INDIAN JOURNAL OF SCIENCE AND TECHNOLOGY

RESEARCH ARTICLE

Isolation of Restriction Enzyme from Sewage Samples Derived *Bacillus* sp.

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OPEN ACCESS

Received: 23-01-2023
Accepted: 18-06-2023
Published: 13-09-2023

Citation: Lalhriatpuii N, Basaiawmoit VC, Damodaran B, Amarnath KP, Arjunan S, Lalremruati F, Zothanpuia (2023) Isolation of Restriction Enzyme from Sewage Samples Derived *Bacillus* sp.. Indian Journal of Science and Technology 16(SP1): 219-227. https://doi.org/10.17485/IJST/v16sp1.msc29

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Funding: None

Competing Interests: None

Published By Indian Society for Education and Environment (iSee)

ISSN
Print: 0974-6846
Electronic: 0974-5645

Abstract

Objectives: The aim is to identify and characterize the restriction enzyme activity of bacteria from a sewage sample. **Methods:** The sample was plated on Nutrient Agar, Luria Bertani, and Tryptone Soy Broth Agar. The selected bacteria were screened for restriction enzyme activity and mass cultured. Extraction and purification of the enzyme were performed using column chromatography (PC 11 column, HTP column, and HPS column) and dialysis. Bacterial identification was performed using Gram's staining and 16S rRNA gene sequencing. **Findings:** A total of 2138 bacterial colonies were retrieved using Nutrient Agar, Luria Bertani, and Tryptone Soy Broth Agar. From the enzymatic screening, bacterial isolate strain no. 8 was selected, and the extraction and purification of restriction enzymes from the selected bacterial strain were performed using column chromatography. After dialysis, the enzyme showed complete activity in 1:6 dilutions; hence, the unit of the enzyme is 6 units. The isolated bacterial strain was found to be a gram-positive rod-shaped *Bacillus* after Gram's staining was performed. The strain was identified using 16S rRNA gene sequencing technology, and it was found out that it has 97.91% homology with *Bacillus* sp. **Novelty:** The study reported sewage water derived *Bacillus* sp. having restriction enzyme.

Keywords: Bacillus sp; Column chromatography; Restriction enzymes; Sewage

1 Introduction

Bacteria are ubiquitous in nature, found in deep-sea vents, the cold regions of the Arctic, and the digestive tracts of humans [(1)]. They can use any organic or inorganic chemical as a food source. Some bacteria are pathogenic to humans, animals, or plants, but most are harmless and beneficial to higher life forms [(2)]. Other bacteria live as symbionts with plants and invertebrates, aiding in nitrogen fixation and cellulose decomposition.

Bacteria are employed in foods, chemicals, and medications [3]. Bacteria are often unicellular, but they may grow attached to one another in clusters, chains, filaments (hyphae), or mycelium (Actinomycetales; higher bacteria). Their shape may be spherical (Coccus), rod-shaped (Bacillus), comma-shaped (Vibrio), or spiral (Spirillum and Spirochaetes) [4]. Bacterial cell machinery makes them well-equipped to survive in almost any habitat; however, they are usually attacked by a group of viruses called bacteriophages [5].

Bacteriophages are widespread in the environment and are the most numerous biological agents on the planet that differ greatly in size, appearance, and genetic structure [6,7]. They are one of the most common organisms on Earth and their genetic material is either in the form of DNA or RNA, encapsulated by a protein coat. The capsid is attached to a tail that has fibres used for attachments to bacterial cell surface receptors. Most of the phages except filamentous phages have polyhedral capsids. Phages from the collection showed a narrow host range, in most cases affecting different strains of the same bacterial species. Bacteriophages can replicate in two ways once attached to the host: lytically and lysogenically [8,9]. Bacteria can protect themselves against the attack of bacteriophages in various ways. Preventing phage attachment, blocking DNA entry, restriction-modification systems, and abortive infection.

Sewage water harbours many different types of microorganisms, which are often capable of handling the stressful situations present there. Sewage water contains bacteriophages in abundance, which are more resistant than *E. coli* to disinfection, water treatment, and natural inactivation in freshwater and seawater [10], so the bacteria present here should have some sort of defense mechanism for fighting against various viruses or bacteriophages attacking them with the help of restriction endonucleases.

