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Rheological characteristics of filamentous cultivation broths and suitable model fluids

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HIGHLIGHTS

- Broths of *A.niger*, *L.aerocolonigenes*, *A.namibiensis* exhibit non-Newtonian rheology.
- Suitable rheometer geometry and measuring method varies for each cultivation broth.
- Broth viscosity and flow consistency factor increase with biomass growth.
- Broth viscosity increases with increased pellet roughness and decreased compactness.
- Xanthan gum solutions can be used as model fluids for *A.niger* and *A.namibiensis*.

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ABSTRACT

The rheological characteristics of cultivations with filamentous microorganisms define many other crucial process parameters in the system, such as the cell morphology and the productivity. The present study aims to determine the rheological changes of broths during batch cultivations of three filamentous microorganisms: the fungus *Aspergillus niger*, and the two actinomycetes *Lentzea aerocolonigenes* and *Actinomadura namibiensis*. Focus is given on establishing measurement methodologies to characterize these heterogeneous filamentous systems. For the rheological characterisation the Ostwald-de Waele power law approach is used. All examined biological filamentous broths exhibit a non-Newtonian shear thinning behavior. The effect of biomass concentration and cell morphology on the rheology of the broth is further enlightened. Finally, the rheology of the cultivation broths is compared with that of various model fluids to test the suitability of the last ones to mimic rheologically the biological broths in further technical applications.

1. Introduction

Filamentous microorganisms are commercially used as cell factories in multiple sectors such as pharmaceutical, food and beverage, paper and pulp or textile industry [1,2]. Knowledge of the broth properties, specifically the broth rheology is of paramount importance for the efficient performance of the cultivation process [3]. The rheological properties influence turbulence characteristics of the fluid and, therefore, heat and mass transfer processes [4], the mixing behavior [5] and the fluid mechanical stress [6], defining the productivity of the cultivation and thus, the overall performance of the cultivation process. Finally, scale-up tasks and downstream processes also require a good understanding of rheological properties [7].

During cultivation, rheology can be drastically affected by the consumption of viscous substrates, the growth of filamentous microorganisms or the production of viscous products (e.g., biopolymers). This work deals exclusively with the rheological changes linked to biomass growth and the morphology of filamentous microorganisms. It is generally acknowledged that the non-Newtonian flow behavior of filamentous cultivation broths is caused by the presence of microorganisms with complex cell morphology [8]. The formation of highly branched mycelia can increase the viscosity and often leads to non-

Abbreviations: CMC, carboxymethyl cellulose solution; DSMZ, German collection of microorganisms and cell cultures; PEG, polyethylene glycol; PAA, polyoxpolyacrylamide

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Nomenclature

Latin Symbols

d_{av} [m] Average Diameter based on the Maximum Feret Diameter
BDW [g/L] Biomass Dry Weight concentration
c [g/L] or [g/kg] or [% w/w] Concentration
t [s] Cultivation Time
$El = \frac{N_1(\dot{\gamma})/\tau(\dot{\gamma})}{\rho \cdot N \cdot d^2/\mu(\dot{\gamma})}[-]$ Elasticity Number
where N_1 first normal stress difference,
ρ fluid density
d impeller diameter
n [-] Flow Behavior Index
K [Pa·s^n] Flow Consistency Factor
s [m] Gap Size
$N[s^{-1}]$ Impeller Speed
K_{MO} [-] Metzner-Otto Constant
C_K *or* C_n [-] Parameter
Re [-] Reynolds Number

Greek Symbols

μ_{app} [Pa·s] Apparent Viscosity
α [°] Cone Angle
Φ_{eff} [−] Effective Volume Fraction
κ [μS/cm] Electrical Conductivity
μ [Pa·s] Newtonian Dynamic Viscosity
α [−] Parameter, exponent in the power law approach
$\dot{\gamma}$ [s^{-1}] Shear Rate
τ_0 [Pa] Yield Stress

Newtonian behavior of aqueous nutrient solutions [9].

Shear rate dependent viscosity is usually handled using the apparent viscosity

$$\mu_{app} = \tau/\dot{\gamma} \quad (1)$$

μ_{app} can be determined by measuring shear stress τ as a function of shear rate $\dot{\gamma}$. In stirred tank reactors and impeller rheometers, a broad distribution of shear rates can be applied.

For the laminar flow regime, Metzner and Otto [10] developed a simple approach to calculate a representative shear rate by using a constant K_{MO} depending on the geometry of the stirring set-up and the stirring rate *N*:

$$\dot{\gamma} = K_{MO} \cdot N \quad (2)$$

The non-Newtonian behavior of filamentous cultivation broths is most commonly described using the Ostwald-de Waele power law approach [11–14]

$$\tau = K \cdot \dot{\gamma}^n \quad (3)$$

due to its simplicity and since it has proven to adequately describe flow curves over a wide range of shear rates [7]. Furthermore, it is the most frequently and successfully used model in transport correlations [7]. The flow consistency factor *K* stands for the inner friction and the flow behavior index *n* can be interpreted as a qualitative information on the stability of the fluid's inner structure. Other rheological models used in literature to describe filamentous cultivation broths include the Herschel-Bulkley correlation with the yield stress τ_0, the Bingham (ideal plastic, $n = 1$) and Casson model (shear thinning, $1/3 < n < 2/3$). The choice of the rheological model is mostly subjective and depends on the experimental condition. Rheological models must be understood as empirical correlations and not as physical laws [15].

Rheology of the broth is influenced decisively by the biomass concentration and the morphological state of the mycelium [5]. Depending on the cultivation conditions (e.g., pH-value, temperature, osmolality, reactor geometry, etc.), filamentous microorganisms grow in the following main morphological forms: (a) homogeneously dispersed mycelia with each individual hypha being surrounded by the cultivation medium, (b) pellet-like bio-agglomerates, in which the hyphal agglomerate assembles mostly into a spherical form, and (c) all intermediate types of clumps. The morphological state of the microorganisms affects significantly the productivity of the cultivation. Whether pellet growth or cultivation of freely dispersed mycelia is preferred, depends on the microorganism and strain, the desired product and the specific application [16]. Extensive research was carried out to understand, control and optimize cell morphology of filamentous microorganisms to increase the product concentrations [17].

Several authors studied the influence of both the biomass concentration and the mycelia morphology on the Ostwald-de Waele parameters for different cultivation systems. In those works, the biomass was usually expressed as *Biomass Dry Weight* concentration (*BDW*) and the morphology was expressed in Euclidian or fractal terms of clump roughness and compactness [18], diameter of mycelial aggregate [19], projected area [20], equivalent diameter [12] or the combination of these shape descriptors in the non-dimensional Morphology number, fractal quotient or lacunarity [15]. These parameters are used for the description of different aspects of the pellet morphology. The roughness describes the irregularity of an object perimeter, which is obtained from circularity measurements, whereas the compactness is a measure for the voidage in a pellet or mycelial aggregate (for details see [17] and [21]). An equivalent diameter usually describes the diameter of a circle with the same area (or volume) as the pellet [22]. The equivalent diameter together with the projected area (area of a two-dimensional representation of the pellet) are both measures for the size of pellets [19]. The studies mentioned above focused on the dependency between the rheological data and *BDW* as well as the chosen morphological parameter. Results strongly vary according to the investigated microorganism and cultivation conditions.

Rheological properties are intrinsic parameters of the fluid and should not be influenced by the measurement system. Nevertheless, due to the highly heterogeneous nature of cultivation broths, conducting rheological measurements is challenging and no standard methodology exists [7]. Measurements are mostly done offline, although approaches for online measurements have been proposed [23–26]. In the literature, several measuring geometries were used. Conventional rotational viscometer set-ups such as double gap cells [7,24,27,28], concentric cylinders [7,24,29] or cone and plate [12]. However, there are several typical problems associated with these rheometers. The biopellet structure may be damaged or altered, as the size of the agglomerates often is of the same size or larger than the annulus/gap size of the instrument. Also the heterogeneous nature of the suspension can lead to erroneous measurements due to sedimentation or aligning of pellets [5]. Bongenaar et al. [30] were the first ones employing the impeller viscometer to cultivation broths, which since then has been employed by several authors [7,15,24,31]. Using this indirect impeller method, the investigated range of shear rate has to be chosen carefully, as the governing equations are theoretically only valid in the laminar flow regime. However, also a lower limit of shear rates exists to ensure a homogeneous suspension without pellet sedimentation. In an extensive study for the comparison among various rheometer geometries, it was suggested that eventually different rheometers should be used to characterize successfully a cultivation broth at the different stages of the cultivation process [32].

The rheology of a cultivation broth defines the fluid dynamic conditions in the bioreactor. Studying the hydrodynamic behavior and the mass transfer of various bioreactor configurations can become

Table 1
Employed biological and model fluid media.

System	Description			
Cultivation broth	Cultivation	Biochemical variable	Cell morphology	Cultivation type/ Period [h]
Aspergillus niger	AS0	400 mosmol/kg (without NaCl addition), inoculum concentration 10^6 spores/mL	Exclusively in pellet form with dense core and a thin fluffy region surrounding the core, big pellets	Stirred tank bioreactor/ 53.5 h
	AS1	400 mosmol/kg (without NaCl addition), inoculum concentration 3.9×10^5 spores/mL	Exclusively in pellet form with dense, compact core without fluffy region surrounding the core, big pellets	Stirred tank bioreactor/ 68.5 h
	AS2	1500 mosmol/kg (c_{NaCl} = 32.2 g/L), inoculum concentration 10^6 spores/mL	Mainly in mycelial form, presence of pellets of losing structure, big bio-agglomerates	Stirred tank bioreactor/ 106.5 h
	AS3	2500 mosmol/kg (c_{NaCl} = 64.4 g/L), inoculum concentration 10^6 spores/mL	Mainly in small non-spherical, elongated clumps with high compactness	Stirred tank bioreactor/ 93.5 h
	AS4	pH-shift from 3 to 5 from cultivation time 24–29 h, 400 mosmol/kg (without NaCl addition), inoculum concentration 10^6 spores/mL	Mainly in small mycelial forms, presence of non-spherical, elongated compact clumps	Stirred tank bioreactor/47 h
Lentzea aerocolonigenes	LA	–	Hairy pellets of medium size and many small mycelia	Shaking flask/240 h
Actinomadura namibiensis	AC	–	Mixture of pellets, clumps and dispersed mycelia, pellets with irregular shape	Shaking flask/240 h

Model fluid medium	Concentration, c [g/kg]	Newtonian dynamic viscosity, μ [Pa·s]	Rheological behavior
Inverted sugar syrup solution	10 - 1000	$9.7 \cdot 10^{-4}$ - 0.50	Newtonian
Glycerin solution	10 - 1000	$9.4 \cdot 10^{-4}$ - 1.313	
Luviskol® K90 solution	10 - 200	$2.64 \cdot 10^{-3}$ - 1.704	
Polyethylene oxide-PEG solultion	20 – 500	–	shear thinning
Xanthan gum solution	0.5 – 10	–	
Carboxymethyl cellulose-CMC solution	0.1 – 10	–	

challenging, since the real biological process material is often difficult to work with. Biological broths are usually opaque, prohibiting optical accessibility; they are stable only under specific temperature and pressure conditions, and allowed to be used under specific safety standards, which can consequently increase the cost of the investigations [33]. Therefore, such studies are usually facilitated by the use of non-biological based model systems, i.e., model particulate systems and model fluids. The use of suitable model systems allows faster, reproducible and thus, more cost-effective experimentations towards the optimization of technical relevant reactors. Experiments with the actual biological material are normally carried out only selectively to confirm the laboratory results obtained with model fluids.

