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Bacterial Diversity towards Industrially Important Enzyme Producers from Velliangiri Hills, Western Ghats

Logeswaran. R, Prabagaran. S. R. P and Ramesh. D
Department of Biotechnology, Bharathiyar University, India

Abstract: *The Velliangiri hill of Western Ghats in Tamilnadu was chosen for a comprehensive study on bacterial diversity because there was no previous report on bacterial biodiversity from this region. Soil samples were collected from 7 sub hills, Velliangiri hills. Culture dependent studies were performed to explore bacterial species from collected samples. Overall 101 bacterial isolates were isolated based on their characteristical and microscopical observations. Statistical analysis (Shannon & Simpson) was recorded. From the results of statistical analysis the bacterial colonies were dominant and more diverse in the Aandisunai hill and less diverse in Paambaatti hill. The Simpson's index is always reciprocal to Shannon index. The bacterial isolates which were isolated from Velliangiri hills had the ability to produce different enzymes like amylase, cellulase, pectinase, proteinase, and lipase. Among the isolates, the 10 enzyme producing bacterial cultures were screened based on their ability to produce more than two enzymes. Based on the protein profiling techniques (using MALDI analysis), the isolates were identified. They are Bacillus mycoides, Rhodococcus equi, Sphingomonas koreensis, Bacillus subtilis, Clostridium clostridioforme, Bacillus mojavensis, Pseudomonas alcaligenes, and Paenibacillus glucanolyticus. Among the bacterial isolates, Pseudomonas alcaligenes alone produces all the five enzymes. Hence, the diverse environment of Velliangiri hills provides sufficient diversity to explore the place for various microorganisms for bioprospecting.*

Keywords: *Velliangiri hills, bacterial diversity, statistical analysis, enzyme producer, MALDI*

I. Introduction

The biological diversity of the Indian subcontinent is one of the richest in the world owing to its vast geographic area, varied topography, climate, and the concurrence of several bio geographical regions. Because of its richness in overall species diversity, India is recognized as one of the 17-mega diversity regions of the world. India's contribution to the global diversity is around 8%. The Western Ghats of India is one of the ''mega biodiversity hotspots'' of the world (UNESCO, 2012).

Knowledge about bacterial community structure and diversity is essential to understand the relationship between environmental factors and ecosystem functions. Such knowledge can be used to assess the effect on ecosystems of environmental stress and perturbations like pollution, agricultural exploitation and global changes. It is also realized that the functional and genetic potential of the microorganisms may exceed that of higher organisms, and provide a valuable source for novel products and technologies. Despite their importance, at the present we do not even know the magnitude of the bacterial diversity in most ecosystems. The measure of bacterial diversity describes the qualitative variation among these organisms in a community or an assemblage. Diversity can also be regarded as the amount of information or 'species' richness in a community . More commonly the diversity concept in addition to the richness also includes an evenness component, which take into account how the information is distributed among the individuals in a community.

Soil is a complex habitat, inhabited by a large number of different organisms. Among these, bacteria and fungi are the most important since they are responsible for the vast bulk of decomposition, and also make up the largest part of the biomass in soil. Bacteria are the most abundant microorganism group in soil and can attain concentrations of more than 10^8 cells per gram of soil [1] or 10^{11} per gram organic material. While the number of different species/genomes per gram of soil is generally not known, the estimates from 10^4 to 10^6 have been documented [1]. The supply of carbon is usually considered the limiting factor for growth of the bacterial community, even in soils high in organic matter. However, there are also studies suggesting that nitrogen and phosphorus might be limiting in some soils [2]. Western Ghats contains diverse bacteria from their soil when plated on nutrient agar [3]. Microbial communities are diverse in Western Ghats and almost unexplained reservoir of resources likely to provide innovative applications useful to mankind.

The sampling sites are diverse not only by microorganisms but also by their climatic condition, soil nature, latitude, longitude, and their elevation of the hill. Velliangiri is located in the south side of Western Ghats, which can be easily reached from Coimbatore, Tamil Nadu. There was no previous scientific literature with respect to bacterial diversity in Velliangiri hills. Due to the diverse biotic and abiotic condition of the

Velliangiri hills, microbes may have a major role in their enzymes production in order to protect them from the extreme conditions.

Enzymes have wide variety of applications in many fields. The application of enzymes to organic synthesis is currently attracting more and more attention. Chemical methods of synthesizing enzymes are time and money consuming. Microorganisms can be manipulated for the production of enzymes which are commercially important. Enzymes like amylase, cellulase, pectinase, proteinase, and lipase finds a wide variety of application in organic compound synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation [4] and [5]. Each microbe will be unique for the production of various enzymes of industrial and commercial value. Therefore, in searching of the multi enzymes producing microorganisms will be able to reduce the cost wise and easy to reduce the contamination during the maintenance as pure culture. These enzyme-producing microorganisms were identified based on their ribosomal proteins.

II. Materials and methodology

2.1. Sample collection and sampling sites

Soil samples were collected by using sterile spatula and polythene bags. The collected samples were labelled and transported to the laboratory and processed immediately for isolation of bacterial colonies.

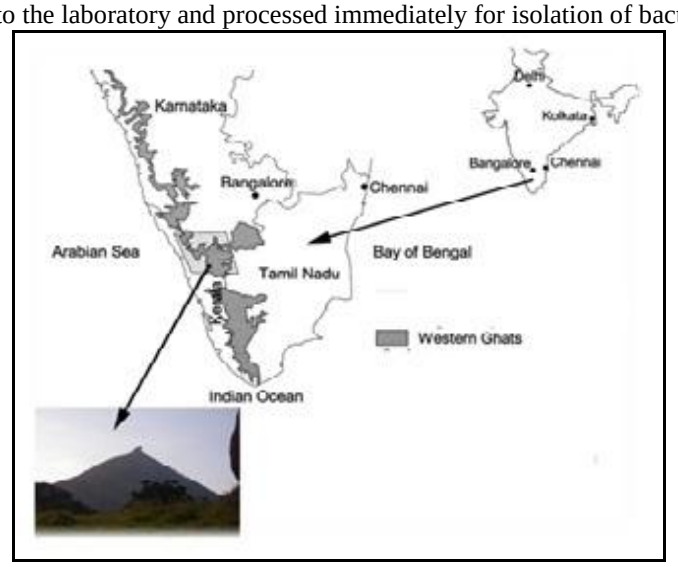

Fig.1. Location of the Velliangiri hill in Western Ghats

2.2. Determination of soil characteristic features

Soil temperatures were measured by using a mercury thermometer at the depth of 10 cm in the hill. Soil pH is a measure of the activity of ionized Hydrogen (H^+) in the soil solution. Alkaline pH rather than acidic [6], influence the diversity of the bacteria. The pH of soil is potentiometrically measured in the supernatant suspension of 1:2 of soils: liquid (w/v) mixture, where the liquid is distilled water. For the determination of soil pH, 5 grams of air-dried soil weighed into a 100 mL beaker. Then 20 mL of distilled water was added in each beaker stirred periodically with a glass rod for a period of 30 minutes. Soil suspension was let to stand for 10 minutes. After that, the pH was readed and recorded.

2.3. Determination of soil moisture

Soil moisture is the measure of the water content (humidity) in the soil sample. The soil moisture content may be expressed by weight as the ratio of the mass of water present in the dry soil to the volume of water to the total volume of the soil sample before drying. To determine any of these ratios for a particular soil sample, the water mass must be determined by drying the soil to constant weight and measuring the soil sample mass after and before drying. The water mass (or weight) is the difference between the weights of the wet and oven dry samples. The criterion for a dry soil sample is the soil sample that has been dried to constant weight in oven at temperature between 100 – 110°C (105^0C is typical). It seems that this temperature range has been based on water boiling temperature and does not consider the soil physical and chemical characteristics

Empty container was weighted, and recorded this weight and mentioned as tare. Soil sample was taken for about 10g and weighed (wet soil+tare container). Sample was placed in the oven at 105^0C and dried it for 24 hours or overnight. Sample was again weighed, and recorded this weight as weight of (dry soil + tare container). Samples were returned to the oven and dry for several hours, and determine the weight of dry soil + tare container. Again, step was repeated until there is no difference between any two consecutive measurements of the weight of dry soil+ tare.

