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Isolation and characterization of psychrotrophic cellulolytic bacteria from landfill site under temperate climatic conditions

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Abstract

In temperate regions due to lower temperatures the decomposition of cellulosic material is slow due to reduced metabolic activities of microbes. However, psychrotrophic bacteria could be isolated and used for enhanced decomposition of accumulating biodegradable municipal waste. Hence the present study was aimed at the isolation and characterization of psychrotrophic cellulolytic bacteria with efficient enzyme activities. By following serial dilution and spread plate technique a total of 8 psychrotrophic cellulolytic bacteria were isolated on carboxy methyl cellulose agar media (CMC) at pH of 7.0 and temperature of 15°C after 48 hours. The isolates were screened for carboxymethyl cellulase (CMCase) activities qualitatively through congo red dilution assay and quantitatively through Dinitrosalicylic acid (DNS) method at different temperatures and incubation periods. Qualitative analysis revealed significant enzyme productions by isolates through formation of hydrolysis zones on CMC agar media with the isolate CB_2 producing maximum hydrolysis zone diameter of 16mm after 72 hours. Quantitative CMCase analysis was in accordance with qualitative test and the isolate CB_2 again showed highest CMCase activity of 2.33UmL^{-1}.Based on morphological, biochemical and molecular characteristics (16SrRNA analysis) the isolate CB_2 showed 96% similarity with *Bacillus flexus*. It was concluded that the study taken was a new work from the region and the isolates showed good enzyme activities at lower temperatures that could be either used for enhanced waste decomposition or could have industrial applications.

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Introduction

Kashmir valley is a temperate climatic region of state Jammu and Kashmir, India and Srinagar city is the summer capital of the valley. The city generates about 575 tones of municipal solid waste per day with biodegradable waste content of 55% (CPCB, 2011) that is disposed at Achan sanitary landfill site that lies between 34° 7' 00" North and 74° 47' 38.08"East and has surface area of 67.5 acres. Generally the biodegradable waste fraction contains about 40-50% of cellulose (Gautam *et al*, 2010) however, the decomposition of cellulosic waste slows down during cold environmental conditions due to slower and altered microbial activity. The literature reveals that, the psychrotrophic microbes are not present only in the areas that are permanently cold but also inhabit the areas that experience seasonal variations in temperatures during late fall and spring (Baghel *et al*, 2005). Therefore, the present site has the probability of habiting some psychrotrophic cellulose degrading bacteria that could be used for enhanced decomposition of cellulosic materials of the waste and could have industrial applications. Hence, the interest was developed to study the nature of extracellular enzymes secreted by these cold adopted bacteria. Cellulose is the most abundant biomass on the earth and considered as one of the most important sources of carbon with annual biosynthesis by both land and marine plants of 0.85×10^{11} tonnes per annum (Rani *et al*, 2004; Das *et al*, 2010; Venkata *et al*, 2013). Cellulose is a valuable renewable energy source that has attracted the attention of scientific community and made the cellulose hydrolysis the subject of intense research and industrial interest (Nowak *et al*, 2005). The degradation or hydrolysis of cellulosic material is a complex process performed by the participation of enzymes called cellulases that are secreted by cellulolytic bacteria isolated from cellulose rich habitats (Doi *et al*, 2008; Gautam *et al*, 2010, Shaikh *et al*, 2013). The potential cellulase producing bacteria are *Bacillus spp., Cellulomonas, Pseudomonas, Thermoactinomycetes* (Godana *et al*, 2007). However a very little work has been done for the isolation and characterization psychrophllic bacteria that show metabolic functions at low temperatures by incorporating unique features in their proteins and membranes (Georlette *et al*, 2004).

These microbes also show the synthesis of cold-shock or antifreeze proteins as the temperature drops that encourages their more efficient enzyme activity. Hence the interest was developed to study the isolation and characterization of psychrotrophic cellulolytic bacteria that could be used for the development of efficient bacterial consortium for rapid composting of biodegradable solid waste under temperate conditions. Further no such work has been done to the best of our knowledge and characterization of the isolates would serve as a base line data for characteristics of cold adapted cellulase producing microorganisms.