A wide range of model fluids is reported in the literature. The model fluids used in laboratory must behave rheologically in a manner representative of the actual process fluid. They often need to be transparent for optical accessibility. Low cost and easy to follow safety standards, as well as a safe and economical disposal are also required. Characteristics such as the stability of the fluid, slow decomposition and temperature sensitivity need to be considered [33]. Model fluids have been used in investigations of flow visualization, power input, fluid mechanical stress, mixing times, solid-liquid mixing (solid suspension and distribution), liquid-liquid dispersion and gas-liquid mixing (gas hold-up, volumetric mass transfer coefficient, bubble size and specific interfacial area), heat and mass transfer measurements [33–44].

Typical examples from the literature include water, glycerol [34,35,43], sucrose [42], sugar syrup [36], silicon and mineral oil [36], castor and rapeseed oil [38], aqueous polyvinyl pyrrolidon solution [40], polyethylene glycol (PEG) [43] and Na_2SO_4 solutions [37] as Newtonian fluids. As non-Newtonian model fluids carboxyl methylcellulose (CMC) solutions [35–39,42], aqueous solutions of kaolin and polyoxpolyacrylamide (PAA) [36], Carbopol [42], Xanthan gum [39,42,43], soluble starch [34], neoponite, wallpaper paint and laponite solutions [36] have been used. Due to the complexity of rheological measurements, the choice of model media is often based on an inadequate characterization of the real cultivation broth. A direct comparison has seldomly been conducted and the choice of model media was to some extent done arbitrary in the literature [28].

As pointed out by many authors [30,45], measuring the rheology of filamentous cultivation broths is challenging due to their strong inhomogeneity. Therefore, this study intends to investigate the suitability of rheometer configurations and measurement methodologies to characterize the rheological properties of three filamentous microorganisms. In particular, the study deals with the fungus *Aspergillus niger* and the bacteria *Lentzea aerocolonigenes* and *Actinomadura namibiensis*. Additionally, the work aims at the rheological characterization of the batch cultivations of these three microorganisms based on the Ostwald-de Waele power law model, enriching the database of the reported rheological characterization for such cultivation systems. For the bacterial cultures, rheological properties are determined for first time. The rheology of *A. niger* broths has been investigated by several authors, but partially contradictive findings are reported [7], requiring further systematic investigations. Flow curves were measured considering cultivation time, biomass concentration and morphological shape descriptors as the key parameters influencing the rheology of the cultivation broth. However, several studies did not consider all influencing parameters leading to erroneous conclusions. Finally, as mentioned before, many technical applications in literature employ non-biological model fluids instead of the original biological matter, but the choice of model fluids is often based on inadequate rheological comparison with the cultivation broths. Therefore, the last aim of the current study is to compare the biological rheological behavior with that of the model fluids widely used in the literature. This way the suitability of such model fluids to mimic the rheology of cultivation broths in further technical applications is tested.

2. Materials and methods

2.1. Filamentous microorganisms and cultivation systems

In this study, the rheological behavior of three filamentous microorganisms was investigated: the eukaryotic fungus *Aspergillus niger* and the prokaryotic actinomycetes *Lentzea aerocolonigenes* and *Actinomadura namibiensis*.

Aspergillus niger SKAn 1015 is a recombinant strain carrying the suc1 (fructofuranosidase) gene leading to the production of fructofuranosidase under the control of the constitutive pkiA (pyruvate kinase) promoter [46]. Cultivations were conducted in a stirred tank bioreactor (Applikon, Schiedam, The Netherlands) with 2.2 L filling volume. The tank was equipped with three baffles and two six-bladed Rushton turbine impellers (diameter 45 mm and impeller spacing 70 mm). Inoculation and cultivation were carried out as described by Wucherpfenning et al. [46]. An aeration rate of 1 L/min, an agitation speed of 300 1/min, a temperature of 37 °C and a pH value of 5.0 were set as operational conditions. The mycelial morphology was adjusted mainly by NaCl salt addition (with exception the *A. niger* AS4 cultivation) affecting the osmolality of the system, as described by Wucherpfenning [21], and presented in Table 1.

Lentzea aerocolonigenes DSM 44217, purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), was cultivated in 250 mL shaking flasks with four baffles and 50 mL filling volume. So far, a method to cultivate *L. aerocolonigenes* in bioreactors successfully has not been established. The shaking flasks with GYM-Medium (4 g/L glucose, 4 g/L yeast extract, 10 g/L malt extract, pH = 7.2) were incubated in an orbital shaker (Certomat BS-1, Sartorius, Göttingen, Germany) with a shaking diameter of 50 mm and a shaking speed of 120 1/min at 28 °C in darkness. Pre-cultures were inoculated with 1 mL of frozen culture (biomass of a two-day pre-culture frozen at −80 °C in 30 % glycerol) and incubated for two days. The main-cultivation was then inoculated with 300 µL pre-culture and incubated for 8 days at the same conditions as the main-culture.

The filamentous strain *Actinomadura namibiensis* DSM 6313 used in this study was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was cultivated as described by Tesche et al. [47]. Main cultivations were inoculated with 5 mL of the pre-culture and cultivated in 500 mL shaking flasks without baffles and 100 mL working volume for 240 h at 30 °C and 180 1/min (50 mm orbital shaking diameter) in darkness.

2.2. Model fluids

Seven types of products were tested for their suitability as model fluid media: Inverted sugar syrup (72.7/66, Grafschafter Krautfabrik Josef Schmitz, Meckenheim, Germany), Glycerin (≥ 98 % water free, Carl Roth, Karlsruhe, Germany), Luviskol® K90 Powder (BASF, Ludwigshafen, Germany) (all Newtonian flow behavior), Polyethylene oxide-PEG (molecular weight (MW) > 5 000 kDa, Alfa Aesar, Ward Hill, USA), Xanthan gum (Food grade E415, Fufeng., Shandong, PRC), Carboxymethyl cellulose solution (CMC Blanose, Ashland, Wilmington, USA) (all shear thinning flow behavior). Aqueous solutions of the abovementioned substances were prepared using ultrapure water (electrical conductivity $\kappa = 0.055$ µS/cm, PURELAB flex 2 system, ELGA Labwater, Celle, Germany). All solutions were prepared on a weight basis (Table 1) and used within one day after preparation.

2.3. Rheological Measurements

Due to the strong inhomogeneity of the cultivation broths, their rheological characterization cannot be considered trivial. The main challenges concern sample preparation and handling, settling or slip conditions as well as sample alteration or damage during the measurement [32,48]. Therefore, the first goal of this work was to

determine appropriate measuring configurations and methods for the rheological analysis of different filamentous cultivation broths.

Cultivation broths were analyzed using a tempered rotational rheometer with air bearing (Kinexus lab+, Malvern Panalytical, Kassel, Germany) and different measuring geometries: a plate-plate, a cone-plate and an impeller configuration with a four-paddle. For each biological sample, a measuring geometry as well as the shear rate range for accurate measurements were selected, depending strongly on the broth viscosity, biomass concentration and most importantly the morphological state of the biological matter, as it will be analytically discussed in Chap. 3.1. To select the most appropriate measuring geometry for each examined microorganism and sample, transparent acrylic glass duplicates of the lower plate of the plate-plate configuration and the cylindrical vessel of the impeller configuration were built. These transparent set-ups ensured visual accessibility during the measurement. The pellet suspension stage, any possible pellet settling and sample alteration during the measurement were recorded by taking images of the transparent set-ups with a digital camera. In the case of the transparent plate-plate system, images were taken through an inclined mirror positioned below the plate. Images of the transparent vessel for the impeller set-up were taken from one side of the vessel.

Dimensions of the rheometer geometries are summarized in Table 2. The impeller rheometer configuration was pre-calibrated by the manufacturer with fluids of known viscosity using the Metzner-Otto approach as applied in several works [12]. This set-up requires significantly larger probe volume (32 mL) compared to the other measuring systems (< 5 mL). For the impeller system, measurements were performed in six logarithmic steps per decade from high to low shear rates to obtain a well-mixed suspension. All other measuring protocols included increasing shear rates. The measuring range of shear rates varied depending on the sample properties as it will be analytically discussed in Chap. 3.1.

Treatment of the sample before measurement was shown to be important [5]. Samples were withdrawn during the cultivation and stored temporarily in a heat-bath at the optimum temperature for each microorganism. Finally, they were poured on the measuring device and directly measured. Measurements were carried out at ideal cultivation temperature of the microorganisms which was 37 °C for *A. niger*, 28 °C for *L. aerocolonigenes* and 30 °C for *A. namibiensis,* respectively. All measurements were performed in duplicate.

To determine the dependency of the rheological behavior on biomass concentration without taking into consideration any morphological variations, selectively chosen broth samples were either diluted with defined volumes of the respective cultivation medium or concentrated by removing an amount of the supernatant fluid to create samples in a wide range of biomass concentration while the morphology remained constant from sample to sample.

Rheological behavior of the model fluids was investigated using a tempered cone-plate rheometer with air bearing (MCR 302, Anton Paar, Graz, Austria). Measurements were carried out at 20 ± 0.1 °C in the range of shear rate $\dot{\gamma}$ = 0.01 - 5000 s^{-1}.

2.4. Quantification of biomass dry weight concentration and cell morphology

The biomass dry weight concentration (*BDW*) was determined gravimetrically. Therefore, a pre-defined volume of the cultivation broth was filtered through pre-dried and pre-weighted (24 h at 105 °C) filter discs (Filter Discs Grade 389, Sartorius, Göttingen, Germany), rinsed with deionized water and dried for 48 h at 105 °C. The sample was then re-weighted. *BDW* was finally calculated as the dry weight of the biomass in the sample volume. All measurements were performed in duplicate.

The culture cell morphology was analyzed under an EVOS xl microscope with 4.5 µm/pixel (Thermo Fisher Scientific, Waltham, USA). The bio-suspension sample was diluted in a petri dish to allow individual pellets to be examined. For each sample at least 100 pellets or mycelial clumps were analyzed. Image analysis was conducted using ImageJ 1.52 h (National Institute of Health, Bethesda, USA). The images were first converted to 8-bit grayscale format and then binarized using the thresholding tools in ImageJ. The particle analysis tool was applied to calculate the desired morphological parameters of each pellet or mycelial structure. Objects exceeding the edge of an image were not considered for evaluation as they could not be analyzed as full objects. Different morphological parameters can be obtained with this method, however, only the maximum Feret diameter, defined as the maximal distance between two points along the selection boundary of the analyzed object, was considered for further analysis in the study. The average bio-agglomerares diameter d_{av} for each sample was calculated as the arithmetic mean of the maximum Feret diameter of every pellet.

3. Results and discussion

3.1. Measuring method for the rheological characterization of filamentous cultivation broths

The first goal of this experimental work is to establish appropriate measuring methodologies for the rheological analysis of different filamentous cultivation broths. Three rheometer set-ups were tested for their suitability: a cone-plate, a plate-plate and an impeller configuration. The main challenges concern the avoidance of phase separation, pellet settling (sustaining the particulate matter uniformly distributed) and morphological alterations/damages of the bioagglomerates during the measurement. The investigation of three strains with different rheometers allows a comparative understanding of the influence of the broth viscosity, biomass concentraion, pellet size and cell morphology on the applicability of a measuring geometry and the respective suitable shear rate measuring range.

3.1.1. Aspergillus niger

Samples of the cultivation AS2 of mycelial grown *A. niger* were initially measured with the plate-plate configuration by adjusting the gap size between the plates to 1 mm and 1.5 mm. Fig. 1 shows microscopic images of *A. niger* before (Fig. 1A) and after (Fig. 1B) a measurement with the plate-plate configuration (1.5 mm gap size) and shear rates up to 500 s^{-1}. Severe sample damage and aggregation occurred as proven by the microscopic images (Fig. 1B) as well as by the visual observation of the lower plate (Fig. 1C) after the measurement. Biomass aggregates were formed, rolling into filaments, which were separated from the media while shearing. Malouf [32] has referred extensively to such morphological alterations, where the solid phase builds aggregates,

Table 2
Dimensions of used rheometer configurations.

