

From Screen to Structure using the Crystal Former

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PURPOSE: Innovative, simple and effective methods are needed for growing beam-ready protein crystals. The Crystal Former (Microlytic, Denmark) is a device designed to facilitate crystallization by combining unique surface chemistry with highly efficient fluid mixing in a simple to use format. The Crystal Former can be used in initial screening efforts using conventional sparse matrix screens leading to improved crystallization hit rates. In this study we evaluate the feasibility of harvesting and cryo-protecting crystals grown in the Crystal Former channels using a sparse matrix screen for subsequent X-ray diffraction studies.

METHODOLOGY: The protein Xylanase XYNII (Hampton Research, USA) was diluted to a concentration of 18 mg/mL in deionized water. Using the JCSG plus screen (Molecular Dimensions, England) crystallization experiments were set up using the Crystal Former. Crystallization experiments were incubated at room temperature for 7 days and crystallization hit rate was evaluated by manual inspection. Selected channels containing crystals were harvested from the Crystal Former. Crystals were cryo-protected and extracted using both cryo loops (Hampton Research, USA) and microtools (Mitegen, USA). Crystals were mounted at the X6A beamline at NSLS at Brookhaven National Labs and diffraction data was collected.

RESULTS: We obtained 11 crystal hits using the Crystal Former for the protein Xylanase XYNII. Crystals grown under varying conditions were harvested, cryo-protected and full datasets were obtained. The resolution of the refined structure was 1.45 Å.

CONCLUSIONS: Using a set of 6 Crystal Former chips we were able to go directly from initial screening using a standard sparse matrix screen to the structure of the protein Xylanase XYNII to 1.45 Å resolution. The Crystal Former can be used as an efficient and self sufficient crystallization tool allowing users to more efficiently explore the protein crystallization phase space, while enabling users to collect full diffraction datasets from crystals grown within the Crystal Former microchannels.

INTRODUCTION: Obtaining high quality crystals of biological macromolecules remains a bottleneck in structural biology. The fluid physics, at the microscale, allows gentle mixing of solutions by diffusion, facilitating the crystallization process. While various microfluidic devices have been demonstrated to improve protein crystallization output (1-5), wide scale adoption of these devices has been slow due to issues related to reliable crystal harvesting and obtaining crystals that are immediately suitable for X-ray diffraction studies. The Crystal Former is designed to enable gentle diffusive mixing of protein and precipitant inside microchannels resulting in significantly higher crystallization hit rates. In addition to the improved mixing kinetics, the Crystal Former allows harvesting of crystals from individual reaction chambers without disturbing other reaction chambers. In this way crystals can be harvested from individual experiments and be subjected to X-ray diffraction analysis. In principle, these advantages enable growth of crystals suitable for solving the structure of a biological macromolecule without need for using other crystal growth methods. We wanted to test if we could obtain a high resolution structure for the protein Xylanase XYNII using the Crystal Former as the sole device for obtaining crystals.

METHODS: Xylanase XYNII of *Trichoderma* sp. (Hampton Research, USA) was diluted in deionized sterile water to a concentration of 18 mg/mL (final protein buffer contained: 21.5 % glycerol, 90mM Na/K phosphate pH 7). Using the sparse matrix screen JCSG plus (6) (Molecular Dimensions, UK) crystallization trials were set up for Xylanase using 6 Crystal Former devices. Devices were incubated at room temperature for 1 week and inspected for crystal formation. Crystals produced in specific channels of the Crystal Former were harvested by simply peeling back the film covering the underside of the channels. Scouring the film with a scalpel allows the film to be easily lifted from the channel area exposing the channel and crystal for extraction. Once the channel is exposed, 5 µL of cryo-protectant is added to the open channel to prevent drying of the crystal during manipulations and protect the crystal during the subsequent flash cooling. Cryo-protectant was made up by combining equal volumes of the precipitant condition yielding crystals and 50 % glycerol. Crystals were harvested from microchannels using conventional nylon loops

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(Hampton Research, USA) as well as various microtools (Mitegen, USA). In general it is best to use loops or microtools with diameters of 100 μm or less to manipulate the crystals within the 150 μm channel. Crystals were flash frozen and stored in liquid nitrogen for eventual data collection. Diffraction properties were evaluated at the X6A beamline at Brookhaven National Laboratory and full data sets were collected for suitable crystals.

All crystals were randomly mounted on the goniostat. A complete data set consisting on 300 frames, 1 degree oscillation each was recorded using the ADSC Q210 detector on a single crystal. The exposure time was 30 s and the distance was set at 200 mm. Data processing and scaling was performed with HKL3000 (7). The structure was solved from data from a single crystal by the molecular replacement method using the program MOLREP (8) and the 1.50 \AA resolution Xylanase-II structure with PDB ID 2JIC as the search model (9). Refinement consisted of repeated cycles of model building in COOT (10) and refinement in REFMAC (11). On the Ramachandran plot, 91% of the residues were in the most favored regions and 9% in the additionally allowed regions. Detailed data collection and refinement statistics are given in Table 1.

