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Isolation and characterization of novel and efficient protease producing bacteria from drinking water resources

S. Jeevan Chandra, A. Shiva Shanker and Pavan Kumar Pindi

ABSTRACT

An attempt has been made to explore the stability of protease enzyme (isolated from *Bacillus* sp.) by statistical method. More than 100 isolates were screened for extracellular protease activity derived from various potable water samples of Mahabubnagar district, Telangana State, India. The activity of protease is found to be varying from sample to sample, the highest being reported by the isolate from water sample of Kalwakurthy mandal, Mahabubnagar district and therefore was selected for further studies. The 16S rRNA (ribosomal ribonucleic acid) gene sequence of the isolate showed closest similarity with *Bacillus* sp. and the sequence was submitted to National Center for Biotechnology Information (NCBI) gene bank (accession number GU566359) and the culture was deposited in three international culture deposition centers (KCTC-13725: MTCC-10465: JCM-16713). The present study revealed that, this *Bacillus* sp. showed a greater amount of protease production with the inherent characters of thermo, alkali and oxidant stability which makes it a potential alternative protease producing strain in various industrial applications.

Key words | *Bacillus* sp., kinetic stability, protease, purification and RS

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INTRODUCTION

Proteases (EC: 3.4. 21-24, 99) are enzymes and about 2% of the entire genes code for proteases in higher organisms (Barrett *et al.* 2001). Since the plant and animal proteases could not meet the ever growing demand, more emphasis is given on microbial proteases globally (Sakinala *et al.* 2016). Bacteria are the promising source of protease enzyme for billion dollars of international trade. Microbial proteases are known for stability at broad range of pH, temperatures, and nature of substrates.

However, wild strains are unable to secrete huge amounts of extracellular enzymes. Several attempts like induced mutations by physical and chemical mutagens render to enhance the capacity. Recently, the statistical approaches gained prominence over a collection of independent variables (Kennedy & Krouse 1999). Industrial production requires less investment and more profit. Therefore, at least a 10–20% reduction in the input is considered to be cost-effective (Alnahdi 2012).

Enzyme purification is a critical task; the steps involved in all types of systems are strictly applied to minimize protein contamination. In light of the above aspects, an effort was made in the present investigation to assess the protease produced by the isolate. In order to specify the kinetic behavior of the protease, purification was done by employing a two-tier approach, ammonium sulfate or acetone solvent precipitation and homogenization by salting out with dialysis followed by polyacrylamide gel electrophoresis (Gupta *et al.* 2005).

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MATERIALS AND METHODS

In the present study, drinking water samples were collected from different primary health centers (rural health care facilities which are run by government) of Mahabubnagar district for the screening of protease producing bacteria. About 1 L of potable water sample was collected in a manner to avoid the contamination of atmospheric microorganisms (Pindi *et al.* 2013a, 2013b, 2014).

Plating and isolation

0.1 mL of sample was layered on skimmed milk agar (SMA: poly peptone 5.0 g/L; yeast extract 5.0 g/L; glucose 10.0 g/L; KH_2PO_4 1.0 g/L; $MgSO_4$ 0.02 g/L; skimmed milk 2.0 g/L; agar 18.0 g/L) using sterile spreader under aseptic condition. The plates were labeled and incubated for 24–72 h at 25–30 °C (Chandra *et al.* 2015a).

Primary screening

The qualitative screening of the alkaline protease producing bacteria was performed by the method suggested by Chandra *et al.* (2015a). The proteolytic potential was calculated by the formula:

$$Hz = Tzd - Cd / Cd$$

where Hz = Hydrolytic zone, Tzd = Total zone diameter, Cd = Colony diameter.

The enzyme producing efficiency of the selected isolates was screened and isolated. The colonies were maintained in SMA at 4 °C, and the efficiency for each isolate was tested at each subculture.

Cultural, biochemical, and molecular characterization

The characterization of the isolate was carried out according to the guidelines of *Bergey's Manual of Determinative Bacteriology* (Holt *et al.* 1994).

Enzyme assessment

Enzyme assay was carried with casein as the substrate by the method described in Chandra *et al.* (2015b).

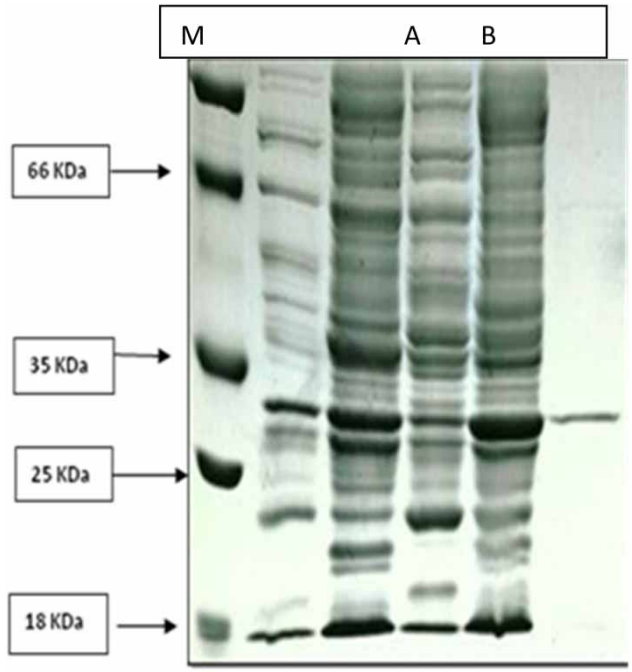

Figure 1 | SDS-polyacrylamide gel electrophoresis of protease *Bacillus* sp. RS; where, lane M, protein markers; lane A, crude enzyme; lane B. Purified enzyme.

Purification of alkaline protease

The proteases were purified by ammonium sulfate or acetone solvent precipitation and homogenization by salting out with dialysis followed by polyacrylamide gel electrophoresis for the determination of their molecular weights (Anbu *et al.* 2013) (Figure 1).

Evaluation of enzyme kinetics

The purified proteases were evaluated for pH, temperature stability, solvent, oxidizing agents, and substrate specificity by varying the one component at a time (Palmer & Bonner 2007).

RESULTS AND DISCUSSION

Screening and characterization of isolates

More than 100 extracellular protease producing isolates were isolated from different potable water samples of Mahabubnagar district, Telangana State. Among them, the isolate RS from Kalwakurthy Civil Hospital drinking water

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sample has exhibited superior extracellular proteolytic activity based on primary screening and was selected for further studies. The 16S rRNA gene sequence results revealed that the isolate RS showed closest similarity with *Bacillus* sp. (Figure 2). The gene sequence was submitted to NCBI Genbank and the accession number was GU566359, the culture was deposited in three international culture deposition centers (KCTC-13725: MTCC-10465: JCM-16713). Biochemical characterization of isolate RS confirmed that the organism is Gram positive, filamentous rod shaped, and motile bacteria. Out of all the *Bacillus* species, the superior protease activity with a greater degree of stability and strength was shown by isolate RS. Further investigations were carried out with RS isolate to secure stronger scientific evidence to support the study.

Design of experiments for enhanced production and bioprocess optimization

Bioprocess optimization by a conventional statistical approach is indispensable and widely applied for optimization of various industrially important microbial products like enzymes and chemicals. In this research few methods like OVAT (one variable at a time) and RSM (response surface methodology) were adopted based on the principle of design of experiment to obtain the optimal product. After OVAT experiment the yield was 470 ± 02 EU/mL and PBD (Plackett-Burman design) followed by RSM protease production yield was 15,000 EU/mL. The resulted OVAT increased to 31.9 fold which is highly significant in regulating the production of protease enzyme.

Based on the principle of design of experiment (DoE), the methodology encompasses use of various types of experimental designs, generation of polynomial equations, and mapping of the response over the experimental domain to determine the optimum product.

In contrast, Haddar *et al.* (2010) reported that the 14 fold increase in protease production by RSM with PBD followed by face centered composite design (FCCD). The other experiments performed by different authors including Sen & Satyanarayana (1993), Tari *et al.* (2006), Anbu *et al.* (2013) and Sakinala *et al.* (2016) also showed similar results of protease enzyme activity.

