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Evaluation of *in vitro* Anti-proliferative Activity of L-arginine deiminase from Novel Marine Bacterial Isolate

Rahamat Unissa[1,2*], M. Sudhakar[1,2] and A. Sunil Kumar Reddy[3]

[1]Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Malla Reddy College of Pharmacy, Maisammaguda, Dhulapally, Secunderabad, Telangana, India.
[2]Osmania University, Telangana, India.
[3]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Bharat Institute of Technology Pharmacy, Jawaharlal Nehru Technological University of Hyderabad, Ibrahimpatnam, Hyderabad, Telangana, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author RU designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors RU, MS, ASKR managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

L-Arginine deiminase is a therapeutic l-arginine depleter found to counteract various arginine auxotrophic cancer cells (do not express ASS/OCT). The aim of the present study was to evaluate the anti -proliferative activity of the purified l-arginine deiminase from *Vibrio alginolyticus* 1374. Production of the enzyme was carried out by shake flask method under optimal conditions. The enzyme thus produced was purified to near homogeneity by ammonium sulphate fractionation followed by ion exchange and gel filtration chromatography. The enzyme was purified to 529.43 fold and showed final specific activity of 280.6 IU/mg with 43.5% yield. SDS-PAGE revealed that

**Corresponding author: E-mail: srunissa@gmail.com, syedaunissa37@gmail.com;*

the purified enzyme had a molecular weight of 48 kDa. The purified enzyme tested against A375-C6, MCF-7, HCT-113 and Jurkat, clone E6-1cell lines showed I.C.50 value of about 5. 21, 6.3,8 and 3.13 U/ml respectively. ADI obtained from *Vibrio alginolyticus* 1374 has several beneficial properties needed for its use as a therapeutic and industrial enzyme. It was active over wide range of pH (4-10) and temperature (25°-50℃), had high s ubstrate specificity etc. Moreover it showed broad range of activity against cancer cells lines of human origin. Further animal model studies must be carried out to develop ADI as a potential drug.

Keywords: Vibrio alginolyticus 1374; ornithine carbamoyl transferase; argininosuccinate synthetase; A375-C6; MCF-7; HCT-113 and jurkat; clone E6-1cell lines.

ABBREVIATIONS

OCT : Ornithine carbamoyl transferase
ASS : Argininosuccinate synthetase,
ADI : L-Arginine deiminase,
IEC : Ion Exchange Chromatography,
GFC : Gel Filtration Chromatography.
DAMO : Diacetyl monoxime
TSC : Thiosemicarbazide
A375-C6 : Melanoma cell lines
MCF-7 : Breast cancer cell lines
HCT-116 : 6Colon cancer cell lines

1. INTRODUCTION

Cancer is a major public health problem, causing approximately 7 millions of deaths every year globally [1,2]. More than 80% of cancer deaths are due to carcinoma in lung, breast, prostate, colorectal and pancreas [3]. Lung cancer and colorectal cancers are responsible for the first and third most cancer related deaths in men and women. Breast cancer in women and prostate cancer in men rank second [4].

Among various treatments available, nutrient depletion therapy is also equally effective. Most of the cancer cells require continuous supply of l-arginine for their growth and survival [5]. They use these amino acids as a source of nitrogen for the synthesis of their cell components. In experiments where l-arginine metabolism in cancer cells has been specifically compared with that in normal cells of the same origin, l-arginine metabolism in the cancer cells has been found to be considerably faster. Hence demand for l-arginine (nutrient) is high in case of cancer cells [5-7]. Depletion of which starve them to death.

Exploration of novel marine bacteria for therapeutic compounds has been notable in the past few years. A very limited literature is available on marine bacteria and its production of l-arginine deiminase. Although ADI production has been reported from many microbial sources, amounts obtained from them were inadequate. Moreover, they were associated with problems of incompatibility. Therapeutic enzymes obtained from terrestrial bacterial sources, which is used currently for the treatment of leukemia is known to cause several side effects and hence there is need for an alternative enzyme drug that is compatible to human blood and immunologically induce less or no side effects in the patient [8].

