"PRODUCTION AND PARTIAL PURIFICATION OF ARGININE DEIMINASE ISOLATED FROM BACTERIA

Afshaan Naaz Khaleed Shaikh., R.M. Khobragade
Department of Microbiology,
Dr. Babasaheb Ambedkar Marathwada University Sub-campus, Osmanabad,
M.S., India (413501).

Abstract:

An arginine deiminase (EC 3.5.3.6)is an hydrolytic enzyme that catalyzes the arginine and produce citruline as a product. Hence it has been studied as a potential anti-tumor drug for the treatment of arginine-auxotrophic tumors, in the treatment of tumors susceptible to arginine deprivation, such as malignant melanoma and hepatocellular carcinoma, arginine deiminase (AD), an arginine-degrading enzyme, has been used. In addition, tests of human lymphatic leukemia cell lines show that ADI is a potential anti-angiogenic agent and is effective in leukemia therapy. In a broad range of microorganisms, including bacteria, actinomycetes, yeast and fungi, ADI is present throughout nature. Soil samples for the isolation of bacteria have been used in recent research work. . 11 bacteria isolates were collected and tested for ADI, activity was only seen in 2 isolates labeled ADI 9 and ADI 11. In order to verify the efficiency of ADI production, these 2 isolates were further screened. ADI 9 provided the highest ADI enzyme (83 IU/ml). Via ammonium sulpate precipitation and dialysis, partial enzyme purification was performed. It was found that the optimum pH was 7, the optimum temperature was 37ºC and the maximum enzyme production of glucose as a carbon source was provided by the effect of pH, temperature and carbon sources on the enzyme. ADI 9 isolate was identified with the help of Gram character, morphological character and biochemical test. The ADI 9 bacteria have been shown to belong to *Bacillus sps.*

Key words: Arginine deiminase (ADI), Anticancer, dialysis, *Bacillus sps.*

INTRODUCTION

Some tumors need additional cellular sources of certain amino acids which, due to metabolic deficiencies, are considered non-essential in normal cells(1). While some tumor cells are auxotrophic to arginine, extracellular arginine depletion could be used to treat certain tumors by means of arginine degrading enzymes(2). Therefore, enzymatic degradation of these amino acids may be an effective way for tumor suppression. Arginine deaminase has gained greater interest as a possible anti-cancer agent for arginine-auxotrophic tumors among the enzymes that disrupt arginine(3). One potential way to find new sources of arginine degrading enzymes is to screen, extract and characterize microorganisms. Arginine deiminase (ADI) has been considered an anti-tumor enzyme among the arginine degrading enzymes in the treatment of hepatocellular carcinomas and melanomas that are auxotrophic to arginine(4). In the arginine deiminase pathway, the catabolism of arginine to citrulline and ammonia is catalyzed by this enzyme, which is used by a variety of microorganisms like *Pseudomonas, Mycoplasma, Halobacterium, Lactobacillus, Lactococcus, and Streptococcus* as an energy supply(5).

$$\text{L-arginine} + H_2O \longrightarrow \text{L-citrulline} + NH_3$$

The aim of the current study was to isolate bacterial species which are capable of producing enzymes that degrade arginine and to check the effect of different parameter on enzyme activity and partially purify enzyme. A variety of microorganisms were screened for this purpose from different samples to obtain the species that can produce enzymes that degrade arginine; using a simple and efficient screening technique on phenol red indicator plates. Furthermore, the enzyme activity was measured by Nessler's reagent(6), and the isolated species were characterized on the basis of Gram's nature, morphology and biochemical testing. Seeing as optimizing the production of enzymes will lead to higher amounts of enzymes, the effects of temperature, pH, and different carbon source on enzyme production has been assessed.

MATERIALS AND METHODS

Chemicals

All chemical used in this research work were of analytical grade were purchased from Himedia, Mumbai, India.

Collection of soil sample

Soil sample was collected from Aurangabad region at a depth of 5 to 10 cm. The samples were collected into a sterile polythene bags and carried to departmental research lab for further study. Samples were stored at 4^{o}C, till further use.

Isolation of ADI producer

To the selective screening medium where Arginine was used as the sole nitrogen source, 1 mL of the diluted soil samples was transferred and incubated at 37^{o}C for 24 hours on rotary shaker at 150rpm.