Enzyme purification of alkaline protease

The crude way purification of RS protein was 69.4 mg/mL, the acetone solvent precipitation yield was 115.4 mg/mL,

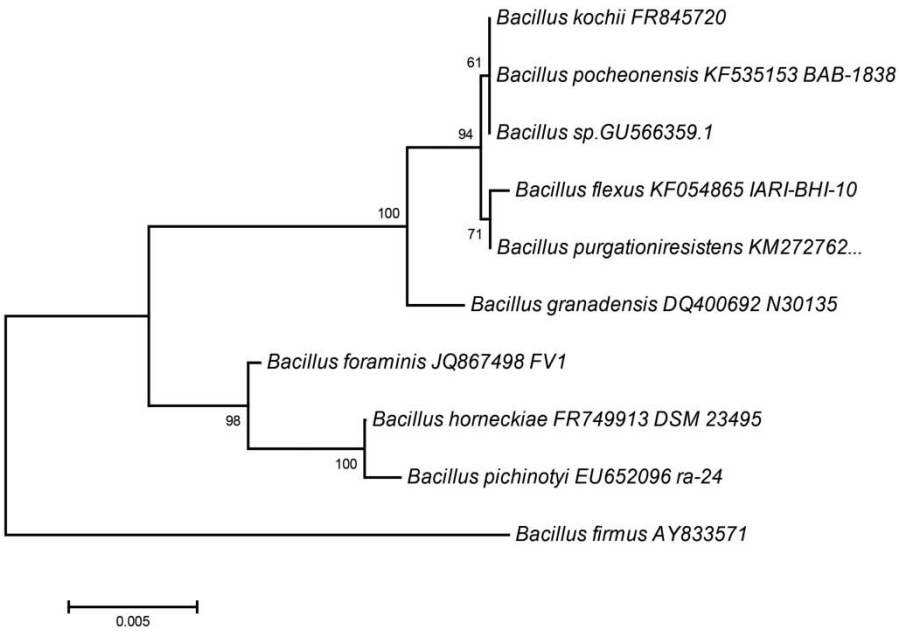

Figure 2 | Phylogenetic analysis shows the isolate RS is *Bacillus* sp. (GU566359).

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the purity was increased to 12.195 U/mg fold, and resulted specific activity per mg of protein concentrate was 63.258 enzyme units. The purity of the enzyme from RS isolate yielded was 20.27% by acetone purification method. Another type of purity method performed was ammonium sulfate precipitation, which showed 124.7 mg/mL of crude total protein with the specific activity per mg of protein concentrate 71.371 enzyme units. The purity of the enzyme with this process of extraction was 13.75 fold and yield percentage was 24.728 which have a more purity when compared to acetone extraction method. Also, the size of the purified enzyme was found to be 28 kDa when compared to the standard marker.

After successful precipitation, salting out was carried out and the amounts were reduced to 10 mL. The dialyzed enzyme was purified with DEAE-C (diethylaminoethyl cellulose column) chromatography.

A critical perusal of Table 1 reveals that, among the different purification strategies employed herein, the crude enzyme of the wild strains exhibited a low amount of protein content in contrast to the acetone and ammonium sulfate precipitation methods. Although the purity was low in DEAE method, interesting fact is that the specific activity of the enzyme was increased to 2,025.6 U/mg of purified protein with 390.51 fold purity and the recovery percentage of yield was 227.88.

Characterization of enzyme

Influence of pH on alkaline protease stability and activity

As in the case of a wild strain of RS, it has shown optimum (100%) of relative activity within the pH 9.0–10.0. In contrast, mutated RS strain has shown more than 80% of specific activity. Interestingly, the stability of the partially purified enzyme was further stably maintained even at an elevated pH 12.0 with 100% of specific activity. These results (Table 2) revealed that the enzymes can withstand the harsh industrial process and perform well.

An alkaline protease from *Bacillus halodurans* with pH optimum of 9.0 was also reported (Dabonné *et al.* 2011). Also, an alkaline stable protease with pH optimum of 9.0 by *B. horikoshi* (Joo & Chang 2005) has been reported. Pandey *et al.* (2012) documented a thermostable alkaline protease from *Nocardiopsis alba* OK-5 with extreme pH tolerance of 10.0–11.0. Salwan & Kasana (2012) have also reported an alkaline protease from *Acenitobacter* sp. MN MTCC (10786) with broad pH range of 7.0 to 11.0.

Influence of temperature on alkaline protease stability and activity

In the present investigation, an attempt has been made to assess the influence of different temperatures. The optimal range was found to be between 40–70 °C. Interestingly, mutant strain of RS has shown more than 70% activity at 30 °C and optimal over a broad temperature range 40–48 °C. Finally, it is concluded that the present enzymes are thermophilic in nature. Hence, they can be used in an industrial process wherein the rise in high temperature is a problem.

The above results are in agreement with Adidi *et al.* (2008) who reported an alkaline protease from *Botrytis cinerea* with an optimum temperature of 50 °C. Dabonné *et al.* (2011) also reported an alkaline protease from *Bacillus halodurans* with pH optima of 9.0 and 65 °C of temperature optima. Genckal & Tari (2005) reported an extreme alkaline serine protease with optimal pH 11.0 and 60 °C of temperature optima by *Bacillus* strain from the highly alkaline environment of Turkey. Chellappan *et al.* (2006) have reported an extreme alkaline protease with broad pH range from 5.0–12.0 and 60 °C temperature optima from *Engyodontium album* BTMFS10. Pandey *et al.* (2012)

Table 1 | Purification regimes and specifications of alkaline protease

Isolate	Type of purification	Total protein mg/mL	Total activity U/mL	Specific activity U/mg	Purification fold U/mg	Yield %
RS	Crude	69.4	360	5.187	1	100
	Acetone	115.4	7,300	63.258	12.195	20.27
	NH_4SO_4	124.7	8,900	71.371	13.75	24.72
	DEAE	40.5	82,037	2,025.6	390.51	227.88

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Table 2 | Performance of the purified protease in different parameters

pH	Relative activity	Temperature	Relative activity	Metal ions	Relative activity	Solvents	Relative activity	Oxidizing agents	Relative activity	Substrates	Relative activity
Control	100	Control	100	Control	100	Control	100	Control	100	Control	100
7	49.03 ± 03	10	10.38 ± 01	KCl	88 ± 02	Methanol	80 ± 03	H_2O_2	105 ± 03	Gelatin	80 ± 03
8	63.45 ± 03	15	47.43 ± 02	$CaCl_2$	80 ± 02	Ethanol	80 ± 03	SDS	80 ± 03	Casein	100 ± 03
9	100 ± 02	20	82.82 ± 02	NaCl	85 ± 02	Propanol	80 ± 03	Tween 20	120 ± 03	Hemoglobin	80 ± 03
10	100 ± 02	25	86.36 ± 03	$FeCl_3$	78 ± 02	Butanol	90 ± 03	Tween 80	117 ± 03	BSA	90 ± 03
11	100 ± 02	30	100 ± 03	$CoCl_2$	75 ± 02	Acetone	95 ± 03	Triton X-100	80 ± 03	Soya protein	95 ± 03
12	63.45 ± 03	35	100 ± 03	$MgCl_2$	130 ± 02	Chloroform	130 ± 03	**Reducing agents**		Gluten	130 ± 03
–	–	40	100 ± 03	$MnCl_2$	78 ± 02	Hexane	82.5 ± 03	Control	100 ± 03	Egg albumin	82.5 ± 03
–	–	45	100 ± 03	$ZnCl_2$	80 ± 02	Diethyl ether	68.8 ± 03	Mercaptoethanol	52 ± 03	–	–
–	–	50	100 ± 03	–	–	–	–	Sodium thioglycolate	130 ± 03	–	–
–	–	55	100 ± 03	–	–	–	–	Inhibitors		–	–
–	–	60	100 ± 03	–	–	–	–	EDTA	68 ± 03	–	–
–	–	65	47.43 ± 03	–	–	–	–	PMSF	00	–	–
–	–	70	17.95 ± 03	–	–	–	–			–	–
–	–	75	17.95 ± 03	–	–	–	–			–	–
–	–	85	10.38 ± 03	–	–	–	–			–	–

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documented a thermostable alkaline protease from *Nocardiopsis alba* OK-5 with extreme pH tolerance from 10.0–11.0 and 60–80 °C of temperature tolerance. Salwan & Kasana (2012) have also reported an alkaline protease from *Acinetobacter* sp. MN MTCC (10786) with broad pH range of 7.0–11.0 with 40 °C of temperature optima.

Effect of various solvents and metal ions on alkaline protease stability and activity

The solvent-stability of proteases were assessed and presented in Table 2. It is evident from Table 2 that the proteases have shown more than 70% of the activity with the solvents tested. However, the purified proteases of all strains were found to be stable in the chloroform solvents with more than optimal (100%) activity. Thus, it is apparent from the Table 2 that the chloroform followed by acetone and butanol are the good solvent systems. Further, these solvents are excellent at 20% V/V ratio for the enzyme activity.

