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Extraction, Optimization, and Antioxidant Evaluation of Arginine Deiminase Enzyme in the *Escherichia Coli* Clinical Isolates

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

Address: Kut City, Wasit, Iraq
Author correspondence: jalmaksusse@uowasit.edu.iq

***Abstract**. Arginine deiminase (ADI) is a promising enzyme with significant therapeutic potential, particularly for its anticancer effects through the depletion of arginine in cancer cells that are auxotrophs. In this study, we aimed to optimize the production of ADI using clinical Escherichia coli isolates and to evaluate its antioxidant activity. A total of 25 E. coli isolates were obtained from 45 hospital samples collected in Wasit Province, Iraq. Optimization of ADI production was performed by systematically testing various factors including culture media, pH, carbon and nitrogen sources, incubation temperature, and time. The antioxidant activity was assessed using the DPPH radical scavenging assay. The highest ADI production was achieved using a modified M9 medium supplemented with 1% w/v sucrose as the carbon source and 5% w/v yeast extract as the nitrogen source. The optimal enzyme activity of 1.6 U/mg protein was observed at pH 7.0, 37°C, and after 24 hours of incubation. The crude ADI extract exhibited high antioxidant activity, with 79.28 ± 1.06% DPPH scavenging at 200 µg/mL, comparable to ascorbic acid, which showed 86.11 ± 1.45% DPPH scavenging. The study successfully optimized the conditions for enhanced ADI production based on clinical E. coli isolates, demonstrating its potential as both an anticancer enzyme and an antioxidant. The dual therapeutic potential of ADI warrants further research, including clinical trials, to explore its application in cancer therapy and as an antioxidant in medical treatments, offering promising avenues for future drug development and improved therapeutic strategies, particularly for targeting cancer and oxidative stress-related diseases.*

***Keywords:** Antioxidant activity, Arginine deiminase, Clinical isolates, Enzyme optimization, Escherichia coli*

1. INTRODUCTION

Arginine deiminase ADI (Greek letter arginine, deiminase) is a vital enzyme as the engagement of deimination by a hydrolytic reaction of un-supplemented L-arginine with L-citrulline and Ammonia that is vital in microorganism and biomedicine metabolism (Wang *et al*., 2023). The enzyme is under the guanidino group-modifying enzyme and has attracted much attention because of its incredible therapeutic potential especially in cancer treatment (Ye *et al*., 2023). The new concentration on the role of ADI is due to its preferential killing of arginine-auxotrophic cancer cells, which are deficient in arginosuccinate synthetase because they do not produce arginine de novo (Dhankhar *et al*., 2022).The *E. coli*, which is a ubiquitous gram-negative bacterium, is a very good model organism to study the microbial enzyme production because of its generous genetics, fast growth, and possibility of genetic manipulation (Kaur & Kaur, 2013).The *E. coli* clinical isolates are an excellent source of naturally occurring enzymes which may have an improved property than those in the laboratory strains. It is shown that the production of ADI in *E coli* depends on the many environmental and nutritious factors such as the composition of medium, pH, temperature, and incubation time (Zarei *et al*., 2019). A great deal of therapeutic importance of ADI has been reported in preclinical and clinical trials, especially in the treatment of the R-auxotrophic cancer,

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hepatocellular carcinoma, melanoma, and some hematological malignancies (Pal *et al*., 2019).The action is achieved through the depletion of arginine which results in apoptosis of cancerous cells which are deficient in biosynthesis of this semi essential amino acid (Glazer *et al*., 2010). A recent study has depicted that pegylated arginine deiminase (ADI-PEG20) has already got into the clinical trials and in phase II reports, it has shown an encouraging outcome in different types of cancer (Lowery *et al*., 2017). In addition to anticancer effects, ADI has been reported to have possible antioxidant activities which might expand its therapeutic use. The bilateral character of enzymes as both a catalyst and antioxidant can be seen as a recent field of study in the spheres of biotechnology and pharmaceutical creation (Kumar *et al*., 2016). The antioxidant enzymes are important in reducing oxidative stress in cells and oxidative stress triggers a wide range of pathological disorders, such as cancer, cardiovascular diseases, and neurodegenerative disorders (Gulcin, 2025). ADI production should be optimized and this will be important in generating therapeutic applications in a cost-effective manner. Several approaches have been utilized to increase the enzyme yield, among them; optimization of media, genetic engineering and variation of the fermentation parameters (Ginesy *et al*., 2015). Familiarizing ourselves on what happens in the clinical isolated *E. coli* with respect to the production of ADI may help to create efficient production mechanisms in terms of therapeutic use. In the proposed study, there is a need to clone and identify the characteristics of clinical isolates of *E. coli* strains producing ADI, to optimize the production conditions by a systematic development of different parameters and evaluate the antioxidant characteristics of raw extract of enzyme. This integrated methodology deals with the optimization of production and the intrinsic therapeutic prospect of ADI of clinical isolates, which leads towards the generation of therapeutic agent that is enzyme-based.

