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PURIFICATION AND CHARACTERIZATION OF ARGININE DEIMINASE FROM *E. COLI* AND ITS DIFFERENTIALLY CYTOTOXIC EFFECT ON NORMAL AND CANCER CELL LINES

Jawad N. K. Makassees[1]
[1]Ministry of Education, General Directorate of Wasit Education, Wasit, Iraq.

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Corresponding Author:
Jawad N. K. Makassees

Email:
jalmaksusse@uowasit.edu.iq

ABSTRACT

In this study, the author is concerned with the purification, extraction and biochemical analysis of arginine deiminase (ADI) of a clinical strain of Escherichia coli to determine its anticancer properties. Ammonium sulfate precipitation followed by two subsequent purification processes such as Ion-exchange chromatography and gel filtration enhanced the purity 9.5 fold and the final specific activity was found to be 16 U/ mg with the molecular weight of 46 kDa . The enzyme was observed to be most active at pH 7.0 and 37°C and lost less than 75% of its trial activities at marginally acidic to neutral pH. Using metal ion analysis and the fact that ADI was a metalloenzyme; Fe^{3+} and Mn^{2+} were found to increase the ADI activity, whereas EDTA suppressed the activity significantly. In vitro cytotoxic testing by MTT technique showed that ADI dose dependently and specifically suppressed the viability of HeLa cancer cells with the IC_{50} of 140.55 ug/mL, whereas the normal HEK293 cells had the IC_{50} of 253.47 ug/mL, with a selectivity index of 1.80. This justifies the therapeutic potential of bacterial ADI as a specific anticancer agent of arginine-auxotrophic tumors

INTRODUCTION

The arginine deiminase (ADI, EC 3.5.3.6) is an imperative enzyme of arginine degradation pathway but its catalytic cleavage of L-arginine into citrulline and ammonia embarks it as a potential cancer instrument in the enzyme therapy approach of the application (Park *et al*., 2003; Kumari & Bansal, 2021). The action of this enzyme is based on a novel mechanism which is depletion of plasma arginine and thus thwarting the growth of cancerous cells which rely on exogenous sources of arginine in order to sustain their life and divide themselves (Chen *et al*., 2024). In recent studies it has been shown that many malignant tumors, such as liver cancer (hepatocellular cancer) and melanoma malignant, do not have arginine succinate synthetase (ASS1), which makes them auxotrophic in the production of arginine and susceptible to therapies that eliminate arginine (Wang *et al*., 2024). Although arginine depleting enzymes have attracted a lot of attention as a potential therapeutic agent, the clinical advancement of these enzymes has

been characterized by a number of challenges that regard issues of thermal stability, immunogenicity, and therapeutic activity (Muth *et al*., 2017). In modern times, it has been noted that some of the sourced arginine deiminase differs in their biochemical properties, which has an impact on their curative aspects (Zarei *et al*., 2019). In this regard, recent studies indicated that purified ADI prepared using Penicillium chrysogenum displayed a purification stack of 17.2 and a specific activity of 50U/mg protein and a molecular weight of 49kDa (Jastrząb *et al*., 2024). The concept of extraction and purification is important in the elaboration of efficacy and stability of the enzymes with the traditional methods of extraction and purification involving the ammonium sulfate precipitation process, ion-exchange chromatography and the molecular sieve filtration (Pulikkottil, 2024).

They have revealed that high purification fold could up to 5.9 with recovery activity of 38.7 percent could be attained by DEAE-cellulose column chromatography (El-Sayed *et al*., 2015). Also, the latest studies revealed striking anticancer effects of purified enzyme on multiple cancer cell types, which further evidences prospects of its application as a promising drug (Teixeira & Sousa, 2021). The mechanism of action of the enzyme includes depletion of circulating arginine leading to a selective pressure of cancer cells having impaired arginine biosynthesis pathway. This is based on the metabolic susceptibility of some tumors which are unable to produce adequate levels of arginine intracellularly (Bathe, 2025). As shown in clinical trials, the arginine deiminase-based treatment can result in the objective response in arginine-auxotrophic tumor patients, i.e., hepatocellular carcinoma and malignant pleural mesothelioma (Chen, 2012). Designation of bacterial sources, especially *Escherichia coli*, as appealing matrices of ADI production owes to their fast growth rates, superlative genetic understanding, and spacious fermentation pipelines. Nevertheless, bacterial ADI has yet to be used clinically until extensive characterization into its biochemical features, such as optimum pH and temperature, necessity of cofactors, and its stabilities are deciphered (Iyer & Ananthanarayan, 2008).

Additionally, the anticancer activity assessment by in vitro cytotoxicity assays yields the necessary initial information on the possible use in treating the diseases (Nga *et al*., 2020). Considering the fact that anticancer agents should be developed urgently, especially those which target cancer cell metabolism, the objective of this study is to extract and purify arginine deiminase in clinical isolate of *Escherichia coli*, biochemically characterize it and pilot the in vitro efficacy of the anticancer agent. The study aims at obtaining a better insight into the mechanisms of actions of this enzyme and its therapeutic potential, which can further lead to finding perspective therapy approaches against arginine-dependent tumors.

METHODOLOGY

Bacterial Strain,Growth Condition and Preparation of Crude Arginine Deiminase ADI

Standard morphology, Gram stain and biochemical (IMViC) tests were employed to identify clinical isolates of *E. coli* obtained in the hospitals of Wasit Governorate and stored as slants of nutrient agar at 4^{o}C. The states of the bacterial cells used were growth in the minimal medium with L-arginine (10 mM) as the only source of nitrogen under shaking conditions (150 rpm; 37 ^{o}C) to late exponential phase. The harvesting of cells was done through centrifugation, and the disruption under sonication, which also followed some washing using phosphate buffer (pH 7). Further purification of the crude enzyme extract was done using -20^{o}C (Westphal & van, 2021).

