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Cloning, Expression, and Sequence Analysis of Diaminopropionate Ammonia Lyase Gene from a Nonvirulent *Salmonella typhimurium* PU011

K. R. Rupesh, K. R. Padma, P. Saravanan, and S. Jayachandran*

Department of Biotechnology, Pondicherry University, Kalapet, Pondicherry 605014, India, E-mail: sr_jayachandran@yahoo.com

Abstract

Background: Seeds of *Lathyrus sativus*, a legume plant, contain 3-oxalyl and 2,3-dioxalyl DAP (O-DAP), neurotoxins which when consumed causes Neurolathyrism or Osteolathyrism, in humans, affecting nervous system and bone formation respectively. Some microorganisms viz virulent and non-virulent *Salmonella typhimurium*, *Salmonella typhi* and Pseudomonad have been shown to detoxify L-α,β-diaminopropionate (DAP), the immediate precursor of O-DAP.

Result: The gene coding for diaminopropionate ammonia lyase (DAPAL) which detoxifies DAP was cloned from nonvirulent *S. typhimurium* PU011 into *Escherichia coli* DH5α and the nucleotides sequenced (1212 bp). Whereas the specific enzyme activity of DAPAL obtained from recombinant *E. coli* PU018 was 0.346 U/mg, the specific activity of the enzyme from nonvirulent *S. typhimurium* PU011 was 0.351 U/mg. The DAPAL corresponding to 43 kDa protein was found both in nonvirulent *S. typhimurium* PU011 and *E. coli* PU018. The Km value was found to be 0.740 m*M* and 0.680 m*M* for *S. typhimurium* PU011 and 0.741 m*M* and 0.683 m*M* for *E. coli* PU018 when grown in minimal medium (MM+DAP) and *L. sativus* seed extracts respectively, indicating that both of them were capable of utilizing the neurotoxins present in *L. sativus* seeds. The biomass, enzyme production and the effect of pH and temperature on DAPAL enzyme activity from both non-virulent *S. typhimurium* PU011 and *E. coli* PU018 were found to be similar.

Conclusion: The recombinant *E. coli* PU018 as well as non-virulent *S. typhimurium* PU011 are as good as pathogenic *S. typhimurium* in detoxifying DAP, the immediate precursor of O-DAP present in *L. sativus* seeds.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: Diaminopropionate ammonia lyase; *Salmonella typhimurium, Lathyrus sativus*; diaminopropionic acid; neurolathyrism.

Introduction

Diaminopropionic acid (DAP), the immediate precursor of the neurotoxins 3-oxalyl and 2,3-dioxalyl DAP (O-DAP), present in *Lathyrus sativus* seeds, a drought resistant legume rich in proteins causes neurolathyrism/osteolathyrism in humans when ingested regularly or in large doses *(1)*. Diamiopropionate ammonia lyase (DAPAL), an enzyme belonging to the Pyridoxal-5-phosphate (PLP) family, catalyses the α-β-elimination reaction of both L-and D-α-β-diaminopropionate to form pyruvate and ammonia *(2,3)*. Vijayalakshmi et al. (1975) *(2)* and Nagasawa et al. (1988) *(3)* have reported the production of DAPAL by Pseudomonads and *Salmonella typhi*, respectively. In their recent publication Khan et al. (2003) *(1)* have reported cloning, overexpression, purification, and initial characterization of DAPAL from recombinant *E. coli* as well as virulent *S. typhimurium*. However, because virulent strains are hazardous, we have been able to successfully identify a nonvirulent strain of *S. typhimurium* PU011 capable of degrading DAP *(4)*.

In order to detoxify DAP present in *L. sativus* seeds for safe consumption by the famine-hit rural population, either that DAPAL in affordable form in bulk is made available to the community or ready-to-use, efficient, nonpathogenic bacteria capable of producing DAPAL, which is capable of detoxifying DAP, is supplied. The other alternative would be perhaps to develop transgenic *L. sativus* plants free of O-DAP. The study of enzymes involved in the biosynthesis and degradation of the neurotoxin is therefore important for developing biotechnological methods for reducing its level in the seeds. In this investigation we report the cloning of gene coding for DAPAL from a nonvirulent *S. typhimurium* PU011 into pBluescript-II (SK-) vector and its expression in *E. coli* DH5α for possible use by the age old conventional solid state fermentation technique to detoxify the toxin present in *L. sativus* seeds for consumption by the hungry millions starved of proteins in their diet.

Materials and Methods

The nonvirulent *S. typhimurium* PU011, *E. coli* DH5α (endA1, recA1, gyrA96, thi, hsdR17, (r_k^-, m_k^+), relA1, supE44, λ^-, Δ (lac-proAB), [F′, traD36, proAB, lac l^qΔZM15]) and plasmid vector BlueScript SK (-) were obtained from the culture collections of our Department. All the chemicals used in media preparation and buffer preparations were obtained from Hi Media Laboratories, India. Other fine chemicals such as TEMED, X-gal, IPTG and enzymes used in this work were procured from Sigma. The restriction enzymes and T_4 DNA ligase enzyme used in this study were obtained from either Bangalore Genei Pvt. Ltd., India or New England Bio Labs or Promega

Inc. The protein molecular weight marker was obtained from Bangalore Genei Pvt. Ltd., India.

