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Final report

Shear stress investigations of the magnetically levitated single-use centrifugal pump PuraLev® 600SU using the protein shear stress model for lysozyme

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Nomenclature

Symbol	Unit	Description
ID	mm	Inner diameter
N_p	-	Number of pumping cycles
p	bar	Pressure
t_p	min / d	Pumping time
$\dot{V}$	L min^{-1} / lpm	Flow rate
V_L	L	Fluid volume
η	min^{-1}	Strain rate

1 Introduction

Based on the experiments of the CTI project "Development of a magnetically mounted single-use centrifugal pump for biopharmaceutical applications" (CTI P-Nr: Flank 153), the investigations are extended using the protein shear stress model for lysozyme for the single-use centrifugal pump PuraLev® 600SU. A 4-piston diaphragm pump is used for comparison. For this purpose, the fluid is pumped in a circuit in order to be able to classify the pumps used with regard to the pump-specific shear stress [1].

The background is the relevance of the mechanical stress of pumps on proteins for downstream processing. In order to investigate the influence of different pump types on protein quality, lysozyme from chicken egg protein was selected as a model protein. This 14.3 kDa (129 amino acids) enzyme is characterized by good availability at moderate prices and has already been used several times in the literature, for example to investigate refolding after mechanical stress [2–4].

In order to determine changes in the enzyme, an activity measurement was carried out. This is based on a bacteriolysis reaction, which is measured photometrically once turbidity starts decreasing. A reduction in lysozyme activity was expected with increasing mechanical stress. In addition, dynamic light scattering (DLS) enabled a statement on structural changes and the aggregation of individual lysozyme monomers. No size exclusion chromatography (SEC) was used as this did not provide any information on lysozyme aggregation in previous experiments [1].

2 Material and methods

The materials and methods used for the tests are shown below. The single-use centrifugal pump PuraLev® 600SU from Levitronix® GmbH and a 4-piston diaphragm pump were used as comparative devices in the experiments to determine their mechanical stress on proteins.

Table 1: Equipment and materials used.

Devices	Type	Vendor
Photometer	Ultrospec 3000 pro	Amersham
Flow sensor	Clamp-on Ultraschall-Sensor	Levitronix® GmbH
Flow/pressure monitor	LCO-i100.1 LCO-i600.1	Levitronix® GmbH
Particle measuring device	Zetasizer	Malvern Instruments
Thermostat	Minichiller-H1	Huber
Storage tanks	Chemap	3.5 L and 12 L stainless steel reactors with double jacket

Table 2: Consumable material.

Material	Type	Vendor	Produkt-No.
Pressure sensors	3/8" Tülle	PendoTECH	PREPS-N-038
Disposable cuvettes	Makro, Semi-Mikro	Greiner bio-one	614101, 613101
Disposable syringes	Omnifix 10 mL	B. Braun	4617100V
Reagent tubes	1.5 mL Safe Lock	Eppendorf	0030 120.086
Tubing for clamp-on flow sensors	Pharma-80 Tubing 0.375" x 0.563"	Dow Corporation	1811070-1015
Tubing for reduction of cross section	Silikon transparent 6 x 2 mm	Maagtechnic AG	10075335
Silicone tubing for container outlet	Cole-Parmer 95623-10 1" ID	Cole-Parmer	95623-10
Tubes	15 mL	Corning	430791

Table 3: Chemicals and substances.

Product	Vendor	Product No.	Lot-No.
Potassium dihydrogen phosphate, monobasic	Sigma-Aldrich	P5655-1KG	SLBQ1072V
Potassium hydroxide	Sigma-Aldrich	P1767-1KG	SZBB0740V
Lysozyme of chicken egg white	Sigma-Aldrich	L6876-100G	SLBQ0509
Micrococcus lysodeikticus, lyophilised	Sigma-Aldrich	M3770-5G	SLBR1182V

2.1 Preparation of the required solutions

A 66 mM phosphate buffer was used for the lysozyme. For this purpose, KH_2PO_4 was dissolved in ultrapure water and the pH was adjusted to 6.24 using 1 M KOH. For the pumping tests, a lysozyme stock solution with a concentration of 20 g L^{-1} was prepared, which was added to the pre-tempered phosphate buffer for the start of the test. The target lysozyme concentration in the storage tank was 1 g L^{-1}, resulting in the required amounts of lysozyme stock solution and phosphate buffer as shown in Table 4. A suspension of lyophilized *Micrococcus lysodeicticus* with a concentration of 0.015 % (w/v) in phosphate buffer was prepared as substrate for the enzyme activity determination.

Table 4: Overview of the required buffer and lysozyme stock volumes with indication of the respective protein concentration.

	PuraLev® 600SU, 4-piston diaphragm pump
Lysozyme stock	20 g L^{-1}
Target concentration	1 g L^{-1}
Overall volume	2 L / 4 L
Volume phosphate buffer	1900 mL / 3800 mL
Volume lysozyme stock	100 mL / 200 mL

2.2 Experimental setup

The experimental setup for the determination of the stress acting on the lysozyme basically corresponded to the configuration of the previous experiments (See Figure 1) [1]. Two identical 3.5 L reactors or two identical 12 L stainless steel reactors with a double jacket were used per experiment. The temperature was controlled by a thermostat via the double jacket to a constant value of 42 °C.

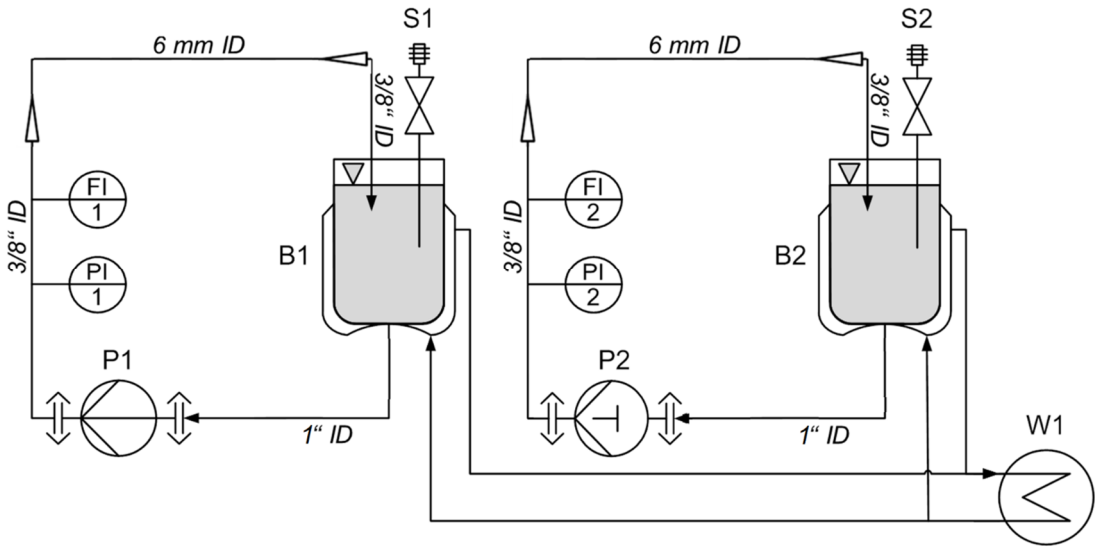

Figure 1: Schematic representation of the experimental setups for the Levitronix® PuraLev® 600SU (P1) and 4-piston diaphragm pump (P2). (S1) and (S2) sampling valve Clave Connector, (B1) and (B2) storage tank, (W1) thermostat, (FI1) and (FI2) flow sensor, (PI1) and (PI2) pressure sensor.

2.3 Pump test

Before the experiments, the reactors were autoclaved empty to inactivate any residual substances. The reactors were subsequently filled with the required volume of phosphate buffer (See Table 4) and tempered to 42 °C at low pump speed. After connecting the measuring devices, the pressure and flow sensors were calibrated with the pumps switched off, and the test parameters (flow rate via pump speed and back pressure via length of tubing with ID 6 mm) were set immediately before starting the experiment. To start the experiment, the required volume of lysozyme stock solution (See Table 4) was added to the temperature-controlled phosphate buffer and the reactor content was pumped for about 2-3 minutes to achieve a homogeneous mixing of the lysozyme solution. During the experiment, pressure, temperature and flow were regularly checked, recorded online and readjusted if necessary.
Sampling was carried out with Luer-Lock syringes via a sampling port on the lid of the reactors. The Luer-Lock port is connected to a tube inside the reactor, which reached into the lysozyme test solution. For sampling, a primary volume of about 3-4 mL was taken and discarded. Subsequently, a 4 mL sample was taken, transferred into a 15 mL Falcon tube and cooled down on ice.

- 1 mL of this sample was pipetted into an Eppendorf tube for the activity measurement and stored at 4 °C
- 1 mL was pipetted into an Eppendorf tube for the dynamic light scattering measurement with the Zetasizer and stored at 4 °C
- 2 mL were stored in reserve at 4 °C

2.4 Overview of experiments

The planned experimental conditions are shown in Table 5 below. Because of the different flow and strain rates of the lysozyme solution with a concentration of 1 g/L, different pumping times resulted. Therefore, all experiments were standardized to an equal number of 40,000 pumping cycles N_P according to equation 1. η represents the strain rate in 1/Minute and t_P the pumping time in minutes or days. The strain rate results from the flow rate $\dot{V}$ in lpm and the total volume V_L in litres.

$$N_P = \eta \cdot t_P \qquad \text{Equation 1}$$

$$\eta = {}^{\dot{V}}/_{V_L} \qquad \text{Equation 2}$$

To ensure a constant strain rate of 5 min^{-1} for all experiments, the volumes V_L were adapted to the desired flow rates $\dot{V}$. The duration per pump experiment was 5.6 days or 133.33 h, whereby due to the use of two reactors, one test approach always consisted of a parallel run of a PuraLev® 600SU and the 4-piston diaphragm pump.

