In Vitro and *in Silico* Analysis of Recombinant Arginine Deiminase from *Pseudomonas Furukawaii* as a Potential Anticancer Enzyme

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Abstract

Arginine deiminase (ADI) is a promising anti-tumor enzyme that can be employed in amino acid deprivation therapy for the treatment of various arginine auxotrophic tumors. In our previous work, *Pseudomonas furukawaii* was identified as a potent producer of ADI with optimum activity at physiological pH and temperature. In this study ADI coding *arcA* gene was cloned and expressed in *E. coli* BL21. Recombinant ADI of *P. furukawaii* (PfADI) was purified using affinity chromatography and its molecular mass was estimated to be ~46KDa. PfADI was found to effectively inhibit the HepG2 cells with an IC_{50} value of 0.1950 IU/ml which is lower than the IC_{50} value of *Mycoplasma hominis* ADI (MhADI) which is in phase III clinical trials. The 3D structure of PfADI was modeled. Immunoinformatics analysis was also carried out to compare the immunogenicity of PfADI with MhADI. The PfADI was found to be less immunogenic in terms of number of linear and conformational B cell epitopes and T cell epitope density. The overall antigenicity and allergenicity of PfADI was also lower as compared to MhADI. Thus the present *in vitro* and *in silico* studies establish PfADI as a potential anticancer drug candidate with improved efficacy and low immunogenicity.

Highlights

- Arginine deiminase (ADI) of *Pseudomonas furukawaii* was cloned and expressed in *E coli*
- *In vitro* anticancer efficacy of rADI was better than ADI in clinical trials
- *In silico* analysis predicted low immunogenicity and allergenicity of rADI

Introduction

Arginine deiminase (ADI, EC 3.5.3.6) is an arginine catabolizing hydrolase that catalyzes the conversion of L-arginine into L-citrulline and ammonia [1]. ADI is the major enzyme of the arginine deiminase pathway in prokaryotes that serve as a non-glycolytic pathway for energy generation [2]. Besides energy production and arginine catabolism, ADI also protects the bacteria from acidic environment by generation of ammonia [3]. The enzyme is widely present in bacteria and few lower protozoa but has not been reported in mammals [4] .

ADI has gained wide importance in the last three decades and emerged as an important therapeutic agent for the treatment of arginine auxotrophic cancers via amino acid deprivation therapy [5]. Arginine is non-essential amino acid for humans (essential for neonates); it is synthesized in the urea cycle with the help of enzymes arginine succinate synthetase (ASS) and arginine succinate lyase (ASL) [6]. However, certain tumors lack these enzymes and rely on surrounding cells for the supply of amino acid arginine. This difference in physiology of tumor cells from normal cells is harnessed in the treatment of such arginine auxotrophic tumors using ADI. ADI depletes arginine in these tumors and consequently the tumor recedes mainly due to protein starvation [7, 8]. The ADI also induces both caspase dependent and independent pathways to inhibit the proliferation of tumor cells [9] .

The anticancer activity of ADI from various sources *viz. Mycoplasma arginini, Mycoplasma hominis, Pseudomonas plecoglossicida, Pseudomonas aeruginosa, Lactobacillus lactis* have been reported earlier [2]. Currently ADI-PEG (pegylated ADI) from *Mycoplasma* is in the late-stage clinical development for the treatment of hepatocellular carcinoma (HCC), melanoma and mesothelioma (NCT01287585, NCT00450372, NCT02709512). Polaris pharmaceuticals, a leading biopharmaceutical company, holds the world-wide rights for ADI-PEG named Pegargiminase (http://polarispharma.com/). In spite of promising preclinical results, the efficacy and safety of *Mycoplasma* ADI is limited due to the immunogenic and allergic reactions. *Mycoplasma* ADI treatment is reported to elicit hypersensitivity reactions ranging from local and systemic allergy to anaphylactic shock [10, 11]. Although the

enzyme is pegylated, PEG has its own limitations and it reduces the overall efficacy of the enzyme. It was observed that drug clearance and toxicity was enhanced due to production of anti-PEG antibodies while using pegylated therapeutic enzymes asparaginase and uricase [12, 13]. Hence, to circumvent these hurdles, it is of great interest to find a suitable alternative ADI with high activity and low immunogenicity.

With the purpose of identifying potent anticancer ADI, in our previous work, we have screened bacterial isolates from environmental samples and identified *Pseudomonas furukawaii* as an alternate source of ADI with optimum activity at human physiological conditions [14]. In this study, the *arc*A gene (gene coding ADI) of *P. furukawaii* was cloned and expressed in *Escherichia coli.* The recombinant *P. furukawaii* ADI (PfADI) was purified and its *in vitro* anticancer activity was assessed. The 3D structure of the enzymatic protein was predicted, and the sequence-structure analysis was performed to identify the putative antigenic epitopes. The immunoinformatics analysis was carried out to compare the immunogenicity and allergenicity of PfADI with the ADI from *M. hominis* (MhADI) (currently in clinical trial) in order to ascertain the potential of PfADI as an anticancer agent.

Materials And Methods

Unless specified all the chemicals and reagents are obtained from HiMedia laboratories (Mumbai).

Cloning of ADI and sequence analysis of *P. furukawaii arc*A gene:

The *arc*A gene coding for ADI enzyme in *P. furukawaii* cells was amplified by polymerase chain reaction (PCR). Primers (Forward primer- 5'ATATCCATGGCGATGTCCAAAGTCAAACTCGG3' and reverse primer- 5'ATTACTCGAGGTAGTCGATCGGATCGCGG3') were designed and the PCR amplification was carried out using Phusion® (Thermo Scientific) and 2% DMSO in the MJ Mini thermal cycler (Bio-Rad). The reaction conditions were as follow: Initial denaturation at 98˚C for 5 minutes followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 58˚C for 45 seconds, extension at 72˚C for 45 seconds with a final extension at 72˚C for 5 minutes.

