Chapter 7
Alternative to Chromatography: Purification of Therapeutic Protein/Peptides by Continuous Crystallization in Millifluidics

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Abstract In the last decade, therapeutic proteins have emerged as an important class of pharmaceuticals due to their excellent target-specificity and low-toxicity profile, making them better equipped to treat certain diseases (e.g., cancer, enzyme deficiency, degenerative diseases) toward which conventional small-molecule drugs are not as effective. Recently, rapid technological advancements in protein/peptide synthesis have enabled pharmaceutical industries to increase production by more than 20-fold. However, this multifold increase in the upstream production capacity has created a bottleneck in the downstream processing, which heavily relies on chromatography. In this project, we aim to replace chromatography with continuous millifluidic crystallization as a more effective and efficient alternative to purify therapeutic protein/peptides in order to reduce the current stress in downstream processing.

Keywords Aspect ratio · Batch crystallization · Continuous crystallization · Reynolds number

7.1 Introduction

7.1.1 Background

I. Therapeutic proteins

Therapeutic proteins are proteins engineered in the laboratory for pharmaceutical use. They are highly effective in vivo and have revolutionized the treatment of diseases. Protein therapeutics permit an individualized treatment approach by supporting a specifically targeted therapeutic process through compensating the deficiency of an essential protein [1].

II. Continuous millifluidic crystallization

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Continuous millifluidic crystallization can potentially provide improved control of crystal properties, improved process reproducibility, and reduced scale-up risk. Liquid and gas are introduced into one end of the tube at flow rates selected to spontaneously generate alternating slugs of liquid and gas that remain stable while cooling crystallization occurs in each liquid slug. Mixing within each stable self-circulating slug is maximized by controlling the slug aspect ratio through specification of liquid and gas flow rates [2].

7.1.2 Research Question and Objective

I. Research Question

How does aspect ratio affect the quality of lysozyme crystal products? In this experiment, the quality of crystals is taken to be their mass, length, and activity.

II. Research Objective

This project will study the effect of aspect ratio on lysozyme crystal products' quality. By tuning the air introducing pump (tuning injection interval to control water slug length, thus, water slug aspect ratio; tuning injection volume to control bubble length, thus, air slug aspect ratio), effects of different slug and bubble lengths on quality of crystal products can be investigated.

7.1.3 Literature Review

Aspects of continuous crystallization

I. Aspect ratio (AR)

The aspect ratio of the slug, referred to as the slug size, is the ratio between the length of slugs and the inner diameter of the tubing. Based on properly selected co-current flow rates of gas and liquid, stable alternating slugs of liquid and gas are spontaneously generated at aspect ratios close to 1, facilitating good mixing for enhanced heat and mass transport without mixing blades.

II. Slug flow

Slug flow in liquid–gas two-phase flow is a type of flow pattern. The slug usually refers to the heavier fluid, in this case the liquid, but it can also refer to the lighter fluid, in this case the gas.

Comparison between batch crystallization and continuous crystallization

Continuous crystallization results in less equipment footprint than batch crystallization, reducing + 20% in capital expenditure. As for process variability, batch

crystallization displays comparably significant batch-to-batch variability regarding physical properties, making continuous crystallization more reproducible and reliable. Thus, continuous crystallization could be more feasible in the large-scale downstream processing of therapeutic proteins. However, continuous crystallization is not as well-researched as batch crystallization. Despite batch processes usually having higher yields in a once-through system, it is possible to achieve higher yields from continuous processes with appropriate recycling strategies. For material traceability, the tracing material process in batch processes is well understood from both an operational and a regulatory perspective while there is still a gap between both perspectives for a continuous process [3].

7.2 Materials and Methods

7.2.1 Materials

The chemicals used for our experiment include: Distilled water, Sodium acetate, Acetic acid, Hydrochloric acid, Sodium hydroxide, Lysozyme, Sodium chloride, Liquid nitrogen, 0.1 M Potassium phosphate, pH 7.0, Micrococcus lysodeikticus cells.

The apparatus used for our experiment include: Culture tubes, Centrifuge tubes, Mini Centrifuge tubes, Magnetic stirrer, Vortex machine, Centrifuge, Freeze dry machine, liquid nitrogen, Vacuum filter, Syringe filter, Pipette, Micropipette, UV-mini Spectrophotometer, UV-Visible Spectrophotometer, Weighing machine, Microscope.

