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Research Article
Isolation and Characterization of Cold Active Bacterial Species from Municipal Solid Waste Landfill Site

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Abstract

Background and Objective: Land-filling is considered as a common method for disposal of Municipal Solid Waste (MSW) and the landfills are regarded as repository of various groups of important microbes growing on organic waste materials. In temperate regions due to lower environmental temperatures the landfill sites are inhabited by a distinct group of bacteria that utilize the wastes as energy sources through the secretion of important cold active enzymes. Hence, the present study was taken to examine physicochemical and bacteriological characterization of organic municipal waste samples collected form landfill site. **Materials and Methods:** A total of 15 waste samples mixed with soil were randomly collected from the landfill site. The waste samples were analyzed for various physicochemical parameters by following available standard methodology. The isolation of bacteria was done by following serial dilution and spread plate method at temperature of 15°C, pH 7.0 and incubation period of 48 h. **Results:** The results of the study revealed that the landfill wastes at the surface were partially decomposed by the presence of diverse group of cold active bacterial isolates. The morphological characterization of the isolates revealed that most of bacteria belonged to genus *Bacillus* (gram+) majority of which produced two important types of cold active enzymes (CMCases, proteases) in response to different substrates (carboxymethyl cellulose and skim milk) under *in vitro* conditions. **Conclusion:** It was concluded that the landfill site acted as the repository of unique group of cold active bacteria most of which showed potential of producing multiple enzymes. The study presents a platform for further scientific investigation through microbial profiling of landfill site that may lead to discovery of some novel microbes of high enzyme producing potential of scientific importance.

Key words: MSW, landfill, cold active bacteria, decomposition, enzyme activity, *Bacillus* sp.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The present world is on the path of rapid development but at the same time it has also become a waste generating world[1]. At global level approximately 1.3 billion t of MSW are generated per year and is expected to increase approximately to 2.2 billion t year^{-1} by 2025. These generation rates represent a significant increase from 1.2-1.42 kg of MSW/capita/day in the next 15 years. In Asian cities this value ranges from 0.5-1.3 kg/capita/day, which has a direct correlation with per capita income of the city. The collection, transportation and disposal of MSW are unscientific and chaotic in India[2].

The environmental risks of huge amount of municipal solid waste has been reduced by advances in waste reduction, recycling and composting techniques, but land-filling is still the most common waste disposal option worldwide and is considered as the store house of many important waste degrading bacteria[3]. The MSW contains major fraction as organic that upon degradation produces odors, sludge, pollution or unsightly mess during degradation. However, these wastes are utilized as source of nutrients by different bacteria and converted into stable end products that are safe to the environment. Since these bacteria are exposed to varied substrates and chemicals, there raises the possibility to screen effective bacterial strains from waste dumping sites with valuable applications[4]. However, the moisture content and pH of the substrates provide ideal conditions for the growth and functioning of bacterial populations. The previous research highlighted the isolation of novel types of bacteria from different environments that can have application in industries and in waste degradation[4,5]. However, the disposal of MSW at high altitudes and snowbound areas is an ever growing problem[6]. These regions contain some special type of bacteria that can grow in response to lower environmental temperatures and the organic waste substrates thereby by causing secretion of cold active enzymes to perform their metabolic activities[7]. The cold-adapted microbes can survive in cold environments normally, which is due to their structure and unique molecular mechanism to adapt the low temperature[8]. As a result, they have attracted increasingly attention with their higher catalytic enzymes at cold ambient temperatures, which could provide huge biotechnological applications for the practical production[9,10]. Therefore, investigation of cold-adapt strains under low ambient temperature is of high interest for their practical application and new scientific investigation. The present study was taken from the landfill site of Srinagar city (summer capital) of state Jammu and Kashmir for the reason that these sites contain diverse microbes that have adapted to harsh ambient conditions. The city is generating an average of 575 tons per day (TPD) of municipal solid waste with an average physical composition of 54% organic matter, 30% silt and about 16% other components[11] and the waste is disposed at Achan landfill site (the only waste disposal facility of the city). The city experiences cold temperatures for many months and hence the idea was developed for the isolation and characterization of cold active bacteria from the landfill waste that could be studied for their potentials of producing cold active enzymes that has lower activation energies of industrial and biotechnological importance. Further the special group of isolates provides a platform for further scientific investigation that will lead to the generation of new scientific knowledge.

