Cloning, Overexpression and Purification of RNase D in *Escherichia coli*

A

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Submitted in partial fulfillment of the requirement

for the degree of

Masters of Science

In

Biotechnology

By

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July, 2019

CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the dissertation entitled **"Cloning, Overexpression and Purification of RNase D in *Escherichia coli*"** in the partial fulfillment of the requirements for the award of the degree of Masters in Biotechnology and being submitted to the Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is my own work during the period of six months from January 2019 to June 2019, under the supervision of **Dr. Tanmay Dutta,** Assistant Professor, Department of Chemistry, IIT Delhi and **Dr. Siddharth Sharma,** Associate Professor, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala.

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This is to certify that the thesis entitled **"Cloning, Overexpression and Purification of RNase D in *Escherichia coli*"** submitted by Poulomi Chandra is an authentic record of work carried out as requirement for the award of the degree of **Master of Science in Biotechnology** at **Thapar Institute of Engineering & Technology, Patiala** under the supervision of **Dr. Tanmay Dutta**, Assistant Professor, Department of Chemistry, IIT Delhi and **Dr. Siddharth Sharma**, Associate Professor, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala during the period of six months from January 2019 to June 2019.

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Dedicated to my Parents.....

ABSTRACT

Cells incorporates a giant assortment of ribonucleases (RNases) that are required in either maturation or targeted degradation of cellular RNAs, these maturation processes put together referred to as RNA metabolism. Some RNases also are well-known to have the ability to degrade important functional RNAs that involves numerous regulatory mechanisms. In *E. coli,* twenty RNases with totally different characteristics are known. It's probably that multiple mechanisms operate to safeguard cellular RNAs. RNases are generally classified into two, the exoribonuclease and the endoribonuclease. The endoribonuclease will act on either single stranded or double stranded RNA depending on the type of enzyme. The exonucleolytic degradation of RNA will occur in each 5' – 3' and 3' – 5' orientation in organism cells, however solely 3' – 5' degradative activity has been known only in microorganisms up to now. Out of the seven exoribonucleases RNase D is the one which is known in *Escherichia coli,* has been seen to play a redundant role in maturation of the 3' ends of tRNAs. It was determinedby its activity on "denatured" tRNAs within the initial observation that exoribonucleases might show a high level of specificity. It was evident from numerous studies that tRNA is the most active substrate for this protein. Later research have demonstrated that RNase D cooperates in the development of 5S rRNA, various small-structured RNAs and tRNA. Most identical feature of RNase D is itspreserved DEDD domain that is very similar to the Klenow fragment exonuclease domain. RNase D conjointly includes two coiled spaces that is structurally similar with no distinguishable arrangement similarity among them. These closely resemble the HRDC domain previously as observed in RecQ-family helicases and various extra proteins engaged on nucleic acids. Curiously, the DEDD catalytic space and also the two coiled spaces close to create a doughnut-form construction. The doughnut-form design of *E. coli* RNase D and also the HRDC spaces probably play a serious role in deciding the substrate particularity of this exoribonuclease. RNase D protein depends on metal ions like Co^{2+}, Mg^{2+}, or Mn^{2+} for its action. The DEDD nucleases are divided into two classes or sub teams, the DEDDh and DEDDy supported whether or not they have essential amino acid (h) or an amino acid (y) in their motif III. RNase D like proteins are absent in archealsequenced genomes however all eukaryotes looks to possessat-least one member of RNase D.

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LIST OF ABBREVIATIONS

Abbreviation	Name
E. coli	*Escherichia coli*
tRNA	Transfer RNA
rRNA	Ribosomal RNA
DEDD	Aspartic acid Glutamic acid Aspartic acid Aspartic acid
HRDC	Helicase and RNase D C-terminal
RNA	Ribonucleic acid
RNase	Ribonuclease
ssRNA	single-stranded Ribonucleic acid
dsRNA	double-stranded Ribonucleic acid
TLRs	Toll-like receptors
rmsd	root-mean-square difference
aa	amino acid
Asp	Asparagine
Glu	Glutamine
Tyr	Tyrosine

exo I	exonuclease I
exo II	exonuclease II
exo III	exonuclease III
NMR	Nuclear magnetic resonance spectroscopy
Arg	Arginine
Lys	Lysine
DNA	Deoxyribonucleic acid
mRNA	Messenger RNA
ORN	Oligoribonuclease
poly (U)	polymer of Uracil
PTGS	post transcriptional gene silencing
WEX	Werner Syndrome-like exonuclease
WRN	Werner Syndrome protein
RNAi	RNA interference
MUT	Mutant
B.subtilis	*Bacillus subtilis*
gRNA	guide RNA
oligo (U)	Uracil oligonucleotide
ssDNA	single-stranded Deoxyribonucleic acid

dsDNA	double-stranded Deoxyribonucleic acid
LB	Luria Broth
rpm	Revolutions per minute
PCR	Polymerase chain reaction
Ni-NTA	Nickel Nitrilotriacetic acid
PMSF	Phenyl Methyl SulfonylFlouride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
IPTG	isopropyl β-D-thiogalactopyranoside
APS	Ammonium per sulfate
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
NCBI	National Center for Biotechnology Information

1. Introduction:

The knowledge of RNA metabolism is necessary for many cellular mechanism which consists of transcription, development, quality control and degradation. RNA is the main macromolecule known to have both instructive and catalytic functions, prompting much theory that it might have been the fundamental chemical intermediate in the improvement of life on this planet. The finding of catalytic RNAs has changed the meaning of the word "enzyme". Numerous RNAs are additionally complexed with proteins, making complicated biochemical machines with a wide diversity of functions.

RNA metabolism requires certain ribonuclease enzymes for processing. Ribonuclease (RNase) is an omnipotent nuclease. Ribonuclease (RNase) are the enzymes that are capable of binding and cleaving RNA molecules [1]. These are usually more than one ribonucleases in a species. This is because different RNases are having affinity towards different targets, while some RNases cleave ssRNA, other have a preference for dsRNA or RNA-DNA duplexes. RNase play a major role in RNA metabolism, it is currently understood in bacteria that most RNA molecules particularly stable RNA are processed by these multiple RNase. In recent year there has been progress in finding out RNases responsible for maturation and degradation of RNAs in *E.coli*. A widely studied example of this is tRNA maturation. 3'-end of t-RNA maturation in *E.coli* involves the action of different exoribonucleases (RNase II, T, PH, D, BN) they functionally overlap *in vivo* such that any one of them can take over the functions of all the others with various efficiencies, among them RNase T and PH are highly efficient [2].

Ribonucleases are classified into two types; endoribonucleases, which cleaves phosphodiester bonds from 5'end to 3' end and exoribonucleases, which cleave phosphodiester bonds from 3'end to 5' end resulting into staggered or sticky ends and 5'monophosphate products[3].The endoribonucleases are represented as a set of clippers and exoribonucleases are generally denoted with a symbol of 'Pacman'[4]. Exoribonucleases are either processive, staying joined to a similar RNA molecule for several rounds of catalysis, or distributive, discharging the substrate with each nucleotide removed. They can either show phosphorolytic or hydrolytic properties that is it can either utilize inorganic phosphate or utilize a molecule of water upon the breakdown of each phosphodiester bond. Ribonuclease is widely used as a therapeutic agent. Antifungal, antiviral, antitumor, and immunosuppressiveproperties are shown by RNases [5]. RNase cause

some genetic damage in cancerous cell and as a result their RNA is destroyed. This results in stimulation of immune sesnsors like toll-like receptors (TLRs) and activated TLRs which causes immunokines to induce the cytokines, growth factors and angiogenic modulators which determines the progression of tumor [6]. Effective tumor killing can be done by the combination of RNase with other anticancer molecule [7]. The first RNases was discovered in the year 1961, since then many RNases have been identified [8].

1.1 Maturation of tRNA:

The biosynthesis of utilitarian tRNA molecules needs the contribution of particular nucleases that eliminates additional nucleotides from the 5' and 3' end of tRNA antecedent. In spite of the fact that the nuclease associated in refining at the 5' terminus, RNase P, was known for various years, the enzyme(s) in charge for definite cutting at the 3' end was not known conclusively initially as shown in Figure 1. Nevertheless, various activities capable of producing tRNA-size molecules from tRNA antecedent that have additional remnants following the -C-C-A sequence (termed type I precursors) which have been relatively refined from *Escherichia coli* antecedent [9]. It was recommended at first that the known exonuclease, RNase II, was the enzyme engaged in refining the 3' end of type I antecedent. Subsequently, Rossmanith *et al*., 1995 recognized another nuclease, RNase PIII, distinguishable from RNase II that was essential for union of *E. coli* su^{+}III $tRNA^{Tyr}$ [10].

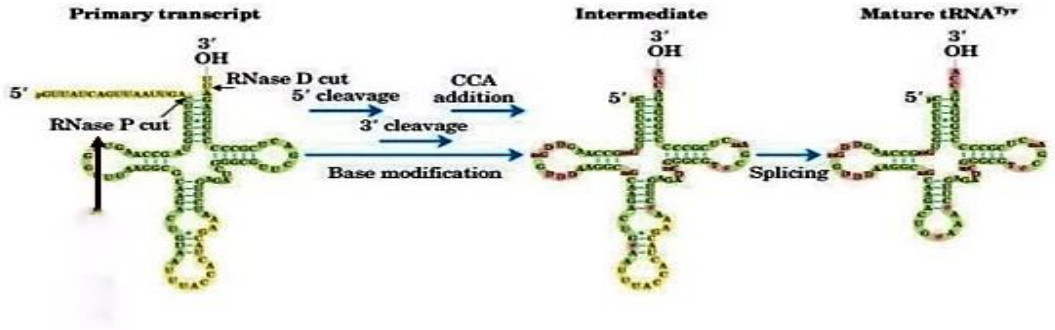


Figure 1: tRNA processing and maturation (Soma *et al.*, 2014).