LB agar containing (per liter of distilled water) 10 g tryptone, 5 g yeast extract, 10 g sodium chloride, and 15 g agar (pH 7.2) was used for screening of transformants. For screening the clones capable of producing DAPAL, minimal medium (per 100 mL of distilled water) containing 0.05 g of yeast extract, 500 µL of mineral salt solution, 50 m*M* of 1 *M* phosphate buffer (pH 8.0) and 2g of agar was prepared and sterilized at 121°C, 15 lb pressure for 20 min. Thereafter, to the minimal medium at 60°C filter sterilized DAP at 0.3% was added.

Cloning of Genes Coding for DAPAL

Total DNA from *S. typhimurium* PU011 was isolated as per the method of Kronstad et al. (1983) *(5)*. Restriction digestion, ligation and transformation were carried out following standard procedures *(6)*. The transformants were first screened on LB + ampicillin plates containing X-gal (2%, final conc. 0.4 mg/ml) and IPTG (100 m*M*, final concentration 0.2 mg/mL). The transformants were then plated on minimal medium supplemented with DAP to check whether they are able to utilize DAP as sole source of carbon.

Sonication and SDS-PAGE

Cell-free crude extract of *S. typhimurium* PU011 was prepared by sonicating the pellet resuspended in 0.5 mL extraction buffer (10 mM potassium phosphate buffer pH 8.0, 1 m*M* EDTA, 1 m*M* β-mercaptoethanol, 100 µM Pyridoxal-5-Phosphate (PLP) and 5% glycerol) for 10 min using Branson Sonifier 250 (10 min bursts at 40% duty cycle with intermittent cooling every 2 min in order to maintain the suspension below 5°C). The sonicate was then centrifuged at 12,000g for 10 min at 4°C, which served as the crude extract. Enzyme assay and protein estimation were carried out using this crude extract. For carrying out sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis *(7)*, the same crude extract was used. The DAPAL activity was measured as per the method of Vijiyalakhsmi et al. (2). Protein estimation was done as per the method of Lowry et al. (1951) *(8)*.

Enzyme Assay (2)

DAPAL enzyme activity was spectrophotometrically measured as per the method of Vijayalakshmi et al. *(2)* and described in detail by Rupesh et al. *(4)*.

Determination of Biomass and Enzyme Activity (4)

An aliquot of 25 mL of culture grown in MM+DAP and *L. sativus* seeds (2% w/v in water) was withdrawn and, after centrifugation at 8000*g* for 10 min at 4°C, the pellet was suspended in 0.5 mL of extraction buffer for sonication. After sonication for 10 min using Branson Sonifier 250, the lysate

was centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was used for estimation of DAPAL enzyme activity.

Effect of pH (4)

Different buffer systems with varying pH regimes employed in our study are: Glycine-HCl buffer (pH 3.0), sodium acetate buffer (pH 5.0), Potassium phosphate buffer (pH 7.0), Potassium phosphate buffer (pH 8.0), Glycine-NaOH (pH 9.0), Glycine-NaOH (pH 11.0) and KCl-NaOH (pH 13.0). The optimum pH for maximum DAPAL specific activity was determined. The crude enzyme extract was incubated with any of the above buffers for 30 min. After incubation, the crude extract was precipitated with ammonium sulfate (80% saturation). The precipitate thus formed was resuspended in minimal volume of 10 m*M* potassium phosphate buffer pH 8.0. The influence of pH on DAPAL enzyme activity was measured as described elsewhere.

Effect of Temperature (4)

Potassium phosphate buffer (10 m*M*) pH 8.0 containing the crude enzyme extract was incubated at various temperature regimes ranging between 0°C to 60°C. After 30 min of incubation, the DAPAL activity in the crude extract was analyzed.

PCR amplification of E. coli *PU018*

The size determination of the insert DNA in PU 018 recombinant clone was done by PCR amplification using T3 (5′ AATTAACCCTCACTA AAGGG 3′), T7 (5′ AATACGACTCACTATAG 3′) primers. The reaction mixture employed in the PCR reaction (20 µL) contained the following: Template DNA, 200ng, Taq DNA polymerase 1.0 U, dNTPs 2 m*M*, forward primers 50 pmol, reverse primers 50 pmol, $MgCl_2$ 2.5 m*M*. PCR assays were performed with a *Mastercycler*® (Eppendorf) using T3, T7 primers with initial 5 min denaturation at 94°C followed by 30 cycles of amplification consisting of 1 min denaturation at 94°C, 1 min annealing at 50°C and 1 min of extension at 72°C. After 30 cycles, a final extension step of 10 min at 72°C was done. After completion of PCR, the product was run on 1.5% agarose gel.