7.2.2 Preparation of Buffer Solution

To obtain 0.5 L of buffer solution (pH 4.6), add 2.029 g of Sodium Acetate and 1.517 g of Acetic Acid to 400 mL of distilled water. Adjust solution to pH 4.6 using HCl or NaOH. Add distilled water until volume is 0.5 L.

7.2.3 Preparation of Lysozyme Solution

To obtain lysozyme solution, add a 1:1 ratio of lysozyme-buffer solution to NaCl solution in each test tube. The volume of lysozyme solution in each tube is dependent on the liquid aspect ratios (Table 7.1).

Table 7.1 Parameters of experiment

Inner diameter of tube (cm)	Aspect ratio	Total height of solution (cm)	Liquid volume (ml)	Lysozyme solution volume (ml)
1.15	0.25	4.60	4.64	2.32
1.15	0.5	2.30	2.35	1.18
1.15	1	1.15	1.16	0.58
1.15	2	0.58	0.46	0.23
1.15	3	0.38	0.36	0.18
1.15	4	0.29	0.16	0.08
1.15	5	0.23	0.10	0.05
1.15	6	0.19	0.06	0.03

7.2.4 Crystallization

The culture tubes were placed on 3 separate magnetic stirrers with stirring rates (SR) of 0 rpm, 100 rpm, and 250 rpm, respectively, for 24 h as crystallization took place. For every AR, two sample tubes were set up for each SR. After 24 h, the culture tubes were removed from the stirrers.

7.2.5 Freeze Drying

Transfer a fixed volume of lysozyme solution from each culture tube into small centrifuge tubes and centrifuge the tubes for 10 min. Separate the liquid from the solid crystals. Freeze the solid crystals in liquid nitrogen then freeze dry in the freeze dry machine for 24 h. After 24 h, remove the centrifuge tubes containing the freeze dried crystals from the freeze dry machine (Table 7.2).

7.2.6 Spectrophotometric Analysis

Add 1.98 mL of buffer solution to a cuvette and use that as a blank. Then add 0.02 mL of liquid from centrifuged lysozyme solution and mix well to obtain readings from the spectrophotometer.

Table 7.2 Volume of lysozyme solution (mL), total volume of crystal solution (mL) and volume of crystal solution transferred to small centrifuge tubes (mL) for each aspect ratio

Aspect ratio	Lysozyme solution volume (mL)	Liquid volume (mL)	Volume of liquid transferred (mL)
0.25	2.32	4.64	1
0.5	1.175	2.35	1
1	0.58	1.16	0.5
2	0.228	0.456	0.2
3	0.181	0.362	0.2
4	0.079	0.158	0.1
5	0.04875	0.0975	0.05
6	0.02905	0.0581	0.03

7.2.7 Enzyme Assay

2.0 mg of each freeze dried crystal sample was mixed with 2.0 mL of distilled water to prepare lysozyme crystal solutions of 1.0 mg/ml concentration. The solution was then diluted to 0.25 mg/mL by mixing 250 mL of each crystal sample with 750 mL of distilled water. A Micrococcus lysodeikticus cell suspension was prepared by suspending 12 mg of dried Micrococcus lysodeikticus cells in 40 mL of Potassium Phosphate (K_3PO_4) buffer of 0.1 M at pH 7.0, mixed thoroughly. A UV-Visible spectrophotometer was used for enzyme assay, adjusted to a wavelength of 450 nm and temperature of 25 °C. Using a micropipette, 1.9 mL of K_3PO_4 buffer and 1.0 mL of Micrococcus lysodeikticus cell suspension were added into a cuvette and incubated for 4-5 min to achieve temperature equilibration and establish blank rate. Then, 0.1 mL of lysozyme solution of 0.25 mg/mL concentration was added to the cuvette. Using the spectrophotometer, the change in A450/s from the steepest linear portion of the curve was recorded. All mixing was done thoroughly using a vortex machine.

7.2.8 Crystal Analysis

20 μL of the sample was diluted with 100 μL of saturated lysozyme. 10 μL of solution was then transferred to a glass slide and observed under a microscope. Pertinent images of the crystals were then saved onto a computer for further analysis. The images were then analyzed using a software, Image J, allowing us to obtain crystal lengths accurately to find the average lengths.