Restriction endonucleases (REases) are enzymes present inside the bacterial cells that help cleave the foreign phage DNA and protect the host organism. REases such as EcoRI are familiar to virtually everyone who has worked with DNA [11]. Over 19000 putative REAses are currently stored in REBASE [12]. REases are classified into four main types: Type I, II, III, and IV. Almost all require a divalent metal cofactor, such as $Mg2^{+}$, for activity [13]. Type II REases represent the largest group of characterized enzymes owing to their usefulness as tools for recombinant DNA technology, and they have been studied extensively [14]. Over 300 Type II REases, with more than 200 different sequence specificities, are commercially available. Far fewer Type I, III, and IV enzymes have been characterized, but putative examples are being identified daily through bioinformatic analysis of sequenced genomes [15].

As mentioned above, bacteria can survive in any kind of environment, but the sewage sample was selected in the present study because bacteriophages that attack bacteria are found abundant, accordingly, it was assumed that the bacteria will be more ready to fight back against the phages and have better-equipped systems to combat these phages. The present investigation was undertaken to isolate the restriction enzymes from bacteria present in the sewage samples that have been recognized as useful tools in molecular biology since the early 1950s, such as for DNA characterization, mapping, and modification [16].

2 Methodology

2.1 Sample collection

The samples were collected during the month of November 2018 from the sewage of Geddalahalli (13.0514° N, 77.6433° E) and Byrathi cross (13.0570° N, 77.6482° E), Bangalore, State of Karnataka, South India. The samples were collected in sterile glass bottles that had been previously autoclaved and transferred to the laboratory. The colour and pH of the sample were noted.

2.2 Isolation of bacteria from sewage sample

Serial dilution of the sewage sample collected was performed and up to 10^{-5} dilutions were plated on 3 media: Nutrient Agar (NA), Luria Bertani (LB) Agar, and Tryptone Soy Broth Agar (TSA), in different concentrations: neat (0.5 ml and 0.25 ml), 10^{-1} (1 ml and 0.5 ml), 10^{-2} (1 ml and 0.5 ml), 10^{-3} (1 ml and 0.5 ml), 10^{-4} (1 ml and 0.5 ml) and 10^{-5} (1 ml and 0.5 ml). After incubation for 24 hours at 37 °C, the colonies formed were counted and noted. All the numbered colonies from NA, LB, and TSA were inoculated again in test tubes containing 5 mL of each broth. The inoculated tubes were kept in a shaker incubator overnight. 500μl was transferred from the overnight broth cultures to new vials (these serve as master cultures). The remaining broth cultures were centrifuged at 10000 rpm at 7 °C for 10 minutes. The pellets were then collected and air-dried. 300μl of sonication buffer was added to each vial and suspended gently. This step was carried out in an ice bath. After this, the vials were placed in the sonicator (Time — 5 minutes, Pulse on — 0.3 Sec, Pulse off — 0.4 Sec, Amplitude — 33%).

2.3 Assay for enzyme activity

Activity assays were carried out following Podgórska *et al.*[17] with few modifications, the sonicated sample (5 μL) served as enzyme and 1 μg λ DNA (GeNei) as a substrate in a 50 μL reaction volume for 1 h at 37 °C. After the period of incubation was completed, gel loading buffer was added in order to stop the reaction. The sample was then loaded into 1% agarose gel and viewed in gel documentation system.

2.4 Mass culture and screening of the isolated strain

The colony that showed activity was then mass cultured. A total of 20μl of the bacterial culture was taken from the master culture and added to 100 ml of Luria Bertani broth and incubated at 37 °C for 16 hours. This served as a seed culture. The enzyme activity was checked again to see whether the particular strain had lost enzyme activity or not. The strain was further mass cultured by inoculating 20 ml of the seed culture into each of four 800 ml conical flasks of Luria Bertani broth and incubating for 16 hours at 37 °C. The culture was then centrifuged at 6000 rpm for 8 minutes at 4 °C. The pellet was saved, and its weight was 6.6 grams. The pellet was gently resuspended by adding 66 ml of sonication buffer in ice cold conditions. After every 12 minutes of sonication, the cell activity was monitored by a spectrophotometer. Following sonication, the sample was centrifuged at 6000 rpm for 8 minutes at 4 °C and the supernatant was collected for column chromatography.