Table 5: Overview of the experiments performed regarding the stress exposure on proteins

No.	Pump	Flow rate $\dot{V}$ [lpm]	Pressure p [bar]	Strain rate η [min^{-1}]	Volume V_L [L]	Pumping time t_P [d]	Pumping cycles N_P
P1.1	PuraLev® 600SU	10	1.5	5	2	5.6	40000
P2.1	PuraLev® 600SU	10	2	5	2	5.6	40000
P3.1	PuraLev® 600SU	10	2.5	5	2	5.6	40000
P4.1	PuraLev® 600SU	20	1.5	5	4	5.6	40000
P5.1	PuraLev® 600SU	20	2	5	4	5.6	40000
P6.1	PuraLev® 600SU	19 [a]	2.5	5	3.8 [a]	5.6	40000
P1.2	4-piston diaphragm pump	10	1.5	5	2	5.6	40000
P2.2	4-piston diaphragm pump	10	2	5	2	5.6	40000
P3.2	4-piston diaphragm pump	10	2.5	5	2	5.6	40000
P4.2	4-piston diaphragm pump	20	1.5	5	4	5.6	40000
P5.2	4-piston diaphragm pump	20	2	5	4	5.6	40000
P6.2	4-piston diaphragm pump	19 [a]	2.5	5	3.8 [a]	5.6	40000

a At maximum speed, only 19 L min^{-1} instead of the desired 20 L min^{-1} were achieved, so the volume had to be slightly reduced for a constant stress rate.

2.5 Enzyme activity measurements

The activity measurement of the lysozyme was carried out according to an internal SOP (See Appendix, SOP - Enzyme activity measurement of lysozyme). The assay is based on first order enzyme kinetics, which are assumed for the following reaction.

$$Micrococcus\ lysodeikticus\ (intact) \xrightarrow{lysozyme} Micrococcus\ lysodeikticus\ (lysed)$$

During the reaction, the photometer Ultrospec 3000 pro (Amersham/GE-Healthcare) was used to record the decrease in turbidity of the bacterial suspension due to the lytic effect of the lysozyme. The enzyme activity is determined via the (negative) increase in the extinction curve. This is higher, the faster the turbidity decreases. The measurements of the samples were always carried out after a completed experimental approach. Up to this time the samples were stored at 4 °C.

2.6 Dynamic light scattering method

With the dynamic light scattering method (DLS), the size of suspended particles in liquid can be determined. The method is based on the principle of Brownian molecular motion, whereby larger particles move more slowly and smaller particles move more quickly. With DLS, the speed of particle movement is measured by means of scattered light by detecting fluctuations in light intensity. The resulting diffusion coefficient is used to calculate the hydrodynamic particle diameter (diameter of a sphere with the same diffusion coefficient as the particles).

The measurement is performed using the Zetasizer Nano ZS (Malvern Instruments Ltd), which gives a particle size distribution in the form of histograms. For evaluation, the diameters of the most frequently represented particle sizes are determined for each sample. Since Brownian particle motion is highly dependent on temperature and viscosity, the measurement was carried out at a constant temperature of 20 °C. Since all samples of a test batch were first measured after completion of the same, the samples were stored at 4 °C until this time.

3 Results and Discussion

In the experiments P1.1 and P1.2, the PuraLev® 600SU was compared with the 4-piston diaphragm pump at a flow rate of 10 L min^{-1} and a pressure of 1.5 bar. The results of the activity measurement are shown in Figure 2. A standardized representation was used for evaluation in order to ensure a better comparability of the results. For this purpose, the activity was set to 100 % at the start of the test and the remaining values were related to it. The flow rate and pressure data recorded online during the experiment are shown in Figure 3 for both pumps.

Enzyme activity did not change significantly for the PuraLev® 600SU during the entire test. The fluctuations are within an acceptable margin of error. In contrast to the Levitronix® centrifugal pump, the 4-piston diaphragm pump showed a significant decrease in activity after just 4 hours, which then dropped to zero after about 14,000 pump cycles (48 hours).

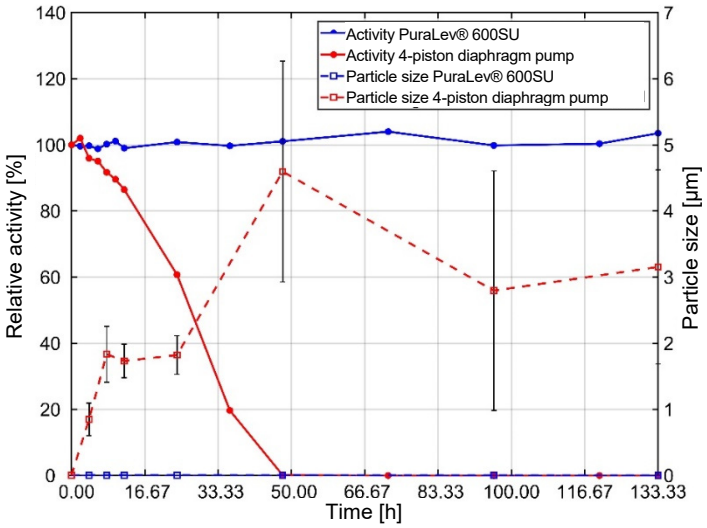

Figure 2: Results of the activity and dynamic light scattering measurements during pump experiments P1.1/P1.2 with the PuraLev® 600SU and the 4-piston diaphragm pump at a flow rate of 10 lpm and a pressure of 1.5 bar.

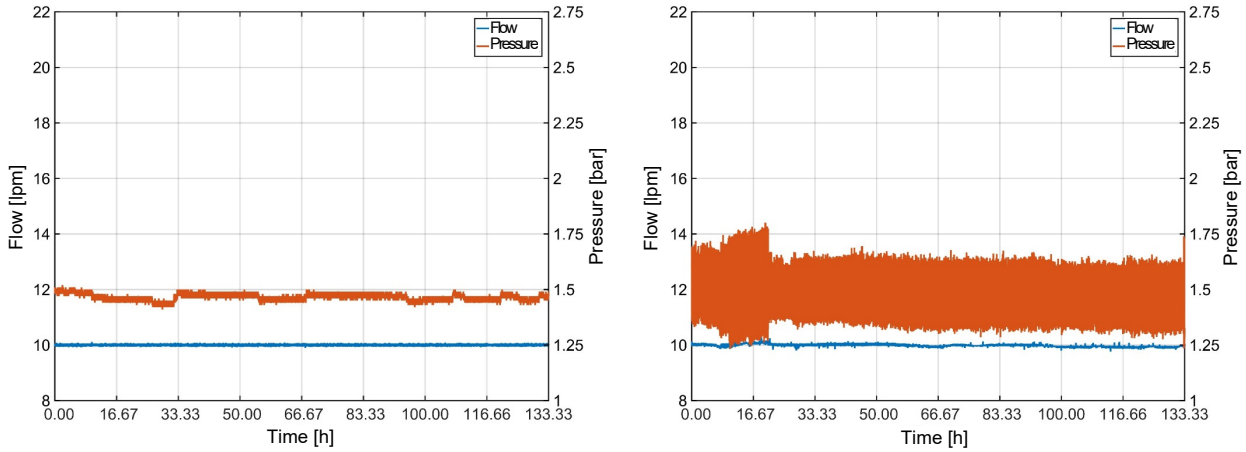

Figure 3: **Representation of the online recorded pressure and flow rates during the experiment for the PuraLev® 600SU (P1.1 - left) and the 4-piston diaphragm pump (P1.2 - right) at a flow rate of 10 lpm and a pressure of 1.5 bar.**

The change in the protein was optically visible as the lysozyme solution in the 4-piston diaphragm pump became increasingly cloudy during the pumping experiment (See Figure 4). This turbidity indicates possible aggregations of proteins or protein components. It can clearly be seen that the amount of sediment or the degree of turbidity increased with the duration of the experiment and remained more or less constant from sample 6 on (48 h). This correlates with the results of the activity measurements, in which no activity could be measured after 48 hours.

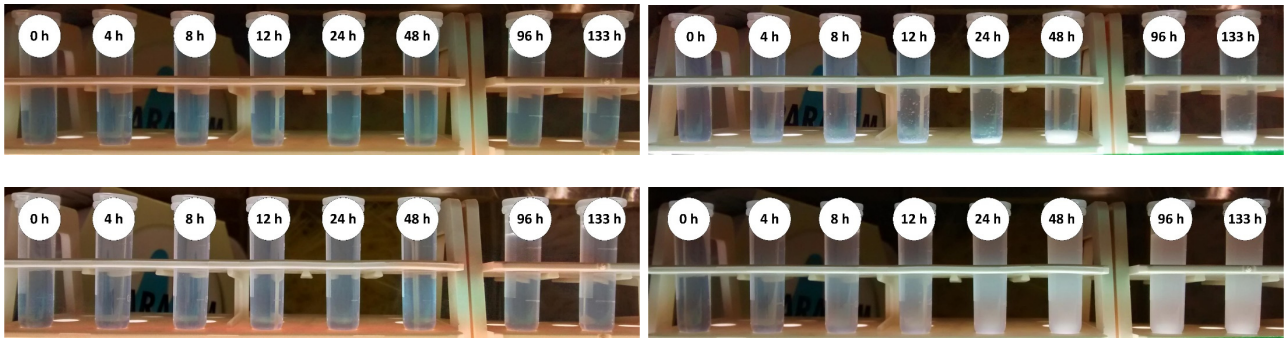

Figure 4: **Visible turbidity of the lysozyme solution in experiment P1.1/P1.2 at 10 lpm and 1.5 bar with the 4-piston diaphragm pump (right). The upper images show the turbidity in sedimented state and those below, after mixing the samples. The samples of the centrifugal pump show no turbidity or aggregates (pictures on the left).**

The results of the experiment show that the PuraLev® 600SU exerts less shear stress on the model protein lysozyme than the 4-piston diaphragm pump. This could be proven by the enzyme activity and the resulting turbidity. In addition to the activity measurements, the DLS method was used to investigate the aggregation of the lysozyme. This resulted in constant particle sizes in the range of 3.6 ± 0.1 nm for the PuraLev® 600SU over the entire period. However, the particle size of the 4-piston diaphragm pump increased strongly within the first 4 h (1.8 ± 0.4 µm), which can be seen from the turbidity of the

lysozyme solution. In the following period, a further increase in particle size to a maximum value of 4.6 ± 1.7 µm was observed.