The amplified fragment was cloned in pET-28a (+) vector at *Nco*I-*Xho*I restriction sites using standard cloning procedures. Fast digest *Nco*I and *Xho*I were obtained from Thermo Scientific (Waltham, MA, USA). The resultant recombinant plasmids were transformed in competent *Escherichia coli* DH5α cells. Colony PCR was performed to screen the colonies with the construct. The positive clones were also confirmed by double digestion of the plasmid isolated from the colonies. The cloning was further confirmed by automated dideoxy DNA sequencing and the homologies of the nucleotide sequences was analyzed using NCBI BLAST.

Construction of phylogenetic tree:

Phylogenetic tree of ADI sequences (retrieved from UniProt https://www.uniprot.org/) of 19 *Pseudomonas* spp. and 2 *Mycoplasma* spp. (Table S1) was constructed by UPGMA method [15] using MEGA X software [16]. The distances were calculated by the Poisson correction method [17].

Expression of PfADI in heterologous host:

pET-*arc*A construct was transformed into *E. coli* BL21 cultured at 37 ℃ in Luria Bertani broth containing kanamycin (50 μg mL^{-1}). The induction conditions for the recombinant PfADI expression were optimized and performed with 1 mM isopropyl β-D-thio-galactopyranoside (IPTG) at $O.D._{600nm}$~ 0.6. The induction was carried out for 6h. The expression of recombinant PfADI was checked on SDS-PAGE and later confirmed by western blotting.

Purification of recombinant PfADI:

Recombinant PfADI was over-expressed as inclusion bodies in the cytoplasm which was purified with the help of Ni^{2+}-NTA affinity chromatography using the manufacturer's guidelines (Qiagen, Germany). The *E. coli* cells with induced recombinant PfADI were harvested and the pellet was dissolved in 8M urea and incubated at room temperature for 2-3 hours till the solution became clear, this step was followed by centrifugation. The pellet was discarded, and the supernatant was incubated with Ni^{2+}-NTA slurry previously equilibrated with a lysis buffer overnight for binding. The bound slurry was passed through the column and the flow through was collected. On column renaturation of protein was performed by decreasing gradient of urea (8M to 0M). The purification steps following renaturation were carried out at 4°C. Later the protein was washed with 20mM and 40mM imidazole and eluted at 200 mM-500mM imidazole concentration in the elution buffer.

Enzyme Assay:

In order to determine PfADI activity, the enzyme assay was performed using the method described by De Angelis and coworkers with certain modifications [18]. The reaction mixture was prepared by adding 150 µl of purified recombinant PfADI into 150 µl of 50 mM substrate (L-arginine) and 1.85 ml of 50 mM acetate buffer. The mixture was then placed in a water bath at 37 °C for a duration of 1 h. The enzymatic reaction was stopped at the end of 1h by adding 2N HCl (500µl)). After the completion of the reaction, the mixture was centrifuged and 100µl of supernatant was taken for the next step i.e. color development. The development of color determines the amount of product (L-citrulline) formation. Color is developed using DAMO-TSC (diacetyl monoxime-thiosemicarbazide) method [19]. In the color development step, 100µl of the previously mentioned supernatant was added in a test tube containing 2 ml acid-ferric solution and 1 ml of DAMO-TSC solution. The mixture was vortexed thoroughly and kept at 100 °C for 10 min. The developed color was analyzed using spectrophotometer by measuring absorbance at 520 nm. 1 U of ADI is defined as the the amount of enzyme required to catalyze the conversion of one micro mole of substrate (L-arginine) into one micro mole of product (L-citrulline) in one minute under the standardized conditions. The amount of protein was quantified using Bradford's assay and the specific enzyme activity was evaluated.

***In vitro* anticancer activity:**

Recombinant PfADI was tested for its anticancer activity on HCC cell lines HepG2. HepG2 cells were procured from ATCC. The cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (fetal bovine serum from invitrogen), 1% antibiotic solution (PenStrep from invitrogen) in 5% CO_2 atmosphere at 37 ^oC. Trypsin-EDTA solution was used to dissociate the cells. The previously cultured cells were adjusted to a cell count of 1.0 x 10^5 cells/ml using DMEM supplemented with 2% FBS. 1 X 10^4 cells/well were seeded in 96 well microtiter plate and was incubated for 24h for the formation of a partial monolayer of cells. The media was removed after 24 h and 100 µl of different concentrations of rPfADI was dispensed in the wells of the microtiter plates followed by an incubation for 72h at 37 ^oC in 5% CO_2 atmosphere. After 72 h, the recombinant PfADI solutions from each well was discarded and 100 µl of MTT solution was added. The plates were again incubated for 4 h. After incubation the supernatant was flicked off and 100 µl of DMSO was added to each well and a gentle shake was given to solubilize the formazan. The absorbance at 590 nm was determined using a microplate reader. The % growth inhibition was evaluated using the given formula and IC_{50} value of recombinant PfADI for the inhibition of HepG2 cells was determined from the dose-response curve computed using GraphPad prism 9. Doxorubicin was used as the control drug.

% Inhibition = (OD_{590} of Control – OD_{590} of sample)/OD_{590} of Control) x 100.

Protein structure prediction of PfADI:

SWISS-MODEL server (https://swissmodel.expasy.org/) was used to predict the 3D structure of PfADI. To predict the structure, the amino acid sequence corresponding to the cloned *arc*A gene (GenBank Accession MK318561, deposited by the authors) of *P furukawaii* was used. The sequence length of PfADI consisted of 416 amino acids. The crystal structure of ADI from *Pseudomonas aeruginosa* (PDB code_2ACI) was used as a template for building the model. The quality of the predicted 3D structure of recombinant ADI was validated by Ramachandran plot obtained for the model (URL: https://swissmodel.expasy.org/assess).