2.5 Protein purification

The supernatant collected was then passed through three columns with some modifications of Bickle *et al.*[18] methods:

Phosphocellulose column: The column was washed with the high-salt buffer, and 66 ml of the sample was loaded into the phosphocellulose (Pc-11) column. The eluate is referred to as a breakthrough. The wash 1 buffer (equilibration buffer) was loaded onto the column to equilibrate the sample, which was followed by the loading of the wash 2 buffer (equilibration buffer) to wash the unbound protein. The column was eluted with elution buffer (high salt buffer and equilibration buffer). 34 fractions were collected. The fractions collected were assayed for enzyme activity and transferred to the next column. The method mentioned above was followed in the next two columns.

***Hydroxyapatite column*:** The peak activity was pooled from pc -11 and transferred into the HTP column. 62 fractions were collected and assayed for enzyme activity.

Heparin sepharose column: The fractions collected from the HTP column were loaded into the HPS column. 53 fractions were collected and assayed for enzyme activity.

***Dialysis*:** Dialysis was performed with some modification of Bickle *et al.*[18] methods, 12 ml fraction from the HPS column (fraction Number 25-36) was collected and 74ul of BSA was added. This sample was stored in the pre-storage buffer for 2 hours followed by storing in the storage buffer for 16 hours. After dialysis, the sample was assayed for the enzyme final unit.

2.6 Bacterial identification by gram staining

Gram's staining was performed using Gram's staining kit for the selected strain, to identify the bacterial colony.

2.7 Bacterial identification by 16S rDNA

***Genomic DNA extraction* :** Genomic DNA was isolated using a genomic DNA extraction kit (cat. no. FC46, Bangalore GeNei, India) and gel electrophoresis was performed.

PCR: The isolated genomic DNA was then amplified using Polymerase Chain Reaction Kadri *et al*[19]. The 50μl PCR mixture volume contains genomic DNA (4μl), forward primer (1μl), reverse primer (1μl), dNTP mix (4μl), 10X assay buffer (5μl), Taq polymerase (1μl) and, distilled water (34 μl). The thermal cycler was set as follows; initial denaturation at 94 °C -98 °C for 5 min followed by denaturation at 94 °C -98 °C for 30 s, annealing at 55 °C -70 °C for 30 s, extension at 68 °C -72 °C for 2 mins and, final extension step at 68 °C — 72 °C for 10 mins. The amplified genomic DNA was run on gel electrophoresis

Recovering DNA from the gel: The gel containing the genomic DNA was extracted using Gel Extraction Kit (Cat No.:6113400011730, Bangalore GeNei, India). The extracted genomic DNA was then sent for sequencing.

3 Results and Discussion

3.1 Sample collection

Sewage samples, sample A and sample B were collected from Gedalahalli and Byrathi Cross, respectively. Sample A is a dark grey, whereas Sample B is a blackish grey. Both the samples had a pungent odour. The pH of sample A was 6.69 and that of sample B was 6.90.

3.2 Isolation of bacteria from sewage sample

Serial dilution was performed to reduce the density of the bacterial cells. This was done to get the maximum number of colonies to be screened for enzyme activity since different bacteria require different media for their growth and these three media are rich media that supports the growth of many different types of bacteria. After incubation, the colonies grew and were counted**.**

3.2.1 ***Number of colonies in different media***

The maximum growth of colonies in both samples A and B was seen in Neat (undiluted sample) and the least growth was seen in 10^{-5} dilutions. This is because, at higher dilutions, the concentration of microorganisms keeps on decreasing. For both sample A and sample B maximum growth was seen in Nutrient Agar followed by Luria Bertani Agar and the minimum growth was seen in Tryptone Soy Broth Agar. When comparing Sample A and Sample B, more colonies was obtained from Sample A, this could be because Sample A was taken from a more polluted source **(Table 1)**