MATERIALS AND METHODS

Site description and collection of samples: The waste samples were collected from municipal sanitary landfill site situated at Achan in Srinagar city of state Jammu and Kashmir, India (Fig. 1, 2). The site lies between 34°7'00" North Latitude and 74°47'38.08" East. A total of 15 waste samples were collected from five sites of the landfill site in-order to find the variability of various parameters. The samples were collected in airtight zip lock bags and labeled. The samples were transported intact at ambient temperature to the laboratory for physicochemical and biological analysis.

Physicochemical characterization of waste samples: The waste samples were analyzed for various physicochemical parameters viz. pH, electrical conductivity, moisture content, organic carbon (%), total nitrogen (%), total potassium (%), total phosphorus (%) by following standard methodology[12].

Isolation and enumeration of cold active bacteria from waste samples: Serial dilution technique was used for the isolation of bacteria from solid waste. In this technique 1 g of waste was taken and serially diluted in 5 test tubes each containing 9 mL of sterile distilled water in the order of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} under aseptic environmental conditions of laminar air flow chamber. From 10^{-1} dilution 1 mL of solution was transferred to 10^{-2}. Then 1 mL was transferred from 10^{-2} dilution to 10^{-3}, then from 10^{-3} to 10^{-4} and then from 10^{-4} to 10^{-5}. From all dilutions 1 mL of solution was taken and transferred onto sterilized nutrient agar plates

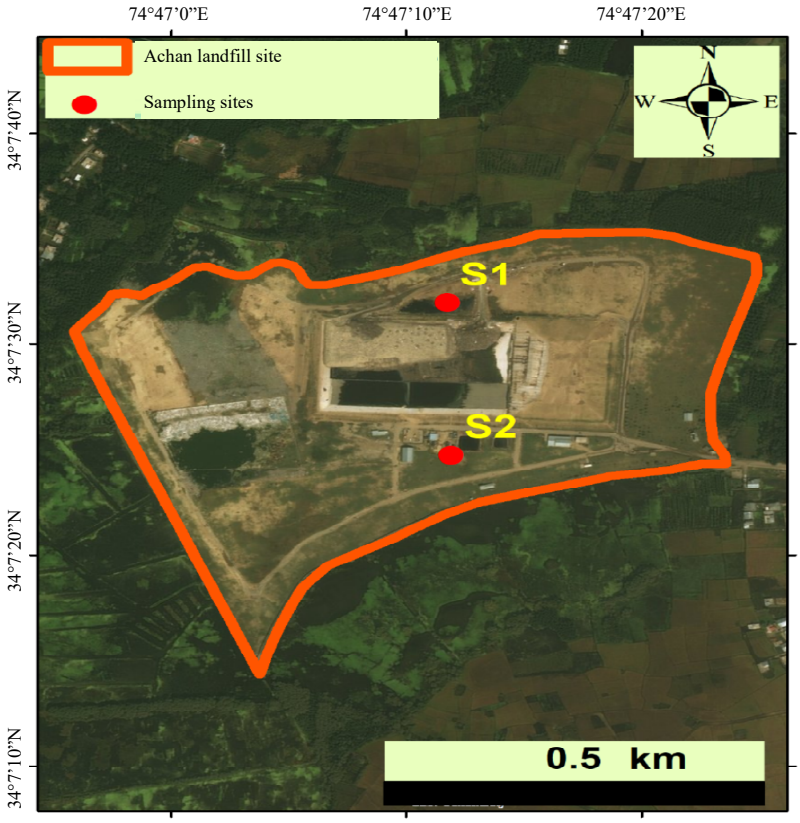

Fig. 1: Location map of Achan Landfill site, Srinagar

Fig. 2: Collection of waste samples from landfill site

and incubated at 15°C for 48 h. At the end of incubation the isolates were observed and enumerated under colony counter meter. The isolates were purified by repeated streaking and preserved in nutrient agar slants at 4°C.

Morphological characterization of bacterial isolates: Identification of bacterial isolates was done on the basis of morphological and cellular characterization by following Bergey's Mannual of Bacteriology[13].

Enzyme activities of bacterial isolates: For the detection of carboxymethyl cellulase and protease activities of the isolates the pure cultures of isolated bacteria were individually spot inoculated on carboxymethyl cellulose (CMC) and casein agar media plates, respectively with the help of inoculation loop. The plates were incubated inverted at temperatures of 15°C for 48 h. The formation of hydrolysis zones on CMC agar media was detected by flooding the plates with 0.1% congo red solution for 20 min[14]. The protease activity of isolates was confirmed by the formation of hydrolysis zone on skim milk agar media.