2. MATERIALS AND METHODS

Isolation and identification of *E.coli*

A total of45 clinical samples were taken on patients visiting hospitals and medical laboratories within the wasit Province in Iraq. The specimens covered a wide range that comprised clients including urine, stool, blood and wound swabs. The sterile swabs were collected in aseptic conditions in sterile containers and carried to the microbiology laboratory under the conditions that will maintain the viability of the bacteria . On receiving, each sample was inoculated on selective and differentiation agar;MacConkey agar and Eosin Methylene Blue(EMB)agar. The inoculated plates were allowed to grow at 37 ºC aerobically in 1824 hours. After incubation, isolated colonies whose morphological characteristics characterized

Escherichia coli were picked and sub cultured on nutrient agar to give clean isolates (Osińska *et al.*,2023). Gram staining was performed preliminarily in order to get cellular morphology and gram reaction.A series of commonly performed biochemical test then ensued to verify the purified isolates. They were; an Indole test, methyl red test, Voges-Proskauer test, citrate utilization test, urease test, catalase test and a Triple Sugar Iron (TSI) agar slant test. All the tests conducted followed the standard microbiological tests.

Screening of Arginine Deiminase (ADI) Production by Quantitative Assay

The production of arginine deiminase (ADI) by *Escherichia coli* isolates was quantified by the use of colorimetric assay, which was based on quantification of the final product of arginine demines hydrolysis, citrulline. The isolates would then be inoculated to a modified arginine-rich minimal medium that contained (per liter): 10g L-arginine, 5 g tryptone, 5 g NaCl, and 1 g KH_2PO_4 that were adjusted to pH 7.0 sterilized by autoclaving. The culture was grown in 37°C temperature in 24 hours at 150 rmp constant shaking. After incubation, cultures were removed at 10,000 x g, 10 min, 4°C to give cell free supernatants. The supernatant was analyzed by measurement of arginine deiminase activity as the level of citrulline formed by the diacetyl monoxime method. In short, 100 mL of the culture supernatant was combined with 100 mL of the substrate solution (0.5 M L-arginine in phosphate buffered saline,pH 7.2), and this was permitted to incubate at 37 °C in 1 hour. Following the enzyme reaction, 1 ml of reagent colour (a mixture of diacetyl monoxime and antipyrine in acid mixture) was added, stirred and incubated in a boiling water bath 10 minutes to bring out the colour. The tubes were allowed to come at room temperature and the absorbances of the solution in the tubes was measured on wavelength 540 nm in a UV-Vis spectrophotometer. Known concentrations of L-citrulline were prepared into a standard curve to determines the level of enzymatic activity. The concentration of citrulline that was synthesized was portrayed in terms of mL of culture supernatant and a blank run in absence of the enzyme was carried out to eliminate background absorbance (Zhao *et al.*, 2024; Saiapina *et al.*, 2024).

Selection of basal medium in manufacturing (ADI).

Six kinds of culture mediums such as Luria Bertani (LB) broth, Nutrient Broth (NB), Tryptic Soy Broth (TSB), M9 Minimal Salt Medium, Brain Heart Infusion (BHI) broth, and GLU-amino acids supplemented (Modified M9 medium) were picked to test the expression of ADI in *Escherichia coli*. To trigger the production of enzymes, all media were added with the arginine in 15 mM. *E. coli* inoculates produced by two times subculture of *E. coli* cells in LB

broth at 37ºC 18-24 hour and inoculum at a concentration of 1% (v/v) were used in the experiment (Patil *et al*., 2016; Wang *et al*., 2022).

Optimization of Arginine Deiminase Production

a. Optimum pH to Produce Arginine Deiminase (ADI)

E. coli isolates were grown in medium rich in arginine under carefully controlled conditions at different initial pH levels. The pH of the production condition was altered to between 5-9 (With the interval of 1.0) with the help of 1 M NaOH before being sterilized. The media were made of (per liter):10g L-arginine, 5 g tryptone, 5 g NaCl and 1 g $KH_2 PO_4$. A 50 mL aliquot of the prepared each isolate was inoculated in a medium and incubated at 37ºC. And constant agitation 150 rpm for 24 hours; thereafter, 5 mL aliquots of each cultivation were again inoculated with 50 mL aliquots of the prepared medium and incubated as before and subsequently used. Cultures were then centrifuged at 10,000 rpm, 4 ºC 10 minutes to separate out cell-free supernatant and further used as the source of enzyme (Dhankhar *et al*., 2019). ADI activity was measured by the diacetyl monoxime based citrulline assay as detailed earlier. Citrulline absorption and concentration were established by absorbancy at a wavelength of 530 nm and was carried out using a standard graph. The largest amount of citrulline was measured to be at the pH that had been seen as the most suitable to use in sampling concerning the generation of enzyme.