In this context, considering the fact that marine environment, particularly sea water, which is saline in nature and chemically closer to human blood plasma, it is anticipated that they could provide enzymes that are compatible and less toxic to human. This had led to screening of marine samples from various sources for isolation of potential microbes, which has ability to produce desired enzyme [9].

Our preliminary findings showed that marine *Vibrio alginolyticus 1374* is a potential source of extracellular ADI. In our another article we have explained various optimization procedures for maximal production of ADI [10]. Since the enzyme produced from various sources differs in terms of physiological, biochemical, catalytic and immunological properties. ADI isolated from *Vibrio sp.* must also be evaluated for anti-proliferative activity. Hence the present study deals with isolation, purification and evaluation of anti -proliferative activity of purified ADI against various cell lines.

2. MATERIALS AND METHODS

2.1 Chemicals

Ingredients used for the preparation of bacteriological medium were procured from Hi-Media Laboratories, Mumbai, India. Remaining chemicals were purchased from Sigma Aldrich, Bengaluru, India.

2.2 Bacterial Strain

Fresh marine samples were collected from different locations of coastal areas of Andhra

Pradesh. The samples were then diluted up to five times and were inoculated in a sterile nutrient agar medium. Upon incubation various colonies were formed. Colonies differing in macroscopic characters were picked up individually and screened for ADI activity on minimal arginine medium by dye based method [9]. Further the enzyme production was confirmed by estimating the levels of l-citrulline formed. Colony showing higher enzyme activity was picked up and identified as *Vibrio alginolyticus 1374* by basic biochemical tests and sequencing method [9].

2.3 Inoculum

Sufficient amount of sterile water was added into 48hrs fresh culture of *Vibrio alginolyticus 1374* and mixed well. 5 ml of the resulting suspension was inoculated into 45 ml of sterilized nutrient broth and incubated at 37℃ for 24 hrs. 2% of the suspension was used as an inoculum.

2.4 Fermentation Medium and Fermentation Conditions for ADI Production [10]

2% of the inoculum was added into the optimal production medium and the production process was carried out. The conditions which were found optimal for enzyme production in our previous studies were pH 8, temperature 37℃, incubation time 120 h, agitation rate of 120 rpm and inoculum size 2%.

Table 1. Composition of optimal production medium

Ingredients	Quantities (gms/100 ml)
Soya bean meal	2
L-Arginine	2
NaCl	3
KH_2PO_4	0.1
K2HPO_4	0.1
$MgSO_4.7H_2O$	0.05
$CaCl_2$	0.01
$NaNO_3$	0.01
Tri sodium citrate	0.01
Yeast extract	0.05
Sea water	100 ml

2.5 Purification

Cells from the culture medium were separated by process of centrifugation at 10,000 rpm for 30 min. The supernatant thus obtained was subjected to step by step process of purification by ammonium sulphate fractionation followed by ion exchange and gel filtration chromatography. The purity of enzyme was further confirmed by SDS-PAGE as described by Laemmli [8].

2.5.1 Ammonium sulfate precipitation

Ammonium sulphate fractionation method was carried out in an ice bath at 4℃ under mild stirring conditions. Certain quantities of solid ammonium sulphate was added to the enzyme solution to get 10% and then successively raised up to 20-70% saturation. Upon precipitation the sample was centrifuged for 45 minutes at 5000 rpm. The pellet was then dissolved in phosphate buffer saline. The protein content, enzyme activity and specific activity of pellet as well as supernatant was quantified after each step of ammonium sulphate precipitation. The fractions thus obtained were pooled and concentrated by dialysis.

2.5.2 Ion exchange chromatography

The dialysate from the previous step was passed through DEAE-Sephacel column (1.5 X30 cm), then eluted with 0.01 M Tris HCL buffer pH 7.0 containing NaCl (0.1– 0.5 M) in gradient. The volume of fraction was 1ml at flow rate 1 ml / min. Each fraction thus collected was analysed for enzyme and protein concentration. Active fractions were pooled and concentrated by lyophilization.