DNA Sequence Analysis

PCR products were sequenced with Automated DNA sequencer employing T3 and T7 primers. From the nucleotide sequence, amino acid sequence was deduced using Gene Tool Lite version 1.0 (Advanced Bioinformatics Solution, Biotools Inc.) package. The complete nucleotide sequence of the DAPAL gene of nonvirulent *S. typhimurium* PU011 has been submitted to GeneBank and given accession no. AY651169. Also, the open reading frame (ORF) and motifs in the nucleotide sequence of PU 018 were analyzed using the same program.

Multiple Sequence Alignment Using CLUSTAL X

Multiple sequence alignment of the nucleotide and amino acids was carried out using the CLUSTAL X program *(9)* and BioEdit sequence alignment editor program *(10)*. The multiple sequence alignment program Clustal X was used to align the DAPAL sequence of PU 018 with the sequences of all other species containing the DAPAL retrieved from the NCBI site (http://www.ncbi.nlm.nih.gov/) The aligned sequences were then manually checked for gaps. Further, the original sequence data set was resampled 1000 times and subjected to bootstrap analysis to obtain the confidence values. The multiple distance matrices thus obtained were used to construct trees using the maximum-likelihood, neighbor-joining method *(11,12)* as compiled in the Phylogeny Inference Package (PHYLIP package, version 3.5, Tree view *[13]*).

Results

Cloning of DAPAL Gene

A genomic library was constructed using chromosomal DNA from nonvirulent *S. typhimurium* PU011 into the plasmid pBlueScript-II (SK-) vector and the library was screened for identifying clone(s) that contains the gene coding for DAPAL enzyme. Out of a battery of transformants, the clone, *E. coli* PU018, that grew well similar to that of wild type non-virulent *S. typhimurium* PU011 on minimal medium containing DAP, vis-à-vis capable of producing DAPAL was chosen for further studies. This clone on PCR amplification was found to contain an approx 1.2-kb DNA fragment corresponding to DAPAL gene (data not shown). In contrast, *E. coli* DH5α when subjected to PCR amplification for the presence of DAPAL gene proved negative.

Protein Profile

A protein band corresponding to 43 kDa was observed in wild-type nonvirulent *S. typhimurium* PU011 (Fig. 1B) and *E.coli* PU018 (Fig. 1C) but not in wild-type *E. coli* DH5α (Fig. 1D).

Enzyme Activity in Crude Extracts

Enzyme activities in the crude extracts of wild type *S. typhimurium* PU011, recombinant *E. coli* PU018 and *E.coli* DH5α are shown in Table 1. The specific activity of the enzyme in all the above categories of bacteria excepting *E.coli* was near similar.

Biomass and Enzyme Production

The kinetics of production of biomass and DAPAL activity by *S. typhimurium* PU011 and *E. coli* PU018 are shown in Fig. 2. A continuous

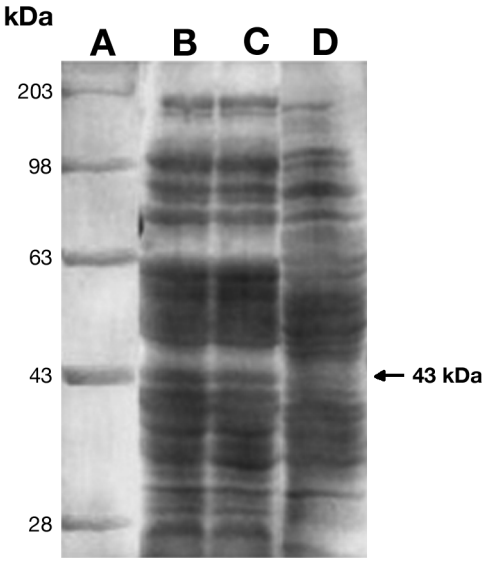

Fig. 1. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of diaminopropionate ammonia lyase. Cells after growth for 16 h in MM+DAP were harvested, sonicated and run on 12% SDS-PAGE. **A**, High-molecular-weight protein marker; **B**, *Salmonella typhimurium* PU011; **C**, *Escherichia coli* PU018, and **D**, *E.coli* DH5α

Table 1
Diamino Propionate Ammonia Lyase Activity From Different Bacterial Strains

Bacteria	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
Salmonella typhimurium PU011	219 ± 3	42.71 ± 0.37	0.351 ± 0.02
Recombinant *E. coli* PU018	209 ± 4	39.63 ± 0.52	0.346 ± 0.05
E. coli DH5α	113 ± 6	0.020 ± 0.009	0.020 ± 0.007

Recombinant *Escherichia coli* PU018 contains 1.215 kb of insert DNA from *Salmonella typhimurium* PU011 coding for DAPAL. *E. coli* DH5α served as the recipient to clone DAPAL gene from *S. typhimurium* PU011. The values are mean of 3 replicates.

increase in biomass and specific activity of enzyme with increase in incubation period was observed, the maximum reaching at 16 h postinoculation.