The moisture content in weight percentage is obtained by:

$$\text{Moist \%} = \frac{A-B}{B - \text{tare container}} \times 100$$

Where,
A: Weight of tared moisture tin and air-dried soil sample
B: Weight of tared moisture tin and oven-dried soil sample
Tare container: Weight of the aluminium container

2.4. Enumeration and Isolation of Bacteria.

Bacteria were isolated after serial dilution of soil samples and plating on nutrient agar medium (HiMedia, India) prepared in distilled water. Different types of media (Table 1) were used for Nutrient medium was Peptone 1g, Yeast extract 0.5g and Sodium chloride 1 g; 1.5 g of agar per 100ml of distilled water. The plates were incubated for 24 hours at 37^{o}C. Morphologically different isolates from the soil samples were isolated and purified for MALDI analysis and for enzymatic studies.

Table 1. Enrichment media and their composition

	Acetic acid medium (g)	Tryptone soy agar (g)	Peptone agar (g)	Gelatin agar (g)	Nutrient agar (g)		
					1X Nutrient agar g/L	1/2X	1:100 X
Peptone	5	-	1	4	5	2.5	10 ml of nutrient broth(1X)
Tryptone	-	15	-	-	-	-	-
Yeast Extract	5	-	-	1	3	1.5	-
Glucose	5	-	-	-	-	-	-
Beef Extract	-	-	-	-	2	1	-
Dipotassium phosphate	-	-	-	-	-	-	-
Sodium chloride	-	5	1	-	5	2.5	-
Magnesium sulphate	1	-	-	-	-	-	-
Soy peptone	-	5	-	-	-	-	-
Gelatin	-	-	-	15	-	-	-
Dist. H2O	1000 ML	1000 ML	1000 ML	1000 ML	1000 ML	1000 ML	990 ML
Agar	20	20	20	20	20	20	20

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2.5. Morphological characterization of bacteria

2.5.1. Colony morphology

A pure culture of bacteria was cross-streaked on a nutrient agar plate and incubated at 37^{o}C for 24 hrs. The colony colour, form (i.e., whether it is punctiform, circular, irregular or mucoid, etc.), elevation (flat, elevated or convex, etc) and the nature of the margin (entire, undulate, lobate or smooth, etc) were noted. Further, the opacity of the colony (opaque, transparent or translucent) was also scored.

2.5.2. Cell morphology

A freshly grown culture of bacteria in the log phase was used for determining the cell morphology. For this purpose, 10µl of the culture was spotted on a slide and observed under the microscope on oil immersion at 100X. The shape of the cell (rod, cocci, curved, spiral, coccobacillary or pleomorphic etc.) was noted down.

2.5.3. Gram staining

Gram staining is an extremely important character based on which all bacteria can be differentiated into two distinct groups: the Gram-positive and the Gram-negative bacteria. Christian Gram first developed the technique in 1884 and is based on the principle that the Gram-positive bacteria retain the crystal violet stain after alcohol wash due to the presence of teichoic acid in the cell wall whereas Gram–negative do not retain the stain.

2.6. Calculation of Diversity Index

A range of diversity indices have been used with bacterial communities, in particular the ubiquitous Shannon index, the evenness indices derived from it, and Simpson's dominance index and its equitability index [7], [8] and [9].

The Shannon index (H) is calculated by taking the proportion of species *i* relative to the total number of species (*pi*) is calculated, and then multiplied by the natural logarithm of this proportion (*lnpi*). The resulting product is summed across species and multiplies by -1.

Shannon's equitability (E_H) is calculated by dividing H by H_{max} (here H_{max} = ln S). Equitability assumes a value between 0 and 1, with 1 being complete evenness [10].

The Simpson's index is calculated by taking the proportion of species *i* relative to the total number of species (*pi*) calculated and squared. Simpson's Index measures the probability that two individuals randomly selected from a sample will belong to the same species.

Simpson's equitability (E_D) is calculated by taking Simpson's index (D) and expressing it as a proportion of the maximum value D could assume, if individuals in the community were completely evenly distributed.

Table 2. Diversity index measures

Measures	Formula	Reference
Shannon H' Index	$H' = -\sum_{i=1}^{S} (p_i \ln p_i)$ H'=the value of the Shannon diversity index *pi*= the proportion of the *i*th species ln = the natural logarithm of *pi* *S* = total number of species in the community ∑ = sum from species 1 to species S	Case *et al*., 2007
Equitability E_H	$E = H'/H = H'/\ln S$ H'= the value of the Shannon diversity index *pi*= the proportion of the *i*th species ln = the natural logarithm of *pi* *S* = total number of species in the community ∑= sum from species 1 to species S	Oguntoyinbo *et al.*, 2007
Simpson's D index	$D = \sum_{i=1}^{S} p_i^2$ D = the value of the Simpson's diversity index p_i^2=the proportion of the *i*th species and squared *S* = total number of species in the community ∑= sum from species 1 to species S	Ahmad *et al.*, 2009
Equitability E_D	$1 - D = 1 - \sum_{i=1}^{S} p_i^2$ D = the value of the Simpson's diversity index p_i^2=the proportion of the *i*th species and squared *S* = total number of species in the community ∑= sum from species 1 to species S	Magurran, 2004

2.7. Screening of enzyme producers

The isolated strains were plated on respective enzyme substrates like amylase – 0.2% soluble starch; gelatinase – 0.4% gelatin; pectinase – 0.1% pectin; cellulase – 0.2% carboxyl methyl cellulose, lipase – 1% Tributyrin) and incubated 37°C for 24 to 48 hours. After incubation, the plates were treated with respective reagents, the zone of clearance around the bacterial colonies, which represents the positive enzyme producers [11].The cultures that showed growth or clearing zones when tested with respective reagents were considered as positive cultures. The isolates were also tested for enzyme activity at pH 3 to 11 and temperature 10°C to 60°C. Data from the three stations were pooled for a homogenous representation of Velliangiri region.

Table 3. Media composition for screening enzyme producers. g indicates grams

	Starch casein agar (g)	CMC agar (g)	Gelatin agar (g)	Pectin agar (g)	Tributyrin agar (g)
Ammonium sulphate	-	-		2	-
Starch	20	-	-	-	-
Casein	10	-	5	-	-
Sodium chloride	10	-	5	-	-
Yeast extract	-	0.5	2	1	-
Peptone	-	0.02	-	-	-
Carboxyl methyl cellulose	-	5	-	-	-
Potassium chloride	-	1	-	-	-
Magnesium sulphate	-	0.5	-	-	-
Dipotassium hydrogen phosphate	-	1	-	-	-

Sodium nitrate	-	1	-	-	-
Glucose	-	1	-	-	-
Disodium hydrogen phosphate	-	-	-	6	-
Potassium hydrogen phosphate	-	-	-	3	-
Pectin	-	-	-	5	-
Gelatin	-	-	3	-	-
Calcium chloride	-	-	0.2	-	-
Tributyrin agar	-	-	-	-	23
Tributyrin	-	-	-	-	10
Agar	20	20	20	20	5
Dist.H2O	1000ML	1000ML	1000ML	1000ML	990ML

2.8. Whole cell protein fraction based identification

Bacterial isolates from agar plate can be directly used for identification. Cultivation conditions have little effect for spectra generation. Media like nutrient agar was used for growth of the culture. Most often freshly grown culture was used, as the cultivated plates stored at 4^{o}C reduces the quality of the spectra. Culture was applied as a thin film directly on MALDI steel target plate. It was overlaid with 1µl of Matrix solution and dried for MALDI Biotyper analysis. Results obtained as spectra were matched with the database of Bruker (version 2.0), which provides the identification of the isolate.

