Macroparticle-enhanced cultivation of *Lentzea aerocolonigenes*: Variation of mechanical stress and combination with lecithin supplementation for a significantly increased rebeccamycin production

Kathrin Schrinner<sup>1,2</sup> | Marcel Schrader<sup>2,3</sup> | Jana Niebusch<sup>1</sup> | Kristin Althof<sup>1</sup> | Friederike A. Schwarzer<sup>1</sup> | Paul-Frederik Nowka<sup>1</sup> | Anna Dinius<sup>1,2</sup> | Arno Kwade<sup>2,3</sup> | Rainer Krull<sup>1,2</sup>

<sup>1</sup>Institute of Biochemical Engineering, Technische Universität Braunschweig, Braunschweig, Germany
<sup>2</sup>Center of Pharmaceutical Engineering, Technische Universität Braunschweig, Braunschweig, Germany
<sup>3</sup>Institute for Particle Technology, Technische Universität Braunschweig, Braunschweig, Germany

Correspondence
Rainer Krull, Institute of Biochemical Engineering, Technische Universität Braunschweig, Rebenring 56, 38106 Braunschweig, Germany.
Email: r.krull@tu-braunschweig.de.

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Abstract
The actinomycete *Lentzea aerocolonigenes* produces the antitumor antibiotic rebeccamycin. In previous studies the rebeccamycin production was significantly increased by the addition of glass beads during cultivation in different diameters between 0.5 and 2 mm and the induced mechanical stress by the glass beads was proposed to be responsible for the increased production. Thus, this study was conducted to be a systematic investigation of different parameters for macroparticle addition, such as bead diameter, concentration, and density (glass and ceramic) as well as shaking frequency, for a better understanding of the particle-induced stress on *L. aerocolonigenes*. The induced stress for optimal rebeccamycin production can be estimated by a combination of stress energy and stress frequency.

In addition, the macroparticle-enhanced cultivation of *L. aerocolonigenes* was combined with soy lecithin addition to further increase the rebeccamycin concentration. With 100 g L<sup>−1</sup> glass beads in a diameter of 969 µm and 5 g L<sup>−1</sup> soy lecithin a concentration of 388 mg L<sup>−1</sup> rebeccamycin was reached after 10 days of cultivation, which corresponds to the highest rebeccamycin concentrations achieved in shake flask cultivations of *L. aerocolonigenes* stated in literature so far.

KEYWORDS
beads, *Lentzea aerocolonigenes*, macroparticle-enhanced cultivation, mechanical stress, rebeccamycin, soy lecithin

Abbreviations: CDW, cell dry weight concentration (g L<sup>−1</sup>); <i>d</i><sub>gm</sub>, grinding medium diameter (m); DOT, dissolved oxygen tension (%); <i>ds</i>, shaking flask diameter (m); <i>E<sub>s</sub></i>, specific energy (J); <i>f<sub>s</sub></i>, shaker frequency (s<sup>−1</sup>); <i>n</i><sub>b</sub>, number of beads (−); <i>q<sub>r</sub></i>, specific rebeccamycin productivity (mg g<sup>−1</sup> d<sup>−1</sup>); <i>SE</i>, stress energy (J); <i>SE<sub>b</sub></i>,<i>sh</i>, stress energy of beads inside a shaking flask (J); <i>SE<sub>gm</sub></i>, stress energy of the grinding medium (J); <i>SF</i>, stress frequency (s<sup>−1</sup>); <i>SF<sub>b</sub></i>,<i>sh</i>, stress frequency of beads inside a shaking flask (s<sup>−1</sup>); <i>SN</i>, stress number (−); <i>t</i>, time (s); <i>u<sub>s</sub></i>, shaking velocity (m s<sup>−1</sup>); <i>u<sub>t</sub></i>, stirrer tip speed (m s<sup>−1</sup>); <i>x<sub>32</sub></i>, mean bead diameter (m); <i>ρ<sub>gm</sub></i>, grinding medium density (kg m<sup>−3</sup>).

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1  |  INTRODUCTION

Filamentous actinomycetes are often a source of bioactive natural products valuable for pharmaceutical industry and medical application (Genilouid, 2017). Therefore, their cultivation is considered quite worthwhile although certain challenges arise in the process. The filamentous actinomycete Lentzea aerocolonigenes for instance, produces the antitumor antibiotic rebeccamycin. Rebeccamycin is a topoisomerase inhibitor and thereby interferes with DNA replication. Despite this activity rebeccamycin itself is not suitable for use in the human body due to the low water-solubility (Bush et al., 1987; Nettleton et al., 1985). To overcome this issue different analogs with increased water-solubility were developed that can easily be derived from rebeccamycin by chemical transformation. The analog becatacarin has already been successfully tested in clinical Phase I and II studies for the treatment of refractory breast cancer, metastatic colorectal cancer, and small-cell lung cancer (Burstein et al., 2007; Goel et al., 2003; Schwandt et al., 2012). In the study of Schwandt et al. (2012) 4200 mg m² becatacarin were used for the treatment of small-cell lung cancer. For a person of 1.8 m and 85 kg this would correspond to a total of 8.7 g of substance. Regarding rebeccamycin titers in the literature mostly concentrations of under 100 mg L⁻¹ are achieved in small scales of around 100 ml (Pommerehne et al., 2019). Hence, cultivations of L. aerocolonigenes in the bioreactor scale with high productivities are desired. However, the complex cellular morphology of filamentous microorganisms, ranging from freely dispersed mycelium to dense pellets, provides challenges during the cultivation process. Each cell morphological form has certain advantages and disadvantages. The appropriate cell morphology for high productivities strongly depends on the microorganism and the desired product (Walsisko et al., 2015; Whitaker, 1992; Wucherpfennig et al., 2010).