The protease of RS wild strain has shown more than 90% of the activity with the metal ions. However, $CaCl_2$, $MgCl_2$, and $MnCl_2$ have enhanced the enzyme activity. Both $CaCl_2$, and $MgCl_2$ at 0.5 and 0.4 mM, respectively, were enough to enhance 130% of specific relative activity. Interestingly, the protease of RS mutant strain has shown more than 90% of the activity with the metal ions. $CaCl_2$, $MgCl_2$, and $MnCl_2$ have enhanced the enzyme activity. The $CaCl_2$ at 0.5 mM was enough to 142.82% of specific relative activity.

The findings of present investigations with regard to the influence of metal ions and solvents are in accordance with many reports (Usami *et al.* 2005; Akolkar *et al.* 2008; Habib *et al.* 2011).

Effect of various oxidizing, reducing, surfactants and inhibitors on alkaline protease activity and stability

Of the two oxidizing agents tested, H_2O_2 at 0.5 mM concentration has enhanced the proteases with more than 100% of relative activity. Moreover, SDS showed an inhibitory effect with less than 85% of specific relative activity. It is evident from Table 2 that the surfactants also greatly enhanced the enzymatic activity to over the 100% specific relative activity in all the cases except Triton X-100, which was inhibitory and resulted in less than 85% specific residual enzymatic action. It is noticeable that the reducing agents also greatly influenced the enzymatic catalysis over 100% specific residual activity in all the cases except mercaptoethanol, which is strongly inhibitory in the action and resulted in 52%, even zero of the specific residual enzymatic action. In all the cases, ethylene diamine tetra acetic acid (EDTA) significantly increased the enzymatic catalysis except phenyl methyl sulfonyl fluoride (PMSF), this is strongly inhibitory in action and resulted in zero of the specific relative activity. Hence, it can be concluded that all the above enzymes belong to serine proteases.

Thus, the observations on the characterization of alkaline protease are in accordance with the reports of the work done by Akolkar *et al.* (2008) and Habib *et al.* (2011).

Substrate specificity of the partially purified enzyme

The enzyme activity of the purified protease was tested with different substrates, including cytochrome C, hemoglobin, casein, egg albumin, bovine serum albumin, gelatin, and soybean protein, and the results are presented in Table 2. Among all, cytochrome C was found to be the preferred substrate with high activity (110%), followed by casein (100%), hemoglobin (75%) gelatin (70%), wheat gluten (50%), egg albumin and bovine serum albumin (50%) and finally soybean meal (48%). Similar results were also made (Adinarayana *et al.* 2003; Habib *et al.* 2011).

Enzyme kinetics

The rate of the reaction was tested by incubating the reaction mixture (1:1 ratio of [S]:[E] W/W) at varying different parameters.

Influence of substrate concentration rate of reaction

Critical analysis of the enzyme showed a steady steep; after attaining the optimum range it showed a steady state, further increase in the substrate and steadiness in the activity was observed.

Michaelis–Menten behavior of the enzymes has been studied. Further analysis of the nature of the kinetic data revealed that as the K_m increased, an increase in the V_{max}

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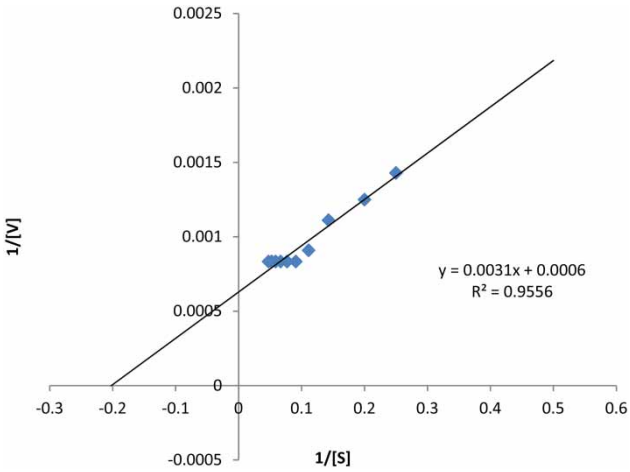

Figure 3 | L.B plot kinetics of the purified protease of Rs *Bacillus* sp. (GU566359).

has been observed at RS required 11–19 mg [s] of initial [s] concentration, on solving the K_m it would be 5.1 mg/S for RS. The quadratic fit of the kinetic data also support the hypothesis and the estimated correlation coefficient R^2 was found to be more than 95.56% for the alkaline protease. Hence, the data are validated. The data presented in Figure 3 confirm the LB plot of enzyme kinetics. Critical analysis of Figure 3 reveals that the alkaline proteases of RS strains have K_m 5.1 mg/S for RS.

CONCLUSION

The challenges which demand attention include loss of enzyme activity over a period of time due to the harsh conditions during industrial processes. The results confirmed the importance of optimization of the production parameters to achieve maximum yield during production of industrially important enzymes. The current four-way optimization resulted in the design of an economical medium with less investment and more yield. The purification by ammonium sulfate yields to a good amount of active protein. The kinetic properties of the enzyme of the isolate RS have gained prominence over other reported proteases by exhibiting thermo, alkali, and oxidant stability. Further screening for agro-based cheap materials and validation of the industrial process is sought. In view of the above-made unusual findings and properties exhibited by the current enzyme, more focus on experiments for industrial applications is in progress.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors declare that they have no conflicts of interest.

Ethical approval: This article does not contain any studies concerning human participants or animals by any of the authors.

REFERENCES

Abidi, F., Limam, F. & Nejib, M. M. 2008 Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: assay as biodetergent. *Proc. Biochem.* **43** (11), 1202–1208.

Adinarayana, K., Ellaiah, P. & Prasad, D. S. 2003 Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *AAPS Pharm. Sci. Tech.* **4** (4), 440–448.

Akolkar, A. V., Deshpande, G. M., Raval, K. N., Durai, D., Nerurkar, A. S. & Desai, A. J. 2008 Organic solvent tolerance of *Halobacterium* sp. SP1(1) and its extracellular protease. *J. Basic. Microbiol.* **48** (5), 421–425.

Alnahdi, H. S. 2012 Isolation and screening of extracellular proteases produced by new isolated *Bacillus* sp. *J. App. Pharm. Sci.* **2** (9), 071–074.

Anbu, P., Annadurai, G. & Hur, B. 2013 Production of alkaline protease from a newly isolated *Exiguobacterium profundum* BK-P23 evaluated using the response surface methodology. *Biologia* **68** (2), 186–193.

Barrett, A. J., Rawlings, N. D. & O'Brien, E. A. 2001 The MEROPS database as a protease information system. *J. Struc. Biol.* **134** (2–3), 95–102.

Chandra, S. J., Pallavi, P., Anuradha, B. S. & Reddy, S. R. 2015a Optimization of bioprocess for enhanced production of alkaline protease by a *Bacillus subtilis* SHmIIIa through Plackett-Burman design. *African J. Microbiol. Res.* **9** (28), 1738–1744.

Chandra, S. J., Pallavi, P., Reddy, K. & Reddy, R. 2015b Alkaline protease bioprocess optimization through response surface

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methodology for alkaliphilic *Bacillus subtilis* SHmIIIa mutant strain from Warangal-Telangana. *American J. Curr. Microbiol.* **3** (1), 35–45.

Chellappan, S., Jasmin, C., Basheer, S. M., Elyas, K. K., Bhat, S. G. & Chandrasekaran, M. 2006 Production, purification and partial characterization of a novel protease from marine *Engyodontium album* BTMFS10 under solid state fermentation. *Proc. Biochem.* **41** (4), 956–961.

Dabonné, S., Ahi, A. P., Gonnety, J. T. & Kouamé, L. P. 2011 A soluble aggregated thermophile metallo amino peptidase produced by an alcalophile strain of *Bacillus halodurans*. *Curr Res. J. Biological Sci.* **3** (1), 5–30.

Genckal, H. D. & Tari, C. 2005 Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitat. *Enzyme. Microb. Technol.* **39**, 703–710.

Gupta, A., Roy, I., Patel, R. K., Singh, S. P., Khare, S. K. & Gupta, M. N. 2005 One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. *J. Chromatogr. A* **1075** (1–2), 103–108.

Habib, S. M. A., Fakhruddin, A. N. M., Begum, S. & Ahmed, M. M. 2011 Production and characterization of thermo-alkaline extracellular protease from *Halobacterium* sp. AF1. *Asian J. Biotechnol.* **3** (4), 345–356.

Haddar, A., Fakhfakh-Zouari, N., Hmidet, N., Frikha, F., Nasri, M. & Kamoun, A. S. 2010 Low-cost fermentation medium for alkaline protease production by *Bacillus mojavensis* A21 using hulled grain of wheat and sardinella peptone. *J. Biosci. Bioengg.* **110** (3), 288–294.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. 1994 *Bergey's Manual of Determinative Microbiology*. Williams and Wilkins, MD, USA.

