



# 读 书 报 告

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# Hot springs of Indian Himalayas: potential sources of microbial diversity and thermostable hydrolytic enzymes

[Authors](#)

[Authors and affiliations](#)

Harmesh Sahay, Ajar Nath Yadav, Atul Kumar Singh, Surendra Singh, Rajeev Kaushik, Anil Kumar Saxena 

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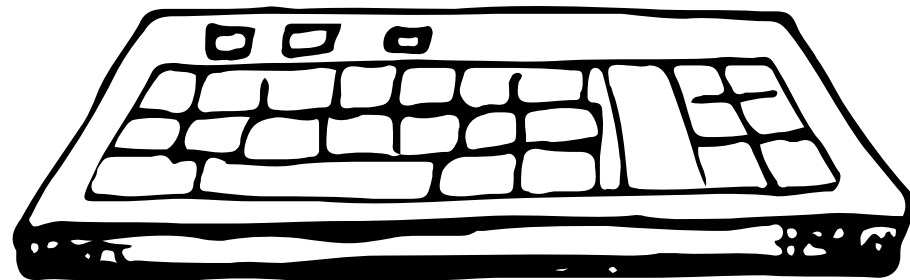
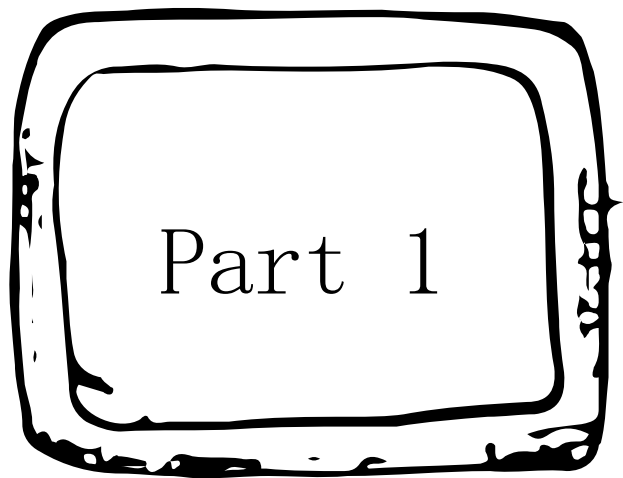
*CONTENTS*