Kinetic Properties of DAPAL

The Km value for the substrate, DL-DAP, based on the Line weaver Burk plot (Fig. 3) was found to be 0.740 m*M*, 0.680 m*M*and 0.741 m*M*, 0.683 m*M* for *S. typhimurium* PU011 and *E. coli* PU018, respectively when grown on MM+DAP or *L. sativus* seed extracts. The percent relative activity of DAPAL

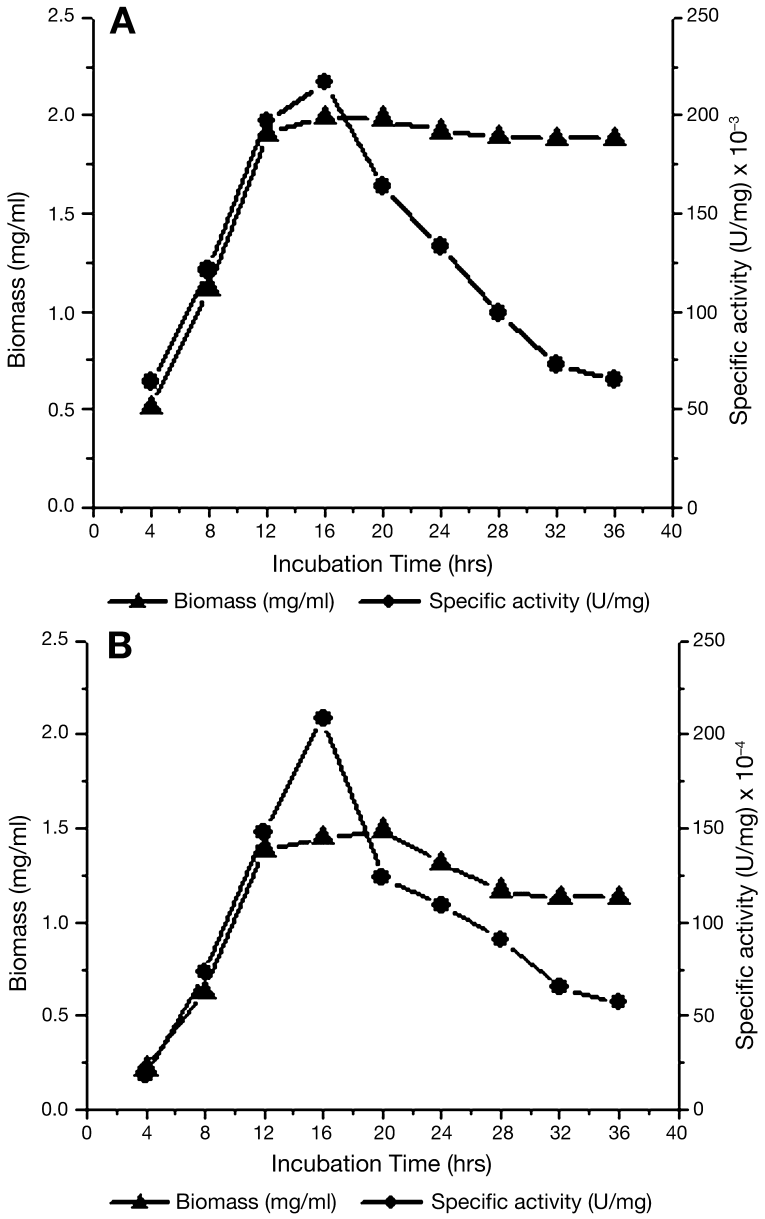

Fig. 2. Biomass and diaminopropionate ammonia lyase production in **(A)** *Salmonella typhimurium* PU011 and **(B)** *Escherichia coli* PU018.

from *S. typhimurium* PU011 and *E. coli* PU018 at various pH regimes has revealed (Fig. 4) that the enzyme from both the bacteria had a sharp pH optimum of 8.0 in phosphate buffer and any pH less than 6.0 or more than 11.0 has resulted in drastic reduction in enzyme activity. With regard to temperature optima for the DAPAL synthesized by *S. typhimurium* PU011 and *E. coli* PU018, the enzyme retained 100 % activity up to 45°C (Fig. 5);