Screening

The screening medium (pH-7) consisted of: 0.01% glucose, 1.5% L-arginine, 0.002% NaCl, 0.075% KH2PO4, 0.05% MgCl2, 0.01% MnCl2, 0.0005% FeSO4, 0.1 M CaCO3, 1.7% agar and 0.005% phenol red, as the pH indicator(7). The plates were prepared and spot inoculated with isolated cultures. All plates were incubated at 37^{o}C for 24hours.After incubation. Formation of purple zone around colony indicates arginine degrading species(8). Out of 11 isolates only 1 strain were ability to produce ADI and were further confirmed by checking enzyme activity.

Enzyme assay

The selected strain was subjected to further activity analysis using Nessler's reagent to calculate the ammonia concentration of arginine degrading enzymes as the product of arginine degrading enzymes. Cultured in 50 mL of the specified medium, the bacteria were incubated at 37oC for 24 hours, during which 2 mL of each growth medium was collected and centrifuged in order to determine the ammonia concentration in the supernatant. Then 50 µL of Nessler's reagent was transferred to 1 mL of the supernatants collected, and the concentration of the ammonia formed was quantified by reading the absorbance at 480 nm by using UV/Vis spectrometer(9).

Optimization of Growth Conditions for ADI Production

Effect of time duration on enzyme production After every 24hrs for 4 days, specified media inoculated with bacterial isolate and ADI activity were calculated using the above-described assay process.

Effect of different temperature on enzyme production The inoculated production medium was incubated at different temperatures (RT (28), 20, 37, 50, 60, 70 ^{o}C) for temperature optimization, and the enzyme activity was calculated using the above process.

Effect of different carbon source on enzyme production The same assay is conducted using various sources of carbon, i.e. glucose, galactose, fructose and sucrose, with 0.2 percent test sugar supplemented for this production medium.

Partial purification of ADI

With ammonium sulphate salt, the ADI was partly purified. For 15 minutes, the fermented broth was centrifuged at 10,000 rpm(10). Supernatants were extracted after centrifugation.The volume of 10 ml of supernatant was taken and by applying pinch wise, ammonium sulphate was taken to saturate 80 percent according to the volume. This process was conducted at a temperature of 4^{o}C, and the broth was allowed to settle overnight after completion of saturation(11). The pellet was then separated by centrifugation. At a minimum quantity of 0.08M potassium phosphate buffer of pH7.33, the pellet was carefully dissolved(12).

Dialysis

The dialysis was used to remove salt protein, and get purified form of enzyme(13)

Protein estimation

Protein estimation was carried out by Folin-Lowery method(14) of crude enzyme, ammonium sulfate precipitate enzyme and purified enzyme.

Identification of strain

The ADI producing strains cultural, biochemical, sugar fermentation and morphological characteristics were studied.

RESULTS

Isolation and Screening

From the soil sample, 11 bacteria were isolated and identified as ADI 1 to ADI 11. 11 isolates have been tested for ADI production in primary screening. Just ADI 9 and ADI 11 out of 11 isolates exhibit growth in the screening medium. For secondary screening, these 2 isolates were chosen. Just one isolate (i.e. ADI 9 isolate) showed good findings in secondary screening of 2 screened cultures. For further analysis, this strain was chosen and identified as *Bacillus sps.*

Effect of time duration

The higher production of enzymes was seen at 24 hours after which the activity declines after 48, 72, 96, 120 hours, maintaining 71%, 54%, 28% and 17% activity respectively.

Effect of different temperature

The higher production of enzymes was shown at 37ºC. Further rise or decrease in temperature retardation of the enzyme activity i.e. 20ºC, 28ºC, 37ºC, 50ºC, 60ºC, 70ºC, 11 %, 58 %, 31 %, 22 % and 11% respectively.

Effect of pH

Higher enzyme production was seen at pH 7 and change in pH affect enzyme activity i.e. 6, 8, 9, 10 pH enzyme retaining activity 27%, 41%, 77%, 81% respectively.

Effect of carbon sources

Higher enzyme production was accomplished with the use of glucose, and much less activity was observed using an alternative carbon source, so glucose was used as a carbon source in the work.

Partial purification of Enzyme:

The impurities contained in crude extract were extracted by a step-by-step purification procedure, i.e. precipitation of ammonium sulphate accompanied by dialysis. The specific crude extract activity prior to purification was 35 U/ml. The basic activity was found to be increased by 1.86 fold after precipitation with ammonium sulphate and the overall protein content was found to be reduced by 1.16 fold. This suggests that the ammonium sulphate fractionation range used for precipitation was sufficiently successful in the elimination of contaminant proteins, resulting in a lack of overall activity. Enzyme activity after dialysis was enhanced by 2.16 times.