Table 1. Number of colonies observed from samples A & B in different media

Media	Dilutions	No. of Colonies Sample A	No. of Colonies Sample B	Media	No. Of Colonies Sample A	No. of Colonies Sample B	Media	No. of Colonies Sample A	No. of Colonies Sample B
NA	Neat (0.5ml)	218	76	LB Agar	38	84	TSA	93	37
	Neat (0.25ml)	267	51		25	84		93	37
	10^{-1} (1ml)	155	56		69	25		3	23
	10^{-1} (0.5ml)	180	34		61	10		10	20
	10^{-2} (1ml)	37	26		54	8		22	19
	10^{-2} (0.5ml)	28	20		57	6		4	8
	10^{-3} (1ml)	11	15		11	6		5	10
	10^{-3} (0.5ml)	4	10		7	5		2	7
	10^{-4} (1ml)	3	7		1	5		5	18
	10^{-4} (0.5ml)	2	4		1	4		3	1
	10^{-5} (1ml)	6	1		Nil	1		2	10
	10^{-5} (0.5ml)	1	1		2	2		1	5
	Total = 912		**Total 301**		**Total 326**	**Total 223**		**Total 195**	**Total 181**

Colonies selected were then incubated into their respective broth and allowed to grow at 37 °C overnight. The bacterial culture from the broth was then centrifuged to separate the bacterial cells from the broth, after this, the cells were then sonicated. This was done to lyse the bacterial cell membrane in order to extract their enzyme.

3.3 Assay for enzyme activity

The sonicate containing the bacterial enzymes was then incubated with lambda DNA to check the enzyme activity. After incubation, gel electrophoresis was performed and Isolates No 8 from Sample A which was grown in nutrient agar was found to show activity and it was then cultured in a different broth and was again checked for enzyme activity. This was done to check which media will ensure maximum enzyme activity. After restriction digestion, enzyme activity was observed by forming a band when the bacteria were cultured in Luria Bertani broth **(Figure 1).**

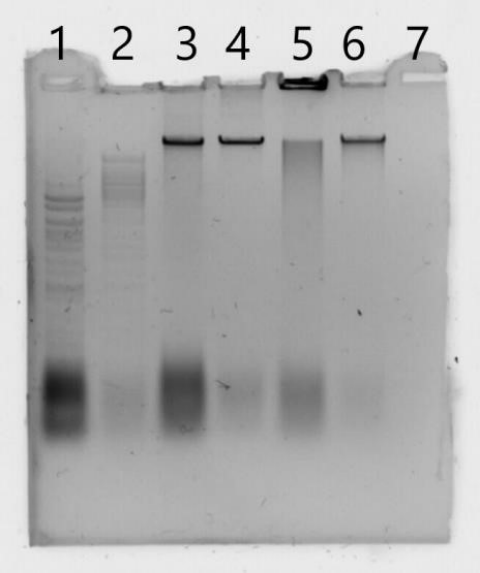

Fig 1. Isolate no. 8 in different media: Lanes 1, 2- LB media, Lanes 3, 4- NB media, Lanes 5, 6- TSB media

3.4 Mass culture and screening of the isolated strain

The resultant strain which showed activity was then mass cultured in Luria Bertani broth. Mass culturing was done to increase the concentration of the enzymes to be extracted. After mass culturing, the bacterial pellet was obtained and it was found to be 6.6g. The pellet was resuspended in a sonication buffer and was sonicated. In order to ensure proper lysis of the bacterial cells, Optical Density (OD) of the sample was taken at an interval of 12 mins using a spectrophotometer and constant OD values indicate the completion of the process. After sonication, it was once again centrifuged using an ultracentrifuge to obtain a clear supernatant free from the bacterial membrane debris. The sonicated pellets were stored **(Figure 2)** and the enzyme activity was again checked after sonicating and it was found that the strain still retains its restriction activity after mass culturing **(Figure 3)**.