2.5.3 Gel filtration chromatography

The concentrated enzyme from previous step was applied to the Sephacryl –S-200 column (2.5 x 55cm) that was pre equilibrated with 0.01 M Tris HCL buffer at p H7.2. The enzyme was eluted with same buffer conditions, then 1 ml fractions were collected in each tube with flow rate of 1 ml/min and quantified for enzyme and protein content. Active fractions were concentrated by dialysis and lyophilized.

The purity of the enzyme was further confirmed by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) as described by Laemmli [11].

2.6 Analytical Techniques

Citrulline concentrations were used to measure the enzyme activity colorimetrically using

modified version of the method described by Oginsky et al. [12]. Reaction mixture containing of 1 part of 80 mM DAMO and 2 mM thiosemicarbazide in DI water and 3 parts of 3 M phosphoric acid, 6M sulphuric acid and 2 mM ferric ammonium sulphate dodecahydrate with DI water was prepared. 200 µl of reaction mixture was added to 60 µl of test sample mixture. Test sample mixtures consisted 40 µl extract and 20 µl 10 mM arginine solution. Test tubes were then sealed with scotch tape. For enzyme activity measurements, test sample mixture (60 µl) in tube was incubated at 37℃ for 30 to 60 minutes, after which the reaction was terminated. Arginine deiminase activity was assayed after drawing the standard curve of citrulline (enzyme product). Color development was measured using absorbance in a spectrophotometer at 530 nm. The enzyme activity was expressed in International unit (IU).

2.6.1 Total protein

The concentration of the protein present in the sample was determined by Lowry's method by taking absorbance values at 660 nm and the values were expressed in mg [13].

2.6.2 Evaluation of antiproliferative activity *in vitro*

The purified enzyme thus obtained was evaluated for in vitro anti-proliferative activity against A375-C6, MCF-7, HCT-116, Jurkat clone E6-1 cell lines by MTT assay.

2.6.3 Cell culture

Cancer cell lines were obtained from American Type Culture Collection. Cells were maintained in RPMI – 1640 medium in CO_2 incubator at 37℃ with 98% humidity and 5% CO_2 gas environment. Details of the cancer cells are given under Table 2.

The indigenous ADI produced by *Vibrio alginolyticus 1374* were tested for anti-proliferative against A375-C6 , MCF-7,HCT-116 and Jurkat cell lines by using MTT assay as described by Mossman, 1983 [14]. Stock samples were diluted with RPMI medium to required concentrations of ADI ranging from 0.01 to 100 IU/ml. 100 µL of cells were added in 96 well plate at the density 5 × 105 cell/ml and incubated at 37℃ in 5% CO2, 95% air for 24 hrs. Then the cells were treated with various concentrations of samples in total volume (200 µl/well) for 24 hr. At 21 hrs, cells were centrifuged at 2000 rpm for 10 minutes and resuspended with 180 µL RPMI medium to rinse treated samples. A volume of 20 µl MTT solution (5 mg/ml) was added to each well and incubated at 37℃ for another 3 hrs. Then the medium was aspirated to about 180 µL from each well. The formazan crystals formed were dissolved with 180 µL of dimethyl sulfoxide (DMSO). An optical density (OD) of formazan was detected by a dual wavelength UV spectrometer at 570 -650 nm reference wavelength. The percentage of cytotoxicity was compared with the untreated cell as control and determined with the equation as below.

$$\text{Percentage cytotoxicity (\%)} = (OD_{\text{of test sample}} / OD_{\text{of control}}) \times 100\ \%$$

The plot of per cent cytotoxicity versus sample concentration was used to calculate the concentration lethal to 50 per cent of the cells (IC_{50}).

Data are reported as the mean ± standard deviation (S.D) for at least three replicates. The percentages of cell viability were presented graphically using Microsoft excel computer program.

Table 2. Details of cancer cell lines

Cell line	Morphology	Origin	Species	Supplier
A375-C6	Epithelial like	Skin from human	Human	American type culture collection
MCF-7	Epithelial-like	Breast adenocarcinoma	Human	American type culture collection.
HCT-116	Epithelial	Colon	Human	American type culture collection
Jurkat, clone E6-1	Lymphoblast	Peripheral blood	Human	American type culture collection.