b. Optimization of (ADI) Production Using Different Carbon Sources

In order to maximize production of arginine deiminase (ADI), different carbon sources were analyzed in regard to the production of the enzyme. The carbon sources chosen were carbon glucose, maltose, galactose, sucrose and starch. A basal fermentation medium was prepared; each carbon source was added individually resulting in a final concentration of 1% (w/v) and used in place of the normal carbon source. The inoculated T-flasks were allowed to incubate at optimized conditions of growth. Harvesting of the cultures was done after incubation and the cell free supernatant was obtained after centrifuging the culture at 10,000 rpm/10 min/4 ºC. Subsequent quantitative analysis of the obtained supernatants was done by use of enzyme assays in determination of the ADI activity (Fan *et al*., 2014). Each experiment was carried out 3times in order to have a reproducible and reliable information.

c. Optimization of (ADI) Using Different Sources of Nitrogen

Various types of nitrogen were tested to maximize the production of arginine deiminase (ADI) by addition of separate nitrogen sources, viz. ammonium sulfate, peptone, tryptone, casein and yeast extract to the basal production medium. All nitrogen sources were added

individually to afinal concentration of 5 % (w/v) to replace the standard nitrogen component of the medium. A standardized bacterial suspension was inoculated onto the fermentation medium and the medium was allowed to incubate under conditions previously determined as optimum concerning the pH, temperature and agitation. After the incubation time, the cultures were centrifuged at 10,000 rpm at 4°C and in place of 10 minutes. The supernatant thus obtained was free of the cells. ADI enzyme activity in the supernatant was determined by standard colorimetric assay of enzyme responsible of the citrulline production (Fasim *et al.*, 2021). The experimental designs were carried out three times to make the results reproducible and substantially valid.

d. Optimization of (ADI) Using Different Incubation Periods

A time-course study was made to find out the best incubation duration which would give the highest yield of arginine deiminase (ADI). The template amount of an inoculum was added into flasks filled with basal production medium within already optimized pH, temperature and agitation levels. The flasks that were inoculated were incubated in various time periods;12,24,36 and48 hours. Each incubation was terminated by centrifugation at10,000 rpm within 10 min at 4ºC to obtain cell-free supers in the end of the period (Wang *et al.*, 2023). A quantitative colorimetric assay that targeted the level of citrulline production was used to determine the ADI activity across the supernatant. Each experiment was done in triplicate so that the results could become accurate and repeatable.

e. Optimization of (ADI) Using Different Temperatures of Incubation

To determine the influence of the incubation temperatures on the synthesis of arginine deiminase (ADI), the bacteria cultures were cultivated under four different temperatures salutary, 27 °C, 32 °C, 37 °C and 42 °C. A common inoculum was used in the basal production medium whereas all other conditions like pH, agitation speed and time of incubation were maintained at constant level. After the incubation, at the mentioned temperatures, the cultures were centrifuged at 10,000 rpm 10 minutes at 4 °C which yields the cell-free supernatant. The activity of ADI was measured in the supernatants by a quantitative colorimetric method on the basis of formation of citrulline with the arginine (El-Shora *et al.*, 2024). All the experimental conditions were carried out three times to attain reliability and reproducibility of the data.

Antioxidant Properties in Crude ADI Extract

The crude extract of arginine deiminase (ADI) obtained in *Escherichia coli* was investigated considering the effectiveness of antioxidant potential by the quantification of the capability of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging. In brief, 1 mL of

methanolic solution of enzyme extract was added into 1mL of 1 mM methanolic DPPHsolution.The mixture was then incubated in darkness at normal room temperature and after 30minutes the absorbance was measured in UV-Vis spectrophotometer at a wavelength of 517nm. Ascorbic acid was attributed with a positive control. Percentage Depletion of DPPH radical the calculation to obtain the Sustaining scavenging activity was according to the following formula (Silva *et al*., 2024).

$$Scavenging\ Activity\ (\%) = (\frac{sampleA\ \ control\ A}{ControlA}) \times 100$$

The results were reported as the means standard deviation because the analysis was carried out in three replicates. What is implied by the antioxidant activity of ADI is a possible biomedical application that extends beyond its enzymatic ability.