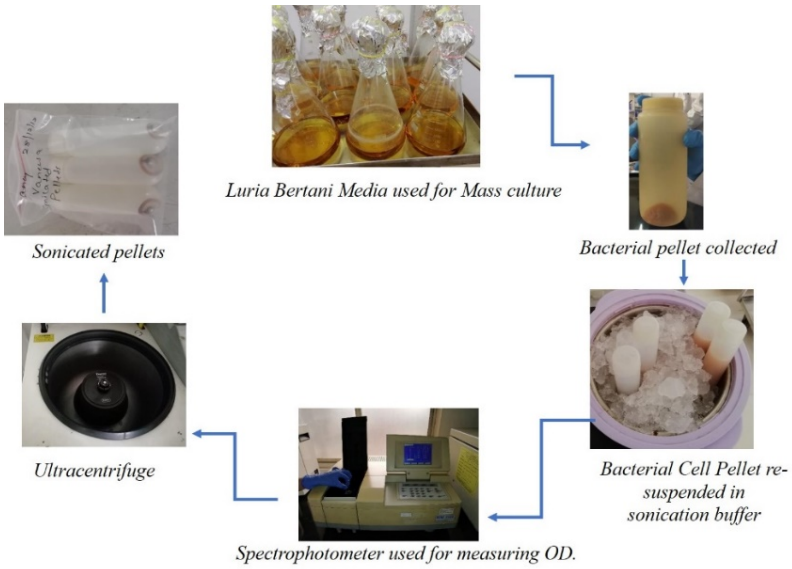

Fig 2. Mass culturing to validate the enzyme activity

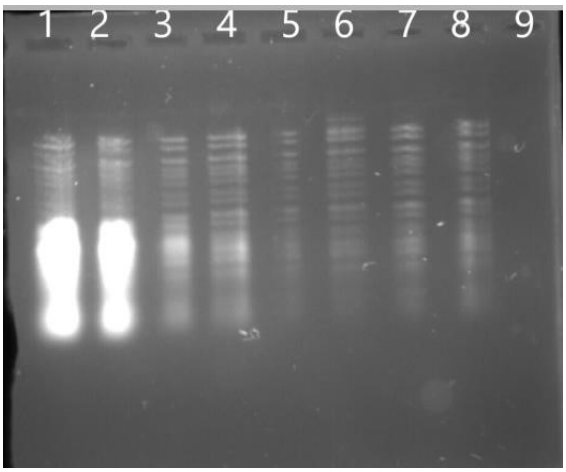

Fig 3. Crude enzyme samples after sonication: Lane 1-5 ul neat, Lane 2- 3 ul neat, Lane 3- 1 ul, Lane 4-1:2 dilution, Lane 5- 1:4 dilution, Lane 6-1:6 dilution, Lane 7- 1:8 dilution, Lane 8- 1:10 dilution

3.5 Protein purification

After sonication is completed, the supernatant is purified using column chromatography. Column chromatography was performed using Pc-11, HTP and HPS columns. These columns have more affinity to restriction endonucleases hence they are used for purification. The samples passed through each of these columns were collected using a fraction collector. Enzyme activity was again checked by incubating the fraction samples collected with lambda DNA after passing through each column. Multistep purification on various matrices is used to achieve high-purity restriction endonuclease which agreed with our result [20].

3.5.1 ***Fractions collected from PC 11:***

Restriction enzyme activity is seen in Lane 3,5,6,7 and 14 which corresponds to fraction number 6,10,12,14 and Breakthrough **(Figure 4)**

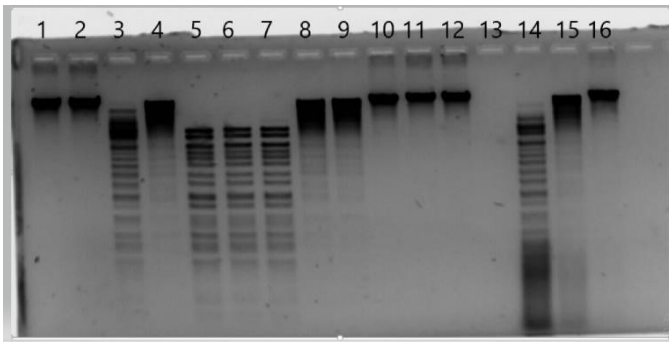

Fig 4. Fractions collected from PC 11: Lanes 1-12 contain fraction samples 2,4,6,8,10,12,14,16,18,20,22,24 and 26 respectively. Lane 14- Breakthrough, Lane 15- Wash 1, Lane 16- Wash 2

3.5.2 ***Fractions collected from HTP and HPS column:***

Restriction enzyme activity is seen in lanes 13,14,15,16,17 and 18 which corresponds to fraction number 25,28,31,34,37,40 from HPS column. Activity is also seen in lanes 24,25,26,27,28,29,30 and 31 which corresponds to 5 ul neat, 3 ul neat, 1ul neat, 1:2, 1:4, 1:6, 1:8 and 1:10 dilutions from HTP column (**Figure 5**).