1.2 RNase D:

Among the seven different exoribonucleases, RNase D (RND) is the one which is distinguished in *Escherichia coli* [11]. It is encoded by *rnd* gene [12]. In many eubacteria and eukaryotes near analogs of RNase D have been found [13]. RNase D displays maturation of 3'end of tRNA. RNase D is a 3'to 5'hydrolytic exoribonuclease which has a place in the DEDD superfamily. individuals of DEDD superfamily have four acidic extremely preserved remnants which further binds two divalent metal ions and other protected characteristic are dispensed in three protected motifs [14]. It can act on "denatured" tRNAs which demonstrates that exoribonucleases have a high level of particularity [15]. RNase D plays an important role by maturing tRNA, 5S rRNA, and other little- organized RNAs [16]. The strains of *E. coli* which are without RNase D grows critically traditionally. When some of the primary nucleases like RNase II, BN, T and PH are removed, RNase D plays an essential role in viability suggesting that it can act as a subsidiary enzyme when these nucleases are absent [17]. RNase D is bivalent metal ion dependent (e.g., Co^{2+}, Mg^{2+}, or Mn^{2+}) and generates ribonucleoside 5'-monophosphate products [18].The DEDD nucleases are separated into two sub groups: DEDDh contains a histidine (h) in motif III and DEDDy which contains a tyrosine (y) in motif III [19]. RNase D belongs to DEDDy subgroup. The Klenow fragment is very similar to the DEDD residues of RNase D. Two α- helical domains are present in RNase D which are structurally similar and have no recognizable arrangement homology between them. The HRDC domain found at C-end of RecQ-family helicases is very similar to the two α- helical domains. A ring shaped construct of *E. coli* RNase D is formed by the DEDD reactive area and the two α- helical spaces.

1.2.1 General structure of *E. coli* RNase D:

RNase D DEDD Domain: This domain is structurally very alike to the Klenow fragment exonuclease area with root-mean-square difference (rmsd) of 1.63Å for 159 equivalent Cα atoms. These two domains belongs to the DEDDy sub group having 17% sequence similarity over 188 amino acids (aa). In this structure six strands of β sheet are present in the center with α helices surrounding on both sides. As a result an open pocket is formed on one side that is covered by β sheet and a closed, dense structure is present on the other side. The conserved

DEDDy residues are present inside this open pocket which forms the reported functional center of RNase D. The most important are those three exo-motifs in which the DEDDy remnants are placed. (Asp28 and Glu30 in exo I, Asp85 in exo II, and Tyr151 and Asp155 in exo III) [20]. A schematic representation of RNase D is shown in Figure 2 and Figure 3.

HRDC Domain, a Reported Nucleic Acid Binding Area: This area was initially discovered at C terminus of RNase D and in numerous RecQ helicases by arrangement evaluation [20].The NMR study reveals that the HRDC domain bends like a bunch of five helices (α1-α5), and the last two helices (α4 and α5) are presented as a single helix with a twist. The most common feature of all proteins having HRDC is their interplay with RNA or DNA, suggesting its role in binding of nucleic acid [20]. No preserved basic residues are present in these HRDC domains. There is no pattern identity except for some preserved hydrophobic remnants, among these HRDC spaces.

Middle HRDC Domain, HRDC1, in RNase D: This area is also known as HRDC1, is an initiating representative of this domain [20]. It contains positively charged patches which are composed by the basic lysine and arginine remnants which includes Arg226, Lys227, Arg231, Arg240, Arg269, Lys273, and Lys281. This domain is validly conserved in sequence.

C-Terminal Helix Domain, HRDC2: This area has a very fragile conservation and is mainly preserved in analogous organisms. Another α-helical HRDC domain is formed by C terminus of RNase D, as demonstrated by using DALI server [21]. There is no distinguishable arrangement uniformity observed among RNase D HRDC2 area and other known spaces of HRDC. The back bone rmsd value of both HRDC1 and HRDC2 domains is of 1.66Å due to very similar folding. This domain contains positively charged surface patches.

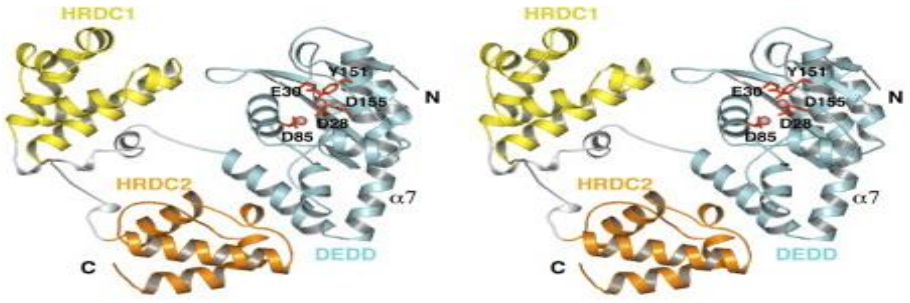


Figure 2: This is the RNase D crystal structure of *Escherichia coli*. The three structural domains in the figure are color coded differently: The N-terminal DEDD domain is shown by Cyan color; the first HRDC domain (HRDC1) is shown by Yellow color and the Orange color represents the C- terminal domain (HRDC2). The red sticks represent the DEDDy remnants which is a conserved domain and the balls represents the bound metal ion (Zuo *et al.*, 2005).

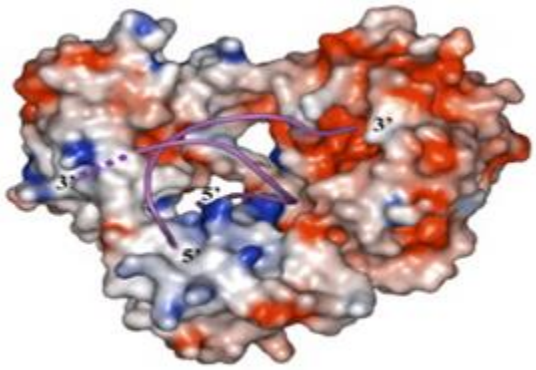


Figure 3: This is the RNase D molecular surface which is observed by local electrostatic potential by using GRASP (Nicholls *et al.*, 1991). Red color and the blue represents the negative positive potential respectively.

1.3 Overexpression:

Overexpressing of RNase D in strains mutated for tRNA nucleotidyl transferase is known to slow growth in *E. coli* [22]. Consistent with this deleterious effect, the expression of this RNase is very tightly regulated by an unconventional UUG start codon [23]. Converting this codon to an AUG (Methionine) results in an eleven-fold increase in expression from single-copy plasmids. An upstream hairpin structure seems to negatively influence RNase D expression, but to a lesser extent than the UUG codon [24]. Microarray data suggests that RNase D is expressed almost exclusively during exponential phase. Cells lacking RNase D makes more effort to exit lag phase than the cells in which RNase D is available and that this imperfection is not due to abnormal development of ribosomal RNAs.

RNase D protein has also been shown to be produced most abundantly during exponential growth. Tight regulation for a potentially toxic RNase, its low *in-vivo* activity on tRNA and its expression pattern makes RNase D a curiosity among the RNases of *E. coli*. All eukaryotes contains minimum one family member of RNase D. Yeast exosome part RRP6 and the human PM/ScI 100kDa auto-antigen are near analogs of RNase D [19]. RNases act as indicated by the necessities of development in adjustment to nature. They play a critical role in adding to the reusing of ribonucleotides, and also carry out reconnaissance; devastating tRNAs that would create unfavorable proteins. There are two extra RNases that have no known essential job in RNA metabolism – RNase D and RNase BN. The exonucleolytic pathway required for the tRNA maturation involves different RNases as shown in Figure 4.

Activity of different RNase, RNase T = RNasePH>RNaseII>RNaseD>RNaseBN/Z.

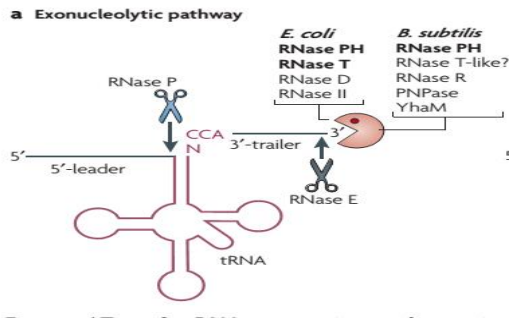


Figure 4: Transfer RNA maturation pathways, where RNase D works on 3' trailer (Condon *et al.*, 2007).

2. Review of Literature:

Research in this area is rapidly expanding and is comprehensively summarized in several reviews.

According to central dogma of biology deoxyribonucleic acids (DNA) are the template for ribonucleic acids (RNA) which is further used to make protein. Everything is known about DNA, RNA and proteins. Even the processes in which they participate in living organisms is also known [25].

The link between protein and DNA is RNA metabolism. Many components are involved to bridge the gap as RNA is not only the molecule which contains information from which the proteins are translated, but it contains the molecular machinery which assembles the protein. The components that are involved in the journey from DNA to protein formation contains ribozymes at their catalytic center but in case of tRNA and ribosomes it is almost completely made of RNA. These phenomena forms the basis for the theory that RNA was the original molecule of life and are required for replication in living systems [26].

The conclusion of decades of research into this topic from molecular biology, biochemistry to genetics has given a detailed knowledge of the relationships between various RNases and RNA in *Escherichia coli*. **This dissertation focuses on cloning, overexpression and purification of RNase D in *E. coli.***

The degradation of RNA occurs when there is a defective RNA substrate or it is no more needed within the cell. RNA is broken down by RNase which mainly depends on the RNA amount of secondary structure in RNA and sometimes on the sequence [27]. The RNAs which are highly structured like tRNA and rRNA are degraded by PNPase and RNase R. The former links with an RNA helicase in vivo so that it can overcome the secondary structure. RNase R on the other hand needs a single stranded 3'overhang of four or more nucleotides for the digestion of structured RNA and can resolve the secondary structure without the help of a helicase. RNAs having less secondary structure are degraded by RNase II which is a close homolog of RNase R. RNase II is sensitive towards the secondary structure in potential substrates with no helicase activity of its own associated with the enzyme [28]. The RNA degradation in *E. coli* is mainly due to these three exoribonucleases.

Many deletion mutants have been characterized and it was found that RNase D plays no obvious role in RNA metabolism in *E. coli* during rapid growth [29] but still these genes are present within *E. coli* chromosome and further RNA and functional proteins are being synthesized. These proteins are therefore needed for some as yet unknown function within *E. coli*.