The results of the following experiments P2.1 - P6.1 and P2.2 - 6.2 with higher pressures of up to 2.5 bar and flow rates of up to 20 lpm are shown in Figure 5 and demonstrate comparable results to experiments P1.1 and P2.2. Only experiments P4.2 (20 lpm and 1.5 bar) and P6.2 (19 lpm and 2.5 bar) showed a slower decrease in enzyme activity. Furthermore, a sudden decrease in the flow rate to 18.3 lpm was observed in experiment P6.2 after a pumping time of 41 h, which in the subsequent run slowly dropped to approx. 17 lpm (See Appendix, Raw data P6.1/P6.2 (19 lpm, 2.5 bar)). However, there was no apparent explanation for this circumstance, nor for the difference between the slower decrease in enzyme activity at 20 lpm and 1.5 bar and 19 lpm and 2.5 bar compared to all other tests.

In summary, it can be concluded that the Levitronix® PuraLev® 600SU does not cause any damage to the model protein lysozyme under the operating conditions tested, while a change in the protein with loss of activity occurred with the reference pump. In this respect, the experiments to extend the investigations carried out in the CTI project "Development of a single-use magnetic bearing centrifugal pump for biopharmaceutical applications" (CTI P-Nr: Flank 153) provide a useful supplement. The high stress exerted on the protein by the 4-piston diaphragm pump could be due to its functional principle and the hydrophobicity of the material used [1,2].

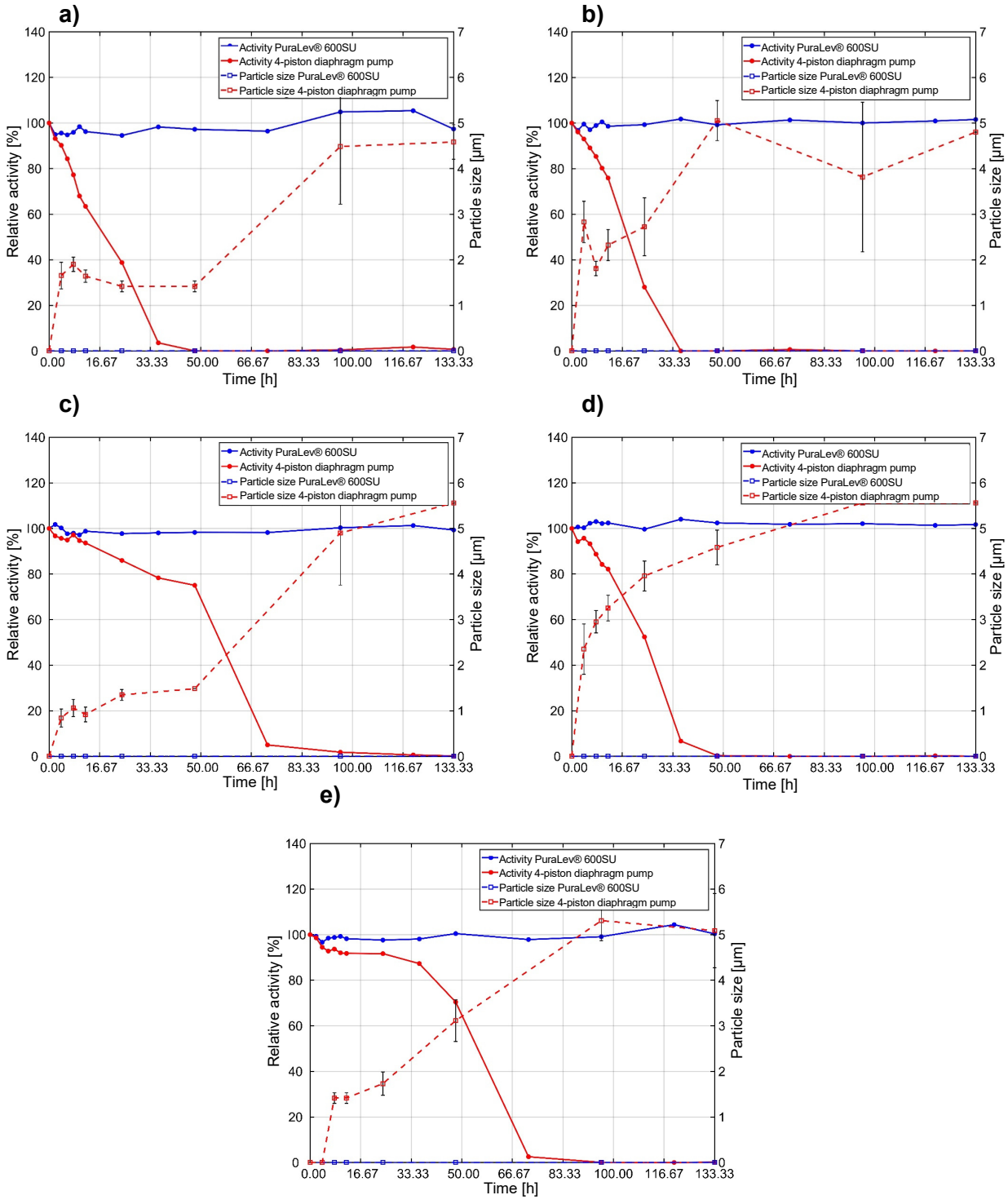

Figure 5: **Results of the activity and dynamic light scattering measurements during the pumping tests with the PuraLev® 600SU and the 4-piston diaphragm pump at a flow rate of 10 lpm and 2.0 bar (P2.1/P2.2 - a), 10 lpm and 2.5 bar (P3.1/P3.2 - b), 20 lpm and 1.5 bar (P4.1/P4.2 - c), 20 lpm and 2.0 bar (P5.1/P5.2 - d) and 19 lpm and 2.5 bar (P6.1/P6.2 - e).**

List of figures

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Literature

1. Löffelholz C, Blaschczok K, Dittler I, Lehmnann N, Kaiser SC, Eibl D, et al. KTI Abschlussbericht (KTI P-Nr: Flank 153) - Entwicklung einer magnetgelagerten single-use Zentrifugalpumpe für biopharmazeutische Applikationen. Wädenswil/Zürich; 2013.

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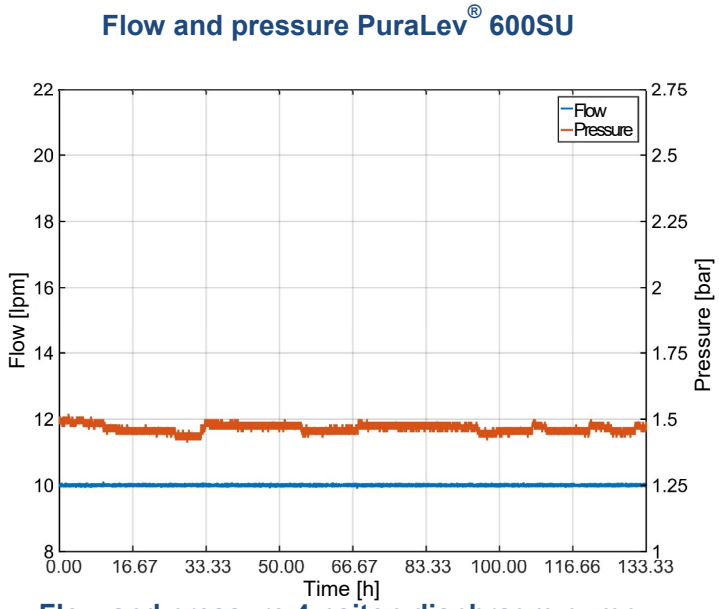
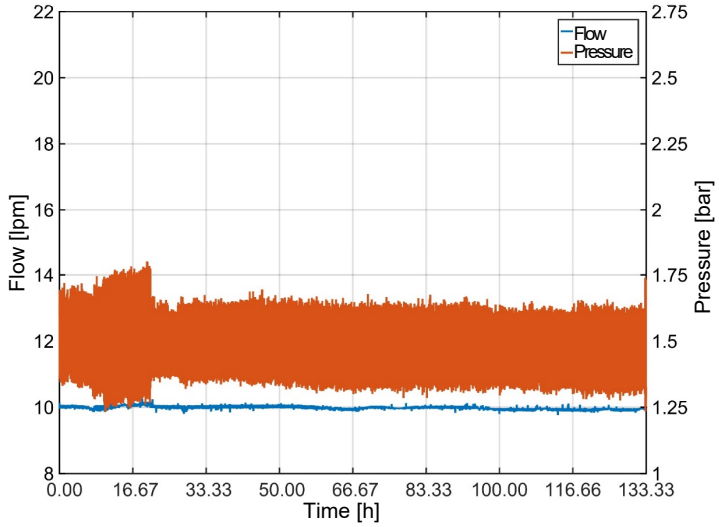
3. Simon S, Krause HJ, Weber C, Peukert W. Physical degradation of proteins in well-defined fluid flows studied within a four-roll apparatus. Biotechnol Bioeng. 2011;108(12):2914–22.

4. Ashton L, Dusting J, Imomoh E, Balabani S, Blanch EW. Shear-induced unfolding of lysozyme monitored in situ. Biophys J. 2009;96(10):4231–6.