Material and methods

Site description and collection of samples

Kashmir valley is a temperate region and Srinagar city is the summer capital of the valley with average annual minimum and maximum temperatures of 7.1°C and 19.5 °C (Indian Meteorological Department) respectively. The sampling site for the study was Achan landfill site located in Srinagar city that lies between 34° 7' 00" North and 74° 47' 38.08" East (Fig.1). The waste samples mixed with soil were collected aseptically and transported intact at ambient temperature in sealed polythene bags to the laboratory for analysis.

Isolation of Cellulolytic Bacteria

The bacteria were isolated on carboxy methyl cellulose (CMC) agar media containing 1% CMC, 1% peptone, 0.25 % $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.03 % $MgSO_4.7H_2O$, 2% agar and pH of 7.0.One gram of waste was taken and diluted in a test tube containing 9ml of sterilized distilled water, shaken thoroughly and serial dilution was performed in order of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5},10^{-6}.After dilutions, 1 mL inoculum of serially diluted samples was spread on CMC plates and were incubated at 15°C for 48 hours. The bacterial colonies with clear hydrolysis zones around were purified by repeated streaking and preserved at 4°C on slanted agar media for further analysis.

Fig. 1. Location map of Achan landfill site.

Screening of isolated strains through detection of carboxymethyl cellulase (CMCase) activity

The isolated bacterial strains were screened for CMCase activity qualitatively and quantitatively by performing congo red assay and carboxymethyl cellulase (CMCase) assay respectively as under:

Congo Red Assay for CMCase Producing Activity of Bacterial Isolates

For zone determination, cultures were grown in circular batches on carboxymethyl cellulose agar media plates and were screened for this enzyme activity. The plates were incubated inverted at 15°C for different incubation periods viz. 24 hours, 48 hours and 72 hours. After incubation CMC agar plates were flooded with 1 % congo red and allowed to stand for 15 min at room temperature. 1M NaCl was used for counterstaining the plates. Clear zones were observed around growing bacterial colonies indicating cellulose degradation. The diameter of the hydrolysis zone were measured in order to select the highest CMCase producer (Arrifin *et al*, 2006).

Preparation of inoculums and CMCase production media

For preparation of standard bacterial inoculums, the selected bacterial isolates were individually cultured in different flasks each containing 20 ml inoculums media with composition 1% CMC, 1% peptone, 0.25 % $(NH4)_2SO_4$, 0.2 % K_2HPO4, 0.03 % $MgSO_4.7H_2O$ and pH was adjusted to 7.0 and incubated at 15°C for 48 hours to obtain an average viable count of 2-3.5 × 10^6CFU /ml of culture media.

These were used as inoculums for CMCase production media. Then 5% of each standard inoculum was separately inoculated in 250 ml Erlenmeyer flasks containing production media with similar composition as that of standard inoculums. The flasks were incubated at 0°C, 5°C, 10°C, 15°C and 20°C on a rotary shaker at150rpm for 72 hours.

Preparation of crude enzyme

After incubation the different cultures were withdrawn and centrifuged at 14000× g for 10 minutes at 4°C and the supernatant served as crude enzyme extract. The crude enzyme solution was

utilized for the estimation of enzyme activities as under:

Carboxymethyl cellulase (CMCase) assay of bacterial isolates

CMCase activity was determined as per the standard method (Miller, 1959) by estimation of reducing sugars (glucose) released from carboxy methyl cellulose (CMC) substrate. 1ml of crude enzyme was added to 1ml of 1 % CMC in 0.05 M phosphate (pH.7.0) buffer and incubated at temperature of 15°C for 60 minutes. After incubation, the reaction was stopped by the addition of 3ml of DNS reagent and boiled at 100°C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm and by using glucose calibration curve. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1μmol of glucose per minute per ml under standard assay conditions. Following formula was used to calculate the enzyme activity (Pokhrel *et al*, 2014).

$$\frac{\text{Units/ml}}{\text{ml}}\ \text{Enzyme(U)} = \frac{\mu\text{moles of Glucose released}}{\text{Time of assay} \times \text{Molecular weight of glucose} \times \text{volume of substrate}} \times 1000$$

Identification of isolated bacterial strains

The bacteria were identified by morphological, biochemical and molecular characteristics as under:

Morphological and biochemical characterization**:** Bacterial isolates were identified on the basis of morphological and biochemical characterization by using standard identification tests given in Bergey's Manual of Determinative Bacteriology (Logan *et al*, 2009).