3. RESULT AND DISCUSION

Identification and Isolation of *Escherichia coli*

The forty-five clinical samples, obtained by several sources, e.g. urine, stool, blood and wound swabs, a total of 25 isolates were provisionally identified as *Escherichia coli* by their colony morphology on selective media. Colonies having pink to red pigment on MacConkey, and metallic green sheen appearing on EMB agar were assumed to be distinctive of *E. coli* and were sub cultured off to perform additional investigation. Gram negative, rod-shaped, bacteria were observed under microscope and they were arranged singly or in midst size chains. Urease-negative and catalase-positive were all isolates. Biochemical studies further supported that the isolates were negative to indole, methyl red positive, Voges Proskauer negative and citrate negative, which is the typical identification pattern of the *E. coli*. Identification was also supported by Triple Sugar Iron (TSI) test that reacted as acid/acid with gas formation and produced no Hydrogen Sulfide. Those being isolation and identifications of *E. coli* in various clinical samples the omnipresent characteristic of this organism in human infections where stool gave the highest rate of isolation (83.3 %). as revealed in the table-1.

Table 1. Distribution of *Escherichia coli* isolates by sample type

Sample Type	Number of Samples Collected	Number of *E. coli* Isolates	Percentage %
Urine	10	6	60.0%
Stool	6	5	83.3%
Blood	5	3	60.0%
Wound swab	4	2	50.0%
Total	25	16	64.0%

This observation reflects findings of the earlier studies that show natural occurrence of *E. coli* in the human gastrointestinal tracts and may induce infections when translocated to the extraintestinal locations (Yang *et al*., 2023). Identification based on the classical microbiological techniques proves that the classical tests, that is, biochemical tests, in the characterization of *E. coli* are also reliable; even with the current techniques of molecular techniques, the role of the traditional tests still has a bearing in the field of clinical microbiology.

Quantitative Screening of Arginine Deiminase (ADI) Activity

Isolations of *Escherichia coli* demonstrated varying enzyme production parameter. Depending on the colorimetric determination of citrulline, the isolates displayed the different enzymatic activity, where some of them were found to be highly productive in terms of ADI, whereas others were moderately and weakly productive. Such differences indicate that expression of ADI in clinical *E. coli* is heterogeneous under the standard growth conditions that were tested. (Figure 1).

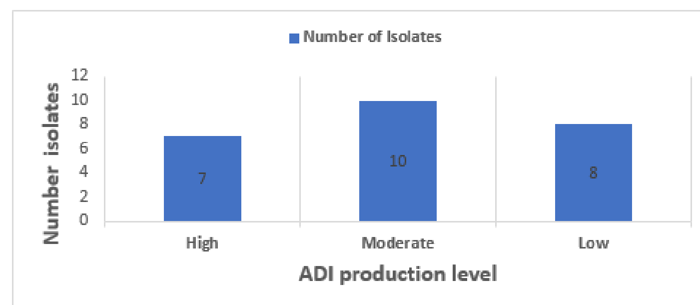

Figure 1. Levels of Arginine Deiminase Production in 25 *E.coli* Isolates.

Some literatures have shown variable expression of arginine deiminase (ADI) in bacterial species such as *Escherichia coli* meaning that there is strain specific regulation of arginine deiminase as well as strain specific metabolic adaptation. Example of scientists who have proven this can be seen in the case of few *E. coli* clinical isolates increasing its expression during acidic or anaerobic conditions that lead to stress response and pathogenicity (Gruening *et al*., 2006).Moreover, screening of related *Enterobacterocea* showed variety of enzyme-types which were growth-media and environmental-need dependent (Lillie *et al*., 2024). These results prove the existence of heterogeneity in ADI production among clinical isolates as it is currently observed.

Effect of Different Basal Media on (ADI) Production

Such a study was performed with *Escherichia coli* and six different culture media to which the addition of 15 mM arginine was made. The findings stated that there was evident change in ADI specific activity across the tested media. ADI activity was the highest in

modified M9 medium with glucose and amino acids followed by M9 Minimal Salt Medium and Tryptic Soy Broth (TSB). On the contrary, reduced activity of ADI production was determined in Luria Bertani(LB), Nutrient Broth(NB) and Brain Heart Infusion(BHI) broth. All these indicate that, minimum media supplemented with defined carbon and nitrogen sources are more supportive in ADI expression as opposed to complex nutrient-rich media (Figure 2).

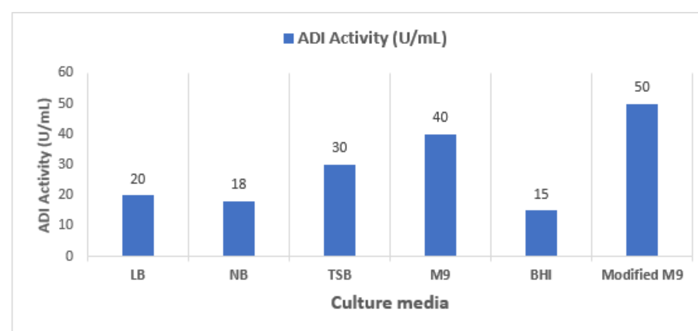

Figure 2. Effect of different cultures media on arginine deiminase (ADI)activity in *E. coli.*