Introduction

Materials and Methods

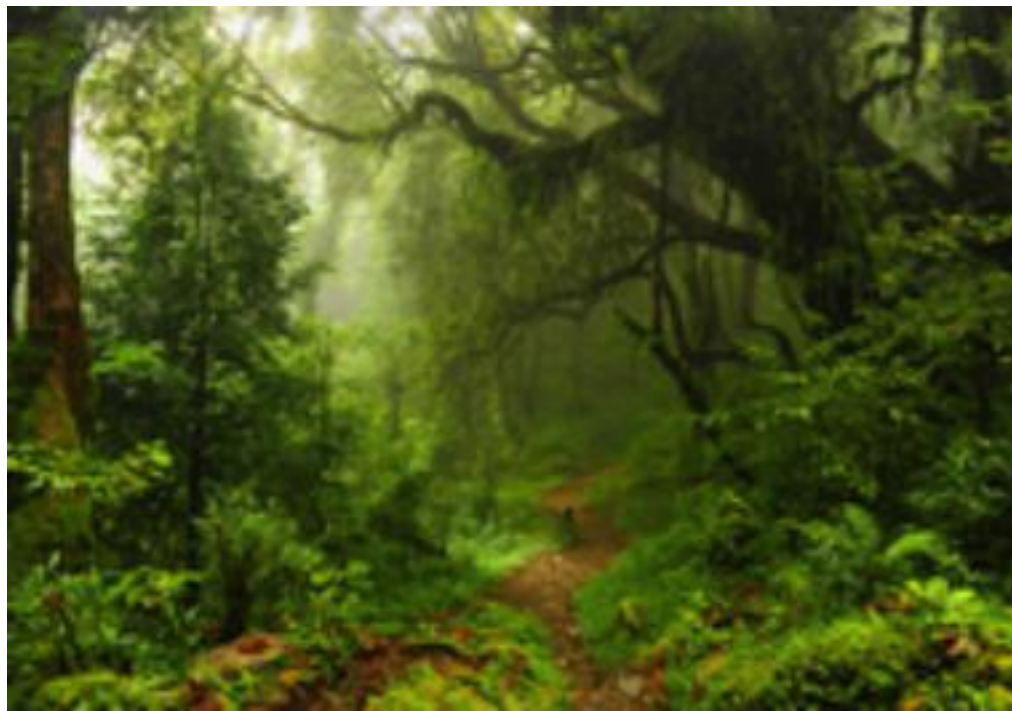
Results and Discussion



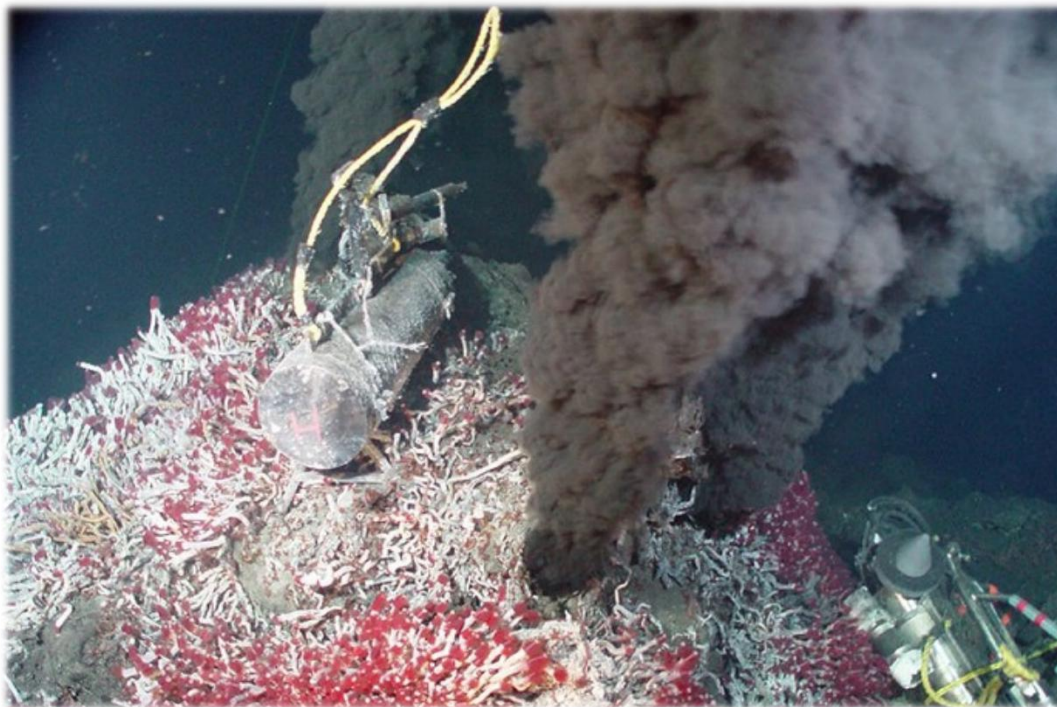
Introduction



# Introduction



## Introduction

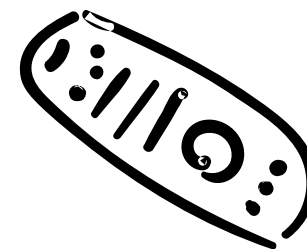
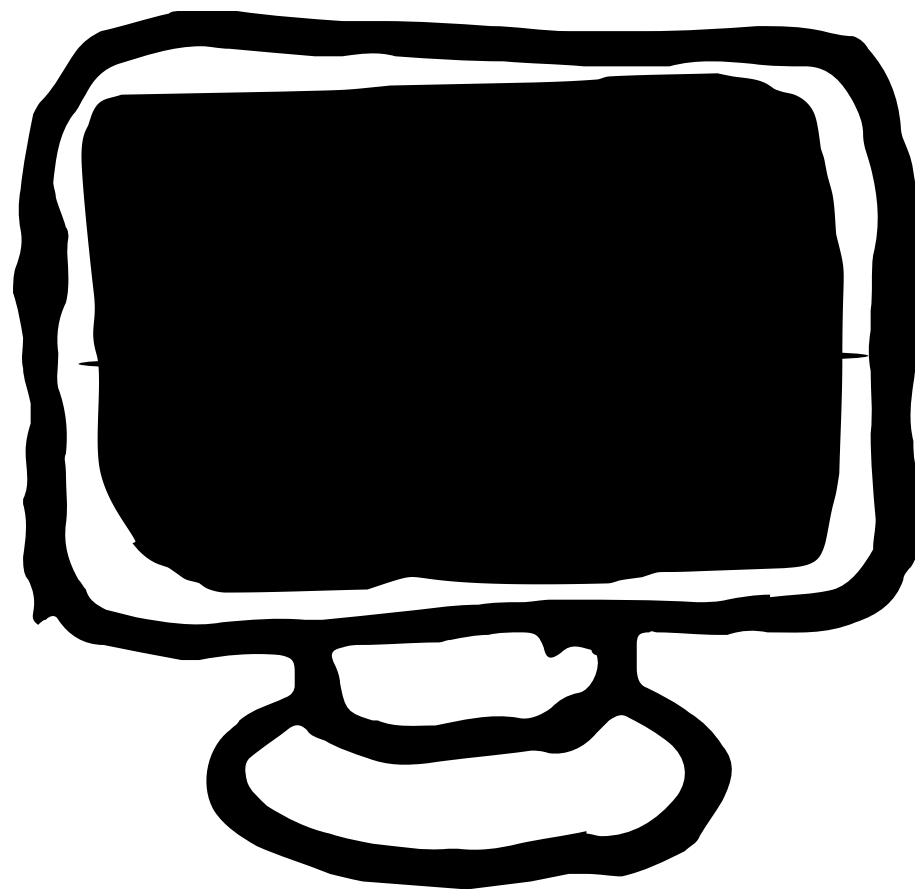