Molecular characterization: The pure culture of the efficient bacterial isolate was grown overnight in nutrient broth media for the extraction of Deoxyribose nucleic acid (DNA). The bacterial DNA was isolated by using HiPurA™ Bacterial Genomic DNA Purification Kit and the 16SrRNA of the DNA was amplified by Thermocycler by using bacterial universal primers 27F (5' AGAGTTTGATCCTGGCTCAG3 ') and 1492 R (5 '-GGTTACCTTGTTACGACTT- 3'). The polymerase chain reaction (PCR)\was carried out in a final volume of 50 µL reaction mixture containing 1µL DNA, 2 µL of forward and reverse 20 picomole/ µl primer, 1µL dNTP mix (10 mM) , 3 µL $MgCl_2$ (25 mM) , 5 µL tris-buffer (10X) , 0.3 µL Taq DNA polymerase (5 units/µL) and 38 µL dionized sterilized water. PCR mixture was mixed gently and centrifuged for few seconds. The reaction mixture was thermocycled (Bioneer, Korea).The PCR conditions were as follows: Denaturation at 94°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 55°C for 45 seconds and 72°C for 45 seconds and final extension of 72°C for 10 minutes. The electrophoresis of amplified PCR products was carried on an agarose gel prepared by dissolving 1.5 g of agarose and 2-5 µL of ethidium bromide in 100ml TAE buffer and heating the solution on an oven. A 1500 bp DNA ladder was used as molecular weight size markers. The electrophoresis was performed for approximately 45 min at 80 mA and visualized. Polymorphic bands were observed by high performance UV transilluminator (Bioneer, Korea) and images of the gels were visualized by GEL DOC/Bio-Imaging System affiliated digital camera. The amplified product was sent for sequencing to Sci Genom Labs Pvt. Ltd., Cochin, Kerala, India). The unknown organism was identified using the maximum aligned 16SrRNA sequences available in the Gen Bank of NCBI through BLAST search. The best sequence alignment results were noted.

Statistical analysis

The enzyme activity of each isolate was performed in triplicates and the results were reported as mean. The complete random design was used to calculate the standard error and critical difference and the values were considered significantly different from each other at P≤ 0.05.

Results and discussion

Isolation of cellulolytic bacteria

A total of 20 morphologically different bacteria were obtained and out of these bacteria only 8 isolates were cellulolytic by forming hydrolysis zone on CMC agar media as shown in Table 1 and Fig.2.

All the bacteria isolated in the study were psychrotolerants (cold tolerant) as they were able to grow at 0°C and showed optimum growth between 15-20°C (Morita *et al*., 1997). The presence of cellulolytic bacteria could be attributed to the presence of cellulosic material in the municipal solid waste (Sivakumar *et al*, 2016, Das *et al*, 2010) and this view was also sheared by Sivakumar *et al*., 2016 and Das *et al*., 2010. In a similar study 50 mesophyllic cellulolytic bacteria were isolated from landfill soil (Masngut *et al*., 2017). In another study cellulolytic bacteria were isolated and enumerated from landfill refuse at 36°C (Pourcher *et al*, 2001).

Table 1. Isolation of bacteria from waste.

Sample No	Number of bacterial isolates	Number of cellulolytic bacterial isolates
S_1	21	6
S_2	19	4
S_3	20	5
S_4	17	7
S_5	25	8
S_6	23	7
S_7	21	3
S_8	20	4
S_9	22	7
S_{10}	20	8
Total	20	8

Table 2. Mean diameter (mm) of hydrolysis zone of isolates at 15°C after different incubation periods.

Isolate	Incubation period		
	24hours	48hours	72hours
CB_0	0	0	0
CB_1	8	9	11
CB_2	11	13	16
CB_3	6	6	8
CB_4	7	8	11
CB_5	3	4	7
CB_6	6	7	10
CB_7	2	3	6
CB_8	9	11	13
SE(±)	1.59	1.625	1.87

SE = standard error

CD= critical difference

mm = millimeter.