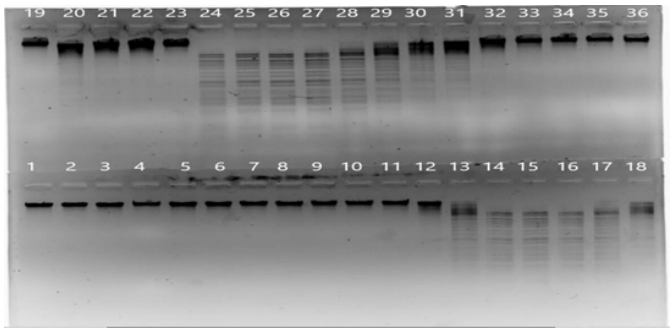

Fig 5. Fractions collected from HTP and HPS column: Lanes 1- 23 are fractions obtained from HPS column. Lane 1- Breakthrough, Lane 2- Wash 1, Lane 3- Wash 2, Lanes 4,19 - control, Lanes 5–23 contain fraction samples from 1,4,7,10,13,16,19,22,25,28,31,34,37,40,43,46,49,52 respectively. Lanes 24 onwards are samples eluted from HTP column; Lane 24- 5 ul neat, Lane25- 3 ul neat, Lane 26- 1 ul neat, Lanes 27-36 contain enzyme units from 1:2 till 1:100 dilutions respectively

3.5.3 ***Final enzyme units after dialysis****:*

Dialysis is usually used to change the salt (small molecule) composition of a macromolecule-containing solution. The solution to be dialyzed is placed in a sealed dialysis membrane and immersed in a selected buffer; small solute molecules then equilibrate between the sample and the dialysate[21]. As Bickle *et al.*[18] suggest, BSA was added to the enzyme to prevent inactivity and stabilize it. The enzyme obtained after dialysis was pure and produced a clear gel pattern **(Figure 6)**. Complete restriction enzyme activity is seen up to lane 6, so the units of the enzyme are 6 units. Moreover, 21,000 units of the enzyme were recovered from 6.6 g of pellets, which is equivalent to 127,272.727 units from 40 g of cells, which is higher than the yield of Taq I endonuclease purified in another study, where 120,000 units were recovered from 40 g of cells[18].

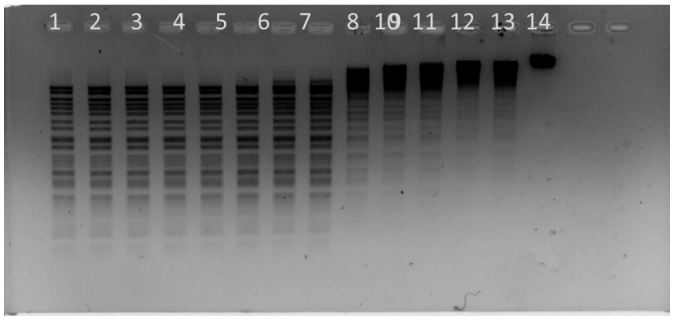

Fig 6. Final enzyme units after dialysis: Lane1- 5 ul neat, Lane 2- 3 ul neat, Lane 3- 1 ul neat, Lanes 4- 13 contains dilutions from1:2,1:4,1:6,1:8,1:10,1:20,1:40,1:60,1:80,1:100. Lane 14-Lambda DNA

3.6 Bacterial identification

After performing Gram staining, the strain was found to be gram-positive rod shape bacteria **[Figure 7(A)].** The bacterial strain was further molecularly identified using 16S rRNA gene sequencing: Genomic DNA was extracted and amplified using PCR and the PCR product was run on a gel. The genomic DNA was then eluted out from the gel and was sent for 16s rDNA sequencing. After 16s rDNA sequencing, it was found that the isolated microorganism has 97.91% homology with *Bacillus oceanisediminis* **[Figure 7 (B)]**.