嗜热生境下的原核生物群体经历了高温和化学胁迫的生理适应，这些微生物群落不仅在生物技术前景方面有重大研究意义，而且有助于了解早期地球的生命起源。

# Introduction



低海拔地区热泉中的微生物群落已在世界范围内得到广泛研究，但对高海拔地区热泉微生物多样性知之甚少。特别是喜马拉雅山脉，它既有极端'冷'，也有'热'，但由于人类活动无法进行，许多有价值的基因库尚未开发。



Materials and Methods





# 1. Study sites and sample collection

**Table 1** Geographic details and physico-chemical characteristics of collection sites

S. no.	Sampling location	No. of samples	Altitude (meter)	Latitude: longitude	Temperature (°C)	pH
1	Manikaran hot springs	6	1760	32°02'00"N: 77°20'48"E	89–95 °C	7.8–8.2
2	Yumthang hot springs	6	3564	27°49'36"N: 88°41'45"E	43–63 °C	6.5–6.8

收集每个热泉的六个不同样品并保存在无菌的恒温瓶中，在最短时间内运送到实验室。



## 2. Isolation and enumeration of thermophilic bacteria

富

water samples : 将10mL每种水样品直接接种到50mL营养肉汤中

sediment samples : 将5g沉淀物与10mL无菌水混合并接种到50mL营养肉汤中

集

60°C 孵育2小时

分离

取100 $\mu$ L各富集样品适当稀释并涂布在六种不同的培养基平板上

60°C 孵育6 - 15天



<b>TM (thermus medium)</b>	1L
Yeast extract	4 g
polypeptone peptone	8 g
NaCl	2 g

<b>TTM (Thermus thermophilus medium )</b>	1L
Yeast extract	4 g
proteose peptone	8 g
NaCl	2g

<b>YET (yeast extract tryptone)</b>	1L
Casein enzymic hydrolysate	6 g
yeast extract powder	3 g

<b>nutrient agar</b>	1L
Peptone	5 g
NaCl	5 g
beef extract	1.5 g
yeast extract	1.5 g



TEA (thermus enhanced agar)	1L
Yeast extract	2.5 g
tryptone	2.5 g
nitrilotriacetic acid	100 mg
CaSO <sub>4</sub> ·2H <sub>2</sub> O	40 mg
MgCl <sub>2</sub> ·6H <sub>2</sub> O	200 mg
Fe-citrate (0.01 M)	0.5 mL
trace element solution	0.5 mL



Nitrilotriacetic acid	12.8 g	FeCl <sub>2</sub> ·4H <sub>2</sub> O	1.0 g;
MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.5 g	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.3 g;
CuCl <sub>2</sub> ·2H <sub>2</sub> O	50 mg	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	50 mg;
H <sub>3</sub> BO <sub>3</sub>	20 mg	NiCl <sub>2</sub> ·6H <sub>2</sub> O	20 mg
phosphate buffer	0.5 mL		
(KH <sub>2</sub> PO <sub>4</sub> , 5.44 g; Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O, 43 g/L)			

R <sub>2</sub> A	1L
Casein enzymic hydrolysate	0.25g
peptone	0.25g
casein acid hydrolysate	0.5 g
yeast extract	0.5g
glucose	0.5 g
starch soluble	0.5 g
K <sub>2</sub> HPO <sub>4</sub>	0.03 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.5 g
sodium pyruvate	0.03 g



## 2. Isolation and enumeration of thermophilic bacteria

富

water samples : 将10mL水样品直接接种到50mL营养肉汤中

sediment samples : 将5g沉淀物与10mL无菌水混合并接种到50mL营养肉汤中

集

60°C 孵育2小时

分离

将100 $\mu$ L各富集样品的等分试样适当稀释并铺展在六种不同的培养基平板上

60°C 孵育6 - 15天

挑菌纯化保藏



### 3. Culture conditions and growth pattern of bacterial isolates

研究温度和pH对所有代表性分离物的生长的影响

在40℃下孵育24小时，将300μL细菌培养液接种到具有TM培养基的微孔板中，于自动微生物生长分析系统中测量600nm处的吸光度值 (n=3)，得到生长曲线，并在平台期测量每种菌的CFU。



#### 4. Screening for thermostable enzymes producing bacterial strains

基础培养基 1L

Yeast extract 1 g

$\text{KH}_2\text{PO}_4$  1 g

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 g

$\text{CaCl}_2 \cdot \text{H}_2\text{O}$  0.05 g

$\text{NaCl}$  5 g

$\text{Na}_2\text{CO}_3$  10 g

agar 15 g

starch 2.5g/L

xylan 10 g/L

carboxy methyl cellulose (CMC) 5 g/L

skimmed milk (10% w/v)