Screening of isolated strains for cellulolytic activity

Congo Red Assay for CMCase activity

All the isolates showed significant CMCase activity over control (0) by producing hydrolysis zones around the colonies after different incubation periods and at 15°C of temperature as shown in Table-2 and Fig.3. As evident from Table 2 all isolates showed increased CMCase activity with increase in incubation period from 24 hours to 72 hours at temperature of 15°C. The diameter of hydrolysis zone ranged from 2

to 16mm and the isolate CB_2 was most efficient by producing largest hydrolysis zone of diameter 16mm. Congo red shows a specific interaction with the hemicellulosic substrates and when the substrate is broken down by the action of cellulases, the congo red no longer has the affinity to the substrate and a hallo will appear on the medium (Teather *et al*, 1982). The highest enzyme activity of the isolate CB_2 at lower temperature might be due to its psychrotrophic nature (Ahamd *et al*, 2013) and high potential for the production of CMCases (Ghimire *et al*, 2016). In the previous study the diameter of hydrolysis zones of isolates ranged between 1 to 13mm at 37°C after 48 hours (He *et al*, 2015). Thus it can be concluded that the isolates of present study were efficient CMCase producer at lower temperatures that reflects their industrial and biotechnological demand.

Table 3. CMCase activity (Uml^{-1}) of bacterial isolates at different temperatures after 72 hours of incubation period.

Temperature	Isolates								
	CB_0	CB_1	CB_2	CB_3	CB_4	CB_5	CB_6	CB_7	CB_8
Control	0.001	0.15	0.5	0.09	0.13	0.05	0.1	0.05	0.21
5°C	0.002	0.31	0.9	0.3	0.42	0.25	0.315	0.2	0.47
10°C	0.004	0.7	1.5	0.6	0.77	0.5	0.61	0.4	0.8
15°C	0.006	1.13	2.3	1.07	1.103	0.75	1.081	0.65	1.5
20°C	0.008	1.17	2.33	1.06	1.121	0.77	1.1	0.67	1.54
SE (±)	0.002	0.07	0.09	0.0661	0.033	0.025	0.065	0.0019	0.051
CD (=0.05)	0.011	0.15	0.21	0.103	0.101	0.09	0.	0.07	0.185

U = μ mol glucose/ minute /ml

SE= standard error

CD= critical difference.

Carboxymethyl Cellulase (CMCase) Assay

Data presented in Table -3 reveals that all isolates produced significant quantities of CMCases in comparison to control (0.008 Uml^{-1}). The isolates showed a good CMCase activity at 0°C that ranged between 0.050 Uml^{-1} to 0.500 Uml^{-1}. However, maximum activity was shown by all isolates at 20°C that ranged between 0.67 Uml^{-1} to 2.33 Uml^{-1}. Among all isolates , the isolate CB_2 showed highest CMCase activity of 2.33 Uml^{-1}followed by CB_8, CB_1, CB_4, CB_6, CB_3, CB_5 and CB_7 with values of 1.54 Uml^{-1}, 1.170 Uml^{-1}, 1.121 Uml^{-1}, 1.100 Uml^{-1}, 1.060 Uml^{-1}, 0.770 Uml^{-1} and 0.670 Uml^{-1} respectively.

Table 4. Morphological, Biochemical and Molecular Characteristics of efficient isolate.

Isolate	Morphological characteristics								
	Colony features							Cell features	
	Colony color	Colony shape	Margin	Elevation	Surface	Visual characteristic		Grams nature	Cell shape
CB2	White	Round	Entire	Raised	Smooth	Opaque		Gram +	Long bacilli
	Biochemical characteristics								
	Indole	M.R	Citrate	Oxise	Catalase	Casein Hydrolysis	Starh hydrolysis	Urease	V.P
	-	+	-	+	+	+	+	+	+
	Molecular characteristics.								
	Max. Score	Percent Identity	16SrRNA Sequence Length			Best match using BLASTn		Accession Number	
	1986	96%	823			*Bacillus flexus* gene for 16SrRNA		MH266216	

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 (Sethi *et al*, 2013). However, the increased CMCase activities of isolates from 0 to 20°C could be attributed to the psychrotrophic nature of the bacterial isolates and the changes in physical membrane structure with increased temperatures that influences the secretion of extracellular enzymes (Rasul *et al*, 2015).