Walsisko et al. (2017) added glass beads with a diameter between 0.25 and 2.1 mm to cultivations of L. aerocolonigenes and thereby increased the rebeccamycin production. The addition of a glass particle concentration of 80 g L⁻¹ with a size range of 0.25–0.5 mm caused a rebeccamycin concentration of 116 mg L⁻¹ which was the highest rebeccamycin concentration achieved in this cultivation approach and a 19-fold increase compared to an unsupplemented control which produced only 6 mg L⁻¹ rebeccamycin. Supplementation of coarser glass particles led to smaller pellets of L. aerocolonigenes and lower rebeccamycin concentrations. The mechanical stress induced by the glass beads was considered to be responsible for this effect, with the proper dose of mechanical stress being an important factor (Walsisko et al., 2017). The addition of glass macroparticles to cultivations of L. aerocolonigenes proved to be beneficial in regard to rebeccamycin formation in further studies. Schrader et al. (2019) investigated the influence of different particle diameters ranging from 0.2 to 2.1 mm on the final rebeccamycin concentration, causing differences in mechanical stress. In this case, the addition of glass beads with a mean diameter of 969 µm led to a higher rebeccamycin concentration of approximately 70 mg L⁻¹ than the addition of smaller glass beads with a mean diameter of 540 µm (similar to the beads in Walisko et al. (2017)) with only 57 mg L⁻¹ rebeccamycin. The different effects of glass bead diameters in these two studies arise from different shake flask geometries. In Schrinner et al. (2020) the differences between glass bead supplemented and unsupplemented cultivations over time were considered and additionally cellular morphological changes were investigated.

Macroparticle addition has already been used with other filamentous microorganisms (e.g., Dobson et al., 2008; Lee et al., 2010). Ochi (1984) used 3 mm glass beads for the homogenization of spores and biomass of Streptomyces sp. Sohoni et al. (2012) added glass beads of different diameters between 0.75 and 4 mm to cultivations of Streptomyces coelicolor in microtiter plates. In that study, the cell morphology of the microorganism varied with glass bead size. Until a glass bead diameter of 2 mm pelleted growth was observed, whereas larger glass particles induced mycelial growth. Cultivation of S. coelicolor with glass beads of 3 and 4 mm led to the most reproducible cellular morphology which was accompanied by an enhanced product concentration of actinorhodin and undecylprodigiosin (Sohoni et al., 2012). In the study of Dobson et al. (2008) even larger glass beads with a diameter of 5 mm were added to the main culture of S. hygroscopicus var. geldanamycin. With increasing glass bead number pellet size decreased and the geldanamycin concentration increased (Dobson et al., 2008). Holtmann et al. (2017) used glass beads with a size range of 250–500 µm in cultivations of S. avidini for the production of streptavidin. The glass bead addition did not increase the final streptavidin concentration, but the production was accelerated. After 72 h of cultivation a 2.2–3.2-fold higher streptavidin concentration in the supplemented approaches compared with an unsupplemented cultivation was achieved, whereas after 120 h of cultivation the streptavidin concentration was similar for all approaches (Holtmann et al., 2017). Hotop et al. (1993) supplemented 4 mm glass beads in pre-cultures of Penicillium chrysogenum producing penicillin V to decrease the pellet size, causing an increased product titer. In another study, 3 mm glass beads were added to cultivations of Acremonium chrysogenum to enhance cephalosporin C production (Lee et al., 2010). Different numbers of glass beads between two and six beads were added to the shaking flasks and a 30% increase in cephalosporin C production compared to an approach without glass beads was observed.

Since the mechanical stress induced by the glass beads in cultivations of L. aerocolonigenes was proposed to be responsible for the described enhanced rebeccamycin production in different cultivations, Walsisko et al. (2017) made a rough estimation of the mechanical stress induced by glass beads based on a stress model for grinding in a ball mill (Kwade, 2003). In this model two characteristic values were determined, the stress energy (SE) (Equation (1)) and the stress frequency (SF) (Equation (2)), describing the comminution in stirred media mills. SE quantifies the maximum amount of energy transferred which is provided for stressing within a single stress event.

\[
SE \propto SE_{gm} = d_{gm}^2 \times u_t^2 \times \rho_{gm}
\]  
(1)

where \(d_{gm}\) is the diameter of the grinding medium, \(u_t\) is the stirrer tip speed, and \(\rho_{gm}\) is the density of the grinding medium. The stress frequency SF describes the number of stress events (SN) per time.

\[
SF = \frac{SN}{t}
\]  
(2)

Varying either SE or SF in a reasonable range, the mechanical stress induced by particles over time can be influenced, especially
how many stress events take place to transfer a certain overall energy to a certain product mass. However, both process parameters can be varied to balance each other. An increase in \( SE \) by an increased bead density, for example, can be compensated by a decrease in \( SF \) caused by a reduced number of beads.

As stated above, this approach can only be used for a rough estimation since the conditions in ball mills and especially stirred media mills essentially differ from those in macroparticle-enhanced cultivation in a shake flask. For example, the degree of filling with beads as grinding media differs. In the case of the stirred media mill, the filling degree is usually 70%–85% of the grinding chamber (Kwade & Schwedes, 1997), corresponding to a volume concentration of 42%–51%, whereas in the shaking flask cultivations of this study volume concentrations of beads between 0.1% and 15% were used.