RESULTS: Xylanase crystals were grown in 11 out of the 96 conditions tested (JCSG plus # 7, 15, 23, 52, 57, 58, 60, 70, 71, 74, 76). All crystals were verified to be protein crystals by X-ray diffraction. Resolution ranged from 1.4 to 4 \AA . A crystal from JCSG plus condition 76 (0.2 M trimethylamine N-oxide, 0.1 M TRIS pH 8.5, 20 % w/v PEG 2000mme) was used to collect a full data set (Figure 1 and 2). This crystal diffracted to 1.45 \AA resolution and belongs to primitive monoclinic P21 space group. Final refined structure had a resolution of 1.45 \AA (Table 1 and Figure 2). There are 2 complete xylanase

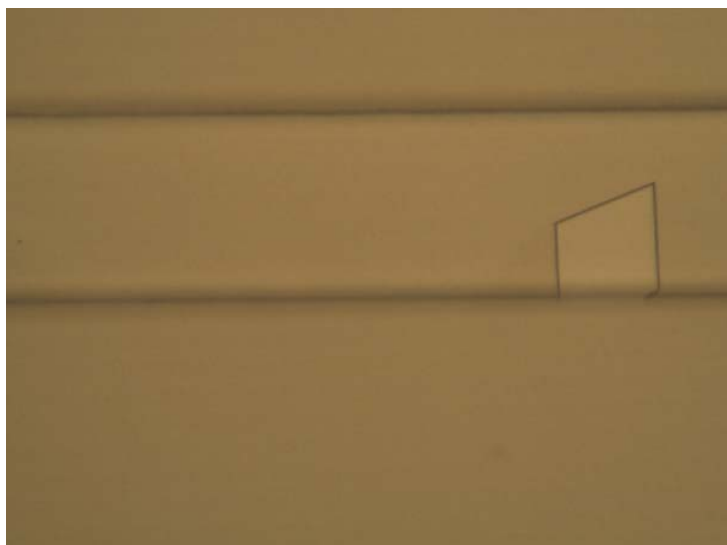


Figure 1 | Crystal of Xylanase XYNII of *Trichoderma* sp. grown in the Crystal Former channel using 0.2 M trimethylamine N-oxide, 0.1 M TRIS pH 8.5, 20 % w/v PEG 2000mme as the precipitant after 7 days of incubation at room temperature. The crystal was harvested from the Crystal Former device and used for collection of full diffraction data set (Figure 2).

Table 1 | X-ray crystallographic data collection statistics.

Data reduction	Xylanase
Wavelength (\AA)	0.95370
Space Group	P21
Resolution (\AA)	20.00-1.45 (1.47-1.45)
Cell Params. a, b, c (\AA) α , β , γ ($^\circ$)	56.48 38.94 80.60 90 95.005 90
I/sigI	33.6 (3.5)
Completeness (%)	99.4 (93.3)
Rmerge (%)	4.0 (25.4)
Redundancy	3.4 (2.3)
Mosaicity ($^\circ$)	0.7
B /Solvent Content (from truncate)	14.7 / 32
# Frames / Osc. per fr.	600 / 0.3
Refinement	
Resolution (\AA)	20.00-1.45 (1.49-1.45)
Rwork / Rfree (%)	13.5 / 15.8
# Protein Res. / Atoms	388 / 3034
# Lig Atoms	30 (2 PCA)
# Waters	433
Average B \AA^2	14.68
Prot B \AA^2	12.76
Lig B \AA^2	21.16
Wat B \AA^2	27.64
RMS Bonds / Angle (\AA / $^\circ$)	0.009 / 1.303

molecules per asymmetric unit, 3 glycerol molecules were identified in the sugar binding domain of one of the 2 monomers and 2 glycerol molecules in the second. The root mean square deviation on the C- α between the 2 monomers is 0.15 Å.

CONCLUSIONS: We used the Crystal Former to grow crystals of Xylanase XYNII of *Trichoderma* sp. using a commercially available sparse matrix screen. We have demonstrated that crystals grown in the microchannels can be readily harvested and flash frozen using standard cryo loops or microtools. Crystals grown in and harvested from the Crystal Former showed excellent diffraction properties and allowed for collection of full datasets from individual crystals and subsequent high resolution structure determination. This work demonstrates the utility of using the Crystal Former as a self contained crystallization screening tool for growing high quality crystals immediately suitable for X-ray diffraction studies.

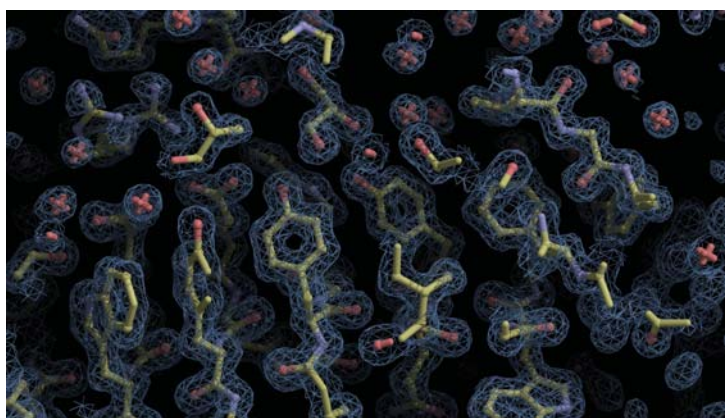


Figure 2 | Structural model of Xylanase XYNII of *Trichoderma* sp. Full data set collected on individual crystal grown in the initial crystallization screen and harvested directly from Crystal Former channel. Final structure has a resolution of 1.45 Å.

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