Fig. 2. Isolates forming hydrolysis zone on CMC agar media.

The reductions in enzyme activities of the isolates at temperatures and incubation higher than 20°C and 72 hours respectively could be attributed to reduction in nutrients and cell death in the media (Arimurti *et al*, 2017). In a similar study CMCase activity of isolate SM-3Mn (8) was found as 3.547 IU/ml at 40°C after 72 hours of incubation period (Rasul *et al*., 2015).

In another study the highest CMCase activity of 0.37U/ml of the isolate PA_2 was reported at 37°C after 48 hours of incubation (Ahmad *et al*, 2013).

Based on these results it was concluded that the isolates of the present study showed effective CMCase activities at lower temperatures that offers their great applicability for waste management at colder regions and industrial use.

Identification of efficient bacterial isolate

As clear from Table 4 the morphological characteristics of the isolate showed that the colonies were white in color, round in shape, margins were entire, smooth surface, visually opaque and the cells were rod shaped and violet colored.

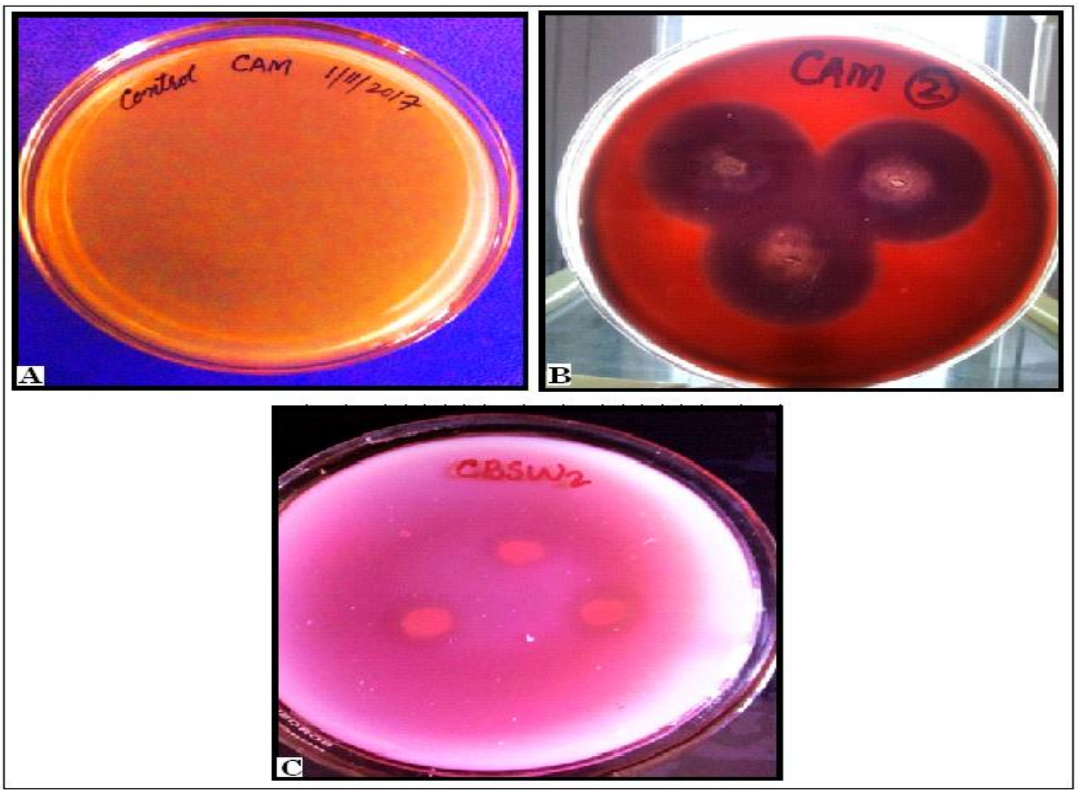

Fig. 3. Control (A) and hydrolysis zone of isolates on CMC congo red agar media (B&C).