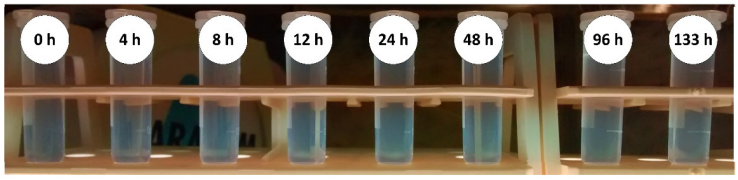
Appendix

Raw data P1.1/P1.2 (10 lpm, 1.5 bar)

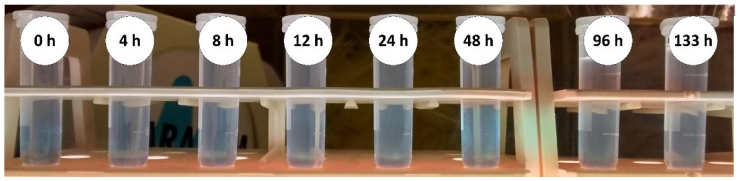




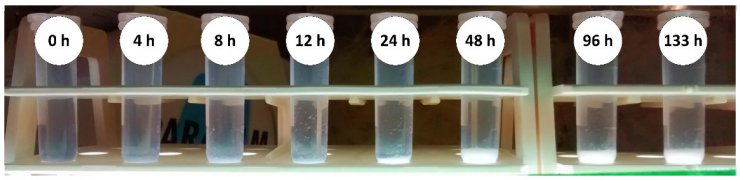
Samples PuraLev® 600SU sedimented



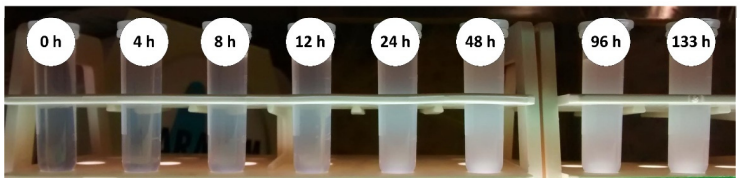
Samples PuraLev® 600SU suspended



Samples 4-piston diaphragm pump sedimented



Samples 4-piston diaphragm pump suspended



Measuring data of DLS method

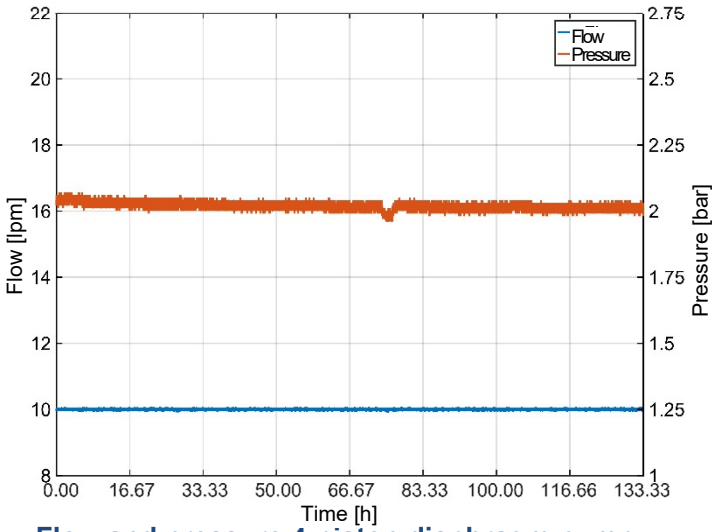
10 lpm; p=1.5 bar 4-piston diaphragm pump				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	2.9	0.8	0.0
2	4	848.7	246.3	1200.0
4	8	1835.7	423.0	2400.0
6	12	1730.7	253.2	3600.0
7	24	1821.3	292.1	7200.0
9	48	4596.3	1669.1	14400.0
11	96	2795.7	1810.6	28800.0
13	133.33	3153.3	1466.8	40000.0
10 lpm; p=1.5 bar PuraLev® 600SU				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	2.4	0.6	0.0
2	4	3.6	0.0	1200.0
4	8	3.6	0.0	2400.0
6	12	3.6	0.0	3600.0
7	24	3.6	0.0	7200.0
9	48	3.0	0.5	14400.0
11	96	2.7	0.4	28800.0
13	133.33	2.1	0.3	40000.0

Data of enzyme activity measurements

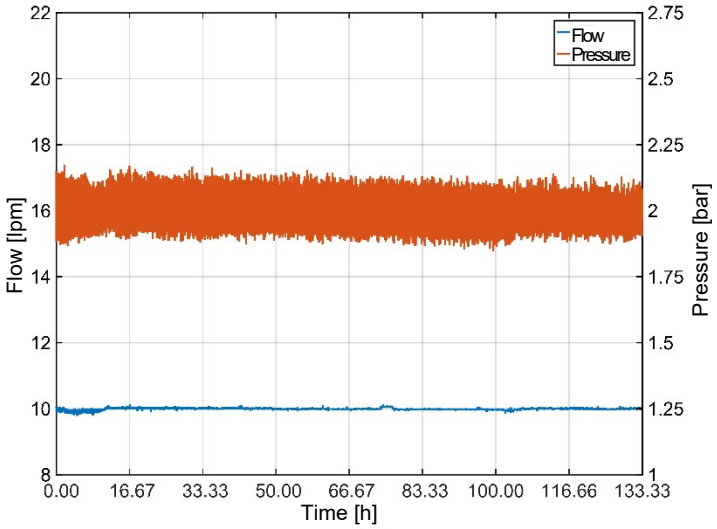
Time[h]	PuraLev® 600SU				4-piston diaphragm pump				Buffer
	Number of cycles [-]	Slope A_{450}/min	Abs. activity [10^3 U/mL]	rel. activity [%]	Number of cycles [-]	Slope A_{450}/min	Abs. activity [10^3 U/mL]	rel. activity [%]	Slope A_{450}/min
0	0.0	0.0248	24.4	100.0%	0.0	0.0251	24.8	100.0%	0.000388
2	600.0	0.0247	24.3	99.6%	600.0	0.0256	25.3	102.0%	0.000388
4	1200.0	0.0247	24.3	99.7%	1200.0	0.0241	23.8	95.9%	0.000388
6	1800.0	0.0245	24.1	98.8%	1800.0	0.0239	23.5	95.1%	0.000388
8	2400.0	0.0248	24.5	100.2%	2400.0	0.0231	22.7	91.7%	0.000388
10	3000.0	0.0251	24.7	101.1%	3000.0	0.0226	22.2	89.6%	0.000388
12	3600.0	0.0246	24.2	99.0%	3600.0	0.0218	21.4	86.4%	0.000388
24	7200.0	0.0250	24.6	100.8%	7200.0	0.0154	15.0	60.7%	0.000388
36	10800.0	0.0247	24.3	99.7%	10800.0	0.0053	4.9	19.7%	0.000388
48	14400.0	0.0251	24.7	101.1%	14400.0	0.0004	0.0	0.1%	0.000388
72	21600.0	0.0258	25.4	104.0%	21600.0	0.0001	-0.3	0.0%	0.000388
96	28800.0	0.0248	24.4	99.8%	28800.0	0.0000	-0.4	0.0%	0.000388
120	36000.0	0.0249	24.5	100.3%	36000.0	0.0000	-0.4	0.0%	0.000388
133.33	40000.0	0.0257	25.3	103.5%	40000.0	0.0000	-0.3	0.0%	0.000388

Raw data P2.1/P2.2 (10 lpm, 2.0 bar)

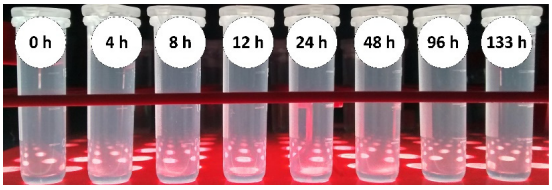
Flow and pressure PuraLev® 600SU



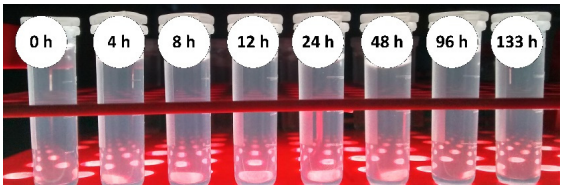
Flow and pressure 4-piston diaphragm pump



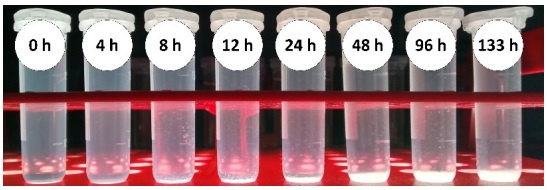
Samples PuraLev® 600SU sedimented



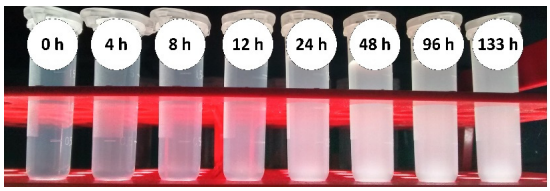
Samples PuraLev® 600SU suspended



Samples 4-piston diaphragm pump sedimented



Samples 4-piston diaphragm pump suspended



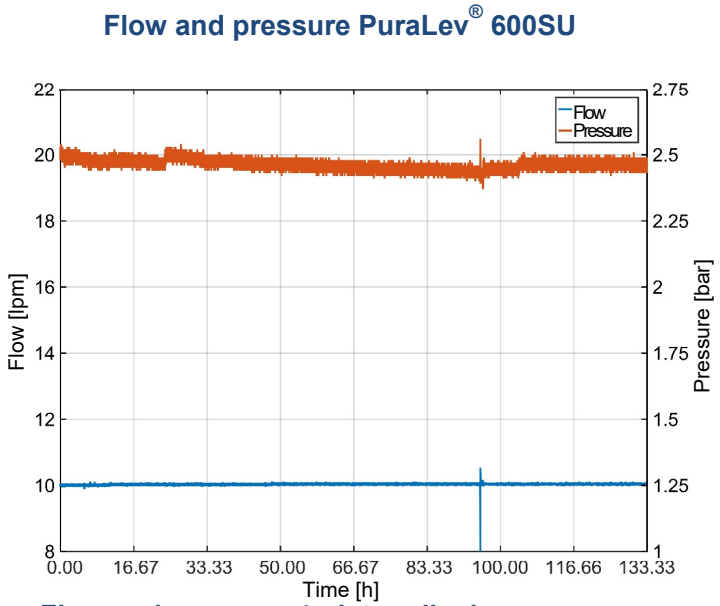
Measuring data of DLS method

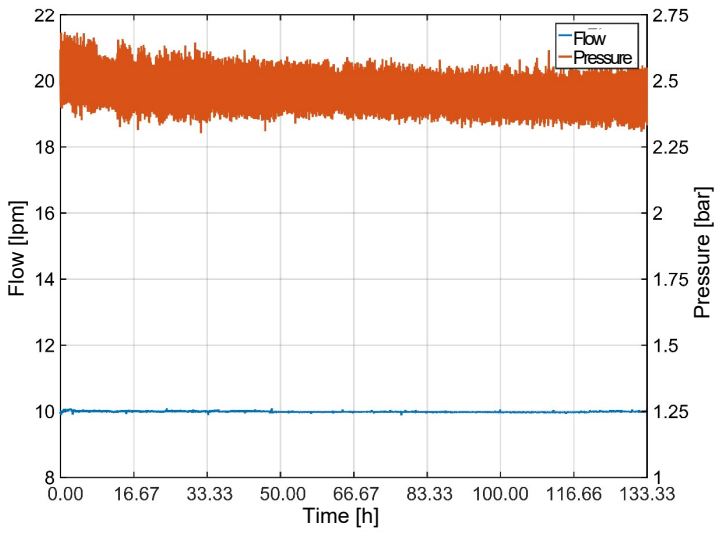
10 lpm; p=2.0 bar 4-piston diaphragm pump				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	3.2	0.7	0.0
2	4	1652.7	292.1	1200.0
4	8	1899.3	157.0	2400.0
6	12	1640.0	135.1	3600.0
7	24	1416.3	117.2	7200.0
9	48	1416.3	117.2	14400.0
11	96	4484.0	1264.7	28800.0
13	133.33	4582.3	378.7	40000.0
10 lpm; p=2.0 bar PuraLev® 600SU				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	0.6	0.3	0.0
2	4	2.5	0.9	1200.0
4	8	2.9	0.7	2400.0
6	12	3.3	0.5	3600.0
7	24	2.5	0.9	7200.0
9	48	3.3	0.5	14400.0
11	96	3.6	0.0	28800.0
13	133.33	3.3	0.3	40000.0