The cells were cultured in six basal media supplemented with 15mM L-arginine and ADI activity was measured after 24 hours' incubation time. The enzyme activity was greatest in modified M9 media whereas the least enzyme activity occurred in BHI and NB. It is found in previous literature that the significant implication of culture media composition on the production of arginine deiminase (ADI) in bacterial systems is significant. BMC Biotechnology research has demonstrated that culture conditions also have significant influence on the in vivo solubility of overexpressed ADI in *Escherichia coli* and in this regard, medium optimization is vital in order to have better enzyme activity (Jamil *et al*., 2015). Research findings on various *E. coli* strains brought out the fact that certain strains such as BL21(DE3) and Rosetta (DE3) have better ADI production potentials because of their different genetics and metabolism (Abdollahi *et al*., 2022). Also, it has been shown that minimal media with explicit carbon sources especially glucose encourages higher metabolite production in *E. coli* than complex media (Rugbjerg *et al*., 2018). The M9 medium has been found to have a buffering capacity that is essential; the modified M9 media has been observed to have an increase of two times the yield of the heterologous proteins (Azatian *et al*., 2019). All these findings undermine the assumption that well designed minimal media and selective nutritional supplements highlight the best bacterial enzyme production systems.

Optimization of (ADI) Production Using Different Carbon Sources

The influence of carbon sources on the synthesis of arginine deiminase (ADI) was studied by the addition of different kinds of sugars into the basal fermentation medium under the

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concentration of 1 % (w/v). The specific activity of ADI gave a wide range variation with different carbon sources used. Substrates tested showed the largest ADI specific activity in sucrose (1.3 U/mg protein), after that, they were glucose (0.9U/mg protein) and starch (0.8U/mg protein). Maltose and galactose were, on the contrary, less active (0.7 U/mg protein each). These findings show the best result in terms of carbon source to increase the ADI production under the experimental conditions is sucrose. Figure 3. Following influence of various carbon sources (1%) on unique action of arginine deiminase (U/mg protein). The greatest enzymatic activity was in sucrose among the substrates tested.

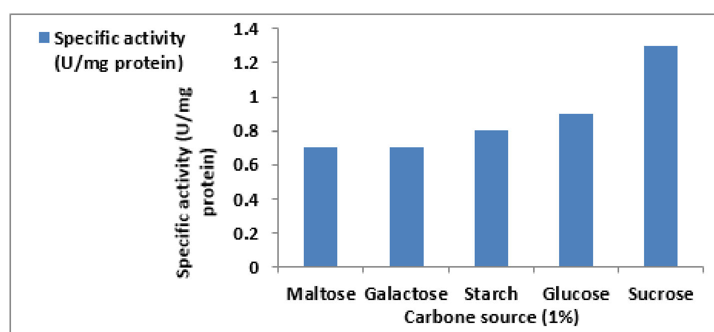

Figure 3. Effect of carbon sources on Arginine Deiminase (ADI) Production.

The character of carbon source highly influences the efficiency with which the microbial enzymes are made. The carbon source can also be sucrose which may be better than glucose in consumer more chemicals and different studies show that they provide more amino acids in *Corynebacterium glutamicum* (Nie *et al*., 2022). In *E. coli* the production of L-isoleucine, it was also established that sucrose was the most promising back-end carbon source in relation to other sugars (Wang *et al*., 2015). The enzyme production has been produced at a low cost following the sucrose addition in *Bacillus subtilis* and showed a better result in obtaining fibrinolytic enzyme (Wu *et al*., 2019).Increased energy efficiency in the production of proteins via sucrose pathways of metabolism means more energy using sucrose as the carbon sources as compared to alternative sources (Boettner *et al*., 2002).These results make sucrose as a suitable carbon substrate to maximize yields in production of bacterium enzymes.

Optimization of (ADI) Production Using Different pH level

The optimum pH of ADI synthesis was to be established by growing *E. coli* isolate on arginine-rich culture under favorable conditions with controlled pH range of 5 to 9. The findings, which can be seen in Figure 4, indicate that the initial pH of the medium greatly affected determination of the specific activity of theADI. The specific activity of enzyme rose steadily between acid pH 5 and pH 7, where the enzyme activity peaked to give the highest

value of 1.6 U/mg protein. More than this, there was a significant reduction in the production of enzymes, with the activity reducing to pH 8 (1.3 U/mg protein) and pH 9 (0.9 U/mg protein). This observation implies that the best condition facilitating the production of ADI in *E. coli* at the tested conditions is neutral pH of about 7.0.

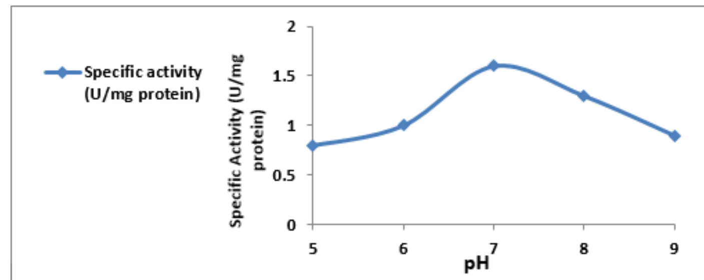

Figure 4. Effect of initial pH on the particular activity of arginine deiminase.