III. Results And Discussion

3.1. Sample collection and Soil Analysis

Bacterial strains were isolated from soil samples obtained from diverse ecological niches of Western Ghats, situated 10^{0} 58'N to 10^{0} 59'N and 76^{0} 41'E to 76^{0} 43'E. The samples mostly include moist and dry soil collected from forest regions.

Table 4. Sampling sites and their global position

Hill	Place	Temperature °C	Height (feet)	Global Position Of Sample Collecting Sites		
				Latitude	Longitude	Altitude(m)
7th hill	Giri (The peak)	10- 20	7349	10° 59' N	76° 41'E	1742
6th hill	Aandisunai	20	5000	10° 59'N	76° 41'E	1513
5th hill	Beeman Kaliurandai	20	4500	10° 58'N	76° 42'E	1376
4th hill	Thiruneer hill / Muzhangaal Muttu	20 – 25	4000	10° 58'N	76° 42'E	1314
3rd hill	Kaithatti hill	20 – 25	3000	10° 58'N	76° 42'E	1218
2nd hill	Paambaatti hill	25 – 30	2000	10° 58'N	76° 42'E	909
1st hill	Vellivinayagar hill	25 – 30	750	10° 58'N	76° 43'E	528

Soil samples were collected from seven different sub hills of Velliangiri hills and subjected to different laboratory tests, which can be useful for the bacterial cultivation.

3.1.1. Soil Characteristic Features

The collected soil samples varied in their colour, structure, texture, moisture condition and consistency (Table 5 and Fig. 3).

Table 5. Morphological characteristic features of soil samples collected from Velliangiri hills

Sample	Colour	Structure	Texture	Soil Consistence	Moisture %
Vellivinayagar hill	Brown	Granular	Gravel	Moist	40 %
Paambaatti hill	Brown	Granular	Silty clay	Moist	55 %
Kaithatti hill	Brown	Granular	Gravel	Moist	45 %
Thiruneer hill	Sandal	Platy	Silt	Dry	5 %
Beeman Kaliurandai hill	Red	Granular	Gravel	Dry	10 %
Aandisunai hill	Thick brown	Granular	clay	Wet	60 %
Giri hill	Brown	granular	Silt	Moist	45 %

Atmospheric temperatures were lower in Velliangiri hills as like the other parts of Western Ghats [12]. The soil texture of Western Ghats and foothills was gravel and silty clay, due to the topography of the hill (Table 5). Moisture content was moderate in these regions when compared to other parts. Soil pH was more acidic in sampling sites than at other parts of Western Ghats (Table 6). The environmental and the edaphic factors influence the colour of the soil. The Species composition in the hill depends on the properties of soil because soil provides sufficient air to the biological systems in the hill (National Trust for Nature Conservation, 2012).

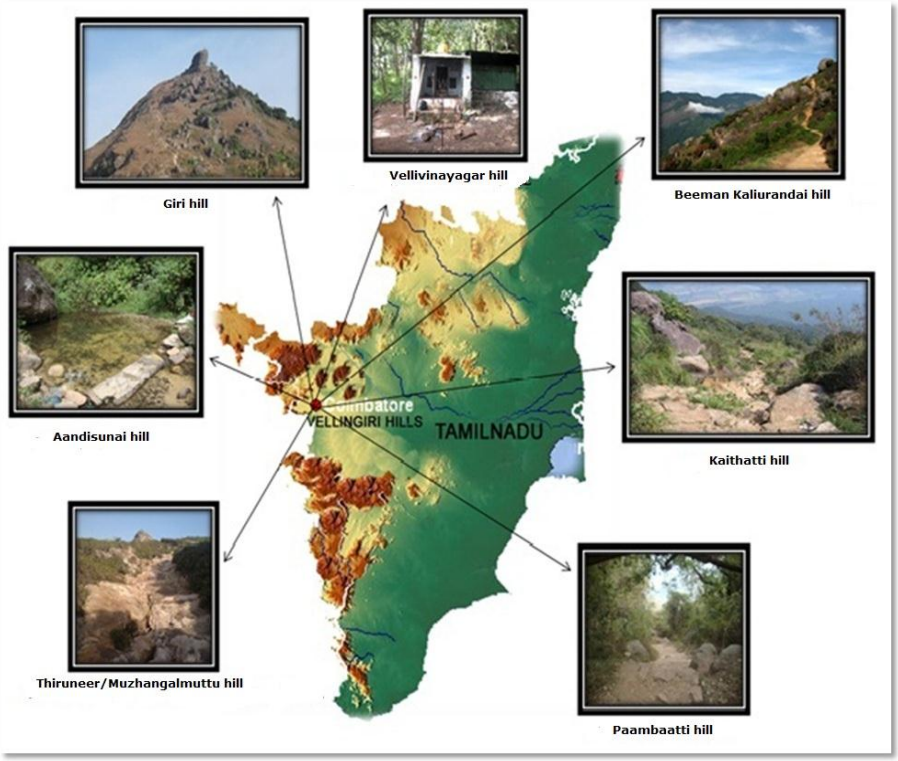

Fig. 2. Location of sampling sites in Velliangiri hills (Western Ghats)

Fig. 3. Soil collected from different hills of Velliangiri

3.1.2. pH

Soil samples are subjected to determination of pH, which ranged from the 4.84 to 6.86 (Table 6).

Table 6. pH of the soil samples collected from Velliangiri hills

Sample	pH of soil
Vellivinayagar hill	6.80
Paambaatti hill	6.71
Kaithatti hill	5.26
Thiruneer hill	4.95
Beeman Kaliurandai hill	5.58
Aandisunai hill	5.64
Giri hill	4.84

3.2. Bacterial Isolation

The total bacterial population in the soil sample was studied using serial dilution plating method. It is well known that the type of bacteria that are recovered by plating is influenced by the type of media used and conditions of growth used in the study, compared to the bacterial diversity from the natural environments [13] and [14]. While it may not be practical to use all possible media compositions and growth conditions for the study of bacterial diversity, culturing in more than one media will support more diverse type of bacteria than the use of a single medium. Totally five different types of media was used. Each media has the capable of producing the heterotrophic bacteria.

Totally 101 isolates were isolated from the seven hills of Velliangiri, Western Ghats based on their morphology and microbiological observations (Fig 4). The type of media used and their growth condition (Table 7) enhanced the isolation of diverse bacteria. High number of bacterial isolates was isolated from the Thiruneer hill, Beeman Kaliurandai hill and Aandisunai hills (24 isolates) compared to the rest. This may be due to their geographical influence and nutrient status of the soil [6]. Among the seven different hills, only Thiruneer hill, Beeman Kaliurandai hill, Aandisunai hill hills showed maximum bacterial isolates. This proves that pH also plays a vital role in the bacterial growth.

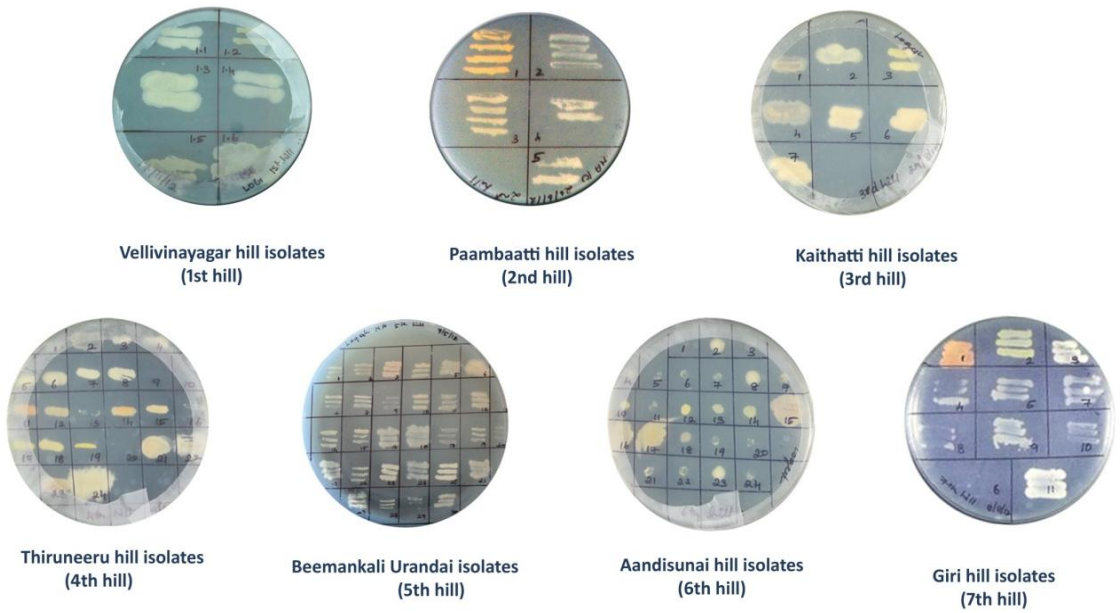

Fig. 4. Bacterial isolates from different regions of Velliangiri hill (Western Ghats)