3. RESULTS

Production of ADI under optimized conditions in an optimal production media gave an overall yield of 360 mg of protein with specific activity of 0.53 U/mg. Results of purification were shown in the Table 3. With ammonium sulphate fractionation there was 1.2 fold purification, 0.637 IU/mg specific activity and 88% recovery with respect to initial value (360 mg of protein). ADI enzyme was precipitated with 60% and 70% saturations of ammonium sulphate. Results of purification were similar to that of ADI from *E. faecalis* [15]. Similar salting out procedure for purification of ADI was followed by Nada Mahdy et al. Jae-Woon Choi et al. and various others [16,17].

3.1 Ion Exchange Chromatography

Enzyme solution from previous step containing 169 IU and 265 mg of protein with specific activity 0.637 IU/mg was loaded in DEAE-Sephacel column (1.5 X30 cm) and elution was carried out with Tris HCL pH 7.0 (0.01 M) with NaCl increasing gradient (0.1-0.5 M). Fractions of 1 ml were collected each time and were quantified for protein and enzyme activity. Sufficient enzyme activity was seen in the fractions between 12-17. Highest activity was seen in the 15^{th} fraction with 112 IU and 0.87 mg of protein with specific activity of 128.7 IU/mg (Fig. 1). In this there was 242.8 fold purification and 58.3% of recovery with respect to initial value from culture filtrate. Elution profile of IEC showed the presence of three peak with only one peak having enzyme activity. Even though different proteins were present in the fraction only one peak showed the arginine deiminase activity. The use of ion exchangers for purification of ADI has also been described by number of workers including Haruo et al. Kim et al. Nada Mahdy et al. [18,19,16].

3.2 Gel Filtration Chromatography

Results in Fig. 2 showed only one peak in the eluted fractions with enzyme activity of 87IU, specific activity of 280.6 IU/mg and protein content. The use of gel filtration chromatography for purification of ADI was also used by B.-S. Park *et al.* Harou et al., Kim et al., Nada Mahdy et al. [20,18,19,16].

3.3 Gel Electrophoresis

The active fractions obtained from IEC showed the presence of three bands in SDS-PAGE (Fig. 3). After GFC, only single band appeared which indicated the purity of the protein. The enzyme was purified to near homogeneity and was found to have a molecular mass of 48 kDa.The ADI purified from *thermophilic Aspergillus fumigatus KJ434941* was found to have specific activity of 26.7 U/mg with a molecular subunit 50 kDa [20]. Whereas the molecular mass of arginine deiminase from *Mycoplasma arthritidis* on SDS-PAGE was approx. 49 k Da and that of arginine deiminase from *Mycoplasma arginini* and Porphyromonas gingivalis on SDS-PAGE was 45 kDa and 46.6 kDa respectively [21-23]. The specific activity of the purified enzyme from *Vibrio alginolyticus 1376* was found to be 280.6 IU/mg which is higher when compared to *Enterococcus* (5.1 IU/mg), *Pseudomonas putida* (76±0.03 IU/mg) and recombinant *E. coli* (30-34 U/mg) [24-26].

3.4 Anti-proliferative Activity

The lyophilized ADI was subjected to cytotoxicity in vitro on the cell lines available (A375-C6, MCF-7, HCT-113 and Jurkat, clone E6-1) using MTT assay. The growth inhibition was expressed as percentage control. The results of anti-proliferative activity were shown in the Figs. 4, 5, 6 and 7. Data obtained from this assay indicated that there is a gradual decrease in viability of cancer cells with increasing doses of l-arginine deiminase.IC_{50} values for A375-C6, MCF-7, HCT-113 and Jurkat, clone E6-1 cancer cell lines were found to be 5.21, 6.3, 8 and 3.13 U/ml respectively. The sensitivity of Jurkat, clone E6-1 cell lines to ADI was higher when compared to other cell lines.

4. DISCUSSION

4.1 Extraction of ADI from Bacterial Cells

ADI isolated from *Vibrio alginolyticus 1374* is an extracellular enzyme. Hence cell disruption methods were not performed. The enzyme was extracted from the supernatant liquid left after centrifugation. For purification of l-arginine deiminase, standard method of purification process involving appropriate chromatographical techniques was followed. Initially the crude extract consisted of large number of impurities and showed specific activity of 0.53 U/mg. Upon purification, specific activity increased to 280.6 U/mg which indicates that the techniques were effective enough in removing proteins which were present in the form of contaminants.