7.3 Results

See Tables 7.3, 7.4, and 7.5.

Table 7.3 Mass of crystals/g under varying aspect ratio and stirring rate conditions (yield)

	Mass/g								
	Aspect ratio								
Stirring rate/rpm	Sample number	0.25	0.5	1	2	3	4	5	6
0	1	0.023	0.028	0.012	0.0060	0.0031	0.0019	0.0017	0.0021
	2	0.037	0.028	0.011	0.0053	0.0036	0.0010	0.0018	0.00080
	Average mass/g	0.030	0.028	0.011	0.0057	0.0034	0.0015	0.0018	0.0015
100	1	0.041	0.019	0.013	0.0085	0.0058	0.0024	0.0023	0.0010
	2	0.042	0.013	0.0095	0.019	0.0092	0.0035	0.0017	0.0017
	Average mass/g	0.042	0.016	0.011	0.014	0.0075	0.0030	0.0020	0.0014
250	1	0.026	0.039	0.017	0.0016	0.0062	0.00090	0.0036	0.0012
	2	0.044	0.028	0.018	0.015	0.0041	0.0026	0.0038	0.0053
	Average mass/g	0.035	0.033	0.018	0.0084	0.0052	0.0018	0.0037	0.0033

Table 7.4 Average length of crystals under different stirring rates

	Stirring rate/rpm		
	0	100	250
Aspect ratio	Average crystal length/μm	Average crystal length/μm	Average crystal length/μm
0.25	7.39	4.34	6.85
0.5	6.64	4.54	4.17
1	5.52	4.09	4.10
2	4.42	3.69	3.45
3	6.35	3.64	3.98
4	5.22	3.03	3.68
5	6.41	3.48	3.35
6	5.56	2.82	3.61

Table 7.5 Average activity/units ml^{-1} under different stirring rates

	Stirring rate/rpm		
	0	100	250
Aspect ratio	Average crystal activity/units ml^{-1}	Average crystal activity/units ml^{-1}	Average crystal activity/units ml^{-1}
0.25	5864.04	7957.36	8042.64
0.5	9376.08	7920.00	7754.72
1	7256.04	7119.96	7872.00
2	7216.08	8512.80	8103.96
3	8527.92	9439.92	8280.00
4	5464.08	6735.96	7632.00
5	5896.08	8007.96	7708.45
6	6359.00	8391.96	7851.00

7.4 Discussion

Firstly, we will discuss batch crystallization as an introduction to continuous crystallization. In batch crystallization, the flow should neither be too turbulent nor too laminar to maximize crystal yield [4]. Laminar flow is denoted by a low Reynolds number (Re) of below approximately 2300 while turbulent flow is denoted by a high Re of approximately 3000 as seen in Fig. 7.1.

The Reynolds number can be calculated using the formula $\mathrm{Re} = \rho u L/\mu$, where ρ is the density of the fluid (SI units: kg/m^3), u is the flow speed (m/s), L is the diameter

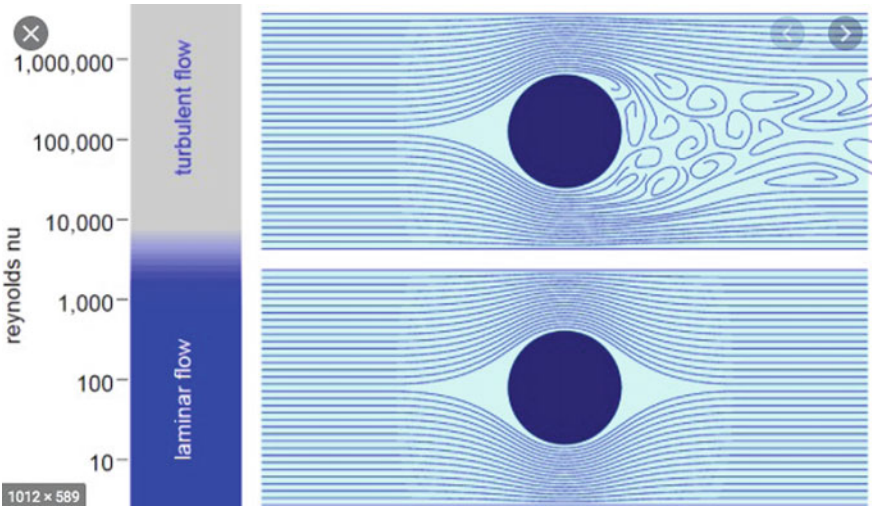

Fig. 7.1 Type of flow corresponding to values of Reynolds number [5]