先将纯化的细菌菌株在含有50mL营养肉汤的150mL烧瓶中培养，然后将5 $\mu\text{L}$ 培养物点接种于不同培养基平板上，并在45,55和65 $^\circ\text{C}$ 孵育3-5天。



## 4. Screening for thermostable enzymes producing bacterial strains

蛋白酶 —— 产生透明圈

木聚糖酶和纤维素酶

在室温下用0.1%刚果红溶液染色15分钟，接着用1M NaCl溶液脱色。

淀粉酶

每个平板中加入0.6% KI溶液，染色30秒。

以透明圈的大小表示酶的活性



#### 4. Screening for thermostable enzymes producing bacterial strains

将选择的菌株接种到含有100mL基础培养基的250mL烧瓶中，并在其各自的最佳生长温度和pH下孵育3-5天。将等份的粗酶（无细胞提取物）在不同温度（45,55,65,75和85°C）下（pH7.0）预孵育30分钟，并在其各自的温度下测量活性。

$$SE = 100 - R_A; \text{ where } R_A(\%) = \frac{E_{TP} - E_{dTP}}{E_{TP}} \times 100$$

$R_A$  represents the percentage of reduced enzyme activity

$E_{TP}$  is the total enzyme activity at its optimum temperature and pH

$E_{dTP}$  is enzyme activity after temperature treatment at its respective optimum pH

Data was subjected to analysis of variance (ANOVA) using software SPSS ver. 10.



## 5. PCR amplification of 16S rRNA gene and phylogenetic analysis

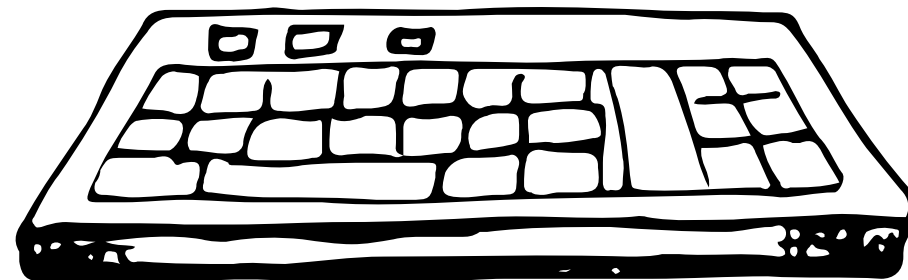
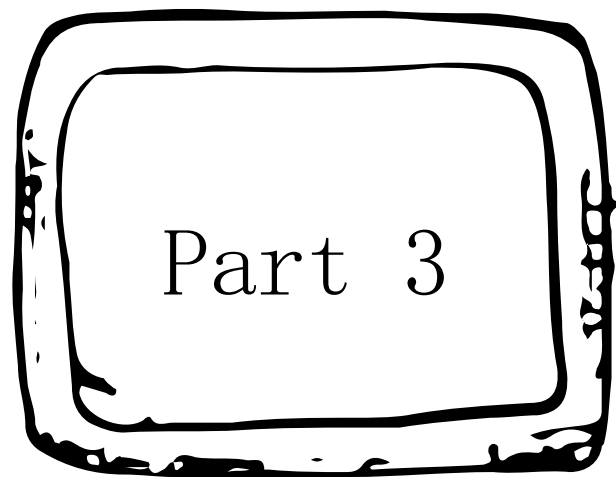
PCR扩增通用引物

pA (5'-AGAGTTTGATCCTGGCTCAG-3')

pH (5'-AAGGAGGTGATCCAGCCGCA-3')