Immunoinformatics analysis for antigenicity prediction

Sequence and structural data: The amino acid sequence of PfADI (416 amino acids) and its modeled 3-D structure were used for immunoinformatics analysis. MhADI amino acid sequence was retrieved from UniProtKB (https://www.uniprot.org/uniprot/P41141) and used for comparative studies.

Prediction of antigenicity and allergenicity: The antigenicity was predicted using VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). VexiJen uses alignment independent approach to predict the antigenicity of the whole protein [20] . The allergenicity prediction was performed using AlgPred (http://crdd.osdd.net/raghava/algpred/) server [21] . The antigenicity and allergenicity of PfADI were compared with MhADI.

T-cell epitope prediction: The MHC-II binding T cell epitopes were predicted using Immune Epitope Database (IEDB) server (http://tools.iedb.org/mhcii/). The reference alleles were chosen on the basis of their wide global frequency. A total of 8 HLA-DRB1 alleles (*01:01,*03:01, *04:01, *07:01, *08:01, *11:01,*13:01, and *15:01) were used to predict the T cell epitopes [22, 23] . The epitope density was obtained by calculating the relative frequency of the predicted epitopes. Relative frequency (fi)= ni/N , where ni is the number of epitopes within the threshold (percentile values ≤ 10) and N represents the total number of predicted epitopes.

B-cell epitope prediction: Linear or continuous B cell epitopes were predicted using BepiPred server (http://tools.iedb.org/bcell/). Conformational B cell epitopes were predicted using modeled PfADI 3D structure. BEpro (previously known as PEPITO) (http://pepito.proteomics.ics.uci.edu/) and DiscoTope2.0 tool (http://www.cbs.dtu.dk/services/DiscoTope/) were employed for the prediction of conformational epitopes [24, 25]. While using BEpro residues with epitope score ≥1 were regarded as conformational B cell epitopes, a threshold of -3.7 was used for prediction by DiscoTope2.0 server.

Results

Cloning of ADI and sequence analysis of *arc*A:

A 1251 bp fragment of *arc*A gene coding for arginine deiminase was amplified from *P. furukawaii* genome.The gene product was purified and ligated into pET28a vectors and was transformed into competent *E. coli* DH5α cells. Colony PCR was performed to screen the transformants, the gene of interest was observed in all the positive clones. Restriction digestion of the plasmid isolated from the colony PCR positive clones with *Nco*I-*Xho*I enzymes further confirmed cloning of *arc*A in pET28a (+) as a fall out was observed at 1251 bp along with the linearized vector backbone of 5.4 k bp. The clones were further verified by automated DNA sequencing and the sequence is submitted in Genbank under accession no. MK318561.

Homology analysis of this gene using BLAST tool showed 100% identity with annotated *arc*A gene present in the complete genome sequence of *P. furukawaii* (Accession no. AP014862.1). The other related sequences were *arc*A gene from *P. resinovorans* (Accession no. AP013068.1), *P. otitidis* (Accession no. AP022642.1), and *P. aeruginosa* (Accession no. CP053917.1) depicting 91.35%, 90.65% and 86.96% similarity respectively.

The UPGMA tree was constructed using ADI sequences from related *Pseudomonas* spp. and two *Mycoplasma* spp. formed three major clusters (Fig 2). *M. hominis* and *M. arginini* formed a distinct group and were most distantly related to the *Pseudomonas* clusters. *P. furukawaii* was most closely related to *P. resinovorans* Amongst the *Pseudomonas* spp. ADI has been experimentally characterized and shown to have anticancer properties in *P. furukawaii* (present study), *P. aeruginosa* [26] and *P. plecoglossicida* [27]. These three species cluster together in Group I. It may be worthwhile to investigate the ADI of the closely related species (*P. putida*, *P. citronellolis* and *P. resinovorans*) for potent anticancer activity.

Expression and purification of recombinant PfADI:

The pET-*arc*A construct was transformed into competent expression host *E. coli* BL21 (DE3). The transformants were successfully induced using 1mM IPTG and the induced protein was visualized on SDS-PAGE. The PfADI was over-expressed in *E. coli* under the control of strong T7 promoter as inclusion bodies. The recombinant PfADI was purified from inclusion bodies using Ni^{2+}-NTA affinity chromatography. The purified ADI protein fractionated on 12% SDS–PAGE and was observed as single band at ~46 kDa (Fig 1A). The recombinant PfADI was purified with a specific enzyme activity of 1.9 IU/ml. Western blotting was done to confirm the presence of His-tagged recombinant ADI using anti-histidine antibodies. A specific band at ~46kda was observed in the induced sample (Fig 1B).

***In vitro* anticancer activity:**

The growth inhibitory effect of recombinant PfADI was tested *in vitro* on HepG2 cells. The anti-proliferative activity was evident by microscopic examination. HepG2 cells were treated with recombinant PfADI in a dose dependent manner. MTT assay was performed and IC_{50} value was determined based on sigmoidal dose response curve. Recombinant PfADI showed IC_{50} value of 0.1950 IU/ml equivalent to 0.007 µg/ml (Fig 3) against HepG2 cells whereas the standard Doxorubicin (drug control) showed an IC_{50} value of 18.71µM.

Protein structure prediction of PfADI:

The three-dimensional structure of PfADI, predicted using SWISS-MODEL server showed tetrameric subunits and the conserved catalytic triad (Glu 222, His 276, Cys 404) typical of prokaryotic ADIs (Fig 4). The template corresponding to the crystal structure of *P. aeruginosa* (PDB code_2ACI) which showed the highest similarity was used to build the model. Ramachandran favored residues for the predicted 3D structure of PfADI by homology modeling was 94.5% and MolProbity score was 1.56 (MolProbity version 4.4) indicating the robustness of the model (Fig. S1). The predicted 3D structure was further used for immunogenicity analysis.