In this study, the stress energy of beads inside a shaking flask \( SE_{b,sh} \) (Equation (3)) was defined with the fluid shaking velocity (Palacios-Morales et al., 2016) as a measure for the maximum bead velocity. The shaking velocity \( u_{sh} \) is calculated from the shaker frequency \( f_{sh} \) and the shaking flask diameter \( d_{fl} \).

\[
SE_{b,sh} = d_{fl}^2 \times u_{sh}^2 \times \rho_b = d_{fl}^2 \times (d_{fl} \times \pi \times f_{sh})^2 \times \rho_b 
\]  

Equation (3)

Furthermore, it is assumed in Equation (4) that \( SF_{b,sh} \) is proportional to the number of beads \( n_b \) and \( f_{sh} \). However, for a more accurate estimation of the macroparticle-induced mechanical stress in shaking flask cultivations, Computational Fluid Dynamics-Discrete Element Method (CFD-DEM)-simulations must be applied.

\[
SF_{b,sh} \propto n_b \times f_{sh} 
\]  

Equation (4)

Previous studies of macroparticle addition to \( L. \) aerocolonigenes mostly investigated the effects of particle diameter variations (Schrad et al., 2019; Walisko et al., 2017). However, the mechanical stress induced by the macroparticles, that is, beads, cannot only be varied by their diameter. Further parameters, such as macroparticle concentration, macroparticle density, and shaking frequency of a shake flask cultivation, as implemented in Equations (1)–(4), also determine the magnitude of \( SE_{b,sh} \) and \( SF_{b,sh} \).

Hence, the aim of the present investigations was the systematic variation of various process parameters such as the bead diameter, concentration and density, as well as the shaking frequency to examine effects of a variation in \( SE_{b,sh} \) and \( SF_{b,sh} \). Moreover, the combination of macroparticle-enhanced cultivation with lecithin supplementation was investigated.

2 MATERIALS AND METHODS

2.1 Strain and cultivation conditions

Filamentous \( L. \) aerocolonigenes DSM 44217 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) and was cultivated in GYM medium (4 g L\(^{-1}\) glucose, 4 g L\(^{-1}\) yeast extract, and 10 g L\(^{-1}\) malt extract). The pH was adjusted to 7.2 using 2 M KOH. Glucose was sterilized separately and subsequently added. 50 ml of GYM medium were filled in 250 ml shaking flasks with four baffles and maximum inner diameter \( (d_{fl}) \) of 81.3 mm. A pre-culture was prepared by thawing 1 ml of frozen biomass (in 30% (v/v) glycerol) followed by inoculation with the same. The shaking flask was incubated in an orbital shaker (Certomat BS-1, Sartorius) with a shaking frequency of 120 min\(^{-1}\) (if not stated otherwise) and an amplitude of 50 mm at 28°C in darkness for 2 days. For the inoculation of the main culture 300 µl of the pre-culture were transferred to another 250 ml baffled shaking flask with 50 ml GYM medium. The main culture was incubated under the same conditions for 10 days. All experiments were conducted in triplicates.

2.2 Determination of dissolved oxygen tension

In some cultivation approaches the dissolved oxygen tension (DOT) in the cultivation broth was determined. For acquisition of these data a shake flask reader (PreSens Precision Sensing GmbH) was utilized. The shaking flasks were equipped with oxygen sensor spots and the DOT data were collected by the shake flask reader software V2.0.0 (PreSens Precision Sensing GmbH) via Bluetooth.

2.3 Rebeccamycin and cell dry weight quantification

The 20 ml of cultivation broth were used for rebeccamycin extraction by adding 5 ml ethyl acetate followed by incubation in an overhead shaker (Intelli-Mixer RM-2 M, LTF Labortechnik) for 60 min. The sample was then centrifuged at 4000 min\(^{-1}\) for 10 min (Heraeus Varifuge 3.0R, Thermo Fisher Scientific) and the ethyl acetate phase removed and used for analytic HPLC measurements as described in a previous study (Schrinner et al., 2020). The cell dry weight concentration (CDW) was determined gravimetrically by filtration through filter papers. Since the beads used in this study sedimented quickly, it was possible to take samples of the culture broth without transferring any beads. The method has been described in more detail previously (Schrinner et al., 2020). For a better overview of the data in the graphs the averaged yield coefficient was calculated by dividing the rebeccamycin concentration by the cell dry weight concentration after ten days of cultivation.

2.4 Macroparticles

Glass (type S and micro glass beads, soda-lime glass) and ceramic (type Z, zirconium silicate) beads as macroparticles were purchased from Sigmund Lindner GmbH. Details on the bead size and density are given in Table 1. For simplicity, the
macroparticles will be referred to by the given mean particle size in the following. Beads were either supplemented in certain weight concentrations, which are indicated with the corresponding results, or at a defined bead number calculated from the bead density and size (Table 1).

2.5 | Lecithin supplementation

Soy lecithin was purchased from MP Biomedicals. A stock solution of 50 g L\(^{-1}\) lecithin was prepared and diluted with water to set the desired concentration in the shaking flask. 40 ml of a concentrated GYM medium with 20% less water were then added to result in a total filling volume of 50 ml. Inoculation and incubation were performed as described above.

3 | RESULTS AND DISCUSSION

3.1 | Variation of stress energy and stress frequency

In this study, shaker frequency, bead size, bead number and bead density were varied to systematically investigate the influence of different \(SE_{b,sh}\) and \(SF_{b,sh}\) on the production of rebeccamycin. An overview of all experimental parameter combinations together with the calculated values of \(SE_{b,sh}\) (Equation (3)) and \(SF_{b,sh}\) (Equation (4)) is given in Tables 2a and 2b. For example, \(SE_{b,sh}\) and \(SF_{b,sh}\) were calculated for different glass bead diameters in a concentration of 100 g L\(^{-1}\) at different shaking frequencies of 100 and 160 min\(^{-1}\) (Table 2a). With increasing glass bead diameter \(SE_{b,sh}\) increases while \(SF_{b,sh}\) decreases. These parameters were furthermore calculated for a constant glass bead number (Table 2a) as well as different concentrations of glass and ceramic beads (Table 2b). The parameters from this table will be discussed in more detail with the results of the experimental approaches below. The number of particles required for the calculation of \(SF_{b,sh}\) was derived from the mass concentration of the beads, the liquid volume, and the mean bead size \(x_{3,2}\).