In this study, diversity of cultivable bacteria was explored using different types of media with varying nutrient conditions viz, double strength, single strength (1X NA), half strength nutrient agar (1/2X NA), in 1:100 dilution, acetic acid medium, tryptone soy agar medium, peptone agar medium, and gelatin agar medium. Among these different types of media used, it appears that the nutritionally poor media 1/2X NA supported the growth of highest number of bacteria even though the number of morphologically distinct colonies was less. Totally 101 strains from all the different types of media (Fig. 4) were picked and preserved as glycerol stock.

Table 7. Different types of media and total number of colonies per gram

Sample	Medium used							No of isolates
	Nutrient agar			Acetic acid medium	Tryptone soy agar medium	Peptone agar medium	Gelatin agar medium	
	1X	0.5X	1:100					
Vellivinayagar hill	255	359	79	183	213	246	161	6
Paambaatti hill	200	212	79	172	212	219	156	5
Kaithatti hill	7	5	8	8	3	8	3	7
Thiruneer hill	125	92	53	82	112	123	73	24
Beeman Kaliurandai hill	76	120	82	22	35	63	49	24
Aandisunai hill	40	63	50	49	31	51	49	24
Giri hill	18	24	5	37	49	19	2	11
Total bacterial isolates								101

3.3. Phenotypic Characteristics

Attempts were made to isolate different morphotypes based on cell morphology and colony morphology. The configurations of most of the bacterial strains were circular except a few that were irregular. Majority of the strains were pale white (Fig. 4), circular, flat and entire. About 90% of the total strains have flat colonies; entire margin was predominant among the strains (Table 8).

Table 8. Microbiological observation of the bacterial isolates from Velliangiri hills.

Sample Site	Sample name	Configuration	Colour	Elevation	Margin	Surface	Grams staining	Shape
Vellivinayagar Hill	VV1	circular	White	Flat	Entire	Smooth	+ ve	Rod
	VV2	circular	pale white	Flat	Entire	Smooth	+ ve	Rod
	VV3	circular	White	Flat	Entire	Smooth	+ ve	Rod
	VV4	circular	White	Flat	Entire	Smooth	+ ve	Rod
	VV5	Irregular	pale white	Flat	Wavy	Smooth	+ ve	Rod
	VV6	Irregular	White	Raised	Wavy	Smooth	+ ve	Rod
Paambaatti Hill	P1	circular	Yellow	Flat	Entire	Smooth	+ ve	Cocci
	P2	Circular	Milky white	Flat	Entire	Smooth	- ve	Rod
	P3	Irregular	Pale yellow	Flat	Wavy	Smooth	+ ve	Rod
	P4	Irregular	Translucent	Flat	Wavy	Smooth	+ ve	Rod
	P5	Circular	Pale white	Flat	Entire	Smooth	+ ve	Rod
Kaithatti Hill	K1	Circular	Brownish white	Flat	Entire	Smooth	+ ve	Rod
	K2	Circular	Pale white	Flat	Entire	Smooth	+ ve	Rod
	K3	Circular	Yellow	Flat	Entire	Smooth	+ ve	Rod
	K4	Circular	White	Flat	Entire	Smooth	+ ve	Rod
	K5	Circular	White	Flat	Entire	Smooth	+ ve	Rod
	K6	Circular	Pale brown	Raised	Entire	Smooth	+ ve	Rod
	K7	Circular	White	Flat	Entire	Smooth	+ ve	Rod
	T1	circular	Pale yellow	Flat	Entire	Smooth	+ ve	Rod
	T2	Irregular	White	Flat	Entire	Smooth	+ ve	Rod
	T3	circular	White	Flat	Entire	Smooth	+ ve	Rod
	T4	circular	Pale yellow	Flat	Entire	Smooth	+ ve	Rod
	T5	circular	White	Flat	Entire	Smooth	+ ve	Rod
Thiruneer Hill	T6	circular	White	Flat	Entire	Smooth	+ ve	Rod
	T7	circular	Pale yellow	Raised	Entire	Smooth	+ ve	Rod
	T8	Irregular	White	Flat	Wavy	Smooth	+ ve	Rod
	T9	circular	White	Flat	Entire	Smooth	+ ve	Rod
	T10	circular	White	Flat	Entire	Smooth	+ ve	Rod
	T11	circular	Orange	Flat	Entire	Smooth	+ ve	Rod
	T12	circular	White	Flat	Entire	Smooth	+ ve	Rod
	T13	circular	White	Raised	Entire	Smooth	+ ve	Rod
	T14	circular	Orange	Flat	Entire	Smooth	+ ve	Rod
	T15	circular	darkish yellow	Flat	Entire	Smooth	+ ve	Rod
	T16	circular	Yellow	Flat	Entire	Smooth	+ ve	Rod
	T17	circular	Yellow	Flat	Entire	Smooth	+ ve	Rod
	T18	circular	Yellow	Flat	Entire	Smooth	+ ve	Rod
	T19	circular	White	Flat	Entire	Smooth	+ ve	Rod
	T20	circular	White	Flat	Entire	Smooth	+ ve	Rod
	T21	circular	Orange	Flat	Entire	Smooth	+ ve	Rod
	T22	Irregular	White	Flat	Wavy	Rough	+ ve	Rod
	T23	circular	White	Flat	Entire	Smooth	+ ve	Rod
	T24	Irregular	Orange	Flat	Wavy	Rough	+ ve	Rod