Table 3. Purification of L-arginine deiminase

Step	Total enzyme activity (IU)	Total protein (mg)	Specific activity (IU/mg)	% Recovery	Purification fold
Crude extract	192	360	0.53	100	1
Ammonium sulphate fractionation	169	265	0.637	88	1.20
Ion-exchange chromatography	112	0.87	128.7	58.3	242.8
Gel filtration chromatography	87	0.31	280.6	45.3	529.43

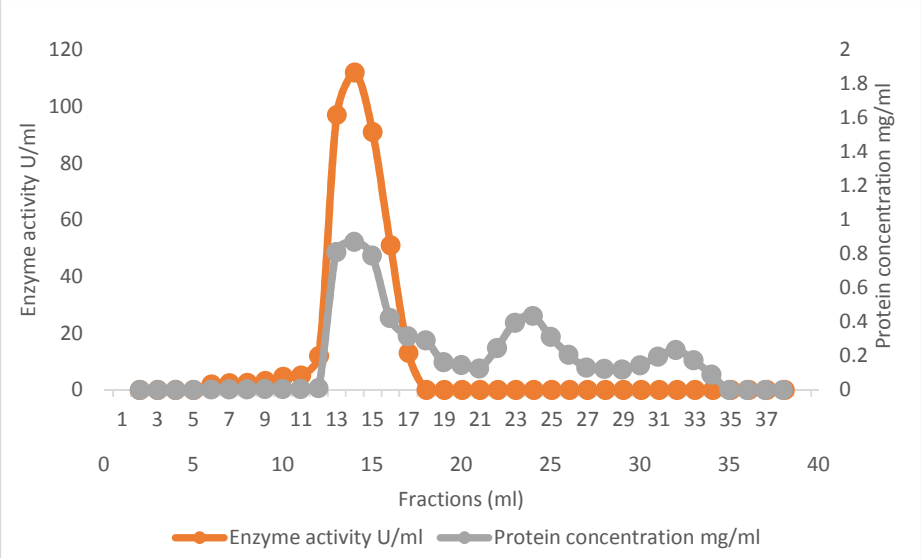

Fig. 1. Ion Exchange chromatography of l-arginine deiminase
Dialyzed ammonium sulphate precipitate was chromatographed on DEAE-Sephacel (1.5 X30 cm) column

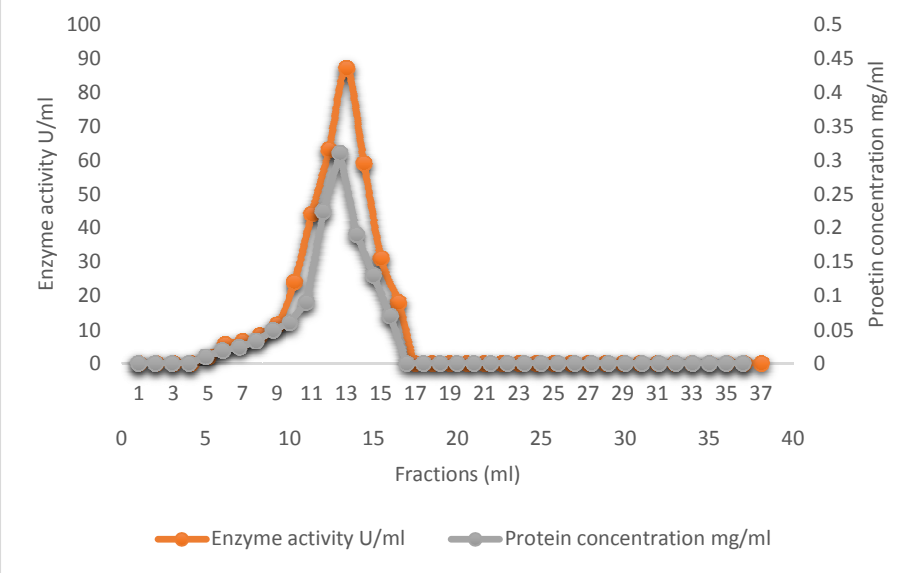

Fig. 2. Gel filtration chromatography of ADI on Sephacryl –S-200 column (2.5 x 55 cm)