DISCUSSION

11 isolates have been isolated from the soil sample in current research work and have been labeled as ADI 1 to ADI 11. Just 2 isolates with positive findings selected for more activity enzyme tests were screened in all 11 isolates for primary study. Just one isolate out of 2 isolates, i.e. ADI 9, showed maximal enzyme activity (15) This strain was selected for further analysis.

The suspected bacterial isolates were positive in Gram staining. In addition, the cells were mainly single spore forming rods. This result agreed with described by(..) Bacillus sps.

Depending on the cultural characterization and results of biochemical tests, isolate was found to be belonging to *Bacillus sps.* (16) revealed that such characteristics usually are coming in agreement with those fitting to *Bacillus sps.*

In present study enzyme arginine deiminase producers were isolated from soil sample of Aurangabad region. Few characteristics of enzyme like optimum temperature, pH, and substrate specificity was determined and few production parameters were optimized. One study reported that arginine deiminase showed optimum activity at 36ºC and 7.5pH (17). The present research work agreed with these temperature optima but optimum pH for my isolates was 7. Also, (18) stated that growth at 36ºC is a characteristic feature of *Bacillus sps*, since most other are

unable to grow at 36°C. Study reported that enzyme shows more activity by using maltose as a sole source of carbon but in present research enzyme is more active in presence of glucose as a sole source of carbon.

The findings show in the first stage of purification that ADI is an enzyme hydrophobic, as it precipitated by high concentrations of salt. The lipid or hydrophobic movement associated with the enzyme may perceive this effect to make it lighter than the aqueous period and thus float on it. ADI enzyme isolated from *Bacillus sps* precipitated with 60% to 70% ammonium sulphate concentration.

CONCLUSION

From present work it can be concluded that there was successful isolation of Arginie deiminase producing isolate identical to *Bacillus sps* and able to produce ADI having optimum activity. Moreover, the study shows that after optimizing fermentation parameters such as fermentation time, temperature, pH and carbon source, maximum ADI enzyme production is achieved. This research suggests that ADI 9 is an arginine deiminase producing potentials strain and that ADI production has an optimum temperature of 37ºC at 7 pH. After 48 hours, the highest production was observed using glucose as an only source of carbon. In the future research, genus level of ADI 9 bacteria will be identified. For optimum enzyme ADI production additional parameter will be considered.

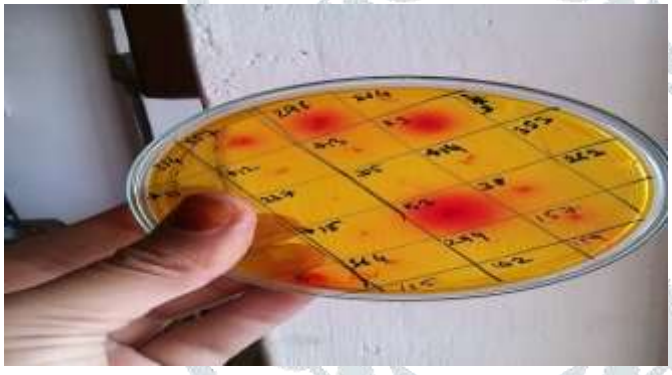

Plate 1 Isolates showing growth on modified media

Identification of the strain:

Table1 Morphological characters

Characters	Pigmentation	Shape	Color	Gram Nature	Motility
Result	Off white	Rod	Pink	positive	Motile

Table2 Cultural Characters

Media	MacConkeys agar	Casein agar	Starch agar	EMB agar
Result	Colorless	zone of hydrolysis	zone of hydrolysis	Colorless

Table3 Biochemical characters

Tests	Indole	VP	Oxidase	Lysine	MR	Citrate	Catalase	Phenylalanine	Urease	Gelatin	H_2S
Result	-	+	+	-	+	+	+	+	-	+	-

Table4 Sugar fermentation

Sugars	Sucrose	Glucose	Fructose	Arabinose	Xylose	Mannitol	Rhamnose	Galactose	Mannose	Lactose
Result	+	+	+	-	-	-	-	-	-	+