Data of enzyme activity measurements

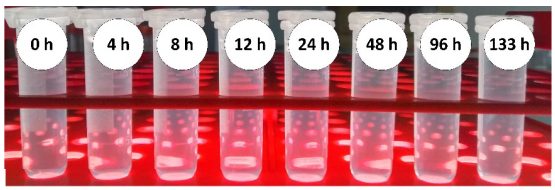
	PuraLev® 600SU				4-piston diaphragm pump				Buffer
Time[h]	Number of cycles [-]	Slope A_{450}/min	Abs. activity [10^3 U/mL]	rel. activity [%]	Number of cycles [-]	Slope A_{450}/min	Abs. activity [10^3 U/mL]	rel. activity [%]	Slope A_{450}/min
0	0.0	0.0204	20.2	100.0%	0.0	0.0202	20.0	100.0%	0.000188
2	600.0	0.0194	19.2	94.9%	600.0	0.0188	18.6	93.2%	0.000188
4	1200.0	0.0195	19.3	95.6%	1200.0	0.0182	18.0	90.2%	0.000188
6	1800.0	0.0193	19.1	94.8%	1800.0	0.0170	16.9	84.3%	0.000188
8	2400.0	0.0196	19.4	95.9%	2400.0	0.0156	15.5	77.2%	0.000188
10	3000.0	0.0201	19.9	98.4%	3000.0	0.0138	13.6	68.0%	0.000188
12	3600.0	0.0196	19.4	96.2%	3600.0	0.0129	12.7	63.4%	0.000188
24	7200.0	0.0193	19.1	94.5%	7200.0	0.0079	7.8	38.7%	0.000188
36	10800.0	0.0200	19.9	98.3%	10800.0	0.0009	0.7	3.5%	0.000188
48	14400.0	0.0198	19.6	97.2%	14400.0	0.0001	-0.1	0.0%	0.000188
72	21600.0	0.0197	19.5	96.4%	21600.0	0.0001	-0.1	0.0%	0.000188
96	28800.0	0.0214	21.2	104.9%	28800.0	0.0003	0.1	0.4%	0.000188
120	36000.0	0.0215	21.3	105.4%	36000.0	0.0005	0.3	1.7%	0.000188
133.33	40000.0	0.0199	19.7	97.4%	40000.0	0.0003	0.1	0.7%	0.000188

Raw data P3.1/P3.2 (10 lpm, 2.5 bar)

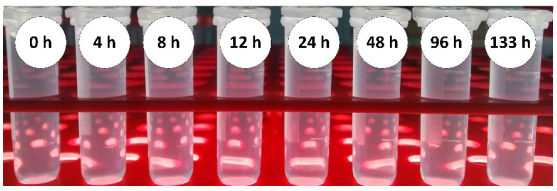




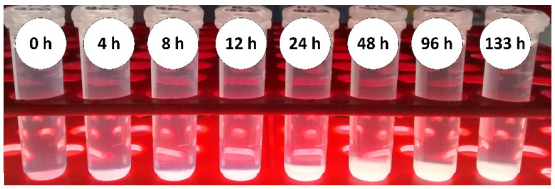
Samples PuraLev® 600SU sedimented



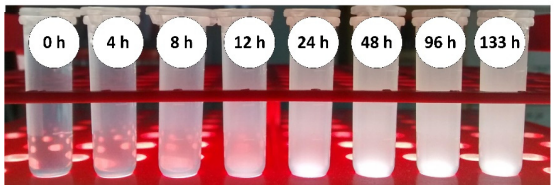
Samples PuraLev® 600SU suspended



Samples 4-piston diaphragm pump sedimented



Samples 4-piston diaphragm pump suspended



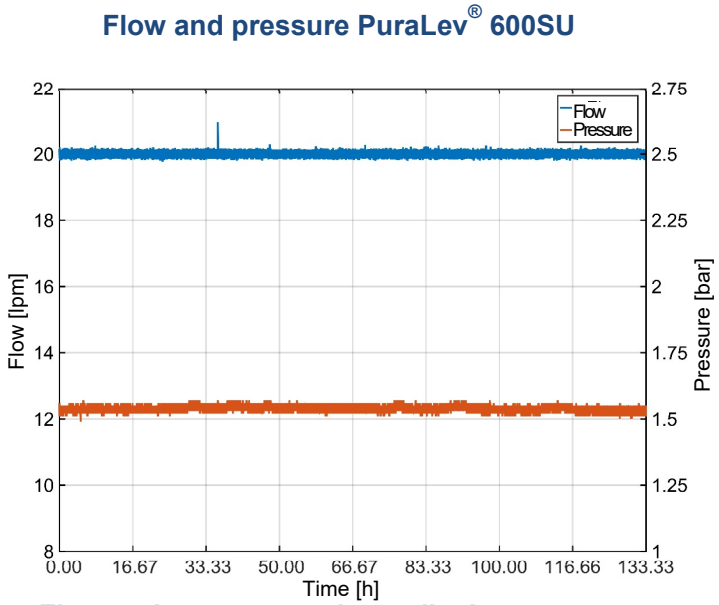
Measuring data of DLS method

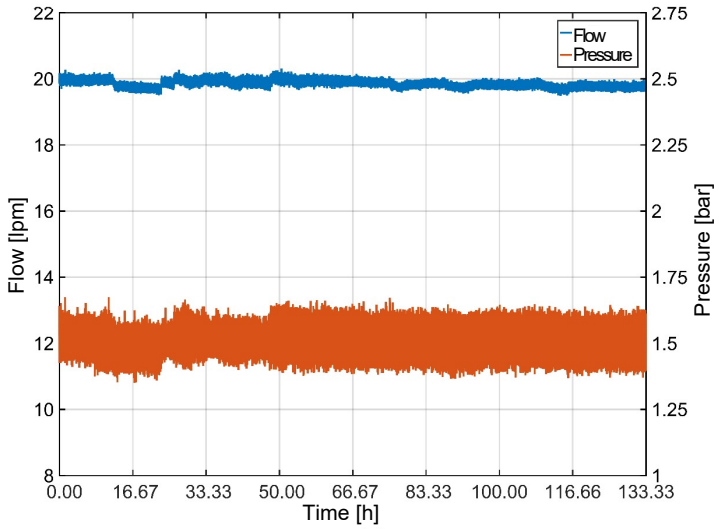
10 lpm; p=2.5 bar 4-piston diaphragm pump				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	3.3	0.3	0.0
2	4	2829.0	453.8	1200.0
4	8	1808.7	157.0	2400.0
6	12	2321.3	339.8	3600.0
7	24	2724.0	635.7	7200.0
9	48	5054.0	438.2	14400.0
11	96	3815.0	1640.2	28800.0
13	133.33	4801.0	0.0	40000.0
10 lpm; p=2.5 bar PuraLev® 600SU				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	2.2	1.7	0.0
2	4	2.7	0.8	1200.0
4	8	3.6	0.0	2400.0
6	12	3.5	0.3	3600.0
7	24	3.5	0.3	7200.0
9	48	3.6	0.0	14400.0
11	96	3.5	0.3	28800.0
13	133.33	3.6	0.0	40000.0

Data of enzyme activity measurements

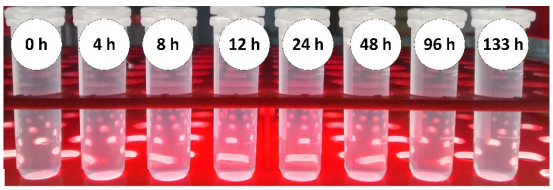
	PuraLev® 600SU				4-piston diaphragm pump				Buffer
	Number of cycles	Slope	Abs. activity	rel. activity	Number of cycles	Slope	Abs. activity	rel. activity	Slope
Time[h]	[-]	A_{450}/min	[10^3 U/mL]	[%]	[-]	A_{450}/min	[10^3 U/mL]	[%]	A_{450}/min
0	0.0	0.0258	25.7	100.0%	0.0	0.0253	25.1	100.0%	0.000144
2	600.0	0.0250	24.8	96.8%	600.0	0.0243	24.1	96.1%	0.000144
4	1200.0	0.0257	25.6	99.5%	1200.0	0.0235	23.4	93.0%	0.000144
6	1800.0	0.0251	24.9	97.0%	1800.0	0.0225	22.4	89.1%	0.000144
8	2400.0	0.0255	25.4	98.9%	2400.0	0.0216	21.4	85.3%	0.000144
10	3000.0	0.0259	25.8	100.5%	3000.0	0.0203	20.1	80.2%	0.000144
12	3600.0	0.0254	25.3	98.6%	3600.0	0.0192	19.1	75.9%	0.000144
24	7200.0	0.0256	25.5	99.3%	7200.0	0.0072	7.0	28.0%	0.000144
36	10800.0	0.0263	26.1	101.8%	10800.0	0.0001	0.0	0.0%	0.000144
48	14400.0	0.0256	25.5	99.2%	14400.0	0.0001	-0.1	0.0%	0.000144
72	21600.0	0.0262	26.0	101.3%	21600.0	0.0003	0.2	0.6%	0.000144
96	28800.0	0.0258	25.7	100.0%	28800.0	0.0000	-0.1	0.0%	0.000144
120	36000.0	0.0260	25.9	100.9%	36000.0	0.0000	-0.1	0.0%	0.000144
133.33	40000.0	0.0262	26.1	101.5%	40000.0	0.0000	-0.1	0.0%	0.000144