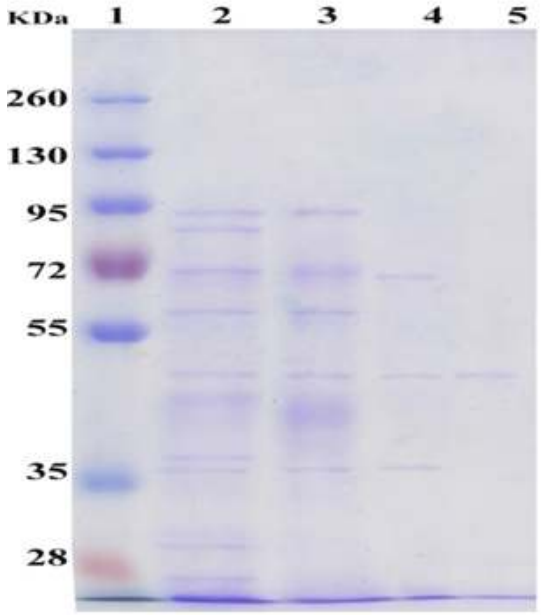

Fig. 3. Results of SDSPAGE

Electrophoretic analysis of ADI produced by Vibrio alginolyticus at various stages of purification.Lane1 molecular weight markers, Lane 2 – crude extract , Lane 3- ammonium sulphate fractionation , Lane 4- GFC , Lane 5- IEC

Ammonium sulphate fractionation studies revealed that the ADI isolated from *Vibrio sp.* is a hydrophobic enzyme because it got precipitated with higher salt concentrations. Cheng-Fu et al also showed that the ADI isolated from *E. faecalis* was precipitated with 60% and 70% saturations of ammonium sulphate [15].

4.2 Ion Exchange Chromatography

In the second stage of purification process, there are three peaks seen out of which there is only one peak showing the enzymatic activity. Hence fractions of this peak were collected for the further purification process. The peak showing enzymatic activity was eluted first, that may be due to the presence of hydrophobic moiety in ADI which reduces its ability to bind with DEAE-Sephacel.

4.3 Determination of Arginine Deiminase Purity

The presence of one band on electrophoresis gel confirmed the purity of ADI enzyme isolated from *V. alginolyticus* that means the purification steps were successful for this enzyme.

4.4 *In-vitro*-cytotoxic Activity

Even though arginine deiminase is widely distributed in the microorganisms. Enzyme isolated from different sources differs in terms of activity, stability, characteristics etc. The characteristics of the enzyme isolated depends upon the genetic nature of the microorganism. Hence it is mandatory to evaluate the activity of the enzyme against various cell lines. Mtt assays performed revealed that the enzyme was active against the cancer cell lines. Kwong-Lam and Chi-Fung et al. in their studies showed that pegylated l-arginase inhibited jurkat cell line with