Arginine Deiminase ADI Activity Assay and Protein Determination

The colorimetric measurement at 466 nm of citrulline freeform with a 2,3-butanedione monoxime reagent defined ADI activity. The reactant before reaction was a mixture of crude enzyme extract and 50 mM of L-arginine substrate (Tris-HCl buffer, pH 7)left to incubate at 37^{o}C over a period of 30min. The unit of enzyme activity was deemed to be that which gave 1 umol of citrulline in

one minute. Bradford assay against BSA was used in determining protein concentration and specific activity was reported in units/mg protein (Takaku *et al*., 1995).

Purification of Arginine Deiminase ADI

Three step procedure was applied to purify ADI: 70% ammonium sulfate precipitation (at 4 ^{o}C), DEAE-cellulose ion exchange chromatography (linear gradient of NaCl, 0-5 M)and Sephadex G-150 gel filtration chromatography. The active fractions were combine and concentrated and were kept in -20 ^{o}C. SDS-PAGE was used to determine purity of proteins and specific activity was determined at each step of purification (Janson, 2011).

Determination of Molecular Weight

SDS-PAGE was applied to determine the molecular weight of purified ADI with 12 % separating gel and 5 % stacking gels. Samples of protein were prepared in sample buffer of 2%SDS, 10% glycerol,5% and 0.002 bromophenol blue, heated at 100 ^{o}C over 5 min and electrophoresed at 120V over 90 min. Assessment of the molecular weight was determined by comparing with standard protein markers and stained with Coomassie Blue R 25 (Sambrook & Russell,2001).

Characterization of Arginine Deiminase ADI Enzyme

Optimum pH of purified ADI was determined in the presence of acetate, phosphate, and Tris-HCl buffers (5.0-9.0), and also determined the optimum temperature between 27-47 ^{o}C. pH and thermal stabilities were also tested after 24h at 4 ^{o}C and 60 min of test temperatures accordingly. The effects of metal ions ($FeCl_3$, $ZnSO_4$, $MgCl_2$) and EDTA (10 mM) were determined 10 min after pre-incubation. Each assay was carried out in triplicate (CV <5%), and reflected as percentage relative activity (Cornish, 2013).

Anticancer and Cytotoxic Activity of Arginine Deiminase (ADI) with MTT Assay

The HEK293 Cells and HeLa were grown in antibiotics and 10% fetal bovine serum on a 37^{o} C, 5% CO_2 in the DMEM media. They cultured the cells (1 x 10^4 /well) in 96-well plate and incubated at 37^{o} C with purified ADI (12.5-800 µg/mL) in arginine free medium after 72h. The calculation of cell viability was based on MTT, the crystalized form of formazan was solubilized in DMSO and calculated to be in absorbance at 570 nm. Data analysis was presented in terms of viability as a percentage to untreated controls (Van *et al*., 2011).

RESULTS AND DISCUSSION

ADI Activity Assay and Protein Determination

Arginine deiminase activity in the crude enzyme extract was quantitatively determined using a colorimetric assay based on citrulline formation. The tested enzyme exhibited activity that could be measured to have a specific activity of 1.7 U/mg protein with a total protein concentration of 0.35mg/mL as registered using Bradford assay of bovine serum albumin as standard. Under optimized assay conditions (50 mM L-arginine substrate, pH 7, 37°C, 30 min), the enzyme exhibited linear kinetics with consistent citrulline production detected spectrophotometrically at 466 nm following reaction with 2,3-butanedione monoxime reagent. The calculated enzyme activity, defined as µmol citrulline formed per minute per mg protein, was 60 U/mg, indicating successful production and extraction of functionally active arginine deiminase from the bacterial culture.

The derived ADI specific activity (60 U/mg) is in agreement with the values, which have been reported earlier on bacterial arginine deiminases. To increase in vivo solubility of over-expressed arginine deiminases in *Escherichia coli*; to evaluate the enzyme properties, *P. putida* ADI was grown under similar specific activity (58.8 U/mg) in vivo culture conditions as the active enzyme in vivo conditions (Y. Wang & Li, 2014). *P. chrysogenum* ADI 50 U/mg Purification and characterization of L-arginine deiminase from *Penicillium chrysogenum* and *E. faecium* ADI 76.65 U / mg(El-Shora *et al*., 2024). These results substantiate the effective purification and

functional integrity of the enzyme, which proves that the bacterial ADI has potential to be used as a treatment as earlier demonstrated in anti-cancer therapy.

Purification of ADI Steps

Ammonium Sulfate Precipitation

Ammonium sulfate precipitation procedure was very useful and efficiency has proved to be large and a sample of 100 ml was reduced to 25 ml with the sample volume reduction being 4-fold and attaining high enzyme concentration. The crude extract had 1.7 U/mg of the specific activity and after precipitation with ammonium sulfate,the specific activity was boosted to4.2 U/mg indicating a 2.5-fold purification factor. In spite of the constant protein concentration at 0.35 mg/ml, activity of the enzyme was succeeded in becoming concentrated to 1.5 U/ml, which is a sign of successful selective precipitation of the target enzyme. The final activity of the enzyme decreased to 37.5 U (as compared to the initial 60 U- a yield of 62.5%, which is satisfactory to this stage of purification as highly successful purification fold was obtained) as show in (Table 1).

This precipitation step was successful in cleaning up the mixture by about 37.5%of the contaminating proteins with little loss in the enzyme activity in order to provide a good basis to conduct further purification of the enzyme using chromatographic techniques. The 2.5-fold purification with a yield of 62.5% obtained by ammonium sulfate precipitation is in agreement with literature findings on purification of ADI. *P. putida* ADI exhibited 1.3-fold purification Farming to enhance in vivo solubility of over expressed arginine deiminases in *Escherichia coli* and enzyme properties(Patil *et al*., 2019). The purification of a dimeric arginine deiminase of *Enterococcus faecium* GR7 as well as investigation of its anti-cancerous activity(Bala *et al*., 2020). These similar outcomes attest to the reliability of the deployment of the ammonium sulfate precipitation to serve as a primary purification protocol, and they meet the requirements to establish a suitable protein concentration and the enrichment of the target enzyme that can serve as a pre-process to the subsequent chromatographic operations.

