Characterization of pterin deaminase from *Mucor indicus* MTCC 3513

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Abstract Pterin deaminase is an amidohydrolase enzyme which hydrolyses pteridines to produce lumazine derivatives and ammonia. Even though the enzyme was shown as early as 1959 for its anticancer efficacy there was a long gap in the communique after that which was in 2013. In our study we have chosen *Mucor indicus* MTCC 3513 which was a promising strain for production of different industrial products. The pterin deaminase enzyme was harvested and extracellular from *M. indicus*. The extracellular sample was partially purified by using ethanol precipitation and ion exchange column (Hi-Trap QFF) in Fast Protein Liquid Chromatography. The molecular weight of the purified pterin deaminase enzyme was apparently determined by SDS-PAGE. The purified enzyme was further biochemically characterized. Molecular docking studies with the predicted sequence showed higher binding affinity towards folic acid interaction. The structure of this protein may open the windows for new drug targets for cancer therapy.

1. Introduction
Having about 1.5 million species, fungi have the second highest number of species after insects [1] with development of biotechnology, fungi have gained a crucial importance in the production of a wide range of products such as unique foods, biofuels, enzymes, antibiotics, and chemicals. Zygomyctota is the most varied and least studied phylum of fungi that are divided into two subphyla, trichomycetes and zygomycetes. Being a source of human food for several hundred years together with ensuring performance in production of different products in the current decades has put zygomycetes at the center of a large number of recent research activities [2]. Among different zygomycetes, Mucor is a genus of about 3,000 members that are widely disseminated in the soil, digestive systems, plant surfaces, and decomposed vegetables. Indeed, *Mucor indicus* were one of the earliest discovered and most studied species of this genus. Followed by discovery in 1860s, different features of biology including morphology, biosynthesis, and metabolism as well as applications of this fungus have been extensively studied [3-10]. However, despite the broad and long term studies,
several aspects of the fungus especially morphology, cell characteristics, and new applications continue to be unknown. A new wave of research on *M. indicus* has been initiated since the beginning of the 21st century, when *M. indicus* was introduced as a promising microorganism with several potential industrial importance such as co-production of ethanol and chitosan from lignocellulosic waste materials [11-16]. Enzyme therapy involves taking enzyme supplements as an alternative form of cancer treatment. They are normal proteins that stimulate and accelerate many biological reactions in the body. Metabolic enzymes build new cells and repair damaged ones in the blood, tissues and organs. Enzymes have specific affinities for binding and catalytic properties towards specific targets with low toxicity make them as ideal drug. Cancer cells are more susceptible to enzymes than a normal cell because enzymes dissolve fibrous coating on cancers cells, allowing the immune system to work [17]. Pterin deaminase is an amidohydrolase enzyme which hydrolyses pteridines to produce lumazine derivatives and ammonia. The enzyme has been reported in several fungi viz., *Aspergillus oryzae* [18], *Aspergillus* Y-8-5 (ATCC NO. 20413), *Mucor lamprosporus* IAM 6114 (ATCC No.20410), *Rhizopus arrhizus* IAM 6052 [19] *Aspergillus tamarii* IFO 4287, *Aspergillus oryzae* var.no 13, *Aspergillus gymnosardae* IAM 2149, *Mucor alboater* IAM 6141, *Rhizopus japonicus* IAM 6002 (ATCC NO. 20409), *Aspergillus* Y-43-3 (ATCC NO. 20414), *Penicillium* Y-70-2 (ATCC No.20411) and *Penticeps* Y-110-2(ATCC No.20412) [20]. The enzyme substrates are involved an important role in the biological activity [21]. This study was conducted to produce and purify enzyme pterin deaminase from *M. indicus* MTCC 3513. Along with this, the study involved the structure prediction of pterin deaminase through bioinformatic tools.