2.1 RNases in *E. coli* :

E. coli contains eleven endoribonucleases and seven exoribonucleases. All these enzymes along with RNA polymerase makes up the bulk of enzymes that are associated with the processes required for RNA metabolism.

The endoribonucleases in *Escherichia coli* includes RNase P, RNase I, RNase III, RNase G, RNase E, RNase BN, RNase HI, RNase HII [30].

RNase I acts as a defense against foreign and potentially infectious RNAs. RNase HII and RNase H I both are involved in replication and repair of DNA. RNase HI functions by degrading the RNA strands of DNA:RNA hybrids. RNase HII functions by cleaving the residues of RNA that have been accidentally incorporated into DNA during replication and repair [30].

RNase P, RNase III, RNase G, RNase E under some special condition, RNase BN- all these participates in the metabolism of RNA via the degradation of mRNA, maturation of stable RNA and quality control of defective RNAs.

The remaining RNases participates in the major elements of RNA metabolism. RNase II is a processive and hydrolytic RNase which is capable in degrading single stranded RNA. It is inhibited due to the presence of secondary structure as it participates in the degradation of mRNA and maturation of stable RNA. RNase R is very much similar to RNase II as it is a close native of RNase II. RNase D, RNase T, and oligoribonuclease (ORN) are the members of DEDD superfamily of RNases. RNase D is a hydrolytic and distributive RNase which participates in the maturation of stable RNA of single stranded substrates and possibly of tRNA [31]. RNase T participates in end- turnover of tRNA and maturation of stable RNA. It cannot invade the secondary structures or junctions in RNAs [32]. It also distinguishes against pyrimidines.

ORN is a hydrolytic RNase and it is a distributive enzyme. It has affinity for short oligoribonucleotides and it is required for the completion of degradation of mRNA in *E. coli.*

RNase BN is also a distributive and hydrolytic enzyme which has preference for tRNA precursors and molecules in which there is altered 3'-CCA sequence.

PNPase and RNase PH both have phosphorolytic activity. PNPase functions in degradation of mRNA and stable RNA and quality control. RNase PH participates in maturation of stable RNA asa single strand specific RNase [33].

2.2 Process of Action and Biological Role of RNase D:

RNase D was the initial exoribonuclease found to have an extreme level of substrate explicitness. Limited functional and mechanistic work have been done on this enzyme. Altered and destructive tRNAs and tRNA antecedent are the known substrates for RNase D. There is just restricted exonuclease action on local tRNA by RNase D [18]. RNase D has non- explicit capacity to cooperate with various nucleic acids. Denatured tRNA hydrolysis by RNase D is hindered by whole and relatively decayed tRNAs, poly (U), salmon sperm DNA and 5S RNA [18].This suggests that RNase D has the ability of binding to a diversity of nucleic acid substrates.

The expression of RNase D seems detrimental to the growth of *E. coli*.This enzyme was expressed distinctly with an exceptionally controlled overexpression plasmid. RNase D chromosomal gene in *Escherichia coli* utilizes an initiation codon UUG and an unusually high state of uncommon codons (<1%) [34], which limits its endogenous expression. The action of RNase D *in-vivo* on tRNAs have detrimental effects on the growth of *E. coli*. This is shown by the decreased level of tRNA on RNase D overexpression [13]. RNase D with local tRNA *in-vitro* has weak exonuclease activity. This may be due to unfavorable conditions or due to absence of extra aspects in the *in-vitro* assays or the genuine local substrates for RNase D are yet to be recognized. RNase D follows up on degraded tRNA however not on single-stranded RNA which recommends that RNase D prefers substrates of RNA with 3'unpaired overhang and with secondary shape. The ring formed structure of RNase D recommends that it may be a processive catalyst, in spite of the fact that it has not been seen in tRNA like substrates [35]. The detrimental

impacts of RNase D expression is might be because of synergetic associations with other RNA deteriorating catalyst [36].

RNase D because of their feeble bonding at the active center do not follow up on short oligonucleotides. As the DEDD space is exceedingly negatively charged it is not susceptible to direct binding of nucleic acids. According to arrangement preservation there is no crucial cooperation between DEDD space and substrates. To display a dinucleotide into DEDD pocket of RNase D the anatomy of a substrate bound to Klenow fragment (PDB ID: 2KZZ) [37] was used suggesting that the DEDD pocket has the ability to hold the dinucleotide (shown in Figure 5A and Figure 5B). Longer substrates are needed to permit cooperation with different areas of RNase D which will give constant presentation and binding of 3'end of the substrate on DEDD catalytic center.

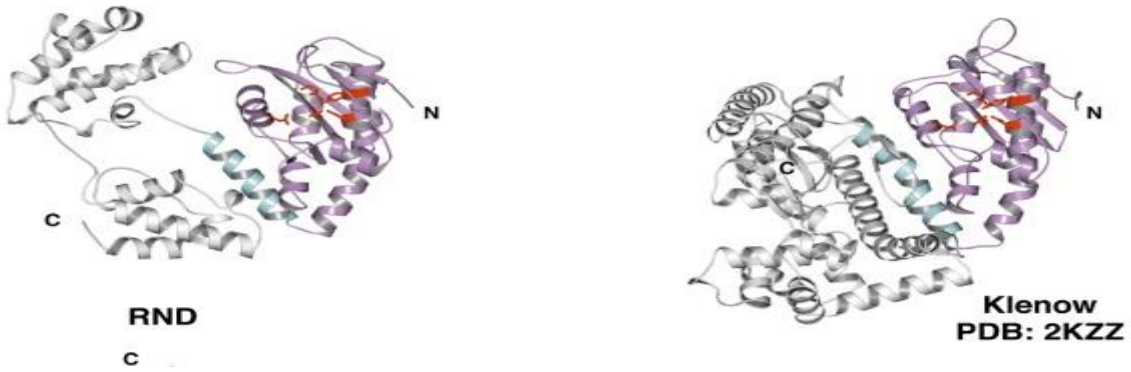


Figure 5A: The RNase D structure of *E. coli* is compared with the structure of Klenow fragment (PDB ID: 2KZZ). The DEDD core domains are purple colored in the ribbon diagram. Both RNase D and Klenow fragment belongs to the DEDDy subgroup. The sticks represents the DEDDh/DEDDy or residues at equivalent positions with red color. The cyan color represents an additional helix shared between RNase D and Klenow fragment. This helix connects their other structural domains to the DEDD domains (Zuo *et al.*, 2005).

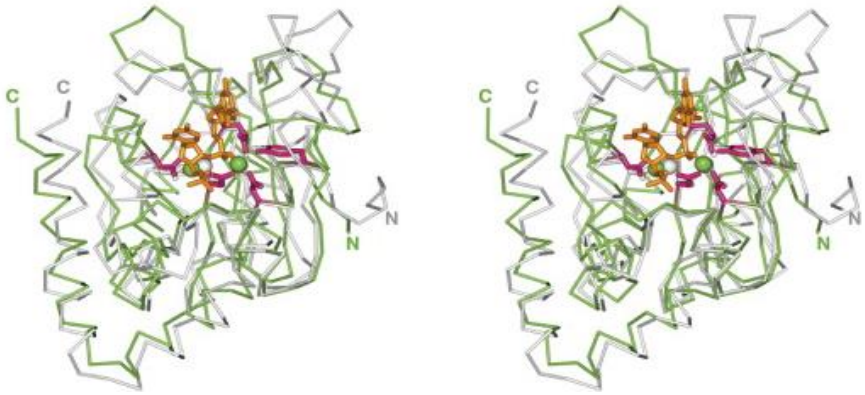


Figure 5B: Superposition of the Cα traces of RNase D (the colored part) and Klenow fragment (in gray color) DEDD domains is shown in stereo. Metal ions (represented by ball structures) and DEDDy residues (shown by red or gray sticks), as well as dinucleotide (represented by orange colored sticks) bound in the klenow fragment crystals are shown. (Zuo *et al*., 2005).

2.3 A gene coding for RNase D exonuclease-like protein is needed for Post-transcriptional gene silencing (PTGS) in *Arabidopsis*:

Post transcriptional gene silencing in which the expression of gene is switched off by the introduction of antisense RNA which blocks the translation of mRNA [38]. In this there is an initial endonucleolytic cleavage of mRNA, resulting into PTGS which are then completely hydrolyzedby RNase D have been studied. Interestingly, many individuals from protein family of RNase D are involved in PTGS in animals as well as in plants. The *Arabidopsis* Werner Syndrome-like exonuclease (WEX) codes RNase D area which is fundamentally the same as the human Werner Syndrome protein (WRN). Wex-1 gene that has a RNase D domain was mutated to show the loss of function for the mutant. Only the wild type can regulate gene expression by gene silencing. The Werner Syndrome causes pre mature ageing, ocular cataracts, diabetes, defects in DNA replication, recombination, repair etc. The *Caenorhabditis elegans mut*-7 gene is crucial for PTGS, RNA interference, and for hushing transposon movement [39, 40]. It encodes an individual from the protein family of RNase D which belongs to the DEDD superfamily of 3'-5'exoribonucleases. The individuals from this family are involved in organizing and

development of tRNA in yeast and *E. coli* [19]. The MUT-7 protein are involved in the breakdown of mRNAs coordinated by PTGS or RNAi.

2.4 Phylogenetic distribution of bacterial ribonucleases:

Ribonucleases are essential for cellular metabolism. Two sorts of RNases are being studied which are exoribonucleases and endoribonucleases. Bacterial ribonucleases have been studied extensively in *B. subtilis* for gram positive bacteria and in *E. coli* for gram negative bacteria. Anaggregate of 17 distinct RNase actions have been identified from 20 unique proteins. The genetic make-up of *E. coli* and *B. subtilis* were compared which results in sharing of six of their characterized RNases suggesting that both gram negative and positive life form have developed with various answers to the problems of RNA decay and maturation [41]. Orthologues of each of the RNases are recommended on the basis of overall arrangementhomology and size contemplation. The different RNases distribution are represented on a phylogenetic tree of 16S ribosomal RNA (rRNA). This shows more clearly the connections between different species of bacteria and the variations between the different bacterial clades.