of the crystallization tube (m) and μ is the dynamic viscosity of the fluid (Pa s). The density of crystal solution used is 1000 kg/m. For continuous crystallization, the flow speed is 0.00252 m/s (3 s.f.) while for batch crystallization at SR 0 rpm, 100 rpm, and 250 rpm, the flow speed is 0 m/s, 0.120 m/s (3 s.f.), and 0.301 m/s (3 s.f.), respectively. The diameter of the continuous crystallization tube is 1.59 mm, while that of the batch crystallization tube is 1.15 cm. The viscosity of the crystal solution is 0.001 Pa·s.

Using these values, the Re of continuous crystallization and batch crystallization at 0 rpm, 100 rpm, and 250 rpm are 6.34 (3 s.f.), 0, 1380 (3 s.f.), and 3460 (3 s.f.), respectively. Based on Fig. 7.1, batch crystallization under SR 0 rpm has a Re of 0 which is extremely laminar, while SR 250 rpm is highly turbulent with a Re of 3460. Among the three SR conditions for batch crystallization, 100 rpm with Re of 1380 is the most suitable for crystallization as it is neither too laminar, nor too turbulent.

Comparing the SRs and ARs under results Table 7.3, the condition with greatest yield in batch crystallization, with greatest average mass of 0.0419 (3 s.f.), is at SR 100 rpm and AR 0.25. Thus, we will compare batch crystallization in this condition with continuous crystallization.

For continuous crystallization, two types of flow ensure the best yield, namely, narrow bore laminar flow and turbulent tubular flow. For a long thin tube less than 1 mm in diameter, laminar flow is most favorable. Since the tube used was 1.59 mm in diameter, narrow bore laminar flow is not favorable. This leads to turbulent tubular flow, which is best for a very long tube, diameter unspecified [6]. Since the tube used is more than 1 mm in diameter, turbulent flow is best. However, according to our Reynolds number for continuous crystallization, the flow is more laminar. Therefore, continuous crystallization does not grant the best yield. Moreover, achieving proper conditions (e.g., tube diameter, flow type) for the best yield proves difficult in continuous crystallization (Fig. 7.2).

Therefore, we can conclude that yield wise, batch crystallization at AR 0.25 and SR 100 rpm is the best condition.

Now, on to the effect of AR on crystals in terms of mass (yield), crystal size (length), and activity for batch crystallization. When comparing the crystal yield against AR, the AR resulting in highest yields at SRs 0, 100, and 250 rpm are AR0.5 at 0.0277 g (3 s.f.), AR0.25 at 0.354 g (3 s.f.) and AR0.25 at 0.0295 g (3 s.f.), respectively. While the AR providing the greatest yields at SR 100 rpm is AR0.5, the results for AR0.25 are considerably close at 0.0274 g (3 s.f.).

When comparing crystal size against AR, the AR resulting in the greatest lengths at SRs 0, 100 and 250 rpm are AR 0.25 at 7.39 μm (3 s.f.), AR 0.5 at 4.54 μm (3 s.f.), and AR 0.25 at 6.85 μm (3 s.f.), respectively. While the AR providing the largest crystals at SR 100 rpm is AR0.5, the results for AR0.25 are considerably close at 4.34 μm (3 s.f.).