在MEGA 4.0.2程序中使用邻接法 (NJ) 构建系统发育树

使用BLASTn程序比对相似性



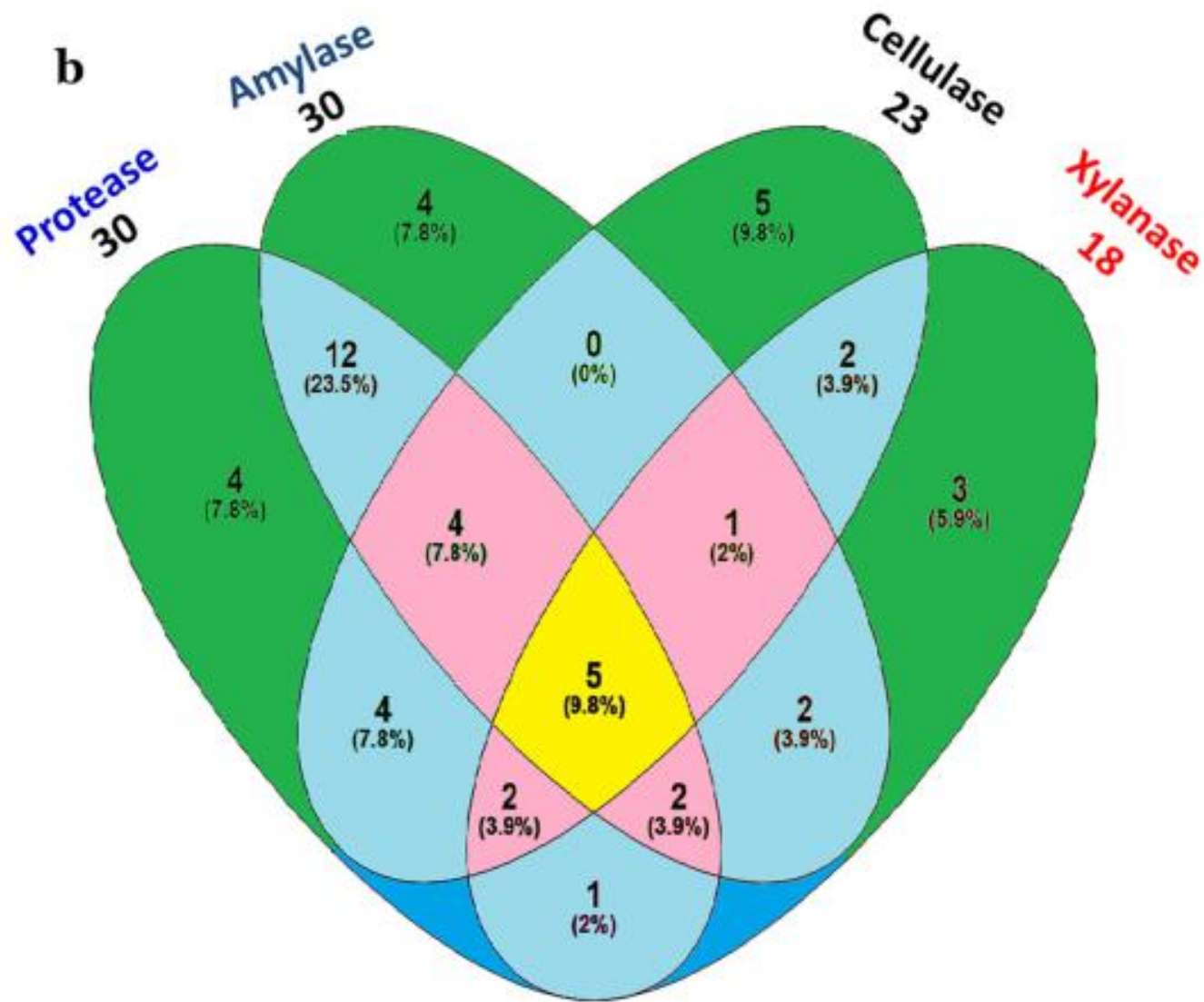
Results and Discussion

**Table 2** Thermotolerance of bacterial isolates obtained from Manikaran and Yumthang hot springs

	(CFU $\times 10^7$ )				
	50 °C	60 °C	70 °C	80 °C	90 °C
Manikaran hot spring					
<i>Bacillus arsenicus</i> NBM47	146.15 $\pm$ 01.03	141.86 $\pm$ 01.0	126.64 $\pm$ 0.89	122.34 $\pm$ 0.86	57.20 $\pm$ 0.40
<i>Bacillus mycoides</i> NBM19	50.25 $\pm$ 0.36	44.97 $\pm$ 0.32	43.65 $\pm$ 0.31	27.44 $\pm$ 0.19	21.49 $\pm$ 0.15
<i>Bacillus pumilus</i> NBM31	50.92 $\pm$ 0.36*	48.61 $\pm$ 0.34	46.95 $\pm$ 0.33	45.30 $\pm$ 0.32	29.76 $\pm$ 0.21
<i>Bacillus subtilis</i> NBM48	93.57 $\pm$ 0.66	93.25 $\pm$ 0.66	84.98 $\pm$ 0.60	83.65 $\pm$ 0.59	30.75 $\pm$ 0.22
<i>Bacillus thermoamylovorans</i> NBM38	54.56 $\pm$ 0.38	50.59 $\pm$ 0.36	45.63 $\pm$ 0.32	43.65 $\pm$ 0.31	40.67 $\pm$ 0.29
<i>Geobacillus</i> sp. NBM49	140.86 $\pm$ 01.0	137.89 $\pm$ 0.97	128.96 $\pm$ 0.91	82.34 $\pm$ 0.58	19.84 $\pm$ 0.14
<i>Paenibacillus glycanilyticus</i> NBM30	133.92 $\pm$ 0.94	132.59 $\pm$ 0.94	126.64 $\pm$ 0.89	125.32 $\pm$ 0.88	82.99 $\pm$ 0.59
<i>Paenibacillus thiaminolyticus</i> NBM71	45.630 $\pm$ 0.32	43.31 $\pm$ 0.31	41.33 $\pm$ 0.29	25.46 $\pm$ 0.18	22.48 $\pm$ 0.16
<i>Planococcus</i> sp. NBM37	148.80 $\pm$ 1.05	113.09 $\pm$ 0.80	110.11 $\pm$ 0.78	98.21 $\pm$ 0.69	42.66 $\pm$ 0.30
<i>Thermonema lapsum</i> NBM28	191.78 $\pm$ 1.35	184.51 $\pm$ 1.30	166.98 $\pm$ 1.18	163.68 $\pm$ 1.15	94.24 $\pm$ 0.66
Yumthang hot spring					
<i>Bacillus pumilus</i> NBY4	3.78 $\pm$ 02.21	3.79 $\pm$ 02.21	4.55 $\pm$ 02.65	4.38 $\pm$ 02.56	1.06 $\pm$ 0.62
<i>Bacillus</i> sp. NBY16	4.23 $\pm$ 02.47	4.10 $\pm$ 02.39	4.10 $\pm$ 02.39	2.17 $\pm$ 01.27	2.08 $\pm$ 01.21
<i>Paenibacillus</i> sp. NBY33	4.32 $\pm$ 02.52	4.67 $\pm$ 02.73	4.67 $\pm$ 02.73	4.77 $\pm$ 02.79	0.68 $\pm$ 0.40
<i>Thermobacillus</i> sp. NBY36	8.61 $\pm$ 05.03	8.39 $\pm$ 04.90	8.55 $\pm$ 04.99	5.44 $\pm$ 03.18	2.56 $\pm$ 01.50