Rheometer	Measuring system	Diameter *d* [mm]	Gap size *s* [mm]	Angle α [°]	Impeller height *h* [mm]
Cultivation broths					
Kinexus lab+, Malvern Panalytical	Plate-Plate	60 (upper plate)/65 (lower plate)	1…1.5	[-]	–
	Cone-Plate	60 (upper plate)/65 (lower plate)	0.03	1	–
	Impeller	25 Cup diameter: 27.5	[-]	[-]	60
Model fluids					
MCR 301, Anton Paar	Cone-Plate	59.978	0.117	1.008	–

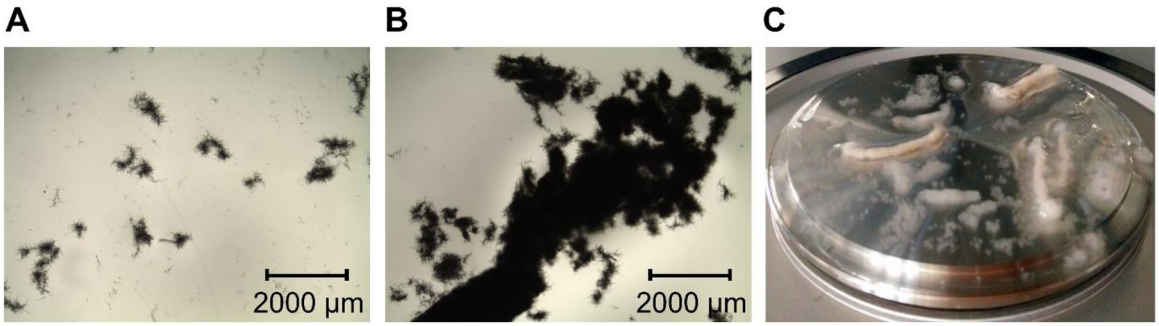

Fig. 1. Sample of the AS2 broth of *A. niger* at cultivation time $t = 27.5$ h, with $BDW = 1.79$ g/L and $d_{av} = 0.74$ mm: (A) Microscopic image of the original sample, (B) microscopic image of the sheared sample after the measurement with plate-plate rheometer, (C) image of the lower plate of the plate-plate configuration after the measurement. *A. niger* is clearly aggregated into filaments.

when the parallel plate geometry was used for samples of *Trichoderma reesei*. Rheological measurements for the case of the pelleted cultivations of *A. niger* (AS0, AS1) were not conducted with the plate-plate system because the pellet size can reach several millimeters (> 2 mm) and this falls far below the required minimum ratio of gap size to pellet diameter of ten given by Barnes [49] and Brummer [50]. Consequently, the plate-plate configuration was found to be unsuitable for rheological measurements of the fungal cultivation broth of *A. niger* independent of its cell morphology, confirming the reported findings from previous studies [32,48]. Similarly, the cone-plate configuration with an even smaller gap size also failed to give reliable rheological data for the cultivation broth of *A. niger* confirming again the literature findings [32,48].

Using the impeller/vane configuration, no sample damage or alteration occurred. This was confirmed by microscopic analysis of the sheared sample after measurement. A change in mean bio-agglomerate diameter of less than five percent was observed in general (Fig. 4). All in all, the impeller rheometer was considered to be appropriate for the rheological characterization of the fungal broth of *A. niger* showing a good compatibility for both the mycelium and the pelleted growth, justifying the common use of this set-up in literature. Nevertheless, a main drawback of the method is the need of large sample volumes. Significantly larger sample volumes (32 mL for the used vessel) were required compared to the other measuring systems (< 5 mL for cone- or plate-plate). Another important challenge of the method is the necessity to keep the biomass in suspension, ensuring a homogeneous sample distribution during the measurement, no phase separation and no formation of less dense layers on the walls of the cylindrical vessel. These points are especially crucial for low viscous broths and they have not been considered sufficiently in literature yet. To face this, in general, measurements were carried out from high to low shear rates to provide a homogeneous pellet distribution. Additionally, to further evaluate the suitability of the method and to identify a reasonable shear rate range for the analysis of the flow curves, measurements were carried out with a transparent vessel as described in Chap. 2.3. The images taken by the camera were analyzed to determine the minimum shear rate $\dot{\gamma}_{min}$ needed to fully suspend the microorganisms. Fig. 2 shows the respective measurements with the transparent vessel for the pelleted cultivation

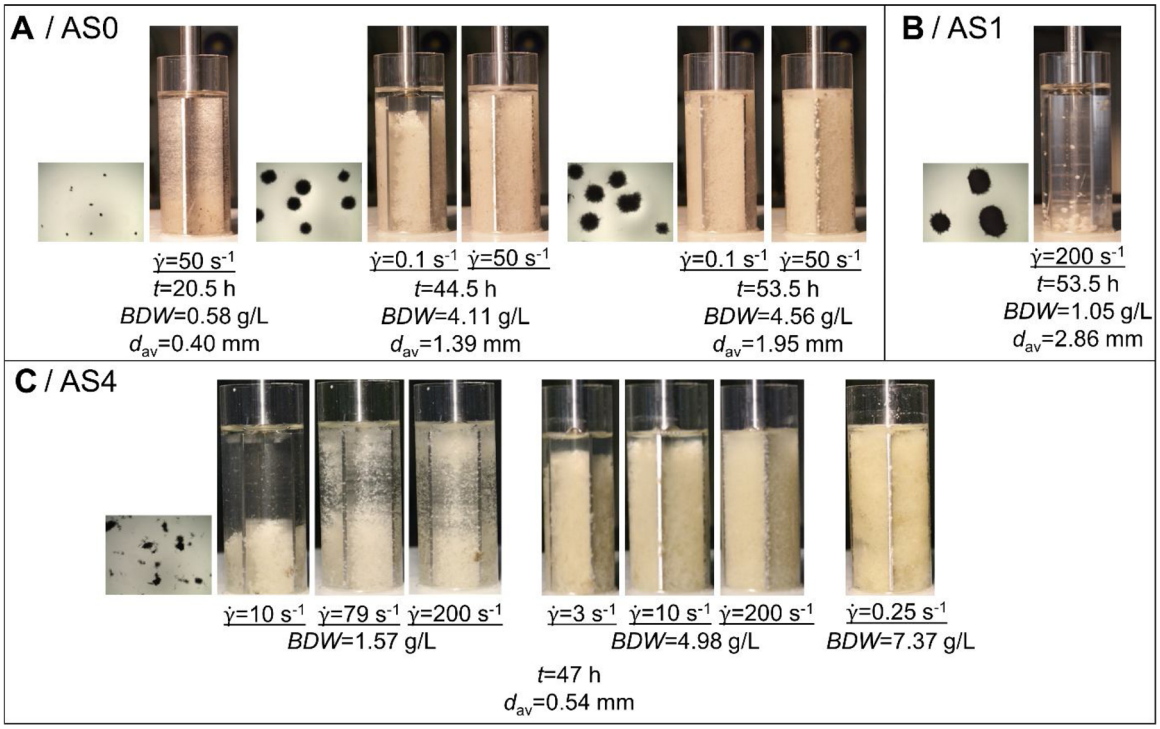

Fig. 2. Evolution of the suspension state of *A. niger* broths with different cell morphology and biomass concentrations in the vane system at different shear rates: (A) Cultivation AS0 (without NaCl addition), inoculum concentration 10^6 spores/mL, (B) Cultivation AS1 (without NaCl addition), inoculum concentration $3.9 \cdot 10^5$ spores/mL, (C) Cultivation AS4, pH-shift.

AS0 and AS1 and the mycelial cultivation AS4 of *A. niger*. Comparative measurements with the same sample in both original set-up and acrylic glass duplicate showed a good agreement.

In Fig. 2 the images depict the suspension state under exemplary shear rates for samples withdrawn at different times of the batch processes. For the pelleted broth of *A. niger* AS0 (Fig. 2A), at cultivation time t = 20.5 h a minimum shear rate of $\dot{\gamma}_{min}$ = 50 s^{-1} was needed to completely suspend the biological matter. At later stages of the batch cultivation (t = 53.5 h), a homogeneous suspension could be reached at lower shear rates allowing measurements even at $\dot{\gamma}_{min}$ = 0.1 s^{-1}. Although pellet size increases over time implying a faster sedimentation velocity for a single pellet, the growth of biomass increases the viscosity of the cultivation broth, as it will be shown in detail in Chap. 3.2.1, resulting in an overall decrease in sedimentation velocity. For the pelleted cultivation of *A. niger* AS1 the bio-agglomerates could not be suspended within the applied range of shear rates even at the latest stages of the process. Fig. 2B shows the respective suspension state during the measurement with the transparent vessel at t = 53.5 h. In this case, the pellets were significantly larger in comparison to the cultivation AS0 with an average size of $d_{av,AS1,t=53.5h}$ = 2.86 mm (instead of $d_{av,AS0,t=53.5h}$ = 1.95 mm). At the same time, the pellet structure was very compact with shorter exposed hyphae surrounding the bio-agglomerate main core and the biomass concentration was significantly lower with $BDW_{AS1,t=53.5h}$ = 1.05 g/L instead of $BDW_{AS0,t=53.5h}$ = 4.56 g/L. Under these conditions, a rapid settling of the pellets was observed independent of the agitation intensity and thus, no suitable measuring method could be identified for the rheological characterization of the *A. niger* AS1 cultivation. Lin et al. [51] compared the settling velocity of *A. niger* pellets with different pellet surface structure but similar pellet diameter. They reported three times higher sedimentation velocities for pellets with compact shape compared to pellets with unstructured and irregular periphery. This confirms that the pellet size as well as other morphological surface characteristics are significant when defining the appropriate measuring range for rheological measurements. Fig. 2C shows the suspension state during measurements of the mycelial broth of *A. niger* AS4. The images depict the suspension state under exemplary shear rates for samples with the same bio-agglomerate morphology (mycelial growth, $d_{av,AS4,t=47h}$ = 0.53 mm), but different biomass concentrations. By visual observation at the lower biomass level of BDW = 1.57 g/L agglomerates accumulated in two distinct zones even at high shear rates of $\dot{\gamma}$ = 200 s^{-1}. As the biomass concentration increases, the minimum shear rate $\dot{\gamma}_{min}$ for a complete dispersion decreased significantly. For the highest biomass concentration of BDW = 7.37 g/L, mycelia appear to be homogeneously distributed throughout the measurement even at a low shear rate of $\dot{\gamma}_{min}$ = 0.25 s^{-1}. Thus, the biomass concentration is another significant parameter when defining the appropriate measuring range for rheological measurements, since it affects dramatically the pellet-to-pellet interaction and so the particle settling.

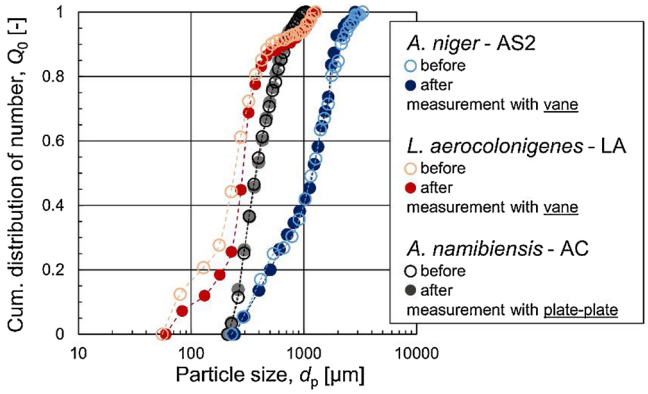

Fig. 4. Particle size cumulative distributions for samples of *A. niger* - AS2 (t = 57.5 h, BDW = 5.83 g/L), *L. aerocolonigenes* – LA (t = 192 h, BDW = 3.73 g/L) and *A. namibiensis* - AC (t = 96 h, BDW = 8.22 g/L) cultivations before and after measurements with the system specific proper rheometers.

