J. (Eds.), *Biology and Biotechnology of Actinobacteria*, Springer International Publishing, Cham 2017, pp. 181–232.
 51. Visser-Luirink, G., De Laat, W. T. A., Klop, J. M., Fermentation of clavulanic acid at a controlled level of ammonia. USP (US 6991925 B2) 2006, 6, 991–925.
 52. Byrne, K. M., Greenstein, M., Nitrogen repression of gilvocarcin V production in *Streptomyces arenae* 2064. *J. Antibiot. (Tokyo)* 1986, 39, 594–600.
 53. Zhu, C. H., Lu, F. P., He, Y. N., Han, Z. L. et al., Regulation of avilamycin biosynthesis in *Streptomyces viridochromogenes*: effects of glucose, ammonium ion, and inorganic phosphate. *Appl. Microbiol. Biotechnol.* 2007, 73, 1031–1038.
 54. Cimburková, E., Zima, J., Novak, J., Vanek, Z., Nitrogen regulation of avermectins biosynthesis in *Streptomyces avermitilis* in a chemically defined medium. *J. Basic Microbiol.* 1988, 28, 491–499.
 55. Lee, M. S., Kojima, I., Demain, A. L., Effect of nitrogen source on biosynthesis of rapamycin by *Streptomyces hygroscopicus*. *J. Ind. Microbiol. Biotechnol.* 1997, 19, 83–86.
 56. Junker, B., Mann, Z., Gailliot, P., Byrne, K. et al., Use of soybean oil and ammonium sulfate additions to optimize secondary metabolite production. *Biotechnol. Bioeng.* 1998, 60, 580–588.
 57. Posch, A. E., Herwig, C., Physiological description of multivariate interdependencies between process parameters, morphology and physiology during fed-batch penicillin production. *Biotechnol. Prog.* 2014, 30, 689–699.
 58. Tiller, V., Meyerhoff, J., Ziele, D., Schügerl, K. et al., Segregated mathematical model for the fed-batch cultivation of a

- high-producing strain of *Penicillium chrysogenum*. *J. Biotechnol.* 1994, 34, 119–131.
59. Anderlei, T., Büchs, J., Device for sterile online measurement of the oxygen transfer rate in shaking flasks. *Biochem. Eng. J.* 2001, 7, 157–162.
60. Anderlei, T., Zang, W., Papaspyrou, M., Büchs, J., Online respiration activity measurement (OTR, CTR, RQ) in shake flasks. *Biochem. Eng. J.* 2004, 17, 187–194.
61. Meier, K., Klöckner, W., Bonhage, B., Antonov, E., Regestein, L., Büchs, J., Correlation for the maximum oxygen transfer capacity in shake flasks for a wide range of operating conditions and for different culture media. *Biochem. Eng. J.* 2016, 109, 228–235.
62. Krull, R., Bley, T. (eds.), Filaments in Bioprocesses, *Adv. Biochem. Eng./ Biotechnol.* 2015 149, Springer, Cham Heidelberg New York Dordrecht London, Springer International Publishing, Switzerland.

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