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*Original Research Article*

# Crystallization Studies of a Low Molecular Weight Xylanase from *Scytalidium thermophilum*

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## ABSTRACT

**Xylanases are hydrolytic enzymes which lately attracted increasing attention due to their broad use in different industrial processes. *Scytalidium thermophilum* xylanase was previously shown by our research group to be a potential candidate for the industry. Therefore, the aim of this study was to optimize the crystal growth conditions of xylanase for further structural studies. Initial crystallization conditions were screened by using commercial kits with sitting drop vapor diffusion method. Consequently, optimization studies were carried out by changing the xylanase concentration, pH, reservoir solution to xylanase ratio in the drop and precipitant concentration. As a result of the study, the best xylanase crystal was obtained from the solution containing 1.8 M ammonium citrate dibasic, 0.1 M sodium acetate trihydrate at pH 4.6 and 2 mg/ml enzyme at 18°C with streak seeding. The largest and well shaped single xylanase crystal at a size of 320 µm with a light-brown color, which has the potential as a starting point of further X-ray diffraction analysis and structural studies, was obtained.**

**Key words: *Scytalidium thermophilum*, Xylanase, Protein crystallization**

## INTRODUCTION

Proteins are one of the most important macromolecules in living systems, therefore, determining the three-dimensional structure of proteins to reveal their structure-function relationship is one of the most popular research topics of current biology and biochemistry sciences. The most comprehensive class of proteins is enzymes which play a role in almost every chemical reaction in a cell. For this reason, understanding cellular processes is mainly based on the three-dimensional structure data of enzymes and other macromolecules (Drenth, 2007). Protein crystallography is directly involved in pharmaceutical research and the enhancement studies of industrial enzymes (Judge *et al.* 2002; Rondeau and Schreuder, 2015). Today, every major biopharmaceutical company establishes a protein crystallography unit for structure-based drug discovery studies (Brader, 2017). Several methods could be employed to solve the structure of proteins, where X-ray diffraction (XRD) and nuclear magnetic resonance (NMR) spectroscopy are the most widely used techniques. Currently (July 2017), more than 131,000 structure data have been deposited to Protein Data Bank (PDB) and about 89,5% of the solved structures have been predicted by X-ray diffraction analysis.

The starting point of X-ray protein crystallography method is the production of the target protein molecule (Chayen and Saridakis, 2008). Today, by the use of genetic engineering techniques including over-expression of the target protein in a host microorganism, the amount of accessible protein is not the limiting step (Ochi *et al.*, 2009). In the second step, the protein must be purified to homogeneity. The term "purity" not only refers to the absence of the contaminants, but also the purity in terms of protein conformation (Ducruix and Giege, 2004). Finally, the pure protein must be crystallized to achieve a crystal because X-ray crystallography is not a direct imaging technique. A protein crystal is necessary to obtain diffraction images which carry information about the unit cell content of the crystal (Büttner *et al.*, 2015).

Xylanases are hydrolytic enzymes which degrade the polymeric xylan structure and produce xylooligomers of different lengths. Xylan, which constitutes the major component of hemicelluloses, is a polymer of xylose units and it is the most-abundant non-cellulosic polysaccharide in nature (Beg *et al.*, 2001). Hydrolytic function of xylanases is applicative in industry due to the high value of xylan degradation products and the necessity of the degradation of lignocellulosic structure. Based on these two functions, xylanases have a

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wide usage area in food, feed, textile and paper-pulp industries, as well as in waste management processes and biofuel and fine chemicals production (Collins *et al.*, 2005; Thomas *et al.*, 2017).

As previously reported, *S. thermophilum* is a thermophilic microorganism which produces a low molecular weight (21 kDa) xylanase with an optimum working temperature of 65°C (Sutay Kocabaş *et al.*, 2015). Due to its thermostability and high-pH preference, the enzyme was regarded as a potential actor for the food and paper-pulp industries. The low molecular weight, which allows effortless penetration into the lignocellulosic network and effective degradation of xylan, is another advantage of the enzyme. The enzymatic hydrolysis studies showed that *S. thermophilum* xylanase is able to degrade the lignocellulosic structure of corn cob, which is considered as an important raw material for the production of second generation biofuels (Sutay Kocabaş *et al.*, 2015). Because of the industrial application potential of *S. thermophilum* xylanase, prediction of its three dimensional structure and understanding its structure-function relationship became important. Therefore, the aim of the current study is to determine the crystallization conditions of a low molecular weight xylanase from *S. thermophilum* for further X-ray analysis and structural studies.