Beeman Kaliurandai Hill	BK1	circular	Rose	Flat	Entire	Smooth	+ ve	Rod
	BK2	circular	White	Flat	Entire	Granular	+ ve	Rod
	BK3	circular	White	Flat	Entire	Smooth	+ ve	Rod
	BK4	circular	Yellow	Flat	Entire	Smooth	+ ve	Rod
	BK5	Irregular	Yellow	Raised	Wavy	Rough	- ve	Rod
	BK6	circular	White	Flat	Entire	Smooth	+ ve	Rod
	BK7	circular	White	Flat	Entire	Smooth	+ ve	Rod
	BK8	circular	White	Flat	Entire	Smooth	+ ve	Rod
	BK9	circular	Rose	Flat	Entire	Smooth	+ ve	Rod
	BK10	circular	Yellow	Flat	Entire	Smooth	+ ve	Cocci
	BK11	circular	White	Flat	Entire	Smooth	- ve	Rod
	BK12	circular	White	Flat	Entire	Smooth	+ ve	Rod
	BK13	circular	Rose	Flat	Entire	Smooth	+ ve	Rod
	BK14	Irregular	White	Flat	Entire	Smooth	+ ve	Rod
	BK15	circular	White	Flat	Entire	Smooth	- ve	Rod
	BK16	circular	White	Flat	Wavy	Smooth	+ ve	Rod
	BK17	circular	Yellow	Flat	Entire	Smooth	+ ve	Rod
	BK18	circular	Rose	Flat	Entire	Smooth	+ ve	Rod
	BK19	circular	White	Flat	Entire	Smooth	+ ve	Rod
	BK20	circular	White	Flat	Entire	Smooth	+ ve	Rod
	BK21	circular	White	Flat	Entire	Smooth	+ ve	Rod
	BK22	Irregular	Yellow	Flat	Entire	Smooth	+ ve	Rod
	BK23	circular	Pale rose	Flat	Entire	Smooth	+ ve	Rod
	BK24	circular	White	Flat	Entire	Smooth	+ ve	Rod
Aandisunai Hill	AS1	circular	White	Flat	Entire	Smooth	- ve	Rod
	AS2	Irregular	shiny white	Raised	Wavy	Smooth	+ ve	Cocci
	AS3	circular	White	Flat	Entire	Smooth	+ ve	Rod
	AS4	circular	Yellow	Flat	Entire	Smooth	+ ve	Cocci
	AS5	circular	White	Flat	Entire	Smooth	- ve	Rod
	AS6	circular	Yellow	Flat	Entire	Smooth	- ve	Cocci
	AS7	circular	White	Flat	Entire	Smooth	+ ve	Rod
	AS8	Irregular	shiny white	Flat	Wavy	Smooth	- ve	Cocci
	AS9	circular	Yellow	Flat	Entire	Smooth	- ve	Rod
	AS10	circular	Orange	Raised	Entire	Smooth	+ ve	Rod
	AS11	circular	Pale yellow	Flat	Entire	Smooth	+ ve	Rod
	AS12	circular	Yellow	Flat	Entire	Smooth	+ ve	Cocci
	AS13	circular	White	Flat	Entire	Smooth	+ ve	Rod
	AS14	circular	White	Flat	Entire	Smooth	+ ve	Rod
	AS15	Rhizoid	White	Flat	Wavy	Smooth	- ve	Rod
	AS16	Irregular	White	Flat	Wavy	Smooth	+ ve	Rod
	AS17	Irregular	White	Flat	Wavy	Smooth	- ve	Cocci
	AS18	circular	brick red	Flat	Entire	Smooth	+ ve	Rod
	AS19	circular	White	Flat	Entire	Smooth	- ve	Cocci
	AS20	circular	Yellow	Flat	Entire	Smooth	+ ve	Rod
	AS21	circular	White	Flat	Entire	Smooth	+ ve	Rod
	AS22	circular	Pale yellow	Flat	Entire	Smooth	- ve	Cocci
	AS23	Irregular	White	Raised	Wavy	Smooth	+ ve	Rod
	AS24	circular	White	Flat	Entire	Smooth	- ve	Rod
Giri Hill	G1	circular	Reddish orange	Flat	Entire	Smooth	+ ve	Rod
	G2	circular	Pale yellow	Flat	Entire	Smooth	+ ve	Rod
	G3	circular	White	Flat	Entire	Smooth	+ ve	Rod
	G4	circular	White	Flat	Entire	Smooth	+ ve	Rod
	G5	circular	White	Flat	Entire	Smooth	+ ve	Rod
	G6	circular	White	Flat	Wavy	Smooth	+ ve	Rod
	G7	circular	blackish brown	Flat	Entire	Smooth	+ ve	Rod
	G8	circular	Pale yellow	Flat	Entire	Smooth	+ ve	Rod
	G9	circular	Pale yellow	Flat	Entire	Smooth	+ ve	Rod
	G10	circular	Pale yellow	Flat	Entire	Smooth	+ ve	Rod
	G11	Irregular	White	Raised	Wavy	Rough	+ ve	Rod

3.3.1. Gram Staining

Nearly 87% of the isolates were Gram positive, among them 83% were rod and 4% were cocci. Among the rest Gram negative bacteria, it was with 7% rod and 6% cocci (Fig.5 and 6).

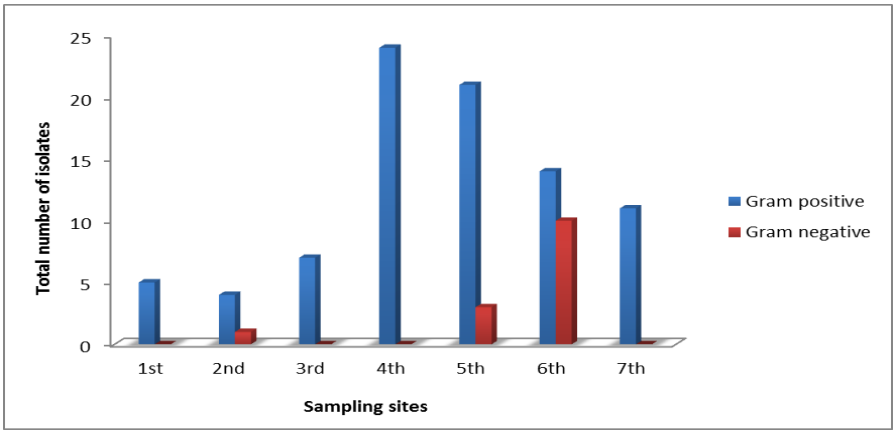

Fig. 5. Graphical representation of Gram positive and gram negative results of the isolates.

Isolation of bacteria using conventional plate methods implies the prevalence of Gram-positive bacteria [15]. Gram-positive bacteria as a group are common soil organisms. The distribution of Gram positive and Gram-negative bacteria was investigated and varying among the different types of soil.

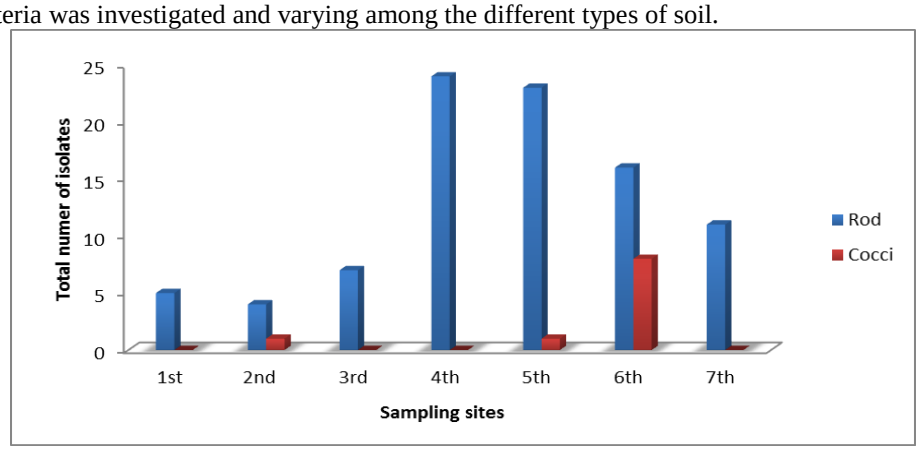

Fig. 6. Graphical represents the Gram staining results of the rods and cocci of the isolates. The number 1 to 7 on the sampling sites indicates the hills from Vellivinayagar to Giri hill.

Gram-positive bacteria were generally dominating the Gram-negative bacteria that are probably due to their ability to form endospores and develop other stresses [16]. Such dominance of Gram-positive bacteria over Gram negative in soil was also observed [17]. The Gram-positive rod dominates by the Thiruneer and the Beeman kaliurandai hill soil samples.

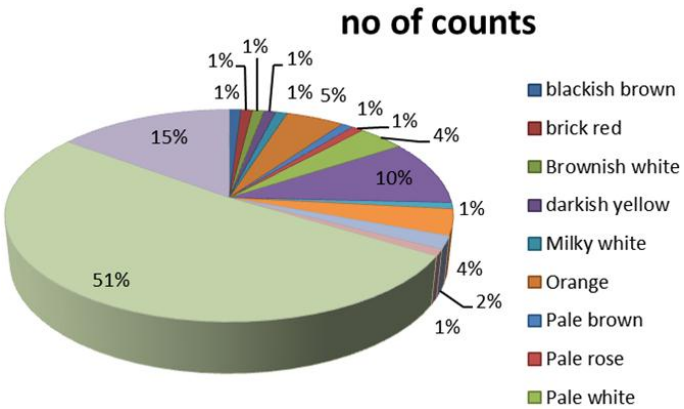

Fig. 7. Pie chart represents the pigmentation of the bacterial isolates**.**

The production of the pigment was predominantly on long period of incubation. From the observation the pale white colour bacterial isolates dominates the remaining cultures.