These characteristic numbers can currently only provide a first qualitative comparison between different cultivations, since the exact dependencies of the characteristic numbers from experimental parameters are not known yet. It should be noted that the modeled \(SE_{b,sh}\) are a measure for the maximal value of \(SE\) as it was also shown to be very useful in comminution processes (Beinert et al., 2015; Kwade, 2003). In reality, \(SE_{b,sh}\) is determined by the distribution of the relative velocities between two colliding beads or a bead and the shaking flask wall. Therefore, the distribution of \(SE\), as well as the

### Table 1

| Particle size range (µm) | Mean particle size \(x_{3,2}\) (µm) | Mass concentration (g L\(^{-1}\)) | Volume concentration (ml L\(^{-1}\)) |
|--------------------------|-----------------------------------|----------------------------------|-----------------------------------|
| Glass beads (\(ρ = 2500\) kg m\(^{-3}\)) | | | |
| 200–300                  | 282                               | 2.5                              | 1.0                               |
| 400–600                  | 540                               | 17.3                             | 6.9                               |
| 500–750                  | 658                               | 31.3                             | 12.5                              |
| 750–1000                 | 969                               | 100.0                            | 40.0                              |
| 1000–1300                | 1183                              | 182.0                            | 72.8                              |
| 1250–1650                | 1513                              | 380.8                            | 152.3                             |
| 1550–1850                | 1746                              | 585.3                            | 234.1                             |
| 1700–2100                | 1932                              | 792.9                            | 317.2                             |
| Ceramic beads (\(ρ = 3800\) kg m\(^{-3}\)) | | | |
| 800–1000                 | 918                               | 25–100*                          | 6.6–26.3*                         |

*See Table 2b.

### Table 2a

| Shaking frequency (min\(^{-1}\)) | 100 | 120 | 160 |
|---------------------------------|-----|-----|-----|
| Bead concentration (g L\(^{-1}\)) | 100 | Varying (see Table 1) | 100 |

| Glass bead size \(x_{3,2}\) (µm) | \(SE_{b,sh}\) (µJ) | \(SF_{b,sh}\) (s\(^{-1}\)) | \(SE_{b,sh}\) (µJ) | \(SF_{b,sh}\) (s\(^{-1}\)) | \(SE_{b,sh}\) (µJ) | \(SF_{b,sh}\) (s\(^{-1}\)) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 282                             | 0.010           | 283,879         | 0.015           | 8400            | 0.026           | 454,206         |
| 540                             | 0.071           | 40,430          | 0.103           | 64,687          | 0.183           | 11,195          |
| 658                             | 0.129           | 22,346          | 0.186           | 35,754          | 0.330           | 6152            |
| 969                             | 0.412           | 6997            | 0.594           | 1055            | 1.055           | 1914            |
| 1183                            | 0.750           | 3845            | 1.080           | 6152            | 1.920           | 6152            |
| 1513                            | 1.569           | 1838            | 2.259           | 6152            | 4.017           | 2941            |
| 1746                            | 2.411           | 1196            | 3.472           | 6152            | 6.173           | 1914            |
| 1932                            | 3.267           | 883             | 4.704           | 6152            | 8.363           | 1412            |

Abbreviations: \(SE\), stress energy; \(SF\), stress frequency.
associated mean values, will be determined by using CFD-DEM simulations in the future.

### 3.1.1 | Variation of particle number

For the variation of $SF_{b,sh}$, with at the same time constant $SE_{b,sh}$, the concentration (or number) of particles in a cultivation of *L. aerocolonigenes* can be varied. Thus, a mean glass bead diameter of 969 µm was chosen to be used in different mass concentrations between 25 and 150 g L$^{-1}$ corresponding to volume concentrations between 10 and 60 ml L$^{-1}$, respectively (see Table 2b), since this glass bead diameter generally led to a high rebeccamycin concentration in earlier studies under the same conditions (Schrader et al., 2019; Schrinner et al., 2020). The resulting rebeccamycin and CDW concentrations are presented in Figure 1. An unsupplemented control cultivation (glass bead concentration of 0 g L$^{-1}$) was added for comparison. During the addition of glass beads, the CDW increased. A maximal CDW of 4.3 g L$^{-1}$ at a 50 g L$^{-1}$ glass bead concentration was achieved. Hereinafter the CDW was rather constant and decreasing at a glass bead concentration of 150 g L$^{-1}$. The rebeccamycin concentration increased until a glass particle concentration of 100 g L$^{-1}$ with approximately 121 mg L$^{-1}$ rebeccamycin and decreased for 125 and 150 g L$^{-1}$ glass bead concentrations. A similar course can be observed for the averaged yield coefficient. Without glass bead supplementation a rebeccamycin titer of only 45 mg L$^{-1}$ was achieved. By variation of the glass bead concentration, $SF_{b,sh}$ was increased with increasing glass bead concentration. At first the rebeccamycin concentration was increasing correspondingly. After a certain point, in this case beyond a particle concentration of 100 g L$^{-1}$, $SF_{b,sh}$ was probably too high causing more stress events in shorter intervals. This either deteriorated growth or metabolic processes in *L. aerocolonigenes*, which in turn caused a decrease of the rebeccamycin concentration. The lower CDW at a glass bead concentration of 150 g L$^{-1}$, at which the rebeccamycin concentration was already decreased, suggests the occurrence of cell destruction or growth inhibition.