Ion Exchange Chromatography

Elution profile displayed a distinct connotation of two protein peaks at absorbance of 280 nm (Figure 1). Peak 1 was a protein peak at 10-20 fractions with a maximum absorbance of about 0.25, and peak 1 had no significant activity of arginine deiminase. The second principal protein peak appeared at a fraction between 45-60 with the maximum absorbance of 0.40 exact in the peak of the enzyme activity. The arginine deiminase activity could be detected only in fraction 45-55 and the highest activity of 2.0 U/ml was found at the fraction 48-50, which shows agood correlation between the elution of proteins and the enzymatic activity. The enzyme purity was tremendously enhanced through pooling of the actively concentrated fractions (45-55). Remarkable 7-fold purification achieved in the DEAE-cellulose chromatography step raised specific activity to 12.0 U/mg after the present 3.0-fold purification factor raised the activity to 4.2 U/mg. Even though the total volume was minimized to 21 ml and the enzyme activity concentrated to1.2 U/ml, the Protein concentration was decreased significantly to 0.1 mg/ml which shows that the contaminating proteins were removed efficiently. The overall enzyme activity was not reduced significantly (25.2 U) and about 42 %of the crude extract was recovered, constituting an acceptable amount of recovery given that significant purification was attained. The separation in this chromatography was rather effective to purify the target enzyme due to the distinct elution pattern and the large increase in specific activity, which creates a great basis of the final purification.

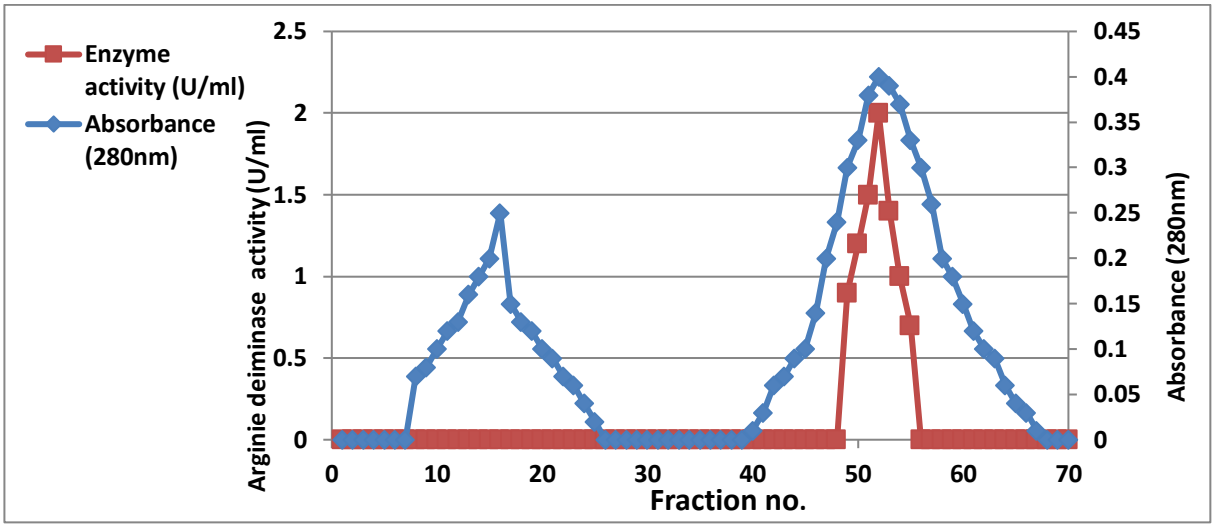

Figure1. Ion Exchange Chromatography of Arginine Deiminase Produced by *E.Coli* DEAE-Cellulose Column (2.5x20 Cm) With a Flow Rate of 30ml/hrs.

The DEAE-cellulose chromatography proved to be of utmost efficiency in purification of arginine deiminase as this resulted in incredible purification of 7-fold with a 42 *%* recovery. The elution pattern as unique and the comparison of the protein peaks with the activity of the enzyme is similar to what other ADI purification studies have found(Thakker & Rajnish, 2025).

In which the highly resolving capacity and wide applicability of ion exchange chromatography to enzymes Interestingly, in the ion-exchange chromatography of enzymes(Wallace & Rochfort, 2023). Allows the selective separation of the target proteins over contaminants. The large change of specific activity (12.0 U/mg) proves that the interfering proteins were removed well, and it places a great basis to the following purification milestones.

Gel Filtration Chromatography

The elutions gel filtration conditions provided a single and sharp protein peak being observed at 280 nm with maximum absorbances at 0.25 of fraction 18-20 (Figure 2). This symmetrical peak meant homogenous protein elution and avoided the accumulation and degradation product of proteins. All the arginine deiminase activities were found in fractions 16-22 with the highest interaction P = 0.82 at fraction 18, indicating that protein elution and activity were very strong. The pure final enzyme preparation was confirmed by the narrow elution profile, and perfect overlap of the protein absorbance and the enzyme activity. Pooling of the active fractions (16-22) was done and smaller quantities concentrated to create an end result of highest purity of enzymes in the purification process.

The Sephadex G-150 chromatography step had increased the purification factor 9.5-fold and the specific activity was improved, i.e., 12.0 U/mg to 16.0 U/mg. The last preparation of enzyme was 18 ml in vol and 1.3 U/ml in enzyme activity whereas the protein concentration was 0.08 mg/ml which reflects truly the super removal of contaminating proteins. The overall enzyme activity was maintained at 23.4 U thus the final yield was 39 % of the original crude extract (Table 1).