Joo, H. S. & Chang, C. S. 2005 Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. *Process Biochem.* **40** (3–4), 1263–1270.

Kennedy, M. & Krouse, D. 1999 Strategies for improving fermentation medium performance: a review. *J. Indus. Microbiol. Biotechnol.* **23** (6), 456–475.

Palmer, T. & Bonner, P. L. 2007 *Enzymes: Biochemistry, Biotechnology, Clinical Chemistry*, 2nd edition.

Pandey, S., Rakholiya, K. D., Raval, V. H. & Singh, S. P. 2012 Catalysis and stability of an alkaline protease from a haloalkaliphilic bacterium under non-aqueous conditions as a function of pH, salt and temperature. *J. Biosci. and Bioeng.* **114** (3), 251–256.

Pindi, P. K., Raghuveer, Y. P. & Kodaparthy, A. 2013a Bacteriological and physico-chemical quality of main drinking water sources. *Pol. J. Environ. Stud.* **22** (3), 825–830.

Pindi, P. K., Raghuveer, Y. P. & Shiva, S. A. 2013b Identification of opportunistic pathogenic bacteria in drinking water samples of different rural health centers and their clinical impacts on humans. *BioMed Res. Int.* 1–10.

Pindi, P. K., Raja Srinath, R. & Pavankumar, T. L. 2014 Isolation and characterization of novel lipase gene *LipHim1* from the DNA isolated from soil samples. *J. Microbiol.* **52** (5), 384–388.

Sakinala, J. C., Anuradha, B. S., Reddy, K. & Reddy, S. R. 2016 Screening and optimization of physical parameters for enhanced alkaline protease production by alkaliphilic *Bacillus subtilis* SH2 isolate. *American J. Curr. Microbiol.* **4** (1), 55–65.

Salwan, R. & Kasana, R. C. 2012 Purification and characterization of an extracellular low temperature-active and alkaline stable peptidase from psychrotrophic *Acinetobacter* sp. MN 12 MTCC (10786). *Indian J. Microbiol.* **53** (1), 63–69.

Sen, S. & Satyanarayana, T. 1993 Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. *Indian J Microbiol* **33**, 43–47.

Tari, C., Genckal, H. & Tokatlı, F. 2006 Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. *Process Biochem.* **41** (3), 659–665.

Usami, R., Fukushima, T., Mizuki, T., Yoshida, Y., Inoue, A. & Horikoshi, K. 2005 Organic solvent tolerance of halophilic archaea, *Haloarcula* strains: effects of NaCl concentration on the tolerance and polar lipid composition. *J. Biosci. Bioengg.* **99** (2), 169–174.

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Research Paper

Exploration of coliform diversity in drinking water resources by culture-independent approaches

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ABSTRACT

The coliform group has been widely used as an indicator of water quality and has historically led to a public health protection concept. Presence of pathogens in drinking water may raise several health problems in humans from mild illnesses to serious waterborne diseases. In spite of several measures taken, water quality is always a pertinent issue prevailing in diverse water systems. So far, coliform contamination and diversity could not be adequately explored as traditionally used culture-dependent methods have a limited capacity to characterize microbiota from their respective sources. The study was designed for assessment of microbial diversity by culture-independent approaches placing emphasis on exploring the total coliform diversity in two drinking water reservoirs, Raman Pahad and Koilsagar of Mahabubnagar district, Telangana, India. Principal analysis based on 16S rRNA gene clone libraries revealed that Raman Pahad library clones belonged to genus *Enterobacter* (41.5%), followed by *Citrobacter* (25.03%), *Klebsiella* (17.86%), *Escherichia* (12.20%), and the least being *Hafnia* (3.39%). The clones in Koilsagar belonged to genus *Enterobacter* (46.42%) as the most predominant, followed by *Citrobacter* (32.14%) and *Escherichia* (21.42%). Comparatively, *Enterobacter* was observed to be the most predominant (representing 50%) of the total clones in both reservoirs.

Key words | 16S rRNA, coliform, contamination, drinking water, public health, reservoir

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HIGHLIGHTS

- The study was designed for assessment of microbial diversity by culture-independent approaches laying emphasis on exploring the total coliform diversity in two drinking water reservoirs, Raman Pahad and Koilsagar of Mahabubnagar district, Telangana, India.
- Principal analysis based on 16S rRNA gene clone libraries revealed that, Raman Pahad library clones belonged to genus Enterobacter (41.5%), followed by Citrobacter (25.03%), Klebsiella (17.86%), Escherichia (12.20%) and the least being Hafnia (3.39%); whereas the clones in Koilsagar belonged to genus Enterobacter (46.42%) as the most predominant followed by Citrobacter (32.14%) and Escherichia (21.42%).
- Comparatively, Enterobacter was observed to be the most predominant (representing 10%) of the total clones in both reservoirs.

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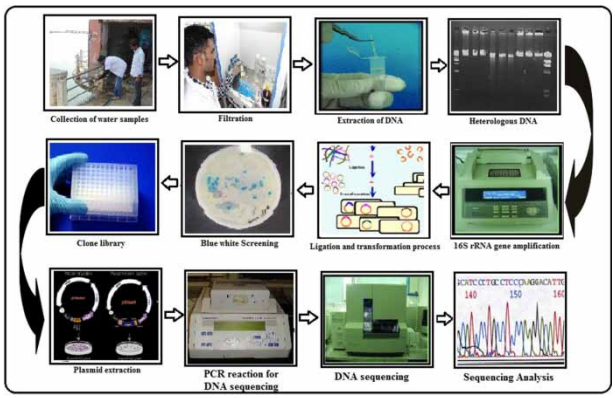
GRAPHICAL ABSTRACT



INTRODUCTION

Water is a pertinent component of life and its main sources include groundwater, surface and rain water. Potable water is defined as having a satisfactory quality in terms of its physical, chemical, and bacteriological parameters so that it can be securely used for drinking and cooking (Jain *et al.* 2010). It is reported that 91% of the global population uses improved water sources which is an increase from 76% in 1990 (Dos Santos *et al.* 2017). The most common and widespread health risks associated with drinking water in developing countries are due to the presence of various microbes (Shakoor *et al.* 2018), and approximately 663 million people continue to lack access to improved drinking water sources (WHO/UNICEF 2015). In 2011, the WHO reported that few opportunistic bacteria along with enteric pathogens persevere in the environment, the presence of which could be unsafe to the elderly, infants, and immunosuppressed individuals. The WHO/UNICEF Joint Monitoring Programme (JMP) for Water Supply, Sanitation and Hygiene presents updated national, regional, and global estimates for water, sanitation, and hygiene (WASH) in households in its 2019 update report, stating that the population using safely managed drinking water services has increased from 61 to 71%. Safely managed sanitation services have increased from 28 to 45 and 60% of the global population has basic hand washing facilities with soap and water at home.

Acute microbial diarrheal diseases are a major public health problem in developing countries as these countries have the lowest financial resources and poorest hygiene facilities. Among all age groups, children under the age of five are the most affected by microbial diseases transmitted through water (Seas *et al.* 2000; Vetrimurugan *et al.* 2013). Some of the diseases that are transmitted through contaminated drinking water are typhoid, cholera, infectious hepatitis, and disease caused by *Shigella* spp and *Escherichia coli* O157 (Lenart-Boroń *et al.* 2017).

Given that abundant pathogens occur in feces, water is therefore monitored for microbial contamination using indicator organisms such as total coliforms and *E. coli* (Stelma & Wymer 2012). Total coliforms are frequently encountered in the aquatic environment, in soil, vegetation, and universally in huge numbers in the feces of warm-blooded animals (Cabral 2010). An important subgroup of this collection is the fecal coliform bacteria, the main member being *E. coli*. These organisms differ from the total coliform group by their ability to grow at higher temperatures. Insufficient treatment of surface waters, defective water distribution pipelines of the drinking water supply, and faulty sewage collection systems have led to contamination of potable water by enterohemorrhagic *E. coli* (EHEC) and other pathogenic bacteria (Ram *et al.* 2008, 2011). *E. coli*, thermotolerant coliforms, and/or intestinal

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enterococci are considered as main fecal indicator microbes for testing the quality of water (Van der Wielen & Medema 2010).