Different amounts of glass beads were already supplemented in cultivations of *S. hygroscopicus var. geldanus* for geldanamycin production (Dobson et al., 2008). In this case, the highest number of 55 glass beads with a diameter of 5 mm led to the highest geldanamycin concentration. The course was similar to the results in the present study, with increasing particle concentration or number, the product concentration increases. Only after a certain point, which in this case was a concentration of 125 g L$^{-1}$, the product concentration was decreasing. However, larger amounts than 55 glass beads to examine for further production increases were not tested by Dobson et al. (2008).

### 3.1.2 | Variation of macroparticle diameter

As mentioned above, Schrader et al. (2019) already investigated the effects of different glass bead diameters. However, in that case not only the glass bead diameter was varied but also the glass bead number since the same weight concentration was used for each size. Hence, both $SE_{b,sh}$ and $SF_{b,sh}$ were varied.

| Shaking frequency 120 (min$^{-1}$) | Glass beads (969 µm) | Ceramic beads (918 µm) |
|-----------------------------------|-----------------------|------------------------|
|                                   | Bead concentration    | $SE_{b,sh}$ (const.)   | $SF_{b,sh}$ | Bead concentration    | $SE_{b,sh}$ (const.) | $SF_{b,sh}$ |
| (g L$^{-1}$)                      | (ml L$^{-1}$)         | (µJ)                   | (µJ)        | (g L$^{-1}$)          | (ml L$^{-1}$)       | (µJ)        | (µJ) |
| 25.0                              | 10.0                  | 0.594                  | 2099        | 25.0                   | 6.6                  | 0.767       | 1624 |
| 50.0                              | 20.0                  | 4198                   | 50.0        | 13.2                   | 3248                 |
| 75.0                              | 30.0                  | 6297                   | 75.0        | 19.7                   | 4872                 |
| 100.0                             | 40.0                  | 8396                   | 100.0       | 26.3                   | 6497                 |
| 125.0                             | 50.0                  | 10,495                 | 125.0       | –                      | –                    | –          |
| 150.0                             | 60.0                  | 12,595                 | 150.0       | –                      | –                    | –          |

Abbreviations: $SE$, stress energy; $SF$, stress frequency.
To investigate the influence of the glass bead diameter without changing $SF_{b,sh}$, the same number of particles for each diameter needs to be supplemented. Figure 2 shows the CDW and rebeccamycin concentrations with 4200 glass beads added per shaking flask for different diameters up to a mean diameter of 1513 µm. No coarser glass beads were added since particles already took up a very large volume of 152.3 ml L$^{-1}$ at this point (compare Table 1). An unsupplemented control cultivation (mean glass bead diameter of 0 µm) was added for comparison. The CDW did not show a distinct trend and mostly fluctuated around 3.5 g L$^{-1}$. However, the rebeccamycin concentration increased up to a mean glass bead diameter of 969 µm and decreased afterward. This can also be observed for the averaged yield coefficient. A similar course as observed in Schrader et al. (2019). In the present study, the differences between the rebeccamycin concentrations using different glass bead diameters were much more pronounced due to the constant bead numbers. This was likely caused by a lower $SF_{b,sh}$ for smaller glass beads due to lower glass bead numbers compared to the approach by Schrader et al. (2019). In that study, the decreasing $SE_{b,sh}$ with decreasing glass bead diameter was surely partially compensated by an increasing $SF_{b,sh}$.

$SE_{b,sh}$ increases with increasing glass bead diameter as can be seen in Table 2a. Since the glass bead number was equal in all approaches, the differences were mainly caused by changes in $SE_{b,sh}$. In Equation (3) the glass bead diameter affects $SE_{b,sh}$ to the power of three, meaning that the glass bead diameter is a significant influencing factor. The addition of glass beads with a mean diameter of 282 µm resulted in about 24 mg L$^{-1}$ rebeccamycin while the same number of 969 µm glass beads led to around 98 mg L$^{-1}$ rebeccamycin. Hence, the alteration of $SE_{b,sh}$ by different diameters with a constant particle number greatly affects rebeccamycin titers.

### 3.1.3 Variation of macroparticle density

In a further approach, the particle density was changed by adding ceramic beads ($\rho = 3800$ kg m$^{-3}$) with an approximately 1.5-fold higher density (Table 1) than that of glass beads ($\rho = 2500$ kg m$^{-3}$) to the cultivation. Ceramic beads with a mean diameter of 918 µm were used in different concentrations between 25 and 100 g L$^{-1}$ (6.6–26.3 ml L$^{-1}$, see Table 2b) (Figure 1). This particle size was chosen due to the similar size of the glass beads with a mean diameter of 969 µm used in Figure 3. An unsupplemented control cultivation (ceramic bead concentration of 0 µm) was added for comparison. The CDW of *L. aerocolonigenes* was decreasing with increasing ceramic bead concentration. The rebeccamycin concentration was increasing until a ceramic bead concentration of 50 g L$^{-1}$ with 56 mg L$^{-1}$ rebeccamycin and was decreasing for higher ceramic bead concentrations. For the addition of similarly sized glass beads, a concentration of 100 g L$^{-1}$ indicated to be most beneficial in regard to rebeccamycin titer. For ceramic beads the optimal mass concentration of particles with a similar size was only half the value. Considering volume concentrations, the differences are even larger with 40.0 ml L$^{-1}$ for glass beads and only 13.2 ml L$^{-1}$ for ceramic beads (Table 2b). Therefore, if the particle density is changed and the mass concentration is constant, the particle number also differs. This means both $SE_{b,sh}$ and $SF_{b,sh}$ are influenced. For the same particle number of ceramic beads which are included in 100 g L$^{-1}$ glass beads, a concentration of approximately 130 g L$^{-1}$ would be required (due to the slightly smaller mean diameter of the ceramic beads it is not the 1.5-fold mass concentration). Hence, increased $SE_{b,sh}$ by an increased particle density can be partly compensated by the reduction of another parameter, for example, the particle number, for maximizing rebeccamycin titers at this $SE_{b,sh}$. However, the difference between $SE_{b,sh}$ (Table 2b) for glass beads with 0.594 µJ and for
ceramic beads with 0.767 µJ seems not high enough to create this kind of compensation. In this case, the use of a rather simple approach of the comparison of $S_{E_b,sh}$ is not sufficient to describe the differences of glass and ceramic beads in cultivation as probably the motion behavior of the beads depends on their density. Here, CFD-DEM simulations could shed light on the underlying differences during cultivation, as these take into account the increased centrifugal forces at higher particle densities.