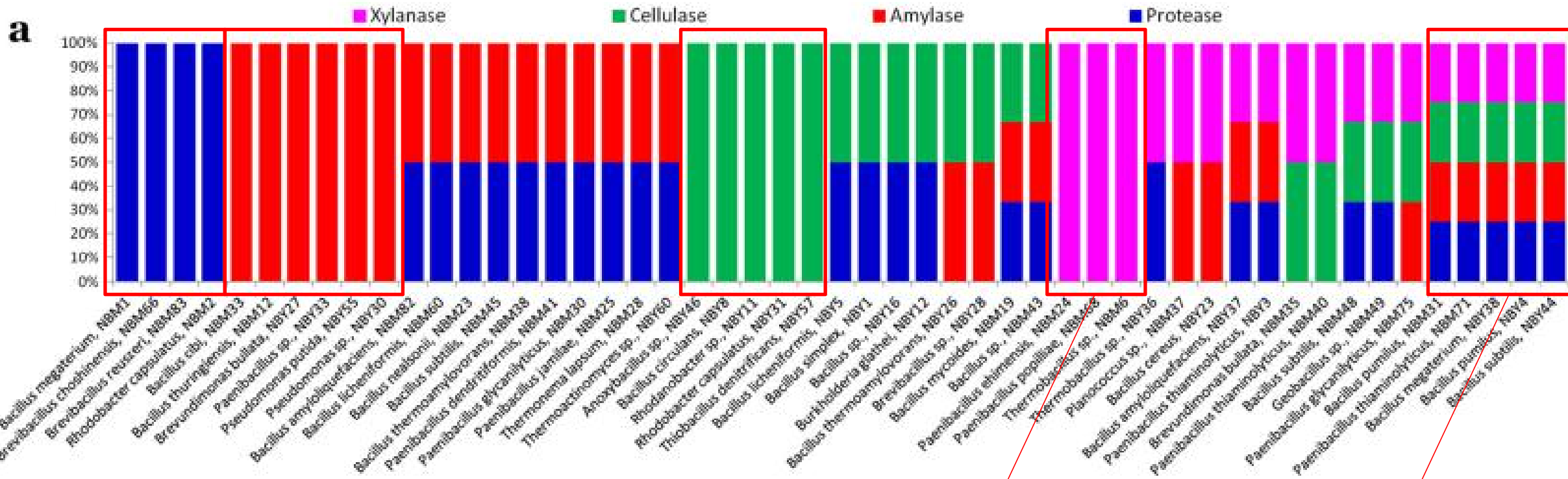
\* Data expressed as mean of average of three replicates given, along with standard deviation