Comparative immunoinformatics analysis of PfADI and MhADI

Prediction of overall antigenicity and allergenicity: Immunogenicity was predicted using VaxiJen server and the antigen probability score for PfADI was found to be less (0.3098) as compared to the predicted score for MhADI (0.4229) (Table 1). The comparison of the allergenicity showed a similar pattern as predicted by the amino acid composition based SVM module via AlgPred server. The PfADI was shown to be non-Allergen (Score=-1.15579) whereas MhADI was found to be an allergen with the allergenicity score -0.313383 at the threshold of -0.4.

B cell epitope prediction: B cell epitopes were predicted to assess the intensity of humoral immune response against the enzyme. Linear B cell epitopes were predicted using BepiPred. A total of 13 linear epitopes of varied length were predicted (Table 2). The discontinuous or conformational epitopes were also predicted using DiscoTope 2.0 and BEpro servers. Five residues were predicted as conformational epitopes by DiscoTope 2.0 and BEpro identified 19 conformational epitopes (including the 5 predicted by DiscoTope 2.0). Thus, 19 residues out of the total 416 residues were recognized as conformational epitopes (Table 1). The B cell epitopes of MhADI were also analysed using the same tools. 15 linear and 32 conformational epitopes were predicted for MhADI [28]. The linear and conformational B cell epitopes of PfADI are listed in table 2. The various parameters like IEDB score, hydrophilicity and surface accessibility are also mentioned in order to provide a quick reference for future mutagenesis studies for enzyme improvement.

T cell epitope prediction: MHC-II binding T cell epitopes corresponding to the eight global alleles were predicted using IEDB server. The obtained data was used to calculate the relative frequency in order to estimate the epitope density. The epitope density for MhADI was also calculated in the similar manner. The T-cell epitope density was also low (0.0677) for PfADI as compared to MhADI (0.1044) (Table 1).

Discussion

The present study was undertaken to determine the potential of recombinant L-arginine deiminase from *P. furukawaii* as an anticancer agent. Previously, we have screened 143 ADI producing isolates from pond water and soil samples from different districts of Haryana and Delhi, India. The isolate RS3 which was identified as *P. furukawaii* showed maximum activity at physiological pH and temperature and it was chosen for further studies [14]. In the current study, *arc*A gene coding for PfADI was cloned in *E. coli* with the aim to enhance and ease the production and purification of ADI for assessing its anti-cancerous activity. Further, computational tools were employed to reveal its structure and to predict immunogenic properties. The sequence-structure based immunogenic properties of PfADI were also compared with the ADI currently in the clinical trial (MhADI) to evaluate its suitability for ADI based therapeutics.

ADI genes from various organisms viz *Streptococcus sanguis, lactococcus lactis, Enterococcus faecalis, Mycoplasma arginini* have been cloned and over-expressed in *E. coli* [3, 29, 30, 31] with diverse aims such as to understand its importance in cell growth, arginine metabolism or the role as anticancer candidate [32]. The arcA gene from two species of genus *Pseudomonas* namely *P. aeruginosa* and *P. plecoglossicida* have also been cloned in the *E. coli* host [27, 33]. As compared to the 1251 bp arcA gene in the present study of *P. furukawaii*, the arcA gene in *Enterococcus faecalis* and *Lactococcus lactis* are 1260 and 1399 bp long respectively while in *P. plecoglossicida* CGMCC2039 is 1,254-bp fragment long. The arcA gene in *P. aeruginosa* is 1257 bp long and it codes for ADI composed of 418 amino acids.

The ADI reported previously in other organisms contain almost similar number of amino acids ranging from 406 to 420 amino acids [3]. Our study was also in accordance with these findings and the arcA gene coding a protein of 416 amino acids was observed. However, the molecular weight of ADI differs significantly among different organisms due to the difference in oligomerization pattern of the various ADIs in their native form [3]. ADI of *M. arginini* exists as homo-dimeric form with a molecular weight of 90 kDa whereas the 3-D structure analysis revealed that *P. aeruginosa* arcA folds into a homo-tetramer with a molecular weight of 184 kDa [32]. In the present study ~46kDa band of purified recombinant PfADI band was observed on SDS-PAGE analysis. The oligomerization pattern of *P. furukawaii* ADI is yet to be elucidated.

After the expression and purification, the *in vitro* anticancer efficacy of recombinant PfADI was tested. The success of microbial L-asparaginase as a part of first line therapy for the treatment of acute lymphoblastic leukemia have accelerated the research for the use of other microbial enzymes in cancer treatment [34]. Out of all other arginine degrading enzymes like arginine decarboxylase and arginase, ADI is preferred because of its high substrate affinity, high Vmax, and better stability at physiological conditions [1]. The arginine auxotrophy is prevalent in tumor cells owing to a significant contribution of L-arginine pool in protein synthesis (~40% of total protein synthesis) (Shen et al. 2006). Various cancers like HCC, sarcoma, melanoma, leukemia, retinoblastoma, adenocarcinoma, non-Hodgkin's lymphoma etc are reported to be auxotrophic to arginine [26, 7]. In the present study the anticancer efficacy of rADI was tested *in vitro* against HCC cell lines, HepG2. The rADI exhibited significant anti-proliferation activity against the tested cell lines with IC_{50} value of 0.1950 IU/ml corresponding to a protein concentration of 0.007 µg/ml. Ensor and co workers have earlier tested the *in vitro* anticancer activity of rMhADI against 23 melanoma and 16 HCC cell lines. All the cell lines were sensitive towards ADI treatment. The 50% growth inhibition of melanoma cell lines was observed in the concentration range of 0.01 µg/ml to 0.3 µg/ml, where as the IC_{50} value for HCC cell lines varied from 0.03 µg/ml to less than 0.01 µg/ml. The inhibition of HepG2 was observed at IC_{50} value of 0.01 µg/ml [35]. Thus *in vitro* anticancer efficacy of PfADI (IC_{50} 0.007 µg/ml) was found to be better as compared to with MhADI activity (IC_{50} 0.01 µg/ml) against the HCC cell line HepG2. However the experimental conditions and the cell viability assays were different in the two experiments. Previously, partially purified ADI of *P. plecoglossicida* was also used to inhibit growth of HepG2 cell lines. ADI activity of 0.05 U/ml inhibited the growth of HepG2 cells by 60% where as 93.4% inhibition rate was observed by 0.5 U/ml ADI [36]. In another study, in *vitro* efficacy of recombinant ADI from *M. hominis* showed an IC50 value of 0.036 U/ml on melanoma cell lines G-361[37].