### 3.1.4 Variation of shaking frequency

Another possibility for $S_{E_b,sh}$ variation is the cultivation at different shaking frequencies. In cultivations of *L. aerocolonigenes* in shake flasks presented above, a shaking frequency of 120 min$^{-1}$ was used. To investigate the influence of the shaking frequency with glass bead addition on the rebeccamycin production, cultivations with different shaking frequencies of 100 and 160 min$^{-1}$ were performed. The CDW and rebeccamycin concentrations of these approaches are displayed in Figure 4. At both frequencies different glass bead diameters at a concentration of 100 g L$^{-1}$ were added. The shaking frequencies of 100 and 160 min$^{-1}$ were chosen for cultivation to investigate the effects of lower and higher frequencies in comparison with usual cultivation of *L. aerocolonigenes* with a shaking frequency of 120 min$^{-1}$. With a shaking frequency of 100 min$^{-1}$ (Figure 4a) the induced $S_{E_b,sh}$ and in parallel also the active $S_{F_b,sh}$ are lower compared to equivalent cultivation at 120 min$^{-1}$ (Table 2a). With different glass bead diameters supplemented, a shift of the highest rebeccamycin titer to a larger glass bead size was expected since this might compensate for the reduced shaking frequency. If the mean glass bead diameter of 969 µm is considered to lead to maximal rebeccamycin titers at a bead concentration of 100 g L$^{-1}$ and a shaking frequency of 120 min$^{-1}$ (Schrader et al., 2019), the expected shift can be observed in this approach (Figure 4a). At 100 min$^{-1}$ the addition of 969 µm glass beads resulted in a rebeccamycin concentration of 63 mg L$^{-1}$, whereas the addition of 1183 µm glass beads led to 72 mg L$^{-1}$ rebeccamycin. When looking at the calculated averaged yield coefficient, this shift is also visible. Significant differences in the CDWs for the different particle diameters were not observed.

With a shaking frequency of 160 min$^{-1}$ (Figure 4b), $S_{E_b,sh}$ and $S_{F_b,sh}$ is increased compared with conventional cultivations at 120 min$^{-1}$ (Table 2a). Concluding from the cultivation at 100 min$^{-1}$, a shift to a smaller bead diameter than 969 µm would be expected. However, due to large standard deviations at smaller mean diameters of 658 and 540 µm this shift can neither be clearly confirmed nor disproved. At a mean glass bead diameter of 969 µm 140 mg L$^{-1}$ rebeccamycin were produced. The total values of rebeccamycin titers differ significantly from the results in Figure 4a. However, they are not directly comparable since the cultivations are two different biological approaches from two different pre-cultures. However, what can be clearly observed in this approach are very low CDWs and rebeccamycin concentrations with addition of the largest mean glass bead diameters of 1746 and 1932 µm (13 and 4 mg L$^{-1}$ rebeccamycin, respectively). In cultivations with lower shaking frequencies described above these coarse glass beads did not result in such low rebeccamycin titers compared to the maximum and especially the CDWs were not as low with only 1.9 or 0.7 g L$^{-1}$. These
results indicate that \( SE_{b,sh} \) for these glass bead sizes is too high. \( SE_{b,sh} \) at 120 min\(^{-1}\) of 1932 µm glass beads is 4.704 µJ while for the same glass beads at 160 min\(^{-1}\) \( SE_{b,sh} \) is almost doubled with 8.363 µJ (Table 2a). This could result in cell destruction or even growth inhibition, which in turn reduces production. Since additionally to the application of large glass beads the shaking frequency was increased, both \( SE_{b,sh} \) and \( SF_{b,sh} \) were increased and, thus, were higher compared with a regular cultivation at 120 min\(^{-1}\).

The DOT during cultivation differed for the different shaking frequencies (Figure 4c). The DOT for 100, 120, and 160 min\(^{-1}\) is displayed over a cultivation time of 10 days. The minimal DOT was 75% for 160 min\(^{-1}\), 55% for 120 min\(^{-1}\), and 43% for 100 min\(^{-1}\). No oxygen limitations were observed suggesting that the mechanical stress induced by different shaking frequencies is a main reason for the differences in the cultivations described above.

Overall, the CDW for small and medium bead size measurements is rather similar for different \( SE_{b,sh} \) and \( SF_{b,sh} \). However, for the three coarser bead sizes and, thus, highest \( SE_{b,sh} \) and an increased \( SF_{b,sh} \) compared with a lower shaking frequency of 100 min\(^{-1}\) a decreased CDW was observed. Cell destruction or growth inhibition could be a reason for this effect. The mostly similar CDW in the approaches supports the idea that the effects of glass beads are not biomass-related, as already indicated by Schrinner et al. (2020). Further aspects such as changes in micro-morphology or the inner pellet structure could be of interest in this case (Schrinner et al., 2020). Furthermore, in different Streptomyces spp. a first mycelium is observed, then programmed cell death takes place followed by the differentiation to another secondary metabolite producing mycelium (Manteca & Yagüe, 2018; Manteca et al., 2019). This differentiation could also apply for \( L. aerocolonigenes \) and since it is initiated by programmed cell death, the addition of macroparticles would benefit this process.