3.4. Diversity Index

3.4.1. Shannon Wiener Diversity Index

Among the samples tested, Aandisunai hill records the maximum index value with 3. 03 and Paambaatti hill sample shows the minimum index value with 0.66. Both the number of species and evenness with which individuals distributed among the species contribute to the index values. Low H' indicated low species richness and evenness and higher H' index indicates high species richness and evenness and approximately our results summarize that Aandisunai hill has the highest diversity and Paambaatti hill sample has the lowest diversity. The H' fluctuated less widely across the samples and Pielou (1975)[18] have demonstrated that H' is an elite description of population diversity (Fig. 8).

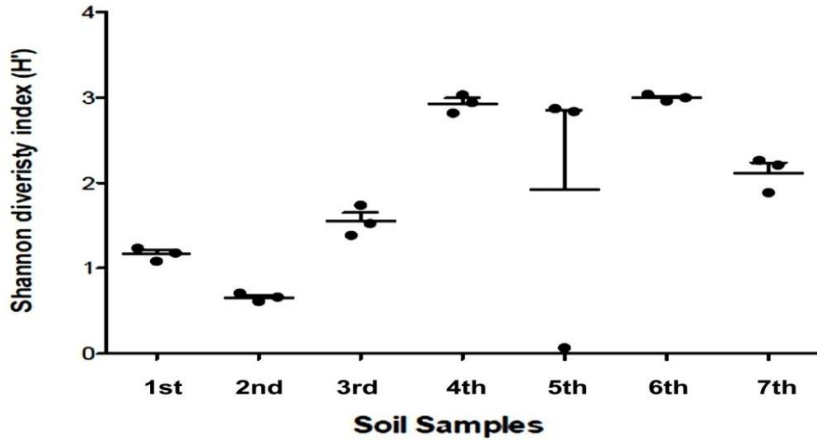

Fig. 8. Shannon Wiener diversity index (H)

3.4.2. Shannon Weiner – Evenness

Evenness values for the samples ranged from 0.43 (Paambaatti hill) to 5 (Vellivinayagar hill). Maximum value was recorded for the Vellivinayagar hill sample implies that the isolates are evenly distributed without any particular species dominations. The lowest value was recorded for Paambaatti hill sample, which shows that there is no even distribution of species and dominance of certain species (Fig. 9).

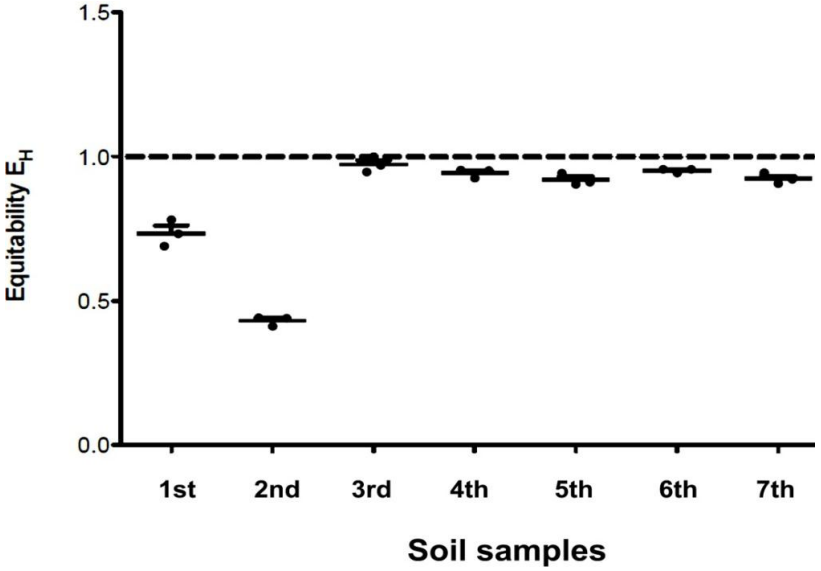

Fig. 9. Shannon Weiner – Evenness (E_H)

3.4.3. Simpsons index (D)

Simpsons index (D) is a measure of dominance rather than diversity it varies inversely with diversity. The Simpsons index (D) represents the likelihood that too randomly chosen individuals will be of the same species, so it varies inversely with diversity. Pertinently Aandisunai hill samples

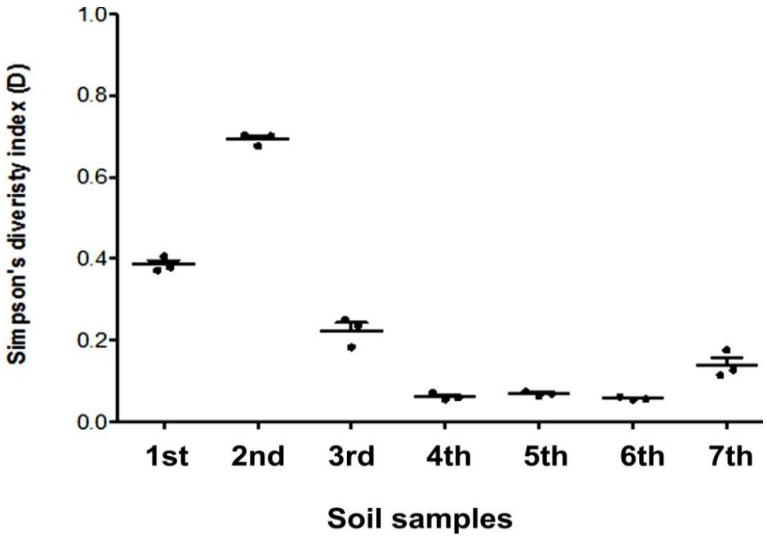

Fig. 10. Simpson's diversity (D)

with greater diversity showed lower concentration of dominance (D = 0.054) and Paambaatti hill sample lower diversity dimulged higher concentration of dominance, D= 0.67 (Fig. 10).

3.4.4. Simpsons index - equitability(ED)

Equitability (ED) value for samples ranged from 0.32 to 0.94. Maximum value was recorded for the samples Aandisunai hill implying that the isolates are evenly distributed without any particularly species dominates. The lowest value was recorded for the Paambaatti hill samples, which shows that there is no even distribution of species and dominance of certain species (Fig. 11).

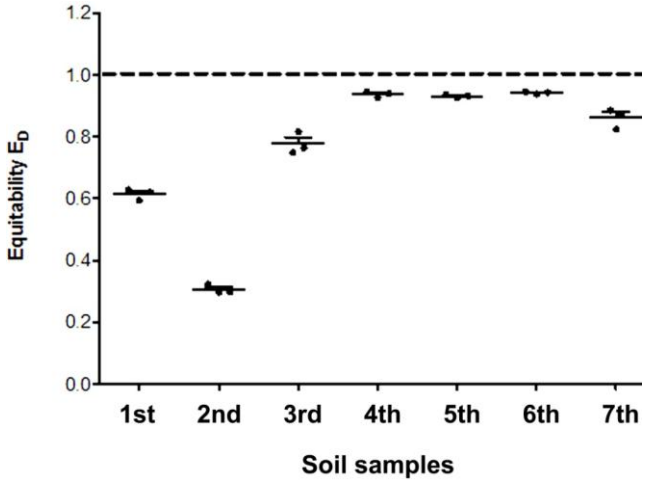

Fig. 11. Simpsons Index- Equitability(ED)

3.4.5. Species richness

From the results obtained through diversity indicated 6th hill samples (Aandisunai hill) has diverse and even distribution of species without dominance of any particularly phylotypes (Fig. 12). Simpson's index is known to put more weight on the abundant members of the community and hence is not affected by the variation in the sample size.

The majority of microorganisms present in various environments may not be readily cultivated by existing technologies and therefore not included in most analysis. Contemporary estimations
indicate that less than 1% of the microorganisms present in various environments are culturable, showing that techniques based on laboratory cultivation may be significantly biased [19].