## MATERIALS and METHODS

### Protein production and purification

Xylanase was purified from the crude culture supernatant of *S. thermophilum* (type culture *Humicola insolens*, ATCC No.16454) using a two-step chromatography procedure consisting of gel filtration and anion exchange, as previously described (Sutay Kocabaş *et al.*, 2015). Protein concentration of the protein sample was determined by the Bradford method using BSA as the standard (Bradford, 1976). The pure xylanase, which is purified 21.8-fold to apparent homogeneity with 9.6% recovery, was concentrated by using Amicon centrifugal concentrators (Millipore, USA) and further used for crystallography trials.

### Screening for crystallization conditions

To determine the appropriate protein concentration for screening trials, Pre-Crystallization Test (PCT) kit (Hampton Research, USA) was used. After predicting suitable protein concentration (3 mg/ml), crystallization screening trials were set up using Crystal, Index, PEG Rx and Salt Rx screen kits (Hampton Research, USA). Crystallization plates with 96 wells were used for screening. The crystal screen solution of 75 µl was pipetted manually into the each well. The purified enzyme of 1.0 µl (3 mg/ml) was mixed with 1.0 µl of the well solution and screening trials were performed using the sitting drop vapor diffusion technique. Plates were covered with an adhesive sealing film (Hampton Research, USA) and incubated at 18 and 25 °C. To evaluate the results of screening experiments, drops were observed under a light microscope for a crystal formation.

### Crystal staining

Before performing further optimization experiments based on a particular crystal-growing condition, it must be proved that the observed crystal is a protein crystal. Commonly, salts in the protein sample which are remained from purification steps and salts that are included in screening kits, may easily crystallize in screening experiments. This problem often happens and belies crystallographers. To determine the nature of the observed crystal, *ca*. 0.5 µl of Izit Dye (Coomassie dye solution) (Hampton Research, USA) was added to the drop. Compared to the salt crystals, protein crystals have larger solvent channels where Izit dye can penetrate. The dye fills the solvent channels in protein crystals and color the crystals blue after an hour of incubation. In contrast, the dye cannot enter into the salt crystal structure and the crystal remains clear.

### Optimization of crystallization conditions

The initial crystallization condition was optimized using hanging drop vapor diffusion technique with 24-well plates by changing the parameters; (1) protein concentration, (2) pH, (3) reservoir solution to xylanase ratio in the drop and (4) the concentration of well (reservoir) solution. The well solutions for optimization trials were prepared in the laboratory. The crystallization solution (1 ml) was pipetted into the well and 1 µl of purified

xylanase was mixed with 1 µl of the well solution on the cover slip. Inverted cover slip was placed over the siliconized reservoir and incubated at 18 °C. To evaluate the results of crystallization, drops were observed daily under a light microscope during the first week and weekly thereafter.

**Seeding**

Seeding is an alternative technique to alter the size and quality of the crystals. The seeding tool (Hampton Research, USA) was used for streak seeding. To obtain fresh seeds, an existing crystal was destroyed using the natural fiber mounted on the seeding tool. The seeds were rapidly introduced into a pre-equilibriated drop by running the probe in a straight line in the drop.

**Verification by SDS-PAGE**

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed to check the crystal purity and to prove that the obtained crystals are *S. thermophilum* xylanase. For this purpose, the xylanase crystal in the drop is dissolved by addition 20 µl of ammonium sulphate dibasic buffer. The solution is mixed with 180 µl of SDS-PAGE sample buffer and boiled for 5 minutes to complete denaturation. Electrophoresis was performed in Mini Protean Tetra Cell (Biorad, Germany) system at 200 V constant voltage (Laemmli, 1970). The gel was calibrated using Spectra™ Multicolor Low Range Protein Ladder marker proteins (Thermo Fisher Scientific, USA). The gel is stained by silver nitrate staining method (Blum *et al.*, 1987).

## RESULTS and DISCUSSION

**Screening for crystallization conditions**

Among the tested screening kits, the best crystal was observed in B4-well of Salt Rx screen at 18°C on 28$^{th}$ day of incubation (Figure 1a). The crystallization condition of this well was 1.8 M ammonium citrate dibasic and 0.1 M sodium acetate trihydrate at pH 4.6. To check the nature of the crystal (protein or salt), it was stained with Izit dye solution. The crystal was observed on 28$^{th}$ day and staining was applied on 40$^{th}$ day incubation to ensure that the crystal growth is ended. After staining, the crystal has completely absorbed the dye which indicates the protein nature of the crystal (Figure 1b).