It is important to mention here that the rate of inhibition of the cancer cell lines differs not only because of the source of the enzyme but also it differs with different types of cancers. Further, the inhibitory activity of the same enzyme also varies for the different cell lines of the same type of cancer. Such observation is because of the discrepancy in the expression of enzyme arginosuccinate synthase. As mentioned earlier, the absence of ASS1 is the hallmark for arginine auxotrophy. McAlpine et al performed the western blot of ASS1 protein in various HCC cell lines and then analysed the band intensity in order to understand the level of ASS1 expression in these cell lines [38]. The study designated all the cell lines as ASS1 high, medium, low or negative depending on the level of ASSI expression. The Sk-Hep1 cell line was ASS1 negative, HepG2 cells showed medium level expression of ASS1 whereas Malhavu has high ASS1. It was observed that the inhibition rate of these cell lines was inversely proportional to the ASS1 levels inside them. A concentration of 5 µ g/mL ADI-PEG20 inhibited ASS1-negative Sk-Hep1 cell line by 95-90%, 50% inhibition was observed in HepG2 cells, while ASS1-high Malhavu cells were fully resistant to ADI treatment. The recombinant PfADI showed significant activity against a medium level ASSI expressing cell line (HepG2), and thus is expected to show even better results with low or nil ASS1 expressing tumors. Hence, extensive studies are required to explore a spectrum of cancer types with low or nil ASS1 levels that can be targeted via AADT using ADI.

Immunogenicity and allergenicity are the major problems associated with the protein therapeutics. Thus, for the development of PfADI as a therapeutic protein accessing its immunogenicity is an important prerequisite. In the present study we used *in silico* immunoinformatics approach to analyse the antigenicity and allergenicity of the PfADI and compared it to MhADI which is under phase III clinical trials. In contrast to the time consuming and expensive experimental techniques, bioinformatics offer many algorithms which are publically available and are highly preferred for the immunogenic and allergenic predictions [39]. Previously, other therapeutic enzymes like asparaginase and uricase were subject to immunoinformatics analysis and the results are in line with the experimental observations [40, 41]. In the present study, the antigenicity of PfADI was found to be less than MhADI

using several prediction softwares. *In silico* analysis for allergenicity prediction showed PfADI as a non-allergen and thus suitable to be used as protein therapeutics.

The linear and conformational B cell epitopes were also predicted for both the ADIs. B cell epitopes represent the precise region of the protein where the paratope of the antibodies generated by the host immune system binds. In the present study it was observed that there are fewer B cell epitopes in PfADI as compared to MhADI. This observation indicates that there is less probability of interaction between PfADI and antibodies as compared to MhADI, hence PfADI is expected to be more stable in the human host.

Another measure of host immunogenic reaction is estimating the T-cell epitope density. The experimental reports on different proteins have confirmed that the response of the immune system is directly proportional to the epitope density [41, 42]. Thus, a higher T cell epitope density of MhADI with respect to MHC-II binding molecules indicates a high rate of immunogenic reaction in comparison to PfADI. Hence the preliminary analysis suggests that the PfADI is superior to the ADI in the clinical trials as it is low immunogenic and non-allergic while having the promising anticancer efficacy *in vitro*. Thus with further investigation and detailed study PfADI could be developed as an suitable alternative to the MhADI, similar to the *Erwinia chrysanthemi* asparaginase which was discovered as an alternative to the commonly used *E. coli* asparaginase, as some patients were found to develop allergic reaction against the latter [34] .

In conclusion, we have obtained a recombinant ADI from *P. furukawaii* which can be a promising anti-tumor agent in arginine deprivation therapy for cancer treatment. This novel PfADI offers lower immunogenicity and allergenicity as compared to the MhADI which is in the clinical trials. PfADI can not only act as an alternative to MhADI, its consecutive administration along with MhADI might help in reducing the immunogenic response due to the variable antigenic properties of both the enzymes. Further, our *in silico* predictions of the T cell and B cell epitopes of ADI from *P. furukawaii* can provide a framework for designing mutagenesis experiments in order to deimmunize the protein further.

Declarations

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Data availability statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contribution statement: PG, RD, VG and AM conceived and planned the experiments. RD and VG performed the experiments. PG supervised the project and received the funding. RD wrote the manuscript. AM did the supervision of bioinformatics investigation. PG,AM and VG did the reviewing and editing.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1: Comparative immunoinformatics analysis of arginine deiminase from *P furukawaii* and *M hominis*

Sr No.	Organism	Allergenicity*	Overall immunogenicity (VaxiJen score)	B cell epitope			T-cell epitope density
				Linear epitopes	Average IDEB Score	Conformational epitopes	
1.	*P furukawaii*	Non-Allergen Score=-1.1557941	0.3098	13	0.477	19	0.0677
2.	*M hominis*	Allergen Score= -0.31338318	0.4229	15	0.472	32	0.1044

*[Threshold=-0.4]

Table 2: Linear and Conformational B cell epitopes of PfADI.