The cultural characteristics interpreted from Bergey's manual of Systematic Bacteriology (Logan *et al*, 2009) were indistinguishable, hence the isolate could be identified as *Bacillus sp*.

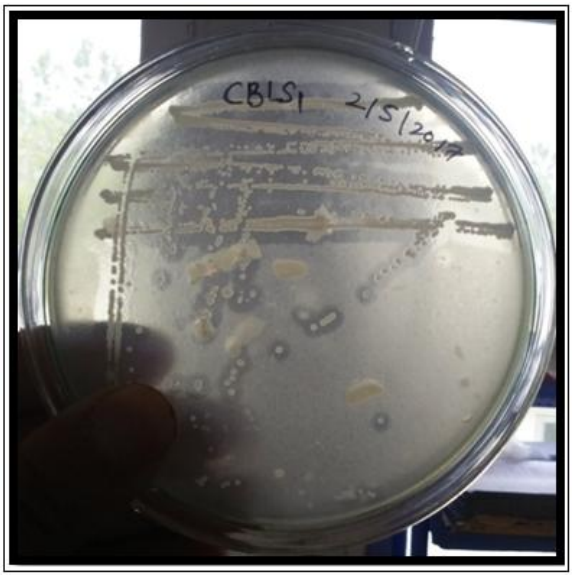

Fig. 4. Pure culture of isolate CBAW2 with identical colonies.

The results of biochemical characteristics of the isolate were also found similar to those demonstrated by Logan *et al*, 2009) and both the results confirmed it as *Bacillus sp*. All the biochemical test were not carried due to the limited resources available and hence the genus of the isolate could not be fully determined. However from the previous studies biochemical characteristics identified the bacterial isolates as Bacillus sp. (Rasul *et al*, 2015; Ahamd *et al*., 2013).

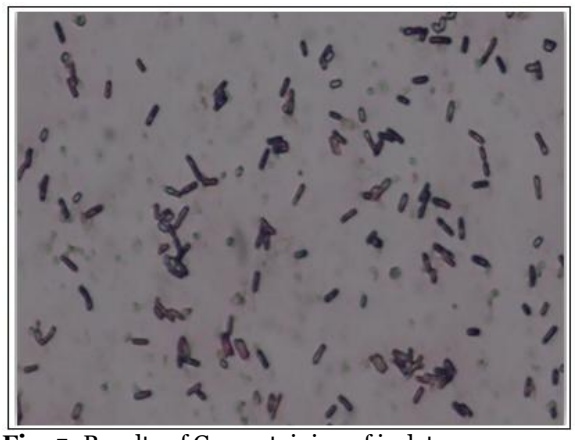

Fig. 5. Results of Gram staining of isolate.

The results could identify the strain only upto genus level and hence molecular characterization was employed which is best identification method (Arimurti *et al*, 2017) and can characterize the strains upto species level. Based on 16SrRNA partial sequencing the isolate showed 96% similarity with *Bacillus flexus* (Accession no. MH266216).

In previous study the isolation of *Bacillus flexus* was reported at higher temperatures (80^{o}C) (Lin *et al*, 2017) but the same species was isolated at lower temperature conditions that might be due to the adaptations of the bacterial species to different environments (Baghel *et al*, 2005). This bacterial species is rarely present in landfill wastes and other studies reported different bacterial sp. of *Bacillus subtilis* (Pokhrel *et al*., 2014; Egbere *et al*, 2017), *Mycobacterium testacium* (Vimal *et al*, 2016). The bacteria isolated from the waste and screened for their cellulose degrading potential could be used for accelerated composting of municipal solid waste during the cold conditions.

Conclusion

During the investigation a number of cold active cellulolytic bacteria were isolated that showed significant cellulase activities at lower temperatures. The best cellulolytic bacteria showing maximum carboxymethyl cellulase activity was identified as *Bacillus flexus* with 96% similarity. The isolate is a rarely found cellulose degrading bacteria that could be used for the development of bacterial suspension for enhanced composting of organic wastes at lower temperature conditions.

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isolation and culturing of different bacteria is also duly acknowledged.

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