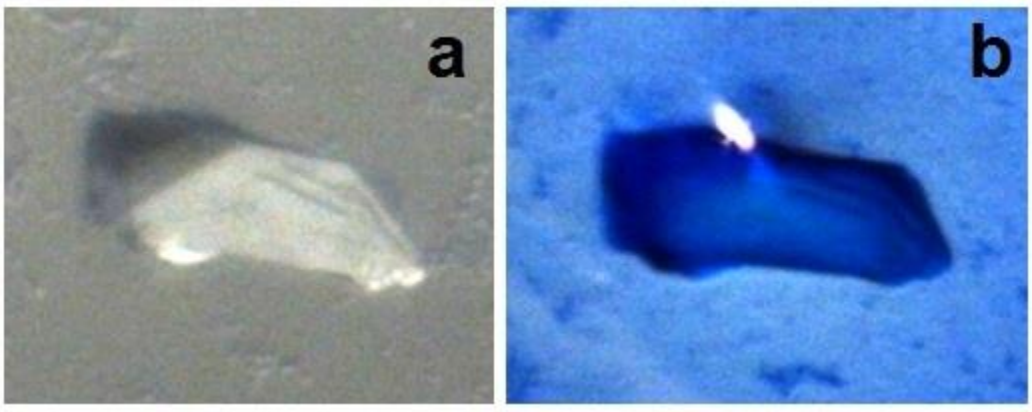



**Figure 1.** Protein crystal; a) observed in B4-well of Salt Rx screen at 18°C on 28$^{th}$ day of incubation with 1.8 M ammonium citrate dibasic and 0.1 M sodium acetate trihydrate at pH 4.6, b) after staining with Izit dye solution on 40$^{th}$ day of incubation.

**Optimization of crystallization conditions**

Based on the initial crystallization conditions, optimization studies were carried out within the ranges of xylanase concentration, pH, reservoir solution:xylanase ratio and precipitant concentration, as given in Table 1.

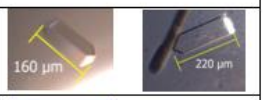
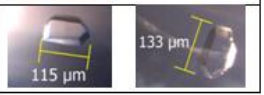
The tested parameters were slightly shifted to lower or higher levels to obtain the best medium for getting bigger and proper crystals for further practices.

**Table 1.** Optimization parameters and corresponding test ranges.

| Optimized parameter | Initial condition | Test range | |
|---|---|---|---|
| | | Lower limit | Higher limit |
| Xylanase concentration (mg/ml) | 3.0 | 2.0 | 3.5 |
| pH | 4.6 | 4.5 | 4.7 |
| Drop content (reservoir solution to xylanase ratio) | 1:1 | 1.5:1 | 1:1.5 |
| Ammonium citrate dibasic concentration (M) | 1.8 | 1.2 | 2.5 |
| Sodium acetate trihydrate concentration (M) | 0.1 | 0.04 | 0.14 |

Several xylanase crystals were observed in optimization trays, mostly at 18°C. The variation of crystal sizes with changing crystallization conditions are given in Table 2. Even some crystal formations reached to 88 days, most of them have been seen at first three weeks. Results have demostrated that 0.1 M concentration of sodium acetate trihydrate pH 4.6 is very critical for xylanase crystal growth. Even small changes in 0.1 M sodium acetate trihydrate concentration has blocked crystal formation in optimization trials. Xylanase crystal could tolerate ammonium citrate dibasic concentration in a range of 1.4 to 2.2 M. The lowest enzyme concentration for crystal growth was found as 3 mg/ml, that is, no crystal was observed at xylanase concentrations lower than 3 mg/ml.

**Table 2.** The variation of crystal sizes with changing crystallization conditions (trays were set at a constant condition of; 1:1 drop content (ratio of reservoir solution to xylanase, 3 mg/ml xylanase concentration, 18°C).

| Ammonium citrate dibasic concentration (M) | Sodium acetate trihydrate (pH 4.6) concentration (M) | Crystal size (µm) | Crystal picture |
|---|---|---|---|
| 1.4 | 0.06 | 160-220 |   |
| 1.8 | 0.10 | 100-135 |   |
| 1.9 | 0.10 | 80-94 |   |
| 1.9 | 0.10 | 183 |  |
| 2.2 | 0.10 | 89 |   |
| 2.2 | 0.12 | 115-133 |   |

## Seeding

Seeding is a practical tool for the growth of large single crystals which diffract to higher resolution (Ducruix and Giege, 2004). It is also useful to bypass the need for nucleation and focus only on the crystal growth. As shown in Table 2, several xylanase crystals with the size of 100-200 µm were obtained after optimization trials. To achieve larger crystals which probably will diffract to higher resolution, the streak seeding technique was applied.