Statistical analysis: The physicochemical and biological parameters of each sample was determined in triplicates and the results were reported as mean. The standard error and critical difference was calculated among the samples and the values were considered significantly different from each other at $p \leq 0.05$.

RESULTS

A total of 5 organic waste samples mixed with soil were collected from Achan landfill site and analyzed for various physicochemical parameters as shown in Table 1. The average pH value of the samples showed a slight acidic value of 6.86. The average moisture content of the samples was 55.09%. The organic carbon and total nitrogen of the samples was 20 and 0.48%, respectively. The total phosphorus and total potassium of the samples was found to be 0.32 and 0.37%, respectively.

Isolation and enumeration of cold active bacterial isolates: By following serial dilution method the total culturable cold active bacterial count ranged from 3.3×106-4×106 c.f.u. (g dry wt.)$^{-1}$ in all the collected samples. Based on morphological characteristics a total of 17 different bacteria were isolated and the results are shown in Table 2.

Morphological characterization of bacterial isolates: The bacterial isolates were characterized for colony shape, color, margins, elevation (Table 3) and cellular characteristics (Table 3, Fig. 3).

The results showed that out of 17 bacterial isolates 10 isolates were Gram-positive *Bacillus* sp. and 7 isolates belonged to Gram-negative *Bacillus* class.

Enzyme activities of bacterial isolates: Out of 17 isolates about 10 isolates showed the ability of producing CMCases

Table 1: Physicochemical characteristics of solid waste samples

Sites	Moisture content (%)	pH*	Organic carbon (%)*	Total nitrogen (%)*	Total phosphorus (%)*	Total potassium (%)*
S_1	57.5	6.83	20.55	0.45	0.35	0.40
S_2	54.44	6.89	19.4	0.55	0.32	0.35
S_3	55.49	6.95	23.5	0.5	0.29	0.37
S_4	57.39	6.79	20.47	0.43	0.30	0.35
S_5	53.3	6.81	18.55	0.47	0.31	0.42
Mean	55.09	6.86	20.05	0.48	0.32	0.37
CD (0.05)	0.009	0.006	0.008	0.007	0.005	0.010
SE (m)	0.005	0.003	0.007	0.004	0.001	0.002