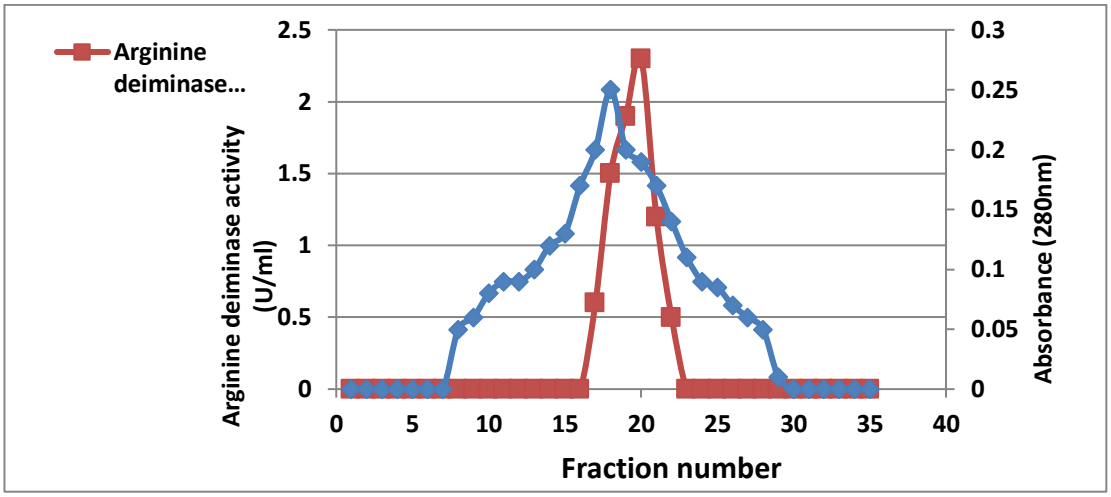

Figure2. Gel Filtration Chromatography of Arginine Deiminase Produced by *E.Coli* (1.5x35cm) On Sephadex G150 Column (1.5cmx35cm) with a flow rate of 30ml/hrs.

This chromatographic step (last one) was sufficient to complete the three-step purification process that resulted in the production of highly purified arginine deiminase with a single-protein peak and demonstrated an optimal enzyme activity (Table 1). The purified enzyme preparation was concentrated and could be kept in -20^{o} C exhibiting very good stability with retention of catalytic activity applicable in the study of comprehensive biochemical characterization.

Table 1. Purification Steps For Arginine Deiminase Produced by *E.Coli.*

Purification Steps	Volume (ml)	Enzyme Activity (U/ml)	Protein Concentration (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification (folds)	Yield (%)
Crude Enzyme	100	0.6	0.35	1.7	60	1	100
Ammonium Sulphate Precipitation 70%	25	1.5	0.35	4.2	37.5	2.5	62.5
DEAE-cellulose	21	1.2	0.1	12	25.2	7	42
Sephadex G150	18	1.3	0.08	16	23.4	9.5	39

The purification step 9.5-fold using Sephadex G-150 gel filtration concurs with other results. On *Penicillium chrysogenum*, we found the effective purification of ADI where separation by sequential purification and finally using Sephadex G-200 was performed, as reported by Abdel-Megeed and El-Sayed (2024) with 17.2-fold purifications and homogeneous preparation of the enzyme(Marini & Didelija, 2015). In the same way, *Enterococcus faecium* GR7 ADI was cleansed to homogeneity by utilizing molecular sieve before subjecting it to Sephadex G-100 gel filtration Purification and characterization of L-arginine deiminase from *Penicillium chrysogenum*(Caro et al., 2015). Recent researches demonstrated similar purification factors (5.9-fold) and 38.7% recovery of arginine deiminase which supports our 39% recovery and argues the effectiveness of gel filtration as a last step in purification(Lada, 2014).

Molecular weight determination for arginine deiminase by SDS- PAGE

The purity of enzymes was verified by performing SDS-PAGE that demonstrated the presence of one band of protein (Figure 3). Relative mobility plotting following exposure to standard protein markers (66, 45, 46 and 35 kDa) identified recognition of molecular weight as around 46 kDa which is related to bacterial arginine deiminase recovered in other sources.

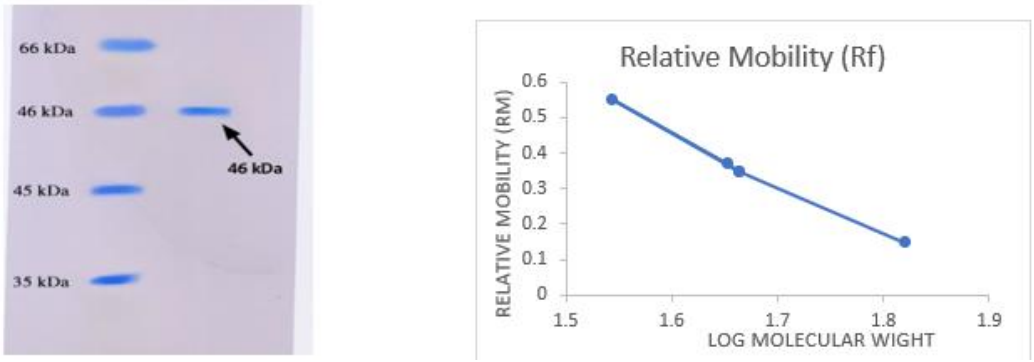

Figure3: Log Molecular Weight of Arginine Deiminase Produced by *E.Coli* After Sds-Page Electrophoresis

The calculated molecular weight of 46 kDa of purified arginine deiminase is concurring with published reports. Liu et al. (2009) published the ADI of *Pseudomonas plecoglossicida* whose molecular weight was calculated as 46.5 kDa and having 417 amino acids(Ni *et al*., 2009). Likewise, ADI produced in *Corynebacterium crenatum* showed molecular weight of 46.8 kDa that had been determined using SDS-PAGE analysis(Qianni *et al*., 2017). According to recent studies, validated by Babaei *et al*. (2020) using *E. coli* BL21(DE3) results of high ADI expression exhibiting molecular weight of 46 kDa(Qianni *et al*., 2017). This is agreement with our results of effectual purification and molecular weight determination.