Bacterial diversity surveys of natural waters are significant approaches currently employed to assess the relation between various enteric bacteria, to support management policies or to sustain risk assessment studies (Molina *et al.* 2015). Culture-dependent methods have been constantly used to optimize, identify, and quantify the presence of organisms relevant in terms of quality control and public health (Leclerc & Moreau 2002; Mossel & Struijk 2004). These methods reveal the enormous diversity of uncultured organisms, and thus, highlight the call for alternative approaches for the analysis of water bacterial diversity (Amann *et al.* 1995; Alain & Querellou 2009). A number of scientific and technological developments, such as molecular fingerprinting (Dewettinck *et al.* 2001), metagenomic and gene library (Cottrell *et al.* 2005), PCR-DGGE (Wu *et al.* 2006) and FISH (Bottari *et al.* 2006), but above all, the inexpensiveness of the nucleic acid sequencing methods, have brought noticeable improvements to bacterial diversity studies. 16S rRNA gene clone libraries, fluorescence *in situ* hybridization (FISH) or denaturing gradient gel electrophoresis (DGGE) are some of the methods used nowadays to explore the bacterial diversity in waters (Lyautey *et al.* 2005; Piterina & Pembroke 2013; Shanker *et al.* 2019). More recently, the potential of the high-throughput 454 pyrosequencing to explore the environmental diversity has been emphasized (Bae *et al.* 2019). The use of culture-independent approaches to complement culture-dependent methods is much preferred for inferring the significance of a specific taxonomic group in the entire community (Shivaji *et al.* 2011).

In view of the above-mentioned facts and witnessing the routine water-borne outbreaks in and around Mahabubnagar district, an attempt was made to decipher the actual problem for several diarrheal outbreaks with the most reliable and advanced molecular methods. Uthappa *et al.* (2015) reported that several diarrheal outbreaks observed on a frequent basis may be attributed to the poor quality of potable water supplied mainly from the reservoirs in the Mahabubnagar district. Therefore, the work reported herein was designed to assess the total coliforms and bacterial diversity of two water reservoirs of Mahabubnagar, Telangana, India by 16sRNA sequencing.

MATERIALS AND METHODS

This study was carried out from March, 2017 to February, 2018 in the combined/erstwhile Mahabubnagar district of Telangana State, India. Sampling locations selected for the above study were Koilsagar village of Deverakadra Mandal in Mahabubnagar district and Kothakota mandal of Wanaparthy district. Both the sampling sources were water reservoirs, i.e., Raman Pahad balancing reservoir (coordinates: 16°22′04″N latitude 77°52′20″E longitude) shown in Figure 1(a) and Koil Sagar reservoir (coordinates: 16°44′ N latitude and 77°45′E longitudes) shown in Figure 1(b).

Sample collection

The drinking water samples were collected at four points, namely, north, east, west and south of each reservoir in sterile bottles at a depth of about 30 cm with the bottle's mouth facing the current and ensuring that the water entering the bottle had no contact with the hand and air. The utmost care was taken to avoid contamination of water with atmospheric bacteria. The sample bottles were capped and labeled appropriately with all necessary details such as source, time, and date of collection. Samples were stored at 4 °C and shipped to the laboratory. All the samples were initially passed through membrane filters of 0.45 μm in order to trap bacteria and were assessed within 6 hours of collection for their bacteriological quality using molecular studies.

Determination of bacterial diversity by culture-independent method

Extraction of total DNA from water and PCR amplification of the 16S rRNA gene

Total DNA was isolated from the drinking samples essentially according to the methods described earlier. Primers 1492r primer (5′-TACCTTGTTACGACTT) and 27f (50-GGC GGTGTG TAC AAG GCC C-30) were used to amplify 1.5 kb 16S rRNA gene. Amplification was done following a method reported by Shivaji *et al.* (2009). The polymerase chain reaction (PCR) amplicon was electrophoresed on a 1.0% agarose gel and visualized following staining with ethidium bromide (0.3 μg/mL). The PCR product was purified

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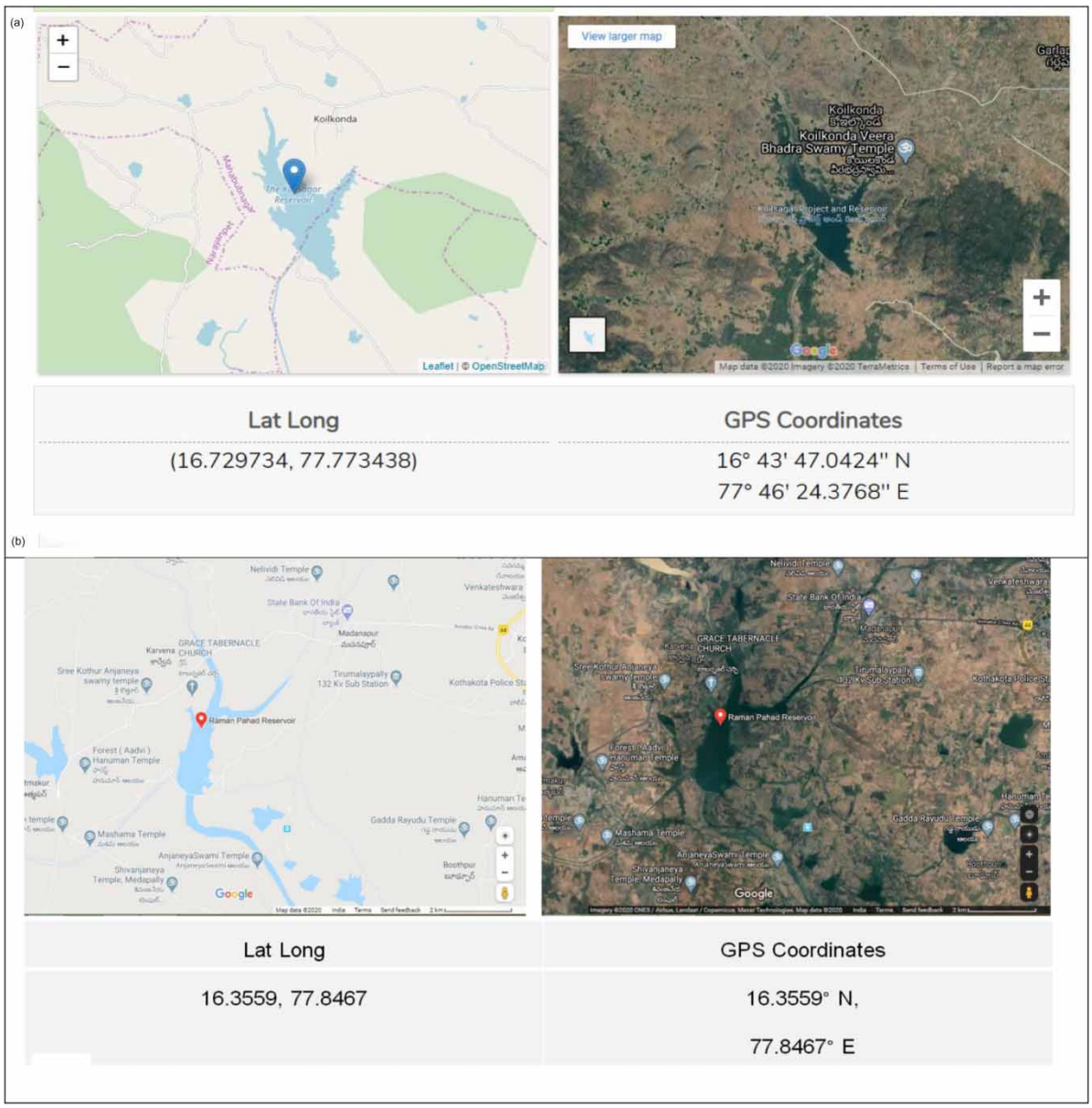

Figure 1 | Location of (a) Koil Sagar reservoir and (b) Raman Pahad reservoir.

with the Quia quick PCR purification kit (Qiagen Inc, Chatsworth, USA) according to the instructions provided.

16S rRNA gene cloning and library construction

The purified PCR product obtained earlier was cloned into pMOS Blue Blunt End vector system (Amersham Biosciences, New Jersey, USA) following the instructions of the manual. Transformants were selected on a LB agar plate containing 40 μg/mL X-gal and 12.5 μg/mL ampicillin and incubated at 37 °C overnight. Clones were maintained on LB agar plates containing X-gal and ampicillin.

16S rRNA gene sequencing and phylogenetic analysis

For 16S rRNA gene sequencing, DNA was prepared using the Mo Bio microbial DNA isolation kit (Mo Bio Laboratories Inc., Solano Beach, CA, USA) and sequenced as

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described previously (Lane 1991). The resultant, almost complete sequence of the 16S rRNA gene contained 1,502 nucleotides. The 16S rRNA gene sequence of the isolate was subjected to BLAST sequence similarity search and EzTaxon to identify the nearest taxa. The entire related 16S rRNA gene sequences were downloaded from the database (http://www.ncbi.nlm.nih.gov), aligned using the CLUSTAL_X program and the alignment was corrected manually. Phylogenetic tree was constructed using tree-making algorithm and the maximum likelihood (ML) using the PhyML program.