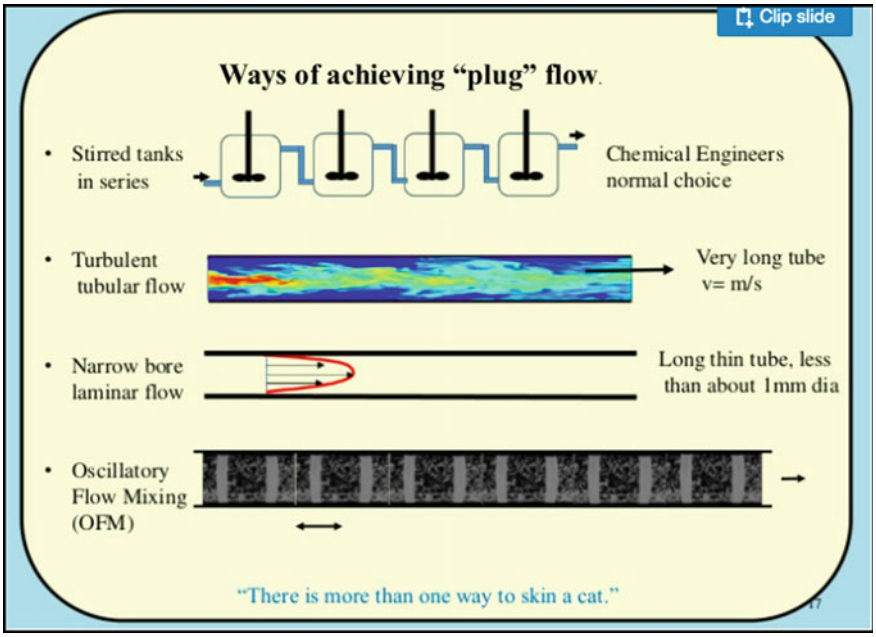

Fig. 7.2 Methods to achieve plug flow [6]

When comparing crystal activity against AR, the AR resulting in greatest activity at each SR is AR 0.5 at 9376.08 units ml^{-1}, with AR 3 next at 8527.92 units ml^{-1} at 0 ^{rpm}, 9439.92 units ml^{-1} at 100 ^{rpm}, and 8280 units ml^{-1} at 250 rpm. As seen, AR 0.25 is most effective in producing greatest crystal yields and sizes while AR 3 produces highest activity. Since AR 0.25 is most favorable for the majority of aspects determining crystal quality, AR 0.25 is most favorable in batch crystallization. In conclusion, batch crystallization proves to be more effective than continuous crystallization; and in batch crystallization, aspect ratio of 0.25 is most favorable, as it produces the best quality of crystals.

Appendix

See Figs. 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 7.10, 7.11 and 7.12.

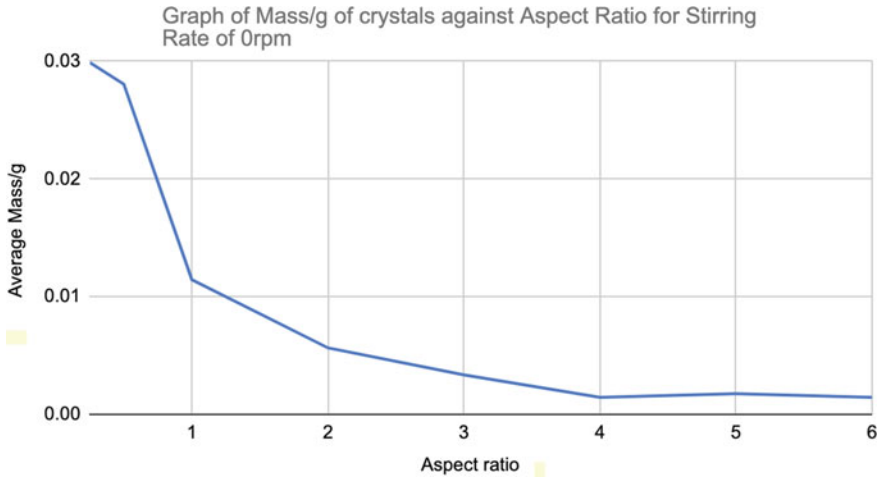

Fig. 7.3 Graph of mass/g of crystals against aspect ratio for stirring rate of 0 rpm

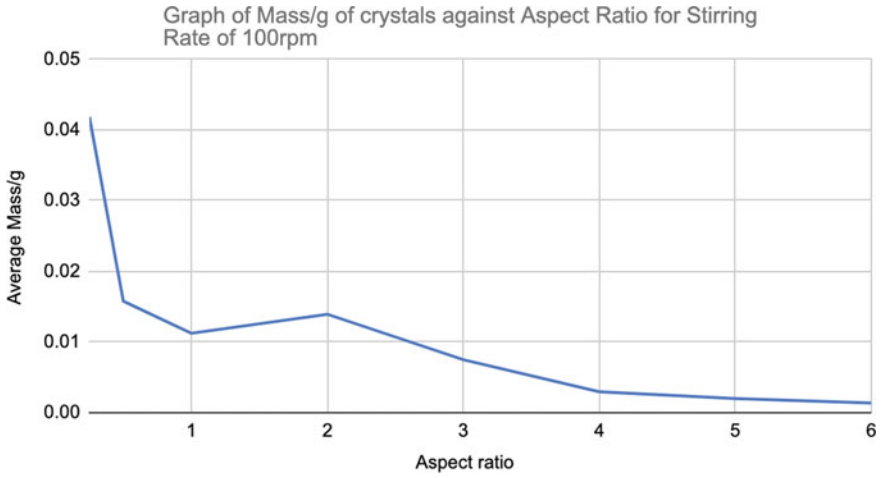

Fig. 7.4 Graph of mass/g of crystals against aspect ratio for stirring rate of 100 rpm