A crystal obtained from a solution composed of 1.8 M ammonium citrate dibasic, 0.1 M sodium acetate trihydrate at pH 4.6 and 3 mg/ml xylanase at 18°C was used as the donor crystal. The condition of the seeding drop was the same as the donor crystal drop, except xylanase concentration (2 mg/ml). A lower protein concentration was used because seeding into the metastable region (lower protein and reagent concentration) favors growth, not nucleation. Nuclei were provided by the donor crystal and their growth was the primary target.

Seeds were collected from the donor crystal by disrupting it with the probe and they were transferred to the recipient drop. The streak seeding method was applied by running the probe tip in a straight line across the middle of the recipient drop. The seeded drop was incubated at 18°C and examined daily for the crystal growth. Seeding application has drastically improved the size and shape of the xylanase crystal and shortened the crystallization period from several weeks to 5 days. The largest xylanase crystal at a size of 320 µm having the best morphology was observed at 5$^{th}$ day of incubation after seeding (Figure 2).

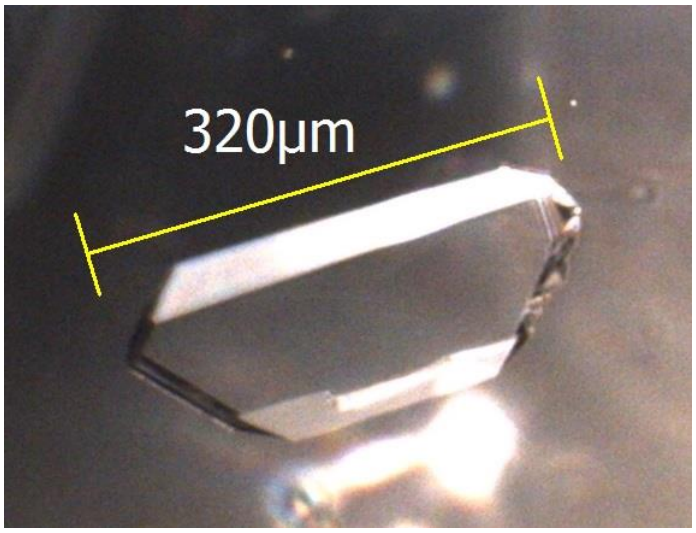



**Figure 2.** Xylanase crystal at a size of 320 µm, obtained from a solution containing 1.8 M ammonium citrate dibasic, 0.1 M sodium acetate trihydrate at pH 4.6 and 2 mg/ml enzyme at 18°C after streak seeding.

**Verification studies**

The xylanase crystal was dissolved and examined by SDS-PAGE. The crystal was detected as a single protein band on SDS-PAGE gel with a molecular weight of 21 kDa (Figure 3), which is in agreement with our previous findings (Sutay Kocabaş *et al.*, 2015). By the help of SDS-PAGE, it is proven that the crystals observed are pure *S. thermophilum* xylanase crystals.