Figure 1 Effect of time course on enzyme production

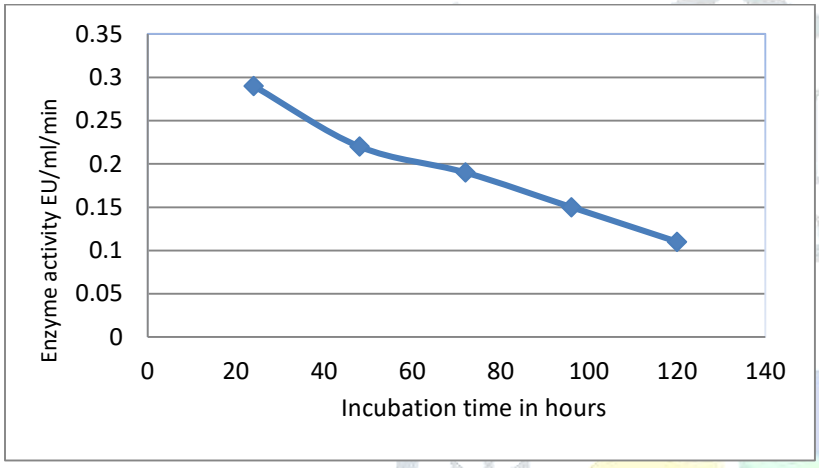

Figure2 Effect of temperature on enzyme production

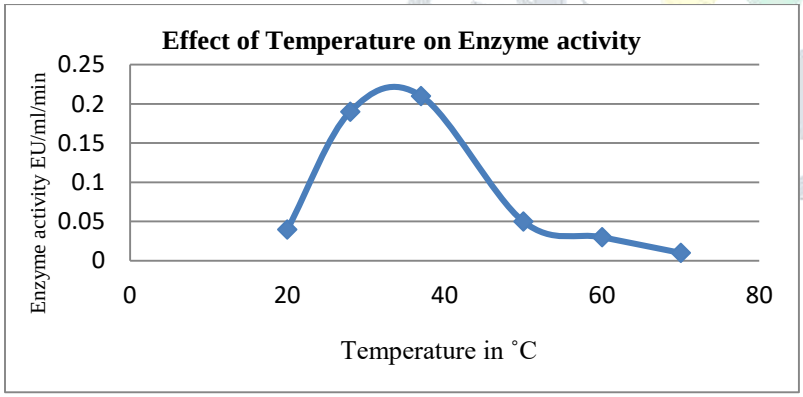

Figure3 Effect of pH on enzyme activity

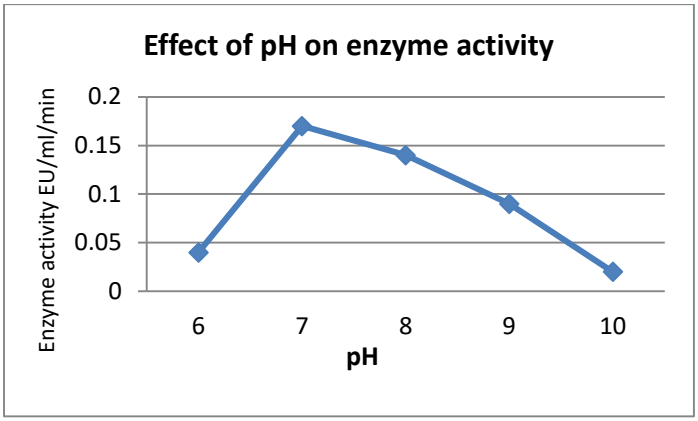

Figure4 Effect of carbon sources on enzyme production

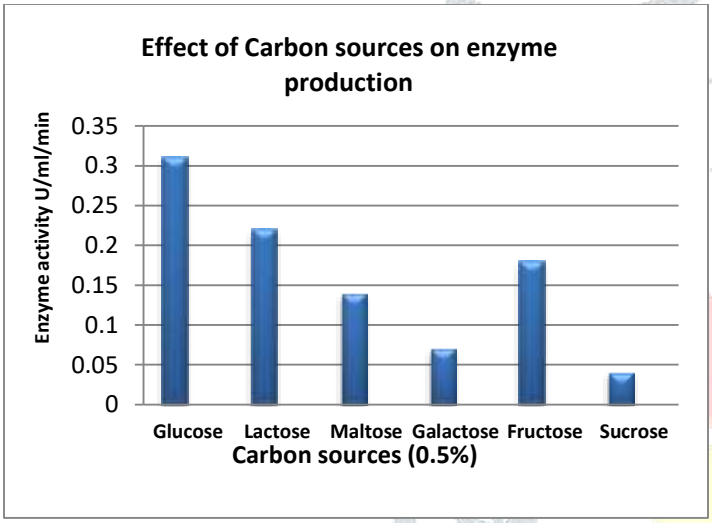

Table6. Specific activity of enzyme

Steps	Enzyme(ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity	Total purification
Crude enzyme	15	32.00	83	0.3855	1 fold
(NH4)2SO4	10	51.00	71	0.7183	1.86fold
After dialysis	5	82.00	58	1.5471	2.16 fold