共有51株菌产酶

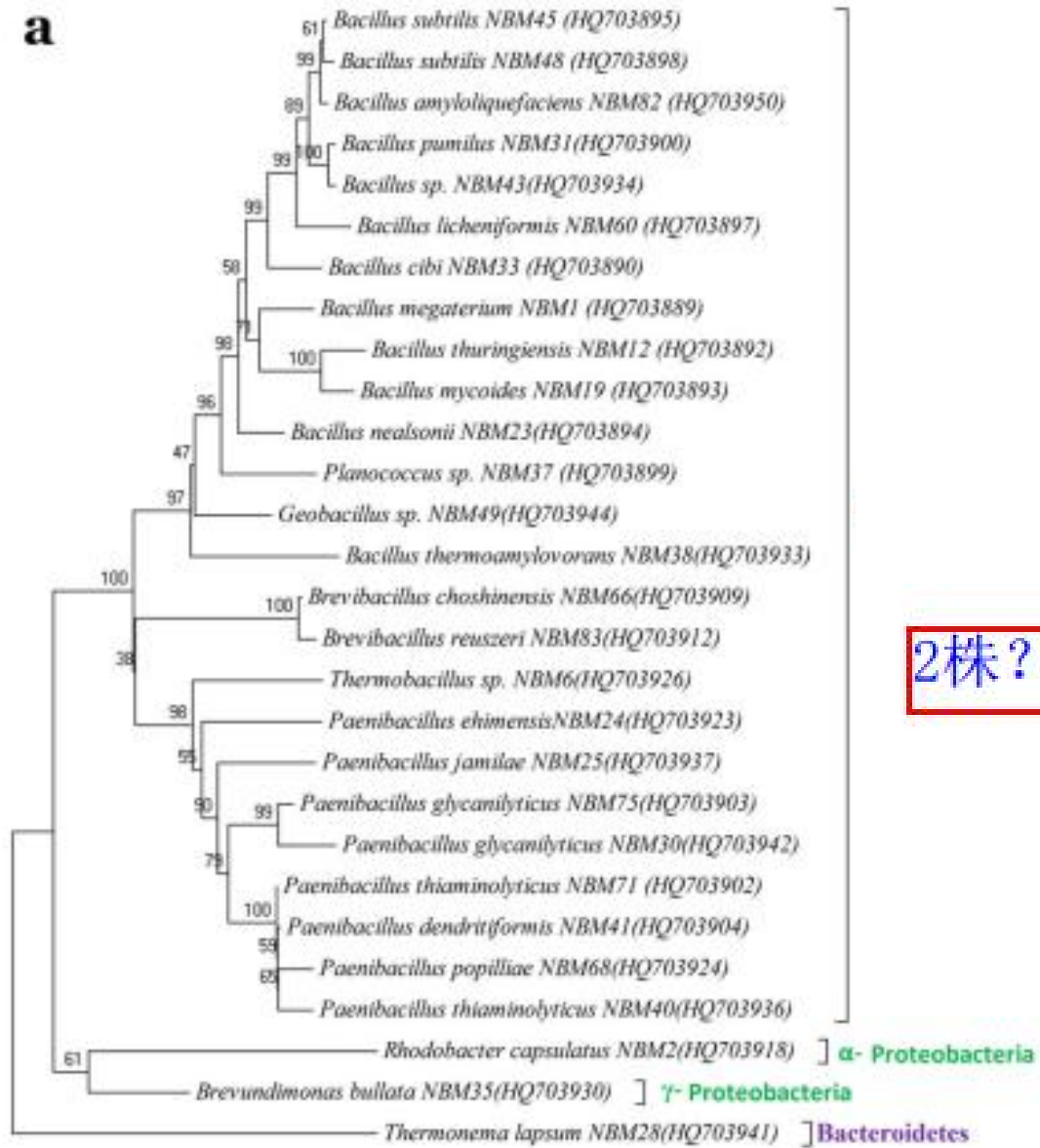
**Fig. 1 a** Diversity and distribution of 51 bacterial isolates for four different hydrolytic enzymes production at high temperature; **b** the *Venn diagram* illustrates the number of extracellular hydrolytic enzymes producing bacteria



*Thermobacillus sp* NBM6  
*Paenibacillus ehimensis* NBM24  
*Paenibacillus popilliae* NBM68