Characterization of Purified Arginine Deiminase

Effect of pH on Enzyme Activity

The findings were that the purified ADI enzyme was pH dependent with the typical bell-shaped curve of activity (Figure 4). The basal enzyme activity was of 1 U/ml at pH 5. The enzyme activity then slowly raised with rises in pH to 1.2 U/ml at pH 6. The activity of the enzyme was still increasing and reached its maximum level 1.3 U/ml at pH 7.0, which shows that the optimum pH of ADI enzyme activity is 7.0. The above the ideal pH caused the enzyme activity to decrease with increments in ph. The activity between PH of 8.0 and 8.5 was at 1.1 U/ml which was about 85 percent of maximum activity. A further elevation of pH to 9.0 led to a strong decline in the enzyme activity to just 0.8 U/ml which is equivalent to about 62 percent of the optimal activity.

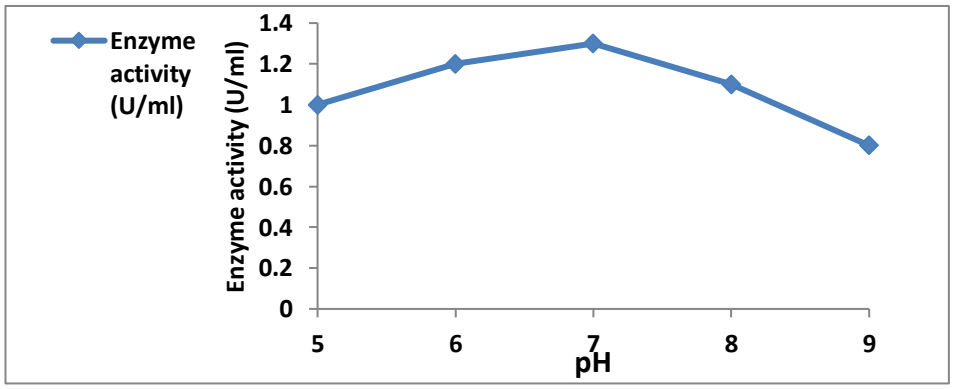

Figure 4. Effect of pH on The Activity of Purified Arginine Deiminase Produced by *E.coli*

The best pH of purified ADI is 7.0 ascribable to characteristic of bacterial enzyme optimal pH of Streptococcus pyogenes M22 ADI was 6.8(Starikova et al., 2016). Knodler *et al*. (2013) identified 6.5 as optimal pH of *S. pyogenes* M49 AD(Hering et al., 2013). Recent researches indicated that *K. pneumoniae* ADI was most active at pH 6.0 and stable at pH 5-9(Hassan *et al*., 2024). showing species differences in the distribution of pH preference in the neutral region.

PH Stability for Arginine Deiminase

It has been shown that the purified ADI enzyme fails to have any considerable stability features that are pH dependent (Figure 5). The enzyme was found moderately stable in mildly acidic conditions since 80 % of its original activity was still present at an acidic pH 5.0. With an increment in pH, there was an increase in the enzyme stability till residual activity of 90 % was achieved at pH 6.0. The highest stability was the one recorded at pH 7, where the enzyme preserved itself with 100 % of its activity, and thus, it is under the neutral pH that the enzyme is most stable not only in terms of activity but also as a structural element. This pH level is an optimum of enzyme stability and it is the physiological balance. At higher pH than the optimum pH, the enzyme lost a great deal of stability. The remaining activity was at 75%, which is 25% of the enzymatic activity at pH 8.0. Another increment of the pH to the range of 9.0 exposed the enzyme to a high level of inactivation with 60 % of initial activity remaining, still meaning that the enzyme lost 40 % of its stability.

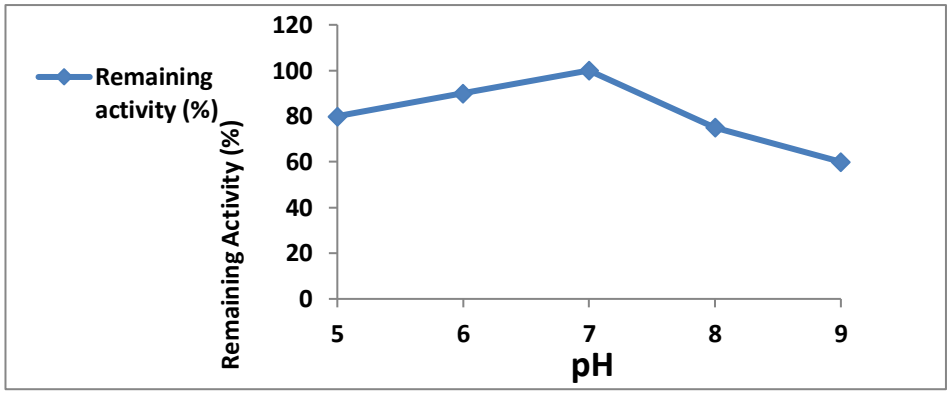

Figure 5. Effect of pH on Stability of Purified Arginine Deiminase Produced by *E.coli*

The highest pH stability 7.0 (no activity loss) fits into the ADI parameters of bacteria. ADI lost only 74 percent of its relative activity with treatment of pH indicating that ADI is bacterial-resistant to pH(W. Wang *et al*., 2023). The acid toleration that is evident at pH 5.0 (80%) is in line with the protective effects of the arginine deiminase system in the survival of bacteria in an acidic condition immobilization of ADI obtained *P. chrysogenum* improved stability of the enzyme over a wide range of pH(Xu *et al*., 2016). Advocating on the flexibility of the enzyme to adjust to circumstance changes in PH in order to tap biotechnological uses.