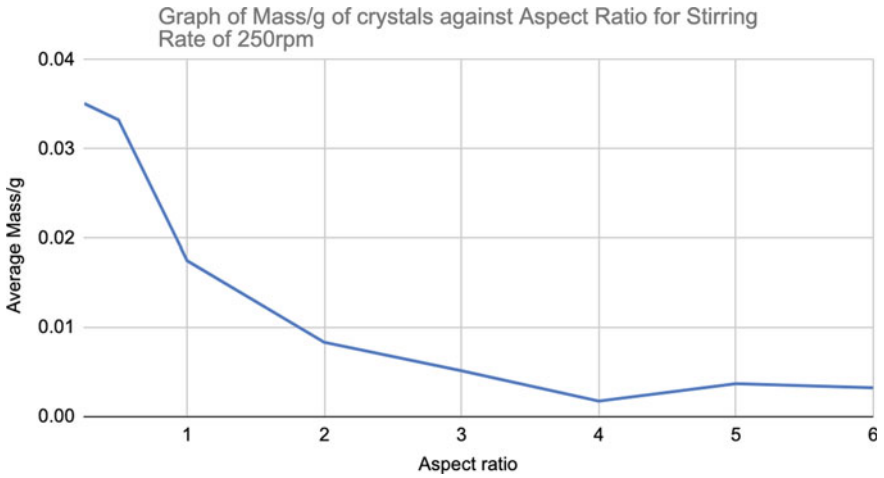

Fig. 7.5 Graph of mass/g of crystals against aspect ratio for stirring rate of 250 rpm

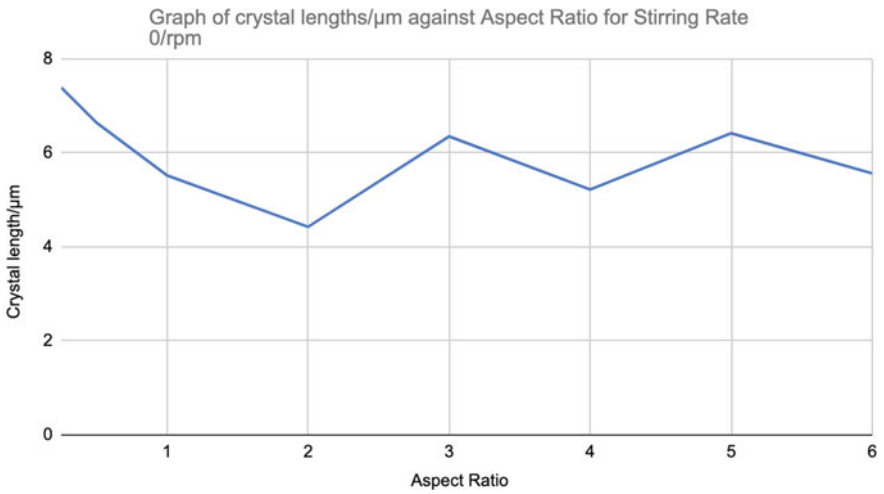

Fig. 7.6 Graph of crystal lengths/μm against aspect ratio for stirring rate 0/rpm