Linear B cell epitopes					
Sr No.	Residue No	Epitope			
1	10-13	SEAG			
2	25-48	LAHTRLTPNNCDELLFDDVIWVSQ			
3	50-51	KR			
4	54	F			
5	79	Q			
6	81-86	KDALKW			
7	92-119	ITNDQVGVGLVNEVRSWIEGLEPRKIAE			
8	122-155	IGGVAGSDLPESEGVSAIKMYRDYLGHSSFILPP			
9	180-184	WPARR			
10	196-219	FHPVFTGAEFEVWYGDPDKDHGMS			
11	296-322	VSEIVPFVLRPDESRPYGIDIRREEKD			
12	332-354	GLKQLRVVQTGGDAFEAEREQWD			
13	374-375	TY			
Conformational B cell epitopes					
Sr No.	Residue No	Residue	IEDB score	Hydrophilicity	Surface accessibility
1	62	E	0.291	2.814	1.153
2	81	K	0.059	2.557	1.85
3	111	G	0.224	-0.543	0.634
4	113	E	0.377	3.443	2.044
5	132	E	1.471	4.386	1.211
6	181	P	0.515	-0.5	2.358
7	213	D	1.95	6.514	4.68
8	214	K	1.896	6.514	2.773
9	215	D	1.724	4.486	1.775
10	216	H	1.383	5.114	1.424
11	217	G	0.955	4.429	1.028
12	307	D	1.11	3.657	5.49
13	308	E	1.262	5.271	4.334
14	309	S	1.74	4.4	4.392

15	310	R	1.418	4.914	2.603
16	311	P	1.448	2.343	1.053
17	320	E	-0.009	4.357	4.057
18	348	A	1.056	3.229	2.125
19	349	E	0.684	3.786	4.25

Figures

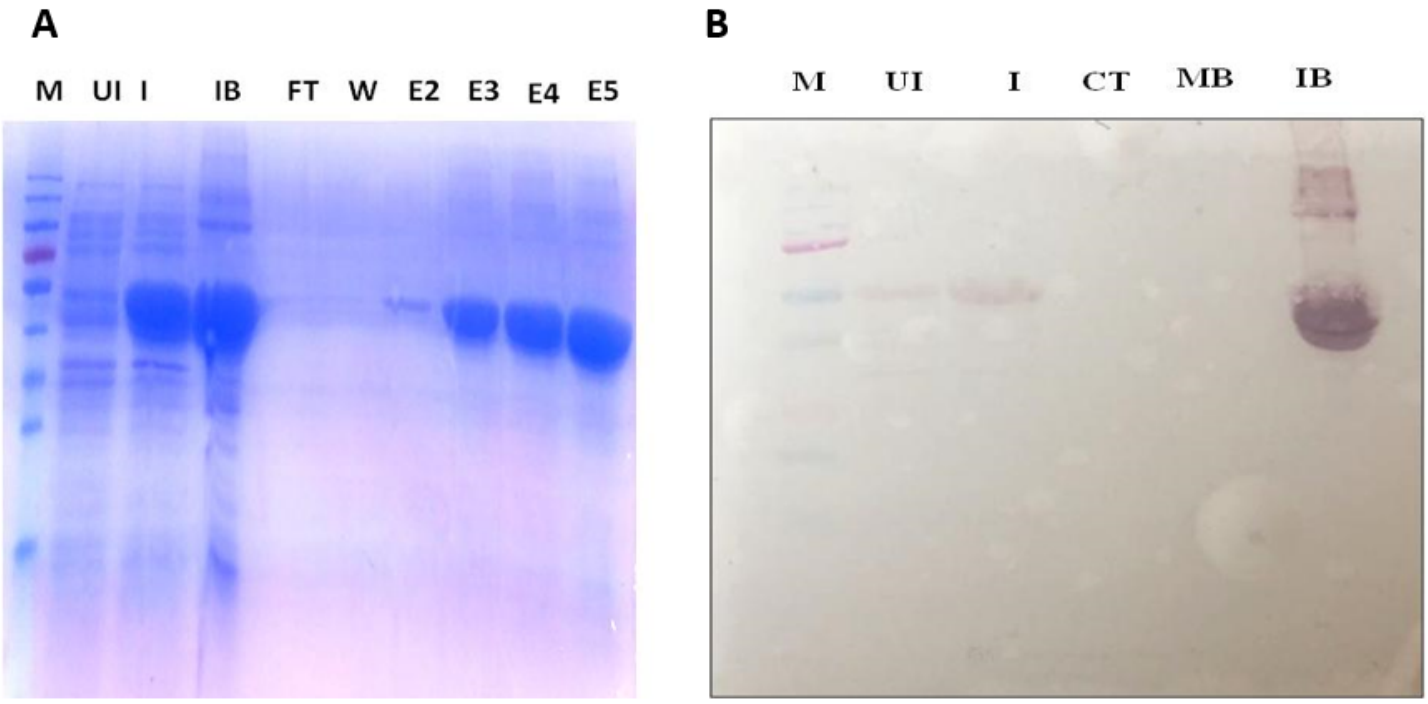

Figure 1

Purification of recombinant PfADI and confirmation using western blotting. A. SDS-PAGE gel showing purification of protein from inclusion bodies B. Western blot. (M- protein marker, UI- uninduced, I-induced, CT- cytoplasmic, MB- membrane bound, IB- inclusion bodies. FT- flow through, W -wash, E2-E5- elutions).