Effect of Temperature on Arginine Deiminase Activity

The findings showed a typical temperature profile of activity showing a particular optimum (Figure 6). The enzyme had a rather low activity of 0.7 U/ml at 27^{o}C, corresponding to about 54 % of the maximum possible activity. After raising the temperature to 32 ^{o} C, enzyme activity increased substantially and attained a value of 0.8 U/ml, which means that catalytic capabilities improved with elevation of temperature. An enzymatic activity was also maximized to a great extent with increased temperature of 37^{o}C at which the enzyme obtained a value of 1.3 U/ml which remains the optimum temperature of enzyme activity attained. This temperature is a standard condition at a physiological level and it implies that the enzyme would perform a well at regular body temperature conditions.

Further increasing the temperature past the optimum marked a continued decrease in the activity of the enzyme which is attributed to thermal denaturation of the activity. The activity reduced to 1.0 U/ml at 42^{o}C which is about 77 % of the maximum activity. The decrease implies that the enzyme becomes threatened by thermal stress, which negatively influences its structural stability as well as the catalytic power thereof. Further heating to 47^{o}C led to the wave of enzymatic activity as the activity was found to be 0.6U/ml and that too with 46 % of the optimal activity. Such a

tremendous drop is indicative of the enzyme sensitivity to a higher temperature and can be interpreted to mean thermodynamic denaturation of the protein.

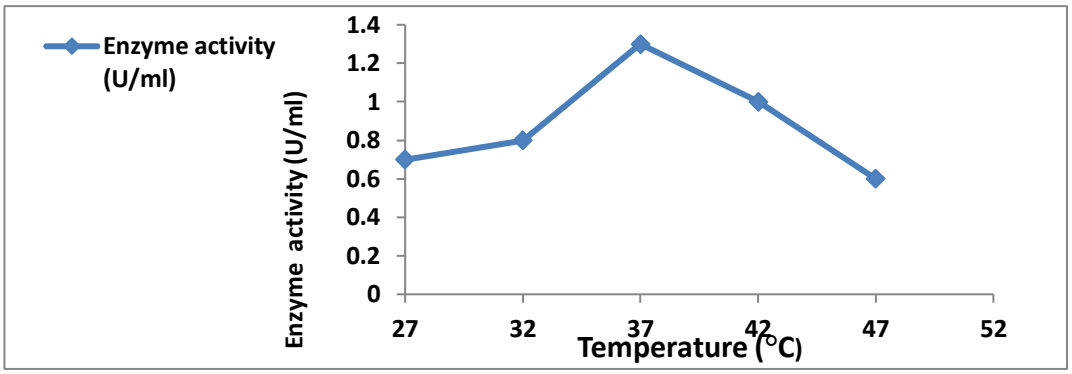

Figure 6. Effect of Temperature On The Activity Of Purified -Arginine Deiminase Produced by *E.coli*

Optimal sustained temperature is 37 ºC, which is close to physiological condition and features of bacteria enzyme with purified ADI of *Penicillium chrysogenum* with the optimal temperature of 40 ºC and thermostability experiments of temperature-related decrease in activity(Kim *et al*., 2007). Conversely, characterized thermophilic member of the genus *Halothermothrix orenii* ADI with maximum activity at 55 ºC and retained 74 % relative activity during thermal stress(Bhattacharya & Pletschke, 2014). The need of thermal characterization in biotechnological applications was emphasized by recent studies that used semi rational design to thermostabilized *Enterococcus faecalis* ADI with enhanced industrial tolerance of up to 3 hours at 70 ºC(Cai *et al*., 2018).

Temperature Stability of Arginine Deiminase

This thermal stability profile of arginine deiminase indicated that there was enzymatic activity preservation in arginine deiminase at specific temperatures. The enzyme was found to retain 100 % activity in 32ºC depicting optimal stability under this temperature (Figure 7). It showed excellent thermostability which did not degrade to make it inactive up to 37ºC. There was a remarkable decrease in enzymatic activity at 42ºC the residual activity was recorded to be about 79%. The temperature seems to be a point of no-return in terms of the thermal denaturation process when the tertiary structure of the enzyme and its catalytic activity are starting of being distorted. The figure also decreased to 75% at 47ºC indicating progressive opening up of active site of enzyme. Almost half of its activity (40 %) was lost at a temperature of 52 ºC and at this then the enzyme was still able to produce60%of its original activity. Such 40% of activity loss implies a partial denaturation of the enzyme structure, probably the destruction of the hydrogen bonds and hydrophobic interactions that form the enzyme structure needed to support the enzyme conformation.

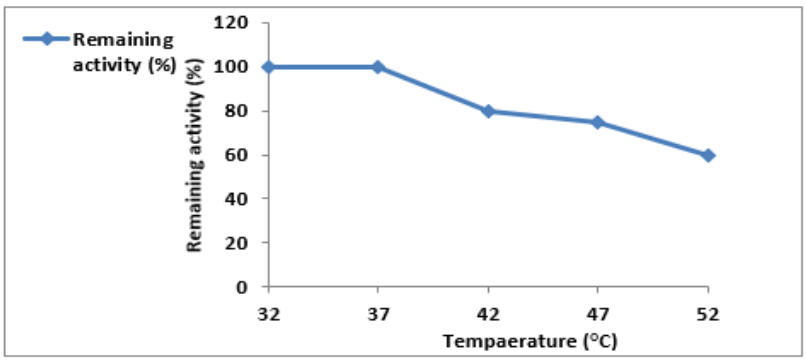

Figure 7. Temperature Stability of Arginine Deiminase Produced by *E.coli*

Thermal stability, which is an important aspect that determines arginine deiminase (ADI) applicability in therapeutic and industrial purposes, is different in various microbial based-sources. ADI purified in Escherichia coli in the present experiment was 100 % active over the temperature range 32-37 ºC, and was stable up to 37 ºC, though slowly losing activity at higher temperatures. where activity of ADI of *Pseudomonas plecoglossicida* expressed in *E. coli* was optimal at 30-37 ºC and decreased with higher temperatures probably by structural denaturation(Peti & Page, 2007). *S. pyogenes* ADI was also found to have lower catalytic activities above 40 ºC reinforcing the idea that ADI has a tertiary structure that is susceptible to heat(Freiberg *et al*., 2020). These similarities indicate that ADI in the *E. coli* shown in the present study has thermal functionality similar to other bacterial ADIs, and therefore it is a potential candidate to be applied in the settings that demand moderate temperatures.