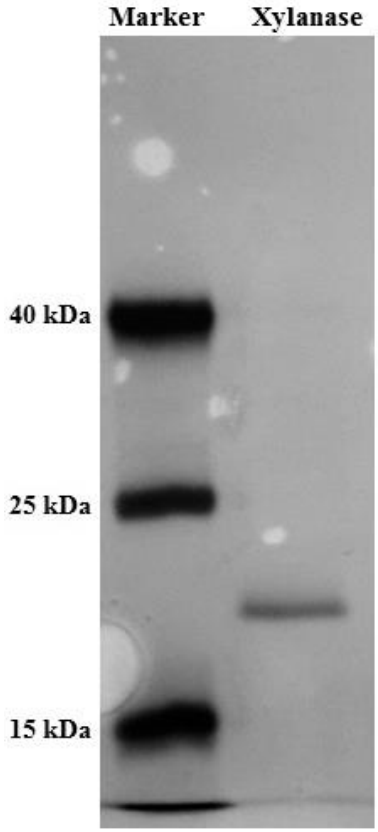



**Figure 3.** SPS-PAGE gel; Lane 1: marker proteins, Lane 2: xylanase crystal.

Due to their broad use in different industrial processes, xylanases attracted considerable attention in recent decades. Besides food, feed, textile, pharmaceutical and paper-pulp industries, xylanases play an important part in lignocellulosic biomass processes to obtain sustainable value-added chemicals and renewable energy by the conversion of xylan (Silva *et al.*, 2015). In parallel to its growing importance, several xylanase structures have been deposited to the PDB, where most of the xylanases were isolated from bacteria. *S. thermophilum* is a fungus (eukaryotic microorganism) and xylanase structures from eukaryotic microorganisms have been studied in lesser extent. Structural studies of xylanases from fungal sources such as *Aspergillus niger* (Krengel, 1996), *Penicillium simplicissimum* (Schmidt *et al.*, 1998), *Thermoascus aurantiacus* (Natesh *et al.*, 2003), *Scytalidium acidophilum* (Michaux *et al.*, 2010), *Fusarium oxysporum* (Dimarogona *et al.*, 2012) and *Talaromyces cellulolyticus* (Kataoka *et al.*, 2014) have been reported but no structural or crystallization data is available for *S. thermophilum* xylanase in literature. It has been previously shown that *S. thermophilum* xylanase has an optimal working temperature of 65°C and is active at alkaline pHs, with the absence of cellulase activity (Sutay Kocabaş *et al.*, 2015). This profile of the enzyme answers the concerns of the paper and pulp industry and makes the enzyme a potential candidate for paper and pulp production process (Harris *et al.*, 1997; Silva *et al.*, 2015). In addition, the hydrolysis ability of the enzyme on lignocellulosic substrates could be the basis for bioethanol production studies.

The production of a xylanase crystal having smooth faces and sharp outlines was achieved in this work. The best crystal was obtained from a solution composed of 1.8 M ammonium citrate dibasic, 0.1 M sodium acetate trihydrate at pH 4.6 and 2 mg/ml xylanase, at 18°C after streak seeding. It took only 5 days for the growth of a large single crystal at this condition. There are several studies in literature about xylanase crystallization from different microorganisms where each of them reports different crystallization conditions. Unfortunately, there is no relationship between the chemical composition of the enzyme and its crystallization

conditions. Each protein has its own unique crystallization characteristics which can be found only by a trial and error procedure (Camara-Artigas and Gavira, 2016).

A light-brown xylanase crystal with 320 μm size was obtained after optimization trials in this study. It is well known that the size of the protein crystal drastically affects the X-ray diffraction quality; larger crystals diffract stronger. Crystals within a size range of 300-500 μm are regarded as optimal in crystallography studies where crystals having dimensions of at least 10 $\mu m^3$ could also be analyzed (Chayen 2004; Drenth 2007). A protein crystal is a lattice of protein molecules and the X-rays scattered by the crystal is collected for structural analysis. Since a single protein molecule cannot diffract a strong X-ray beam for detection, more molecules which are arranged in a repeating pattern (a lattice) is required for a detectable diffraction (Gulerez and Gehring, 2014). Thus, because a larger crystal is consisted of higher numbers of protein molecules, diffracts stronger. Therefore, *S. thermophilum* xylanase crystal with the size of 320 μm has a potential to scatter strong X-rays and to provide collection of high-quality structural data.

Diffraction-quality crystals which are reproducible and ordered in all crystal directions, are required for a successful structure determination (Gorrec, 2014). Seeding of crystals in experiments containing fresh protein sample is sometimes necessary to improve the quality of the crystals since it is not always possible to simultaneously optimize the conditions for nucleation and growth of the crystal (Ochi *et al.*, 2009). Crystallization is known as a phase transition phenomenon which depends on the saturation level of the crystallization solution. At moderate supersaturation state, crystal nucleation starts and protein concentration in the drop decreases. This state is called as metastable zone where no further nucleation takes place. The metastable zone is the most suitable environment for the growth of well-ordered single crystals. During this process, nucleation is the key step and the most common technique to avoid its difficulty is to avoid it completely by seeding. It is possible to take seeds from existing solutions and transferring them directly to metastable zone to obtain better crystals (Chayen, 2004). In agreement with this fact, seeding technique has provided a positive effect in our study, where the average crystal size of 100-200 μm has been increased to 320 μm after seeding. This study has revealed a xylanase crystal ready for X-ray diffraction trials.

## CONCLUSIONS

*S. thermophilum* xylanase has a great potential for industrial applications. Therefore it is important to solve its three-dimensional structure and crystallization studies are vital for further X-ray analysis. We believe that prediction of the best crystallization conditions of *S. thermophilum* xylanase in this study will be the starting point of further X-ray diffraction analysis and structural studies.

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