2. Materials and Methods
2.1 Collection of strain and the Preparation of extracellular enzyme from *M. indicus*
Lyophilized culture of *M. indicus* (MTCC 3513) was procured from Microbial Type Culture Collection and Gene Bank (IMTECH). The Fungal cells were grown in modified PDB (Potato dextrose broth) for 7 days under aerobic conditions. After incubation period the cells were harvested by centrifugation at 10,000 rpm at 4°C for 30 min. The supernatant was collected from the broth culture and served as crude enzyme. It was followed by enzyme precipitation using ethanol. The enzyme activities (IU ml⁻¹) against the substrate 0.01M folic acid were estimated in triplicates in extracellular culture filtrates by Nesslerization [22].

2.2 Protein precipitation
An initial step in purification of protein is concentration of protein sample which may be achieved by precipitation. Required volume of ethanol was cooled at -20°C. The protein sample was placed in ethanol compatible tubes. Different concentration of ethanol ranging from 3 to 9 volumes was added to the tubes. The tubes were vortexed and incubated at 4°C overnight. After incubation the tubes were centrifuged at 10,000rpm for 10 min at 4°C. The supernatant was properly discarded without disturbing the protein pellet. The ethanol was allowed to evaporate from the uncapped tube at room temperature for 30 min. The protein pellet was suspended with 0.01M potassium phosphate buffer (pH-7) for further analysis. The ethanol concentration which yielded higher protein and enzyme activity was chosen for further precipitation studies.

2.3 Ion exchange chromatography (FPLC)
Chromatographic process was performed at 4°C.A pre-packed Hi Trap Q FF (1ml) column a strong anion Exchanger, which was connected to the ÄKTA FPLC system. The column was equilibrated with 10 column volumes (CV) of binding buffer (buffer A: 0.01M PBS pH 7). The precipitated sample was injected to the column through a sample loop. Then the column was washed with 10 CV of buffer A to remove unbound or weakly bound particles. The adsorbed particles were eluted with 70 CV of final elution buffer with 0 %–100% NaCl (buffer B: 1M NaCl) in gradient mode. Flow through and elute
were collected with a fraction collector included in the system (Frac - 920) at fixed volumes (1ml/min) [23]. The peak fractions were collected and assayed for enzyme activity and protein concentration was estimated. The selected fractions were analysed by SDS-PAGE [24]. Silver staining was done for the chosen fraction by the method of Switzer.1979 [25].

2.4 Enzyme characterization
Optimum pH was determined by incubating the reaction mixture in various pH ranging from 6-8 and optimum temperature for enzyme activity was ascertained by incubating the reaction mixture in various temperatures ranging from 10ºC-70ºC. Ammonia liberation in all the reactions were calculated by Nessler’s Reagent for calculating enzyme activity.

2.5 Inhibition assay
The effect of inhibitors on pterin deaminase was determined by using different inhibitors such as Ethylene diamine tetra acetic acid (EDTA), Dithiothreitol (DTT), Phenyl methyl sulfonyl fluoride (PMSF), SDS and Triton X100. The enzyme was incubated for 30min with various concentrations of inhibitors like 10mM, 5mM and 1mM. After incubation, the enzyme activity was estimated by assaying the ammonia released by enzyme by deamination the substrate folic acid by a modified method of Wurster and Butz. 1980 [26].

2.6 In silico analysis of pterin deaminase
A complete genome of one of the zygomyceta family species Malassezia sympodialis and a sequence of Pterin deaminase (WP_012652141.1) were retrieved from NCBI through Z28140.1 identifier (NCBI-Gene sequence). Pterin deaminase perfectly matched with a small part of the sequence present in the Malassezia sympodialis. Further folic acid structure was retrieved from Pubchem database with ID of CID_6037. Using Prime modules of Schrödinger suit 2016, a model protein structure of pterin deaminase was predicted and validated. The modelled structure of pterin deaminase and folic acid from Pubchem database was used for interaction studies by glide modelling followed by extra precision method and rigid docking parameter.

3. Results
3.1 Extraction of extracellular enzyme
M. indicus MTCC 3513 used for this study was procured from Microbial Type Collection Centre (MTCC), Chandigarh and maintained in PDA medium. The extracellular enzyme was extracted from the fungal cells through centrifugation and the crude enzyme retained in the supernatant was used as crude enzyme extract. The protein content of the extracellular enzyme was found to be 3.3 mg/ml and the enzyme activity using folic acid as substrate was found to be 9 U/ml.