Raw data P4.1/P4.2 (20 lpm, 1.5 bar)

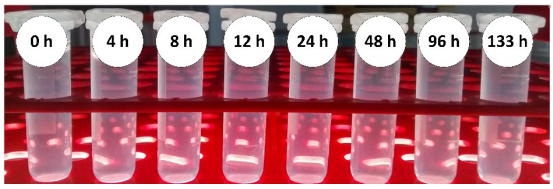




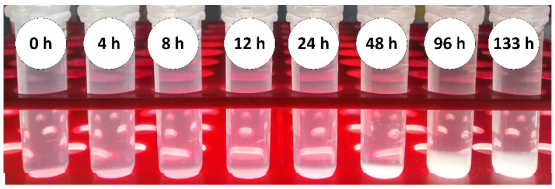
Samples PuraLev® 600SU sedimented



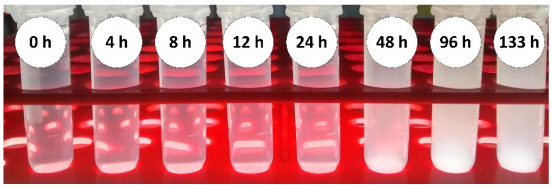
Samples PuraLev® 600SU suspended



Samples 4-piston diaphragm pump sedimented



Samples 4-piston diaphragm pump suspended



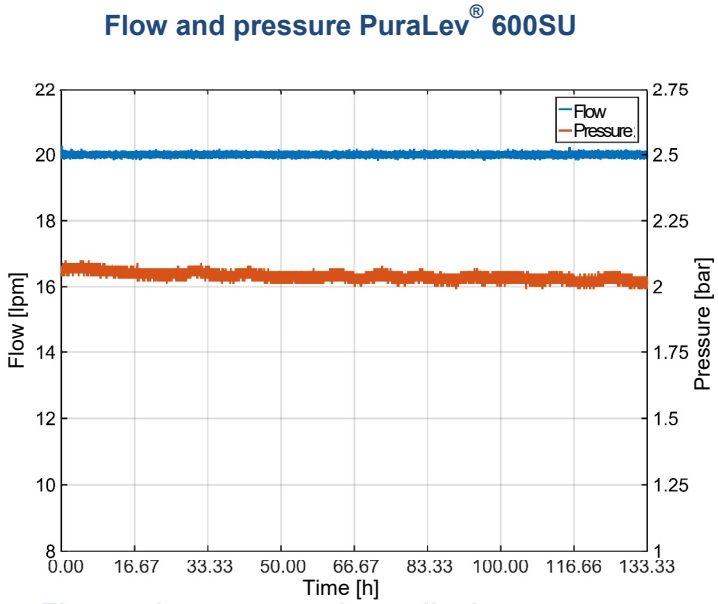
Measuring data of DLS method

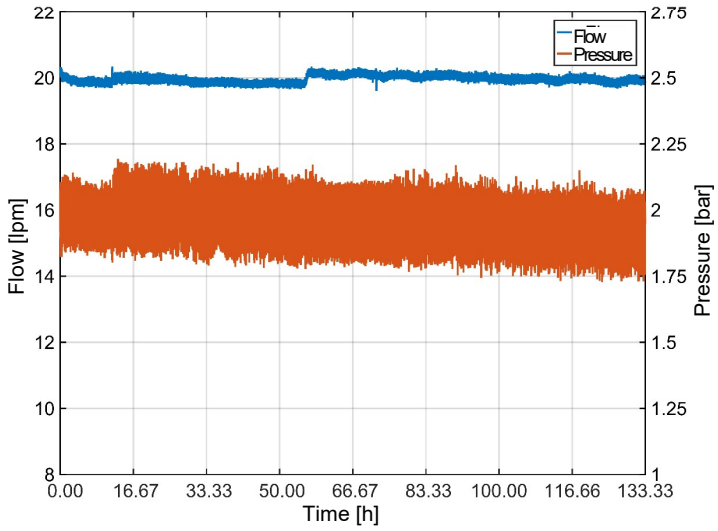
20 lpm; p=1.5 bar 4-piston diaphragm pump				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	3.5	0.3	0.0
2	4	842.0	196.5	1200.0
4	8	1063.9	188.0	2400.0
6	12	918.7	162.2	3600.0
7	24	1348.7	117.2	7200.0
9	48	1484.0	0.0	14400.0
11	96	4900.0	1143.2	28800.0
13	133.33	5560.0	0.0	40000.0
20 lpm; p=1.5 bar PuraLev® 600SU				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	3.1	0.0	0.0
2	4	2.2	1.0	1200.0
4	8	3.1	0.0	2400.0
6	12	3.1	0.0	3600.0
7	24	3.0	0.2	7200.0
9	48	2.7	0.0	14400.0
11	96	3.2	0.7	28800.0
13	133.33	3.1	0.5	40000.0

Data of enzyme activity measurements

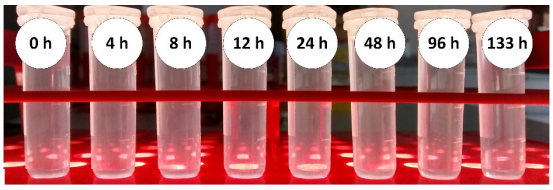
	PuraLev® 600SU				4-piston diaphragm pump				Buffer
	Number of cycles	Slope	Abs. activity	rel. activity	Number of cycles	Slope	Abs. activity	rel. activity	Slope
Time[h]	[-]	A_{450}/min	[10^3 U/mL]	[%]	[-]	A_{450}/min	[10^3 U/mL]	[%]	A_{450}/min
0	0.0	0.0264	26.4	100.0%	0.0	0.0262	26.1	100.0%	0.000075
2	600.0	0.0269	26.8	101.7%	600.0	0.0254	25.3	96.7%	0.000075
4	1200.0	0.0265	26.4	100.2%	1200.0	0.0251	25.0	95.7%	0.000075
6	1800.0	0.0258	25.7	97.7%	1800.0	0.0249	24.8	94.9%	0.000075
8	2400.0	0.0259	25.8	97.9%	2400.0	0.0255	25.4	97.2%	0.000075
10	3000.0	0.0257	25.6	97.2%	3000.0	0.0248	24.7	94.7%	0.000075
12	3600.0	0.0261	26.0	98.8%	3600.0	0.0245	24.5	93.6%	0.000075
24	7200.0	0.0258	25.8	97.7%	7200.0	0.0225	22.5	86.0%	0.000075
36	10800.0	0.0259	25.9	98.1%	10800.0	0.0205	20.5	78.3%	0.000075
48	14400.0	0.0260	25.9	98.3%	14400.0	0.0197	19.6	75.0%	0.000075
72	21600.0	0.0260	25.9	98.2%	21600.0	0.0014	1.3	5.0%	0.000075
96	28800.0	0.0265	26.4	100.3%	28800.0	0.0005	0.5	1.8%	0.000075
120	36000.0	0.0268	26.7	101.3%	36000.0	0.0002	0.2	0.6%	0.000075
133.33	40000.0	0.0263	26.2	99.3%	40000.0	0.0001	0.1	0.2%	0.000075

Raw data P5.1/P5.2 (20 lpm, 2.0 bar)