Effect of Metal Ions and Inhibitors on Arginine Deiminase Activity

The findings show that arginine deiminase has a variable activity to the various divalent metal ions (Table 2). Fe^{3+} ions had the strongest activating effect where the enzyme activity was 120% of the control activity. This 20 % increase would indicate Fe^{3+} could be involved in maintaining the enzyme in an active conformation; or in copper binding. The Mn^{2+} ions also depict a high degree of activation increasing the activity to 115 % of the control. The activation suggests a role of Mn^{2+} as a cofactor or as an allosteric activator of the enzyme. Mg^{2+} ions were found to be moderately activated (110%) implying that they favor the enzyme structure or its catalytic performance. On the contrary, Zn^{2+} had slight inhibitory effects, which led to activity levels of 90 % of the control levels. This inhibition can be caused by competitive binding with the active site or, by hampering with the catalytic mechanism of the enzyme. EDTA had high inhibition, with the activity of enzyme showing 65% as compared to the control value. Such a drastic loss of activity is a very serious indication that arginine deiminase is a metalloenzyme whose activities depend on metal co-factors to perform well as a catalyst. The chelation with EDTA a metal-chelating agent, shows that when the required metal ions are removed out of structure of the enzyme, its catalytic activity is severely undermined.

Table 2. Metal Ions and Inhibitors Effect on Arginine Deiminase Activity Produce by *E.coli*

Reagent	Concentration(mM)	Remaining activity(%)
Control(Enzyme)	-	100
$FeCl_3$	10	120
$ZnSO_4$	10	90
$MgCl_2$	10	110
$MnCl_2$	10	115
EDTA	10	65

It already has been established that the ADI activity highly depends on metal ions, which are ADI cofactors or structural stabilizers. The activity of Mn^{2+}, Mg^{2+} and Fe^{3+} have been reported to be improved and Zn^{2+} and EDTA decreased which are the characteristics of metalloenzymes. The activity of *E. coli*-derived ADI behaves similarly to what these studies report, particularly of its powerful inhibition by EDTA(Hao *et al*., 2013). These results are quite consistent with the loss of activity which has been observed here which serves to validate the requirement of ADI to the presence of divalent metal ions as ameans to its retaining catalytic dependence.

Cytotoxic Activity of Arginine Deiminase Using MTT Assay

Arginine deiminase demonstrated a considerable level of cytotoxicity toward the HeLa cancer cells that was dose-dependent (Table 3). Only 44.7± 2.36 % of HeLa cells were viable at the maximally tested concentration (800 µg/mL^{-1}), which means that 55.3% of the cells died. The cytotoxic activity decreased as the concentration lowered where cell viability was found to be 53.87± 1.70% at 400 µg/mL^{-1}and 61.50 ±1.01% at 200 µg/mL^{-1}. The cell viability was 77.30 ± 2.59 % at 100 µg/mL^{-1}and 87.30 ± 2.55 % at 50 µg/ mL^{-1}. At the lowest concentration of tested

(25 and 12.5 µg/ mL^{-1}), cell viability levels were above 95%, which shows that cell toxicity was minimal. ADI cytotoxic effect on HEK293 normal kidney cells was less than to HeLa cancer cells. Cell viability at 800 µg/$mL^{-1}$was recorded as 60.13±1.60 which indicates 39.87 cell death. The dose-response correlation was observed in all the concentrations tested and the cell viability disclosed 69.1 ±1.95%; 77.10 2.05%; 85.80± 2.05% cell viability at 400 µg/mL^{-1},200 µg/mL^{-1}, 100µg/mL^{-1}respectively. With the concentration of 50 µg/mL^{-1}, there were still 92.13 ± 1.85 % viable HEK293 cells and at the lowest concentrations (25 and 12.5 µg/mL^{-1}), cell viability was more than 96 %.

Table 3. Cytotoxicity Effect of Arginine Deiminase on HeLa and HEK293 Cell Line

Concentration µg/mL-1	**Mean of cell viability (%) ± SD**	
	ADI on HeLa cell line	ADI on HEK293 cell line
800	44.7 ± 2.36	*60.13* ± 1.*60*
400	53.87 ± 1.70	69.1 ± 1.95
200	61.50 ± 101	77.10 ± 2.05
100	77.30 ± 2.59	85.80 ± 2.05
50	87.30 ± 2.55	92.13 ± 1.85
25	95.13 ± 4.29	96.97 ± 1.15
12.5	96.97 ± 1.50	96.07 ± 1.75

The findings indicated that HeLa cancer cells were more sensitive to ADI induced cytotoxicity than HEK293 normal cells at all the concentrations that were tested. At 800 µg/mL^{-1}, HeLa cells displayed 15.43%reduced viability in comparison to HEK293 cells, which means higher sensitivity to treatment with ADI. Such a difference in sensitivity was exhibited across the concentration scale, and it was found that HeLa cells displayed 10-20 % reduced survival in comparison to HEK293 cells all the time. Such trend indicates a possible selective cytotoxicity against cancer cells of ADI, which is an appealing attribute of therapeutical purpose.

Response curves plotting cell viability percentages against log concentration of ADI enabled IC 50 values which were calculated (Figure 8). The findings revealed that HeLa cancer cells were much more sensitive to ADI treatment as opposed to HEK293 normal cells. IC_{50} of HeLa cells was 140.55ug⁄mL^{-1} whereas that of HEK293 cells was 253.47ug⁄mL^{-1}. This 1.8-fold variation of IC_{50} values denotes selective cytotoxicity of ADI against cancerous cells, and selectivity index is 1.80, implying therapeutic usage with less harmful effect on normal cells.