3.1.2. Lentzea aerocolonigenes

Low reproducibility was recorded using the plate-plate configuration for the broths of *L. aerocolonigenes* (LA) as depicted in Fig. 3A. By conducting measurements on the transparent plate, phase separation was observed during the measurement (Fig. 3B and C). In Fig. 3A the deviation between the flow curve obtained with the acrylic glass plate and the ones obtained with the original Kinexus lab + plate might be the result of the broth inhomogeneity during the shearing process. Due to the difficulty to ensure a reliable usage of the plate-plate rheometer configuration for the broth characterization, further measurements were conducted with the impeller/vane system, which showed reproducible results. No morphological damage occurred during the measurement as confirmed by the microscopic analysis of the sheared sample (Fig. 4). In this case, the transparent vane vessel was used again to determine the minimum shear rate $\dot{\gamma}_{min}$. Comparative measurements with the same sample in both the original vessel and the acrylic glass duplicate showed a good agreement (compare Fig. 3A). An increase in biomass concentration again resulted in lower $\dot{\gamma}_{min}$ for a complete dispersion, as a result of the higher viscosity of the broth at higher BDW. The impact of morphology on $\dot{\gamma}_{min}$ was not examined for *L. aerocolonigenes*. This is because, due to the low broth volume (50 mL in shaking flask), samples for the rheological characterization were taken only at the end of the cultivation when the microorganism is in the production phase and thus all samples of *L. aerocolonigenes* had the same morphological state.

3.1.3. Actinomadura namibiensis

For the cultivation of *A. namibiensis* (AC), investigations with the transparent vane geometry showed a rapid settling of the bio-

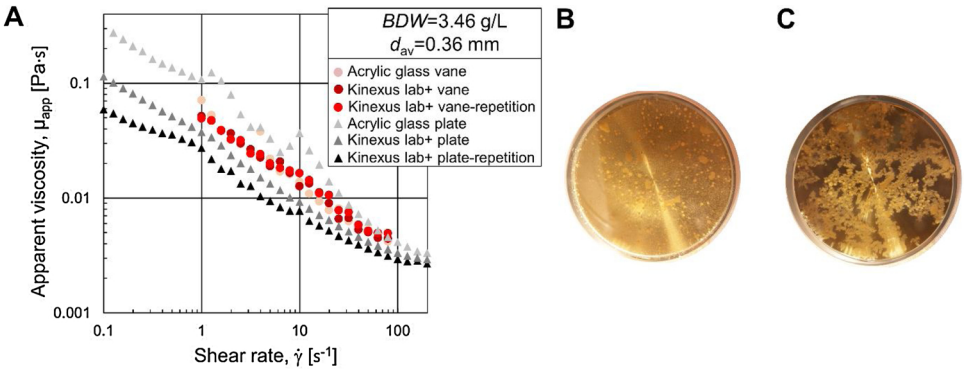

Fig. 3. (A) Rheograms of *L. aerocolonigenes* LA broth measured with different configurations. Top view image of broth sample on the transparent plate (B) before and (C) during the measurement.

agglomerates of broth samples taken within the first two to five days of the cultivation, resulting in low reproducibility. Only for the final stages of the cultivation the sedimentation velocity was low enough allowing the pellets to be suspended. Unlike *A. niger* and *L. aerocolonigenes*, the average pellet diameter of *A. namibiensis* decreases with time. This bacterium develops a highly heterogenous mycelial cell morphology. During the cultivation time the amount of pellets decreases, whereas the fraction of freely dispersed mycelia and loose aggregates increases as reported by Tesche et al. [47]. The change in cell morphology and the increase in biomass concentration explain the significant lower sedimentation velocities after approximately five days of cultivation. Nevertheless, measurements with the vane geometry even at these later stages were treated critically. The impeller rheometer was not further used for this biological system.

On the contrary, due to the smaller average pellet size of *A. namibiensis* ($d_{av} = 0.28 - 0.44$ mm) compared to *A. niger* (d_{av} up to 3 mm depending on the cultivation batch), the plate-plate configuration was considered a reasonable option. Although the ratio of gap size to pellet diameter falls below the recommended minimum value of ten [49,50], the flow curves showed good reproducibility. This should be attributed additionally to the structural properties (micro-morphology) of this bacterium, that apparently ensure high stability of the formed pellets. Using the acrylic glass duplicate of the plate-plate configuration a homogeneous distribution of the biomass was seen. Since stainless steel and acrylic glass have different surface properties, the adhesion in the replica system may have differed from the original plate-plate system. However, the obtained values of μ_{app} were very similar for both original and acrylic plate-plate systems. Therefore, it is assumed that the distribution of the biomass is similar in the original and the replicated plate-plate system. To assess, if bio-agglomerates changed in size or structure, image analysis was performed before and after a measurement. Fig. 4 shows the pellet-size distribution before and after a measurement with the plate-plate rheometer. No significant change was observed. Based on the findings, the plate-plate rheometer was considered a suitable measurement system for *A. namibiensis* for the entire cultivation period.

3.1.4. Conclusions: measuring methods

The impeller rheometer allows a precise rheological characterization of *A. niger* and *L. aerocolonigenes* cultivation broths providing results with an acceptable accuracy and reproducibility in most cases. It was proven that this set-up prevents alteration of the morphology of the biological sample.

Based on the measurements with the transparent vane vessel, care must be taken when choosing the appropriate measuring range of shear rate. The impeller rheometer can offer reliable data only for a limited range of shear rate that depends strongly on the cell morphology and biomass concentration of the broth. In most cases, the minimum shear rate should be as high as $\dot{\gamma}_{min} \geq 10\ s^{-1}$ to avoid sedimentation and phase separation. At the same time, the measurements can be conducted as long as turbulent effects are avoided for the Metzner-Otto concept to be valid. Thus, the upper shear rate limit depends on the viscosity of the sample (see Fig. A1). The more viscous the broth, the higher the shear rates that can be applied. By measuring model fluids of known rheology, $Re < 150$ was found as a suitable criterion to avoid turbulent effects. Therefore, in the case of the biological samples, the maximum shear rate $\dot{\gamma}_{max}$ for the evaluation was chosen according to this Reynolds number limit for each sample. Even though the laminar flow regime ranges up to $Re \approx 10$, the Metzner-Otto concept can still be valid in the transitional regime and therefore at $Re < 150$ [52]. Sedimentation cannot be avoided for low viscous samples or large particles with compact and smooth surface structure. Therefore, there are limitations when measuring low viscosities associated especially with the first samples of a cultivation. The problem was already mentioned in the literature by Malouf [32], who suggested the usage of a concentric cylinder for the measurements of the early obtained broth samples. In preliminary tests the use of such system showed low reproducibility and significant morphological alteration for *A. niger*, mainly due to the significant larger bio-agglomerate sizes in comparison to the cultivations of Malouf [32] with $d_{av,max} \approx 100\mu m$.

The impeller rheometer did not seem appropriate for a precise rheological characterization of the *A. namibiensis* cultivation broth. On the contrary, the plate-plate rheometer configuration provides results with an acceptable accuracy and reproducibility. The use of this system should be interpreted as the result of both macro- (size, shape) and micro- (structure) morphology of the bacteria that ensure the avoidance of pellet disintegration/alteration during shearing.

3.2. Rheological characterization of filamentous cultivation broths

For all samples of the different cultivations the measuring data of shear stress as a function of the shear rate were plotted in double logarithmic plots and fitted to the power law model. Despite the fact that the power law cannot describe yield stresses, this model was chosen for all following results, because of its simplicity and since measurements at very low shear rates (to determine yield stresses) would be considered at least critical if not unreliable as described in Chap. 3.1. For shear thinning samples, the flow consistency factor K and the flow behavior index n of the power law model were determined by linear regression. For each experimental point, at least two measurements (each with new sample) were carried out to ensure the reproducibility of the recorded flow curves (exemplarily see Fig. A1). The error bars in the following graphs depict the deviations between multiple samples.

3.2.1. Cultivation of Aspergillus niger

A. niger was cultivated under controlled level of osmolality (cultivation AS0, AS1, AS2, AS3) or pH (AS4) to obtain a desired fungal morphology (see Table 1). Based on the work of Wucherpfennig et al. [46], in this study the osmolality level at the various cultivations was increased from 400 mosmol/kg (without NaCl addition) to 1500 mosmol/kg ($c_{NaCl} = 32.18$ g/L) and further to 2500 mosmol/kg ($c_{NaCl} = 64.43$ g/L), resulting in a change of the fungal cell morphology from the pelleted to a mycelial form and decreasing bio-agglomerate sizes. The obtained morphological growth was in a good agreement with that described in Wucherpfennig et al. [46].

The dynamic viscosity of the Newtonian culture medium as measured at the beginning of the cultivation was $\mu = 0.844$ mPa·s at 37 °C. At various time points during the cultivation, samples of the broth were filtered to separate the biomass and the supernatant viscosity was measured. It was proven that during the cultivation the viscosity of the continuous phase does not change, but remains at water-viscosity level, meaning that the rheological changes described in the following paragraphs are caused exclusively by the presence of bio-agglomerates and not by viscous metabolites released to the medium.

3.2.1.1. Broth rheology during the cultivation. At an osmolality of 400 mosmol/kg (cultivation AS0), *A. niger* grew in large spherical pellets. The growth phase of the cultivation lasted approximately 55 h before reaching the stationary and later the lethal phase. In this time period, the evaluation of the rheological measurements succeeded from a shear rate of $\dot{\gamma}_{min} = 1\ s^{-1}$ and at the latest stage of the cultivation ($t \geq 50$ h) from a shear rate of $\dot{\gamma}_{min} = 0.1\ s^{-1}$ to avoid sedimentation of the pellets. For the pelleted cultivation broth AS0, at $BDW \leq 0.5$ g/L the low viscosities did not allow to obtain reasonable measuring data. At this initial process stage, the flow behavior index was considered to be near $n \sim 1$, dominated by the Newtonian behavior of the cultivation medium. At $BDW > 0.5$ g/L the broth showed shear thinning behavior and higher viscosities. Once the stationary phase was reached, basically no further change of the flow curves was recorded. Even though in literature it is often claimed that pelleted broths present

Newtonian rheological behavior and low viscosities [20,53], the findings of the present work do not confirm this statement, which seems to be rather generalized. In the past, other authors have also reported non-Newtonian rheology of pelleted broths [11,27,28,54–56]. In Fig. 5A the development of the rheological parameters, the flow consistency factor K and the flow behavior index n, the *BDW* and the morphological characteristic (average pellet diameter d_{av}) are plotted as a function of cultivation time for the pelleted AS0 broth. As a result of the increase in *BDW,* the growth of the pellets and thus the increasing pellet-to-pellet interactions [57], an increase in K is observed. For pelleted AS0 broth (inoculum concentration 10^6 spores/mL) in the examined cultivation period, i.e., t = 20–53.5 h and level of biomass, i.e., *BDW* = 0.58–4.69 g/L, the flow consistency factor varied in a range of $K = 0.01 - 0.65$ Pa·s^n. The flow behavior index after t = 20 h appears to reach a plateau, i.e., $n = 0.24 \pm 0.03$, without being directly influenced by the evolution of the other system properties.