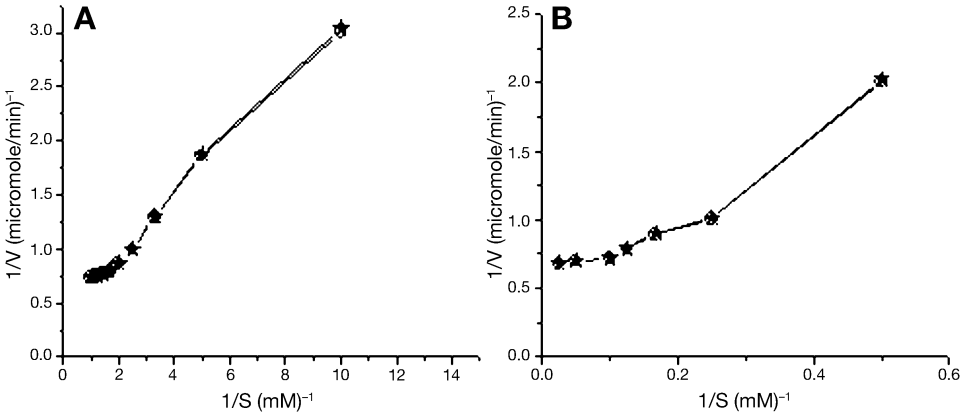

Fig. 3. Determination of Km value for the enzyme diaminopropionate ammonia lyase (DAPAL) obtained from *Salmonella typhimurium* PU011 and *Escherichia coli* PU 018 grown in **(A)** MM+DAP and **(B)** *L. sativus* seed extracts. The Km values of DAPAL from both *S. typhimurium* PU011 (—○—) and recombinant *E. coli* PU018 (—★—) were measured in MM+DAP and seed extracts from *Lathyrus sativus* as per the procedure outlined by Rupesh et al. *(4)*.

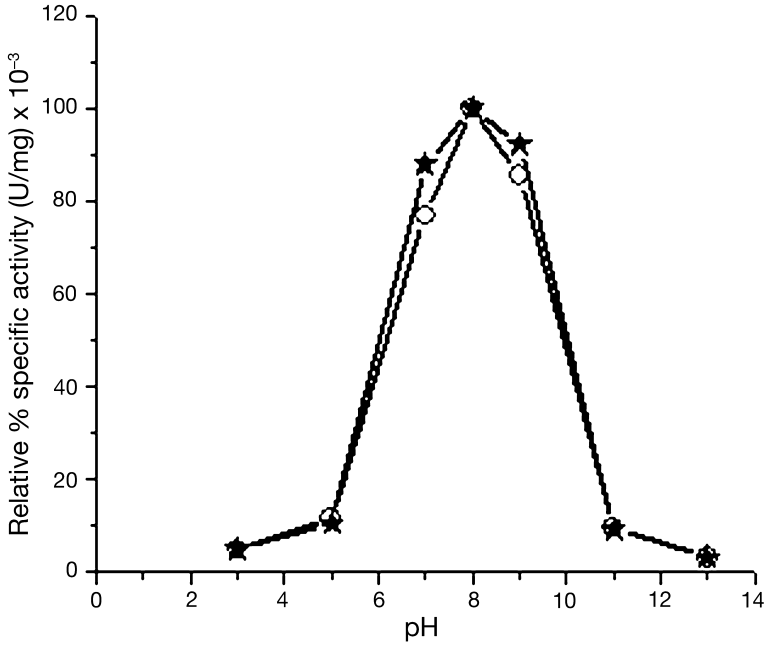

Fig. 4. Effect of pH on the stability of diaminopropionate ammonia lyase. The crude enzyme extract of *Salmonella typhimurium* PU011 (—○—) and *Escherichia coli* PU018 (—★—) were incubated in 10 m*M* phosphate buffer at different pH regimes and the relative percent specific activity (U/mg) $\times$ 10^{-3} measured.

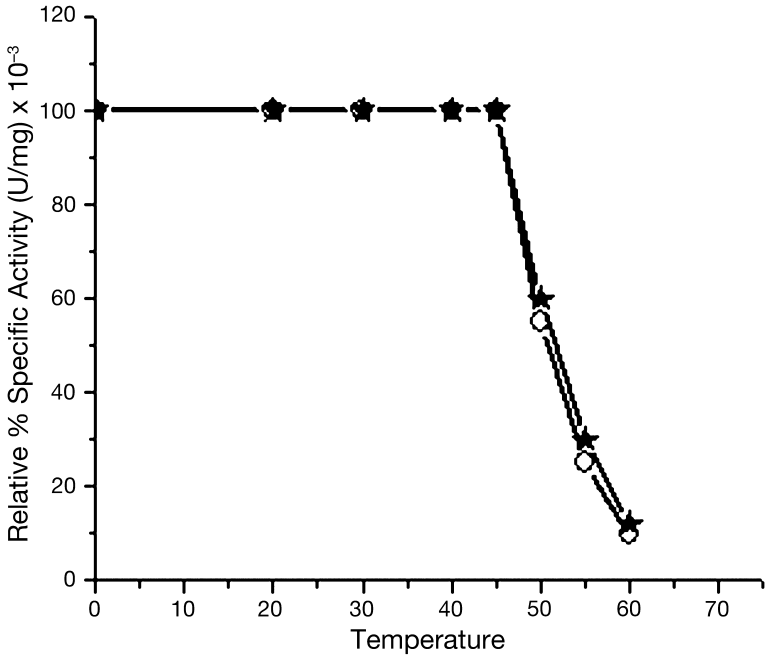

Fig. 5. Effect of temperature on the stability of diaminopropionate ammonia lyase. The crude enzyme extract *Salmonella typhimurium* PU011 (—○—) and *Escherichia coli* PU018 (—★—) were incubated at various temperatures ranging between 20°C to 60°C in 10 m*M* phosphate buffer and the enzyme activity (U/mg) $\times 10^{-3}$ measured.

however, for every 5°C increment thereafter, a decrease in enzyme activity was evident with minimal activity at 60°C.