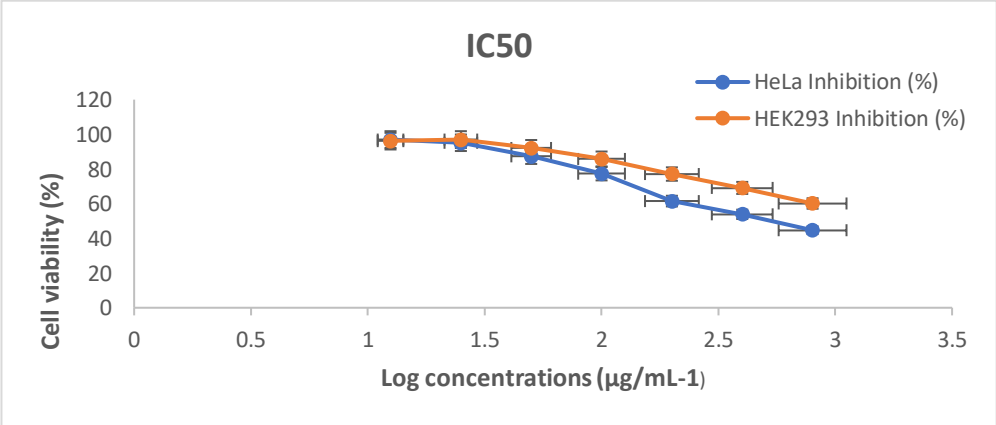

Figure 8. IC_{50} Effect of Arginine deiminase on HeLa and HEK293 Cell Lines

In the present study, was a purified enzyme arginine deiminase by using *Escherichia coli* expression system. The data obtained in this work show that purified arginine deiminase has a dose-dependent cytoxic effect and that the same substance has a significantly greater effect on

HeLa cancer cells than HEK293 normal cells. The measured IC_{50} values; 140.55µg/mL and 253.47µg/mL in HeLa and HEK 293, respectively, implies a selectivity index (SI) of 1.80 which forms favorable toxicity against cancer cells. Our discovery corresponds to the results of a series of other works, which point to the anticancer properties of microbial ADI through the deprivation of arginine, especially in those types of tumors in which arginosuccinate synthetase (ASS1) has an incorrect structure, a major enzyme in the synthesis of arginine (Riess *et al.*, 2018). The distinctive viability of HeLa cells in all the concentrations used could be due to the fact that HeLa cells are arginine auxotrophs which makes them more sensitive to the effect of depletion of arginine as divine by ADI(Delage *et al*., 2010).

On the contrary, higher survival of HEK293 indicates less dependence on extracellular arginine metabolisms, possibly owing to preserved arginine synthesis pathways. In addition, the application of ADI of *E. coli* origin is specifically hopeful because of considerable expression yield and the subsequent level of catalytic activity. Further, lactic acid bacteria-derived ADI have been shown to effectively exhibit cytotoxicity on cancer cell lines (PC3 and A375) with IC_{50} of 68-136 ug/mL and little to no toxicity to normal cells(Gutef, 2022). As an example, how cytosolic overexpression of the tumor suppressor ADI elicits mitochondrial apoptosis in but not of ASS1-deficient cancer cells by inhibiting ferritin synthesis and chromatin autophagic maturation demonstrates a specific mechanism of cytotoxicity(Feng *et al.*, 2020). The cytotoxicology that is present here has justified its use as a therapeutic compound, where it is possible to utilize techniques to make it stable and less immunoreactive like PEGylation. Joining together, these results strengthen therapeutic implications of ADI and selective tumor arginine-dependent targeting of such compounds that should be explored in preclinical and clinical settings.

CONCLUSION

This experiment achieved the success of extracting and purifying a arginine deiminase enzyme by a three-step purification approach on the clinical isolates of *Escherichia coli*. The efficiency of the used protocol can also be proven by the fact that the entire purification achieved a 9.5-fold purification factor with 39 % retention of original enzymatic activity. The last specific activity value was 16.0 U/mg of protein and this can be correlated to results that have been obtained in the researches of other sources of microbes. The analyses of the purified enzyme indicated that the characteristics were optimal with pH optimum of 7.0 and temperature optimum of 37 ºC, which falls under physiological conditions. The stability of the enzyme was excellent under the pH condition of 6.0- 8.0 and it was also retained more than 75 % of the original activity. Purity of the enzyme by SDS-PAGE identified subunit molecular weight as 46 kDa, which is in accordance with its structural features of known bacterial arginine deiminases. Kinetics demonstrated that the enzyme was deemed to be a metalloenzyme with the maximal of its activity in the presence of metal ion and iron and manganese ions stimulated it by 120 and 115 %, respectively whereas EDTA inhibited it severely amounting to 35 %. The results are informative in giving profound understandings about the mechanism and optimum requirements of the enzyme activity. The present study produced the most substantial result in promising results of anticancer activity, as the purified enzyme showed selective cytotoxicity against cervical cancer cells (HeLa)with an IC_{50} value of 140.55 µg/ml, whereas the corresponding analysis on normal cells (HEK293) was identified at 253.47 µg/ml. The selectivity indication of this compound with an index of 1.80 denotes that this enzyme can be used in the development of a good therapeutic tool, with minimal safety to normal cells. The work would help to increase our knowledge on the therapeutic opportunities of arginine deiminase as an anticancer agent and would offer us an effective method of extraction and purification of the enzyme using clinical bacterial sources. Its findings favor the course of coming up with specific enzyme-based treatment in arginine-driven cancer. To continue advancing the research forward, the report suggests that preclinical studies to test toxicity and efficacy in animals be done, and attempts be made to come up with strategies of

modification of the chemistry so as to enhance stability and bioavailability in this case possibly leading to the creation of a novel drug in the sphere of cancer enzyme therapy.

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