2.5 A new member of the RNase D Exoribonuclease family works in mitochondria and directs RNA metabolism:

For mitochondrial gene expression RNA production and RNA altering are crucial. RNA production is directed by RNA 3' adenylation and uridylation. Not much is known about mitochondrial regulation. A mitochondrial exoribonucleases, Tb RND was identified whose expression is extremely synchronized. The Tb RND shares arrangement homology with family of RNase D and possess CCHC Zinc finger space. Tb RND displays 3'-5' exonuclease activity *in-vitro* having specificity towards uridine homopolymers, which includes guide RNAs (gRNAs) having 3' oligo (U) tails. It provides arrangement data for altering of RNA. *In-vivo* Tb RND functions in gRNA metabolism. Both *in-vitro* and *in-vivo* study of the novel mitochondrial exoribonucleases Tb RND have been presented which is the initial organellar individual from RNase D sub family of DEDD nucleases.

2.6 NrnC, an RNase D like protein from *Agrobacterium* is a Octameric Nuclease which Decays dsDNA but not dsRNA:

NrnC gene from *Agrobacterium tumefaciens* is a nuclease which contains a DEDDy area similar to RNase D exonuclease space (shown in Figure 6). According to nuclease tests NrnC have diverse substrate particularity. Rather than RNase D which cleaves both ssRNA and dsDNA, NrnC only hydrolyzes ssRNA, dsDNA, ssDNA with great efficiency but does not cleaves dsRNA [42]. The two divalent metal ions Mn^{2+} and Mg^{2+} are the cofactors for NrnC. This protein have been cloned, overexpressed and purified for the extensive study of crystallization of protein.

RNase D was mostly studied by Cudny, Deutscher, Malhotra and his group. They found that the RNase D is an exoribonuclease which initiates attack at the 3'end of a RNA molecule and gives 5' mononucleotides.

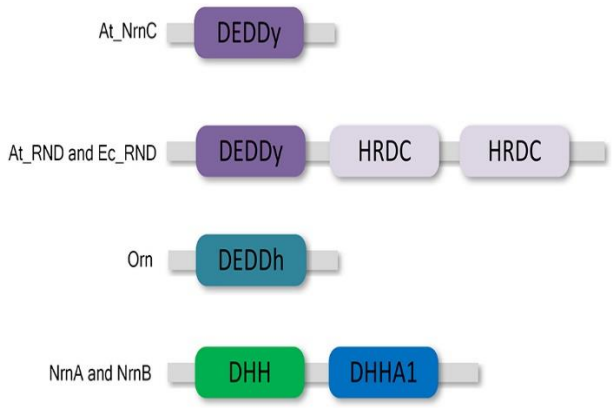


Figure 6: Domain organization of *Agrobacterium* NrnC and other DEDD exonuclease. *Agrobacterium* NrnC contains a single DEDDy domain, whereas Orn consists of a single DEDDh domain. RNase D from *E. coli* (Ec_ RND) and its near analogue from *Agrobacterium fabrum* (At_ RND) consists of DEDDy domain and two extra HRDC domains. NrnA and NrnB contains DHH and DHHA1 domains respectively (Yuan *et al.*, 2019).

The substrate specificity of RNase D makes well to be a 3'-processing nuclease. Its action on additional base pairs following the -C-C-A sequence would allow rapid processing of extraneous sequences, and its relatively poor activity on intact tRNA coupled with a random mode of hydrolysis would permit aminoacylation of the mature tRNA prior to degradation [2]. Studies with the mutants are

in progress to determine whether RNase D actually functions as a processing enzyme in vivo or not.

Also, why RNase D stops processing after the CCA sequence remains hidden. Even after its discovery since more than 40 decades, the mode of action and even basic question like the active site of RNase D remain obscure.

The main question being- If RNase D's absence does not have any deleterious effects on the cell, then why it has been preserved evolutionarily?

3. Objectives:

The experimental objectives have been setup in the following order:

1. Primer designing of RNase D gene
2. Sub-Cloning of RNase D in pET22b
3. Over-expression of RNase D in *E. coli* BL21 (DE3).
4. Purification of RNase D

4. Methodology:

Materials and methods:

Materials: All chemicals were bought from Hi-media. The kit used for DNA/plasmid isolation and gel extraction was obtained from QIAGEN. Primers and restriction enzymes were obtained from IDT & PROMEGA. The whole PCR reaction component and Taq polymerase were purchased from New England Biolabs and GoTaq® Green Master Mix was purchased from PROMEGA. The DNA ladder and T4 DNA ligase Inc. calf alkaline phosphatase dNTP and ATP were obtained from Bio Lit.

Methods:

The RNase D strain MG1655 was grown in LB media.

LB media contents

- Tryptone
- Yeast extract
- NaCl

Growth conditions: Temperature - around 37°C at 180 rpm.

4.1 DNA Isolation:

- DNA was isolated manually and also by using **PROMEGA DNA ISOLATION KIT.**
- 1ml of bacterial culture was added to a 1.5ml micro-centrifuge tube.
- 600µl of Nuclei Lysis Solution was added. Pipetting was done until the cells were resuspended.
- It was incubated at 80ºC for 5 minutes for the lysis of cells and was then cooled to room temperature.
- 3µl of RNase solution was added to the cell lysate and the tube was inverted for 2-3 times for proper mixing.
- It was then incubated at 37ºC for 15 minutes and was cooled to room temperature.
- 200µl of Protein Precipitation solution was added to the RNase treated cell lysate and was vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate.

- The sample was incubated on ice for 5 minutes.
- It was centrifuged at 13000-16000 x g for 3 minutes.
- The supernatant containing the DNA was transferred to a clean 1.5ml micro-centrifuge tube containing 600µl of isopropanol.
- It was gently mixed by inversion until the thread like strands of DNA formed a visible mass.
- It was again centrifuged at 13000-16000 x g for 2 minutes.
- The supernatant was carefully poured off and the tube was drained on a clean absorbent paper. 600µl of 70% ethanol was added and the tube was gently inverted for several times for washing of DNA pellet.
- It was centrifuged at 13000-16000 x g for 2 minutes and was aspirated with ethanol.
- The tube was drained on a clean absorbent paper and the pellet was allowed to air dry for 10-15 minutes.
- The DNA was rehydrated by incubating the solution overnight at room temperature or at 4ºC.
- The DNA was stored at -20ºC.

4.2 Quantitative and Qualitative analysis:

Qualitative analysis was performed by Agarose gel electrophoresis.

Quantitative analysis was performed by using Nano-drop spectrophotometer.

Materials used: Agarose (Sigma), 5X TBE buffer (tris base boric acid, 0.5M EDTA, pH 8.0), DNA sample(s), DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose/glycerol in water), DNA ladder (NEB), EtBr staining solution, Gel tray and Gel caster,

Gel comb, Horizontal electrophoresis unit, Power pack (BIORAD), Glass flask (250ml), Graduated cylinder (1000ml), Gel Doc (BIO-RAD), Micropipettes, sterilized tips.

Quality of the DNA was checked by 1%agarose gel electrophoresis.

Quantitative amount of the DNA was measured by using Nano-drop spectrophotometer and the purity was calculated by using 260/280 ratio.

4.3 Primer Designing for amplifying the Gene of Interest:

Through PubMed we have searched the RNase D gene, by using oligo Calc and NEB cutter software primers was designed.

The gene sequence is as follows:

>EG10858 rnd RNase D, processes tRNA precursors

TTGAATTACCAAATGATTACCACGGACGATGCGCTGGCTTCTTTGTGTGAAGCC
GTCCGTGCCTTTCCGGCGATAGCCCTGGATACTGAATTTGTTCGTACGCGCACT
TATTACCCGCAGCTGGGGTTGATTCAACTTTTCGATGGCGAGCATCTGGCGCTA
ATCGATCCACTCGGGATCACCGACTGGTCACCGCTGAAAGCGATCCTGCGCGAT
CCGTCCATCACAAAATTTCTCCATGCAGGCAGTGAAGATCTGGAAGTGTTCCTC
AATGTCTTTGGCGAATTACCACAACCCTTGATTGACACGCAAATCCTTGCTGCCT
TCTGCGGACGCCCGATGTCATGGGGTTTCGCTTCCATGGTGGAAGAGTATTCCG
GCGTTACGCTGGACAAGAGTGAATCGCGCACCGACTGGCTGGCCAGACCGCTG
ACCGAACGTCAGTGTGAATACGCAGCGGCGGATGTCTGGTATCTGTTACCGATC
ACCGCCAAGCTTATGGTAGAAACGGAGGCCTCCGGCTGGCTACCTGCGGCGCT
GGATGAATGCCGCCTGATGCAAATGCGTCGTCAGGAAGTCGTTGCGCCGGAAG
ATGCCTGGCGTGATATCACCAATGCCTGGCAATTACGCACACGCCAACTGGCCT
GTCTGCAACTGTTAGCCGACTGGCGACTGCGCAAGGCGCGAGAGCGCGATCTG
GCGGTGAACTTTGTCGTGCGTGAAGAGCATTTGTGGTCGGTAGCGCGTTATATG
CCGGGAAGTTTAGGCGAACTGGACAGCCTGGGTTTATCCGGTAGCGAAATCCG
CTTTCACGGTAAAACGCTGCTAGCGCTGGTGGAAAAAGCGCAGACATTGCCGG
AAGATGCCTTACCGCAGCCGATGCTTAACCTGATGGACATGCCGGGTTATCGTA
AAGCGTTTAAAGCGATTAAGTCGCTGATTACTGACGTGAGCGAAACGCATAAGA
TCAGCGCCGAATTGCTGGCATCGCGTCGGCAAATCAACCAACTGCTGAACTGG
CACTGGAAACTGAAACCGCAGAACAATTTGCCGGAGCTGATTTCCGGCTGGCG
TGGTGAGCTGATGGCGGAAGCATTACACAATTTATTGCAGGAATATCCGCAGTA

Forward Primer: 5' GGATCCTTGAATTACCAAATGATTACCACGGAC 3'
Reverse Primer: 5' CTCGAGTTACTGCGGATATTCCTGCAA 3'

Underline sequences represents the restriction enzyme site:

Bam HI - GGA TCC

XhoI - CTC GAG

4.4 Amplification of Desired Gene by PCR:

Polymerase chain reaction (**PCR**) is a method widely used in molecular biology for amplification of single or a few copies of a segment of DNA across several orders of magnitude, producing thousands to millions copies of a particular DNA sequence. It was used to amplify the specific gene containing *rnd* gene sequence.100ng DNA was used from the dilutions (20ng/µl) which were prepared.