Nucleotide and Amino Acid Sequence Analysis of E. coli *PU018*

The insert DNA coding for DAPAL was sequenced and the sequence analysis is given in Fig. 6. For a total of 1215 nucleotide bases, the deduced 405 amino acids contained no stop codons in between. Interestingly, a sequence of nucleotides corresponding to Hind III restriction site were seen between nt 650 to 655.

Determination of Homology of DAPAL in E. coli *PU018 by Multiple Sequence Alignment With Other Microorganisms Containing DAPAL*

The multiple sequence alignment of *E. coli* PU018 with other microorganisms containing DAPAL done using the CLUSTALX alignment program and PHYLIP output are shown in Fig. 7. The nucleotide sequence of DAPAL of *E. coli* PU018 showed 100% percent homology with that of the DAPAL sequence retrieved from the NCBI database for *S. typhimurium*, *S. typhimurium* LT2 and other *E. coli* strains including *Bordetella parapertussis*. With regard to amino acid sequence homology of DAPAL from *E. coli*

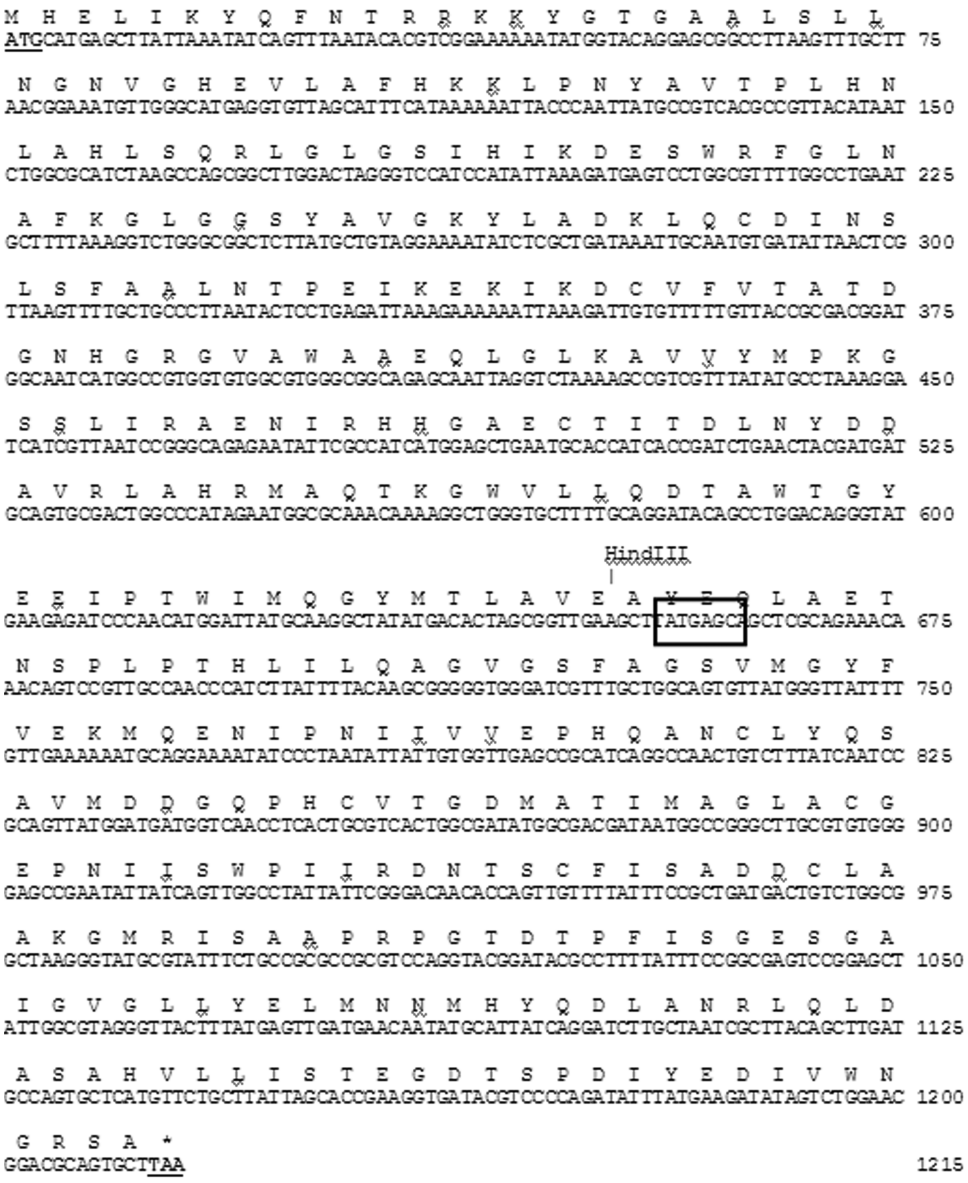

Fig. 6. Nucleotide sequence of the recombinant *Escherichia coli* PU018 containing diaminopropionate ammonia lyase (DAPAL)-coding genes and the amino acid sequence deduced from the open reading frame of the DAPAL gene. DNA bases (bottom line) and amino acids (one-letter code) (top) are listed and numbered to the right of the sequences. The ATG initiation codon and stop codon (TAA) are boldface and underlined. The HindIII restriction site falling between 650th and 655th basepair is blocked.