Maximum extracellular enzyme was precipitated (15.36 U/mL) with 3 volumes of ethanol. The highest concentration of protein being precipitated at 1:3 ratio (Crude Extract: Ethanol) validated 3 volumes as the preferred concentration of ethanol.

| Purification Steps                  | Volume (ml) | Enzyme activity (U/ml) | Protein Concentration (mg) | Specific activity (U/mg) | Purification (fold) |
|------------------------------------|-------------|------------------------|----------------------------|--------------------------|--------------------|
| Crude                              | 50          | 9                      | 3.3                        | 2.72                     | 1                  |
| Ethanol precipitation              | 30          | 15.36                  | 2.35                       | 6.56                     | 2.411              |
| Hi Trap Q FF (Ion-Exchange Column) | 2           | 47.84                  | 0.40                       | 119.6                    | 18.231             |

Table 1. Purification of pterin deaminase.
The ethanol precipitated enzyme, after suspension with potassium phosphate buffer was estimated to have 15.36 U/ml of pterin deaminase activity and protein content was measured to be 2.35 mg/ml. The specific activity was increased from 2.72 U/mg to 6.56 U/mg and the purification fold was increased to 2.411 (Table 1).

The ion exchange column purification process increased the purity of the enzyme which was revealed by the increase in enzyme activity to 47.84 U/ml with the specific activity of 119.6 U/mg. The purifications fold was increased to 18.231 fold. Even though the chromatogram revealed 5 active peaks, the fractions from 39 to 43 under the peak alone exhibited enzyme activity corresponding to 60% NaCl gradient solution (Table 1; Figure 1).

The protein profile of the crude enzyme showed more than 4 bands and appeared very thin due to very low concentration of loaded protein. Single band was visualized upon PAGE analysis of the active fractions (pooled) of ion exchange chromatography and apparent molecular weight of the enzyme was approximately 60 kDa (Figure 2).

![Figure 1. Ion Exchange chromatogram of the purified pterin deaminase](image-url)
Figure 2. SDS-PAGE (Silver staining)

Lane 1- Marker, Lane 2- Purified and concentrated Fraction (15µl), Lane 3- Purified and concentrated Fraction (20µl), Lane 3- Purified and concentrated Fraction (30µl)

3.2 Optimization of purified pterin deaminase

The optimum temperature was found to be 30°C which corresponded to the maximum enzyme activity of 51 U/ml (Figure 3) whereas alkaline pH 7 was optimized condition for pterin deaminase activity (Figure 4). The molecular weight of the purified protein 60kDa determined in silver staining.

Figure 3. Effect of temperature on enzyme activity
3.3 Effect of inhibitors

To predict the nature of the protein, inhibitor studies were carried out with pure protein. The enzyme was inhibited by EDTA at 10mM, 5mM and 1mM concentrations (Figure: 5). Triton X 100 also partially inhibited the enzyme activity at 10mM concentration. EDTA disodium salt inhibited the enzyme activity which indicated that the enzyme may be a metalloenzyme [27].
3.4 Insilco analysis of Pterin deaminase (Homology Modelling of Protein Sequence Retrieved)
Retrieved sequence was then subjected to perform Homology modelling against Protein Databank which has only structural conformation of each atom's configuration. 3-Dimensional structure was predicted for translated protein sequence by homology modelling technique. The template structure was retrieved from crystallographically studied protein in protein data bank. The template ID is 4R7Y_A. By translating six frame translation method. It was taken first frame which is higher in number of protein sequence.