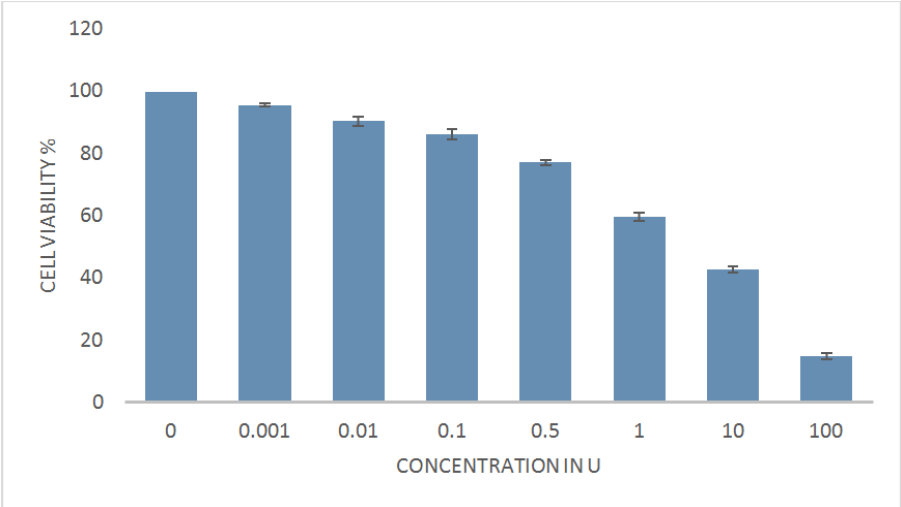

Fig. 4. Cell viability in percentage and inhibition of growth in percentage

IC_{50} estimation in the A375-C6 cell line was 5.21 U/ml for ADI

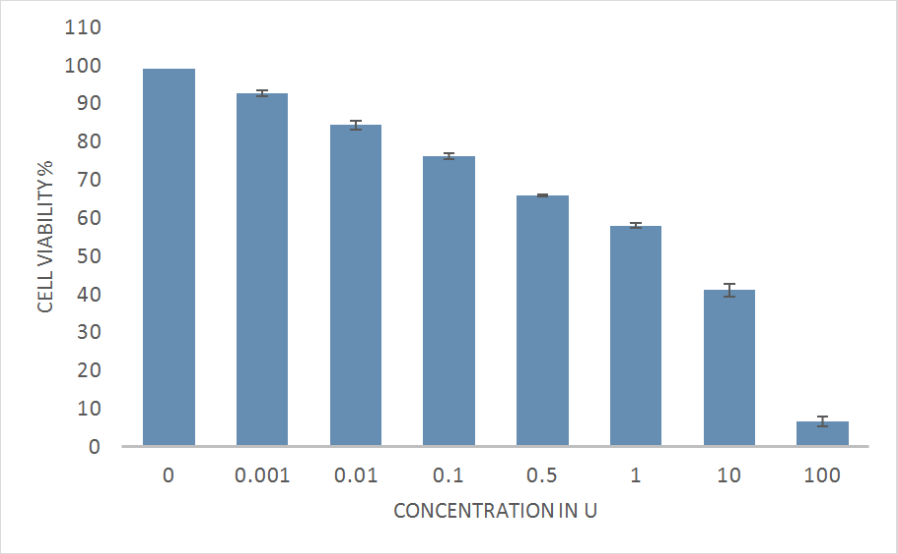

Fig. 5. Cell viability in percentage and inhibition of growth in percentage
IC_{50} estimation in the MCF-7 cell line was 6.3 U/ml for ADI

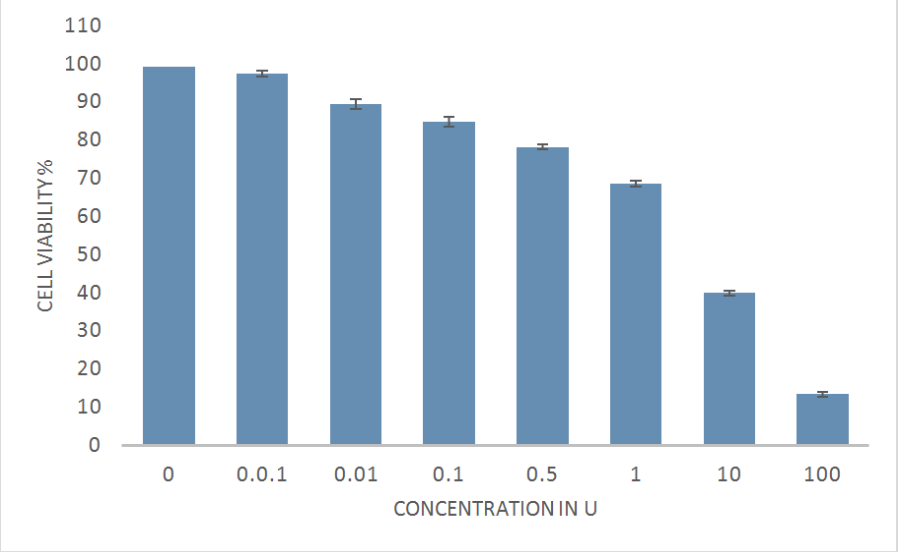

Fig. 6. Cell viability in percentage and inhibition of growth in percentage
IC_{50} estimation in the HCT116 cell line was 8U/ml for ADI