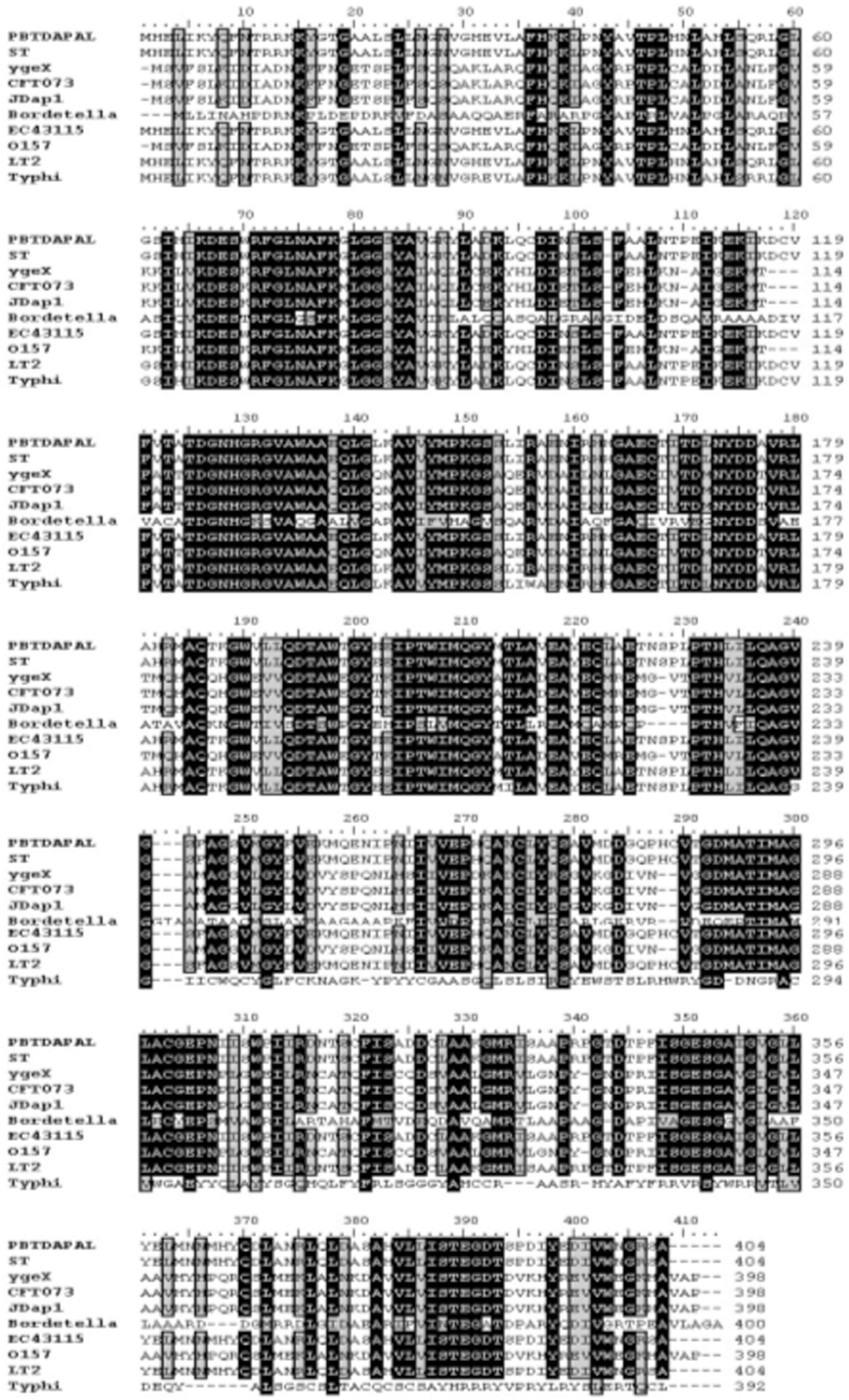

Fig. 7. CLUSTAL alignment of the deduced amino acid sequences encoding diaminopropionate ammonia lyase enzyme from recombinant *Escherichia coli* PU018, *Salmonella typhimurium*, *E. coli* ygex, *E. coli* CFT073, *E. coli* jdapl, *Bordetella parapertussis*, EC 4.3.1.15, *E. coli 0157*, *S. typhimurium* LT2, and *S. typhi*. Amino acids identical in all species are indicated by blocking.

PU018, while cent percent homology was observed for the enzyme DAPAL (EC 4.3.1.15) obtained from *S. typhimurium* and *S. typhimurium* LT2, only 85% homology was observed with *B. parapertussis* and other *E. coli* strains.