3.5 Model protein Vs Folic acid interaction
Interaction profile of Model protein with folic acid showed that the ligand interacted at 6 sites of the Protein having residual atom types ARG 626 (H), ARG 626 (H), ARG 624 (H), PHE 371 (H), ARG 624 (H), HIS 370 (H), ARG 126 (H), ASP 369 (O), ASP 369 (O) & ASP369(O) receptor via H (Hydrogen) and O (Oxygen) and atom types forming bond distance 1.715Å, 2.482Å, 2.325Å, 2.196Å, 1.653Å, 2.307Å, 2.072Å, 2.309Å, 2.309Å & 1.72Å respectively (Table 2, Figure 6 and 7). The higher interaction of Modelled protein with folic acid can be noticed from the Glide score -6.74 kJ/mol as higher interaction profile (Table 2).

| Protein                | Ligand    | Bond atom | Ligand atom | Protein Residue name | Residue No | Bond Length |
|------------------------|-----------|-----------|-------------|----------------------|-----------|-------------|
| Pterin Deaminase Modelled | Folic Acid | Hydrogen  | Oxygen      | ARG                  | 626       | 1.715       |
|                        |           | 2         | Hydrogen    | ARG                  | 626       | 2.482       |
|                        |           | 3         | Hydrogen    | ARG                  | 624       | 2.325       |
|                        |           | 4         | Hydrogen    | PHE                  | 371       | 2.196       |
|                        |           | 5         | Hydrogen    | ARG                  | 624       | 1.653       |
|                        |           | 6         | Hydrogen    | HIS                  | 370       | 2.307       |
|                        |           | 7         | Hydrogen    | ARG                  | 126       | 2.072       |
|                        |           | 8         | Oxygen      | Hydrogen             | 369       | 2.309       |
|                        |           | 9         | Oxygen      | Hydrogen             | 369       | 2.309       |
|                        |           | 10        | Oxygen      | Hydrogen             | 369       | 1.72        |

Table 2. Ligand (folic acid) and protein interaction position.
Figure 6. Ligand (folic acid) and Protein 2D- interaction

Figure 7. Ligand (folic acid) and Protein 2D- interaction
The docking studies revealed that the protein sequence taken for study had folic acid binding capacity with highest Glide score. Hence using this as base sequence the genes responsible for pterin deaminase in *M. indicus* may be identified by further recombination and expression studies.

4. Discussion

The potential of pterin deaminase to possess antitumor activity has been recorded since early 1980’s [28]. In spite of its immense biological significance, there is but little information available about the enzyme and its biological role. The present study was designed to purify and characterize pterin deaminase from *M. indicus* MTCC 3513. The extracellular enzyme was extracted from the fungal cells by centrifugation at high speed. Maximum extracellular enzyme was precipitated with 3 volumes of ethanol. Ethanol is the most commonly used alcohol compound for precipitation of proteins and nucleic acid. Also ethanol induces conformations changes which results in the formation of inter-helical interaction allowing the proteins to aggregate [20].

Protein purification is essential for characterization of protein of interest. The purification process may separate proteins based on molecular weight or interaction with the charged resins. Ion exchange chromatography separates compounds according to their nature and degree of ionic charge. Anion exchange resins packed in the column have a positive charge and are used to retain and separate negatively charged compounds. Literally, there were no reports based on the purification of pterin deaminase enzymes in fungi whereas Kusakabe, 1976 [20] reported the enzyme was purified from fungi Aspergillus Y8-5 (ATCC 20413) by DEAE cellulose column chromatography and obtained 84,975 units of pterin deaminase with the enzyme activity 825 units/mg of powder (1428 units/mg of protein). In this study, ethanol precipitated protein sample was subjected to purification by ion exchange column chromatography. The chromatogram revealed 5 active peaks, the fractions from 39 to 43 under the peak alone exhibited enzyme activity corresponding to 60% NaCl gradient solution.

SDS-PAGE analysis was utilized to check the purity of protein by separating the protein according to their sizes based on their electrophoresis mobility. SDS-PAGE was made according to the method proposed by Laemmli,1970 [24]. Silver staining was performed for protein staining as it is more susceptible and is capable of staining less than 1 ng of protein. Through the purity of protein was not confirmed the single band formation around 60 kDa, denoted that the molecular weight of the enzyme may be apparently 60 kDa. The enzyme molecular weight reported by Takikawa, 1979 [29] was considered to be around 110kDa, which was higher than that obtained in the present study.