Restriction enzymes are an integral part of any bacterial cell that protects them from the attack of bacteriophages. Bacteriophages especially the ones thriving in sewage water are very infectious as they are resistant to many chemicals, hence, bacteria present in sewage samples would have a better-equipped system of protecting themselves against these invading phages with the help of restriction-modification systems namely restriction enzymes. In this study, we have isolated a bacterial strain *Bacillus* sp., that shares 97.91 % sequence homology with the microorganism isolated from the deep sea of China[22]. It is a Gram-positive bacterium and it helps in the process of decomposition. After performing restriction digestion with lambda DNA as substrate, it was found that this particular strain shows restriction enzyme activity. The strain was then mass cultured

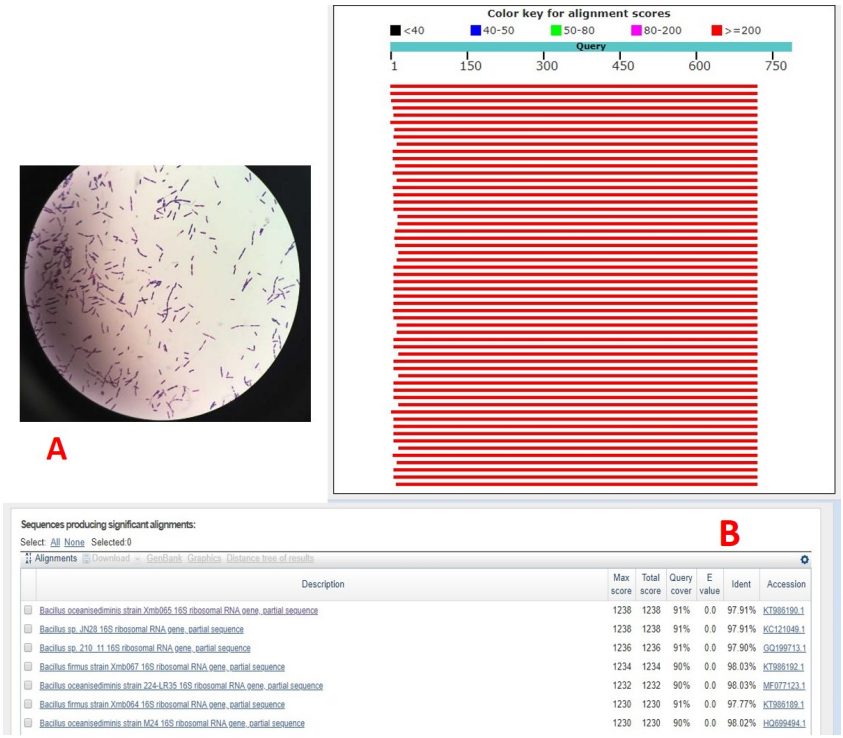

Fig 7. (A) Gram staining showing gram-positive bacilli (B) Nucleotide BLAST results of 16S rRNA gene sequencing

and the enzyme was purified by using column chromatography and the enzyme units were found to be 6 units.

4 Conclusion

This study has given us insight into the prevalence of useful enzymes derived bacteria such as restriction enzymes in sewage samples. Undertaking projects concerning restriction endonucleases has led to various developments in the field of science and technology. These enzymes are also called molecular scissors and are useful in techniques such as recombinant DNA technology. More research can be done to verify the base pair cutting and cofactor requirements, as well as to determine which class this restriction endonuclease belongs to.

5 Declaration

Presented in 4th Mizoram Science Congress (MSC 2022) during 20th & 21st October 2022, organized by Mizoram Science, Technology and Innovation Council (MISTIC), Directorate of Science and Technology (DST) Mizoram, Govt. of Mizoram in collaboration with science NGOs in Mizoram such as Mizo Academy of Sciences (MAS), Mizoram Science Society (MSS), Science Teachers' Association, Mizoram (STAM), Geological Society of Mizoram (GSM), Mizoram Mathematics Society (MMS), Biodiversity and Nature ConservationNetwork (BIOCONE) and Mizoram Information & Technology Society (MITS). The Organizers claim the peer review responsibility.

6 Acknowledgement

The authors are thankful to GeNei laboratory, Bangalore for all the support to carry out the project work.

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