Discussion

Recently, Khan et al. *(1)* have reported the cloning, sequencing, and partial characterization of DAPAL from virulent *S. typhimurium* and *E. coli*. We have reported earlier *(4)* on the production of DAPAL by a nonvirulent strain *S. typhimurium* PU011 utilizing DAP as substrate. However, the question that remains is whether there exists a difference in the genetic sequences that code for DAPAL enzyme in the virulent and nonvirulent strains of *S. typhimurium*. Therefore, an attempt was made to sequence the gene coding for DAPAL in the nonvirulent strain of *S. typhimurium* PU011 to find out the degeneracy of the code, if any, and the degree of homology in the nucleotide/ amino acid sequence in the DAPAL coding gene so as to determine the conserved regions on the DNA.

Based on our earlier *(4)* as well as present studies, it is evident that the 1.2-kb long insert DNA in *E. coli* PU018 codes for a 43-kDa protein (Fig. 1B). Vijayalakshmi et al. *(2)*, Nagasawa et al. *(3)*, and Khan et al. *(1)* have shown the presence of the 43-kDa protein corresponding to DAP ammonia lyase in *Pseudomonas sp.*

With regard to DAPAL enzyme activity, Vijayalakshmi et al. *(2)* have shown a specific activity of 0.10 U/mg in the crude extract of the enzyme in Pseudomonads. Whereas Nagasawa et al. *(3)* have reported the catalysis of α-β elimination of L-DAP to pyruvate at the rate of 46.9 μmol/min/mg of proteins at 30°C from *S. typhimurium*, Uo et al. (2002) (14) reported a specific activity of 0.56 U/mg in the crude extract obtained from their recombinant *E. coli*. Khan et al. *(1)* reported that the specific activity of purified eDAPAL was 84 ± 20 U/mg and the yield was 30–50 mg/500 mL culture. However, the specific activity of DAPAL obtained from *S. typhimurium* was 190 ± 20 U/mg and the yield was 40–50 mg/500 mL of culture. In our studies the specific activity of the enzyme in PU 018 was 0.35 U/mg of protein.

Vijayalakshmi et al. *(2)* and Nagasawa et al. *(3)* have identified Pseudomonad and *S. typhimurium* respectively capable of producing DAPAL that degrades the precursor of neurotoxin present in *L. sativus* seeds. Interestingly, in our work a direct correlation between biomass yield and enzyme activity up to 20 h post inoculation was observed. Nagasawa et al. *(3)* have also observed a similar pattern of growth and enzyme activity in *S. typhimurium*.

Vijiyalakhsmi et al. *(2)* and Nagasawa et al. (3) have reported Km values at 0.267 m*M* and 0.12 mM for DAPAL enzyme obtained from Pseudomonad and *S. typhimurium* respectively. In our studies, the km value for DAPAL produced by *E. coli* PU018 was 0.741 m*M*. The differences in Km values could be attributed to D or L or DL forms of DAP used as substrate in the estimation of enzyme activity. Our observations, wherein, maximum

DAPAL activity was observed at pH 8.0 is in correspondence with the results of Vijiyalakhsmi et al. *(2)* who have also observed maximum DAPAL activity at pH 8.0 produced by *Pseudomonas*. Uo et al. *(14)* from their studies have reported that DAPAL had maximum activity with both D- and L-DAP as substrate at pH 8.0. The loss in DAPAL activity with increase in temperature beyond 45°C observed in our studies is the same as that reported earlier for the *Salmonella sp.* by Nagasawa et al. *(3)*.

The nucleotide sequence of DAPAL gene of *E. coli* PU018 revealed an ORF of 1212 nucleotides (Fig. 6), which encoded a peptide of 404 amino acids. The fact that this open reading frame was in correspondence with the 404 amino acids deduced from the sequenced DNA indicates that the 1212-bp-long region could be the only functional domain. In reverse frame (3′–5′) as expected no other functional domain was seen.

A high degree of amino acid sequence conservation was revealed when the DAPAL of *E.coli* PU018 was aligned with several bacterial DAPAL amino acids by means of CLUSTALX multiple alignment program. DAPAL from *E. coli* PU018 was 55% identical in sequence to the N-terminal domain of L-threonine dehydratase. Uo et al. (14) have reported that their recombinant clone showed only 20% identity in sequence to the N-terminal domain of L-threonine dehydratase. However, Khan et al. *(1)* in their clone showed 51% similarity with threonine dehydratase.

The results presented in our study clearly indicate that the enzyme DAPAL produced by the recombinant *E. coli* PU018 behaves similar in all respects to that of the enzyme produced by the nonvirulent *S. typhiumurium* PU011 thus holding much promise for applicative purposes. This discovery can be considered as a hallmark, because all the earlier studies with regard to DAPAL production involved virulent *S. typhiumurium* restricting its use in the community.

Conclusion

The results presented in our study clearly indicate that the coding sequences of the DNA from non-virulent *S. typhiumurium* PU011 for production of DAPAL are the same as that in virulent *S. typhiumurium* thus offering broader scope and great potential for community level applications under field conditions for detoxifying DAP, the immediate precursor of O-DAP present in *L. sativus* seeds.

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