The cultivation *A. niger* AS1 (not shown) had again an osmolality of 400 mosmol/kg (no NaCl addition), but the inoculum spore concentration was decreased compared to AS0 cultivation to $3.9 \cdot 10^5$ spores/mL. A pelleted growth was obtained again, but the pellets were significantly larger in comparison to AS0 cultivation (compare Fig. 2A,B, average particle diameter d_{av} up to 2.9 mm at AS1, in contrast to $d_{av} < 2$ mm at AS0), the pellet structure was more compact with shorter hyphal length and the *BDW* was approximately four times lower than for AS0. The low viscosities and the fast sedimentation of the pellets made any quantified rheological characterization of the broth impossible as previously described in Chap. 3.1.

At an osmolality of 1500 mosmol/kg (cultivation AS2), *A. niger* grew in the form of freely dispersed mycelia. In this case, the cultivation had a total duration of 107 h (Fig. 5B) and the rheological data used for the evaluation included shear rates higher than $\dot{\gamma}_{min} = 10\ s^{-1}$. The vane measuring system could be used successfully only at *BDW* > 2 g/L. The rheological data reported below this level should be treated with care, since high deviations were observed during the measurements, due to the low viscosities of the samples. At higher biomass concentrations, the broth exhibited a pseudoplastic behavior, which was attributed to hyphal entanglement during the growth [54]. Similar to the pelleted cultivation AS0, again in this case an increase in K was reported during the cultivation. At approximately 80 h the cultivation reached the stationary stage. It is remarkable that the growth period for the *A. niger* AS2 cultivation lasted longer in comparison to the *A. niger* AS0 cultivation, since the mycelial forms with low compactness are more active and suffer less from mass transport limitations. After 80 h of cultivation, a decrease in K was observed. This decrease indicates the lethal phase of the cultivation and it was observed for all cultivations of Table 1. The decreased viscosities at these stages seem to be the result of hyphae fragmentation and lysis. The structural changes of the cells at the late cultivation stages make them less resistant to shear forces. At the same time, the formation of small spores, which typically appear at the branched hyphae and have much lower viscous properties than the filaments, results in a drop in the flow consistency factors K [14,32,57]. Again, no clear correlation of n with the evolution of the *BDW* or the hyphae size is reported. For the examined cultivation period and level of biomass, i.e., t = 43–107 h and *BDW* = 3.7–6.3 g/L, the flow consistency factors varied in a range of $K = 0.4$–2 Pa·s^n and the flow behavior index reached a plateau of $n = 0.27 \pm 0.07$ for the broth of the freely dispersed mycelia (Fig. 5B).

At an osmolality of 2500 mosmol/kg (cultivation AS3, inoculum concentration 10^6 spores/mL, not shown), *A. niger* grew in small elongated clumps. In this case, the system reached the stationary stage after 80 h of cultivation. The maximum level of biomass determined was approximately *BDW* ~ 3.0 g/L and *A. niger* built compact agglomerates of average sizes in the range of $d_{av} \sim 0.4 - 0.7$ mm. For this cultivation broth the use of the vane geometry was significantly limited due to apparent pellet sedimentation. Even at the latest stages of the cultivation, the flow consistency factors lay at the level of $K \sim 0.05$ Pa·s^n. At these low broth viscosities, the rheological data cannot be used reliably. In their study with *Aspergillus oryzae,* Müller et al. [58] connected also small size clumps and low biomass concentrations to low viscosities. A plausible explanation could be that small clumps possess shorter hyphae that cannot contribute significantly to pellet-to-pellet interactions and so the viscosity remains at low levels independent of the *BDW* level.

To understand better the rheological behavior of small elongated clumps, the cultivation *A. niger* AS4 (without NaCl addition, inoculum concentration 10^6 spores/mL) was carried out. In this case, the pH was used to adjust the agglomerate morphology at the desired level (pH-shift from 3 to 5 from cultivation time 24 h–29 h). The advantage of this cultivation in comparison to the AS3 cultivation is that the biomass grew up to *BDW* = 4.98 ± 0.31 g/L at t = 47 h. Thus, even though at a biomass concentration of up to 2.5 g/L similar problems were faced during the measurements as for the AS3 samples, at higher *BDW* rheological data were reliably obtained and they will be discussed in the following section.

3.2.1.2. Influence of biomass on broth rheology. Fig. 6 depicts the evolution of K and n as a function of *BDW* under identical morphological state; with d_{av} = 1.95 mm for the pelleted broth AS0

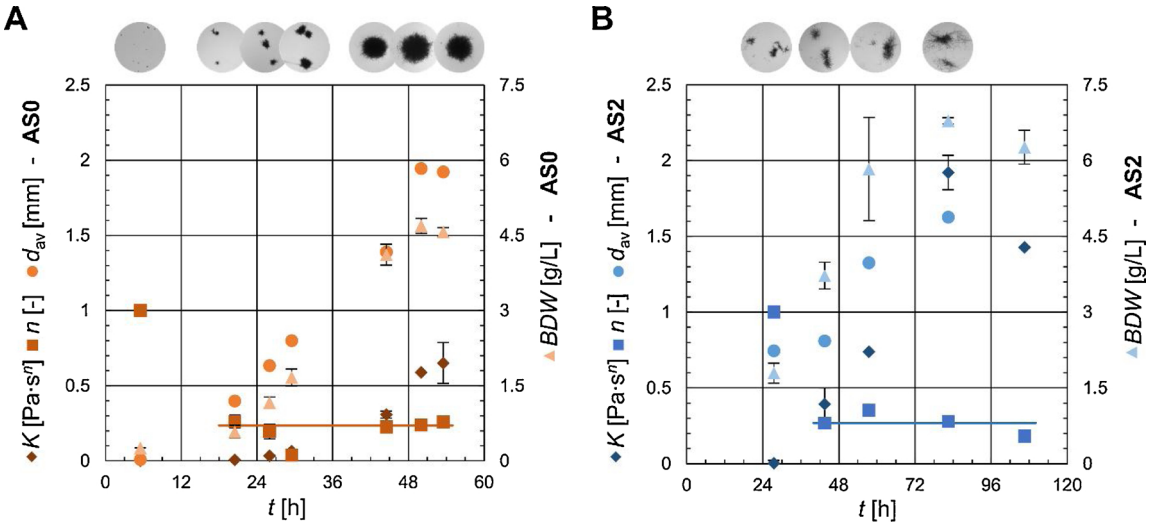

Fig. 5. Evolution of the broth properties during the cultivation of *A. niger* for (A) the pelleted AS0 broth and (B) the mycelial AS2 broth.

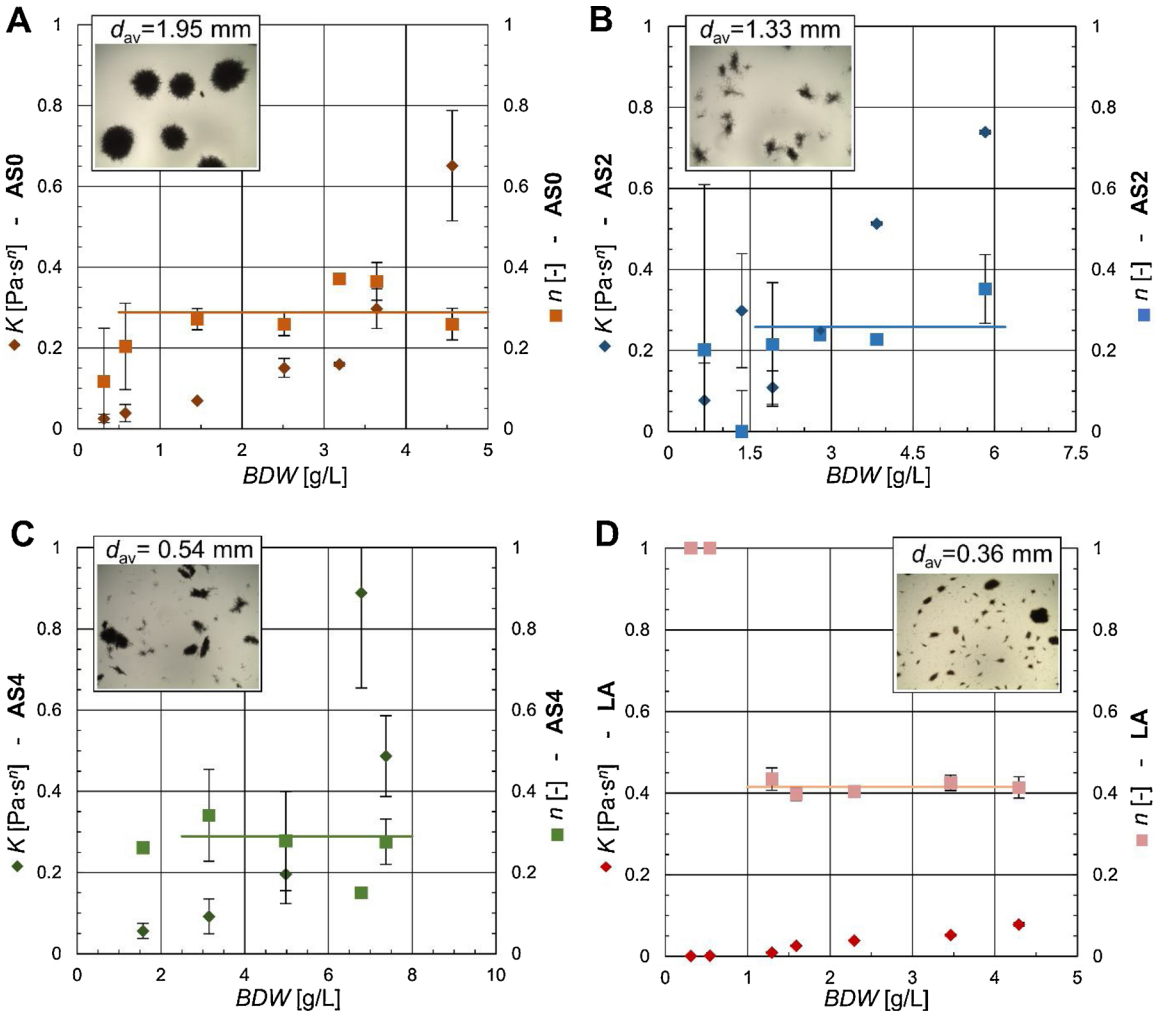

Fig. 6. Impact of biomass concentration *BDW* on the flow consistency factor *K* and flow behavior index *n* for (A) the pelleted AS0 broth, (B) the mycelial AS2 broth, (C) the mycelial AS4 broth of *A. niger* and (D) the pelleted LA broth of *L. aerocolonigenes*.

(Fig. 6A), d_{av} = 1.33 mm for the mycelial broth AS2 (Fig. 6B) and d_{av} = 0.54 mm for the mycelial broth AS4 (Fig. 6C). For these measurements an original sample taken from the stirred tank bioreactor during the cultivation was diluted (with cultivation medium at different ratios) or/and concentrated (removal of supernatant after biomass sedimented), to obtain broth samples in a wide range of *BDW*.

Here, the clear significant dependence of *K* on *BDW* is shown. With increasing *BDW*, the *K* value rises. When trying to build a mathematical relationship with a power law approach to describe the dependence of *K* on *BDW* - in the form of $K = C_K \cdot BDW^{\alpha}$- for each cultivation, an exponent α in the range of α = 1.65–1.8 was determined. However, such simple mathematical relationships in the form of a power law approach should be treated carefully, because the amount of available data may not be sufficient for a statistically safe mathematical modeling. Also, as pointed out by Allen and Robinson [13], such correlations should be treated as system specific and not as "universally" applicable models. Thus, even though the exponent α found in this work seems to be in general agreement to similar approaches in literature; Albaek et al. [59] reported α = 1.5–1.56 for *A. oryzae*, Olsvik et al. [25] reported α = 1.3 for *A. niger*, Gabelle et al. [41] reported α = 1.5 for *T. reesei*, and Pamboukian and Facciotti [55] reported α = 2.03 for *Streptomyces olindensis*, a comparison with literature correlations is generally difficult because of the differences in cultivated strain, cultivation conditions, cell morphologies and measuring methods.