**9.8%**

a



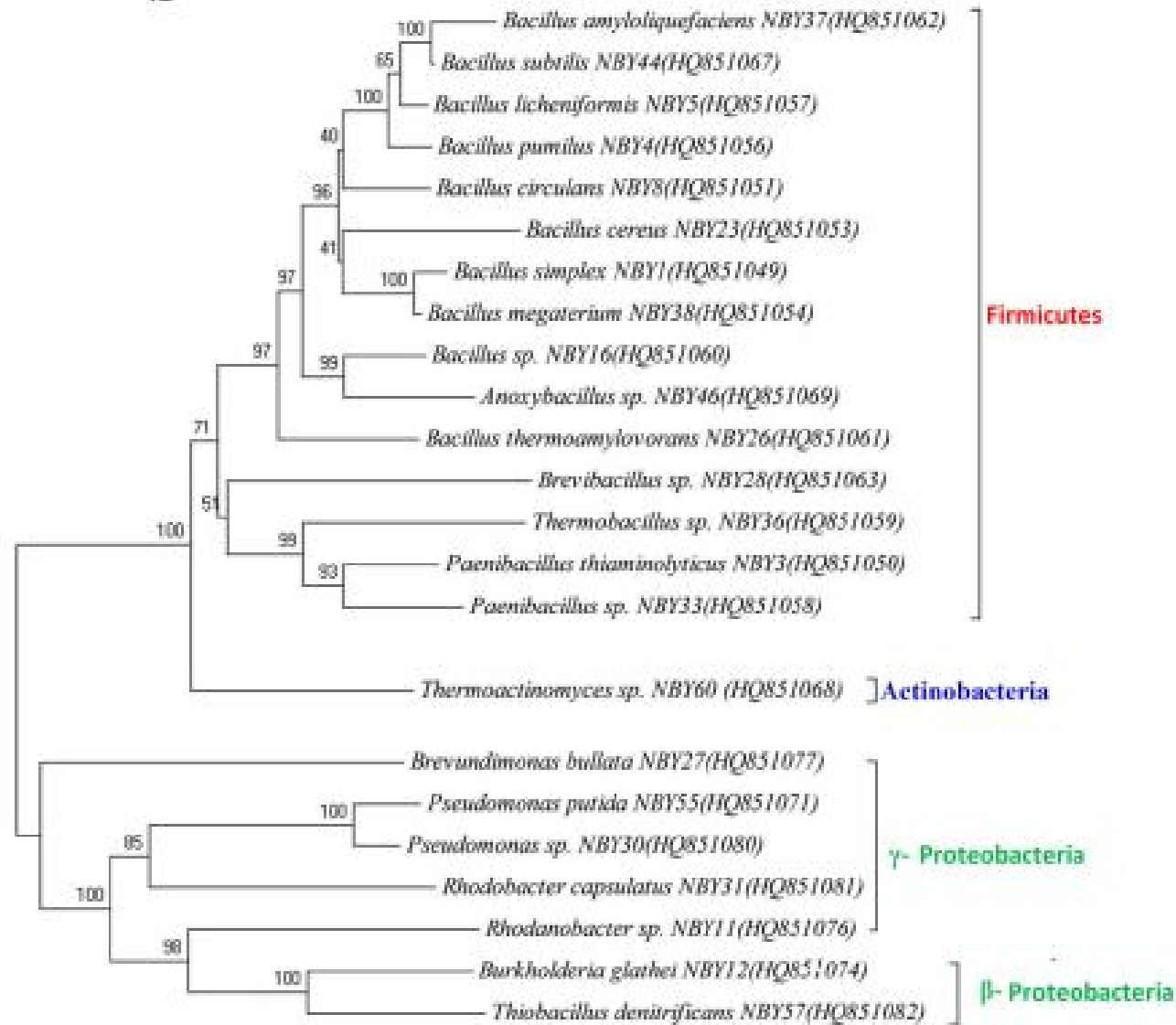
100% similarity with the sequences within the GenBank database. The phylogenetic trees were constructed to determine the affiliations for 28 and 23 bacteria isolated from thermal springs of Indian Himalayas (Fig. 2a, b). Analysis of the 16S rRNA sequences from Manikaran hot spring revealed that 25 strains belonged to Firmicutes (86%), 3 strains to Proteobacteria (10%) and 1 strain to Bacteroidetes (4%) (Fig. 2a). Yumthang hot springs bacterial isolated were grouped into four phyla namely Firmicutes (65%), Proteobacteria (30%) and Actinobacteria (4%) (Fig. 2b). Overall all identified bacteria belong to 37

2株?

Manikaran hot springs



**b**



厚壁菌门 *Firmicutes* (65%)

变形菌门 *Proteobacteria* (30%)

放线菌门 *Actinobacteria* (4%)

0.02

Yumthang hot springs



## 小结

总体上所有确定的细菌属于14个不同属的37个种。

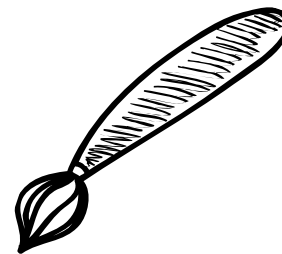
本研究提供的证据表明，芽孢杆菌和芽孢杆菌属在恶劣的温度环境下普遍存在，且是主要的。

Further, in general the bacteria belong to the genera *Bacillus* and *Thermus* were mostly reported as aerobic, heterotrophic thermophiles and found in thermal systems with neutral to alkaline pH (Spanevello and Patel 2004). *Thermus spp.* may be predominant heterotrophs in many hot springs (Hjorleifsdottir et al.2001)

**In present investigation none of the *Thermus* species were obtained.**

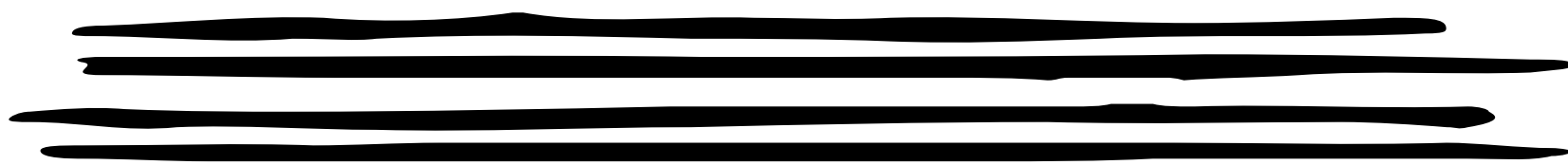


# Conclusion



可用于各种工业、食品、农业和医药用途

为研究热/高温生境中生命适应性和生物技术开发潜力提供了机会







请 各 位 老 师 同 学 批 评 指 导