Bacteria enzyme production and activity critically depend on the pH of culture medium. In vitro directed evolution experiments on arginine deiminase proved that variants with an increased pH optimum at 7.0 had a greater activity at physiologic pH and temperature(Zhu *et al*., 2010). It has been found out that the majority of bacteria, including *E. coli* is neutrophilic and grows best in neutral pH (almost 7.0) (Duffy *et al*., 1999). Analysis of *E. coli* metabolism showed that glycolysis is highly sensitive to fall of intracellular pH below 7 and the activity of enzymes is only 20 % activity at pH 6 compared to pH 7.5 (Foster, 2004). Enzyme stability as well as cellular metabolism require optimal pH because severe pH conditions may denature proteins and inhibit their metabolic routes (Che, 2023).The above results combine together to favor a neutral pH as the ideal environment in which to maximize bacterial enzyme production and to preserve enzyme activity.

Optimization of the Production of (ADI) Using Different Sources of Nitrogen

Various nitrogen sources which affected the synthesis of arginine deiminase (ADI) were tested under conditions where the basal media were supplemented with various nitrogen compounds at a concentration of 0.5 percent (w/v). Figure 5 depicts that the specific activity of ADI changed extensively according to the applied source of nitrogen. Of the tested sources, yeast extract appeared to have the highest specific activity (1.5 U/mg protein), followed by casein (1.2 U/mg protein), tryptone had a moderate and equal activity (1 U/mg protein) and peptone had the same activity (1 U/mg protein). The inorganic source of nitrogen, ammonium nitrate, yield the lowest activity of enzyme (0.8 U/mg protein). The results indicated enhanced biosynthesis of ADI with organic nitrogen source especially yeast extract and casein under the conditions tested.

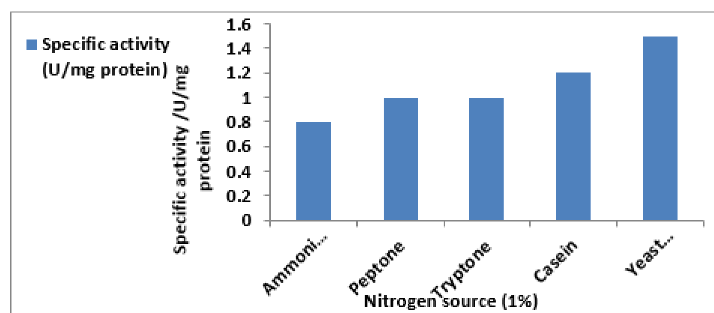

Figure 5. Effect of nitrogen sources on arginine deiminase specific activity.

The composition of the nitrogen source used has major effects on *E. coli* recombinant proteins production and that complex organic nitrogen compounds and particularly yeast extract-containing mixtures of organic nitrogen play a major role and tryptone mixtures resulted in increased ADI solubility and expression compared to their synthetic counterparts during arginine deiminase expression studies (Wang *et al.*, 2006). It was found that when seeking to optimize the synthesis of recombinant proteins the organic sources of nitrogen have the potential of supplying the necessary amino acids and growth factors which will facilitate enzyme production (Rosano & Ceccarelli, 2014). Machine learning was used to establish the truth in the hypothesis that there is a direct impact on differences observed in the protein yield when machine learning was applied using yeast extract having different compositions to act on *E. coli* (Tachibana *et al.*, 2021). Research done on engineering basis revealed that a perfect selection of carbon and nitrogen source is indispensable to maximize production of recombinant protein and limit metabolic stress (Lozano Terol *et al.*, 2019). The combination of all these outcomes allows concluding that organic complex sources of nitrogen, especially yeast extract prove superior as compared to inorganic sources in the case of bacterial optimization of enzyme production.

Optimization of (ADI) Using Different Incubation Periods

In an attempt to determine the best incubation time leading to maximum arginine deiminase (ADI) synthesis, the cultures of *E. coli* were allowed to incubate under the already optimized conditions of 12, 24, 36 and 48 hours. As it was revealed in Figure 4, the activity of ADI changed significantly with the time of incubation. The maximum enzyme activity was found after 24 hours (1.6 U/mg protein), hence, at this moment in time the maximum ADI biosynthesis is achieved. Contrastingly, different shortened (12 h) and prolonged incubation (36 and 48 h), the enzyme activity produced highly decreased and the specific activities measured the values 0.7, 1.0, and 0.8 U/mg protein, correspondingly. It is also possible that activity decrease after 24 hours is due to either degradation of enzymes, exhaustion of nutrients

during fermentation process, or buildup of inhibitory metabolic end products in the culture medium (Figure 6).