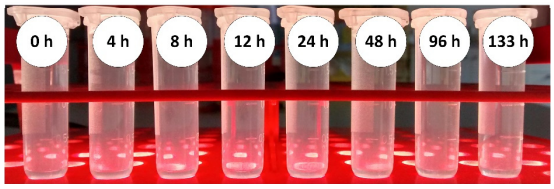




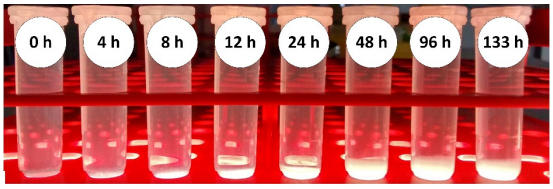
Samples PuraLev® 600SU sedimented



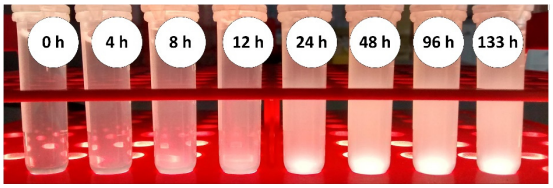
Samples PuraLev® 600SU suspended



Samples 4-piston diaphragm pump sedimented



Samples 4-piston diaphragm pump suspended



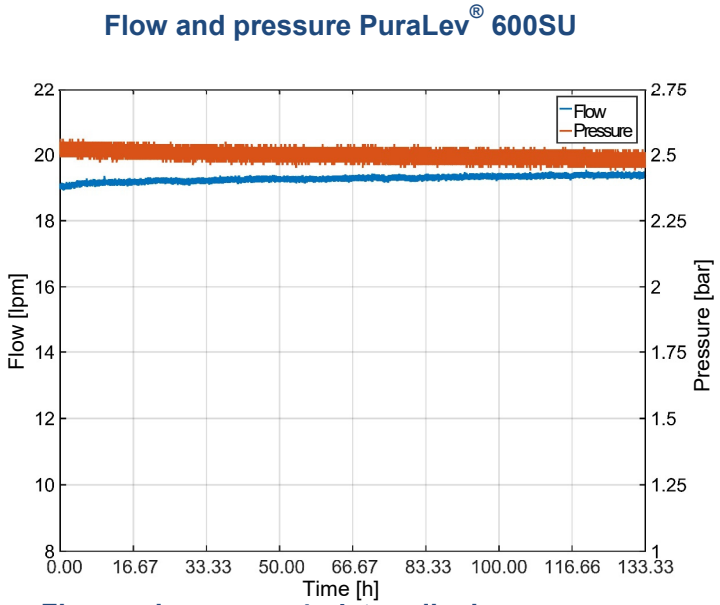
Measuring data of DLS method

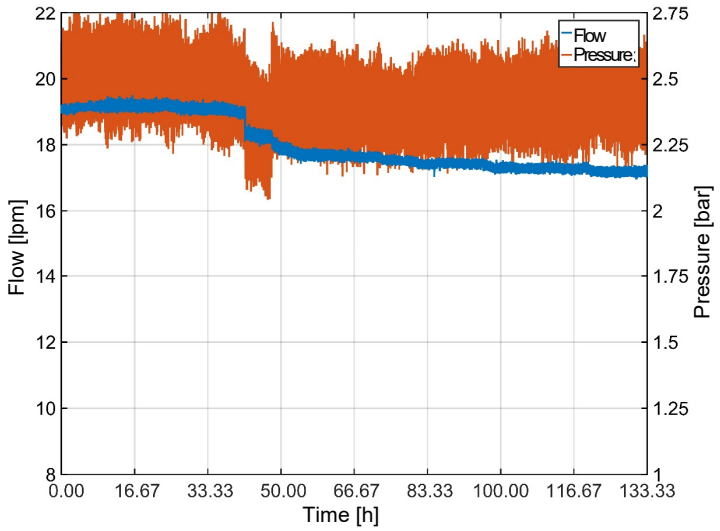
20 lpm; p=2.0 bar 4-piston diaphragm pump				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	3.1	0.0	0.0
2	4	2352.0	549.1	1200.0
4	8	2950.3	243.6	2400.0
6	12	3254.0	282.3	3600.0
7	24	3956.7	326.2	7200.0
9	48	4582.3	378.7	14400.0
11	96	5560.0	0.0	28800.0
13	133.33	5560.0	0.0	40000.0
20 lpm; p=2.0 bar PuraLev® 600SU				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	2.4	1.3	0.0
2	4	2.1	1.3	1200.0
4	8	2.6	1.7	2400.0
6	12	3.0	0.6	3600.0
7	24	2.4	1.2	7200.0
9	48	3.1	0.5	14400.0
11	96	2.6	0.9	28800.0
13	133.33	1.9	1.5	40000.0

Data of enzyme activity measurements

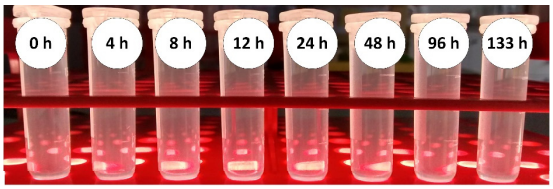
	PuraLev® 600SU				4-piston diaphragm pump				Buffer
	Number of cycles	Slope	Abs. activity	rel. activity	Number of cycles	Slope	Abs. activity	rel. activity	Slope
Time[h]	[-]	A_{450}/min	[10^3 U/mL]	[%]	[-]	A_{450}/min	[10^3 U/mL]	[%]	A_{450}/min
0	0.0	0.0260	25.9	100.0%	0.0	0.0271	27.0	100.0%	0.000143
2	600.0	0.0262	26.0	100.6%	600.0	0.0255	25.4	94.2%	0.000143
4	1200.0	0.0261	26.0	100.3%	1200.0	0.0259	25.8	95.7%	0.000143
6	1800.0	0.0266	26.5	102.3%	1800.0	0.0253	25.1	93.3%	0.000143
8	2400.0	0.0268	26.7	103.0%	2400.0	0.0240	23.9	88.7%	0.000143
10	3000.0	0.0266	26.4	102.2%	3000.0	0.0228	22.7	84.2%	0.000143
12	3600.0	0.0266	26.5	102.4%	3600.0	0.0223	22.1	82.1%	0.000143
24	7200.0	0.0259	25.8	99.6%	7200.0	0.0143	14.1	52.4%	0.000143
36	10800.0	0.0271	26.9	104.0%	10800.0	0.0019	1.8	6.7%	0.000143
48	14400.0	0.0266	26.5	102.4%	14400.0	0.0002	0.1	0.3%	0.000143
72	21600.0	0.0265	26.3	101.8%	21600.0	0.0001	0.0	0.0%	0.000143
96	28800.0	0.0266	26.4	102.1%	28800.0	0.0001	-0.1	0.0%	0.000143
120	36000.0	0.0264	26.2	101.3%	36000.0	0.0002	0.1	0.2%	0.000143
133.33	40000.0	0.0265	26.3	101.7%	40000.0	0.0002	0.0	0.0%	0.000143

Raw data P6.1/P6.2 (19 lpm, 2.5 bar)

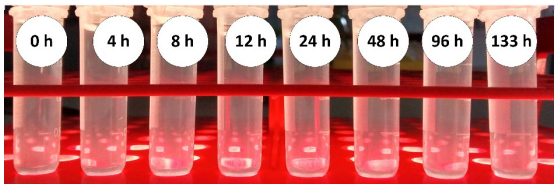




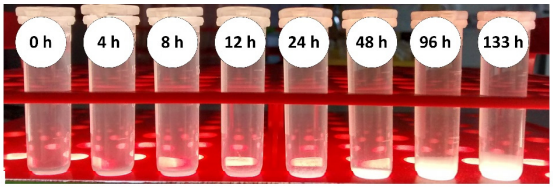
Samples PuraLev® 600SU sedimented



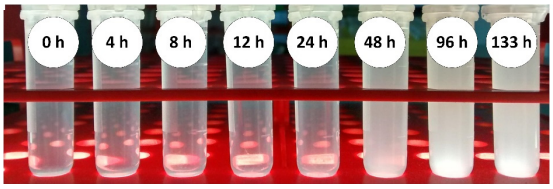
Samples PuraLev® 600SU suspended



Samples 4-piston diaphragm pump sedimented



Samples 4-piston diaphragm pump suspended



Measuring data of DLS method

19 lpm; p=2.5 bar 4-piston diaphragm pump				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	3.3	0.3	0.0
2	4	2.9	0.8	1200.0
4	8	1416.3	117.2	2400.0
6	12	1416.3	117.2	3600.0
7	24	1730.7	253.2	7200.0
9	48	3113.3	455.9	14400.0
11	96	5307.0	438.2	28800.0
13	133.33	5088.3	817.0	40000.0
19 lpm; p=2.5 bar PuraLev® 600SU				
Sample	Time [h]	Average Vol. [nm]	STABW Vol. [nm]	Pump cycles
0	0	3.1	0.0	0.0
2	4	2.9	0.5	1200.0
4	8	3.3	0.3	2400.0
6	12	3.6	0.0	3600.0
7	24	3.1	0.0	7200.0
9	48	3.1	0.0	14400.0
11	96	3.5	0.3	28800.0
13	133.33	3.3	0.3	40000.0

Data of enzyme activity measurements

	PuraLev® 600SU				4-piston diaphragm pump				Buffer
	Number of cycles	Slope	Abs. activity	rel. activity	Number of cycles	Slope	Abs. activity	rel. activity	Slope
Time[h]	[-]	A_{450}/min	[10^3 U/mL]	[%]	[-]	A_{450}/min	[10^3 U/mL]	[%]	A_{450}/min
0	0.0	0.0265	26.4	100.0%	0.0	0.0277	27.5	100.0%	0.000143
2	600.0	0.0263	26.2	99.3%	600.0	0.0273	27.1	98.6%	0.000143
4	1200.0	0.0257	25.5	96.7%	1200.0	0.0261	26.0	94.5%	0.000143
6	1800.0	0.0261	26.0	98.5%	1800.0	0.0257	25.5	92.8%	0.000143
8	2400.0	0.0262	26.1	98.8%	2400.0	0.0259	25.8	93.7%	0.000143
10	3000.0	0.0263	26.2	99.2%	3000.0	0.0255	25.3	92.0%	0.000143
12	3600.0	0.0261	25.9	98.2%	3600.0	0.0254	25.2	91.8%	0.000143
24	7200.0	0.0259	25.8	97.6%	7200.0	0.0254	25.2	91.7%	0.000143
36	10800.0	0.0260	25.9	98.1%	10800.0	0.0242	24.0	87.3%	0.000143
48	14400.0	0.0266	26.5	100.4%	14400.0	0.0195	19.4	70.4%	0.000143
72	21600.0	0.0260	25.8	97.8%	21600.0	0.0008	0.7	2.5%	0.000143
96	28800.0	0.0263	26.1	99.1%	28800.0	0.0002	0.0	0.1%	0.000143
120	36000.0	0.0277	27.5	104.4%	36000.0	0.0000	-0.1	0.0%	0.000143
133.33	40000.0	0.0266	26.5	100.4%	40000.0	0.0002	0.0	0.1%	0.000143

SOP - Enzyme activity measurement of lysozyme

Life Sciences und Facility Management	Enzymaktivitätsbestimmung von Lysozym	**SOP**
	Bioverfahrens- und Zellkulturtechnik	

Titel	**Enzymaktivitätsbestimmung von Lysozym**
Objekt/ Projekt	KTI-Projekt Levitronix
Zweck	Zweck dieser Standard-Arbeitsanweisung ist die Regelung des allgemeinen Vorgehens zur Bestimmung der Enzymaktivität von Lysozym.
Geltungsbereich	Diese SOP gilt in allen Laboren der Gruppe Bioverfahrenstechnik.
Status	Version 01
Mitgeltende Unterlagen	Bedienungsanleitung Ultrospec 3000 pro Assay-Vorschrift von Sigma

-	Erstellung	Prüfung	Freigabe	Ersetzt	Version
Name	K. Blaschczok				01
Datum					
Visum					Seite 1 von 9

Inhalt

1 Definition

Um Schädigungen von Proteinen durch Scherstress zu evaluieren, wird die Enzymaktivität des Modellproteins Lysozym bestimmt. Dazu wird mittels Photometer die Kinetik der Bakteriolyse aufgenommen, wobei die Abnahme der Extinktion einer *Micrococcus lysodeikticus*-Suspension bei 450 nm gemessen wird.