### 3.2 Macroparticle-enhanced cultivation combined with soy lecithin supplementation

#### 3.2.1 Soy lecithin supplementation with \( L. aerocolonigenes \)

The addition of lecithin to cultivations of different actinomycetes isolated from soil was beneficial in previous studies regarding product formation (Adelson et al., 1957; Brock, 1956; Choi & Cho, 2004; Schatz et al., 1956). Due to the positive effects for similar microorganisms as \( L. aerocolonigenes \) lecithin supplemented cultivations were performed.

The addition of soy lecithin to the cultivation of \( L. aerocolonigenes \) was first conducted in different concentrations between 0 and 10 g L\(^{-1}\) (Figure 5). This provides an overview of whether lecithin is beneficial and how much lecithin needs to be reasonably added for further cultivations that are described below.

The CDW increased with increasing lecithin concentration. The addition of 2.5 g L\(^{-1}\) lecithin nearly doubled the biomass compared to an unsupplemented control, 10 g L\(^{-1}\) lecithin even resulted in an almost three-fold CDW. The rebeccamycin titer, however, increased until 7.5 g L\(^{-1}\) lecithin (with about 103 mg L\(^{-1}\) rebeccamycin) and is significantly lower for 10 g L\(^{-1}\) lecithin. The rebeccamycin concentration of 18 mg L\(^{-1}\) for the approach with the addition of 10 g L\(^{-1}\) lecithin was similar to the rebeccamycin concentration in the unsupplemented control with 15 mg L\(^{-1}\) rebeccamycin. This course can also be observed for the averaged yield coefficient. The increasing CDW indicates the metabolization of lecithin as an additional carbon source leading to enhanced growth. Higher CDWs can produce higher amounts of rebeccamycin, explaining the increasing rebeccamycin titer with increasing lecithin concentration. The decreased rebeccamycin titer for 10 g L\(^{-1}\) lecithin might be explained with a prolonged exponential growth due to larger amounts of substrate. Since rebeccamycin is a secondary metabolite and is only produced after substrate deprivation, this could lead to a delayed start of rebeccamycin production. To further investigate this hypothesis, the growth kinetics of cultivation without and with 5 g L\(^{-1}\) soy lecithin were compared (Figure 6). Additionally, 100 g L\(^{-1}\) of 969 µm glass beads were supplemented in both approaches.

Both approaches showed a similar course of CDW, but the lecithin supplementation resulted in higher overall concentrations (Figure 6a). The glucose consumption was slower for the lecithin supplemented approach. The glucose was fully depleted after 3 days, whereas without lecithin the glucose was nearly completely depleted after 2 days (Figure 6b). This influenced the start of the rebeccamycin formation. Without lecithin addition the first amounts of rebeccamycin were measured on Day 4 of cultivation while the addition of lecithin led to a delayed rebeccamycin formation starting on Day 5 (Figure 6b). Although this delay in glucose depletion and rebeccamycin formation is proposed to be caused by lecithin acting as an additional substrate, further effects, such as feedback inhibition, cannot be excluded from the presented results.
However, in the lecithin supplemented approach the rebeccamycin concentration then increased faster and to significantly higher concentrations (259 mg L⁻¹ vs. 79 mg L⁻¹ on Day 10). The increased biomass growth with lecithin was in accordance with a greater decrease in the DOT (Figure 6c). The minimal DOT value without lecithin was approximately 65% whereas more biomass due to lecithin addition resulted in a minimal value of about 45%. Furthermore, the increase of the DOT at the end of the exponential growth phase between Day 2 and 3 was slower for the lecithin addition. Therefore, a direct comparison with and without glass beads and different concentrations of lecithin starting from the same pre-culture is not entirely reasonable. Therefore, a direct comparison with and without glass beads and different concentrations of lecithin starting from the same pre-culture was conducted for reliable results (Figure 7). The CDW increased with increasing lecithin concentration. For the addition of 5 g L⁻¹ of lecithin, the CDW of the cultivation with glass beads is lower than of the cultivation without glass beads, whereas for 10 g L⁻¹ lecithin a similar level was observed. The rebeccamycin concentration for the cultivation with 5 g L⁻¹ lecithin and no glass beads was around 220 mg L⁻¹ and was therefore significantly higher than the cultivation without lecithin and no glass beads with only 59 mg L⁻¹ rebeccamycin. However, a combination of 5 g L⁻¹ lecithin and 100 g L⁻¹ glass beads (ϕ = 969 µm) further increased the rebeccamycin concentration up to a very high value of 388 mg L⁻¹. For 10 g L⁻¹ lecithin addition the rebeccamycin concentration was clearly compared with an unsupplemented approach was observed, but also a higher maximum specific productivity.

Brock (1956) investigated the influence of different oils and fatty acids on the filipin production in S. filipinensis. Among others, the effect of the addition of a vegetable and animal-based lecithin was investigated. Both supplementations led to an increased product concentration during cultivation compared to the cultivation approach with glucose added as a carbon source. The vegetable lecithin even led to a two-fold higher filipin concentration than the animal lecithin. The lecithin, which was chosen in the current study to increase rebeccamycin production was derived from soybean and might therefore potentially lead to higher product titers than animal lecithin. Lam et al. (1989) investigated different carbon sources for L. aerocolonigenes and found starch, which is also a plant-based compound, to be most beneficial in regard to rebeccamycin production. Choi and Cho (2004) added lecithin to cultivations of L. aerocolonigenes and found starch, which is also a plant-based compound, to be most beneficial in regard to rebeccamycin production. Adelson et al. (1957) used lecithin in cultivations of a soil actinomycete as it provided fast and abundant growth accompanied by increased oxygen uptake, as also observed for L. aerocolonigenes in this study.