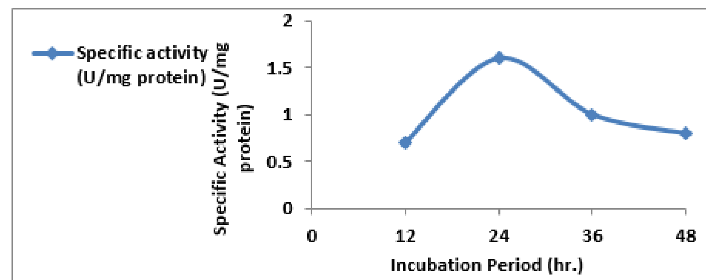

Figure 6. Effect of incubation period on specific activity of arginine deiminase.

Other studies have showed that the best utilization of enzymes in bacterial fermentation is through a period of the range of 24hrs incubation. Studies into arginine deiminase overexpression in *E. coli* suggest that the growth environment has a considerable effect on solubility and activity of the enzyme which tested five *Escherichia coli* derivative strains as the medium of arginine deiminase over production (Wang & Li, 2014). Prolonged incubation after the optimum periods of incubation results in the denaturation of proteins and the loss of catalytic activities since all proteins degrade with time evolutionary engineering of methylotrophic *E. coli* allows rapid growth on methanol (Moni *et al*., 2024).

Optimization of (ADI)Using Different Temperatures of Incubation.

In order to investigate the impact of incubation temperatures on the arginine deiminase (ADI) production, the growth was done at four different temperatures (27ºC, 32ºC, 37ºC, and 42ºC) keeping other conditions unchanged. The figure 7 shows that the incubation temperature significantly affected the specific activity of ADI. Optimal temperature 37 °C (1.6 U/mg protein) was determined where the enzyme showed the greatest activity (1.6 U/mg protein) thus making this the ideal temperature to carry out the ADI biosynthesis reaction. The activity was moderate at both temperatures 32 ºC and 42 ºC (0.9 U/mg protein) whereas at 27 ºC very low production was recorded (0.4 U/mg protein). The results indicate that the optimal temperature to express an enzyme is the 37 ºC because at this temperature, metabolism of the cell as well as protein synthesis is probably highest.

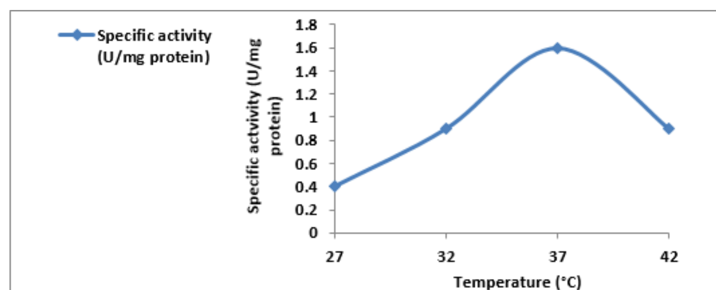

Figure 7. Effect Incubation temperature upon the specific activity of arginine deiminase.

Past research results have agreed that the most ideal temperature of enzyme production and cellular metabolism in *E. coli* was 37 ºC. The typical arginine deiminase activity assays are carried out at 37 ºC, since this temperature is the best one to give optimum enzymatic conversion rates production of arginine deiminase (ADI) of *Pseudomonas plecoglossicida* CGMCC2039 in *E. coli* and its anti-cancer efficacy (Ni *et al*., 2009). Studies concerning protein synthetic capacity of *E. coli* marked that ribosomal cellular machinery functions optimally at a range of temperatures between 25-37 ºC and at 37 ºC as the optimal temperature (Nieh *et al*., 2024). The optimum growth temperature of *E. coli* is 37 ºC corresponding to body temperature of humans where, in *E. coli*, cellular machinery is at its most effective allowing fast cell division and high metabolic capacity efficient renaturation of the anti-tumor enzyme Mycoplasma arginine deiminase which had been expressed at high levels in *Escherichia coli*(Misawa *et al*., 1994).

Antioxidant Activity of Crude ADI Extract

DPPH radical scavenge assay was used as a quantitative method to determine the antioxidant activity of crude arginine deiminase (ADI) extract obtained in *Escherichia coli* compared against ascorbic acid which acts as a control (standard reference antioxidant). The results included in (Table 1) showed that ADI extract exhibited a concentration-dependent scavenging activity as well as ascorbic acid. The ADI extract showed scavenging activity of (79.28 ± 1.06%), percent at the greatest concentration possible (200 µg / mL) that was similar to ascorbic acid (86.11 ± 1.45%) The scavenging effect was gradually reduced at lower concentrations till 12.5 µg/mL of ADI which was reduced to (37.46 ± 4.14%) and that of ascorbic acid stood at (42.32 ± 1.01%) Table 1. Although the overall scavenging efficacy measured of the ADI extract was slightly lower than that of ascorbic acid, it orally continued to pose substantial antioxidant potential, especially in higher concentrations. At free radical neutralization half levels (50 µg/mL), statistical inference indicated no significance ($p > 0.05$), indicating that the performance of neutralizing free radicals at these levels was relatively similar (figure 8). Based on these findings it is possible to assume, that crude ADI has some

antioxidant action, which can prove its possibility of biomedical application outside of its effect in arginine metabolism.