Nucleotide sequence accession numbers

The representative 16S rRNA gene clone library in this study was deposited in the GenBank database. The accession numbers for the clones of Raman Pahad were KR612007-KR612060 and for Koilasagar were KR612049-KR612071.

RESULTS AND DISCUSSION

Analysis of coliform bacterial diversity

The water samples collected from the Raman Pahad and Koilsagar water reservoirs were monitored for total coliforms and *E. coli*. The study revealed the presence of fecal colifrom, i.e., *E. coli* in RP and KS, respectively, of 12.20 and 21.42%. Whereas total coliforms were in the ratio *Enterobacter*:*Citrobacter*:*Klebsiella*:*Escherichia*:*Hafnia* of 41:25:17:12:3 for RP reservoir and *Enterobacter*:*Citrobacter*:*Escherichia* in 46:32:21 for KS. Enumeration of this population in the microbial aquatic ecosystem has been universally applied to certify the sanitary quality of water. The samples yielded about 30–80 μg DNA per 100 mL concentrated water. About 200 ng of DNA was used for constructing a 16S rRNA gene library. The libraries constructed from DNA isolated from Raman Pahad and Koilsagar samples consisted of 785 and 323 clones, respectively, with an insert size of approximately 1 kb. The affiliation of each and every clone to the nearest phylogenetic neighbor is based on 16S rRNA gene sequence. Thus, 16S rRNA gene could prove to be a constructive diagnostic tool for identifying the presence of pathogens in water samples (Srinivasan *et al.* 2015).

Diversity within clone libraries

BLAST sequence similarity analysis of the clone libraries of Raman Pahad and Koilsagar indicated that clones belonged to the phylum Proteobacteria (Figures 2 and 3). Of the 785 clones examined in the Raman Pahad library, the predominant sequences were from the genus *Enterobacter* (41.5%), followed by *Citrobacter* (25.03%), *Klebsiella* (17.86%), *Escherichia* (12.20%), and the least being *Hafnia* (3.39%); whereas among the 323 clones in Koilsagar the genus *Enterobacter* (46.42%) was prevalent followed by *Citrobacter* (32.14%) and *Escherichia* (21.42%). Dufour (1977) and Leclerc *et al.* (2001) reported *E. coli*:*Citrobacter*/*Enterobacter*:*Klebsiella* in the ratio of 94:4:2 in natural habitats. According to Patel *et al.* (2016), fecal coliforms were in the ratio of 88.89:4.78:5.11:1.52 for *E. coli*:*Enterobacter*:*Citrobacter*:*Klebsiella*, respectively. Earlier reports also proved the presence of these coliforms in groundwater, surface. and drinking water samples (Omari & Manu 2012; Somaratne & Hallas 2015).

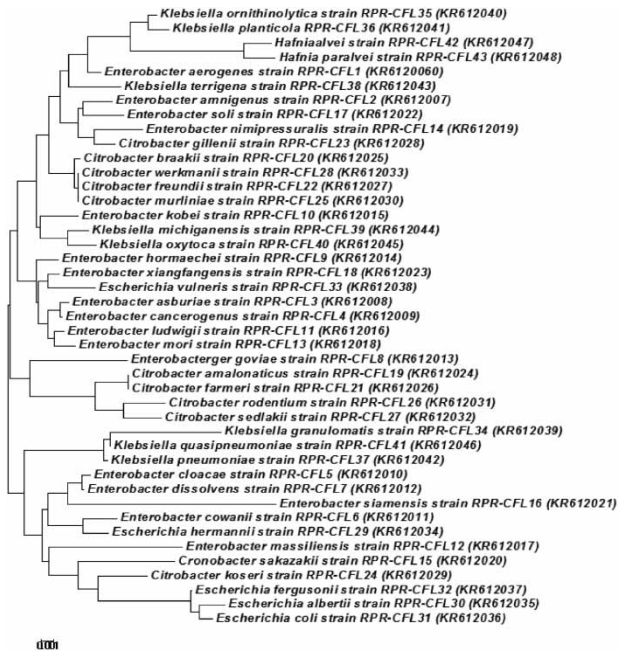

Figure 2 | Phylogenetic analysis of bacterial 16S rRNA in Ramanpadu water sample.

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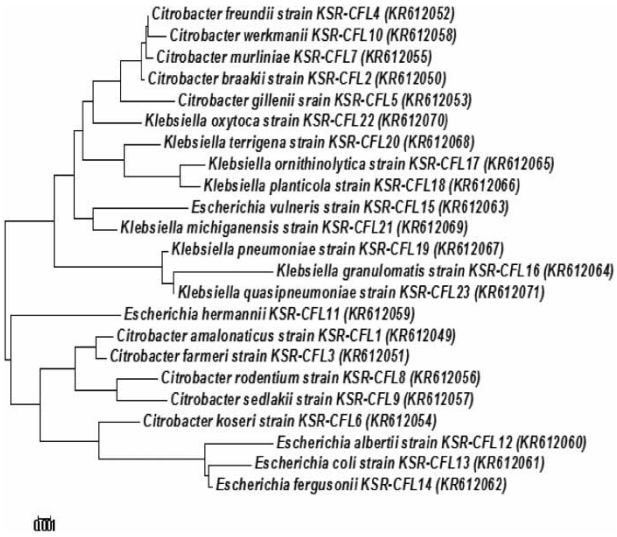

Figure 3 | Phylogenetic analysis of bacterial 16S rRNA in Koilsagar water sample.

The present study revealed the predominance of *Enterobacter*, the only representative in the order Enterobacteriales of the class Gamaproteobacteria in the phylum *Proteobacteria* (representing 10%) clones in Koilsagar rather than in Raman Pahad, indicating that the former had greater bacterial burden than Raman Pahad water.

Diarrheagenic *Escherichia coli* (DEC) are important bacteria responsible for a number of waterborne gastroenteritis outbreaks (Swerdlow *et al.* 1992), and has been detected in various ecological niches ranging from mammalian intestines to various aquatic environments, such as surface water and groundwater (Coleman *et al.* 2013). In general, an outbreak is likely to be attributed to a single *E. coli* clone as an etiological agent; however, multiple DEC strains are occasionally involved in a waterborne outbreak (McCall *et al.* 2010; Lienemann *et al.* 2011). In the present study, 12.20% and 21.42% *E. coli* were found in Raman Pahad and Koil Sagar reservoirs, respectively. Similar results were reported from environmental samples in waterborne gastroenteritis outbreaks, mainly caused by DEC, hemorrhagic colitis (Park *et al.* 2018) *E. coli* is universally used as a bacterial indicator of fecal contamination in water, the isolation of which from reservoir samples indicate that the water might have been mixed with feces (Saxena *et al.* 2015).

Citrobacter is named for its ability to utilize citrate as the sole carbon source. *Citrobacter* is reported to occur in environments such as water, sewage, soil, and food. These species can be isolated from different clinical sites, in particular, *C. freundii* is an intestinal inhabitant of humans that may sometimes produce an enterotoxin and thus become an intestinal pathogen (Frederiksen 2005). *C. amalonaticus, C. freundii*, and *C. koseri* (*C. diversus*) were reported in the feces of Australian mammals. This genus was also reported in the water of Paradi (Gordon & FitzGibbon 1999; Mukherjee *et al.* 2016). In the present study, around 25.03% and 32.14% *Citrobacter* was found in Raman Pahad and Koil Sagar reservoirs, respectively.

In spite of being recognized as naturally occuring species in the environment, the virulence properties of aquatic-borne *Klebsiella pneumoniae* which resemble clinical strains remain principally mysterious (Podschun *et al.* 2001). This bacteria causes a wide range of infections, including pneumonias, urinary tract infections, bacteremias, and liver abscesses and is the leading cause of neonatal sepsis in developing countries (Paczosa & Mecsas 2016). In the present study, *Klebsiella* was found to be around 17.86% in Raman Pahad reservoir. Oropharyngeal colonization could act as the main reservoir for nosocomial outbreaks caused by *K. pneumoniae* (Ballén *et al.* 2015).

Hafnia sp. (formerly *Enterobacter hafniea*) in the present study was found only in Raman Pahad reservoir. It resides in the gastrointestinal tract of humans and many animal species and is also found in various ecological samples. However, it is not a common human pathogen but is often associated with gastroenteritis (Janda & Abbott 2006; Aishvarya *et al.* 2017). There have been numerous reports involving diarrhea in the isolation of *H. alvei* from stool samples because the organism is part of normal fecal microbiota. However, in a case-control study of Finnish tourists returning from Morocco, the pervasiveness of *H. alvei* in those with diarrhea was notably greater than in those without diarrhea, substantiating a possible etiologic role (Ridell *et al.* 1994).