REFERENCES:

1. Chidambaram, K.V., Kuppanan, N., Kannan, K., Perumalsamy, L. 2009. Production of L- asparaginase by *Serratia marcescens* SB08: optimization by response surface methodology. Iran J. Biotechnol., 7:10 30
2. Crow, V.L., Thomas, T.D. (1982). Arginine metabolism in lactic *Streptococci*. J. Bacteriol., 150:1024–1032.
3. Kaur, B., Kaur, R. 2013. Application of response surface methodology for optimizing arginine deiminase production medium for *Enterococcus faecium sp*. GR7. Sci. World J., .doi.org/10.1155/2013/892587.
4. Oudjama, Y., Tricot, C., Stalon, V., Wouters, J. 2002. Over-expression, purification, crystallization and preliminary X-ray crystallographic analysis of *Pseudomonas aeruginosa* L-arginine deiminase. Acta Cryst. D58, 2150-2152.

5. Yoon, C.Y., Shim, Y.J., Kim, E.H., Lee, J.H., Won, N.H., Kim, J.H., Park, I.S., Yoon, D.K., Min, B.H. 2012. Renal cell carcinoma does not express arginino succinate synthetase and is highly sensitive to arginine deprivation via arginine deiminase. Int. J. Cancer 120:897-905.
6. Sharma, A., Husain, I. 2015. Evaluation of antitumor activity of glutaminase-free periplasmic asparaginase from indigenous bacterial isolates as candidates for cancer therapy. Proc. Nat. Acad. Sci. India., DOI 10.1007/s40011-015-0681-z
7. Rodriguez, S.B, Stitt, B.L., Ash, D.E. 2009. Expression of peptidylarginine deiminase from *Porphyromonas gingivalis* in *Escherichia coli*: Enzyme purification and characterization. Arch. Biochem. Biophys., 488:14–22
8. Rodriguez, S.B, Stitt, B.L., Ash, D.E. 2009. Expression of peptidylarginine deiminase from Porphyromonas gingivalis in *Escherichia coli:* Enzyme purification and characterization. Arch. Biochem. Biophys., 488:14–22
9. El-Sayed, S.S.A., Hassan, M.N., Nada, H.M.S. 2015. Purification, immobilization, and biochemical characterization of L-arginine deiminase from thermophilic *Aspergillus fumigatus* KJ434941: Anticancer activity in vitro. Biotechnol. Prog., 31:396-405.
10. Hering, S., Sieg, A., Kreikemeyer, B., Fiedler, T. 2013. Kinetic characterization of arginine deiminase and carbamate kinase from *Streptococcus pyogenes* M49. Protein Expr. Purif., 91:61-68.
11. Mansour, N.M., and Mostafa, F.A. (2016). Molecular identification and characterization of an arginine catabolizing *Entercoccus faecium* NM216 via arginine deiminase enzyme (ADI) pathway and evaluation its effect in vitro on tumor cell lines. Research Journal of Pharmaceutical, Biological and Chemical Sciences 7, 1646–1653
12. Sharma, A., & Husain, I. (2015). Evaluation of Antitumor Activity of Glutaminase-Free Periplasmic Asparaginase from Indigenous Bacterial Isolates as Candidates for Cancer Therapy. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 87, 997-1004.
13. Sharma, Kiran Bala and Islam Husain., (2017).Optimization of Arginine Deaminase Production from Indigenous Bacterium *Pseudomonas aeruginosa* PS2. Int.J.Curr.Microbiol.App.Sci 2017: 6 (11) : 3621-3632
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193:265-275.
15. Rasheed,M;Latteef,N;Jassim,A; Nader,M.(2016). Rapid Identification of *Pseudomonas aeruginosa* by Using Real Time PCR. Journal For Veterinary Medical Sciences 7: 1
16. Sousa, A. M.; Machado, I.; Nicolau, A. and Pereira, M. O. (2013). Improvements on colony morphology identification towards bacterial profiling. Journal of Microbiological Methods. 95: 327–335
17. Jaafar, Z. M., Dhahi, M. A. R., Abd, A. K. H., & Jaafar, S. M. (2014). Molecular identification and antibiotics resistance genes profile of *Pseudomonas aeruginosa* isolated from Iraqi patients. African Journal of Microbiology Research, 8(21), 2183-2192.
18. Rasheed,M;Latteef,N;Jassim,A; Nader,M.(2016). Rapid Identification of *Pseudomonas aeruginosa* by Using Real Time PCR. Journal For Veterinary Medical Sciences 7: 1