Materials used: Thermal cycler (BIO-RAD), DNA samples (template), Go Taq® Green Master Mix (PROMEGA), Forward and Reverse primer (ordered from IDT), Distilled water, PCR tubes, Micropipettes, Sterilized tips.

Reaction volume	25 µl
GoTaq® Green	12.5 µL
DNA	3.5 µL
Forward Primer (10µM)	1.25 µL
Reverse primer (10µM)	1.25 µL
Nuclease free water	7.5 µL

Table 1: Working concentrations of PCR chemicals for 25µl of reaction

4.4.1 PCR optimization:

PCR reaction was carried out at different annealing temperatures to optimize temperature condition for PCR. Also different number of cycles for PCR were used to determine the best protocol to get proper amplification. Different volumes of PCR reactions were also optimized to get maximum amplification product.

STEP	TEMPERATURE (^oC)	TIME
Initial denaturation	98	2 minutes
Denaturation	98	20 sec
Annealing	58	30 sec
Extension	72	2 minutes
Final extension	72	5minutes
Infinite Hold	4	

Table 2: Conditions for PCR

4.5 PCR purification:

The gel extraction kit was used according to the manufacturer's instructions for PCR product purification and to extract DNA fragments from agarose gels. After amplification the desired band which we got is extracted from agarose gel via **QIAGEN GEL EXTRACTION KIT** and the procedure is as follows:

- The DNA fragment was excised from agarose gel with a clean, sharp scalpel.

- The gel slice was weighed in an eppendorf. 3 volumes of Buffer QG was added to 1 volume gel (100 mg ~ 100 ml). The maximum amount of gel slice per spin column is 400 mg; for gel slices >400 mg, add 6 volumes of Buffer QG.
- Incubated at 50ºC for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.
- After the gel slice has dissolved completely, check that the color of the mixture is yellow.
- 1 gel volume of isopropanol was added to the sample and mix.
- A QIAquick spin column was placed in a provided 2ml collection tube.
- To bind DNA, the sample was applied to the QIAquick column, and centrifuged for 1 min.
- The flow-through was discarded and the QIAquick column was placed back in the same collection tube.
- To wash, 0.75 ml of Buffer PE was added to QIAquick column and centrifuged for 1 min.
- The flow-through was discarded and centrifuged the QIAquick column for 1 min at ~13,000 rpm.
- The QIAquick column was placed into a clean 1.5ml microfuge tube.
- To elute DNA, 50 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) or water was added to the center of the QIAquick column and centrifuged for 1 min at maximum speed. Alternatively, for increased DNA concentration, 30 µl Buffer EB was added to the center of the QIAquick column, let stand for 1 min, and then centrifuged for 1 min.

4.6 Plasmid DNA Isolation (pET-22b Plasmid containing cells):

It is a vector that contains an expression cassette, which enables expression of the cloned protein in transformed cells. Artificial plasmids are widely used as vectors in molecular cloning, serving to drive the replication of recombinant DNA sequences within host organisms.

It was isolated with the help of **QIAGEN KIT**. Quantitative and qualitative estimation of the isolated plasmid was done using Nano drop and agarose gel electrophoresis respectively. The protocol of **QIAGEN KIT** is as follows:

- 1-5 ml of overnight bacterial culture was pelleted by centrifugation at >8000rpm for 3 minutes at room temperature.
- The pelleted bacterial cells were resuspended in 250µl Buffer P1 and was transferred into a micro-centrifuge tube.
- 250µl of Buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times until the solution becomes clear.
- 350µl of Buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4-6 times.
- It was then centrifuged at 13000 rpm for 10 minutes in a table top micro-centrifuge.
- 800µl of supernatant from step 5 was added to the QlAprep 2.0 spin column by pipetting.
- It was centrifuged for 30-60 seconds and the flow-through was discarded.
- The QlAprep 2.0 spin column was washed by adding 0.5ml of Buffer PB and centrifuged for 30-60 seconds. The flow-through was discarded.
- The QlAprep 2.0 spin column was washed by adding 0.75ml of Buffer PE. It was centrifuged for 30-60 seconds and the flow-through was discarded.
- It was again centrifuged for 1 minute to remove the residual wash buffer.
- The QlAprep 2.0 spin column was placed in a clean 1.5ml micro-centrifuge tube. The DNA was eluted by adding 30µl of Buffer EB.

- 20µl of Buffer EB was again added and spinned in a micro-centrifuge. It was repeated.
- Again it was spinned for 1 minute and the isolated Plasmid DNA was stored at -20ºC.

4.7 Restriction Digestion of Plasmid and PCR product:

A restriction enzyme that cuts DNA near specific recognition nucleotide sequences known as restriction sites. These enzymes are found in bacteria and provide a defense mechanism against invading viruses.

All restriction digestions were done with enzymes from **PROMEGA**, following the protocols recommended by the supplier. In separate tubes, the plasmid vector (2µg) and the DNA were digested with the appropriate restriction enzymes. The pET22b + PCR product were digested with *BamHI* and *XhoI* at 37°C for 3hrs in a 30µl reaction. After the reaction, the sample was mixed with loading buffer, and immediately loaded on an agarose gel and run, in order to stop the reaction.

Material used: amplified DNA product, Restriction Digestion PROMEGA KIT, micropipettes and sterilized tips.

Components	For pET 22b	For PCR	Control
pET 22b	5 µl	_	5 µl
PCR product	_	8 µl	8µl
10X RE buffer	3 µl	3 µl	3 µl
Restriction enzyme	3µl (1.5µl of each enzyme)	3µl (1.5µl of each enzyme)	_
Nuclease free water	19 µl	16 µl	14 µl

Table 3: Conditions for restriction digestion

4.8 Ligation of Digested Plasmid and PCR Product:

10X ligase buffer	2 µl
Double digested PET vector	5 µl
Double digested PCR product	5 µl
DNA ligase	1 µl
Nuclease free water	7 µl

Table 4: Conditions for ligation

After ligation, ligated product was kept for overnight at 4°C.

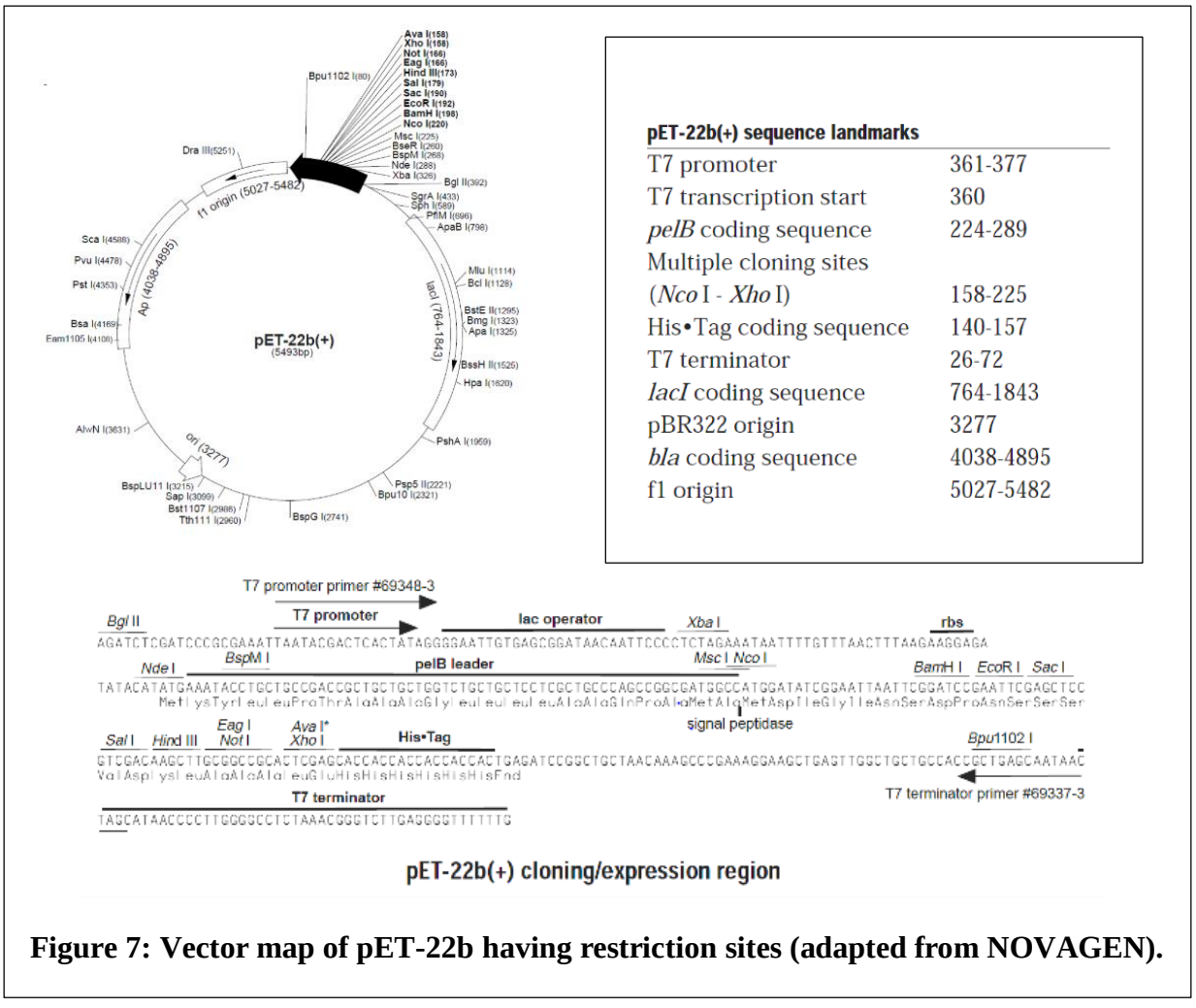


Figure 7: Vector map of pET-22b having restriction sites (adapted from NOVAGEN).

4.9 Competent cell preparation:

Competent cells was used for transformation.