In addition, traditional methods are based on differences in morphology, growth, enzymatic activity and metabolism, but these phenotypic characters are not always enough to define genera and overall species [20].

Several cultivation methods have been untaken and suggested as alternatives to the plate cultivation method, including growth chambers and micro colony growth on filters [21]. These methods have shown to enhance the cultivability and diversity of bacteria, but in spite of this, the agar plate approach is most frequently used due to its simplicity, low cost and the independency of equipment, all allowing several dilutions of sample to be analyzed in many replicates [22].

To understand the roles of microbial communities play vital role in the environment, cultivability of microorganisms and obtaining bacteria in pure culture is still imperative [23]. Factors like season and time scale were not observed, since the primary objective was to provide a first assessment on the diversity of culturable soil bacteria in the southern regions of Western Ghats, from the time of sampling. However, this is likely to have a significant impact on the types of culturable bacteria obtained, just as would different types of culture media. The isolated cultures are to be further identified using molecular methods and to be pondered for their biotechnological applications.

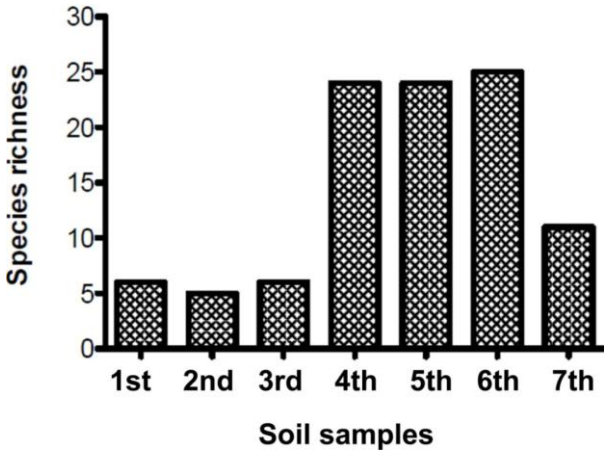

Fig.12. Graphical representation of the species richness in Velliangiri hills

3.5. Enzyme Screening

Screening of enzyme producing strains was determined by agar diffusion method as per the protocols given in manual of general bacteriology [24]. Enzyme activities were tested in nutrient agar medium with pH 7.5 amended with respective substrates (Amylase – 0.2% soluble starch; Gelatinase – 0.4% gelatin; Pectinase – 0.1% pectin; Cellulase – 0.2% carboxyl methyl cellulose, Lipase – 1% Tributyrin). The cultures that showed growth or clearing zones when tested with respective reagents were considered as positive cultures. Data from the three hills (Thiruneer, Beeman kaliurandai and Aandisunai hill) were pooled for a homogenous representation of Velliangiri region.

Table 9. Enzyme producing strains from the isolates of Velliangiri hills.
+: enzyme producers; -: non producers

Sample Site	Sample name	Cellulase	Amylase	Pectinase	Lipase	Proteinase
Vellivinayagar Hill	VV1	-	+	-	-	-
	VV2	-	+	-	-	-
	VV3	-	-	-	-	-
	VV4	-	+	-	-	-
	VV5	-	-	-	+	-
	VV6	-	+	-	-	-
Paambaatti Hill	P1	-	-	-	-	-
	P2	+	-	-	-	-
	P3	-	-	-	-	-
	P4	+	-	+	+	-
	P5	+	-	+	-	-
Kaithatti Hill	K1	+	+	-	+	-
	K2	+	-	-	-	-
	K3	-	-	-	+	-
	K4	-	-	-	+	-

	K5	+	-	-	+	-
	K6	-	-	-	-	-
	K7	-	-	-	-	-
Thiruneer Hill	T1	-	-	-	+	-
	T2	-	-	-	-	-
	T3	+	-	-	+	-
	T4	-	-	-	+	-
	T5	+	-	+	+	-
	T6	-	-	-	-	-
	T7	-	-	-	+	-
	T8	+	-	+	+	+
	T9	-	-	+	+	+
	T10	-	-	+	+	-
	T11	-	-	-	+	-
	T12	+	-	-	-	-
	T13	+	-	-	-	+
	T14	-	-	-	-	-
	T15	-	-	-	-	-
	T16	-	-	-	+	-
	T17	-	-	-	+	-
	T18	-	-	-	-	-
	T19	-	-	+	-	-
	T20	-	-	+	-	+
	T21	-	-	+	-	+
	T22	+	+	-	+	+
	T23	-	-	+	-	-
	T24	-	-	-	+	-
Beeman Kaliurandai Hill	BK1	+	-	-	-	-
	BK2	+	-	-	+	-
	BK3	+	-	-	-	+
	BK4	+	+	-	+	-
	BK5	+	-	-	-	-
	BK6	+	-	+	+	+
	BK7	-	-	-	-	-
	BK8	+	-	-	+	+
	BK9	+	-	+	-	+
	BK10	+	+	-	+	-
	BK11	+	-	-	+	+
	BK12	+	-	-	-	-
	BK13	-	-	-	-	-
	BK14	+	+	+	-	-
	BK15	+	-	-	-	-
	BK16	+	-	+	+	-
	BK17	+	+	+	+	+
	BK18	-	-	-	-	-
	BK19	-	-	-	-	-
	BK20	-	-	-	-	-
	BK21	+	+	+	+	-
	BK22	+	-	-	-	-
	BK23	+	-	-	-	-
	BK24	+	-	-	+	-
Aandisunai Hill	AS1	+	-	+	-	+
	AS2	+	-	-	-	-
	AS3	+	-	-	-	-
	AS4	+	-	+	-	+
	AS5	+	-	-	-	-
	AS6	+	-	-	-	-
	AS7	+	-	-	-	-
	AS8	+	-	-	+	+
	AS9	+	-	-	-	-
	AS10	+	-	-	-	-

	AS11	+	-	-	-	-
	AS12	-	-	-	-	-
	AS13	-	-	-	-	-
	AS14	+	-	-	-	-
	AS15	+	-	-	-	-
	AS16	+	-	-	-	-
	AS17	+	-	-	+	-
	AS18	-	-	-	-	+
	AS19	-	-	-	-	-
	AS20	-	-	-	-	-
	AS21	-	-	-	-	-
	AS22	+	-	-	-	+
	AS23	-	-	-	-	+
	AS24	+	-	-	+	+
Giri Hill	G1	-	-	-	+	-
	G2	+	-	+	+	+
	G3	+	-	-	-	+
	G4	+	-	-	+	+
	G5	+	-	-	+	-
	G6	+	-	-	-	+
	G7	+	-	+	-	+
	G8	+	+	+	-	-
	G9	+	-	-	-	-
	G10	+	-	+	-	+
	G11	+	-	+	+	+

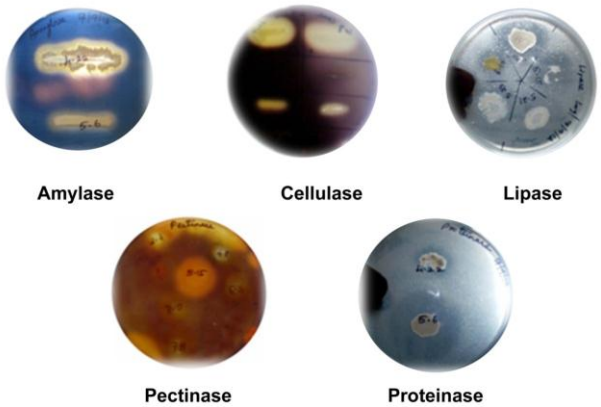

Fig. 13. Screening of the bacterial isolates which producing extracellular enzyme. Amylase activity on starch casein agar; Cellulase activity on CMC agar; lipase activity on Tributyrin agar medium; Pectinase activity on pectin agar; Protease activity on gelatin agar plate.

3.5.1. Extracellular enzyme producer and its activity

From the results obtained it concludes that 50% of the isolates expressed cellulase, 5% of amylase, 5% of pectinase, 10% of protease and 30% of lipase activity in agar plates.