No clear correlation between *n* and *BDW* was recorded for any cell morphology of *A. niger*, with *n* being almost independent of the biomass level. The average value of *n* for the measured range of biomass concentration was *n* = 0.29 ± 0.07 for the pelleted *A. niger* AS0 cultivation (*BDW* = 0.58–4.56 g/L), *n* = 0.26 ± 0.06 (*BDW* = 1.91–5.83 g/L) for the mycelial-growing AS2 broth and *n* = 0.29 ± 0.07 (*BDW* = 1.57–7.37 g/L) for the mycelial AS4 broth, respectively (Fig. 6A–C).

Various studies have reported mathematical modeling - usually also in the form of the power law approach of $n = C_n \cdot BDW^{\alpha}$ - to correlate *n* with *BDW*. However, other studies claim that such a relationship is not so straightforward, as confirmed by the present work as well. In the study of Riley et al. [20] for the tested cultivations of *P. chrysogenum*, the *n* value was principally stable with an average value of 0.35 for *BDW* > 10 g/L with exceptions of the values measured at the earliest and latest cultivation phases. Similar findings were reported earlier by Pedersen et al. [28] for *P. chrysogenum* with *n* appearing to be approximately constant and independent of the *BDW* reaching a plateau at 0.4 − 0.5 at the examined biomass range. Müller et al. [58] also concluded that in the cultivations of *A. oryzae* no correlation could be found between *n* and *BDW* or the examined strains of *Aspergillus*, with the recording range of *n* values to fluctuate strongly in a range of 0.03 − 0.38 under varying pH cultivation conditions and morphological states of the different employed strains. The early work of Allen and Robinson [24] stated that for the filamentous cultivation broths of *A. niger*, *P. chrysogenum* and *Streptomyces levoris*, with increasing biomass concentration *n* drops off rapidly from 1 to values between 0.2 − 0.4. In agreement with that, in the work of Pollard et al. [7], the filamentous

fungi *Glarea lozoyensis* also showed that *n* dropped rapidly at the initial stages of cultivations to constant values in a range of 0.35 − 0.4. Pamboukian and Facciotti [55] characterized batch and fed-batch cultivations of *S. olindensis* and reported a sharp decrease in *n* after the initial 24 h of cultivation, which then remains relatively stable ($n \sim 0.3$), while the cell morphology had hardly an influence on the *n* values. Similar findings have been recorded by Gabelle et al. [41] for cultivations of *T. reesei* where *n* was found constant ($n = 0.4 \pm 0.1$) for *BDW* > 7 g/L. Petersen et al. [12] claimed no correlation between *n* and biomass concentration, batch time, agglomerates size or feed mode for a pilot scale cultivation of *A. oryzae*. Finally, Olsvik et al. [25] in their work with *A. niger* could not find any strong dependence of *n* on the cultivation conditions.

3.2.1.3. Influence of macro-morphology on broth rheology. Throughout the experimental study, it was observed that the effect of the bio-agglomerate size on the broth viscosity was not as strong as the effect of the biomass concentration and the effect of other morphological characteristics (e.g., compactness, roughness, hyphal length surrounding the pellet core). This point was first reported in Chap. 3.1 when a comparison was made between AS0 and AS1 cultivation broth. Despite the bigger pellet size of the *A. niger* AS1 cultivation, the lower biomass concentration, the high pellet compactness and the lower pellet roughness made the cultivation broth less viscous than the AS0 broth and led to faster pellet settling. In the current chapter, by comparing Fig. 5A and B (rheological characterization at different points of time during the cultivation) with Fig. 6A and B (rheological characterization at specific cultivation point with concentrated/diluted samples of the same bio-agglomerate morphology) respectively (see also Fig. A2A), again it can be observed that the impact of the biomass concentration on the viscosity of the system and thus the flow consistency factor *K* is significantly stronger than the one of the bio-agglomerate size. Figure A2A shows that with the same biomass concentration the recorded *K* values are very similar independent of the agglomerate diameter.

This allows merging the data of Fig. 5 and Fig. 6 (see Fig. A2B) and draw conclusions for the effect of other morphological factors (e.g., shape, compactness) on flow consistency factor *K* under consideration of the same biomass concentration. As depicted in Fig. A2B only a slight difference in the flow consistency factor *K* was recorded between the AS0 (pellets) and the AS2 (mycelia) cultivations. At the same biomass concentration, independently of agglomerate size, the AS2 broth was slightly more viscous (higher *K* values) than the AS0 broth. In both broths, *A. niger* built agglomerates with fluffy, rough external surface, which contributes to the pellet-to-pellet interactions and the increase in viscosity. On the other side, the elongated compact clumps of the AS4 cultivation with the short hyphae hair (decreased roughness) had clearly lower *K* and, therefore, lower viscosities in comparison to the AS0 and AS2 cultivations. Thus, the external pellet surface properties seem to be crucial for pellet-to-pellet interaction and so the viscosity of the system. The inner core of the bio-agglomerate seems to affect indirectly the viscosity of the system, by defining the biomass growth. The pellets of the AS0 broth had a compact core, which limited the oxygen and nutrient transport and finally led to an earlier fungi death and lower total biomass levels. *A. niger* of the AS2 cultivation had a highly loose inner structure leading to better mass transport, prolonged growth and eventually higher biomass concentrations, at which significantly higher viscosities were recorded. Olsvik et al. [25] in their work with *A. niger*, after proving that the biomass is the most crucial system variable affecting the flow consistency factor, they examined systematically the influence of various morphological parameters, i.e., roughness, compactness, clump area and overall percentage of mycelial in form of clumps, on *K*. It was reported that at the same biomass concentration, the flow consistency factor was strongly correlated to the clump roughness, to lesser extent to the compactness, while *K* was not significantly interrelated with other morphological parameters. This statement is in agreement with the findings of the current work.

Despite the distinct cell morphologies (diameter, shape, roughness, compactness etc.) of *A. niger*, no significant difference in flow behavior index *n* was recorded for the AS0, AS2 and AS4 cultivations as seen in Fig. 6. Indisputably, this result, referred previously also in other studies such as the ones of Olsvik et al. [25], Pamboukian and Facciotti [55] and Müller et al. [55], indicates the need to relate the degree of pseudoplasticity more to micro-morphology, the inner structural properties and the inner stability of a specific strain and not to macro-morphology.

At the same time, the results of *A. niger* under salt exposure (AS2) are in contrast to Wucherpfennig et al. [15]. The authors, who were the first to rheologically investigate this specific strain of *A. niger,* reported a strong influence of *BDW* on the flow behavior index *n* and distinguish three different areas: shear-thickening behavior ($n > 1$) for low *BDW* (0.5–2.5 g/L), Newtonian behavior ($n = 1$) for medium *BDW* (2.5–4.0 g/L) and shear-thinning flow behavior ($n < 1$) for high *BDW* (4.0–9.0 g/L). A shear-thickening behavior seems very unusual and the data generated in this work with $n \leq 1$ for all cultivation times do not confirm the findings of Wucherpfennig et al. [15]. Furthermore, the *K* values determined between 0.07 and 0.75 $Pa{\cdot}s^n$ (compare Fig. 6B, AS2, addition of 32.18 g/L NaCl) are much more plausible than the previously determined K values [15], which are at levels far below the viscosity of water. The deviations between the current work and that one of Wucherpfennig et al. [15] clearly show how critically rheological investigations of filamentous systems have to be evaluated and that a great effort of research is still needed to improve the accuracy and reproducibility of such investigations.

In literature authors have reported predictive correlations that describe the interrelation among broth rheology, biomass concentration and cell morphology. For *A. niger,* such correlations describe the flow consistency factor *K* as a function of the biomass concentration and the pellet roughness [5,25] or the non-dimensional Morphology number [15]. Difficulty arises to use these correlations, among others due to the variety of methods and parameters used to describe the moprhological characteristics of the bio-agglomerates.

For the prediction of such sophisticated dependencies though, the concept of the effective volume fraction Φ_{eff} might be of interest. This concept considers the solid volume fraction and the liquid that can be embedded between solid agglomerates. This amount of liquid is temporarily immobilized in the agglomerates and it should therefore not be considered in the liquid volume fraction [60]. The solid volume fraction in this study is composed of biomass, which significantly influences the viscosity of the cultivation broth. The effective solid volume fraction is potentially even higher, especially in case of pellets or mycelia with a rough and irregular surface, which tend to entangle. At high biomass concnentrations, the potential for entaglement and embedded liquid volume fraction is increased, since a lot more particle contacts are possible. At lower biomass concentrations, however, the interactions between pellets or mycelia are lower due to larger distances in between. Such behavior was described for the viscosity of suspensions by Nguyen et al. [61]. Accessing the effective volume fraction is not easy for such complex biological systems and thus it was not investigated in detail in this study. Nevertheless, this concept can be promising for further research.

3.2.2. Cultivation of Lentzea aerocologineses

As described in Chap. 3.1 the vane geometry was used for this biological system. The batch cultivation of *L. aerocolonigenes* lasted in total 8 days. However, due to the limited broth volume (50 mL in shaking flask), samples for the rheological characterization were taken only at the end of the cultivation, since the microorganism is in the production phase at this point. The rheology of the LA cultivation broth at $t = 192$ h was determined. Additionally, the influence of the biomass on the broth rheology was investigated by following the same methodology of diluting and concentrating the originally obtained sample as in the case of the *A. niger* cultivations, thus, creating samples with the

same cell morphology but varying solid content. The dynamic viscosity of the Newtonian culture medium was measured to be $\mu = 0.93$ mPa·s at 30 °C. At $t = 192$ h, a sample of the broth was filtered to separate the biomass and the supernatant viscosity was determined resulting in $\mu = 0.96$ mPa·s, indicating that the increased viscosities and the pseudoplasticity of the broth depicted in Fig. 6D was entirely due to the biomass growth and not the aqueous culture medium or extracellular metabolites.

The rheological data used for fitting included shear rates higher than $\dot{\gamma}_{min} = 10\ s^{-1}$ for $BDW < 1.5$ g/L. Above this biomass level the minimum shear rate taken into consideration was $\dot{\gamma}_{min} = 1\ s^{-1}$. As shown in Fig. 6D the cultivation broth is characterized by low viscosity, since significantly small values of K were determined for all samples. When $BDW < 1.0$ g/L, the broth has Newtonian behavior. Above this biomass level, shear thinning properties were recorded. An increase of BDW led to a clear increase of the K factor. At the same time, BDW had no effect on n, similarly to what was reported in Chap. 3.2.1 for the broths of *A. niger*. The average value of n for the measured range of BDW was $n = 0.41 \pm 0.01$ ($BDW = 1.29$–4.29 g/L). In comparison to *A. niger*, *L. aerocologineses* shows a less pronounced shear thinning behavior (higher n values) and lower apparent viscosity (lower K values) at the same BDW. This might be explained by the difference in cell morphology. The surface structure of *A. niger* has higher hyphal gradients in the outer pellet layer compared to *L. aerocolonigenes* pellets with a higher circularity/compactness surface structure.

3.2.3. Cultivation of Actinomadura namibiensis

As described in Chap. 3.1 the plate-plate geometry was used for *A. namibiensis*. The batch cultivation of *A. namibiensis* lasted in total 10 days and this bacterial strain develops a highly heterogeneous mycelial morphology. A transition from pellet to dispersed morphology takes place during the cultivation, with the pellet to disintegrate into looser fragments. Fig. 7 shows the rheological properties, the BDW concentration and the particle size d_{av} during the AC cultivation. The cultivation broth develops a shear thinning behavior. For the first 120 h of the cultivation, as the biomass increased, the viscosity and thus the flow consistency factor K increased. At this point in time, the pellet structure started getting loose, the pellet size decreased and the amount of freely dispersed mycelial increased. From this point on, no further significant change in the rheology of the system was observed and the measured flow curves were almost identical with $K = 0.39 \pm 0.02$ Pa·s^n and $n = 0.40 \pm 0.02$ for $t = 144$–220 h.