*Indicates mean values

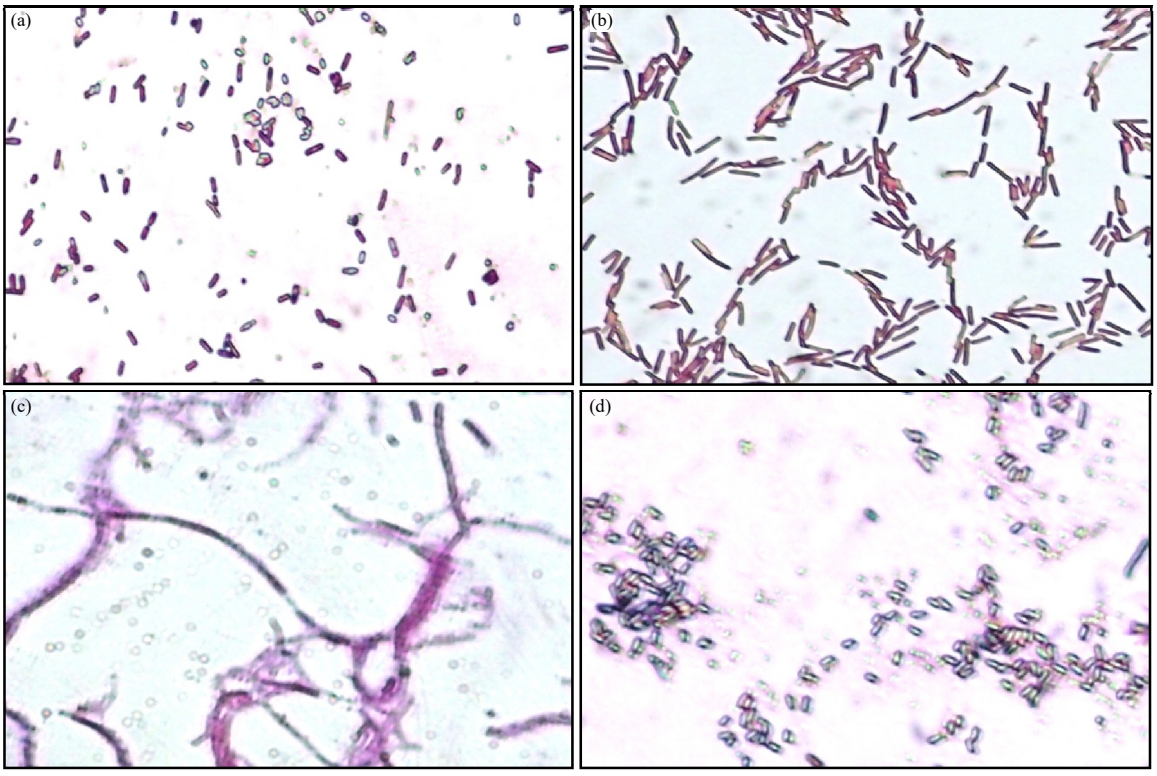

Fig. 3: Continued

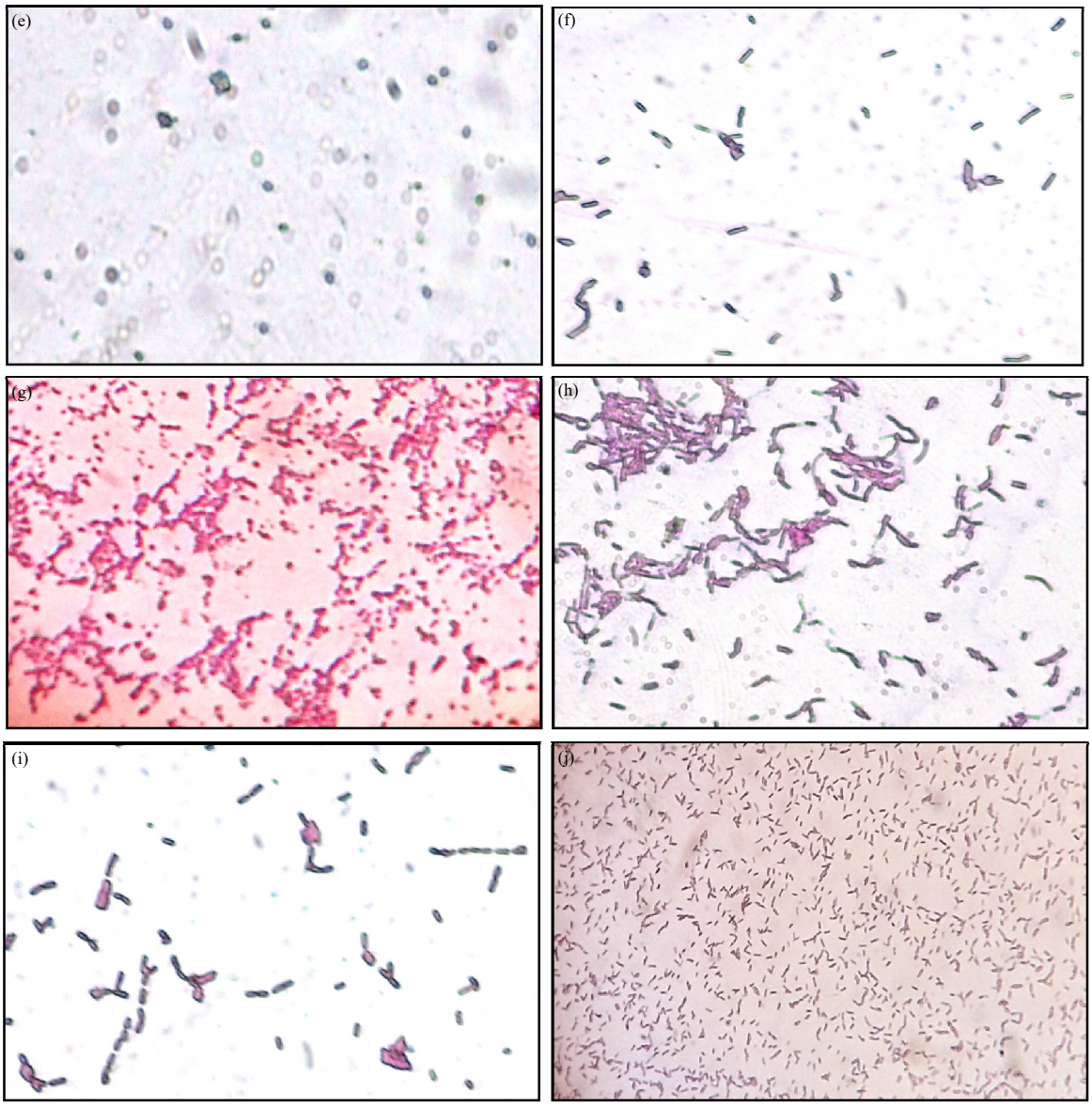

Fig. 3(a-j): Microscopic characteristics and Grams reaction of bacterial isolates, (a) Gram+ *Bacillus* sp., (b) Gram- *Bacillus* sp., (c) Gram+ *Bacillus* sp., (d) Gram+ *Bacillus* sp., (e) Gram+ *Coccus* sp., (f) Gram+ *Bacillus* sp., (g) Gram- *Coccus* sp., (h) Gram+ *Bacillus* sp., (I) Gram+ *Bacillus* sp. and (j) Gram- *Bacillus* sp.

Table 2: Colony forming units (c.f.u g^{-1}) and No. of bacteria form the waste samples

Sites	Colony count ($\times 10^6$)*	No. of bacteria*
S_1	3.3	15
S_2	3.5	17
S_3	4.0	22
S_4	3.8	20
S_5	3.5	16
Mean	3.7	18

*Represents mean values

and proteases by forming hydrolysis zones on different media after 72 h of incubation and the results were shown in Fig. 4 and 5, respectively.

DISCUSSION

In the present study the solid waste samples were collected form Achan Landfill site, Srinagar during the winter

Table 3: Morphological characteristics of isolated bacteria

Isolates	Colony color	Colony shape	Surface	Margins	Elevation	Transparency	Grams reaction	Cell shape	Cell arrangement
I1	White creamy	Round	Smooth	Erose	Raised	Opaque	Negative	Short bacilli	Single
I2	Creamy	Round	Rough	Entire	Raised slopy edges	Opaque	Positive	Bacilli	Diplobacilli
I3	Oily and dew drop	Irregular	Smooth	Entire	Convex	Transparent	Positive	Bacilli	Streptobacilli