Enterobacter found in Raman Pahad reservoir were rarely found as pathogens, but these organisms are now increasingly encountered, causing nosocomial infections such as urinary tract infections and bacteremia. *E. amnigenus* has been mostly isolated from water, but some strains have been isolated from clinical specimens of the respiratory tract, wounds, and feces (Grimont & Grimont 2005).

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CONCLUSION

Despite the high genetic variability of the total coliform group, this study showed that it is possible to use molecular assays to detect total coliforms in drinking water. The 16S rRNA molecular-based assay proved to be as sensitive as recommended, culture-based methods. In this study, the identification of bacterial communities in Raman Pahad and Koilasagar water samples of Mahabubnagar district, Telangana, was performed using culture-independent methods based on the 16S rRNA gene clone library. Data revealed that the Raman Pahad water has a greater diversity of coliforms as compared to the water samples from Koilsagar reservoir. The results obtained in the present study are applicable only to drinking water samples. Results could be diverse with other types of water.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

Aishvarya, N., Malviya, M. K., Tambe, A., Sati, P., Dhaka, K. & Pandey, A. 2017 Bacteriological assessment of river Jataganga, located in Indian Himalaya with reference to physico-chemical and seasonal variations under anthropogenic pressure: a case study. *J. Environ. Microbiol.* **1** (1), 10–16.

Alain, K. & Querellou, J. 2009 Cultivating the uncultured: limits, advances and future challenges. *Extremophiles* **13**, 583–594.

Amann, R. I., Ludwig, W. & Schleifer, K. H. 1995 Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59** (1), 143–169.

Bae, S., Maestre, J. P., Kinney, K. A. & Kirisits, M. J. 2019 An examination of the microbial community and occurrence of potential human pathogens in rainwater harvested from different roofing materials. *Water Res.* **159**, 406–413.

Ballén, V., Sáez, E., Benmessaoud, R., Houssain, T., Alami, H., Barkat, A., Kabiri, M., Moraleda, C., Bezad, R., Vila, J., Bosch, J., Bassat, Q. & Soto, S. M. 2015 First report of a *Klebsiella pneumoniae* ST466 strain causing neonatal sepsis harbouring the $bla_{CTX\text{-}M\text{-}15}$ gene in Rabat, Morocco. *FEMS Microbiol. Lett.* **362** (1), 1–4.

Bottari, B., Ercolini, D., Gatti, M. & Neviani, E. 2006 Application of FISH technology for microbiological analysis: current state and prospects. *Appl. Microbiol. Biotechnol.* **73** (3), 485–494.

Cabral, J. P. 2010 Water microbiology. Bacterial pathogens and water. *Int. J. Environ. Res. Public Health* **7**, 3657–3703.

Coleman, B. L., Louie, M., Salvadori, M. I., McEwen, S. A., Neumann, N., Sibley, K., Irwin, R. J., Jamieson, F. B., Daignault, D., Majury, A., Braithwaite, S., Crago, B. & McGeer, A. J. 2013 Contamination of Canadian private drinking water sources with antimicrobial resistant Escherichia coli. *Water Res.* **47**, 3026–3036.

Cottrell, M., Waidner, L. A., Yu, L. & Kirchman, D. L. 2005 Bacterial diversity of metagenomic and PCR libraries from the Delaware river. *Environ. Microbiol.* **7** (12), 1883–1895.

Dewettinck, T., Hulsbosch, W., van Hege, K., Top, E. M. & Verstraete, W. 2001 Molecular fingerprinting of bacterial populations in groundwater and bottled mineral water. *Appl. Microbiol. Biotechnol.* **57**, 412–418.

Dos Santos, S., Adams, E. A., Neville, G., Wada, Y., de Sherbinin, A., Mullin Bernhardt, E. & Adamo, S. B. 2017 Urban growth and water access in sub-Saharan Africa: progress, challenges, and emerging research directions. *Sci. Total Environ.* **607–608**, 497–508.

Dufour, A. P. 1977 *Escherichia coli*: the fecal coliform. In: *Bacterial Indicators/Health Hazards Associated with Water* (A. W. Hoadley & N. J. Dutka, eds). American Society for Testing Materials, Philadelphia, PA, USA, pp. 48–58.

Frederiksen, W. 2005 Genus citrobacter. In: *Bergey's Manual of Systematic Bacteriology*, 2nd edn, Vol. 2 (D. J. Brenner, N. R. Krieg & J. T. Staley, eds). Springer, New York, USA, pp. 651–656.

Gordon, D. M. & FitzGibbon, F. 1999 The distribution of enteric bacteria from Australian mammals: host and geographical effects. *Microbiology* **145**, 2663–2671.

Grimont, P. A. D. & Grimont, F. 2005 Genus *Enterobacter*. In: *Bergey's Manual of Systematic Bacteriology*, 2nd edn, Vol. 2 (D. J. Brenner, N. R. Krieg & J. T. Staley, eds). Springer, New York, USA, pp. 661–669.

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Jain, C. K., Bandyopadhyay, A. & Bhadra, A. 2010 Assessment of ground water quality for drinking purpose, District Nainital, Uttarakhand, India. *Environ. Monit. Assess.* **166**, 663–676.

Janda, J. M. & Abbott, S. L. 2006 The genus Hafnia: from soup to nuts. *Clin. Microbiol. Rev.* **19**, 12–18.

Lane, D. J. 1991 16S/23S rRNA sequencing. In: *Nucleic Acid Techniques in Bacterial Systematics* (E. Stackebrandt & M. Goodfellow, eds). John Wiley and Sons, Chichester, UK, pp. 115–175.

Leclerc, H. & Moreau, A. 2002 Microbiological safety of natural mineral water. *FEMS Microbiol. Rev.* **26** (2), 207–222.

Leclerc, H., Mossel, D. A., Edberg, S. C. & Struijk, C. B. 2001 Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. *Annu. Rev. Microbiol.* **55**, 201–234.

Lenart-Boroń, A., Wolanin, A. & Jelonkiewicz, E. 2017 The effect of anthropogenic pressure shown by microbiological and chemical water quality indicators on the main rivers of Podhale, southern Poland. *Environ. Sci. Pollut. Res.* **24**, 12938–12948.

Lienemann, T., Pitkanen, T., Antikainen, J., Molsa, E., Miettinen, I., Haukka, K., Vaara, M. & Siitonen, A. 2011 Shiga toxin producing *Escherichia coli* o100:H(-): stx2e in drinking water contaminated by waste water in Finland. *Curr. Microbiol.* **62**, 1239–1244.

Lyautey, E., Lacoste, B., Ten-Hage, L., Rols, J. L. & Garabetian, F. 2005 Analysis of bacterial diversity in river biofilms using 16 S rDNA PCR-DGGE: methodological settings and fingerprints interpretation. *Water Res.* **39** (2–3), 380–388.

McCall, B. J., Slinko, V. G., Smith, H. V., Heel, K., Culleton, T. H., Kelk, V. R. & Stafford, R. J. 2010 An outbreak of shiga toxin-producing *Escherichia coli* infection associated with a school camp. *Commun. Dis. Intell. Q. Rep.* **34**, 54–56.

Molina, F., López-Acedo, E. & Tabla, R. 2015 Improved detection of *Escherichia coli* and coliform bacteria by multiplex PCR. *BMC Biotechnol.* **15**, 48. https://doi.org/10.1186/s12896-015-0168-2.

Mossel, D. A. A. & Struijk, C. B. 2004 Assessment of the microbial integrity, sensu G.S. Wilson, of piped and bottled drinking water in the condition as ingested. *Int. J. Food Microbiol.* **92**, 375–390.

Mukherjee, N., Bartelli, D., Patra, C., Chauhan, B. V., Dowd, S. E. & Banerjee, P. 2016 Microbial diversity of source and point-of-use water in rural Haiti – a pyrosequencing-based metagenomic survey. *PLoS One*. https://doi.org/10.1371/journal.pone.0167353.

Omari, S. & Manu, D. K. Y. 2012 The study of bacterial contamination of drinking water sources: a case study of Mpraeso, Ghana. *Internet J. Microbiol.* doi:10.5580/2b06.

Paczosa, M. K. & Mecsas, J. 2016 Klebsiella pneumoniae: going on the offense with a strong defense. *Microbiol. Mol. Biol. Rev.* **80** (3), 629–661.