3.3. Use of model fluid systems to simulate the biological rheology

Apart from the rheological characterization of the biological filamentous systems, this work aims to find model fluid substances that can mimic the rheology of the cultivation broth and can be further used for various fluid dynamic and mass transfer investigations in the respective bioreactors. Therefore, various substances were tested as suitable model fluids (compare Table 1). The rheograms were recorded for all model fluids. Fig. 8 depicts exemplarily flow curves of the non-Newtonian model fluids (Xanthan gum, CMC and PEG solutions, Fig. 8C) that were examined, as well as the viscosity of the Newtonian fluids (inverted sugar syrup, glycerin and Luviskol® K90 solutions, Fig. 8D) as a function of the per weight concentration (% w/w) of each model substance in aqueous solutions. It should be underlined that some model fluids depending on the producer could exhibit deviating rheological properties. The results shown in this work are valid for the specific substances as described in Chap. 2.2.

Since all filamentous biological systems exhibit shear-thinning behavior, their flow curves were compared with those of the non-Newtonian, shear thinning solutions: Xanthan gum, CMC and PEG solutions. However, both CMC solutions (Fig. 8B) and PEG solutions (Fig. 8C) show lower pseudoplasticity in comparison to Xanthan solutions (Fig. 8A) as well as the cultivation samples. Therefore, these substances were not further considered as potential model fluids of the examined filamentous cultivations.

Fig. 9 depicts exemplary flow curves of Xanthan gum solutions and the cultivation broths of *A. niger* AS0 (Fig. 9A), AS2 (Fig. 9B) and AS4 (Fig. 9C), *L. aerocolonigenes* (LA) (Fig. 9D) and *A. namibiensis* (AC) (Fig. 9E).

In general, the shear thinning behavior of *A. niger* can be simulated satisfactorily by Xanthan gum solutions. Despite the fact that *A. niger* exhibits a slightly stronger pseudoplastic behavior than the model substance, a satisfying fitting is illustrated between the flow curves of the biological and the model fluid system for a wide range of shear rates (approximately $\dot{\gamma} = 1$ - $400\ s^{-1}$). More specifically, fitting values were observed for samples of AS0 broth with $BDW > 2.5$ g/L with Xanthan sol. of $c_{Xanthan} = 0.05 - 0.1$ % w/w (Fig. 9A), samples of AS2 broth with $BDW > 3.5$ g/L with Xanthan sol. of $c_{Xanthan} = 0.08 - 0.3$ % w/w (Fig. 9B) and samples of AS4 broth with $BDW > 5$ g/L with Xanthan sol. of $c_{Xanthan} = 0.05 - 0.1$ % w/w (Fig. 9C). At the same time though, for the samples of the AS0 and AS4 broth with the highest biomass $BDW_{AS0} = 4.69$ g/L and $BDW_{AS4} = 7.37$ g/L, the measured μ_{app} in the shear rate range $\dot{\gamma} \sim 0.1$ - $1\ s^{-1}$ seem to deviate strongly from the ones of the Xanthan solutions. At this point, to evaluate the significance of such deviations at lower shear rates, an estimation of the shear rate range expected in the bulk of the investigated cultivation system in the stirred tank has to be made.

An approximate estimate of the average shear rate in a stirred tank under laminar flow can be made using the Metzner-Otto approach (Eq. 2). For a typical configuration of a stirred tank equipped with a Rushton turbine the reported K_{MO} values in literature [28,52,62] are in the range of $K_{MO} \sim 10 - 13$. Taking this into account, the average shear rates in the examined lab-scale reactor vary from $\dot{\gamma} \sim 30$ - $150\ s^{-1}$ for operation at typical rotational speeds of 200–600 1/min with a Rushton turbine. Similar estimations have been done by other authors as well, with the reported average shear rate ranges to vary between 1 - 1000 s^{-1} [32], 10 - 300 s^{-1} [63], 50 - 500 s^{-1} [14] and 20 - 180 s^{-1} [28,58]. Sánchez Pérez et al. [64] derived theoretically a correlation to estimate average shear rates under turbulent flow conditions. For the dimensions of the stirred tank used in this work and for medium to low broth viscosities (when the flow is turbulent), this approach would predict average shear rates in the bioreactor of approximately 45-600 s^{-1} (for a broth of $K = 0.15$ Pa·s^n, $n = 0.26$). Based on the flow field analysis conducted by Krämer et al. [65], measurements with particle image velocimetry employing a Xanthan solution (0.05 % w/w) in a stirred

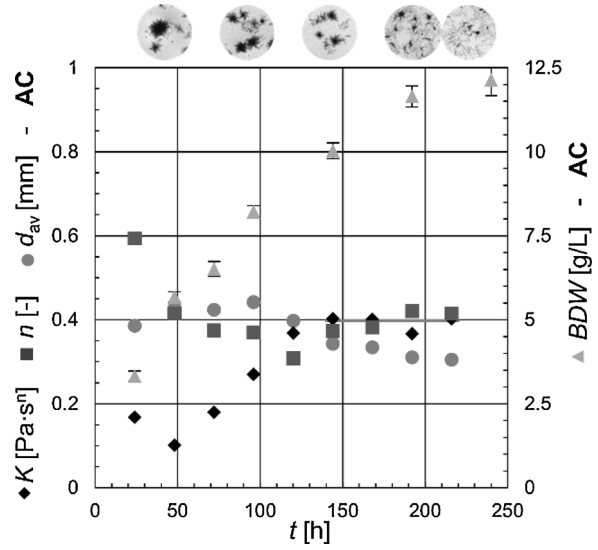

Fig. 7. Evolution of the cultivation broth properties during the cultivation of *A. namibiensis* (AC).

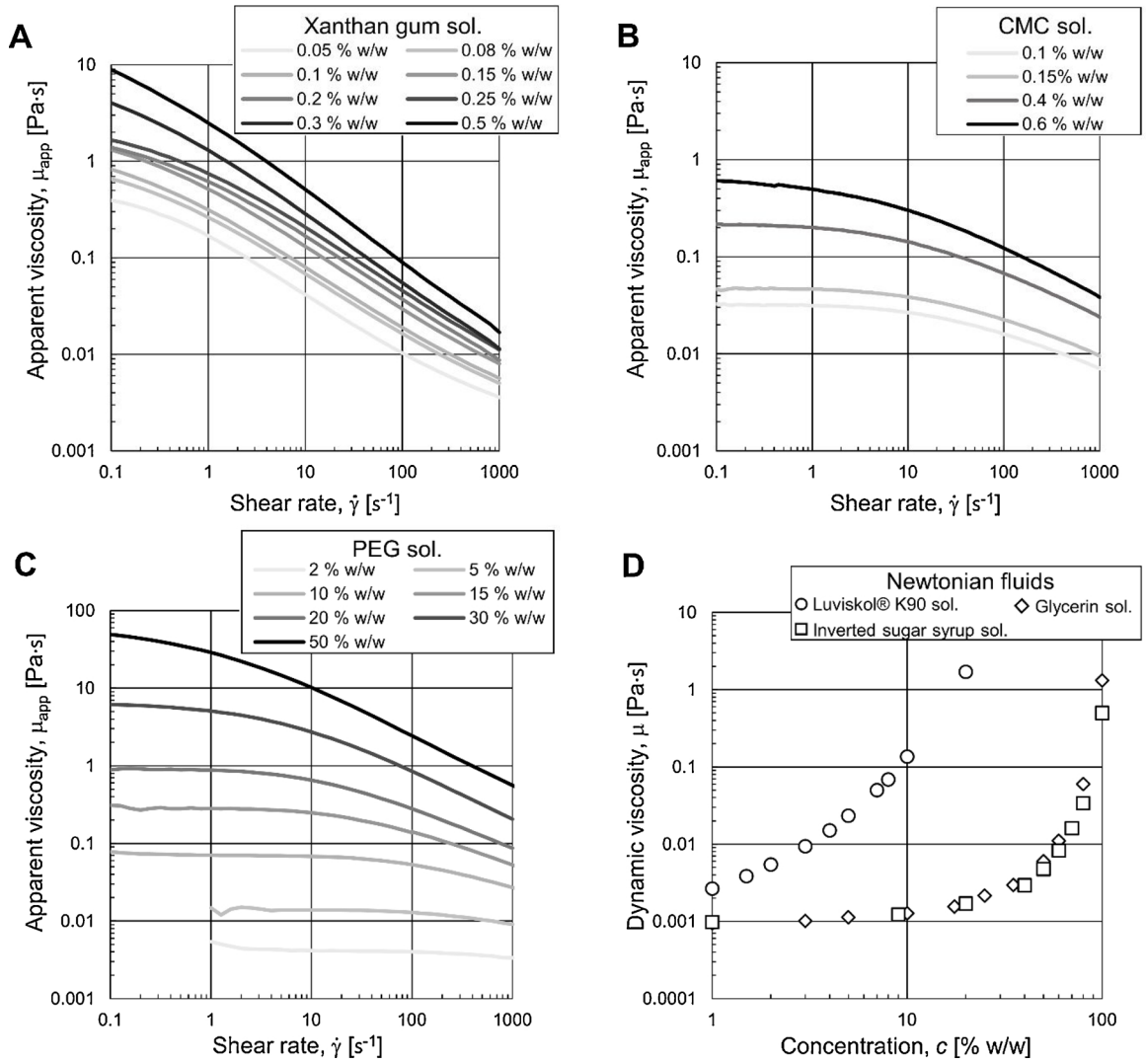

Fig. 8. Flow curves of the non-Newtonian media (A) Xanthan gum, (B) CMC and (C) PEG solutions and (D) the evolution of dynamic viscosity of Newtonian model systems as function of the concentration of the model substances in aqueous solutions.

tank equipped with a Rushton turbine showed that shear rates up to 1 s^{-1} corresponded to less than 15 % of the total amount of shear gradients in the reactor. For more precise estimations, computational fluid dynamics simulations have to be done on a specific system. All in all, it is estimated that the deviations seen in Fig. 9 for the lower shear rate range are not crucial for the use of Xanthan solutions as model fluids of the cultivation broth of *A. niger*. Probable viscoelastic properties of the cultivation broth and Xanthan solutions were not measured, as the influence on the flow field in the stirred tank bioreactors is negligible for typical operating conditions in the turbulent flow regime. A conservative estimation of the elasticity number for the conducted cultivations led to an elasticity number of $El \ll 0.02$, meaning that the flow is dominated by inertia forces [66].

Concerning the broth of *L. aerocologineses* (Fig. 9D), due to the low viscosities no real fitting was observed with the flow curves of Xanthan solution. Even for the highest biomass concentration obtained during experimentation, the biological sample did not seem to be simulated well by any of the tested model fluids. The flow curves lie always lower than that one of the 0.05 % w/w Xanthan solution, which was the less concentrated Xanthan solution used in this study. Nevertheless, eventually in cultivations with enhanced biomass growth, Xanthan in low concentrated aqueous solutions could be a possible model fluid. If the cultivation of *L. aerocolonigenes* in a stirred tank reactor leads to increased biomass concentrations in comparison to the ones obtained in shaking flasks is still a point of further investigation. For *A. namibiensis* (Fig. 9E), at $BDW > 6$ g/L a great agreement with the flow curves of Xanthan solutions of $c_{Xanthan} = 0.05-0.1$ % w/w can be shown.