I4	White	Round	Smooth	Entire	Umbulate	Opaque	Positive	Short bacilli	Single
I5	Waxy	Rhizoid	Rough	Lobate	Flat	Transparent	Positive	Long bacilli	Diplobacilli
I6	White	Irregular	Rough	Lobate	Thick leafy	Opaque	Positive	Bacilli	Single
I7	Cream	Round	Smooth	Wavy	Flat	Translucent	Negative	Short bacilli	Single
I8	White	Punctiform	Smooth	Entire	Flat	Translucent	Positive	Long bacilli	Diplobacilli
I9	White	Irregular	Rough	Wavy	Convex	Transparent	Positive	Bacilli	Single
I10	Oily yellow	Circular	Smooth	Entire	Raised	Transparent	Negative	Short bacilli	Single
I11	Orange	Rhizoid	Rough	Lobate	Thick leafy	Translucent	Negative	Short bacilli	Single
I12	Yellow	Circular	Smooth	Entire	Flat	Translucent	Negative	Coccus	Single
I13	Cream	Filamentous	Rough	Lobate	Flat	Transparent	Positive	Long bacilli	Diplobacilli
I14	Cream	Irregular	Rough	Wavy	Thick leafy	Transparent	Positive	Bacilli	Single
I15	White	Circular	Smooth	Entire	Flat	Translucent	Positive	Bacilli	Diplobacilli
I16	Pale	Filamentous	Rough	Lobate	Flat	Opaque	Negative	Short bacilli	Single
I17	Yellow	Irregular	Smooth	Lobate	Thick leafy	Translucent	Negative	Short cocci	Streptococcus

Fig. 4: Hydrolysis zones around the inoculum on congo red CMC agar media showing the production of Carboxymethyl cellulases

Fig. 5: Hydrolysis zones around the inoculums on skim milk agar media indicating the production of extracellular proteases