Previous reports also show that the enzyme producing bacteria are of great significance as they aid in the processes of modification and transformation of specific substrate compounds [25]. These enzyme activities signify the role of culturable bacteria in the degradation and mineralization of organic matter, their production and activity depend primarily on availability, distribution, type, and concentration of the organic substrates. These complex organic matters may be relatively fresh or partially degraded organic carbon [26].

Of the 101 isolates, only 1 bacterial isolate expressed all the five enzymes namely amylase, cellulase, lipase, protease and pectinase. 6 bacterial isolates produced 4 different enzymes; 19 bacterial isolates were three enzyme producers and 15 bacterial isolates produced only two enzymes. 39 bacterial isolates were produced only one enzyme. Remaining 21isolates were non enzyme producers. Enzymes producing bacterial isolates are mentioned in the table 6. Totally 10 bacterial isolates were identified through screening the maximum enzyme activity on specific substrate for particular enzyme. The enzyme producing isolates were screened with the references of the standard enzyme producers like *B.subtillus* for amylase*; B.pumilus* for protease*; P.aeuruginosa* for lipase*; Paenibacillus cineris* for cellulase*; B.cereus* for pectinase. According to [27], 37°C is the optimum

growth temperature for the *Bacillus* strain and 50^0C is the higher temperature which supported less number of colonies. This could be due to the mesophilic nature of the species. As per earlier report high temperature may inactivate the expression of gene responsible for the starch degrading enzyme. Most of the starch degrading bacterial strain revealed a pH range between 6.0 and 7.0 for normal growth and enzyme activity [28].

Comparing to the standard organism, the enzyme activity from the Velliangiri hill bacterial isolates are in less production. This may due to the sample-collected sites were varied from the standard cultures origin. It is also due to the physiological parameters like optimum temperature, substrate concentration and pH ranges are important for enzyme production by microbes [28] and [29].

Table 10. Screening of maximum enzymes producing strains by the zone of clearance

Table 10. Screening of maximum enzymes producing strains by the zone of clearance

Enzymes	Culture names	Zone of clearance in 48 hrs incubation (mm in dia.)	Increase ▯ /decrease ▯ (Compared to standard in %)
Amylase	*B.subtillus*	31	Standard
	BK6	15	52 % ↓
	BK17	20	35 %
	T22	20	35 % ↓
	G2	18	42 % ↓
Protease	*B.pumilus*	27	Standard
	T22	25	2 % ↓
	BK6	45	66 %↑
	BK17	25	2 % ↓
lipase	*P.aeruginosa*	35	Standard
	T8	13	63 % ↓
	BK17	40	14 % ↑
	T22	25	29 % ↓
	BK14	37	5 % ↑
	BK21	42	20 % ↑
	G11	37	5 % ↑
Cellulase	*Paenibacillus cineris*	35	Standard
	T8	7	80 % ↓
	BK17	15	58 % ↓
	BK5	5	86 % ↓
	BK14	17	51 % ↓
	BK15	15	58 % ↓
	BK21	11	68 % ↓
	BK22	19	46 % ↓
	G2	5	86 % ↓
	G11	8	77 % ↓
Pectinase	*B.cereus*	27	Standard
	BK17	11	60 % ↓
	T8	12	65 % ↓
	BK15	33	22 % ↑
	BK14	6	87 % ↓
	BK5	7	74 % ↓
	G2	11	60 % ↓

3.5.2. Enzyme producing strains activity on different temperature and pH

From the screening results, a total of 10 isolates were selected among the overall bacterial isolates. This bacterial isolates were further subjected to growth in nutrient broth at different pH and temperature. Growth was observed at 48 hour time interval.

Table 11. Growth activity at different pH (range from 3 to 11)

pH	3	4	7	9	11
isolates	48 hrs	48 hrs	48 hrs	48 hrs	48 hrs
T8	-	-	+	+	-
T22	-	-	+	+	-
BK5	-	-	+	+	-
BK6	-	-	+	+	-
BK14	-	-	+	+	-
BK15	-	-	+	+	-
BK21	-	-	+	+	-
BK17	-	-	+	+	-
G2	-	-	+	+	-
G11	-	-	+	+	-

Table 12. Growth activity at different temperature (range from 10° to 60°C)
+ indicates the growth of the bacterial isolates

Temp	10°C	20°C	30°C	50°C	60°C
isolates	48 hrs	48 hrs	48 hrs	48 hrs	48 hrs
T8	-	+	+	+	-
T22	-	+	+	+	-
BK5	-	+	+	+	-
BK6	-	+	+	+	-
BK14	-	+	+	+	-
BK15	-	+	+	+	-
BK21	-	+	+	+	-
BK17	-	+	+	+	-
G2	-	+	+	+	-
G11	-	+	+	+	-

The selected organisms that possess the enzyme activity were grown in different temperature (20, 30, 40 and 50°C) and pH (3, 4, 7, 9, and 11) conditions (Table 11 and 12). The organisms showed good growth at 20°C, and 30°C, but in 50°C the growth was slower and there was no growth in 10°C and 60°C which suggests that the organisms were typically a mesophile where even a moderate increase in temperature diminishes its growth pattern. The observation upholds with the weather conditions prevailing in its habitat (Western Ghats). When the organism was able to grow in the neutral pH range and can tolerate alkaline conditions at pH-7. No growth was observed in acidic condition (pH 3 and 4).

3.6. Bacterial identification based on its whole cell protein fraction

From the results of MALDI analysis the isolates were identified as listed (Table 9). From 10 isolates 5 belong to *Bacillus* species and the remaining were spread among as *Rhodococcus equi*, *Sphingomonas koreensis*, *Clostridium clostridioforme*, *Pseudomonas alcaligenes*, *and Paenibacillus glucanolyticus*.

From the results, the bacterial strain *Pseudomonas alcaligenes*, which is isolated from the Velliangiri hills, has the capacity of producing five different enzymes like amylase, cellulase, lipase, pectinase, protease at the stable temperature of 37^{o}C. Bacterial samples isolated from Saline Belt of Purna River had showed the activity for 3 enzymes like protease, amylase and lipase [30]. Cha *et al*., (2010) also reported on fungus *Aspergillus niger*, which shows the ability of producing four enzymes like phytase, xylanase, cellulase, and mannanase which is required for the production of the animal feeds.

Table 13. Identification of bacterial isolates through MALDI Analysis

S.No	Laboratory Names	Culture name (MALDI analysis)	Score values
1	T8	*Bacillus mycoides*	1.805
2	T22	*Rhodococcus equi*	1.725
3	BK5	*Sphingomonas koreensis*	1.388
4	BK6	*Bacillus thuringiensis*	1.771
5	BK14	*Bacillus subtilis*	1.96
6	BK15	*Clostridium clostridioforme*	1.399
7	BK21	*Bacillus mojavensis*	1.884
8	BK17	*Pseudomonas alcaligenes*	1.301
9	G2	*Paenibacillus glucanolyticus*	1.626
10	G11	*Bacillus mojavensis*	1.775

IV. Conclusion

From the sub hills of Velliangiri, the Aandisunai hill has a rich bacterial diversity whilst the Paambaatti hill has a low bacterial diversity. Totally 101 isolates were isolated from the Velliangiri hills, and on the basis of their morphological and physiological characteristic features, *Sphingomonas koreensis*, *Bacillus mycoides*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Clostridium clostridioforme*, *Bacillus mojavensis*, *Pseudomonas alcaligenes*, *and Rhodococcus equi* were identified using MALDI. The identified organisms can be used in industrial for multi enzyme producing purposes. Among them *Pseudomonas alcaligenes*, from the Beeman kaliurandai hill has the capacity of producing all five enzymes (Amylase, Proteinase, lipase, Cellulase and pectinase). So it can be used as commercially important enzyme producing bacterial strain. From the above results, it is clear that industrially important bacterial strains can be explored from Thiruneer hill, Beeman kaliurandai hill and Aandisunai hill of Velliangiri hills.

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