- MG-1655 cells were inoculated in 10 ml of LB broth.
- At optical density of 0.6, the culture was kept in ice for 30 minutes.
- After 30 minutes, the cells were centrifuged at 5000 rpm for 10 minutes at 4^{o} C.
- The pellet thus obtained was resuspended into 15 ml 0.1M ice cold $CaCl_2$ and was kept on ice for 20-30 minutes.
- The cells were centrifuged again at 5000 rpm for 10 minutes and were again resuspended in 5 ml of ice cold 0.1 M $CaCl_2$.
- The cells were again spun down at 5000 rpm for 10 minutes at 4^{o}C and the pellet obtained was suspended in 200 µl of 0.1 M $CaCl_2$.

4.10 Transformation:

MG-1655 competent cell was used for transformation.

- 100µl of competent cell was added to 10µl of the ligated mixture; mixture was gently mixed and kept on ice for 1 hr.
- Heat shock was given at 42°C for 90 sec -2 min.
- Mixture was incubated on ice for 5min.
- 1ml of LB was added to the mixture and incubated at 37°C for 2hr at 200 rpm.
- The cells were then centrifuged to obtain the pellet and was resuspended.
- Then it was spread on the ampicillin plates and was left overnight.

If any colonies were found on ampicillin plate then it shows that the ligation was successful.

4.11 Plasmid Isolation (MG1655 cloned cells):

It was isolated with the help of **QIAGEN KIT**. Quantitative and qualitative estimation of the isolated plasmid was done using Nano drop and agarose gel electrophoresis respectively.

4.12 Colony and Plasmid PCR to confirm RNase D cloning:

PCR of plasmid along with colony PCR was done.

Component	Plasmid	Colony PCR
Go Taq® Green	12.5µl	12.5µl
Forward primer	1µl	1µl
Reverse primer	1µl	1µl
Plasmid DNA	3.5µl	X
Nuclease free water	7µl	X
Colony mixed with 50µl water	_	10.5µl

Table 5: Conditions for Plasmid and Colony PCR

4.13 Competent cell preparation:

- BL21 (DE3) pLysS cells were inoculated in 10 ml of LB broth.
- At optical density of 0.6, the culture was kept in ice for 30 minutes.
- After 30 minutes, the cells were centrifuged at 5000 rpm for 10 minutes at 4^{o} C.
- The pellet thus obtained was resuspended into 15 ml 0.1M ice cold $CaCl_2$ and was kept on ice for 20-30 minutes.

- The cells were centrifuged again at 5000 rpm for 10 minutes and were again resuspended in 5 ml of ice cold 0.1 M $CaCl_2$.
- The cells were again spun down at 5000 rpm for 10 minutes at 4°C and the pellet obtained was suspended in 200 µl of 0.1 M $CaCl_2$.

4.14 Transformation:

BL21 (DE3) pLysS competent cell was used for transformation.

- 100µl of competent cell was added to 10µl of the ligated mixture; mixture was gently mixed and kept on ice for 1 hr.
- Heat shock was given at 42°C for 90 sec -2 min.
- Mixture was incubated on ice for 5min.
- 1ml of LB was added to the mixture and incubated at 37°C for 2hr at 200 rpm.
- The cells were then centrifuged to obtain the pellet and was resuspended.
- Then it was spread on the ampicillin plate and was left overnight.

If any colonies were found on chloroamphenicol + ampicillin plate then it shows that the ligation was successful.

4.15 Over-expression and Purification of RNase D:

Principle: Nickel Nitrilotriacetic acid (Ni-NTA) Agarose is an affinity chromatography matrix which is used for purification of recombinant proteins having a His-tag sequence. The Histidine remnants of His-tag sequence binds to the empty positions of immobilized nickel ions with high accuracy and affinity in the coordination sphere. The cleared cell lysates are loaded on the matrices and as a result the His-tagged proteins remains bound to the matrix. The other proteins

passes out through the matrix. After washing the bound His-tagged proteins are rinsed in buffer under denaturing conditions.

Chemicals used: Lysis buffer, Elution buffer, Ni-NTA Resin from GE Healthcare.

Composition of lysis buffer: 20mM Tris HCl (pH -7.5), 100mM KCl, 10mM imidazole, 10% glycerol (V/V).

Composition of elution buffer: 20mM Tris HCl (pH-7.5), 100mM KCl, 500mM imidazole, 10% glycerol (V/V).

- Strain carrying plasmid was grown at 37ºC in one litre LB Broth supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol to maintain the plasmid.
- At an A_{600} of 0.6, 1mM isopropyl β-D- thiogalactopyranoside was added and the growth was continued for another 2 hours.
- Cells were harvested by centrifugation and washed with 0.9% NaCl. The cell pellets were frozen at -80ºC until use.
- Frozen cells were resuspended in lysis buffer (10ml) with 1mM Phenyl Methyl Sulfonyl Flouride (PMSF) and lysozyme (10mg/ml) was added and was incubated for 1hour.
- These cells were lysed by sonicating from 30 second pulse for five times.
- The lysed cells were centrifuged at 12000 rpm for 35 minutes.
- The protein concentration was measured by Bradford Assay.
- NTA resin was washed with water.
- First 2 ml of resin was taken and was given a quick spin. The supernatant was discarded and autoclaved water was added and the quick spin was repeated.
- After washing the resin, it was mixed with lysis buffer (1ml) and was incubated for 30 minutes at 4ºC.

- The mixture was centrifuged at 2000 rpm for 10 seconds and the supernatant was discarded.
- The pellet obtained from lysing the cells was discarded and the supernatant was mixed with the NTA resin and was incubated overnight at 4^{o}C on dancing shaker.
- Next day, all resin solution was poured on the column.
- The unbound was collected in a conical flask (non- specific binding).
- This resin was washed again with lysis buffer (10ml).
- Elution buffer (8ml) was added after this to elute the bound protein.
- The size of the protein was analyzed by SDS-PAGE.

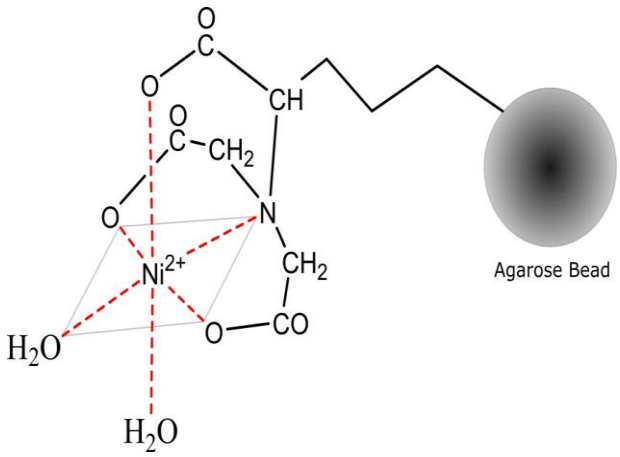


Figure 8: Depiction of Ni-NTA resin (adapted from Generon).

4.16 SDS-polyacrylamide-gel electrophoresis:

SDS-PAGE, is sodium dodecyl sulfate polyacrylamide gel electrophoresis which is a commonly used method for separation of proteins from the sample mixture. When current is applied, proteins migrate towards the positive anode inside the poly-acrylamide gel under denaturing conditions. It is used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein folding, posttranslational modifications and other factors).The SDS gel electrophoresis of samples having identical charge to mass ratios results in fractionation by size and is probably the world's most widely used biochemical method.

Materials required: Acrylamide/ Bis- acrylamide (30%), 1.5 M Tris-Cl; pH (8.8), 0.5 M Tris-Cl; pH (6.8), 10% SDS, 10% ammonium per sulfate (APS), N,N,N',N'-tertramethylethane-1,2-diamine (TEMED), staining solution, destaining solution, running buffer, loading dye.

Composition of Staining solution: 0.1% Coomassie Brilliant Blue, 40% Methanol, 10%Acetic acid, double distilled water.

Composition of Destaining solution: 40% Methanol, 10%Acetic acid, double distilled water.

Composition of Running Buffer: Glycine (14.4g/l), Tris-base (3g/l), SDS (1g/l), double distilled water.

Composition of loading dye: 0.5M Tris-Cl (pH-6.8), 10% SDS, glycerol, 0.5% Bromophenol Blue (W/V), double distilled water, 2-mercaptoetanol added to it at the time of loading.

SDS PAGE Protocol:

Casting of gel:

Make the separating gel (7%):

The casting frames were placed on the casting stands by clamping two glass plates.

The gel solutions were prepared in a separate small beaker.

Suitable proportion of separating gel solution was pipette into the gap between the glass plates. The gap was filled with water until it overflow to make the separating gel be horizontal. Then left it for 20-30 min. for gelation.

Composition of separating gel:

1. Water- 4.15ml
2. 1.5M Tris-Cl (pH 8.8) - 2.5ml
3. 10% SDS- 0.1ml
4. Acryl/Bisacryl – 3.25ml
5. 10% APS - 50µl
6. TEMED - 10µl

Make the stacking gel (3%):

The water is discarded and separating gel was left.

- Stacking gel was pipette into the glass plates until it overflows. The comb was then inserted between the glass plates. Care should be taken that no air bubbles is produced when the comb is being inserted. Then wait for 20-30 min for gelation.
- The glass plates were taken out from the casting frame and was put in the cell buffer dam. The running buffer (electrophoresis buffer) was then poured into the inner chamber of the buffer dam until the running buffer reaches the required level in the outer chamber.

Composition of stacking gel:

1. Water- 3.05ml
2. 0.5M Tris-Cl- 1.25ml
3. 10% SDS - 50µl
4. Acryl/Bisacryl- 650µl
5. 10% APS - 50µl
6. TEMED- 10µl

Preparation of the samples:

- 15 µl samples with 5 µl sample buffer (loading dye). Then samples were heated in boiling water for 5-10 min.
- Samples were loaded into wells.
- The electrophoresis was run at 60 volts for stacking gel and 100 volts for separating gel.

- (SDS-PAGE was stopped when the lower most sign of the protein marker reaches the end of the glass plate.)