season. The samples were analyzed for various physicochemical parameters and biological characteristics. The average moisture content of the samples was found to be 55% that is the primary detrimental factor for the presence of bacterial populations in the waste samples. The regions with high moisture content of 50-75% support the maximum growth and optimum activities of bacteria[15]. The pH of samples of present study was found to be slightly acidic (6.86) that might be due to the reason of degradation of the waste materials. Previous study reported the average pH value of 7.09 of waste samples collected from Pirana MSW dumping site[16]. The organic carbon was found to be lesser than that of initial values of Indian municipal solid waste. The reason might be the partial decomposition of the organic waste portion that has resulted in the loss of carbon in the form of carbon dioxide and conversion into protoplasm of bacterial cells[17]. The level of nitrogen was found to be 0.48% in the waste samples of dumping site. The good quantity of nitrogen in the waste samples indicates the decomposed form of waste and its presence could be due to organic matter which is chemically bound to nitrogen and conversion of ammonium nitrogen to nitrate nitrogen by nitrifying bacteria[18]. In other studies the nitrogen content in waste was found to range[4,19] between 0.05-2.2%, whereas other study reported the nitrogen content of waste dumping site to range between 0.34 and 0.54% that was in agreement with the total nitrogen content of the present study[20]. The phosphorus and potassium contents of the waste samples could be due to the decomposition of the waste materials leading to their release and accumulation[21,22]. The biological analysis of the waste samples showed that the average bacterial colony count was 3.7×10^6 CFU/g at temperature of 15°C. In previous study it was highlighted that among different bacterial species colony count of *Pseudomonas* sp. was maximum (3.38×10^6 CFU) in winter season[23]. The presence of bacteria could be due to the optimum moisture content and the presence of decomposed organic matter[5,4]. Further the bacterial isolates were found psychrotrophic due to their remarkable growth at lower temperatures[21] of 15°C. Based on morphological characteristics of isolates majority were found to belong to genus Gram positive *Bacillus* and the results were supported by the findings of Siddiqqui[5] and He *et al.*[24]. Out of all bacterial isolates only 10 isolates showed ability of producing extracellular carboxymethyl cellulases and proteases at lower temperatures that was confirmed by the formation of hydrolysis zones on respective specific media. The enzyme

activities of isolates at lower temperatures may be due to the presence of cold shock or cold acclimatized proteins and accumulation of polyunsaturated fatty-acids that maintains the semi fluid nature of the cell membranes for easy transport of materials or enzymes[25]. Earlier cellulolytic bacteria were isolated and enumerated from landfill refuse[26] at 36°C. In another study, 108 mesophyllic (37°C for 2 days) bacteria were isolated from MSW out of which 15 isolates exhibited cellulose degrading potential by showing hydrolyzing activity on CMC agar media[27]. Similarly earlier findings reported that a total of 6 bacteria were isolated from kitchen waste and screened on skim milk agar out of which two were demonstrated for transparent circular zones around the colonies indicating protease production[28]. Further the presence of cellulolytic and proteolytic bacteria in the landfill site could be due to the presence of high cellulosic material and protein rich kitchen waste. However, the bacterial isolates of the present study were unique in their characteristics by showing significant growth and enzyme activities at lower temperatures. Further the study also provide new scope for further scientific investigation regarding the factors and mechanisms of the origin and functioning on the cold active enzymes produced by these isolates at the landfill site. The study provides first report of presence of cold active bacteria at landfill site and also paves path for further scientific research for isolation of some novel enzyme producing microbes for surface to the bottom of landfill sites through microbial profiling.

CONCLUSION

The results of the present study concludes that Achan Landfill site, Srinagar, Jammu and Kashmir has cold active bacterial repository at the surface and among the isolates some special bacterial isolates produced multiple cold active enzymes in response to the organic substrates and the environmental conditions that lead to partial decomposition of organic wastes in un-conducive environmental conditions. Thus the study becomes a field of research for investigating the factors that makes the microbial community to survive in harsh environmental conditions that actually proves to have induced the multiple cold active enzyme production potential in the isolates of the present study.

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SIGNIFICANCE STATEMENT

In the early studies from the same site bacteria were isolated that produced only one type of cold active enzyme, however the present study resulted in the isolation of bacterial isolates majority of which produced many enzymes viz. CMCases and proteases. The study presents a new search for the occurrence of cold active bacteria that carry out the decomposition of surface wastes at the landfill site. The cold active enzymes opens a scope for their characterization that will help in understanding the mechanism of their action and will help the scientific community in designing more efficient enzymes for the betterment of humanity like their high applicability in industries that will help in energy conservation etc. Further the study also hypothesizes that the unique characteristics of the bacterial isolates may be due to three factors viz. the bacterial community, the organic waste materials as energy source and the external environmental conditions that may have altered the genetic setup of the bacterial isolates to produce the cold active enzymes to survive. Lastly, the landfill site opens the way for further scientific investigation for microbial profiling that may lead to the isolations of novel microbes adapted to extreme environmental conditions like cold temperatures, high leachate toxicity, etc.

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