Park, J., Kim, J. S., Kim, S., Shin, E., Oh, K. H., Kim, Y., Kim, C. H., Hwang, M. A., Na, K., Lee, J., Cho, E., Kang, B. H., Kwak, H. S., Seong, W. K. & Kim, J. 2018 A waterborne outbreak of multiple diarrhoeagenic Escherichia coli infections associated with drinking water at a school camp. *Int. J. Infect. Dis.* **66**, 45–50.

Patel, C. B., Shanker, R., Gupta, V. K. & Upadhyay, R. S. 2016 Q-PCR based culture-independent enumeration and detection of *Enterobacter*: an emerging environmental human pathogen in riverine systems and potable water. *Front. Microbiol.* **7**, 172. doi.org/10.3389/fmicb.2016.00172.

Piterina, A. V. & Pembroke, J. T. 2013 Use of PCR-DGGE based molecular methods to analyse microbial community diversity and stability during the thermophilic stages of an ATAD wastewater sludge treatment process as an aid to performance monitoring. *Int. Schol. Res. Not.* doi:10.5402/2013/162645.

Podschun, R., Pietsch, S., Holler, C. & Ullmann, U. 2001 Incidence of *Klebsiella* species in surface waters and their expression of virulence factors. *Appl. Environ. Microbiol.* **67**, 3325–3327.

Ram, S., Vajpayee, P. & Shanker, R. 2008 Contamination of potable water distribution systems by multiantimicrobial-resistant enterohemorrhagic *Escherichia coli*. *Environ. Health Perspect.* **116**, 448–452.

Ram, S., Vajpayee, P., Dwivedi, P. D. & Shanker, R. 2011 Culture-free detection and enumeration of STEC in water. *Ecotoxicol. Environ. Saf.* **74**, 551–557.

Ridell, J., Siitonen, A., Paulin, L., Mattila, L., Korkeala, H. & Albert, M. 1994 *Hafnia alvei* in stool specimens from patients with diarrhea and healthy controls. *J. Clin. Microbiol.* **32**, 2335–2337.

Saxena, G., Bharagava, R. N., Kaithwas, G. & Raj, A. 2015 Microbial indicators, pathogens and methods for their monitoring in water environment. *J. Water Health* **13**, 319–339.

Seas, C., Alarcon, M., Aragon, J. C., Beneit, S., Quinonez, M., Guerra, H. & Gotuzzo, E. 2000 Surveillance of bacterial pathogens associated with acute diarrhea in Lima, Peru. *Int. J. Infect. Dis.* **4**, 96–99.

Shakoor, S., Ahmed, I. & Mukhtiar, S. 2018 High heterotrophic counts in potable water and antimicrobial resistance among indicator organisms in two peri-urban communities of Karachi, Pakistan. *BMC Res. Notes* **11**, 350. https://doi.org/10.1186/s13104-018-3461-z.

Shanker, A. S., Goud, R. K. & Pavan Kumar Pindi, P. K. 2019 Exploration of bacterial contaminants in reservoirs of Palamuru by PCR-DGGE method: household treatment. *J. Water Sanit. Hyg. Dev.* **9** (1), 38–48.

Shivaji, S., Chaturvedi, P. & Begum, Z. 2009 Janibacter hoylei sp. nov., Bacillus isronensis sp. nov. and Bacillus aryabhattai sp. nov. from cryotubes used for collecting air from upper atmosphere. *Int. J. Syst. Evol. Microbiol.* **59**, 2977–2986.

Shivaji, S., Kiran, K., Hara, K., Pavan, K. P., Pasupuleti, S. R. & Srinivas, T. N. R. 2011 Vertical distribution of bacteria in lake sediment from Antarctica by culture-independent and culture-dependent approaches. *Research in Microbiology* **162** (2), 191–203.

Somaratne, N. & Hallas, G. 2015 Review of risk status of groundwater supply wells by tracing the source of coliform contamination. *Water* **7** (7), 3878–3905.

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Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S., Nadarajan, R., Brodie, E. L. & Lynch, S. V. 2015 Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One* **10** (2), 22.

Stelma Jr., G. N. & Wymer, L. J. 2012 Research considerations for more effective groundwater monitoring. *J. Water Health* **10**, 511–521.

Swerdlow, D. L., Woodruff, B. A., Brady, R. C., Griffin, P. M., Tippen, S., Donnell Jr., H. D., Geldreich, E., Payne, B. J., Meyer Jr., A., Wells, J. G., Greene, K. D., Bright, M., Bean, N. H. & Blake, P. A. 1992 A waterborne outbreak in Missouri of Escherichia coli o157:H7 associated with bloody diarrhea and death. *Ann. Intern. Med.* **117**, 812–819.

Uthappa, C. K., Allam, R. R., Nalini, C., Gunti, D., Udaragudi, P. R., Tadi, G. P. & Murhekar, M. V. 2015 An outbreak of cholera in Medipally village, Andhra Pradesh, India, 2013. *J. Health Popul. Nutr.* 33. doi:10.1186/s41043-015-0021-1.

Van der Wielen, P. W. J. J. & Medema, G. 2010 Unsuitability of quantitative Bacteroidales 16S rRNA gene assays for discerning fecal contamination of drinking water. *Appl. Environ. Microbiol.* **76**, 4876–4881.

Vetrimurugan, E., Elango, L. & Rajmohan, N. 2013 Sources of contaminants and groundwater quality in the coastal part of a river delta. *Int. J. Env. Sci. Technol.* **10** (3), 473–486.

WHO/UNICEF 2015 *Progress on Sanitation and Drinking Water 2015 Update and MDG Assessment 2015*. Available from: http://www.who.int/water_sanitation_health/monitoring/jmp-2015-update/en/ (accessed 11 July 2017).

Wu, Q., Zhao, X.-H. & Zhao, S.-Y. 2006 Application of PCRDGGE in research of bacterial diversity in drinking water. *Biomed. Environ. Sci.* **19**, 371–374.

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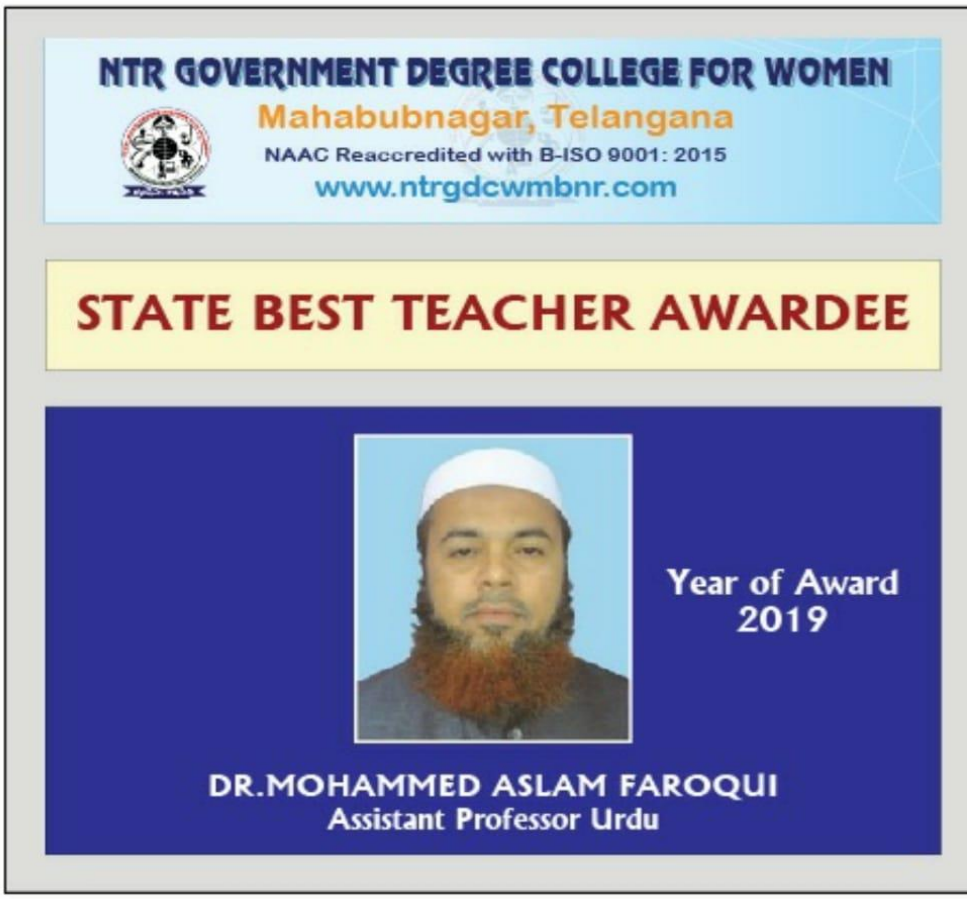

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