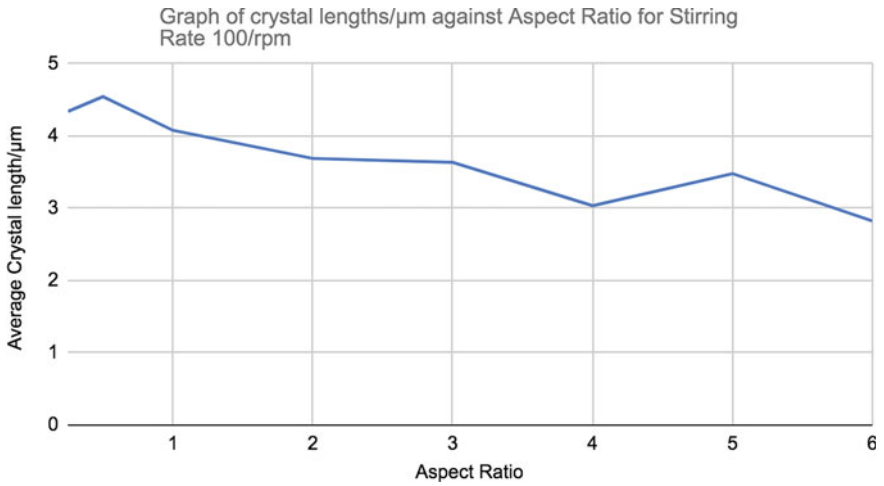

Fig. 7.7 Graph of crystal lengths/μm against aspect ratio for stirring rate 100/rpm

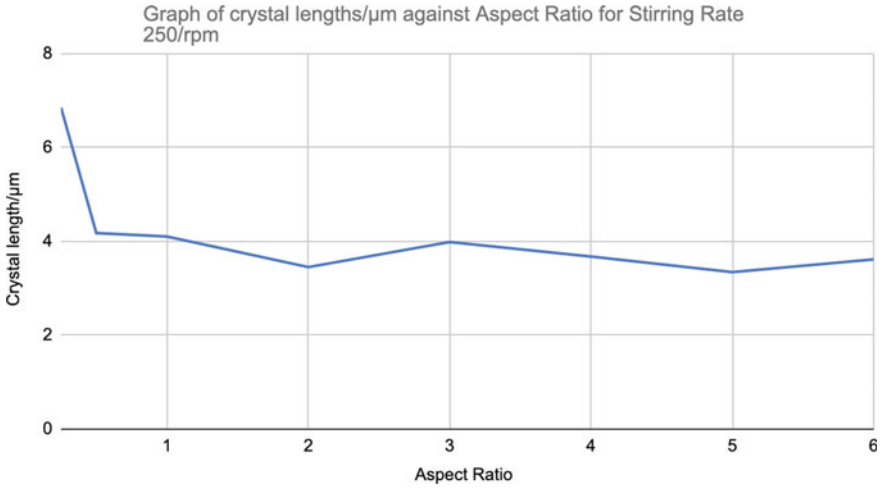

Fig. 7.8 Graph of crystal lengths/μm against aspect ratio for stirring rate 250/rpm