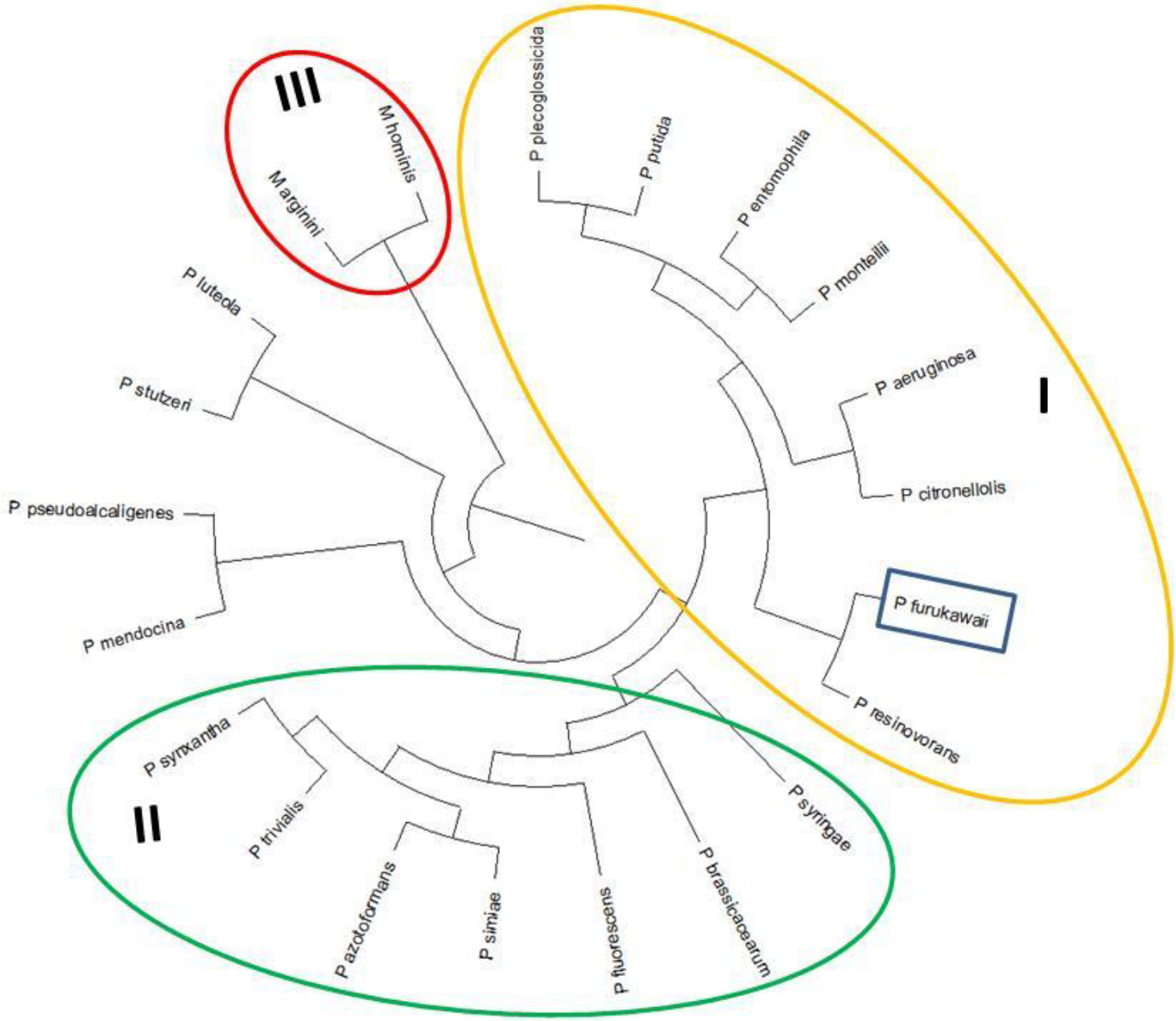

Figure 2

Phylogenetic tree based on Arginine deiminase sequences showing three distinct clusters I, II and III.