2 Materialien

- Photometer Ultrospec 3000 pro
- Temperierbarer Küvettenhalter für Ultrospec 3000 pro
- Feinwaage
- Magnetrührer und Magnetrührstab
- pH-Meter

- Mikroliterpipetten 5000 µL, 1000 µL, 200 µL, Spitzen
- Schottflaschen 100 mL, 500 mL
- Messkolben 100 mL, 500 mL
- Pasteurpipetten, disp.
- Eppendorf-Tubes 2 mL
- Wägeschalen
- Spatel
- Makroküvetten, disp.

- KOH
- Kaliumdihydrogenphosphat, monobasisch, wasserfrei (Sigma, P5379)
- *Micrococcus lysodeikticus*, lyophilisiert (Sigma, M3770)
- Lysozym (Sigma, L6876)

3 Durchführung

3.1 Herstellung der Lösungen

Phosphatpuffer:
66 mM Kaliumdihydrogenphosphat, pH 6.24 (einstellen mit 1 M KOH) bei 25 °C

Substrat:
0.015 % (w/v) *Micrococcus lysodeikticus*-Suspension, in Phosphatpuffer

Enzym:
200-400 U/mL Lysozym, in kaltem Phosphatpuffer

3.2 Einschalten und Vorbereiten des Photometers

a) Falls noch nicht installiert: Einbau des temperierbaren Peltier-Küvettenhalters.
b) Einschalten von Photometer und Drucker am Kippschalter.

c) Gerät führt Selbsttest durch (ca. 15 min). Akzeptieren der Resultate mit „enter". Resultate werden bei eingeschaltetem Drucker automatisch gedruckt.
d) Einschalten der Temperaturregelung mit der Taste „function", Registerkarte „Accessory", 25 eingeben, Häkchen setzen, „enter"
e) mit „stop" zurück ins Hauptmenü

3.3 Extinktionsmessung von Puffer und Substrat

a) Registerkarte „Basic", „enter"
b) Registerkarte „Absorbance"
c) Taste „wave λ", 450 eingeben, „enter"
d) Küvette mit 2.5 mL Puffer füllen, im Küvettenhalter platzieren (Strahlengang beachten)
e) warten bis Temperatur = 25 °C erreicht ist (Symbol in der unteren Leiste des Displays zeigt „<25" oder „>25" an, wenn Temperatur noch nicht erreicht ist)
f) Taste „set ref" drücken, Drucker druckt automatisch
g) Zweite Küvette mit 2.5 mL Substrat füllen, warten bis Temperatur erreicht ist
h) Taste „sample" drücken, Sample Number eingeben, „enter", „print"
i) „stop" drücken, um Menü zu verlassen
j) Der A_{450}-Wert des Substrates gegen Puffer sollte 0.6-0.7 sein.

3.4 Probenvorbereitung

a) Proben mit Puffer verdünnen, sodass 200-400 U/mL vorliegen
b) Substrat, Puffer und (verdünnte) Proben im Wasserbad auf 25 °C temperieren

3.5 Aufnahme der Kinetik

a) Registerkarte „Applications", „enter"
b) Registerkarte „Kinetics", „enter", Setup-Fenster öffnet sich
c) folgende Parameter einstellen:
   Wavelength: 450
   Factor: 1
   Units: –
   Auto set ref: –
d) mit Pfeiltasten zur nächsten Registerkarte wechseln, folgende Parameter einstellen:
   Mode: Serial
   Units: Seconds
   Delay: 0
   Reaction: 300
   Interval: 10
e) In 2 Küvetten je 2.5 mL Substrat pipettieren
f) Blank:
   - eine Küvette im Photometer platzieren, auf 25 °C temperieren
   - 0.1 mL Puffer zugeben, zügig Mischen durch Schwenken oder Enzymspatel, wieder im Küvettenhalter platzieren, Messung sofort starten

- A_{450} während 5 min aufzeichnen
- Werte bei der Messung notieren oder später manuell aus dem gedruckten Diagramm auslesen

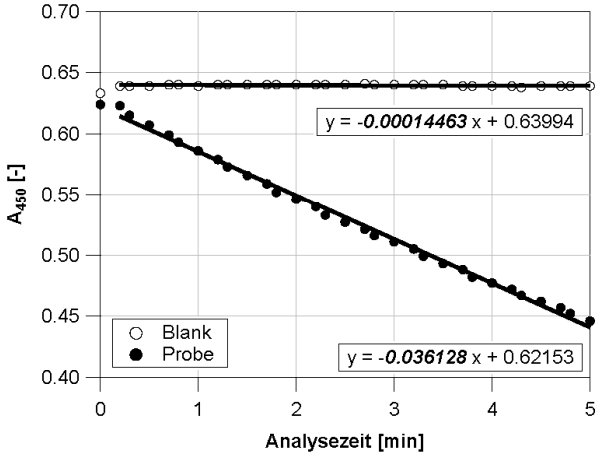
g) Probe:
- zweite Küvette im Photometer platzieren, auf 25 °C temperieren
- 0.1 mL Lysozym zugeben, zügig Mischen durch Schwenken oder Enzymspatel, wieder im Küvettenhalter platzieren, Messung sofort starten
- A_{450} während 5 min aufzeichnen
- Werte bei der Messung notieren oder später manuell aus dem gedruckten Diagramm auslesen

3.6 Auswertung

a) es werden Daten in folgender Form erhalten:

Analysezeit		A_{450} [-]	
[s]	[min]	Blank	Probe
0	0.0	0.633	0.624
10	0.2	0.639	0.623
20	0.3	0.639	0.615
30	0.5	0.639	0.607
40	0.7	0.640	0.599
50	0.8	0.640	0.593
60	1.0	0.639	0.586
70	1.2	0.640	0.579
80	1.3	0.640	0.573
90	1.5	0.640	0.566
100	1.7	0.640	0.559
110	1.8	0.640	0.552
120	2.0	0.640	0.546
130	2.2	0.640	0.540
140	2.3	0.640	0.533
150	2.5	0.640	0.527
160	2.7	0.641	0.521
170	2.8	0.640	0.516
180	3.0	0.640	0.511
190	3.2	0.640	0.505
200	3.3	0.640	0.499
210	3.5	0.640	0.493
220	3.7	0.639	0.488
230	3.8	0.639	0.482
240	4.0	0.639	0.477
250	4.2	0.639	0.472
260	4.3	0.638	0.467
270	4.5	0.639	0.462
280	4.7	0.639	0.457
290	4.8	0.639	0.452
300	5.0	0.639	0.446

b) graphische Darstellung der Absorption gegen die Zeit [min]:



c) maximale Abnahmerate ΔA450/min (Anstieg) für Blank und Probe bestimmen (mind. über 1 min und 4 Datenpunkte)

d) Berechnung der Aktivität [U/mL]:

$$U/mL = \frac{[(\Delta A_{450}/min)_{\text{Test}} - (\Delta A_{450}/min)_{\text{Blank}}] \cdot D}{0.001 \cdot 0.1}$$

D ... Verdünnungsfaktor

0.001 ... Absorptionsänderung bei 450 nm laut Unit-Definition

0.1 ... Volumen (mL) Enzym

Unit-Definition:

1 U entspricht einem ΔA_{450} von 0.001 pro Minute bei pH 6.24 und 25 °C in 2.6 mL Reaktionsvolumen bei Verwendung einer *Micrococcus lysodeikticus* Suspension als Substrat

4 Mitgeltende Unterlagen

Bedienungsanleitung Ultrospec 3000 pro

Sigma Anleitung „Enzymatic Assay of LYSOZYME"

5 Änderungshinweise

Diese SOP wurde neu erstellt.

6 Anhang

SIGMA

ProductInformation

SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of LYSOZYME[1]
(EC 3.2.1.17)

PRINCIPLE:

Micrococcus lysodeikticus Cells (Intact) $\xrightarrow{\text{Lysozyme}}$ Micrococcus lysodeikticus Cells (Lysed)

CONDITIONS: T = 25°C, pH = 6.24, A_{450nm}, Light path = 1 cm

METHOD: Turbidimetric Rate Determination

REAGENTS:

A. 66 mM Potassium Phosphate Buffer, pH 6.24 at 25°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 6.24 at 25°C with 1 M KOH.)

B. 0.015% (w/v) Micrococcus lysodeikticus Cell Suspension (Substrate)
(Prepare 25 ml in Reagent A using Micrococcus lysodeikticus, ATCC 4698 lyophilized cells, Sigma Prod. No. M-3770. The A_{450nm} of this suspension should be between 0.6 and 0.7.)

C. Lysozyme Enzyme Solution
(Immediately before use, prepare a solution containing 200 - 400 units/ml of lysozyme in cold Reagent A.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable cuvettes:

	Test	Blank
Reagent B (Substrate)	2.50	2.50

Equilibrate to 25°C. Monitor the A_{450nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent C (Enzyme Solution)	0.10	------
Reagent A (Buffer)	------	0.10

L6876
SPMICR04
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Enzymatic Assay of LYSOZYME[1]
(EC 3.2.1.17)

PROCEDURE: (continued)

Immediately mix by inversion and record the decrease in A_{450nm} for approximately 5 minutes. Obtain the ΔA_{450nm}/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{450nm}/\text{min Test} - \Delta A_{450nm}/\text{min Blank})(df)}{(0.001)\,(0.1)}$$

df = Dilution factor
0.001 = Change in absorbance at A_{450nm} as per the Unit Definition
0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will produce a ΔA_{450nm} of 0.001 per minute at pH 6.24 at 25°C using a suspension of Micrococcus lysodeikticus as substrate, in a 2.6 ml reaction mixture.

FINAL ASSAY CONCENTRATION:

In a 2.60 ml reaction mix, the final concentrations are 66 mM potassium phosphate, 0.014% (w/v) Micrococcus lysodeikticus cell suspension and 20 - 40 units lysozyme.

REFERENCE:

Shugar, D. (1952) *Biochimica et Biophysica Acta* **8**, 302-309

NOTES:

1. This assay procedure is not to be used to assay Lysozyme, Bovine Recombinant Expressed in Pichia pastoris, Sigma Prod. No. L-9772, Lysozyme, Human, Recombinant Expressed in Pichia pastoris, Sigma Prod. No. L-2026, and Lysozyme Insoluble Enzyme on Agarose, Sigma Prod. No. L-1129.

Enzymatic Assay of LYSOZYME[1]
(EC 3.2.1.17)

NOTES: (continued)

2. This assay is based on the cited reference.

3. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

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