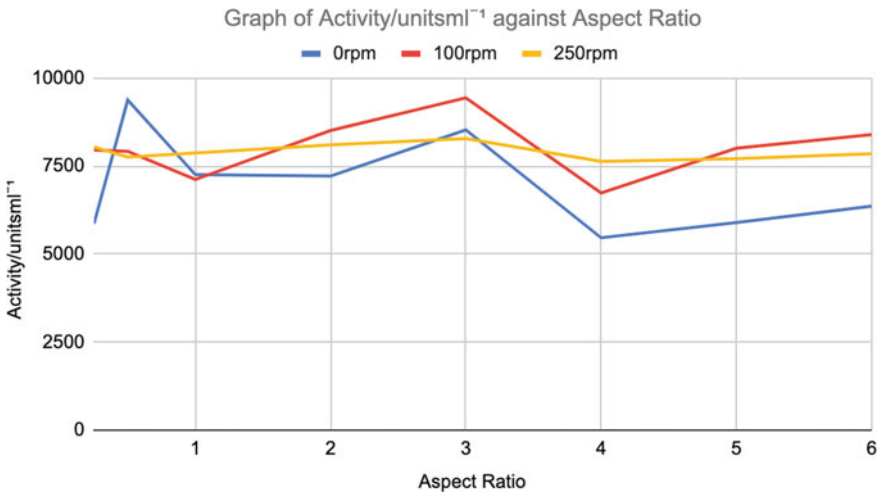

Fig. 7.9 Graph of activity/units ml^{-1} against aspect ratio

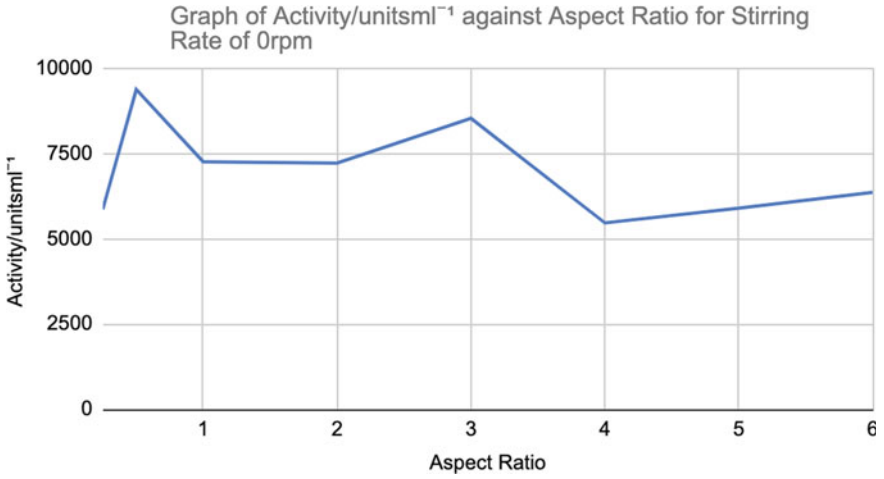

Fig. 7.10 Graph of activity/units ml^{-1} against aspect ratio for stirring rate of 0 rpm

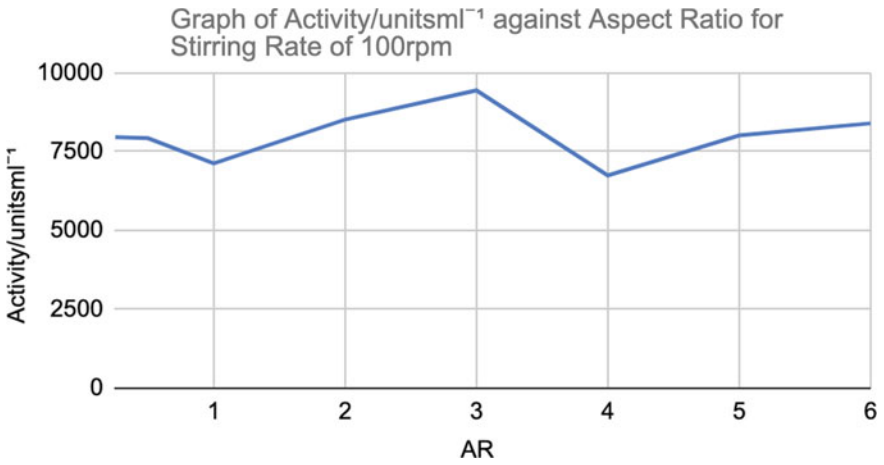

Fig. 7.11 Graph of activity/units ml^{-1} against aspect ratio for stirring rate of 100 rpm

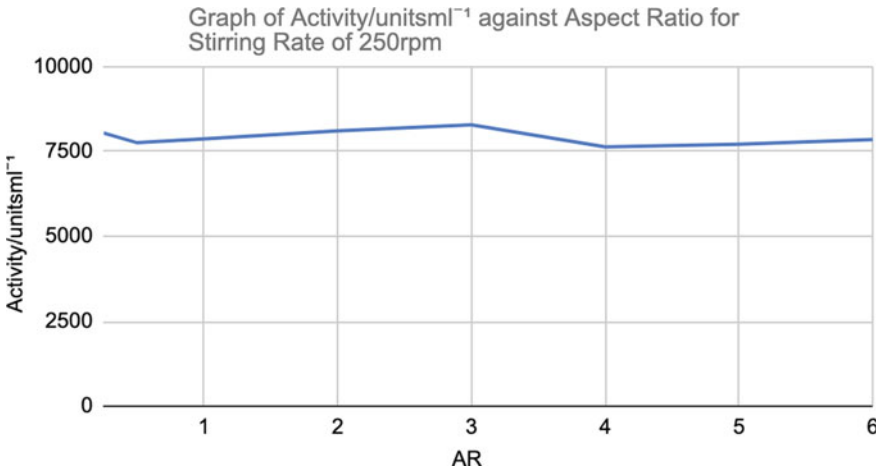

Fig. 7.12 Graph of activity/units ml^{-1} against aspect ratio for stirring rate of 250 rpm

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