In the past, Pedersen et al. [28] showed in a similar way that Xanthan solutions can mimic quite well the rheology of *P. chrysogenum* cultivation broth, while Gabelle et al. [41] reported that the mass transfer coefficients measured in Xanthan solutions fit significantly well to the ones of a *T. reesei* broth. In agreement to these findings, this work illustrates the suitability of Xanthan solutions to mimic at a satisfying degree the rheology of *A. niger* and at a greater degree the rheology of *A. namibiensis*.

4. Conclusions

The present study investigated the rheological behavior of three filamentous microorganisms. The fungus *A. niger* was cultivated in a stirred tank bioreactor in batch with varying cell morphology by the addition of inocula with different NaCl concentrations and different spore concentrations as well as performing a pH shift. The actinomycetes *L. aerocolonigenes* and *A. namibiensis* were cultivated in shaking flasks and they were both rheologically characterized for the first time.

To establish a measurement methodology for the various heterogeneous cultivation broths, multiple rheometer geometries were tested. It was proven that significant attention should be paid when conducting

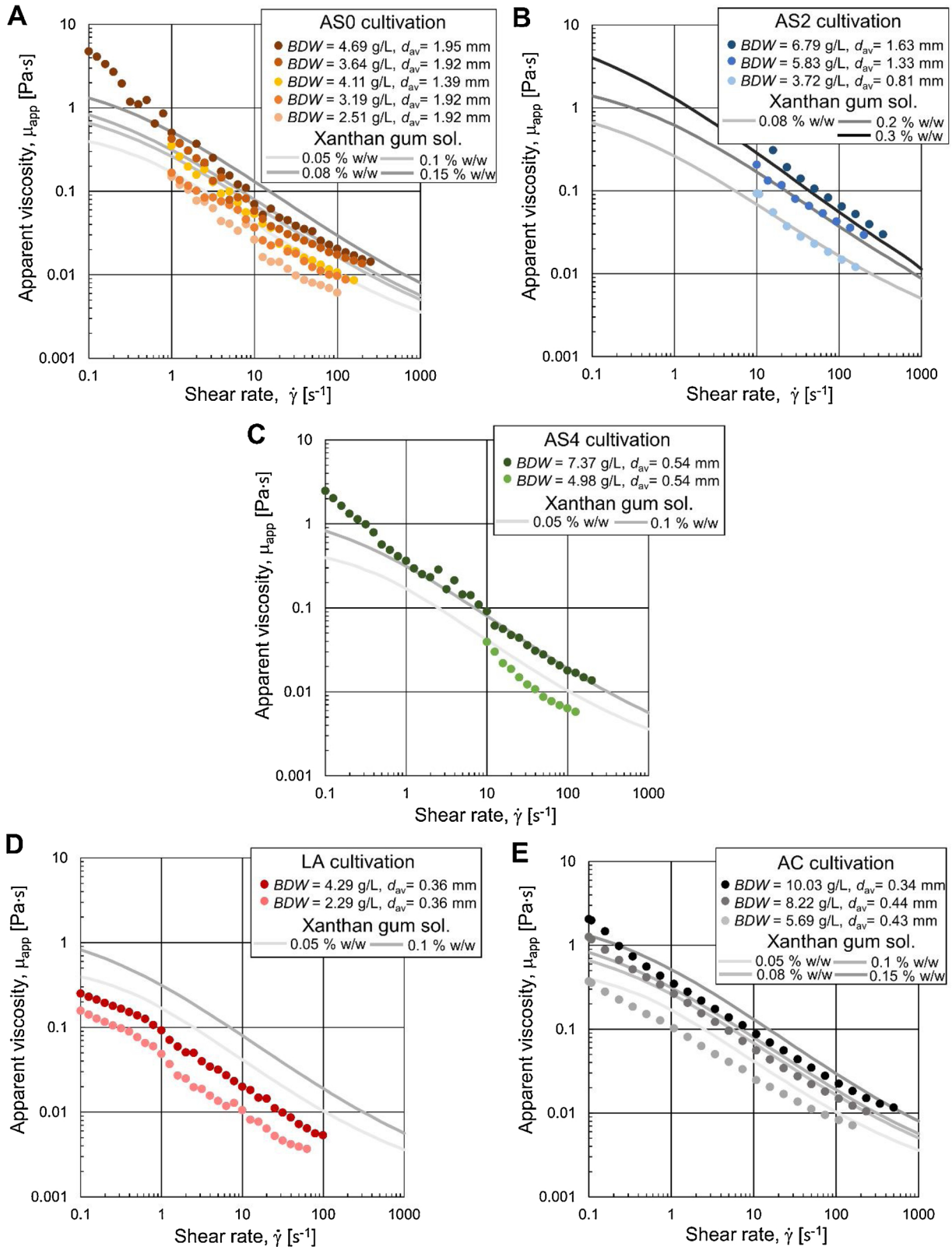

Fig. 9. Comparison between flow curves of the cultivation broths *A. niger* (A) AS0, BDW: 2.51 to 4.69 g/L, (B) AS2, BDW: 3.72 to 6.79 g/L, and AS4, BDW: 4.98 to 7.37 g/L, (C) *L. aerocolonigenes* (LA), *BDW*: 2.29 to 4.29 g/L, and (D) *A. namibiensis* (AC), *BDW*: 5.69 to 10.03 g/L, and the ones of shear thinning non-Newtonian model fluids of xanthan gum (solid lines).

rheological measurements of particulate materials due to settling and/or agglomeration and all conventional methodologies should be re-evaluated before applied to meet the specific characteristics of such complex filamentous biological systems. The impeller geometry is considered suitable for (most of) the cultivation broths of *A. niger* as well as the broth of *L. aerocolonigenes*. The plate-plate-system gives reliable, reproducible results for the cultivation broth of *A. namibiensis*. Acrylic glass replicas of the plate-plate-system and impeller/vane-

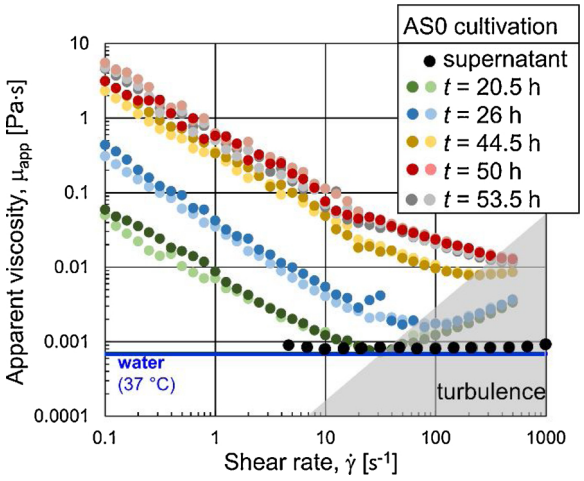

Fig. A1. Reproducibility of the flow curves for different samples taken during the *A. niger* AS0 cultivation, measured with the impeller/vane geometry. The effect of turbulence in the vessel becomes evident as the shear rate increases. The more viscous the sample, the higher the shear rate at which the turbulent effects become dominant.

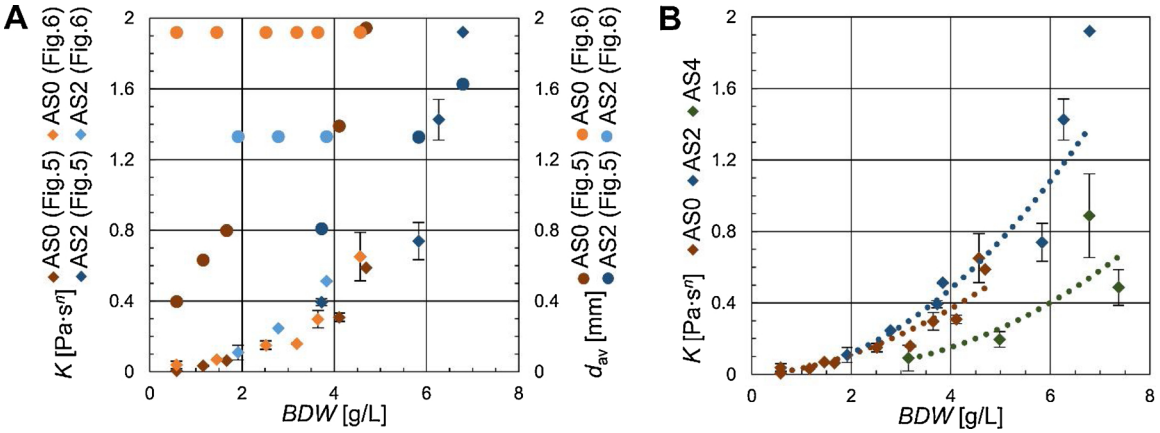

Fig. A2. (A) Comparison of data from Fig. 5 and Fig. 6 for *A. niger* AS0 and AS2 cultivation, (B) evolution of the flow consistency factor *K* with the biomass concentration *BDW* for *A. niger* AS0, AS2 and AS4 cultivation (by merging data from Fig. 5 and Fig. 6 independently of agglomerate size).

system were used to allow optical accessibility throughout the measurements. This gave a significant insight, especially in the usage of the vane rheometer, for which the shear rate region for reliable measurements is strongly limited by both the settling of the bio-agglomerates (lower limit) and turbulent effects under which the Metzner-Otto concept fails (upper limit). It was proven that both the biomass concentration and the agglomerate surface structure (compactness, roughness) impact significantly the pellet-to-pellet interactions and so they are crucial to avoid sedimentation and enable measurements of the broth sample with the vane set-up.

In this study, the Ostwald-de Waele rheological model was applied to describe the rheological characteristics of the filamentous cultivation broths. This most commonly used in literature model fitted the obtained rheological data of this work at a great degree, providing sufficient quality of the resulting trends.

A. niger was cultivated in batches of controlled osmolality, spore concentration and pH to obtain varying morphological development of the fungi. The obtained morphological growth verified the previously reported findings of Wucherpfennig et al. [46]. Independent of the morphological form (pellet or dispersed mycelia) *A. niger* shows clearly a non-Newtonian shear thinning behavior from the second cultivation day on. The more the biomass grows, the more enhanced the pellet-to-pellet interactions and the more viscous the cultivation broth becomes. The surface structure for pellets (e.g., compactness, hyphal gradient in the outer pellet layer) and the intertanglement of dispersed mycelia is another crucial factor defining the pellet-to-pellet adherence and affecting the rheology. The more compact the bio-agglomerates or the shorter their external hyphal length (lower roughness), the less viscous the broth is. The degree of pseudoplasticity depicted by the flow behavior index in the power law approach seems to be independent of the biomass concentration as well as the cell morphology of *A. niger*. It is believed that pseudoplasticity reflects the stability of the inner structure of the respective filamentous system and so it might be a strain-specific property. This will be a topic of further filamentous related research with great potential of new knowledge.

L. aerocolonigenes and *A. namibiensis* were cultivated in shaking flasks. Both broths exhibit non-Newtonian shear thinning behavior but a lower degree of pseudoplasticity in comparison to *A. niger*. *L. aerocolonigenes* is a significantly less viscous system. Again, the effect of biomass concentration on the broth rheology confirmed the conclusions made for *A. niger*.

Finally, a comparison was shown between the rheology of the biological broths and of model fluids that are often used in literature for fluid dynamic investigations in bioreactors. Xanthan gum solutions can be used as suitable model fluid to mimic the rheological behavior of a medium of the filamentous microorganisms *A. niger* and *A. namibiensis*. None of the examined model fluids could mimic the viscous properties of *L. aerocolonigenes*. To create a wider database of possible model fluids, solutions of Xanthan gum in different Newtonian media should be measured and tested as further options. In this way, together with

additional experimentation with cultivation broths of other filamentous microorganisms, the generated results could be further improved and refined.

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CRediT authorship contribution statement

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Appendix A

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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