### 3.2.2 Combination of soy lecithin supplementation with macroparticle addition

In Figure 5 the addition of 5 and 7.5 g L⁻¹ of lecithin led to around 100 mg L⁻¹ of rebeccamycin, which is a rather high product titer compared with literature data with mostly 10–50 mg/L (see Pommerehne et al. (2019)). Since lecithin acts as a suitable additional carbon source, the combination of lecithin supplementation and mechanical stress caused by glass beads could lead to even higher rebeccamycin titers as both methods employ different mode of actions for an increased rebeccamycin production. Glass beads with a diameter of 969 µm were already added in the cultivation approach with macroparticle addition. However, an increased rebeccamycin production was derived from soybean and lecithin. The lecithin, which was chosen in the current study to increase rebeccamycin production was derived from soybean and might therefore potentially lead to higher product titers than animal lecithin. Lam et al. (1989) investigated different carbon sources for L. aerocolonigenes and found starch, which is also a plant-based compound, to be most beneficial in regard to rebeccamycin production. Choi and Cho (2004) added lecithin to cultivations of L. aerocolonigenes and found starch, which is also a plant-based compound, to be most beneficial in regard to rebeccamycin production. Adelson et al. (1957) used lecithin in cultivations of a soil actinomycete as it provided fast and abundant growth accompanied by increased oxygen uptake, as also observed for L. aerocolonigenes in this study.
lower for both approaches. Furthermore, the averaged yield coefficient is higher for both approaches with the combination of lecithin and glass beads compared to approaches in which only lecithin was supplemented. This was likely the effect of a delayed start of the rebeccamycin production due to larger amounts of substrate, as it was suggested by the results shown in Figure 6. However, further effects cannot be completely excluded by the presented results at this point.

In this approach, the favorable substrate soy lecithin was combined with glass macroparticle addition and resulted in an extremely high rebeccamycin concentration. The highest rebeccamycin titer in shaking flasks achieved in literature was 183 mg L$^{-1}$ (Netleton, Jr. et al., 1985; Pommerehne et al., 2019). The given 388 mg L$^{-1}$ rebeccamycin by addition of lecithin and glass particles is more than a two-fold increase compared to this literature value.

Looking at the microscopic images of *Lentzea aerocolonigenes* pellets in Figure 8 some differences between the above-described approaches become apparent. Without glass bead addition, pellets from the unsupplemented cultivation and the cultivation with 5 g L$^{-1}$ lecithin (Figure 8a,b) looked similar. However, the combination of 100 g L$^{-1}$ glass beads and 5 g L$^{-1}$ soy lecithin resulted in pellets that, in some cases, appeared less dense. This can be observed by a lighter color in the microscopic images (Figure 8c). At higher magnification (Figure 8d) a dense core and a less dense peripheral area of these pellets were visible. These pellets suggest changes in the micro-morphology or the inner pellet structure that might be connected to the significantly increased rebeccamycin formation.

### CONCLUSIONS AND FUTURE PERSPECTIVES

The addition of macroparticles (e.g., glass beads) to cultivations of the filamentous growing actinomycete *L. aerocolonigenes* can significantly enhance the rebeccamycin concentration. Walisko et al. (2017) proposed the mechanical stress induced on the microorganism by the beads to be responsible for the increased product concentration. Hence, this study was performed to be a systematic investigation of different parameters for macroparticle addition, such as bead diameter, concentration, and density as well as shaking frequency, for a better understanding of the particle-induced stress on *L. aerocolonigenes*. The proper level of particle-induced stress for optimal rebeccamycin production can be estimated by a combination of stress energy and stress frequency. Different combinations of these parameters are possible to achieve high a rebeccamycin concentration during cultivation. Regarding different concentrations of
969 µm glass beads being added in a cultivation approach, only the stress frequency of beads inside a shaking flask (SF_{b,sh}) is varied. If it was reduced or increased compared to the optimum, the resulting rebeccamycin concentration decreased. In case of a reduction of the shaking frequency from 120 to 100 min^{-1} SF_{b,sh} was reduced but the highest rebeccamycin concentration was achieved at a larger glass bead size (1135 instead of 969 µm), which is an increase of stress energy of beads inside a shaking flask (SE_{b,sh}). Here, a decrease of one and increase of the other parameter are still result in a high rebeccamycin concentration.

This study represents the basis for additional studies to quantify and model SE_{b,sh} and SF_{b,sh} in dependence of the cultivation parameters by the CFD-DEM-simulation method developed by Schrader et al. (2019). In future, these simulation results will allow a direct link of SE_{b,sh} and SF_{b,sh} with the new cultivation results. Based on this, model relationships between mechanical stress and the influence on the cultivation of filamentous microorganisms will be derived.

The combination of macroparticle-enhanced cultivation (100 g L^{-1} glass beads with \( \varnothing = 969 \) µm) and an additional soy lecithin supplementation of 5 g L^{-1} showed to further increase the rebeccamycin titer, resulting in the highest rebeccamycin concentration of 388 mg L^{-1} reported in the literature in the shake flask scale (Pommerehne et al., 2019). Both approaches influence the cultivation in different ways and do not affect each other negatively.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Marcel Schrader  https://orcid.org/0000-0002-5880-615X
Anna Dinius  https://orcid.org/0000-0002-3562-7925
Anno Kwade  https://orcid.org/0000-0002-6348-7309
Rainer Krull  http://orcid.org/0000-0003-2821-8610

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