IC $_{50}$ 0.3125 U/ml which is closer to the results obtained in our study [26]. Ashraf et al studied in vitro cytotoxicity of a novel ADI from the *thermophilic Aspergillus fumigatus KJ* 434941 against various cell lines. They reported IC_{50} value of free ADI as 22, 16.6, 13.9 U/ml and for Dex-ADI as 3.98, 5.18, 4.43 U/ml for HCT, MCF and HEPG-2, cells respectively [21]. IC $_{50}$ of HCT and MCF in our studies were found to be 8 and 6 U/ml which is lower when compared to results of native ADI and slightly higher than that of Dex-ADI. Manca et al. conducted anti-proliferative activity on 25 melanoma cell lines (with a concentration of ADI ranging from 0.4-0.2 mU/ml) and had observed that 21 out of 25 cell lines showed good activity with median IC_{50} value as 1.3 mU/ml [27]. And the remaining 4 cell lines were not effected. ADI used in our studies showed good inhibitory action against A375-C6 cell line with IC50 5.21 U/ml.

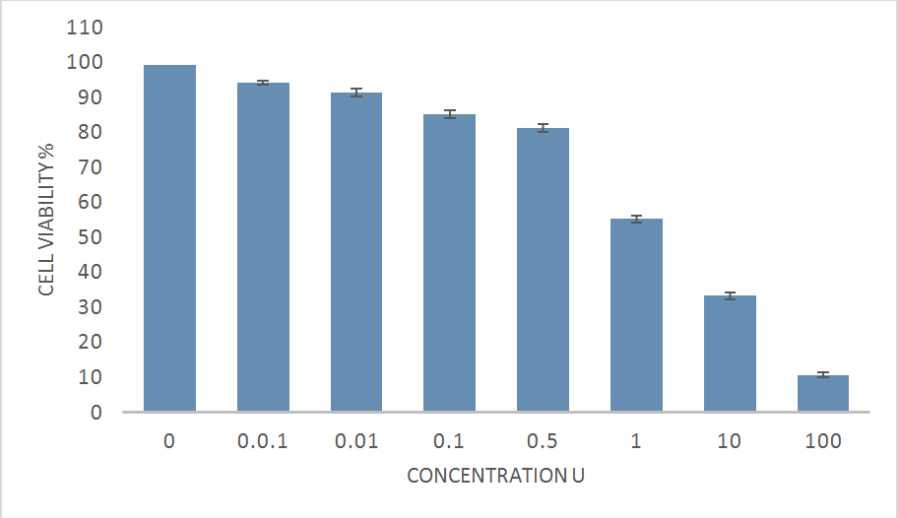

Fig. 7. Cell viability in percentage and inhibition of growth in percentage
IC_{50} estimation in the Jurkat, clone E6-1 cell line was 3.13 U/ml for ADI

5. CONCLUSION

For an enzyme to be ideally suited for a therapeutic purpose, it should meet several criteria. It should be produced from non-pathogenic easily available strain, capable of giving higher yield when grown on simple nutritional media. The enzyme produced should be free from toxins and stable at physiological environment (p H & temperature). It should exhibit broad range of activity against cancer cell lines. ADI produced in the present study meet all of the above criteria. It is produced from a non-pathogenic potential strain, easily grown on simple inexpensive medium. The ADI produced by the organism has several beneficial properties needed for a therapeutic and industrial enzyme. It is active and stable over a wide range of pH and temperature and in highly salt-tolerant. The substrate specificity towards l-arginine is also very high which means that it could be used in low amounts to achieve the desired effect. Moreover, the purified enzyme showed good activity against the A375-C6, MCF-7, HCT-113 and Jurkat, clone E6-1cancer cell lines. From this study, it is concluded that the new marine arginine deiminase is having anti-proliferative activity against various cancer cell lines. Further, animal model studies of this marine ADI for cancer therapy would help to authenticate the use of this enzyme as an anticancer agent.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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