Previous reports have expressed the enzyme activity in terms of micromoles of substrate such as pterin carboxylic acid [30] and 2- amino -4-hydroxypteridine [31], deaminated in one min. The reduction in absorbance at 490nm being the absorbance peak for the substrate was taken for calculating the deaminated product. As per Takikawa [29] for each mole of substrate deaminated, equivalent moles of ammonia are released. Hence in the present study, the ammonia liberated during the deamination process was evaluated and the enzyme activity was expressed as micromoles of ammonia released/ml/min.

The temperature of a system is tantamount to some extend a measure of the kinetic energy of the molecules in the system. Increase in temperature of a system results in the simultaneous increase in the kinetics of the enzyme reaction due to energetic collision between the enzyme and substrate molecules. As the temperature of the system is raised, the internal energy of the molecules of the system will increase. The internal energy includes transitional energy, vibration energy and rotational energy of the molecules. The energy involved in chemical bonding of the molecule as well as the energy involved in nonbonding interactions. If the chemical potential energy increases greater enough, some of the weak bonds that determine the 3D shape of the active protein may be broken. This could result in a thermal denaturation and inactivation of protein. Thus, too much heat can cause the enzyme catalyzed reaction to reduce as the enzyme or substrate becomes denatured and inactive. In the present study, the optimum temperature was found to be 30°C which corresponded to the maximum enzyme activity of 51 U/ml.
Optimum pH values for enzyme activity reflects the point at which bonds within them are influenced by H+ and OH- ions in such a way that the shape of their active site is the most complementary to the shape of their substrate i.e. at optimum pH the rate of reaction is at an optimum. Any change in pH above or below the optimum will quickly cause a decrease in the rate of the reaction, since more of the enzyme molecules will have operational sites whose shape are not complementary to the shape of their substrate. Slight changes in pH above or below the optimum do not cause a permanent change to enzyme, since the bonds can be reformed. However, extreme changes in the pH can cause enzymes to denature and permanently lose their function. In the present study, the optimum pH of 7 was recorded with maximum enzyme activity of 52 U/ml. Pterin deaminase from Rattus norvegicus liver was reported to have an optimum pH of 7. In Bacillus megaterium and Bacillus subtilis, the pterin deaminase enzyme was observed to exhibit an optimum pH of 7.3 [32]. The result of the present study is in accordance with earlier reports wherein alkaline pH of around 7 was optimum.

To predict the nature of the protein, inhibitor studies were performed with pure protein. The enzyme suffered as a result by EDTA at 10mM, 5mM and 1mM concentrations. Triton X 100 also partially inhibited the enzyme activity at 10mM concentration. EDTA disodium salt inhibited the enzyme activity which indicated that the enzyme may be a metalloenzyme [27]. Besides, this study added a further significant unique result of the protein structure of pterin deaminase in eukaryotic organisms M. indicus MTCC 3513. Sequence from reference (ID: gil22086854) was taken and blast against nucleotide sequence database (non-reductant) to find out similar region from zygomycetes. The sequence was modeled and submitted to interact with folic acid. Glide score obtained from the interaction studies revealed that the folic acid has study interaction towards modelled pterin deaminase structure. More recently, one of the authors Fan [33] determined a multidisciplinary approach that integrated homology modeling, molecular docking screens of a metabolite library and physical library screening by kinetic assays in Arad3529 from Agrobacterium radiobacter K84 regarding pterin deaminase.

5. Conclusions

The study highlighted the possibility of extracting pterin deaminase enzyme from Mucor indicus. The enzyme activity and specific activity of the partially purified enzyme has clearly reflected the role of chromatographic separation in protein purification. The current research also throws light on the influence of temperature and pH on the enzyme activity. Molecular docking studies with the predicted sequence showed higher binding affinity towards folic acid interaction. The structure of this protein may open the windows for new drug targets for cancer therapy.

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