Table 2. DPPH Radical Scavenging Ability of crude ADI extract and ascorbic acid.

conc(µg/ml)	Ascorbic acid		Crude ADI Extract	
	mean	**SD**	**mean**	**SD**
200	86.111	1.450483	79.28233	1.060648
100	76.852	3.543266	71.682	2.956229
50	67.168	0.876269	66.20367	1.68097
25	54.70667	0.837114	52.85467	3.347018
12.5	42.32267	1.010981	37.46133	4.135307

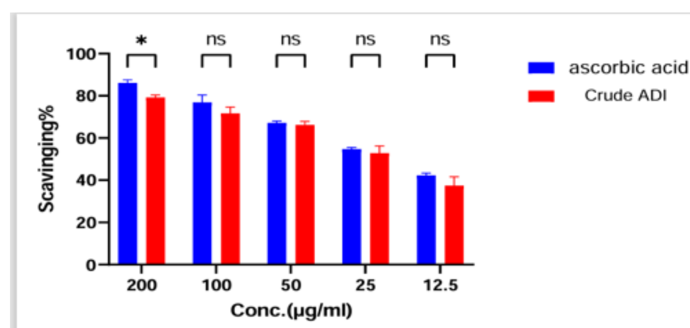

Figure 8. Antioxidant activity of crude ADI extract and ascorbic acid.

In recent studies it has been shown that the bacterial enzymes especially the ones participating in amino acid metabolism show superb antioxidant abilities in addition to their main functional mode of catalytic activity. The research on bioactive peptides has exhibited a significantDPPH radical scavenging effect with an IC_{50} in the micromolar ordinate as evidence of concentration-dependent antioxidant property (Ahn *et al.*, 2014). Study of the microbial enzyme extracts also indicated significant free radical scavenging capacities with a DPPH scavenging capacity of 70-80 at media optimization for recombinant soluble arginine deiminase by response surface methodology for in optimum concentration of *Escherichia coli* (Zarei *et al.*, 2017). Antioxidant defense systems used by biological systems mainly entail enzyme systems that counter antagonistic reactive oxygen species using a variety of pathways evolutionary engineering of methylotrophic *E. coli* allows rapid growth on methanol (Nieh *et al.*, 2024). The extraordinary antioxidant ability of protein hydrolysates has been determined with DPPH scavenging rates higher than 60 % under the favorable circumstances metabolic engineering of *Escherichia coli* for enhanced arginine biosynthesis (Charlier *et al.*, 2019). This observation favors the dual role of arginine- metabolizing enzymes of both catalytic and protective functions on the cellular level.

4. CONCLUSION

This research achieved the isolation and optimization of *E. coli* strains producing ADI out of clinical specimens and thorough optimization of the conditions of the ADI production led to considerable increases of the enzyme yield. In the systematic optimization process, incorporation of Modified M9 medium containing sucrose as the carbon supply as well as yeast extract as the nitrogen supply, pH 7.0, 37 °C temperature and 24 hours incubation was found to be the optimal condition that can be used to obtain maximum ADI. The revelation of a strong ability of crude ADI extract as antioxidant is a new finding that widens therapeutic potential spectrum of this enzyme besides its well-known anticancer activity. This radical scavenging ability presents an excellent potential of being antioxidant with a DPPH activity of 79.28 percent of 200 ug/mL significance which may show clinical effectiveness through a two-pronged antidote effect. The existence of heterogeneity among clinical isolates in the ADI production capacity demonstrates the need to consider strain selection in terms of biotechnology applications. The rationalized production conditions that were developed in this research paper serve as a basis in the development of cost-efficient ADI production systems which can be used as potential therapeutic systems. The scale-up of production process, exploration of the enzyme purification and characterization, in vitro and in vivo experiments, to investigate the therapeutic value of ADI as dual anticancer as well as antioxidant agent should be conducted in future. Further application of such metabolic engineering strategies and the optimized conditions, as they are revealed in the current investigation should contribute to even greater efficiencies of the ADI production. Those results are valuable to the perspective on ADI production optimization and the new insights into the multifunctional properties of this enzyme, which explains the growing interest to work on it as a perspective therapeutic agent to treat cancer and the diseases of the oxidative stress nature.

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