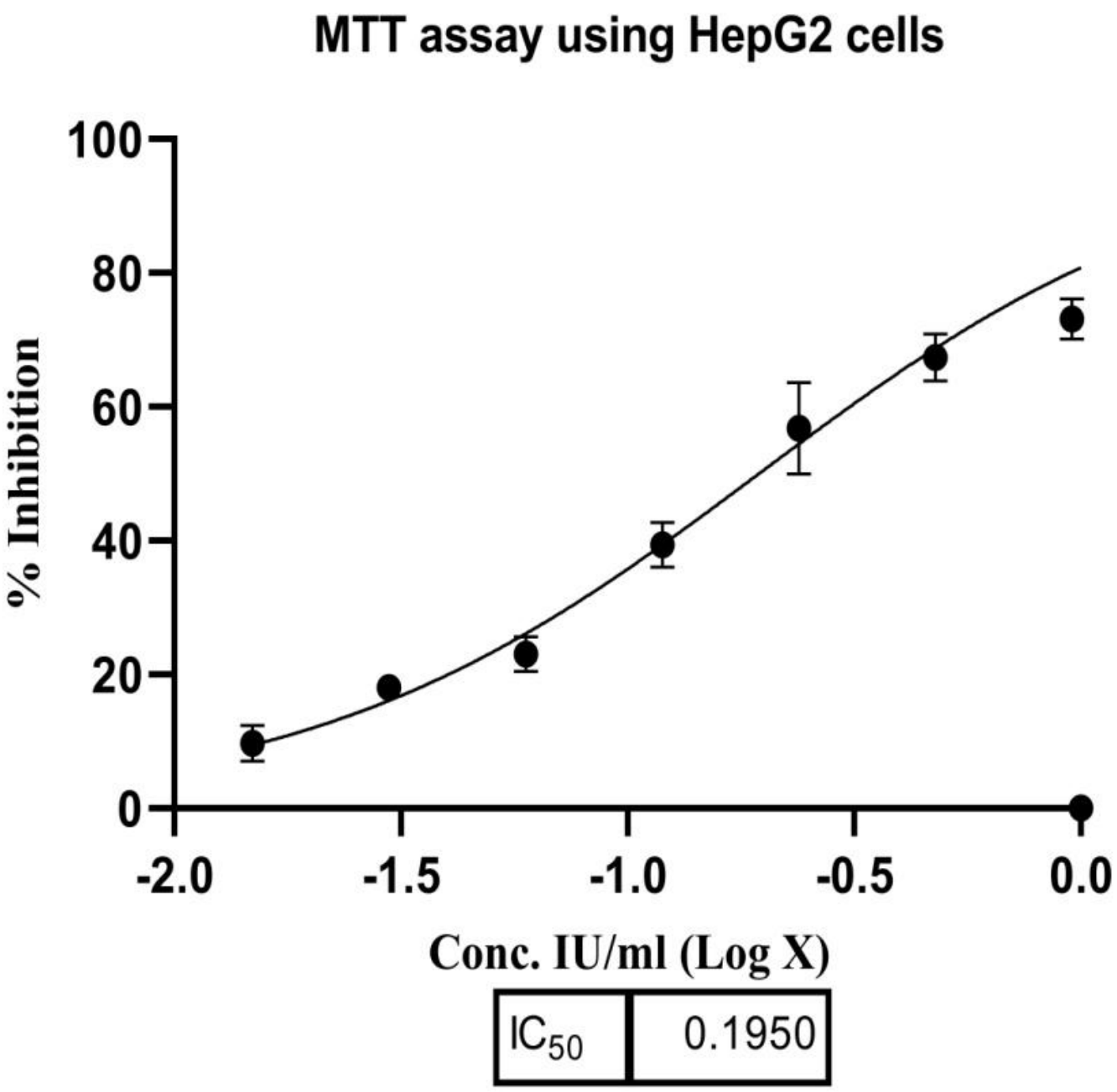

Figure 3

Dose response curve for the inhibitory action of rADI on HepG2 cells

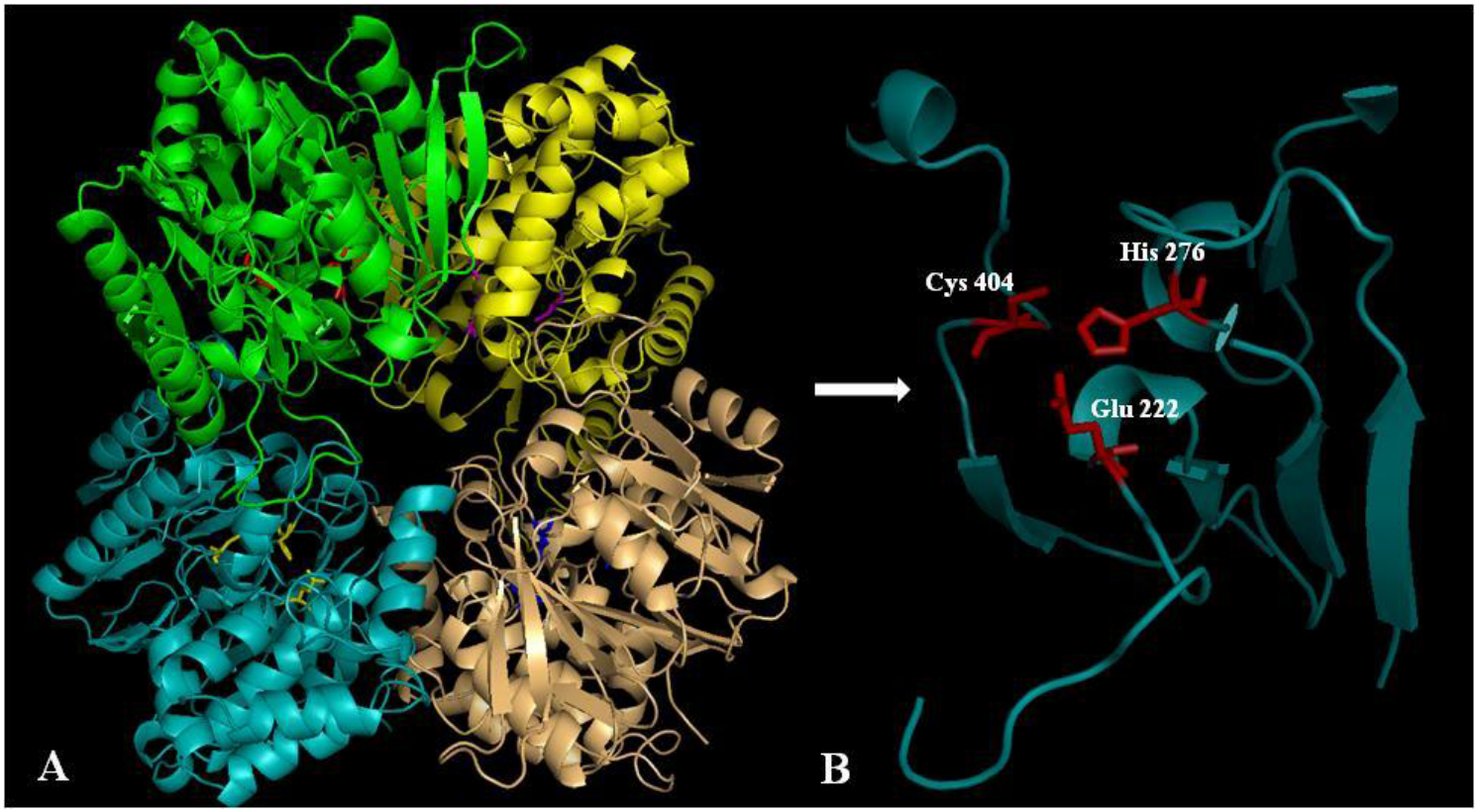

Figure 4

Homology model of Arginine deiminase of Pseudomonas furukawaii showing (A) the tetrameric subunits; (B) conserved catalytic triad (Cys-His-Glu)

Supplementary Files

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