- The staining and destaining solution were prepared.
- The plate was carefully removed with the help of spacer and the gel was placed in the staining solution. Before placing the gel a cut mark should be there for knowing the loading order of protein sample.
- The gel was kept in the staining solution for 1-3hours in a dancing shaker.
- After staining the gel was washed with destaining solution until the dye color was removed.
- When the gel is placed in destaining solution, the solution should be changed several times until the color is removed.
- Then the bands were observed with marker after complete destaining.

5.0 Results and Discussions:

The aim of this present study is to clone, overexpress and purify RNase D in *E. coli.* Cloning was done by digesting both pET 22b vector and gene of interest with the help of two restriction enzymes *BamHI* and *XhoI*. Then the digested products were obtained by gel purification. The purified digested products were ligated with T4 DNA ligase in 1:3 ratio. The ligated products were transformed in *E. coli* (MG1655) to confirm cloning by growing on ampicillin plates. Cloning was successful as transformed colonies were obtained on ampicillin plates. Further, cloning was confirmed by Plasmid PCR and sequencing report.

RNase D was transformed in BL21 (DE3) pLysS for overexpression was successful. It was then purified by Ni-NTA method. An overexpressed and purified band of 43kDa (purified protein with His-Tag) was obtained which was analyzed by SDS-PAGE.

5.1 Isolation of Genomic DNA of *E. coli*:

Figure 9: Genomic DNA of *E.coli* was isolated and run on 1% agarose gel. Lane 1 contains 1Kb ladder and Lane 2, 3 and 4 contains Genomic DNA of *E. coli*.

5.2 PCR Product:

The amplification of the RNase D gene was done by PCR. Figure below shows the quality of PCR product. The PCR product obtained from the samples wasobserved in gel. 1Kb marker was loaded. RNase D gene is 1127bp long. This can also be confirmed by the gel shown below as we get PCR band corresponding to 1.1 kb marker band.

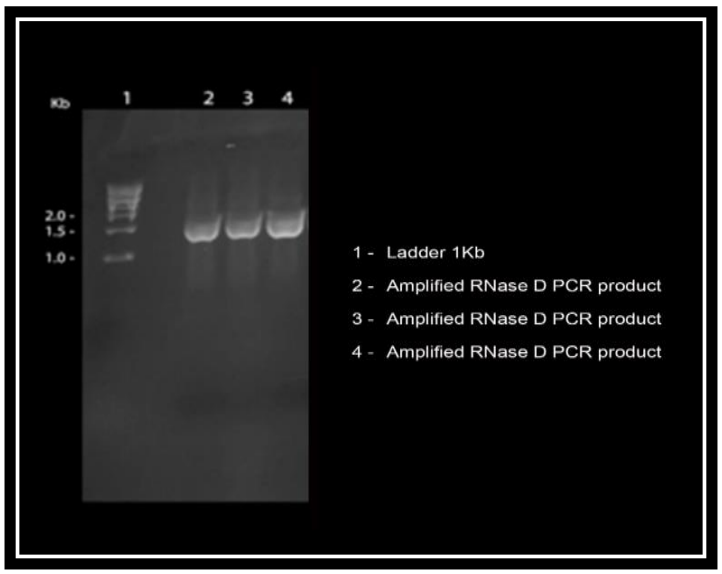


Figure 10: Agarose Gel of RNase D PCR product. Lane 1 contains 1Kb ladder and lane 2, 3 and 4 contains amplified RNase D PCR product.

5.3 Plasmid Isolation:

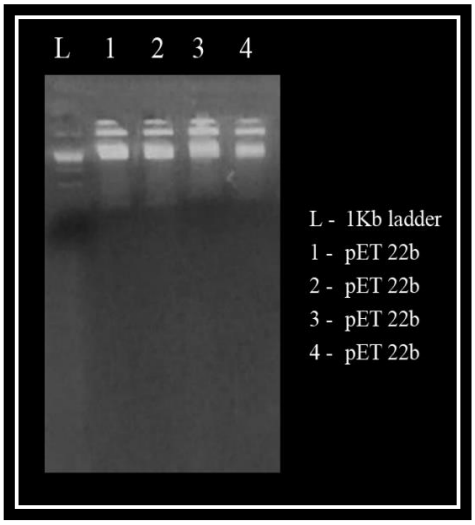


Figure 11: Picture showing pET 22b plasmid.

5.4 Restriction Digestion:

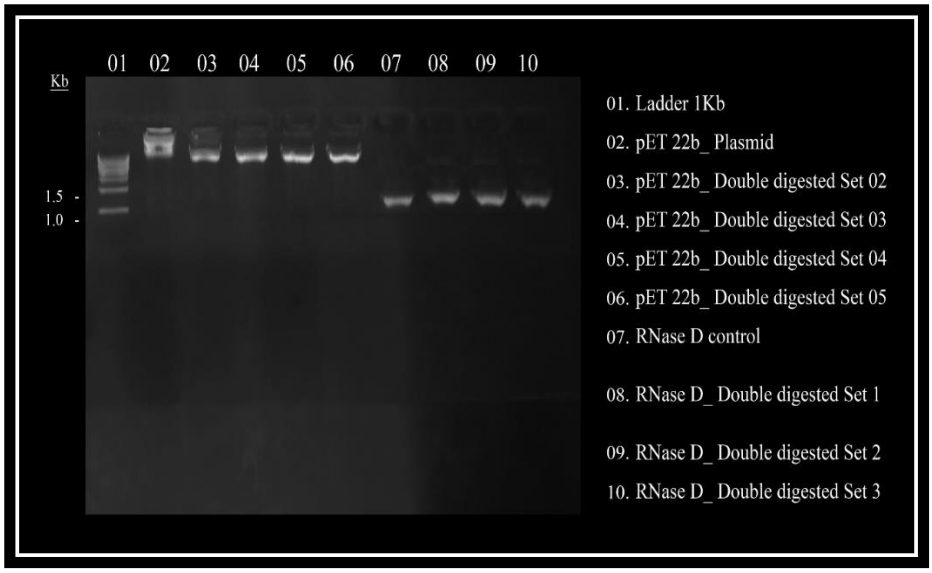


Figure 12: Double digestion of plasmid pET 22b and PCR product of RNase D gene by *BamHI* and *XhoI*. Lane 1 contains 1Kb ladder, Lane 2 contains pET 22b (control), Lane 3-6 contains double digested pET 22b, Lane 7 contains RNase D (control), Lane 8-10 contains double digested PCR product of RNase D.

5.5 Cloning Confirmation:

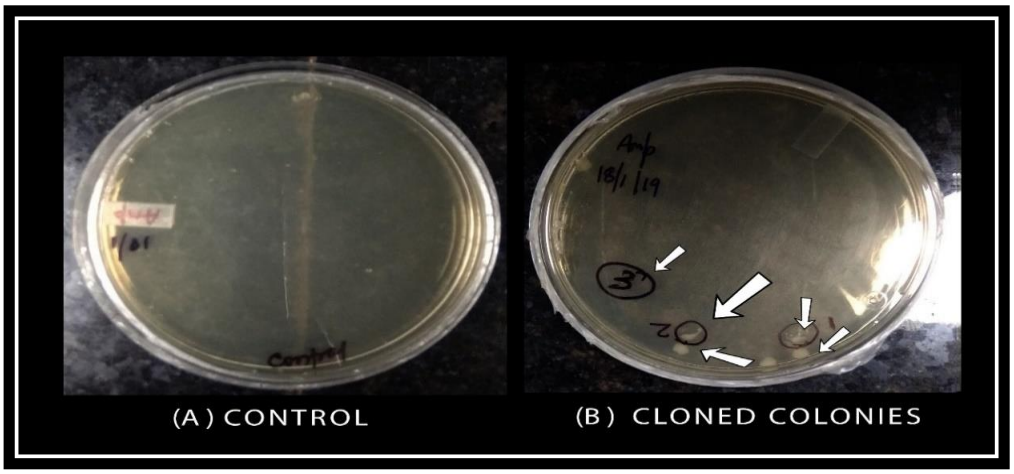


Figure 13: Clone is confirmed by antibiotic (Ampicillin) screening.

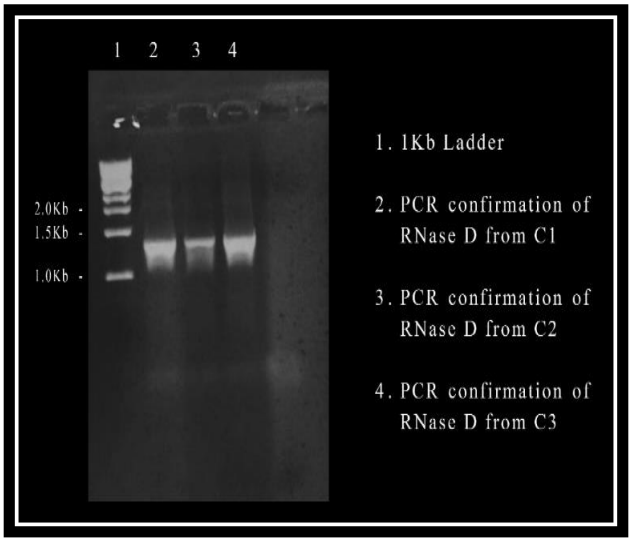
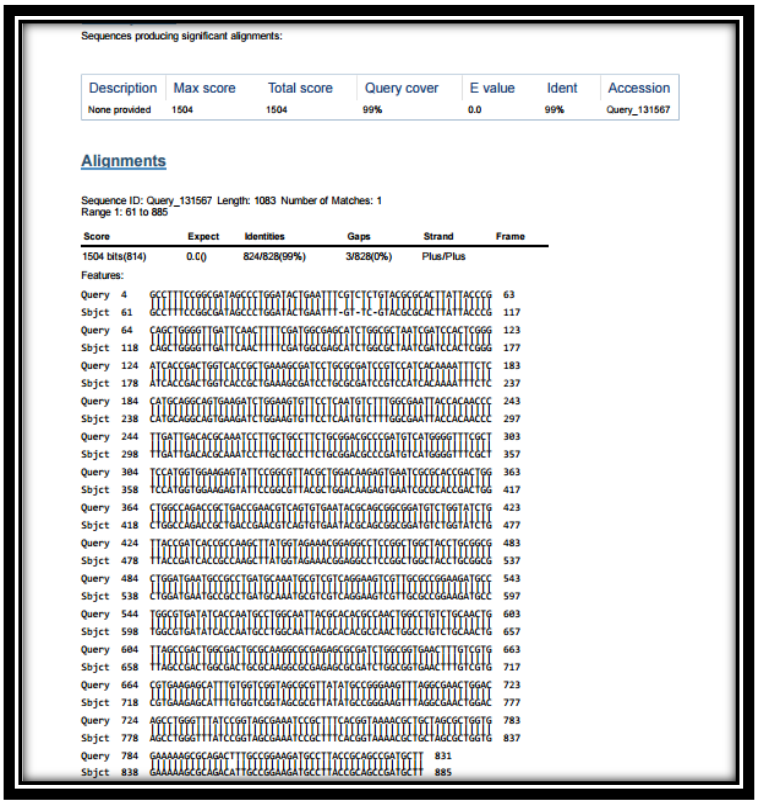


Figure 14: Cloning confirmed by Plasmid PCR using RNase D primer and colonies picked after antibiotic screening. Lane 1 contains 1 Kb ladder, Lane 2-4 contains RNase D from colonies 1, 2 and 3 respectively.



Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
None provided	1504	1504	99%	0.0	99%	Query_131567

Alignments

Sequence ID: Query_131567 Length: 1083 Number of Matches: 1
Range 1: 61 to 885

Score	Expect	Identities	Gaps	Strand	Frame
1504 bits(814)	0.0()	824/828(99%)	3/828(0%)	Plus/Plus	

Features:

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Query  4    GCCTTTCCGGCGATAGCCCTGGATACTGAATTTCGTCTCTGTACGCGCACTTATTACCCG  63
Sbjct  61   GCCTTTCCGGCGATAGCCCTGGATACTGAATTT-GT-TC-GTACGCGCACTTATTACCCG  117
Query  64   CAGCTGGGGTTGATTCAACTTTTCGATGGCGAGCATCTGGCGCTAATCGATCCACTCGGG  123
Sbjct  118  CAGCTGGGGTTGATTCAACTTTTCGATGGCGAGCATCTGGCGCTAATCGATCCACTCGGG  177
Query  124  ATCACCGACTGGTCACCGCTGAAAGCGATCCTGCGCGATCCGTCCATCACAAAATTTCTC  183
Sbjct  178  ATCACCGACTGGTCACCGCTGAAAGCGATCCTGCGCGATCCGTCCATCACAAAATTTCTC  237
Query  184  CATGCAGGCAGTGAAGATCTGGAAGTGTTCCTCAATGTCTTTGGCGAATTACCACAACCC  243
Sbjct  238  CATGCAGGCAGTGAAGATCTGGAAGTGTTCCTCAATGTCTTTGGCGAATTACCACAACCC  297
Query  244  TTGATTGACACGCAAATCCTTGCTGCCTTCTGCGGACGCCCGATGTCATGGGGTTTCGCT  303
Sbjct  298  TTGATTGACACGCAAATCCTTGCTGCCTTCTGCGGACGCCCGATGTCATGGGGTTTCGCT  357
Query  304  TCCATGGTGGAAGAGTATTCCGGCGTTACGCTGGACAAGAGTGAATCGCGCACCGACTGG  363
Sbjct  358  TCCATGGTGGAAGAGTATTCCGGCGTTACGCTGGACAAGAGTGAATCGCGCACCGACTGG  417
Query  364  CTGGCCAGACCGCTGACCGAACGTCAGTGTGAATACGCAGCGGCCGGATGTCTGGTATCTG  423
Sbjct  418  CTGGCCAGACCGCTGACCGAACGTCAGTGTGAATACGCAGCGGCCGGATGTCTGGTATCTG  477
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Sbjct  478  TTACCGATCACCGCCAAGCTTATGGTAGAAACGGAGGCCTCCGGCTGGCTACCTGCGGCG  537
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Sbjct  838  GAAAAAGCGCAGACATTGCCGGAAGATGCCTTACCGCAGCCGATGCTT  885
```

Figure 15: Clone confirmed by sequencing report (Cromas Biotech). Photo showing sequence alignment using NCBI BLAST.

5.6 Overexpression:

Figure 16: Transformed *rnd* cloned gene in Bl21 (DE3) pLysS was successful for overexpression.

5.7 SDS-PAGE gel of RNase D:

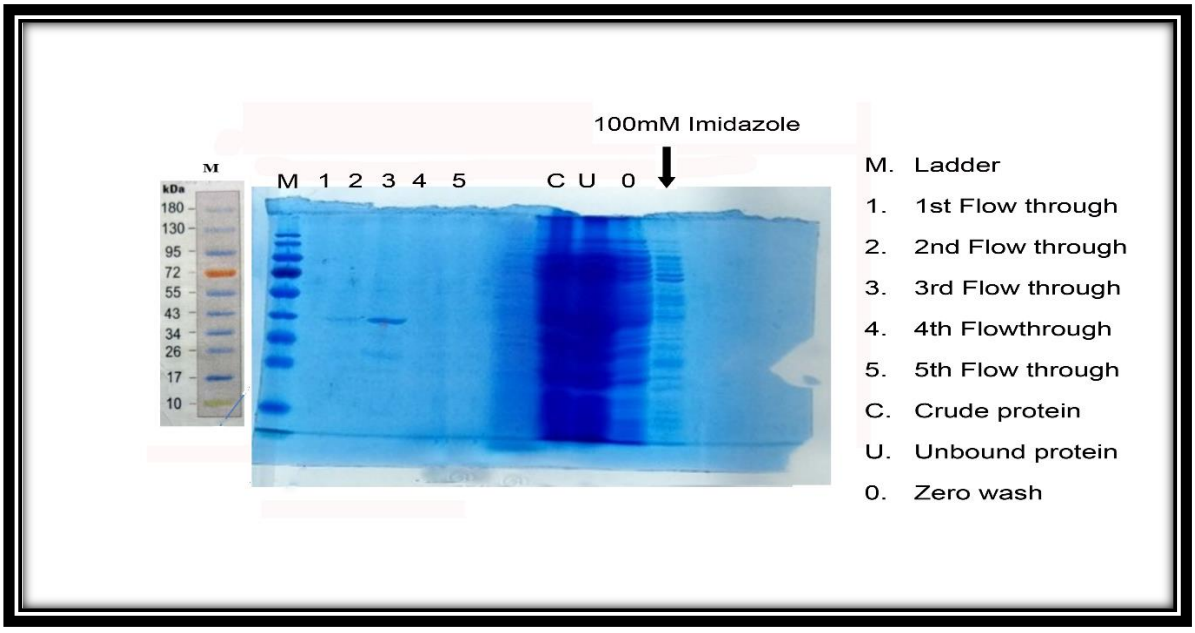

Figure17: Picture showing the protein purified band of 43kDa. The purified protein obtained through different concentration of imidazole (10mM in 0 wash, gradient of 100mM and finally by 500mM imidazole).

Discussion:

The RNase D gene of *E.coli* was amplified by PCR from MG1655 WT and was cloned into the vector of pET 22b. It was cloned between the *BamHI* and *XhoI* sites in pET 22b vector. The clone was confirmed by DNA sequencing report. The rnd cloned gene was then transformed in BL21 (DE3) pLysS for overexpression and it was successful. The RNase D protein obtained have a molecular weight of 43kDa. RNase D was overexpressed by addition of 1mM IPTG to 1L of cells which were grown in Luria Broth (Hi-media) to an absorbance of A_{600} of 0.6 at 37ºC.Cells were recovered by centrifugation and washing with 0.9% NaCl was done and the cell pellet was frozen at -80ºC. For purification of RNase D, frozen cells were resuspended in lysis buffer (20mM Tris-Cl [pH 7.5], 10mM KCl, 10mM imidazole, 10% glycerol) with 1mM protease inhibitor PMSF (phenylmethylsulfonyl fluoride). The cells were lysed by adding lysozyme and sonicated from 30seconds pulse for 5 times. The lysate obtained was cleared by centrifugation and RNase D was chromatographically purified by using the procedure as mentioned in the methodology part. After centrifugation the supernatant was applied to Ni-NTA resin column (Bio-Rad). RNase D was passed out with three different concentration of imidazole (10mM in 0 wash, gradient of 100mM and finally by 500mM imidazole). All steps of centrifugation and chromatography were carried out at 4ºC. However, Zuo *et al.*, 2005 reported that the RNase D gene of *E. coli* was amplified by PCR from pDB14 plasmid and was cloned into vector of pET15b. It was cloned between the *NcoI* and *BamHI* sites in pDB14.For overexpression the cloned *rnd* gene was then transformed into *E. coli* Rossetta (DE3) pLysS (Novagen Inc.) strain. The RNase D protein obtained have a molar mass of 42.7kDa. RNase D was overexpressed by addition of 1mM IPTG to 2 litre of cells grown in Luria Broth to an A_{600} of ~1.0 at 37ºC. The cells were harvested by centrifugation and resuspended in buffer (20mM Tris-Cl [pH-7.5], 10% glycerol, 1mM DDT) and was frozen using liquid nitrogen and stored at -80ºC. For purification of RNase D, the frozen cells were thawed and lysed by French Press at 12,000 psi in the presence of protease inhibitors (0.1mM PMSF and one tablet of Complete Mini Protease Inhibitor Cocktail) and 10µg/ml of DNase I. The lysate obtained was cleared by centrifugation and the supernatant was applied to an Affi-Gel Blue Column in Buffer A and passed out with increasing amounts of NaCl. RNase D was extracted with a linear concentration of potassium phosphate (8-400mM). RNase D passes out at about 250mM NaCl. RNase D

monomer with an apparent size of 45kDa was obtained as compatible with the prior findings [43].

6.0 Conclusion:

Escherichia coli contains RNase PH, RNase T and RNase II as primary ribonucleases and RNase D as a back-up enzyme. RNase D has no effect after knocking out on physiology of *E. coli* cells, but cells still maintain its protein level. It is considered that when the primary ribonucleases are knocked out of the cells, RNase D then displays a vital role in cell physiology. The primary role of RNase D as a secondary enzyme in *E. coli* cells is yet to be discovered. The aim of my study was to clone, overexpress and purify RNase D in *E. coli* so that further biochemical characterization can give some idea about the primary role of RNase D protein.

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